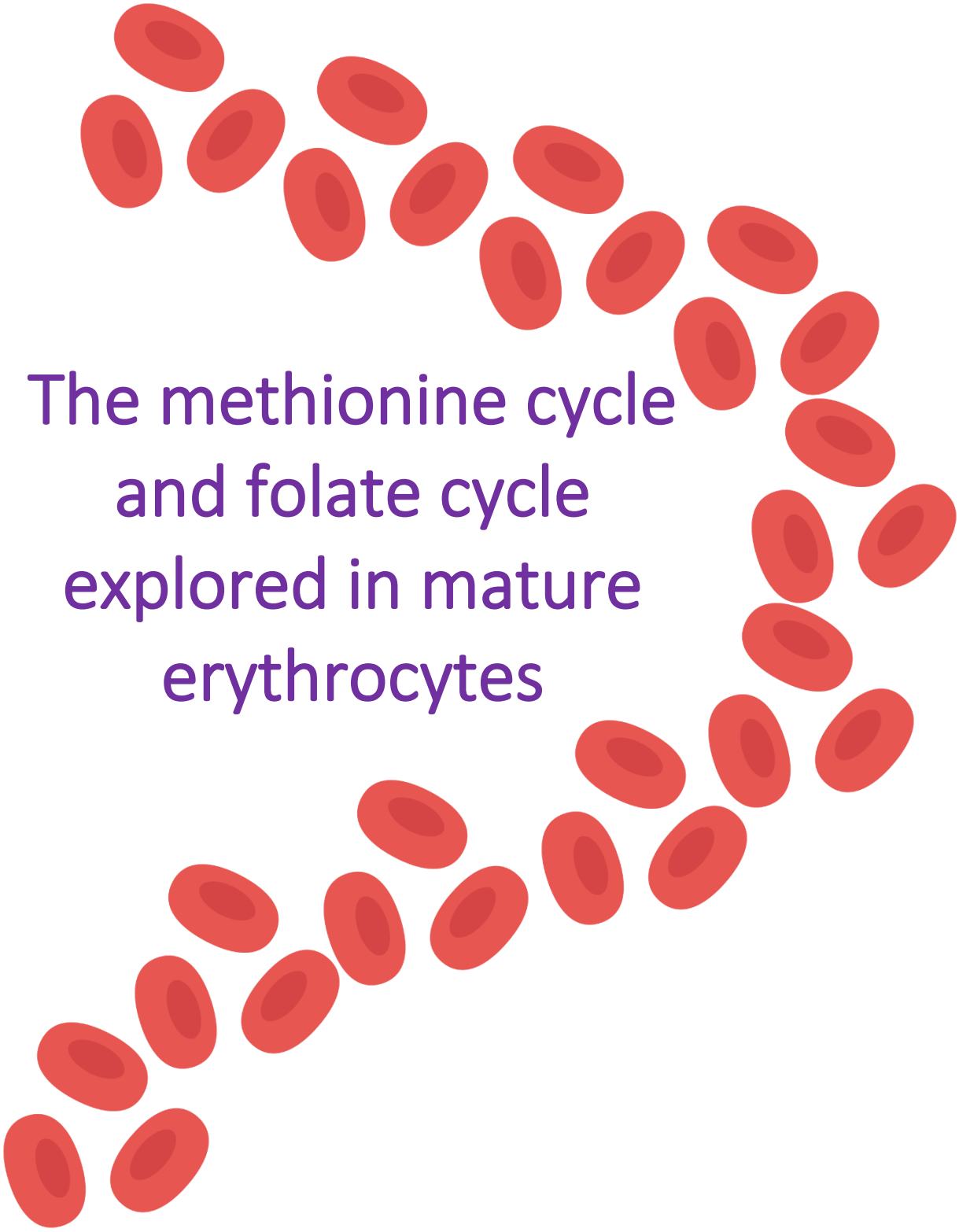

The methionine cycle and folate cycle explored in mature erythrocytes

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LAYMAN SUMMARY – EXPLORING RED BLOOD CELL METABOLISM

Red blood cells (RBCs), play an important role in our body by transporting oxygen from our lungs to tissues and removing carbon dioxide. RBCs are derived from the bone marrow where they mature via precursor cells into RBCs. During this process, the RBC loses its nucleus and mitochondria, the powerhouses of the cell. After maturing, the RBC is released into the blood stream, where it can live up to 120 days. Because RBCs lose their nucleus and mitochondria, they were always thought to be very simple cells with only the crucial chemical reactions.

A series of chemical reactions in cells is called a metabolic pathway and the intermediates in the pathway are called metabolites. Glycolysis and the pentose phosphate pathway (PPP) are important for giving the RBC energy and providing it with nicotinamide adenine dinucleotide phosphate (NADPH) respectively. The oxygen in RBCs can react in the cell and then forms highly reactive compounds, that damage RBCs. NADPH helps in antioxidant reactions to reduce oxidative stress. In the past decade, studies on the metabolic pathways in RBCs revealed that other pathways next to glycolysis and the PPP play a role in RBCs, including the one-carbon metabolism. As RBCs lack a nucleus and mitochondria, the one-carbon metabolism in RBCs is more limited compared to other cells.

Here I give an overview of the one-carbon metabolism in RBCs. The overview has a focus on the methionine cycle and the folate cycle. The methionine cycle can be divided into two pathways: the transmethylation pathway and the transsulfuration pathway. The transmethylation pathway in RBCs is important for donating one-carbon groups (methyl groups) to other metabolites. In this pathway the amino acid methionine is transformed into homocysteine via several metabolites. By this transformation of methionine into homocysteine, the transsulfuration pathway is driven. In the transsulfuration pathway the antioxidant, glutathione is formed. This is one of the main antioxidants in RBCs. The folate cycle is important for forming metabolites for the methionine cycle. In other cells than the RBC, the folate cycle also plays a role in DNA synthesis.

Blood disorders can be caused by mutations in the DNA, or by shortage of metabolites from the diet. Patients with these blood disorders can suffer from a shortage in RBCs, called anaemia. This means a lower capacity for oxygen transport. The anaemia is caused by dysregulation of the metabolism, also including the one-carbon metabolism. This dysregulation can lead to high oxidative stress levels, which can cause damage and early death to the RBC. This shows that the one-carbon metabolism is essential to form antioxidants against the oxidative stress in RBCs. Thus, supplementation with metabolites that can boost the formation of antioxidants, could help to increase the RBC-life span. In addition, controlling oxidative stress in RBCs is important during blood storage because stored RBCs also face high oxidative stress levels.

Future research could focus on unravelling the remaining parts of one-carbon metabolism, including the salvage pathway and the polyamine metabolism in RBCs. And it could improve the understanding of the RBCs metabolism and thereby the further understanding on blood disorder and optimizing blood storage. In conclusion, the one-carbon metabolism in RBCs plays important roles in managing oxidative stress levels to support the survival of the RBC.

ABSTRACT

The glycolytic pathway, the Rapoport-Luebering Shunt, and the Pentose Phosphate Pathway (PPP) are important metabolic pathways in erythrocytes. The glycolytic pathway is the main provider for energy metabolites and the PPP for the antioxidant metabolite, nicotinamide adenine dinucleotide phosphate (NADPH). Through these pathways the erythrocyte is provided with most of the energy and antioxidant metabolites needed for a 120-day survival. However, the erythrocytes metabolism goes beyond glycolysis and the PPP. Metabolomic studies showed that the one-carbon metabolism plays a role in erythrocytes. Here, a literature review is given about the one-carbon metabolism in mature erythrocytes to show the current understanding, to give insights into knowledge gaps, and to discuss future research implications. This overview shows that the transmethylation, transsulfuration, and the folate cycle play important roles in the mature erythrocyte. The main purposes of these three metabolic pathways are to methylate metabolites, and to generate antioxidants for balancing the redox level. Blood disorders often have a defect which involves or affects a part of the one-carbon metabolism, including haemoglobinopathies, enzyme deficiencies, and dietary metabolite deficiencies. These disorders point out the relevance of a solid antioxidant system through the methionine cycle and the folate cycle to maintain the redox balance and promote erythrocyte survival. In addition, regulation of the one-carbon metabolism in blood storage is of crucial importance for an optimal blood transfusion. Altogether, the provided overview of one-carbon metabolism in mature erythrocytes aims to aid in the further understanding of the erythrocytes one-carbon metabolism and its role in blood disorders, and blood storage and transfusion.

1 INTRODUCTION

The red blood cell (RBC), also known as the erythrocyte, is often considered a simple cell, due to the absence of a nucleus, mitochondria, and endoplasmic reticulum[1]. Erythrocytes are equipped with sufficient energy to survive for approximately 120 days, even without mitochondria[2]. The three primary metabolic pathways, the Embden-Meyerhof-Parnas glycolytic pathway (glycolysis), the Pentose Phosphate Pathway (PPP) and the Rapoport-Luebering Shunt, provide erythrocytes with most of the essential metabolites for survival and their primary function of transporting oxygen to tissues[2–4].

In glycolysis, vital energy metabolites are generated through the anaerobic catabolism of glucose. The pathway involves numerous enzymes responsible for converting glucose into lactate, such as kinases and dehydrogenases. The main purpose of glycolysis in erythrocytes is to generate intermediate metabolites, including the main energy metabolite Adenosine Tri Phosphate (ATP). In glycolysis, one glucose molecule yields a net gain of 2 ATP. Within this pathway, pyruvate kinase plays a pivotal role by generating ATP from phosphoenolpyruvate[5]. The primary function of ATP is to ensure the survival of erythrocytes[6].

The glycolytic intermediate 1,3-diphosphoglycerate branches out into the Rapoport-Luebering Shunt[5]. This shunt is specific to the erythrocyte and biphosphoglycerate mutase facilitates the conversion of 1,3-diphosphoglycerate into 2,3-diphosphoglycerate (2,3-DPG). It does so by bypassing phosphoglycerate kinase. 2,3-DPG plays a critical role in the erythrocyte as it regulates the oxygen affinity to haemoglobin (Hb). When 2,3-DPG binds to Hb, it blocks the oxygen binding site, thereby the affinity of Hb for oxygen decreases[7]. In addition, 2,3-DPG functions as an energy buffer. When 2,3-DPG levels are low, it is released from Hb and it can re-enter the Rapoport-Luebering Shunt, where it is converted into the glycolytic intermediate 3-phosphoglycerate by biphosphoglycerate phosphatase. 3-phosphoglycerate can then be metabolized into pyruvate, thereby yielding ATP. Thus, 2,3-DPG is crucial for optimizing oxygen transport via Hb and can generate ATP via glycolysis[5].

In the physiological situation, erythrocytes contain high levels of oxygen. Oxygen is highly reactive and can therefore induce oxidative stress through the oxidation of NADPH by NADPH oxidase and the auto-oxidation of Hb[8–10]. Autoxidation of Hb is crucial, as it causes oxygen to bind the heme molecule of Hb via Fe^{+2} (ferrous). Auto-oxidated Hb then donates an electron to ferrous resulting in a Fe^{+3} (ferric) heme molecule. Consequently, heme releases perhydroxy or superoxide radicals, which are reactive oxygen species (ROS). Superoxide can further react into H_2O_2, thereby contributing to oxidative stress[10]. Additionally, the ferric-heme molecule, also known as methemoglobin (metHb), has an altered electron configuration, and a conformational change of the heme molecule. Accordingly, oxygen cannot bind to this ferric heme anymore[11]. Moreover, erythrocytes take up ROS from the bloodstream, which are secreted by other tissues, especially during hypoxia[9,12]. These two sources of ROS can elevate oxidative stress levels in erythrocytes, and highlight the importance of antioxidants.

NADPH generated in the PPP is a crucial antioxidant molecule in erythrocytes. The PPP branches from glycolysis at glucose-6-phosphate and is the sole source of NADPH in erythrocytes. NADPH is important in maintaining antioxidant levels and thereby controlling ROS. Furthermore, NADPH functions as a cofactor of NADPH-methemoglobin (metHb) reductase for the reduction of ferric to ferrous heme[13]. Without reducing the oxidative stress in erythrocytes, the intracellular damage would accumulate eventually initiating lysis[14].

The metabolic pathways described above have been extensively studied in erythrocytes. In the past decade, several metabolomic studies have been conducted to unravel the erythrocyte's metabolome[15–18]. These studies showed that this metabolome is more complex than anticipated, including a possible role for one-carbon metabolism, amino-acid metabolism, polyamine metabolism, purine metabolism, and carnitine metabolism. While one-carbon metabolism is extensively studied in

hepatocytes, kidney cells and cancer cells[19–21], it remains underexplored in erythrocytes. Therefore, the role of one-carbon metabolism in erythrocytes is discussed in depth in this review.

One-carbon metabolism is a complex biochemical network, which is vital for cellular homeostasis by maintaining methylation with one-carbon units through many metabolic pathways. This biochemical network can be divided into three distinct cycles: the methionine cycle, the thymidylate cycle, and the purine cycle. In addition to these main cycles, one-carbon metabolism is also involved in heme synthesis and in the generation of antioxidants. Furthermore, other metabolic pathways can interact with metabolites of the one-carbon metabolism, making it a complicated metabolic network[22,23]. Moreover, the one-carbon metabolism can be impacted through blood disorders and blood storage. Blood disorders influence the one-carbon metabolism by high oxidative stress levels and often shortage in metabolites, leading to anaemia[24,25]. Blood storage impacts the one-carbon metabolism by high oxidative stress levels and low antioxidant capacity[26,27]. The oxidative stress in these situations inhibit pathways involved in the one-carbon metabolism[4,28]. Here I will focus on 1) the methionine cycle, including the transmethylation cycle and the transsulfuration pathway, 2) other cycles which are in close relation to the methionine cycle, involving the folate cycle, the salvage pathway and the polyamine metabolism, 3) blood disorders in which aspect(s) of the one-carbon metabolism are affected, 4) and the role of one-carbon metabolism in blood storage and transfusion.

2 ONE-CARBON METABOLISM IN ERYTHROCYTES

As previously mentioned, one-carbon metabolism can be divided into the thymidylate, purine, and methionine cycles. The fundamental principle of one-carbon metabolism involves the transfer of one-carbons, such as methyl groups, from a methyl donor to other molecules[29]. S-adenosylmethionine (SAM) is a crucial methyl-donor in one-carbon metabolism, which is generated in the methionine cycle from methionine[30]. The methionine cycle, one of the main cycles of one-carbon metabolism, can be further divided into the transmethylation pathway, the transsulfuration pathway and the salvage cycle[29]. Additionally, the folate cycle is interconnected to the methionine cycle and plays a significant role in generating intermediates of the pyrimidine and purine cycles[31]. The focus here will be on the transmethylation and transsulfuration pathway of the methionine cycle as well as the related folate cycle in mature erythrocytes (Figure 1).

2.1 METHIONINE CYCLE – TRANSMETHYLATION PATHWAY

The central pathway in methionine metabolism is the transmethylation pathway. Methionine is an essential amino acid, acquired from dietary intake[32], and plays a central role in this pathway. Within

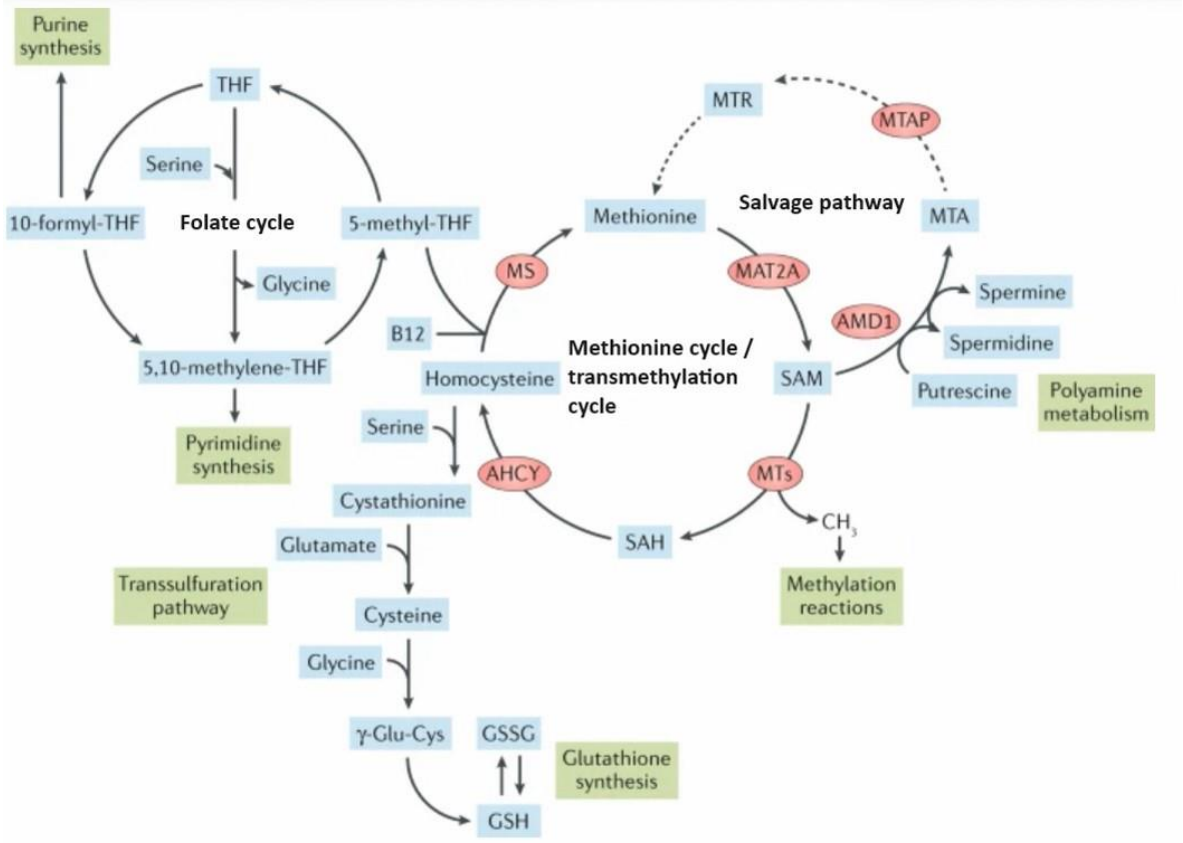

Figure 1. The methionine cycle in one-carbon metabolism. The methionine cycle consists of three parts. The central role is for the transmethylation pathway/methionine cycle. Methionine is converted into homocysteine by methionine s-adenosyltransferase (MAT), methyl transferases (MTs), and S-adenosylhomocysteine (SAH) hydrolase (AHCY). Homocysteine can be converted back to methionine by methionine synthase (MS) and cobalamin (vitamin B12). The transsulfuration pathway branches from homocysteine. Homocysteine is converted into cysteine by cystathionine-β-synthase (CBS) and its coenzyme vitamin-B6, and γ-cystathionase. Cysteine can then be converted into taurine (not depicted here) or glutathione. Glutathione is metabolized by γ-glutamylcysteine ligase and glutathione synthetase. The salvage cycle can regenerate methionine from SAM and also generates polyamines. In close collaboration with the methionine cycle is the folate cycle. The derivative from folate, tetrahydrofolate (THF), is metabolized into 5-methyl-folate by methylenetetrahydrofolate dehydrogenase (MTHFD) and MTHF reductase. 5-methyl-THF is used for the conversion of homocysteine into methionine. The purine and pyrimidine cycles branch from these metabolic cycles. Adapted from Sanderson et al.[132].

the transmethylation pathway, methionine is converted into homocysteine in the cell's cytosol through several steps. In an ATP-dependent methylation reaction, methionine s-adenosyltransferase (MAT) converts methionine into SAM. Subsequently, S-adenosylhomocysteine (SAH) is methylated by SAH hydrolase, resulting in the formation of reduced homocysteine (Figure 1)[33]. Recent findings by Ye et al. described that the transmethylation pathway also plays a role in mature erythrocytes. By studying the homocysteine levels in whole blood samples, the generation of homocysteine in erythrocytes was confirmed[34]. Furthermore, Ye et al. showed the importance of homocysteine in the mature erythrocyte by studying its role as an intracellular antioxidant. Homocysteine contains a free sulfhydryl bond[34], similar as the main antioxidant in erythrocytes, GSH. GSH can form an intramolecular disulfide bond to prevent excessive redox by ROS, like H_2O_2. Furthermore, GSH can be regenerated through reduction by NADPH (Figure 3) [35]. Because of the high levels of oxidative stress in erythrocytes, an antioxidant role for reduced homocysteine can be assumed. Thereby, reduced homocysteine can contribute to damage control of the cell's structure and membrane caused by oxidative stress[34].

Accordingly, erythrocytes treated with methionine generate reduced homocysteine to a higher extend, which has a reducing effect on Hb, causing the reduction of Fe^{4+} to Fe^{3+}. Consequently, methionine treatment reduced ROS via homocysteine in the transmethylation pathway. On another note, the conversion of homocysteine into methionine is thought to take place in remote organs. When oxidized homocysteine is not metabolized to its reduced form, the oxidized homocysteine will be secreted from the erythrocyte to the plasma and be taken up by hepatocytes and renal cells. In these cells, homocysteine can be metabolized back into methionine. Methionine can be secreted into the blood stream and be re-used for, among other things, one-carbon metabolism. Accordingly, it is thought that the conversion of homocysteine to methionine does not, or in a very low extend, occur in mature erythrocytes[34].

The role of SAM is not restricted to the methionine cycle, it also plays a role as methyl donor in reducing protein isoaspartyl damage of amino acid side chains. ROS, generated by high oxygen levels in erythrocytes, can damage especially the side chains of asparagine and aspartate. The side chain of asparagine can be easily affected by deamidation and aspartate by dehydration. These affected side chains are susceptible for degeneration into L-isoaspartyl residues, which influence the protein's structure and function. Moreover, L-isoaspartyl groups can lead to defective and pathologic erythrocytes. To repair this damage SAM can play a role as methyl donor for aspartate and asparagine used by L-isoaspartyl methyltransferase (PIMT). This results in converting the damaged amino acids in aspartate with normal aspartyl groups. Hence, erythrocytes depend on PIMT and SAM to maintain the intracellular redox balance[36].

2.2 TRANSSULFURATION PATHWAY

Another pathway branching from methionine is the transsulfuration pathway. In this pathway, homocysteine is converted to cysteine, the precursor of the antioxidants taurine and GSH[22]. First, homocysteine is condensated by cystathionine-β-synthase (CBS), serine and its coenzymes vitamin-B6 and heme to generate cystathionine[37,38]. Then, cystathionine is converted into cysteine by γ-cystathionase (CSE)[39]. Consequently, via two distinct pathways, cysteine can form GSH and taurine, to function as antioxidants[22].

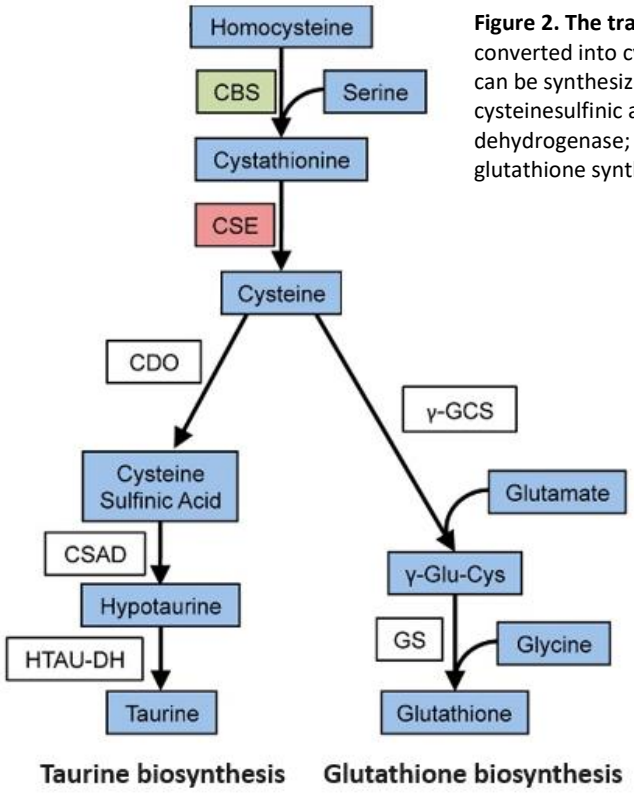

Figure 2. The transsulfuration pathway. Homocysteine is converted into cyteine. From cysteine, taurine or glutathione can be synthesized. CDO: cysteine dioxygenase; CSAD: cysteinesulfinic acid decarboxylase; HTAU-DH: hypotaurine dehydrogenase; *γ -GCS:* γ-glutamylcysteine ligase; GS: glutathione synthetase. Adapted from Sbodio et al. [133].

GSH is synthesized in two steps by γ-glutamylcysteine synthetase and glutathione synthetase, using glutamate and glycine as substrates (Figure 2)[40]. The availability of cysteine was shown to be rate limiting for GSH, moreover glycine is thought to have the same rate limiting properties as cysteine, making glycine an essential amino acid for GSH synthesis[41].

As mentioned before, GSH is important to keep the oxidant stress in balance as a redox buffer, especially to reduce ROS, like H_2O_2[42]. GSH is the reduced form which can be metabolized into the oxidized form (GGSG) by glutathione peroxidase. In turn GSSG can be reduced back into GSH by glutathione reductase and NADPH, which is generated in the PPP. Also resulting in $NADPH+H^+$ (figure 3). The GSH metabolism is crucial for erythrocytes since it reduces ROS and therefore prevents protein degradation and eventually apoptosis[42]. In addition, the oxidative stress drives the transsulfuration pathway, including the biosynthesis of GSH, through activation of CBS, γ-glutamylcysteine ligase and glutathione synthetase[43]. CBS could be regulated by oxidative stress

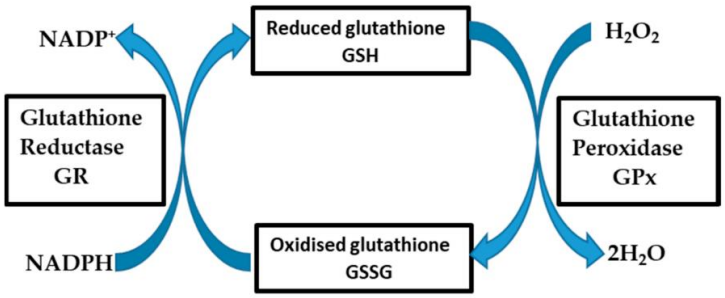

Figure 3. Glutathione (GSH) metabolism. GSH can be reduced by glutathione reductase and NAPDH and can be oxidized by glutathione peroxidase and H2O2 . This is a similar system as for homocysteine[134].

through its coenzyme heme. The redox state of heme determines its activity as cofactor. Ferrous heme showed decreased enzymatic activity of CBS compared to ferric heme[38]. As for γ-glutamylcysteine ligase and glutathione synthetase, enzyme activity increases with elevated oxidant levels[44]. Thus, increased oxidative levels, increase the activity of enzymes in the transsulfuration pathway to elevate the antioxidant levels and maintain the redox balance.

Especially during hypoxic conditions, erythrocytes take up ROS from peripheral cells, which causes a significant increase in total oxidative stress. During this process, erythrocytes especially rely on antioxidants like GSH, because of their reducing capacity. It was shown that erythrocyte GSH levels increased in reaction to hypoxia[45]. Fenk et al. showed that GSH can interact with Hb via one of its thiol groups in a non-covalent manner. In the oxygenated state, four GSH molecules are bound to the Hb-thiol groups faced inwards, increasing the Hb-affinity for oxygen. When Hb gets deoxygenated, Hb conformationally changes, resulting in the thiol group facing outwards and two GSH molecules are released. It was shown that 2,3-DPG and GSH share the same binding site in the central cavity of an Hb subunit. Without GSH bound to the thiol group it can participate in rebalancing the erythrocyte redox state, and 2,3-DPG binds the now available outwardly facing thiol group. Thus, erythrocytic GSH accumulates in the cytosol upon hypoxia because of the deoxygenation of Hb[45].

Taurine, the other derivative from cysteine, is synthesized in three steps. First, cysteine is converted to cysteinesulfinic acid by cysteine dioxygenase. Second, cysteinesulfinic acid is decarboxylated to hypotaurine by cysteinesulfinic acid decarboxylase. Last, hypotaurine is oxidized into taurine by hypotaurine dehydrogenase (figure 2)[46–48]. Taurine plays a role in the antioxidant system in erythrocytes. Bertolone et al. showed that taurine in erythrocytes reduces the oxidative stress from H_2O_2, negatively correlates with haemolysis after oxidant stress, and increases GSH levels. Additionally, taurine boosts the energy and redox metabolism of erythrocytes through preventing ATP breakdown and deamination, stimulating the PPP, and increasing GSH levels[49]. However, it is unclear if the enzymes involved in the biosynthesis of taurine in the transsulfuration pathway are expressed in erythrocytes.

Homocysteine metabolization can also go through choline and betaine in cells containing mitochondria, like erythroid progenitor cells[50]. Choline can be taken up in the erythrocyte via an Na^+ dependent transport system [51]. In the mitochondrion, intracellular choline can be converted to betaine by choline dehydrogenase and betaine aldehyde dehydrogenase[50]. Consequently, betaine is transported to the cytosol where it can replace the function of methylenetetrahydrofolate (MTHF) by providing a methyl group which is transferred by betaine homocysteine methyltransferase to form dimethyl glycine and homocysteine[52]. Dimethylglycine would normally be converted to glycine by dimethylglycine dehydrogenase and sarcosine dehydrogenase, but these enzymes are expressed in mitochondria[53]. Therefore, the conversion of dimethylglycine to glycine is unlikely to occur in the mature erythrocyte.

2.3 Salvage pathway

Another pathway in one-carbon metabolism related to methionine, is the salvage pathway including polyamine synthesis. In this cycle, methionine is regenerated from SAM in several enzymatic steps. The salvage of methionine in erythrocytes has not been studied and might not be necessary, because erythrocytes are permeable to methionine, thus methionine levels can be easily replenished from the plasma[54]. One of the key enzymes in the salvage pathway is SAM decarboxylase (AMD1). AMD1 has a short half-life and would therefore already be degraded in mature erythrocytes (Figure 1)[55]. Thus, the methionine salvage pathway is unlikely to be present in mature erythrocytes[55].

The polyamine metabolism is linked to the methionine salvage pathway (Figure 1). Polyamines play a role in cell proliferation through maintaining DNA structure, regulating gene expression[56,57]. In addition, they are involved in cell signalling, immune function, and neutralizing free radicals[58–60]. Erythrocytes can take up the polyamines: putrescine, spermidine, and spermine, with a more rapid uptake of spermidine and putrescine. No evidence of polyamine interconversion was found[61]. Polyamines in erythrocytes have been described[62], but the exact polyamine cycle in erythrocytes, if present, is still unclear. Therefore, when polyamines are detected in erythrocytes, it is likely due to the uptake of polyamines rather than the synthesis through polyamine metabolism.

2.4 FOLATE CYCLE

A pathway closely related to the transmethylation pathway of the methionine cycle is the folate cycle. Folate drives the methionine cycle through tetrahydrofolate (THF). Dietary folate is taken up by the erythrocyte from the plasma where it is converted to dihydrofolate followed by tetrahydrofolate and both are catalysed by dihydrofolate reductase[63,64]. The folate cycle exists in the mitochondria, the nucleus, and the cytosol. The cytosolic folate cycle depends on one-carbon units generated in the mitochondrial folate cycle[65,66]. In the mitochondrial folate cycle, serine is catabolized into formate through several metabolic steps, with these steps one-carbon units, like glycine, are generated, transported to the cytosol, and used in other metabolic processes[67].

The three cytosolic methylenetetrahydrofolate dehydrogenase (MTHFD) isoenzymes convert THF into 5,10-methyleneTHF (5,10-MTHF) in three reactions[68]. First, 5,10-MTHF is converted into 5-MTHF by MTHF reductase and its coenzyme flavin adenine dinucleotide (FAD) and the electron donor NADPH [69]. 5-MTHF can then be used for the conversion of homocysteine into methionine by methionine synthase with its cofactor vitamin B12 (cobalamin) (figure 1)[70]. Cobalamin has a pivotal role in this step, as it catalyses the reaction. Cobalamin is not synthesized in humans and can only be acquired from dietary intake[71]. 5,10-MTHF can also be converted back to THF, which gives glycine as a byproduct (Figure 1). Additionally, 5,10-MTHF is an important player in the thymidylate cycle [72]. 5,10-MTHF provides one-carbon units for deoxyuridine monophosphate (dUMP) methylation to form deoxythymidine monophosphate (dTMP)[73], and 10-formyl-THF provides one-carbon units for purine synthesis[74]. Both dTMP and purines are essential for DNA biosynthesis[75,76]. For erythropoietic cells, the folate cycle is especially crucial, because of the high demand for DNA synthesis and proliferation. Hence, purine and thymidylate cycles connected to the folate cycle can provide these cells with sufficient DNA building blocks[25,77,78].

It has been shown that erythrocytes store folate throughout their life span[79] and that 5-MTHF is present in mature erythrocytes[80]. All folate cycle enzymes are expressed in erythroid progenitor cells and might therefore still be present in mature erythrocytes. It is important to note that the cytoplasmic folate cycle in cells containing mitochondria uses a derivative of folate, formate, from the mitochondria[72], making the cytosolic folate cycle dependent on the mitochondrial folate cycle. However, recently formate was found in mature erythrocytes, which showed to be permeable for formate. Hence, the cytoplasmic folate cycle in erythrocytes might, to some extend, occur via formate, taken up from the plasma[81]. Because of a major role for the folate cycle in DNA synthesis, mainly important for organelles containing DNA, it is still unclear to what extend the cytosolic folate cycle is active in mature erythrocytes.

In the conversion from THF to 5,10-MTHF, an important one-carbon metabolism metabolite, glycine, is generated[2]. Glycine is a crucial amino acid for erythrocytes, because glycine is necessary for metabolizing GSH, a prominent antioxidant and it is important during erythropoiesis. During erythropoiesis, glycine is used for heme synthesis, heme is the key molecule for oxygen transport throughout the body. One-carbon metabolism is involved in heme synthesis by providing one-carbon units and methyl groups in four of the eight metabolic steps (steps 3-6) [82]. First, glycine and succinyl-CoA are condensated into d-aminolevulinic acid (ALA) by ALA synthase. Second, d-aminolevulinic acid dehydratase catalyses the condensation of two ALAs into porphobilinogen (PBG). In the third step, four molecules of PBG are condensated and deaminated by PBG deaminase, to form tetrapyrrole hydroxymethylbilane (HMB). During the fourth step, HMB is converted into uroporphyrinogen III (URO-III) by uroporphyrinogen III synthase. In the fifth step, coproporphyrinogen is synthesized by URO dehydrogenase. In the sixth conversion, coproporphyrinogen is oxidized by coproporphyrinogen oxidase to form protoporphyrinogen IX (PP). In the seventh step, PP is oxidized by PP oxidase. In the final step of heme biosynthesis, iron is incorporated in PP by ferrochelatase to form heme[82].

The synthesis of glycine is regulated by cytosolic serine hydroxymethyltransferase (SHMT1) together with its coenzyme vitamin B-6. SHMT1 converts serine into glycine where a carbon unit is donated to the folate cycle[83]. In addition, the erythrocyte can acquire glycine from the plasma via several transport routes[84].

As mentioned before, the folate cycle metabolite, 5,10-MTHF is a starting point for purine biosynthesis. 5,10-MTHF contributes as methyl donor in the *de novo* purine cycle to generate purine bases for DNA. Moreover, 5,10-MTHF can be used in thymidylate synthesis[31]. However, erythrocytes lack a nucleus and mitochondria, thus they do not contain DNA. Thus, the *de novo* purine and thymidylate synthesis is not probable to occur in mature erythrocytes. On the other hand, purine salvage pathways have been identified in mature erythrocytes to generate ATP from adenosine and adenine[85], but this would be subject to an entire paper and will therefore not be discussed in detail here.

3 ERYTHROCYTE DISORDERS LINKED TO ONE-CARBON METABOLISM

Blood disorders can be inherited or caused by dietary insufficiency. Inherited blood disorders can be divided into enzyme deficiencies[86], haemoglobinopathies[87], and membrane defects[88]. Blood disorders from dietary insufficiency are generally caused by rapid breakdown of cells or insufficient intake through the diet. The most common erythrocytic dietary insufficiencies are iron, cobalamin, and folate deficiency[24]. Blood disorders are often characterized by anaemia, where the oxygen transport to the tissues is insufficient[25].

3.1 HAEMOGLOBINOPATHIES

Sickle cell disease (SCD) is an autosomal recessive blood disorder and is the most common disorder of the haemoglobinopathies. SCD is caused by a single point mutation in the β-globin gene, resulting in a conformational change of the sickle β-chains (HbS)[89]. This conformational change results in dysfunctional polymerization upon deoxygenation of Hb. The polymerized Hb causes the erythrocytes to sickle, leading to vaso-occlusion[90]. The polymerization also makes the erythrocyte more fragile and more susceptible for erythrophagocytosis, leading to haemolytic anaemia[90]. A recent metabolomics study in blood from patients with SCD showed a distinct metabolic profile compared to blood from healthy controls. Decreased levels of GSSG and GSH, and increased levels of GSH precursors were observed in SCD patients. This is due to high oxidative stress levels, causing a rapid GSH turnover. The high turnover in combination with insufficient cysteine levels for the increased GSH biosynthesis, makes the erythrocyte unable to meet the required GSH levels[91]. Therefore, increasing the cysteine levels in erythrocytes might reduce the oxidative stress in SCD. Nur et al., described a treatment option for SCD via cysteine supplementation with N-acetylcysteine. This treatment showed increased GSH levels and decreased oxidative stress in SCD, which might result in inhibition of haemolytic anaemia[92]. Low NADPH levels would also cause a decrease in metHb reducing capacity, resulting in a decreased Hb affinity for oxygen[13].

Other aspects of one-carbon metabolism affected in SCD are the folate and methionine cycle. Because of the high rate of haemolysis in SCD, there is a high demand for proliferation and the involved metabolic pathways and metabolites. One of these metabolic pathways, is the folate cycle and its crucial metabolite folate. The rapid rate of proliferation causes a folate depletion in SCD subjects. Accordingly, folic acid supplementation can help to replenish the folate stores to promote erythropoiesis[93]. Related to folate deficiency are elevated levels of homocysteine in SCD[94]. As described above, homocysteine is metabolised in the methionine cycle[33]. For the conversion of homocysteine into methionine, the folate cycle plays an important role as it generates 5-MTHF, which can work as a cofactor in this metabolic step[70]. In SCD, this step is inhibited, because of the folate depletion, thereby accumulating homocysteine. Increased folic acid supplementation might overcome the elevated homocysteine levels[94]. Altogether, one-carbon metabolism is affected in SCD patients, due to the high haemolysis rate, leading to increased demand for metabolic processes involved in cell proliferation.

3.2 Β-THALASSEMIA

β-thalassemia is a heterogeneous disease caused by downregulating mutations in the β-globin gene of Hb. This downregulation leads to absent or deficient β-globin synthesis and an excess of α-globins, which can form aggregates[95]. These aggregates stimulate ROS-formation, causing damage to the cell membrane[96]. This damage is also initiated by oxidized Hb subunits, hemichromes. After the disintegrating of heme, toxic iron-species are released promoting ROS-formation[95]. Together, ROS and hemichromes disrupt erythropoiesis and decrease the erythrocytes life span, thereby anaemia is induced[97,98]. In addition, β-thalassemia subjects can present with cardiac dysfunction due to iron overload[99]. Because of the heterogeneity in β-thalassemia, the one-carbon metabolism can also be affected in different ways. High oxidative stress and rapid erythrocyte turnover can cause reduced

folate, homocysteine, and methionine in β-thalassemia subjects due to an increased rate of the transsulfuration pathway[100]. Taken together, because of the β-globin downregulation, the erythrocyte contains excessive ROS levels and cell damage, leading to an increased metabolic rate of the transsulfuration pathway.

3.3 Enzyme deficiencies

Pyruvate kinase deficiency (PKD) is the most common enzyme deficiency in blood disorders. In PKD the final step of glycolysis, and thereby the production of ATP is inhibited. As erythrocytes highly rely on the glycolytic pathway for generating energy rich compounds, this deficiency severely impacts the life span of erythrocytes, causing anaemia. Next to decreased glycolysis, a metabolomic study showed increased hypoxic and oxidant stress metabolites, and accumulated PPP metabolites. As for the one-carbon metabolism, methionine, taurine and several polyamines were increased in PKD. Methionine increased in reaction to the high oxidative stress, the high methionine is used for isoaspartyl damage repair via SAM. The high levels of methionine, taurine and polyamines can aid in oxidative stress reduction[101].

Another disorder compromising the antioxidant system in erythrocytes is glutamate cysteine ligase deficiency[102]. Glutamate cysteine ligase catalyses the second step in the GSH synthesis from homocysteine. Especially mutations in the catalytical subunit of the enzyme inhibit the conversion from cysteine to gamma-glutamylcysteine and prevent GSH synthesis[103]. Subjects with this deficiency present with low GSH levels and high oxidative stress resulting in haemolytic anaemia. All four different identified mutations presented with low GSH levels[102].

Two similar disorders are methionine synthase deficiency and methylenetetrahydrofolate reductase (MTHFR) deficiency. In methionine synthase deficiency the *MTR* gene is mutated[104], and in MTHFR deficiency the *MTHFR* gene is mutated[105]. Methionine synthase and MTHFR are key enzymes in one-carbon metabolism, important for the conversion of homocysteine to methionine (Figure 1) and therefore in preventing the accumulation of homocysteine[104,105]. The enzyme MTHFR catalyses the reaction from 5,10-MTHF to 5-MTHF in the folate cycle. This is a crucial step for the folate cycle to also be able to provide 5-MTHF for the reaction by methionine synthase[105]. Because methionine synthase depends on the metabolic reactions by MTHFR[105], both mutations result in elevated homocysteine levels, in the blood, which can increase the risk for cardiovascular disease, and neurological defects[104]. In addition, both mutations impair the methionine cycle and thus insufficient levels of methionine and of methyl donors, like SAM, are metabolised. Furthermore, folate dependent reactions are inhibited by the *MTR* mutation, because methionine synthase is the only enzyme using 5-MTHF as a substrate. Therefore, 5-MTHF is not further converted into other metabolites, causing a folate trap in the form of 5-MTHF[106]. Patients with the *MTR and MTHFR* mutation are characterized with neurological problems and anaemia[107]. To replenish methionine levels and to reduce homocysteine levels, patients can be treated with folinic acid, betaine, and hydroxocobalamin[108]. The effects on the erythrocyte's life span show the importance of a functional folate and methionine cycle in mature erythrocytes.

3.4 Dietary insufficiencies

Folate and cobalamin are crucial compounds during erythropoiesis, because of their key roles in proliferation through DNA synthesis and repair (figure 1). In most cases, both a cobalamin and a folate deficiency are caused by a dietary insufficiency[109]. Subjects with insufficient cobalamin or folate can present with anaemia due to hampered proliferation and therefore a shortage in erythrocytes[25]. A folate or cobalamin deficiency significantly impacts erythropoiesis, but would it also impact mature erythrocytes?

Whereas erythroid precursor cells are dependent on folate and cobalamin for proliferation, mature erythrocytes are mainly dependent on the folate and methionine cycle, not because of DNA and protein synthesis, but merely because of generating important antioxidants through these

cycles[34,43]. A deficiency of folate, or cobalamin hampers the conversion of homocysteine into methionine[22], thereby affecting the transmethylation pathway of the methionine cycle and the transsulfuration pathway[22]. Subsequently, antioxidant-levels generated in these pathways, like GSH, will decrease, resulting in outbalanced levels of antioxidants and oxidant stress[42]. As mentioned before, this will damage the erythrocyte and initiate apoptosis[14].

For the synthesis of heme, iron is an essential compound. Patients with iron deficiency have a low iron level in their erythrocytes, causing impaired erythropoiesis and iron deficiency anaemia. Iron deficiency can be caused by insufficient dietary intake, blood loss, or insufficient iron absorption[110]. Iron deficiency is not directly linked to the one-carbon metabolism, but iron deficiency is related to excessive oxidative stress levels in the erythrocyte, causing heme degradation and a decreased erythrocyte life span[111]. As seen in the previously described deficiencies leading to oxidative stress, the metabolites, involving pathways important for erythropoiesis are easily deprived, leading to impairments in the methionine cycle, folate cycle and the transsulfuration pathway.

On another note, an inherited disorder, not typically known for affecting erythrocytes, has been described to affect the erythrocytes metabolism. Trisomy 21 (T21) / down syndrome subjects have an extra copy of chromosome 21. This extra chromosome results in many complications, also comprising erythrocytes. Culp-Hill et al. showed age related changes in erythrocyte metabolism in T21 subjects[112]. High antioxidant activity and low methionine levels were determined in these subjects. The low methionine levels observed in erythrocytes relate to a disturbed homocysteine metabolism, which could also affect the transsulfuration pathway and GSH synthesis[113]. The low methionine levels are due to overexpression of cystathionine β synthase in T21 subjects. This overexpression impairs the folate cycle and inhibits resynthesis of methionine[114], and causes elevated levels of homocysteine. These erythrocyte impairments are also shown in the short RBC survival, due to the outbalanced oxidative stress, causing early apoptosis[114]. Altogether, the one-carbon metabolism is distorted in many blood disorders, which highlight the importance of the methionine and folate cycle in mature erythrocytes to fight oxidative stress and to support erythrocyte survival.

4 THE ROLE OF ONE-CARBON METABOLISM IN BLOOD STORAGE AND TRANSFUSION

During blood storage, erythrocytes face several challenges for survival. In the first weeks of storage, 2,3-DPG is excessively consumed due to a decreased glycolytic rate[115], decreased pH in the closed bag system, and oxidation of the rate-limiting enzyme glyceraldehyde-3-phosphate dehydrogenase[4]. As 2,3-DPG decreases, the oxygen saturation increases due to the increased affinity of Hb for oxygen[116]. Simultaneously, ROS levels accumulate[26], which reduces the antioxidant capacity[27,117,118]. Together, this results in protein damage[119,120], and inhibited metabolic pathways, including the transsulfuration, and the PPP[28,118]. Eventually, this will result in haemolysis[121]. Moreover, erythrocytes stored longer than 35 days show increased haemolysis risk with transfusion[122], thus a significantly decreased life span for stored erythrocytes compared to erythrocytes in the bloodstream under healthy conditions. Accordingly, studies are proposing solutions to increase blood storage and transfusion quality.

Especially metabolomic studies gave new insights into improved storage and transfusion. A study on the effect of hypoxic conditions showed that this can improve erythrocyte posttransfusion recovery. By using hypoxic conditions, the quality of erythrocytes improved, and oxidative stress decreased. As for the one-carbon metabolism, due to the low oxygen levels and oxidative stress, levels of GSH turnover and oxidation, and methionine oxidation in hypoxic conditions decreased[123]. A study on metabolic reprogramming showed decreased erythrocyte storage lesion in hypoxic conditions with sufficient levels of 2,3-DPG, ATP and GSH[4]. The high levels of energy rich compounds and antioxidants shows improvement of the redox balance in erythrocytes under hypoxic conditions. Another elaborate metabolomics study showed possible valuable metabolic additives to improve blood storage. Considering the one-carbon metabolism, methionine supplementation, increased SAM levels, and PIMT activity. Hence, methionine supplementation can enhance the repair of oxidatively damaged erythrocytes, scavenge ROS, and increase the generation of methyl donors[124].

Red blood cell exchange for SCD subjects was studied and showed rejuvenated erythrocytes after treatment with phosphate, inosine, pyruvate, and adenine[125]. These four metabolites boost the energy metabolism and replenish antioxidant levels through purine metabolism, the glycolytic pathway, and the PPP [126]. Through increased PPP metabolism, the GSH synthesis also increased. Furthermore, these additives decreased levels of harmful post-transfusion plasticizers[125]. Taken together, unravelling the metabolic processes in erythrocytes can aid in the improvement of blood storage and transfusion.

5 DISCUSSION

Erythrocytes were thought to have a simple metabolism, only needing the glycolytic pathway, the Rapoport-Luebering Shunt, and the PPP for sufficient energy supply and fighting oxidative stress. In this review, a small part of the uncharted territory of the erythrocyte's metabolism was discussed. The one-carbon metabolism showed to have a main role in generating antioxidants through several metabolic pathways, which are crucial in erythrocyte survival. This showed pivotal roles for the methionine cycle, including the transmethylation and the transsulfuration pathways, and the folate cycle. In addition, the discussed blood disorders showed that the erythrocyte's metabolism is easily disrupted and that metabolites from the one-carbon metabolism are crucial in the erythropoiesis process.

First, the relevance of the transmethylation pathway in erythrocytes was discussed. In this pathway important methyl donors are generated, like SAM. SAM donates a methyl group to amino acids, damaged by ROS, to repair the isoaspartyl damage[36]. Furthermore, homocysteine is generated from methionine in the transmethylation pathway. Ye et al., showed that homocysteine possibly has an important antioxidant role, because of its sulfhydryl bond. Methionine treatment showed to have antioxidant effects through reduced homocysteine, which prevents protein damage, membrane deformability and Hb-autoxidation. Therefore, methionine might have potential as a treatment option for metabolic disorders in which the oxidative stress in erythrocytes is elevated[34]. An example of a metabolic disorder with elevated oxidative stress in erythrocytes, is glucose-6-phosphate dehydrogenase deficiency. Here, glucose-6-phosphate dehydrogenase is deficient, causing a less efficient metabolic conversion in the first step in the PPP. This deficiency is heterogenous and can have effects in varying degrees, depending on the specific mutation. Overall, this deficiency can lead to shortage of NADPH in conditions of high oxidative stress. As a result, levels of GSH are low, making it more challenging to reduce the oxidative stress. Subjects with this deficiency can suddenly show a high rate of haemolysis[127]. By treating with methionine, the generation of reduced homocysteine could be increased, which might work as an antioxidant replacement for GSH. But, further research is needed into the exact antioxidant role of reduced homocysteine and its treatment potential.

Similar as the transmethylation cycle, the transsulfuration pathway plays a pivotal role in the generation of antioxidants in erythrocytes, through GSH. As mentioned in section 2.2, the taurine biosynthesis in erythrocytes has not been described. Roy et al. described an increase of taurine in erythrocytes, but it was not specified if the increase of taurine came from mature erythrocytes or erythroid progenitors and if it was increased by the transsulfuration pathway or through uptake from the plasma[101]. Moreover, not all enzymes involved in taurine biosynthesis were identified in erythroid progenitor cells. Therefore, the taurine biosynthesis pathway is unlikely to occur in mature erythrocytes. Nonetheless, taurine from dietary intake could still have effects on the erythrocyte's metabolism through reduction of H_2O_2[49]. As cysteine is an important amino acid in the transsulfuration pathway, cysteine supplementation might boost the biosynthesis of antioxidants. Increasing this biosynthesis can elevate antioxidant levels and help to maintain the redox balance in blood disorders where antioxidant levels are exhausted, for example in SCD[92] and glucose-6-phosphate dehydrogenase deficiency[127].

Two pathways closely related to the methionine metabolism are the salvage pathway and the polyamine metabolism. These pathways are unlikely to occur in mature erythrocytes (section 2.3). However, a study on the effect of radiation on erythrocyte metabolism showed decreases in polyamine levels. Metabolomics measurements were performed on erythrocyte samples, thus including mature erythrocytes. However it is unclear if the polyamine levels in the erythrocytes were synthesized in the erythrocyte or taken up by the erythrocyte from the plasma[62]. Future polyamine fluxomic studies, or polyamine enzymatic studies could point out a possible activity of polyamine metabolism in mature erythrocytes[128]. Labelled methionine could be used to measure the metabolic fluxes of the methionine salvage pathway and the polyamine metabolism. By culturing the erythrocytes in medium containing ^{13}C methionine, the erythrocytes will eventually only contain the

labelled methionine. The kinetics of the isotopically labelled methionine can be measured by liquid chromatography – mass spectrometry[129]. Fluxomics with glucose isotope tracing has previously been used in erythrocytes[124]. Furthermore, polyamine enzymatic activities can be tested by measuring the release of labelled CO_2 by ornithine decarboxylase, or SAM decarboxylase[130]. Information on the synthesis of polyamines in erythrocytes could help in the further understanding of how the erythrocyte manages oxidative stress.

By driving the methionine cycle, the folate cycle plays an important role in one-carbon metabolism. The efficiency of the folate cycle is mainly dependent on the dietary uptake of folate and cobalamin[71], especially during erythropoiesis[25]. As the mature erythrocyte only contains the cytosolic folate cycle, it is dependent on uptake of formate from the plasma[81]. As the folate cycle is only assumed to be similar as in cells with a nucleus and mitochondria, more evidence is needed to show the exact metabolic pathway. To determine the folate metabolism in mature erythrocytes, fluxomics, and enzyme activity measurements with labelled folic acid could provide insight into the exact metabolic reactions (similar method as described for the polyamine metabolism).

Future research could focus on further improving the blood storage and transfusion strategies, with an emphasis on elevating antioxidant levels through metabolites from the transsulfuration pathway. Besides, delving into polyamine metabolism and the salvage pathway may offer valuable insights into optimizing blood storage conditions, particularly focussing on reducing oxidative stress. Comprehensive research into personalized blood storage conditions may provide the most suitable blood storage and transfusion conditions for general transfusion properties and for subjects with blood disorders who have specific metabolic needs. For instance, for thalassemia patients, additives to boost the folate cycle, and the methionine cycle could improve storage and transfusion conditions[100]. Altogether, future research into the metabolism of erythrocytes can help in improving the use of additives in blood storage and transfusion. These metabolic improvements aim to improve erythrocyte quality and survival and eventually enhance transfusion outcomes.

The main limitation of this literature review is that not all aspects of the one-carbon metabolism could be discussed. Here, the focus was on the transmethylation, transsulfuration and the folate cycle. Therefore, maybe with more time and an elaborate literature search, clues for the polyamine metabolism in erythrocytes could be found. In addition, the one-carbon metabolism is also linked to the purine metabolism. The purine metabolism in erythrocytes plays a role in salvaging purine bases, which can be used for the synthesis of high energy compounds, like ATP and GTP, which are also in turn catabolized back into purine bases[131]. Accordingly, reviewing the purine metabolism in erythrocytes might give even more insights into the erythrocyte's energy metabolism and its effects on storage, transfusion and diseases related to the purine metabolism.

In addition, not all studies reviewed here were based on human research. The study by Padmanabhan et al. showed the importance of the folate metabolism in erythropoiesis, but their knock out experiments were tested on mice and therefore not fully representable for the human folate metabolism in human erythrocytes[78].

In conclusion, the one-carbon metabolism showed to have pivotal roles in maintaining the redox balance and promoting the survival of erythrocytes. By shedding light on the transmethylation pathway, transsulfuration pathway, and the folate cycle in mature erythrocytes, the importance of maintaining a redox balance for erythrocyte survival became clear. This also highlighted that further research into the erythrocyte's metabolism can help in the understanding of blood disorders and improve blood storage and transfusion quality.

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