Chapter

Erythrocytes as Messengers for Information and Energy Exchange between Cells

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Abstract

Evolution has created a hierarchy of systems for information and energy using different cells according to messages generated from DNA, RNA, and other sources. Erythrocytes are formed in high speed at about 2×10^6/s to balance dying or not working erythrocytes to maintain optimal energy and information transfer. Important information is handled by nucleotides and distribution of metal ions and phosphates when starting synthesis process. Handling of these processes needs kinases known to be magnesium-dependent. Oxygen delivered by erythrocytes is used by other cells to synthesize ATP and to increase reaction capacity. Complex signals to bone marrow balance erythroblasts before developing into reticulocytes and erythrocytes. We discuss some aspects of erythrocyte communication with other cells of the body with special focus on magnesium and selenium in this process.

Keywords: erythrocyte, magnesium, selenium, reactive oxygen species, glutathione, cholesterol, microRNA, kinase, hierarchy

1. Introduction

Erythrocytes provide oxygen and other necessary compounds to cells of the vascular compartment and other cells of the body. Erythrocytes also provide protection by collaborating in many ways with immune cells and humoral immunity [1, 2]. Erythrocytes communicate with other parts of the vascular compartment and to some extent also other parts of the body (**Figure 1**). Erythrocytes communicate as a transporter or by using signaling components perceived by receptors in other cells. We give some examples of how erythrocytes use magnesium and selenium in a hierarchy for optimal results as part of this communication. Magnesium occurs together with ATP, the energy currency of erythrocytes, and other cells. This makes magnesium necessary for many enzymes like the kinases of glycolysis. Further examples will be taken from lipids, glutathione, sphingosine-1-phosphate, purinergic signaling, RNA, the malaria parasite Plasmodium, and erythrocyte microvesicles.

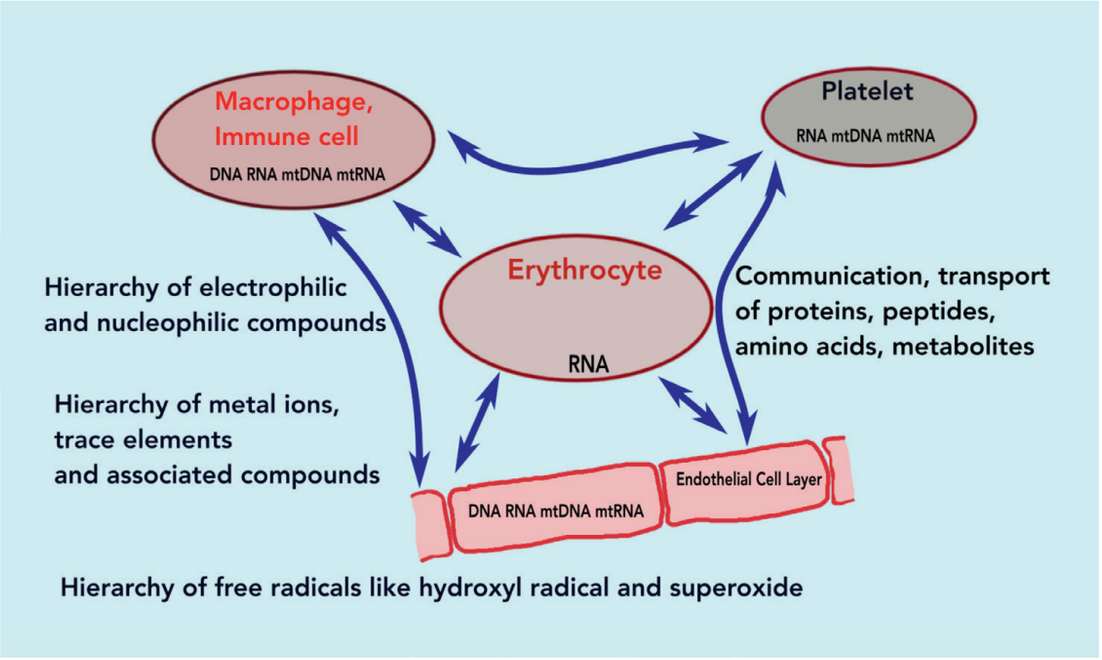

Figure 1.
Communication between erythrocytes and other cells takes place through RNA, proteins, metal ions, and other compounds that each form hierarchies. The first hierarchy involves free radical-induced production of compounds where hydroxyl radical and solvated electron reactions are involved. The second hierarchy involves electrophilic and nucleophilic compounds forming products adequate to the cells. The third hierarchy involves metal ions and ligands dependent on the previous two hierarchies but adapted to cell demand. In erythroid precursors and in other cells, excluding the erythrocyte, compounds and metal ions may reach DNA and RNA not controlled by evolutionary developed genome adapted to cell demand. Small changes of DNA and RNA, including epigenetic changes, may take place. These may become established or restored, but large damages will present symptoms and are difficult to restore.

2. Energy needs of the erythrocyte

Energy needs of the erythrocyte are fulfilled by ATP generated from glycolysis, which comprises several magnesium-dependent enzymes [3]. Magnesium is also necessary as enzyme cofactor or as part of enzyme substrate or product for more than 600 enzymes in the body, although not all these occur in the erythrocyte.

Among the enzymes of glycolysis, or the Embden-Meyerhof pathway, hexokinase, phosphofructokinase, phosphoglycerate kinase, and pyruvate kinase need magnesium as part of the ADP/ATP substrate or product. Aldolase and enolase need magnesium as a fundamental enzyme cofactor for stability and activity. The remaining glycolysis enzymes, glucose-phosphate isomerase, triosephosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, monophosphoglycerate kinase, and lactate dehydrogenase are not magnesium-dependent. Hexokinase, the first enzyme of glycolysis, increases reaction rate by at least 10 orders of magnitude compared to the uncatalyzed reaction, as an example of the very high catalytic demands on glycolytic enzymes [4]. Along with glycolysis, there is also the Rapoport-Luebering shunt producing 2,3-diphosphoglycerate, which is necessary for hemoglobin regulation. Magnesium level within erythrocytes is around 0.3 mM, which is three times higher than in plasma. The necessary levels of magnesium for glycolytic enzyme function are about an order of magnitude less than intra-erythrocyte magnesium concentration, suggesting that magnesium, while necessary for glycolysis, would not have a direct regulatory function in glycolysis [5]. Extracellular glucose can induce

magnesium efflux in erythrocytes [6]. Erythrocytes of type II diabetes patients also have lower magnesium levels as compared to healthy individuals. Glycolysis is sometimes described as two competing mechanisms, the pentose-phosphate pathway and the Embden-Meyerhof pathway. The pentose-phosphate pathway, also called the hexose monophosphate shunt, is used by erythrocytes to generate reducing power in the form of NADPH and is not known to require magnesium. Glycolysis is regulated by positive or negative feed-back or feed-forward loops [7] and by the availability of oxygen. The pentose-phosphate shunt is favored when oxygen is abundant, whereas the Embden-Meyerhof pathway is favored in oxygen-limiting conditions. Erythrocyte glycolysis is also regulated by sphingosine-1-phosphate (S1P), at least in high altitudes [8], and by a circadian rhythm coupled to redox regulations [9].

Erythrocytes also contain insulin receptors at a copy number of about 1000–2000 per erythrocyte, depending on age and health status [10]. Erythrocyte insulin receptors respond to insulin and may regulate glycolysis, probably through phosphorylation of phosphofructokinase and intracellular redistribution of the enzyme [11]. This could be through a magnesium-dependent process, since insulin induces magnesium efflux from erythrocytes [12]. Signaling from the erythrocyte insulin receptor seems to be through the phospho-inositide pathway, since magnesium efflux was inhibited by wortmannin, a known inhibitor of phosphinositide-3-kinase [12]. Insulin receptor signaling also involves magnesium-dependent autophosphorylation of the tyrosine kinase part of the receptor. Erythrocytes are not dependent on insulin for glucose uptake, since they import glucose through the non-insulin-dependent glucose transporter GLUT-1. Insulin in synergism with the insulin C-peptide has been shown to inhibit the release of ATP from erythrocytes that occur as a result of low oxygen levels [13, 14]. This effect could be reversed by a phosphodiesterase 3 inhibitor [15]. Insulin and insulin receptors are also known to regulate the potassium balance of cells. This regulation is thought to mainly be at the expression of the genes encoding the voltage-gated potassium channels [16]. If so, then insulin and the insulin receptor are mainly at work in erythroid precursors for regulating potassium channels, rather than in the mature erythrocyte. High glucose concentration in plasma, also called hyperglycemia, as can be observed in diabetes, can have many effects on erythrocytes [17]. For instance, glucose can be metabolized in the erythrocyte by aldose reductase leading to sorbitol or fructose production through the polyol pathway, which can lead to complications like diabetic neuropathy [18]. Hyperglycemia also leads to glycation of hemoglobin. Glycated hemoglobin has an increased affinity for oxygen and could be expected to be less prone to release its oxygen in a normal way. However, the total oxygen delivery capacity of blood containing glycated hemoglobin is essentially unchanged [19]. Hyperglycemia can on the other hand cause cellular hypoxia by other mechanisms [20].

3. Glutathione and sphingosine-1-phosphate in the erythrocyte

Glutathione and sphingosine-1-phosphate are two compounds synthesized by and exported from erythrocytes. Glutathione is mainly a protector against oxidative damage, whereas sphingosine-1-phosphate is an immune cell communicator. Glutathione is synthesized by the enzymes glutamate-cysteine ligase and glutathione synthetase which are magnesium-dependent enzymes. Glutamate-cysteine ligase uses magnesium-ATP as substrate, and glutathione synthetase crystals show two magnesium ions in addition to ADP, glutathione, and sulfate [21]. Glutathione is the main reducing

agent in erythrocytes and other cells. Supplementation with the glutathione pro-drug N-acetylcysteine or glycine has been shown to improve several health aspects, including insulin resistance and cognition [22]. Erythrocytes and other tissues like liver and brain export glutathione to plasma [23]. Erythrocyte export of glutathione takes place by multi-drug resistance proteins [24]. Extracellular glutathione is of relevance in inflammation and disease [25–27] and can directly regulate immune components in plasma [28]. Intracellular glutathione concentration is usually three orders of magnitude higher than the extracellular glutathione concentration, and an energy-consuming active glutathione uptake mechanism seems not to be known [29]. Therefore, extracellular glutathione is not expected to be taken up by cells. Erythrocyte glutathione synthesis is dependent on the availability of the substrates glutamate and cysteine. Cysteine is imported as cystine through the glutamate/cystine antiporter, also known as SLC7A11 or system Xc-. Glutamate is acquired as glutamine through the ASCT2, also known as SLC1A5 transporter.

Sphingosine-1-phosphate (S1P) is a multifunctional molecule synthesized, stored, and exported to plasma by erythrocytes, platelets, and endothelial cells [30]. The erythrocyte is considered as the main contributor to plasma S1P. The two enzymes sphingosine-kinase-1 and sphingosine-kinase-2 are responsible for the synthesis of S1P. Targeted deletions of the genes coding for these enzymes suggest that erythrocytes and platelets are redundant for S1P synthesis under normal conditions, but necessary in systemic anaphylaxis [31]. Erythrocytes obtain sphingosine from plasma for S1P synthesis, although the precise import mechanism has not been elucidated. S1P is then synthesized by sphingosine-kinase-1, which is dependent on magnesium as shown by the presence of magnesium in the crystal structure of sphingosine-kinase-1 [32]. Evidence for intra-erythrocytic sphingosine synthesis has not been found [33]. The S1P-degrading enzymes sphingosine lyase and sphingosine phosphohydrolase are not present in erythrocytes, leading to some erythrocytic S1P storage capacity [34]. S1P is exported from erythrocytes by the major facilitator superfamily domain 2b (Mfsd2b), whereas export from endothelial cells is mediated through protein spinster homolog 2 (Spns2). S1P also needs apolipoprotein M in complex with high-density lipoprotein for effective export from erythrocytes [35].

S1P has several known effects in plasma. S1P forms a gradient where high levels are found peripherally and low levels are found within lymph nodes. Maturing lymphocytes expressing the S1P-receptor-1 proceed along this gradient, thereby leaving the lymph nodes. S1P1 receptor expression and further downstream signaling in endothelial cells are necessary for blood vessel integrity and vascular tone. Some of these effects are thought to be brought about by nitric oxide signaling [30]. Increased erythrocyte S1P levels have been found in COVID-19 patients [36]. S1P can reprogramme erythrocytes of chronic kidney disease patients to glucose metabolism through the Rapoport-Luebering shunt [37].

4. Erythrocytes, Alzheimer's disease, and other types of dementia

Several studies have indicated changes in erythrocytes of demented patients, including Alzheimer's disease. Binding of amyloid-beta to erythrocytes has been reported, and fibrils supposedly containing amyloid-beta have been visualized on erythrocytes [38]. A metabolomics study of Alzheimer's disease patients and controls identified 750 metabolites of which 7 increased and 24 decreased in erythrocytes of Alzheimer's disease patients [39]. The increased metabolites included argininate,

2-oxoarginine, and N-acetylarginine, all of which are known to form as a result of deficiency of the enzyme arginase. Erythrocyte arginase activity in Alzheimer's disease patients needs to be investigated to follow up on these results. All the 24 decreased metabolites were lipids, or related to lipid metabolism, whereof 10 were in the sphingolipid group like sphingomyelin. A metabolomic study of plasma in Alzheimer's disease similarly found differential presence of many lipids, but in this study sphingomyelin was higher in plasma of Alzheimer's disease patients [40]. Low blood hemoglobin levels and anemia have been associated, possibly in a causal relationship, with Alzheimer's disease and cognitive function [41].

Genome-wide association studies have found association between Alzheimer's disease and complement receptor 1 (CR1), clusterin, complement component 1 s (C1s), and in some ethnic groups also complement factor H [42–44]. CR1 is a transmembrane protein with a short cytoplasmic part, and many extracellular short consensus repeats (SCR) that bind complement component 3b (C3b) in complex with an antibody and its antigen, also referred to as an immune complex. The bound immune complex is then delivered to macrophages for internalization and degradation. CR1 has been localized to the membrane of erythrocytes where 80–90% of all CR1 in the body is estimated to be localized [45]. C3b binds to immune complexes with amyloid-beta, the main suspected protein in Alzheimer's disease. C3b-antibody-amyloid-beta complexes then bind to CR1 on erythrocytes that carry their load for delivery to the Kupffer macrophages in the liver. This process is sometimes referred to as immune adherence [46]. The anti-amyloid-beta antibody Aducanumab is a recent addition to Alzheimer's disease therapy. Aducanumab showed reduction of amyloid-beta in the brain, but no improvement of cognition or functional ability of patients [47]. Since antibodies have low ability to penetrate the blood-brain barrier, the effect of immunotherapy against Alzheimer's disease may involve amyloid-beta clearance from blood via erythrocytes and CR1 [48]. It should be noted that brain expression of CR1 is controversial, and therefore the erythrocyte-based explanation for CR1 involvement in Alzheimer's disease is preferred [45]. Complement has been shown to be active in normal brain function, for instance by performing synaptic pruning during development [49, 50]. The other complement components or complement factors that have been implicated in Alzheimer's disease may therefore be active in the brain. For instance, the C3b-binding complement factor H is present in both brain and plasma and has been shown to protect erythrocytes and other cells against complement-mediated damage [51]. As mentioned, complement factor H has shown association with Alzheimer's disease in some population studies [43]. A functional association between complement factor H and CR1 on erythrocytes in the pathology of Alzheimer's disease can therefore not be excluded. Inspection of CR1 alleles shows that CR1*2 is associated with increased risk for Alzheimer's disease. CR1*2 has an extra short consensus repeat in the extracellular domain of the protein. This length polymorphism is almost always associated with lower expression of CR1*2 on the erythrocyte surface [52]. The patients also show high levels of soluble CR1. The significance of soluble CR1 seems to be largely unknown. Clusterin, also known as apolipoprotein J, and ATP-binding cassette A7, also known as ABCA7, are two additional erythrocyte proteins [53, 54] that are known to be associated with Alzheimer's disease from genome-wide association studies.

Erythrocyte levels of the omega-3 fatty acids docosahexaenoic acid and eicosapentaenoic acid were lower in subjects with dementia in a longitudinal study population [55]. Possibly omega-3 fatty acids offer some protection against dementia as is further discussed in the section on cholesterol and lipids.

Erythrocytes have also been suggested to be a potential link between Alzheimer's disease and diabetes [56]. For instance, erythrocytes in Alzheimer's patients express more glucose transporter 1 (GLUT1) and insulin receptor than control subjects [57]. It is possible that these results are indications of a dysregulated metabolism that influences the availability of oxygen for the brain which might lead to cognitive impairment [58]. It should also be noted that Alzheimer's is more frequent in patients with type 2 diabetes.

5. Magnesium, cognition, and the erythrocyte

Body magnesium levels can be assessed through measurement of erythrocyte or plasma magnesium levels. Since only about 1% of total-body magnesium is found in blood, further tests may be necessary for more thorough evaluation of whole-body magnesium levels. A correlation between intra-erythrocyte magnesium levels and cognition was found in rats [59], and a similar association could be found in a human study involving patients with vascular cognitive impairment [60]. Erythrocytes show significantly diminished intracellular magnesium levels in patients with vascular cognitive impairment, although plasma magnesium levels are normal. This could be interpreted as a measure of whole-body magnesium levels, including the brain. Low magnesium levels in the central nervous system are known to be associated with complications like depression. An explanation for this may be the excitotoxicity caused by the *N*-methyl-D-aspartate (NMDA) receptor, a glutamate-regulated calcium channel present in the plasma membrane of nerve cells. The NMDA receptor requires magnesium as a "gate-keeper" to prevent opening of the channel. Small areas of blood-brain contact can occur in dementia as microbleeds or microhemorrhages, also known as blood extravasations [61]. Communication between brain and the immune complement and blood coagulation systems has been suggested to be part of the pathology in vascular dementia [62]. A meta-analysis of dementia trials showed that vascular dementia was associated with increased levels of fibrinogen, activated factor VII, factor VIII, von Willebrand factor, D-dimer, and homocysteine [63]. When displaying phosphatidylserine on the surface, erythrocytes contribute to coagulation by interacting with the gamma-carboxyglutamyl (Gla) domains on coagulation factors, initiating the formation of thrombin from prothrombin. Phosphatidylserine exposure is regulated by intracellular calcium level and by the enzymes flippase and scramblase. Flippase moves phospholipids from the exoplasmic to the cytoplasmic side of the plasma membrane. Flippase activity in human erythrocytes is performed by ATP11C, a P4-ATPase [64] that has been crystallized together with its interacting protein CDC50, which is also known as TMEM30A [65]. The structure is similar to other human or yeast P4-ATPase flippase structures [66, 67]. Reduction in ATP11C activity leads to increased phosphatidylserine exposure on erythrocytes [68] and in case of deficiency may lead to mild hemolytic anemia [69]. Flippase activity is dependent on ATP and magnesium, and some of the 3D structures also show magnesium ions at the phosphorylation site [66, 67]. Lower magnesium levels in erythrocytes can therefore lead to lower flippase activity and more exoplasmic exposure of phosphatidylserine.

Scramblase moves lipids between the two monolayers of a membrane in either direction, thus evening out differences in lipid composition between the two sides. Human scramblase comes in three different protein families [70]. The scramblase activity of erythrocytes is performed by transmembrane protein 16F (TMEM16F, also known as anoctamin6) [71, 72]. TMEM16F is a calcium-dependent homodimeric

structure with 10 transmembrane alfa-helices and a large amino-terminal cytosolic domain in each subunit [73]. The hydrophilic head of the lipid substrate proceeds in a cavity on TMEM16F, similar to the swiping of a credit card [74]. Evidence for TMEM16F scramblase activity also comes from patients with Scott syndrome [75, 76], a condition involving low coagulation ability of platelets and erythrocytes [77]. Three calcium-binding sites can be seen in TMEM16F high-resolution structures, but so far, crystal structures of the TMEM family seem not to have included magnesium. Since TMEM16F is negatively regulated by magnesium [78], low intracellular magnesium levels, as was found in erythrocytes of vascular dementia patients, may lead to more TMEM16F scramblase activity, phosphatidylserine exposure, and blood coagulation. Magnesium binding to TMEM proteins could involve a magnesium-calcium competition similar to some other calcium-dependent proteins often involving the regulatory protein calmodulin [79]. A direct interaction between TMEM proteins and calmodulin has been suggested but is still controversial [80].

6. Iron and the erythrocyte

Iron is necessary for oxygen transport by hemoglobin in the erythrocyte. In addition, iron is stored in ferritin, which has been shown to occur in the erythrocyte and may be necessary for binding excess erythrocyte iron. Excess erythrocyte iron may be a consequence of hemoglobin oxidation and degradation and may be a normal part of erythrocyte aging. The erythrocyte also contains the iron export protein ferroportin, which may likewise protect the erythrocyte from toxic effects of excess iron. Ferroportin is regulated by the iron hormone hepcidin, which in turn is regulated by interleukin-6. Conditional deletion of ferroportin in mice leads to build-up of intracellular erythrocyte iron and may result in hemolysis [81]. The ferroportin Q248H mutation protects ferroportin from degradation caused by hepcidin and seems to have been selected in African populations possibly due to some protection against malaria conferred by the mutation. Ferroportin functions in erythrocytes and erythroid cells as an export gate for the regulation of total-body iron homeostasis [82, 83]. Iron is dysregulated in ferroptosis, a form of regulated cell death distinct from apoptosis [84]. Ferroptosis is characterized by iron overload, peroxidation of lipids and low activity of the selenium-containing enzyme glutathione peroxidase 4. Ferroptosis has been associated with several pathological conditions including neurological diseases such as Alzheimer's disease [85]. Transcriptomic analysis reveals changed expression levels of many ferroptosis-related genes in Alzheimer's disease patients [86], including the selenium-containing enzyme glutathione peroxidase 4. Neuron ferroportin needs the amyloid precursor protein for stability and localization. Degradation of amyloid precursor protein to amyloid-beta may lead to increased iron levels in neurons and pave the way for ferroptosis-induced neuron death [87]. Some amyloid-beta will reach the vascular compartment by way of the glymphatic system. Amyloid-beta can then bind to erythrocytes in the vascular compartment. Amyloid-beta has been shown to induce morphological changes in erythrocytes [88], affect signal transduction [89], and inhibit production and release of ATP from erythrocytes [90]. Amyloid-beta could potentially affect erythrocytes leading to dysregulation of total-body iron homeostasis, potentially worsening the prodromal phase of Alzheimer's disease, although this remains to be tested.

Hereditary hemochromatosis is another condition also characterized by iron overload in several tissues of the body. Particularly the liver, heart, pancreas, and skin

are affected in hereditary hemochromatosis. Erythrocytes of patients with hereditary hemochromatosis may show morphological changes [91], and the patients can be affected by secondary diseases that affect erythrocytes, such as polycythemia vera or hemolytic anemia [92].

7. The erythrocyte and purinergic signaling

Cells of the vascular compartment communicate with each other by adenosine and its nucleotides ADP and ATP, a process called purinergic signaling. A set of 19 receptor subunits form receptors for adenosine and its nucleotides [93]. The ATP-binding P2X7 receptor is found on erythrocytes and many other cells in the vascular compartment. Binding of the ATP ligand to the P2X7 receptor of erythrocytes leads to phosphatidylserine exposure and clearance by macrophages [2]. ATP generated from erythrocytes can activate the P2X7 receptor on other cells like endothelial and myocardial cells. This can lead to inflammasome activation and may lead to pyroptosis of the cell through activation of gasdermin [94]. Structural studies of the P2X7 receptor show a "leaping dolphin"-like structure, where the head is formed by the extracellular domain and the tail by the transmembrane helices [95]. Unlike other P2X receptors, the P2X7 receptor shows a rather extended C-terminal domain described as the "cytoplasmic ballast" [96]. The cytoplasmic ballast contains one GDP-binding site, two zinc-binding sites and similarity to TNF receptor I and lipopolysaccharide binding domains [97]. When exposed to high ATP concentrations, the P2X7 receptor can form a macropore that allows passage of solutes up to 900 Da in size leading to apoptosis of the cell. The macropore size is sometimes incorrectly stated to be 900 kDa [94, 98, 99]. The macropore may be formed by the P2X7 receptor or together with some other membrane proteins like pannexin or connexin. Some evidence suggests that the P2X7 receptor is inhibited by magnesium [100]. The P2Y12 receptor is expressed on erythrocytes but is most known for its expression on platelets, where binding to ADP is part of the process of platelet activation, which is an important part of blood coagulation. Due to this, the P2Y12 receptor has been the focus of pharmaceutical development for instance leading to the antagonists clopidogrel, prasugrel, and ticagrelor. The P2Y13 receptor is activated by ADP on erythrocytes leading to diminishing of ATP export. Purinergic signaling in erythrocytes is part of the interaction between erythrocytes, platelets, and endothelial cells, both in normal physiological conditions and in pathological conditions such as diabetes [101].

Export of ATP from erythrocytes takes place through the pannexin transmembrane protein. ATP can then be metabolized in the extracellular space, for instance in plasma, to ADP and AMP by ectonucleoside triphosphate diphosphohydrolase, usually called CD39. AMP can then be further metabolized to adenosine by ecto-5′-nucleotidase, usually called CD73. CD39 and CD73 are transmembrane proteins expressed on the plasma membrane of many cell types including endothelial cells [102, 103]. Crystal structures of CD39 and CD73 show calcium and zinc ions [104, 105]. Interestingly, CD39 and CD73 collaborate with heme oxygenase-1 in heme catabolism [106]. Adenosine generated by CD73 increases heme oxygenase-1 in macrophages through stimulation of the adenosine A2A or A2B receptors. Heme and ATP can be generated from erythrocytes as a consequence of hemolysis. The intracellular pool of adenosine can be replenished by equilibrative nucleoside transporter 1 [101], which is a transporter localized in the plasma membrane of erythrocytes and other

cells. AMP can then be regenerated in the erythrocyte from adenosine by adenosine kinase [107], an enzyme in the purine nucleotide salvage pathway [108].

Uric acid, the result of adenosine and guanosine catabolism, is found in plasma where it may have antioxidative properties [109]. Significant correlations between uric acid levels and several erythrocyte parameters have been found, such as mean corpuscular volume, mean corpuscular hemoglobin concentration, and erythrocyte distribution width [109, 110]. However, decreased plasma uric acid levels accomplished by the recombinant urate oxidase Pegloticase did not affect the oxidative status of plasma [111]. A possible interpretation of this result is that the antioxidative properties of erythrocytes could compensate for the loss of uric acid's antioxidative capacity.

8. The erythrocyte, cytokine, and immune cell relation

Erythrocytes have been found to be a reservoir of cytokines, a group of immune signaling proteins. Cytokines include chemokines, interferons, interleukins, and the hormone erythropoietin. Close to 50 different cytokines have been identified in or associated with erythrocytes [112]. Erythrocytes probably due to cytokine storage have a role in defense against pathogens, immune function, and homeostasis [2]. Erythrocytes also interact with the cellular part of the immune system (**Figure 1**). Macrophages are an important part of the cellular immune system that participate in both the birth and death of erythrocytes. Macrophages phagocytose senescent erythrocytes when they pass through liver or spleen. A phagocytosis signal is provided by phosphatidylserine exposure on erythrocytes, recognized by the TIM (T cell immunoglobulin and mucin domain containing) and CD300 receptors on macrophages. The CD47 membrane protein, which is present on erythrocytes, binds to the SIRPalfa protein on macrophages to downregulate phagocytosis [113]. Erythrocytes stored for long time periods can induce M2 macrophage polarization through the immunosuppressive interleukin-10 [114], thereby downregulating immunity. Macrophages are also necessary in erythropoiesis, the formation of new erythrocytes, where they interact with the erythroid progenitors in erythroblastic islands [115, 116]. Macrophages promote erythroblast proliferation and differentiation by secreting growth factors, providing nutrients like iron and finally phagocytosing nuclei of the nascent reticulocytes. Early-stage erythroblasts respond to growth factors like interleukin-3, stem cell factor, and erythropoietin. Several receptor-ligand pairs facilitate macrophage-erythroblast interactions. Vascular cell adhesion molecule1 of the macrophage interacts with integrin alfa4beta1 of the erythroblast and integrin alfaV of the macrophage interacts with intercellular adhesion molecule4 of the erythroblast [115]. Erythroblast macrophage protein of the macrophage interacts with erythroblast macrophage protein expressed on the surface of the erythroblast. CD163 and CD169 are expressed on macrophages and are known to be necessary for erythropoiesis, but the corresponding molecule on the erythroblast has not been identified. Finally, DNase2alfa of the macrophage is necessary for the phagocytosing of the nuclei of the nascent reticulocytes [116]. Selenium and selenoproteins are other factors necessary for erythropoiesis. Mutation of the selenocysteine-transfer-RNA, sometimes abbreviated Trsp, selenoprotein W, and glutathione peroxidase 4 genes in mice led to defective erythropoiesis [117–119]. Selenoprotein W has been suggested to be an adaptor protein to the E3 ubiquitin ligase TRIM21 [120]. Ubiquitin is a protein that delivers proteins for degradation to the proteasome, a protein complex that degrades mainly

damaged proteins marked by ubiquitin. The 20S proteasome and ubiquitin have been detected in reticulocytes and mature erythrocytes. Erythrocytes from patients with Alzheimer's or Parkinson's disease show decreased 20S proteasome activity [121, 122].

Erythrocytes also interact with other cells of the immune system. Erythrocytes have been shown to inhibit T cell activation or activation-induced apoptosis presumably via reactive oxygen species-dependent pathways [123, 124]. Erythrocytes treated with a cancer cell line stimulated T cells to more proliferation and other cytokine secretion profile than if treated with control erythrocytes [125]. The C-C chemokine RANTES (regulated on activation, normal, T cell expressed, and secreted) guides transendothelial migration of eosinophils, an immune cell that is responsible for interleukin-5 production and largely involved in asthma and allergy. Erythrocytes regulate this process by scavenging RANTES in the vascular compartment [126].

9. Erythrocytes and function of the vascular compartment

The erythrocyte is important for several aspects of the vascular compartment, in particular vascular tone and vascular integrity. One aspect is the signaling based on nitric oxide, a gaseous molecule produced in the vascular compartment by erythrocytes, platelets, and endothelial cells by endothelial nitric oxide synthase (eNOS). Erythrocytes both produce and release nitric oxide [127] but can also scavenge nitric oxide by hemoglobin [128]. The synthesis of nitric oxide proceeds from arginine and oxygen-generating nitric oxide and citrulline. Regulation of nitric oxide synthesis is performed by arginase I by degrading the substrate arginine to ornithine and urea. Nitric oxide synthesis can also take place by deoxy-hemoglobin acting as nitrite reductase in hypoxia.

Nitric oxide is a vasodilator based on its effects on the vascular smooth muscle cells that surround the vascular compartment and contribute to vascular tone. The vascular smooth muscle cells contain the nitric oxide receptor soluble guanylate cyclase that produces cyclic guanosine monophosphate (cGMP). Protein kinase G is a further downstream signaling component, but the steps leading to vasodilation seem to be unknown in exact detail [129], although the last step probably involves changes in myosin phosphorylation, performed by Rho-associated kinase, zipper interacting kinase [130], myosin light chain kinase, or myosin phosphatase [131]. Some of these enzymes are also regulated by calmodulin and may therefore also be indirectly regulated by magnesium [79]. Soluble guanylate cyclase and protein kinase G are also present in erythrocytes and platelets, although the precise function of this pathway in erythrocytes is unknown [132]. Nitric oxide has an inhibitory effect on platelet activation, possibly through phosphorylation of the thromboxane receptor [133]. The effects of nitric oxide also include S-nitrosation of proteins and regulation of cGMP-gated ion channels by cGMP [128]. For instance, nitric oxide protects against myocardial infarction through S-nitrosation of the mitochondrial permeability transition pore regulator cyclophilin D [134]. Nitrosation of cysteine-93 of the beta-chain of hemoglobin has been suggested as a transport mechanism for nitric oxide in the erythrocyte. This hypothesis is not currently favored as a result of mutation studies [127]. A magnesium-dependent nitrosation of glutathione, yielding S-nitrosoglutathione, has been reported and could potentially occur in erythrocytes [135]. The physiological significance of S-nitrososglutathione is however unclear.

The relative contribution of different cell types to nitric oxide production in the vascular compartment is not yet fully elucidated. Erythrocytes both produce and

scavenge nitric oxide, which complicates the interpretation their contribution. Several evidences from pathological conditions point to the importance of erythrocytes for generation of nitric oxide bioactivity. Increased expression of erythrocyte arginase in diabetes leads to less nitric oxide production from erythrocytes. ENOS can be monomerized in erythrocytes of type 2 diabetes patients, usually referred to as uncoupling. The monomers then produce superoxide, which is also produced by nicontinamide adenine dinucleotide oxidase (NOX), both in erythrocytes and in endothelial cells. This results in dysfunction of endothelial cells [136], a common phenomenon in type 2 diabetes. As a further example, studies of anemic patients and a mouse model of anemia show that anemia is associated with erythrocyte dysfunction and reduced nitric oxide bioactivity. ENOS activity is then increased in the vascular wall and heart as compensatory mechanisms. If anemia is combined with endothelial dysfunction, the compensatory nitric oxide bioactivity may not be sufficient and could lead to adverse outcomes in myocardial infarction [137, 138].

10. Erythrocytes, cholesterol, and other lipids

The erythrocyte membrane contains lipids like phosphatidylserine, phosphatidylethanolamine, phosphatidylcholine, sphingomyelin, and membrane proteins. The membrane also contains cholesterol, which is important for membrane stability and deformability of erythrocytes [139]. The parasite Plasmodium does not synthesize cholesterol, and vesicles containing cholesterol have been observed to transport from erythrocytes to the Plasmodium parasite [140]. Cholesterol can be chelated by beta-methyl-cyclodextrin, which has been used against Plasmodium infections [141]. Many studies point to an association between lipid constituents of erythrocytes and divers diseases [142]. Many studies have found that the omega-3, also called n-3 fatty acids, are promoting health in various ways, whereas omega-6 (n-6) may be less health promoting and the saturated fatty acids are further less health promoting. Trans-fatty acids are usually not considered health promoting, although this is somewhat controversial considering their occurrence in dairy products. Similar results are found across many studies, irrespective of whether plasma or erythrocyte levels are measured. One reason for less clear health promotion of omega-6 fatty acids is that the omega-6 arachidonic acid is a precursor for pro-inflammatory prostaglandins, thromboxanes, and leukotrienes [143], collectively called eicosanoids. Omega-3 fatty acids on the other hand have been reported to reduce signaling through the pro-inflammatory toll-like receptor 4 (TLR4) [144]. Dietary levels of omega-6 fatty acids are usually well in excess of those of omega-3, which may lead to undesirable enzymatic competition for the fatty acid substrate. Erythrocyte membrane constituents are of interest as diagnostics, since erythrocytes are more long-term indicators than plasma levels, which are influenced by the most recent meal. Erythrocyte levels of omega-3 fatty acids were associated with less cardiovascular events in the Framingham heart study [145]. Erythrocyte omega-6 fatty acid levels showed no association with cardiovascular events in the same study [146]. Erythrocyte omega-3 polyunsaturated fatty acids were negatively correlated with cancer [147]. Erythrocyte omega-3 fatty acids were associated with less risk of islet autoimmunity in children with diabetes [148]. A study of erythrocytes in preclinical Alzheimer's disease found increased levels of the omega-6 fatty acid arachidonic acid and decreased levels of the omega-3 fatty acid docosapentaenoic acid in participants with high neocortical beta-amyloid load prior to cognitive impairment [149]. Decreased omega-3 fatty acid levels

in erythrocyte membranes of schizophrenia patients has given rise to a modified version of the dopamine hypothesis of schizophrenia [150]. A study of erythrocyte fatty acids in schizophrenia patients found that omega-3 polyunsaturated fatty acids were significantly lower and the omega-6:omega-3 fatty acid ratio was significantly higher in the group with dominantly negative symptoms as compared to the group with dominantly positive symptoms [151]. A significant deficit of erythrocyte omega-3 docosahexaenoic acid has been noted in bipolar disorder type I [152], whereas erythrocyte fatty acid profiles had no predictive value in autism spectrum disorder [153].

Trans-fatty acids in erythrocytes were found to be associated with increased risk of coronary heart disease in prospective [154] or cross-sectional studies [155]. However, trans-fatty acids in erythrocytes appear to be decreasing in Europe as seen in samples from 2008 to 2015 [156].

11. Erythrocytes and ribonucleic acid (RNA)

Although ribonucleic acid (RNA) has been reported in erythrocytes since long (**Figure 1**), the purity of blood cell preparations was often questioned [157]. The use of single-cell transcriptomics has provided further confirmation of the presence of RNA in erythrocytes [158]. Around 8000 messenger-RNA transcripts have now been found in erythrocytes. Analysis of messenger-RNA has shown that erythrocytes can be divided into at least seven different categories [158]. These seem to represent different stages of development from reticulocytes to cells in transition to mature and finally senescing erythrocytes. The most abundant messenger-RNA transcripts are related to erythropoiesis and may be seen as a residue from that process. Alternatively, messenger-RNA may serve as template for protein synthesis, which would require the presence of ribosomes and other parts of translation, which may possibly exist at a low level in erythrocytes [159]. Hundreds of different microRNAs (miRNAs) have also been confirmed in erythrocytes [159]. MiRNAs are usually about 22 nucleotides in length and function to reduce, or silence, the expression of genes. Important parts of this mechanism are the RNAse III enzymes Drosha and Dicer, the endonuclease Argonaute, and the RNA-binding protein DGCR8. Drosha, Dicer, and Argonaute are all magnesium-dependent enzymes [160–162]. MiRNA genes are transcribed by RNA polymerase II to a pri-miRNA. Drosha and DGCR8 then process the pri-miRNA to a pre-miRNA which is exported from the nucleus through exportin-5. Once in the cytoplasm, the miRNA is further processed by Dicer and bound to Argonaute2 forming the RNA-induced silencing (RISC) complex. Some of the miRNAs of the erythrocyte like miR-451, miR-144, and miR-486 are involved in erythropoiesis [163]. MiR-451 is also associated with malaria, and miR-144 is correlated with hypoxia at high altitudes [159]. An attractive hypothesis is that erythrocytic RNA enclosed in microvesicles is used as a means of communication between erythrocytes and other cells.

Microvesicles are extracellular vesicles 0.1–1 micrometer in size, formed by budding from the plasma membrane. Microvesicles are distinguished from exosomes, which are smaller and released as preformed vesicles, 10–100 nanometer in size. Microvesicles and exosomes may contain proteins, lipids, and RNA. Microvesicles are particularly known as carriers of miRNA. Erythrocytes are known to form microvesicles under blood storage, but microvesicles are also formed as a normal physiological process [164]. As mentioned, microvesicles may fulfill a function as information carriers between different cells in the body. Microvesicles also function as an efficient protection against proteases or RNases that would otherwise degrade

the content [165]. A connection between erythrocyte microvesicle formation and diseases or pathological conditions have been proposed in several cases. However, a more comprehensive analysis of miRNA content of microvesicles derived from healthy erythrocytes compared to those from patients with disease conditions seems to be needed.

Transport of miRNA or messenger-RNA from erythrocytes to other cells may be a way of regulating gene expression in target cells [159]. The known functions of these miRNAs in other cells of the body could accordingly be relevant also in the context of erythrocytes. MiRNAs are generally edited by the gene silencing mechanism in cells, giving rise to several variants of each miRNA family, often indicated in the nomenclature by extra suffixes, like the expressed strand being indicated by a 3-p or 5-p suffix. Some abundant and notable erythrocyte miRNAs are miR451, miR144, miR16, and let-7. The miR451 family is abundant in erythrocytes and functions in erythropoiesis by downregulating the Ywhaz gene, whose protein product 14–3-3-zeta keeps the transcription factor FoxO3 in the cytosol [166]. FoxO3 positively regulates antioxidant enzymes like catalase and glutathione peroxidase. CRISPR-Cas9-mediated mutagenesis of miR451 confirmed that miR451 is necessary for erythroid differentiation and expression of transferrin receptor 1, also known as CD71 [163]. The miR144 family is expressed together with miR451 and was similarly found to be necessary for erythroid differentiation and CD71 expression [163]. Plasmodium-infected erythrocytes produce microvesicles containing miR451, miR16, and let-7, among others [167–169].

12. Erythrocyte and other organisms, particularly plasmodium

Erythrocytes interact with several organisms like bacteria and Plasmodium. Erythrocytes also interact with viruses, although mainly through adherence of viruses to erythrocyte membrane proteins or glycocalyx. Several erythrocyte membrane proteins are known as receptors for Plasmodium, somewhat dependent on the Plasmodium species. An interesting aspect is that Plasmodium seems to be dependent on iron acquired directly from hemoglobin of the erythrocyte. For instance, Plasmodium cannot obtain iron from heme. The erythrocyte is made dependent on the Plasmodium parasite by the glutathione synthesis of the parasite, whereas the erythrocytes own glutathione synthesis seems to be largely turned off [170]. Glutathione export from the Plasmodium-infected erythrocyte proceeds mainly in the oxidized (GSSG) form probably reflecting conditions of oxidative stress under infection. Erythrocytes infected with Plasmodium produce microvesicles that contain miR451, let7, and Argonaute2 protein together forming a functional RISC complex [167]. Plasmodium does not contain the genes necessary for miRNA production, but human miRNA and Argonaute protein have been detected in Plasmodium, presumably transferred from the erythrocyte by microvesicles. Infected erythrocytes also produce microvesicles that can be transferred to endothelial cells, astrocytes, and microglia [171]. Plasmodium causes profound changes in the erythrocyte plasma membrane, which can become almost devoid of cholesterol and display phosphatidylserine [172]. Absence of cholesterol makes the plasma membrane susceptible to pore formation by granulysin, a pore-forming peptide produced by cytotoxic cells like gamma-delta T cells. Exoplasmic phosphatidylserine may make the cells less susceptible to perforin, another pore-forming peptide. Infected erythrocytes display PfEMP (Plasmodium falciparum erythrocyte membrane protein) which binds to receptors on

endothelial cells and thereby prevents circulation of infected erythrocytes. Although they may display phosphatidylserine, infected erythrocytes are consequently not easily cleared from circulation by spleen macrophages. PfEMP can be recognized by gamma-delta T cells of the host, but the parasite can change the expressed PfEMP, since it contains about 60 different PfEMPs. Although malaria is usually not fatal, cerebral malaria caused by Plasmodium falciparum can be fatal particularly in children. Cerebral malaria involves a partial disruption of the blood-brain barrier [173]. This probably comes about through phagocytosis of infected erythrocytes by endothelial cells in brain capillaries, followed by presentation of Plasmodium antigens on the endothelial cell and attack from alfa-beta Tcells [173].

13. Medical and societal impact of erythrocyte science

Treatments and diagnosis based on erythrocytes have been much sought for, and many have been developed. For instance, several modulators of S1P-receptors have been developed, the first and most well-known being fingolimod. These are now being used as therapy for multiple sclerosis. Fingolimod is first phosphorylated to fingolimod-phosphate by sphingosine-kinase-2, which resides in the nucleus of nucleated cells. The S1P-receptor-1 is then internalized, thus preventing lymphocyte egress along the S1P gradient. The result is reduced damage from the immune system on the central nervous system or other organs. It is likely that new treatments or modulators based on S1P will be developed.

Erythrocyte microvesicles present diagnostic and possibly also therapeutic opportunities. Erythrocyte microvesicles may be used as carriers in pharmaceutical applications, or as alternatives to whole cells in cell therapy applications. Microvesicles are less complex to handle in biological product formulation and production and confer less safety issues than cells [174]. Erythrocyte microvesicles could be used to deliver the miRNAs they harbor naturally or engineered for more specific delivery. Potential challenges include multiple effects of the same miRNA and the existence of many different miRNAs, with different and opposing effects, in the same microvesicle. It is also important to consider the conditions that favor microvesicle formation. Lysophosphatidic acid (LPA) has been shown to induce phosphatidylserine exposure and microvesicle formation [175].

Malaria is the disease caused by the erythrocyte parasite Plasmodium. Malaria is still of considerable importance as a devastating disease in many parts of the world. It is to be expected that new knowledge on erythrocytes and the interaction with Plasmodium could lead to new treatments against malaria. For instance, it may be possible to interfere with the attachment of infected erythrocytes to the endothelium [176]. That strategy may circumvent undesirable side effects of many other approved antimalarial drugs.

Population levels of selenium can be increased by fortification of foodstuff [177] or by fertilization of crops. Foliar selenium fertilization has been shown to increase selenium levels in serum and erythrocyte glutathione peroxidase activity in subjects consuming the crop [178]. That selenium in foodstuff finds it way to the erythrocyte was elegantly shown in the case of selenoneine from the beluga whale that was found in erythrocytes of inuits [179]. Magnesium can be easily replenished by mineral supplements, or by a diet rich in vegetables and some other known sources of magnesium. More reliable diagnostics for assessment of whole-body magnesium status may be desirable to detect subclinical cases of magnesium deficiency. Several

of the diseases or nutritional imbalances mentioned are of substantial importance, especially considering growing human populations, faster human communications, and climate change. All this may contribute to a changed exposure to pathogens like bacteria, virus, and Plasmodium. Erythrocytes as biomarkers of changed element profiles may provide one way to observe early deviations and provide customized diet suggestions and earlier treatment of diseases.

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