1 **Active modulation of human erythrocyte mechanics**

2 Lennart Kuck¹, Jason N. Peart², Michael J. Simmonds¹

3 ¹Biorheology Research Laboratory, Griffith University Gold Coast, Queensland, Australia

4 ²School of Medical Science, Griffith University Gold Coast, Queensland, Australia

5

6

7

8

9

10 Corresponding author

11 Michael J. Simmonds, PhD

12 Biorheology Research Laboratory, Griffith University, Gold Coast, Australia

13 Tel: +61 (7) 5552 8529

14 Fax: +61 (7) 5552 8674

15 Email: mike@thesimmonds.id.au

16

17 *Running title* *Dynamic properties of erythrocytes*

18 *Keywords* *calcium; blood; deformability; haemorheology; nitric oxide; red blood*
19 *cell; signal transduction*

20 *Abstract*

21 The classic view of the red blood cell (RBC) presents a biologically inert cell, that upon
22 maturation, has limited capacity to alter its physical properties. This view developed largely
23 due to the absence of translational machinery and inability to synthesise or repair proteins in
24 circulating RBC. Recent developments have challenged this perspective, in light of
25 observations supporting the importance of post-translational modifications, and greater
26 understanding of ion movement in these cells, that each regulate a myriad of cellular
27 properties. There is thus now sufficient evidence to induce a step-change in understanding of
28 RBC; rather than passively responding to the surrounding environment, these cells have the
29 capacity to actively regulate their physical properties and thus alter flow behaviour of blood.
30 Specific evidence supports that the physical and rheological properties of RBC are subject to
31 active modulation, primarily by the second-messenger molecules nitric oxide (NO) and
32 calcium-ions (Ca^{2+}). Further, an isoform of nitric oxide synthase is expressed in RBC (RBC-
33 NOS), which has been recently demonstrated to have an active role in regulating the physical
34 properties of RBC. Mechanical stimulation of the cell membrane activates RBC-NOS leading
35 to NO generation, which has several intracellular effects, including the S-nitrosylation of
36 integral membrane components. Intracellular concentration of Ca^{2+} is increased upon
37 mechanical stimulation *via* the recently identified mechano-sensitive cation channel, *piezo1*.
38 Increased intracellular Ca^{2+} modifies the physical properties of RBC by regulating cell
39 volume and potentially altering several important intracellular proteins. A synthesis of recent
40 advances in understanding of molecular processes within RBC thus challenges the classic
41 view of these cells, and rather indicates a highly active cell with self-regulated mechanical
42 properties.

43

44

45 *Role of cell mechanics in oxygen delivery*

46 Cellular respiration is vital to sustained metabolism, and therefore life, and is only possible
47 due to the integration of the cardiovascular, pulmonary, and circulatory systems. One of the
48 challenges to oxygen loading, and subsequent transport, within the circulatory system is the
49 extremely small apertures that blood needs to navigate: the smallest capillaries (2-3 μm) in
50 the pulmonary and peripheral circulations are a fraction of the resting diameter ($\sim 8 \mu\text{m}$) of
51 red blood cells (RBC); consequently, the RBC is morphologically agile, with a unique ability
52 among all mammalian cells to change shape and subsequently return to its resting state
53 without any appreciable negative effects (e.g., hysteresis; 10). A pathologically reduced
54 ability of RBC to deform has thus long been considered a precursor for heightened risk of
55 tissue ischaemia at both micro- and macro-vascular scales, given rigid cells: i. have limited
56 capability to perfuse the rich microvascular network of organs (47); and, ii. affect the bulk
57 properties of blood through increasing the resistance to flow by contributing to hyperviscosity
58 (4). Indeed, even the capacity for oxygen dissociation from haemoglobin during tissue
59 perfusion has been reported to be limited by decreased cellular deformability (56). Further,
60 many disease states associated with tissue necrosis, ischaemia, and/or hypoxia are
61 characterised by poor cell mechanics of RBC (53). Thus, despite a vital role for optimal
62 cellular mechanics being well established, the molecular regulation of these physical
63 properties is only recently emerging.

64

65 *Classic physical properties enable erythrocyte deformability*

66 The properties of RBC that were classically recognised to confer their unique capacity to
67 deform in response to mechanical stimulation included the excess surface area relative to cell
68 volume, the intracellular viscosity, and a visco-elastic cellular membrane (10). The excessive
69 surface area of RBC is clearly observed when the cell is compared with a perfect sphere: while
70 the average volume of an RBC ($\sim 90 \mu\text{m}^3$) could be contained in a sphere with only $97 \mu\text{m}^2$ of
71 surface area, the surface area of an RBC is $\sim 44\%$ larger ($\sim 140 \mu\text{m}^2$) and thus yields a surface
72 area-to-volume ratio that approaches 1.5 (10). The resultant excess surface area is provided by
73 the bi-concave structure of the RBC membrane and facilitates significantly greater capacity to
74 deform than a spherical shape. The intracellular viscosity of RBC is largely determined by
75 haemoglobin concentration suspended in the cytosol. The mean intracellular viscosity of
76 RBC is $\sim 7 \text{ mPa}\cdot\text{s}$ at normal haemoglobin content, which is comparatively higher than the
77 viscosity of the surrounding plasma ($\sim 1.2 \text{ mPa}\cdot\text{s}$; 10). This disparity contributes to the
78 deformation of RBC by facilitating an independent rotation of the cellular membrane around
79 the cytoplasm, termed tank-treading (16). While the earliest interpretation of tank-treading
80 indicated that the cell membrane was mostly independent of internal structures (16), advances
81 over the last decades support that various entities complex with the intracellular surfaces of
82 the cell membrane, which indeed may even serve to enhance the mechanical properties of the
83 cell (36).

84 Further, the exceptionally deformable capacity of the RBC membrane has been long
85 observed; this cell provides the template for lipid bilayer models of cell membranes and is
86 noted for its exceptional capacity to deform upon exposure to shearing forces, subsequently
87 reassuming its original bi-concave shape (4). The RBC membrane contains a cytoskeletal
88 mesh-network with integral proteins establishing connections with the lipid bilayer, which
89 stabilises the characteristic bi-concave shape of RBC. Collectively, the properties that permit
90 a remarkable capacity of RBC to deform have been studied (Fig. 1), and until recently the

91 determinants of these properties were thought to be static. Recent advances challenge this
92 perspective and support an ‘active’ regulation of mechanical properties that may acutely alter
93 the physical properties of RBC.

94

95 *The molecular structure of the erythrocyte membrane facilitates cellular deformability*

96 The molecular composition of both the cytoskeletal and lipid bilayer components of RBC has
97 been subject of extensive study during the past century (21, 25). The cytoskeleton consists of
98 spectrin filaments (i.e., α - and β -subunits, which are antiparallel isomers), assembled in
99 conjunction with filamentous actin to form hetero-tetramers in head-to-head manner (Fig. 2).
100 These filaments are interconnected to the lipid bilayer: i. *via* complexation with integral
101 membrane proteins band 3 and glycophorin; or ii. *via* actin and protein 4.1 (52). Smith et al.,
102 (49) recently demonstrated that the spectrin-actin cytoskeleton is stabilised by an isoform of
103 non-muscular filamentous myosin (NMIIA). NMIIA is a hexameric actin-binding ATPase
104 comprised of two heavy chains, two light chains, and two regulatory chains, and importantly
105 is capable of contraction – which in other cell lines has been shown to be vitally important to
106 cell function (46). Knowledge around the role that NMIIA exerts within RBC is still
107 developing, although it has been recently demonstrated that *via* linkage to actin in the
108 cytoskeletal component of the membrane, tension is regulated to maintain the cell’s unique
109 bi-concave shape (49). It follows that the mechanical properties that are promoted by this
110 unique shape are thus also integrally affected by NMIIA (49). Indeed, when NMIIA is
111 inhibited using a NMIIA-specific inhibitor (i.e., blebbistatin), RBC exhibit elongated
112 morphology at rest and lose their membrane curvature (49). How phosphorylation or other
113 post-translational modifications may alter NMIIA-contractility and RBC mechanics,

however, remains to be resolved, although it is a valid area of interest given observations in other cell lines (9, 46, 58)

Collectively, although the molecular identity and structure of the RBC membrane has been largely determined, the interactive functions of numerous proteins remain unresolved, as is the dynamic re-assembly of membrane components during cellular deformation. Moreover, while classically the physical properties of RBC were viewed as the singular determinants of their extensive deformability, post-translational modifications of membrane proteins in particular have been suggested as mechanisms to acutely alter RBC mechanics (13, 24, 32).

Red blood cells are not sedentary: the role of nitrogen species

The concept that the physical properties of RBC may be modulated “actively” – that is, dynamically altered upon exposure to mechanotransductive stimuli – has emerged only over the past decade or so. While the presence of a process responsible for generating intracellular NO in RBC was speculated for some time (38), Kleinbongard et al., (31) only relatively recently employed independent and complimentary methods to demonstrate that RBC contain a NOS isoform that was distinct to the neuronal and inducible forms. Indeed, several key physical features were shown to reflect those of the endothelial-type NOS. The activity of this so-called “RBC-NOS” isoform was shown to be dependent on the substrate L-arginine, was calcium-sensitive, and also sensitive to phosphorylation *via* the PI3-kinase pathway (31) – all properties shared with endothelial NOS. Various groups subsequently confirmed that RBC carry a functional NOS-type enzyme, which facilitates endogenous production of nitric oxide (24, 39, 51, 54). Elucidating potential activation and down-stream targets mechanisms of RBC-NOS was thus of interest. Shear stress – induced either *in vitro* by exposing RBC to fluid shear in a microchannel or *in vivo* by increasing blood flow through exercise – may act

as an activator of RBC-NOS by inducing phosphorylation at its active residue serine 1177 (ser¹¹⁷⁷) (51, 54). Increased phosphorylation of this residue has been reported by various groups to be closely associated with increased RBC deformability (22, 24, 32), while inhibition of RBC-NOS was previously shown to be associated with impaired cell deformability (24). The concrete activation mechanism of RBC-NOS is currently inferred from its alleged structural twin – endothelial NOS – although requires direct evidence. Given RBC and endothelial cells differ significantly in structure and intracellular composition (i.e., RBC lack a nucleus and Golgi apparatus, which are pivotal in activation of endothelial NOS; 44), further elucidation is required to examine the RBC-NOS isoform to discern its molecular mechanisms in higher resolution.

Activation mechanism and down-stream targets of erythrocyte-derived nitric oxide synthase

The primary mechanism currently proposed to elicit activation and mobilisation of RBC-NOS is mechanical stimulation (13, 54); that is, it is thought that physical forces deforming the cell membrane may be transduced into biochemical events that stimulate NO-generation directly, or alternatively *via* an independent method involving Ca²⁺-signalling.

Investigations aimed at discerning the effects of varying shear stress magnitudes on RBC deformability and NO-metabolism in flow led to a dose-response relationship being observed between shear stress and NO-production (27). Moreover, *in vitro* application of shear stress in the physiological range during laminar flow (i.e., comparable to forces present in the human circulatory system) was shown to elicit phosphorylation of RBC-NOS ser¹¹⁷⁷, which occurred in concert with increased cell deformability (32). These data support that shear stress appears to activate RBC-NOS, and lead to predictable increases in intracellular NO production, that may impact cellular deformability. The mechanism for the shear-induced RBC-NOS activation remains elusive, although Ulker et al., (54) demonstrated that while shear exposure increased the phosphorylation of RBC-NOS at ser¹¹⁷⁷, intracellular

concentrations of NO and intracellular Ca^{2+} concentration increased in tandem. Grau et al., (24) independently, demonstrated that NO produced during mechanical stimulation may reversibly incorporate into the cytoskeletal component of the cell membrane, as evidenced by increased S-nitrosylation of integral membrane proteins. Specifically, Grau et al (24) suggested that NO appears to bind the exposed sulphur atoms in α - and β -spectrin filaments forming nitrosylated residues, and they hypothesised that this process may explain the increased cellular deformability in the presence of increased intracellular NO concentration. Further observations to confirm the S-nitrosylation of the cell membrane remain elusive, although provide a tantalising suggestion of a mechanism for shear-mediated modulation of cell deformability with implications in vascular perfusion.

It collectively appears that shear stress activates RBC-NOS, and thus also increases intracellular availability of NO, which may impact the physical properties of the cell membrane *via* S-nitrosylation. How the flux of Ca^{2+} during mechanical stimulation of the cell membrane – such as when RBC pass narrow capillaries to facilitate gas exchange – is related to these events remains a topic of current investigation. While these characteristics infer striking similarities between endothelial NOS and RBC-NOS, it is stressed that these cells maintain fundamental biological differences, no less than the obvious differences in intracellular organelles. Several groups are thus currently exploring the function of RBC-NOS (24, 32, 54), although fundamental studies into the structure of RBC-NOS, and its potential localisation within RBC are ongoing, yet stand to deliver much greater understanding on the regulatory role of this protein.

Modulation of erythrocyte mechanics by intracellular calcium

186 Incubation of RBC with calcium was classically reported to induce membrane rigidification,
187 and thus decreased cellular deformability; at least for experiments employing high
188 concentrations of extracellular Ca^{2+} (45). The mechanism of cellular rigidification, however,
189 has never been well understood, leading to inferences of a hypothetical ionic association of
190 Ca^{2+} with some membrane components (41). It is clear that intracellular calcium of RBC is
191 maintained at a comparatively low concentration ($\sim 40\text{-}60 \text{ nmol}\cdot\text{L}^{-1}$; 6) relative to that of the
192 surrounding plasma ($\sim 1.8 \text{ mmol}\cdot\text{L}^{-1}$; 34), owing to the action of powerful calcium-ATPases
193 (PMCA; 42). Given RBC do not possess mitochondria, and glycolysis is the sole pathway of
194 endogenous ATP-generation, the capacity of RBC to maintain such a tightly regulated
195 intracellular calcium concentration is thus dependent on the metabolic status of the cell.

196 O'Rear and colleagues (41) hypothesised calcium may impair the flexibility of the cell
197 membrane due to electrostatic interaction of this cation with the membrane lipid bilayer – at
198 least in the context of RBC being exposed to supraphysiological shears. Calcium depletes
199 cellular ATP (35), which was initially thought to be essential for maintaining RBC
200 deformability (57), by forcing high PMCA-activity. On the other hand, Clark et al., (11)
201 demonstrated that ATP-depletion and calcium-accumulation have distinct effects on cellular
202 deformability, hypothesising that the primary consequence of increased intracellular calcium
203 is activation of the Gárdos channel (for a review, see 37) and subsequent loss of intracellular
204 fluid. Briefly, the Gárdos channel is a calcium-activated potassium channel embedded in the
205 RBC membrane (19) that facilitates transport of potassium-ions (K^{+}) to the extracellular
206 space (33), and thus also water according to an osmotic shift, leading to a decrease in cell
207 volume (i.e., Gárdos effect; Fig. 3). Of note, while the intracellular fluid leaving the RBC
208 contains chloride ions (Cl^{-}) and water, haemoglobin is unable to leave the cell *via* this
209 mechanism. It follows that intracellular viscosity increases due to the resultant higher relative
210 abundance of haemoglobin, which has been experimentally validated and suggested to induce

impaired cellular mechanics (55). Recent developments have led to a paradigm shift indicating that the Gárdos effect may have valuable contributions to the maintenance of cellular mechanics, at least when shear stresses and/or calcium flux is maintained within physiological limits.

Mechanical stimulation, calcium and volume regulation

Early patch-clamp experiments performed on RBC to examine stretch-activated currents found evidence of a transient Ca^{2+} -flux that occurred in immediate response to mechanical stimulation. A negative pressure was placed onto single RBC using micropipettes to induce a reproducible deformation of the cell while isolating a membrane patch to record currents (14). It was observed that secondary to the Ca^{2+} -trace, K^{+} was transported in the opposite direction across the cell membrane (14). The authors thus hypothesised a possible interplay between a mechanically-activated calcium channel (that was yet to be identified) and the calcium-activated potassium channel, termed the Gárdos channel. Subsequent investigations into stretch-activated ion channels in the RBC membrane became more complicated given that the gold standard method – patch clamping – itself involves applying a mechanical stimulus to the membrane which could evoke currents from mechanically-activated channels (for a review, see 29).

Danielczok and colleagues (13) used *in vivo* and *in vitro* methods to assess transient calcium-increases within deformed RBC, visualised using a fluorescent probe. Upon cellular deformation of the RBC membrane, intracellular calcium transiently increased (13). While the RBC membrane is highly permeable to anions, it is poorly permeable to cations, and thus calcium flux involves transporters/pumps residing within the membrane. The hundreds of both selective, and non-selective transports are now known, with the vast majority of these

235 routes being voltage sensitive (for a review, see 2). Danielczok and colleagues (13) thus
236 examined whether mechanosensitive cation-channels (i.e., piezo1), shown to be expressed on
237 the RBC membrane (8), were critically involved in the transient calcium-response.
238 Application of a specific piezo1-blocker, the peptide GsMTx-4 (1), abolished the expected
239 flux in calcium-content caused by stretching of the RBC membrane. Rheological and
240 biophysical measurements to assess the contribution of this mechanism to tissue perfusion
241 more globally (e.g., quantification of flow velocities, shear stresses, transit times), however,
242 were not performed in these experiments and would present a valuable addition to the
243 observations of Danielczok and colleagues (13). Moreover, quantifying the alterations in cell
244 volume as RBC pass narrow capillaries is required, in conjunction with investigations aimed
245 to discern the reversibility of this phenomenon both on a cellular (e.g., return to normal cell
246 size) and molecular level (e.g., ion-flux during states of reduced cell volume that facilitate a
247 restoration of cellular homeostasis). Integrative studies investigating the interplay between
248 mechanically-activated NO-generation and Ca^{2+} -signalling pathways present ambitious albeit
249 valuable directions for full understanding of the RBC active regulation of its mechanical
250 properties. Given the calcium-dependency for activation of NOS observed in endothelial cells
251 (17), in addition to existing evidence supporting a similar mechanism in the RBC (31, 54), it
252 is plausible to suspect that cross-talk between these two signalling molecules exists.

254 *Relevance in mechanical circulatory support and blood disorders*

255 The mechanics of RBC are known to be impaired in a vast variety of pathologies (e.g., type 2
256 diabetes, coronary artery disease; 48, 53) which manifest in circulatory defects. Further,
257 given these diseases may progress to require interventions facilitated by mechanical
258 circulatory support (e.g., cardiopulmonary bypass; ventricular assist devices), which exert

259 supraphysiological mechanical force on the blood (18), alterations in the sensitivity of
260 second-messenger pathways may be particularly implicated in commonly observed post-
261 surgical complications (30). Impaired transduction of NO- and Ca^{2+} -regulated signalling
262 contributing to the observed rheological impairments in clinical conditions and after high-
263 shear exposure presents a therapeutic opportunity. Stimulating or inhibiting mechanically-
264 activated signalling pathways by pharmacological means could ameliorate the observed
265 deficiencies in sustaining oxygen-delivery.

266 Sick cell anaemia is caused by a single point mutation in exon I coding for beta-globin (12)
267 which results in polymerisation of the haemoglobin beta-chain and thus 'sickling' of RBC
268 under local hypoxia. Repeated collapses of RBC – such as through sickling when traversing
269 the venous circulation – would provide a mechanical stimulus even stronger than shrinking
270 induced by exposure to an hypotonic solution, which has been independently shown to induce
271 piezo-1 activation and calcium-influx (13). The Gárdos effect has been associated with sickle
272 cell pathology (i.e., exacerbating the 'sickling'; 28) and inhibitors of the Gárdos channel
273 (e.g., clotrimazole and senicapoc) have been therapeutically explored, although these agents
274 proved ineffective in reducing vascular crisis events despite decreasing the relative
275 abundance of 'sickled' cells and haemolysis (7, 50). Homozygous piezo1-mutations are
276 expressed in 20% of the sickle cell anaemia population in contrast to 5% in the general
277 population (40); however, preliminary clinical observations of piezo1-mutations in sickle cell
278 anaemia patients did not significantly associate with disease severity (43). Independent
279 studies established abnormal NO-metabolism in sickle cell anaemia patients; that is, sickle
280 RBC present with increased NOS-activation and NO-production which are not associated
281 with improved rheological characteristics (23). It appears, however, that NO may inhibit the
282 Gárdos channel through interaction with cysteine residues on the extracellular side of the
283 RBC membrane, thereby ameliorating the Gárdos effect (3, 5). Alternatively, ionic volume-

regulation via the Gárdos effect could offset NO-dependent increases in membrane flexibility; however, further studies investigating the concrete interplay between these pathways are required.

Mutations in the genes coding for piezo1 and the Gárdos channel have been reported in patients suffering from xerocytosis and other forms of rare anaemia (15, 20). The rheological properties and calcium-homeostasis of RBC were significantly altered, providing a potential mechanistic explanation for the premature haemolysis observed in these patients (26). Collectively, it appears that RBC-volume regulation *via* Ca^{2+} -homeostasis and endogenous NO-generation play pivotal roles in classic blood disorders rooted in abnormal properties of RBC. Given that only symptomatic treatments currently exist for these genetic blood disorders, pharmacological interventions to correct the acute regulation of Ca^{2+} and NO within RBC is a promising approach to restore cellular mechanics and ensure tissue perfusion. For example, specific inhibitors of piezo1-channels (e.g., GsMTx4) may be titrated to prevent intracellular Ca^{2+} -overload, while stimulators of RBC-NOS (e.g., L-arginine) or up-stream kinases (e.g., 740 Y-P) could increase RBC-derived NO and thus improve cell mechanics which ultimately increase tissue perfusion.

Conclusion

Evidence has accumulated over the past decade establishing a significant role for second-messenger molecules, such as NO and Ca^{2+} , in contributing to the *active* regulation of the physical properties of RBC. While several interactions and mechanisms remain to be elucidated, it appears that tight control over the concentration of these messengers is pivotal for ensuring RBC homeostasis, cellular deformability, and ultimately tissue perfusion. Thus while the last decade has provided a new perspective of the *active* regulation of RBC, integration of molecular techniques, electrophysiology, and rheological assessment of blood

appears a fruitful avenue for improved therapeutic approaches for cardiometabolic and haematologic disorders, and further in the refinement of mechanical circulatory support devices that remain plagued by poor complication rates.

References

1. **Bae C, Sachs F, and Gottlieb PA.** The mechanosensitive ion channel Piezo1 is inhibited by the peptide GsMTx4. *Biochemistry* 50: 6295-6300, 2011.
2. **Balach MM, Casale CH, and Campetelli AN.** Erythrocyte plasma membrane potential: past and current methods for its measurement. *Biophys Rev* 11: 995-1005, 2019.
3. **Barodka V, Mohanty JG, Mustafa AK, Santhanam L, Nyhan A, Bhunia AK, Sikka G, Nyhan D, Berkowitz DE, and Rifkind JM.** Nitroprusside inhibits calcium-induced impairment of red blood cell deformability. *Transfusion* 54: 434-444, 2014.
4. **Baskurt OK, and Meiselman HJ.** Blood rheology and hemodynamics. *Semin Thromb Hemost* 29: 435-450, 2003.
5. **Belanger AM, Keggi C, Kanas T, Gladwin MT, and Kim-Shapiro DB.** Effects of nitric oxide and its congeners on sickle red blood cell deformability. *Transfusion* 55: 2464-2472, 2015.
6. **Bogdanova A, Makhro A, Wang J, Lipp P, and Kaestner L.** Calcium in red blood cells-a perilous balance. *International journal of molecular sciences* 14: 9848-9872, 2013.
7. **Brugnara C, Gee B, Armsby CC, Kurth S, Sakamoto M, Rifai N, Alper SL, and Platt OS.** Therapy with oral clotrimazole induces inhibition of the Gardos channel and reduction of erythrocyte dehydration in patients with sickle cell disease. *J Clin Invest* 97: 1227-1234, 1996.
8. **Cahalan SM, Lukacs V, Ranade SS, Chien S, Bandell M, and Patapoutian A.** Piezo1 links mechanical forces to red blood cell volume. *eLife* 4: 2015.
9. **Cai Y, Rossier O, Gauthier NC, Biais N, Fardin MA, Zhang X, Miller LW, Ladoux B, Cornish VW, and Sheetz MP.** Cytoskeletal coherence requires myosin-IIA contractility. *J Cell Sci* 123: 413-423, 2010.
10. **Chien S.** Red cell deformability and its relevance to blood flow. *Annual review of physiology* 49: 177-192, 1987.
11. **Clark MR, Mohandas N, Feo C, Jacobs MS, and Shohet SB.** Separate mechanisms of deformability loss in ATP-depleted and Ca-loaded erythrocytes. *J Clin Invest* 67: 531-539, 1981.
12. **Connes P, Alexy T, Detterich J, Romana M, Hardy-Dessources MD, and Ballas SK.** The role of blood rheology in sickle cell disease. *Blood Rev* 30: 111-118, 2016.
13. **Danielczok JG, Terriac E, Hertz L, Petkova-Kirova P, Lautenschlager F, Laschke MW, and Kaestner L.** Red Blood Cell Passage of Small Capillaries Is Associated with Transient Ca(2+)-mediated Adaptations. *Frontiers in physiology* 8: 979, 2017.
14. **Dyrda A, Cytlak U, Ciuraszkiewicz A, Lipinska A, Cueff A, Bouyer G, Egee S, Bennekou P, Lew VL, and Thomas SL.** Local membrane deformations activate Ca²⁺-dependent K⁺ and anionic currents in intact human red blood cells. *PloS one* 5: e9447, 2010.
15. **Fermo E, Bogdanova A, Petkova-Kirova P, Zaninoni A, Marcello AP, Makhro A, Hanggi P, Hertz L, Danielczok J, Vercellati C, Mirra N, Zanella A, Cortelezzi A, Barcellini W, Kaestner L, and Bianchi P.** 'Gardos Channelopathy': a variant of hereditary Stomatocytosis with complex molecular regulation. *Sci Rep* 7: 1744, 2017.

16. **Fischer TM, Stohr-Lissen M, and Schmid-Schonbein H.** The red cell as a fluid droplet: tank tread-like motion of the human erythrocyte membrane in shear flow. *Science* 202: 894-896, 1978.
17. **Forstermann U, Pollock JS, Schmidt HH, Heller M, and Murad F.** Calmodulin-dependent endothelium-derived relaxing factor/nitric oxide synthase activity is present in the particulate and cytosolic fractions of bovine aortic endothelial cells. *Proc Natl Acad Sci U S A* 88: 1788-1792, 1991.
18. **Fraser KH, Zhang T, Taskin ME, Griffith BP, and Wu ZJ.** A quantitative comparison of mechanical blood damage parameters in rotary ventricular assist devices: shear stress, exposure time and hemolysis index. *Journal of biomechanical engineering* 134: 081002, 2012.
19. **Gardos G.** The function of calcium in the potassium permeability of human erythrocytes. *Biochimica et biophysica acta* 30: 653-654, 1958.
20. **Glogowska E, Schneider ER, Maksimova Y, Schulz VP, Lezon-Geyda K, Wu J, Radhakrishnan K, Keel SB, Mahoney D, Freidmann AM, Altura RA, Gracheva EO, Bagriantsev SN, Kalfa TA, and Gallagher PG.** Novel mechanisms of PIEZO1 dysfunction in hereditary xerocytosis. *Blood* 130: 1845-1856, 2017.
21. **Gorter E, and Grendel F.** On Bimolecular Layers of Lipoids on the Chromocytes of the Blood. *J Exp Med* 41: 439-443, 1925.
22. **Grau M, Friederichs P, Krehan S, Koliymitra C, Suhr F, and Bloch W.** Decrease in red blood cell deformability is associated with a reduction in RBC-NOS activation during storage. *Clinical hemorheology and microcirculation* 60: 215-229, 2015.
23. **Grau M, Mozar A, Charlot K, Lamarre Y, Weyel L, Suhr F, Collins B, Jumet S, Hardy-Dessources MD, Romana M, Lemonne N, Etienne-Julan M, Antoine-Jonville S, Bloch W, and Connes P.** High red blood cell nitric oxide synthase activation is not associated with improved vascular function and red blood cell deformability in sickle cell anaemia. *Br J Haematol* 168: 728-736, 2015.
24. **Grau M, Pauly S, Ali J, Walpurgis K, Thevis M, Bloch W, and Suhr F.** RBC-NOS-dependent S-nitrosylation of cytoskeletal proteins improves RBC deformability. *PLoS one* 8: e56759, 2013.
25. **Gulliver G.** Medical Times and Gazette. London: John Churchill & Sons 1862.
26. **Hertz L, Huisjes R, Llaudet-Planas E, Petkova-Kirova P, Makhro A, Danielczok JG, Egee S, Del Mar Manu-Pereira M, van Wijk R, Vives Corrons JL, Bogdanova A, and Kaestner L.** Is Increased Intracellular Calcium in Red Blood Cells a Common Component in the Molecular Mechanism Causing Anemia? *Frontiers in physiology* 8: 673, 2017.
27. **Horobin JT, Watanabe N, Hakozaiki M, Sabapathy S, and Simmonds MJ.** Shear-stress mediated nitric oxide production within red blood cells: A dose-response. *Clin Hemorheol Microcirc* 2018.
28. **Joiner CH.** Gardos pathway to sickle cell therapies? *Blood* 111: 3918-3919, 2008.
29. **Kaestner L.** Channelizing the red blood cell: molecular biology competes with patch-clamp. *Front Mol Biosci* 2: 46, 2015.
30. **Kirklin JK, Pagani FD, Kormos RL, Stevenson LW, Blume ED, Myers SL, Miller MA, Baldwin JT, Young JB, and Naftel DC.** Eighth annual INTERMACS report: Special focus on framing the impact of adverse events. *J Heart Lung Transplant* 36: 1080-1086, 2017.
31. **Kleinbongard P, Schulz R, Rassaf T, Lauer T, Dejam A, Jax T, Kumara I, Gharini P, Kabanova S, Ozuyaman B, Schnurch HG, Godecke A, Weber AA, Robenek M, Robenek H, Bloch W, Rosen P, and Kelm M.** Red blood cells express a functional endothelial nitric oxide synthase. *Blood* 107: 2943-2951, 2006.

32. **Kuck L, Grau M, Bloch W, and Simmonds MJ.** Shear Stress Ameliorates Superoxide Impairment to Erythrocyte Deformability With Concurrent Nitric Oxide Synthase Activation. *Front Physiol* 10: 36, 2019.
33. **Lauf PK, and Adragna NC.** Properties and membrane transport mechanisms of erythrocytes. In: *Erythrocytes: Physiology and pathophysiology* World Scientific, 2012, p. 57-228.
34. **Lew VL, and Tiffert T.** On the Mechanism of Human Red Blood Cell Longevity: Roles of Calcium, the Sodium Pump, PIEZO1, and Gardos Channels. *Frontiers in physiology* 8: 977, 2017.
35. **Lew VL, Tsien RY, Miner C, and Bookchin RM.** Physiological $[Ca^{2+}]_i$ level and pump-leak turnover in intact red cells measured using an incorporated Ca chelator. *Nature* 298: 478-481, 1982.
36. **Lux SEt.** Anatomy of the red cell membrane skeleton: unanswered questions. *Blood* 127: 187-199, 2016.
37. **Maher AD, and Kuchel PW.** The Gardos channel: a review of the Ca^{2+} -activated K^+ channel in human erythrocytes. *The international journal of biochemistry & cell biology* 35: 1182-1197, 2003.
38. **Mehta JL, Mehta P, and Li D.** Nitric oxide synthase in adult red blood cells: vestige of an earlier age or a biologically active enzyme? *J Lab Clin Med* 135: 430-431, 2000.
39. **Nagarajan S, Raj RK, Saravanakumar V, Balaguru UM, Behera J, Rajendran VK, Shathya Y, Ali BM, Sumantran V, and Chatterjee S.** Mechanical perturbations trigger endothelial nitric oxide synthase activity in human red blood cells. *Sci Rep* 6: 26935, 2016.
40. **Nguetse CN, Purington N, Ebel ER, Shakya B, Tetard M, Kremsner PG, Velavan TP, and Egan ES.** A common polymorphism in the mechanosensitive ion channel PIEZO1 is associated with protection from severe malaria in humans. *Proc Natl Acad Sci U S A* 117: 9074-9081, 2020.
41. **O'Rear EA, Udden MM, McIntire LV, and Lynch EC.** Reduced erythrocyte deformability associated with calcium accumulation. *Biochimica et biophysica acta* 691: 274-280, 1982.
42. **Pasini EM, Kirkegaard M, Mortensen P, Lutz HU, Thomas AW, and Mann M.** In-depth analysis of the membrane and cytosolic proteome of red blood cells. *Blood* 108: 791-801, 2006.
43. **Rooks H, Brewin J, Gardner K, Chakravorty S, Menzel S, Hannemann A, Gibson J, and Rees DC.** A gain of function variant in PIEZO1 (E756del) and sickle cell disease. *Haematologica* 104: e91-e93, 2019.
44. **Sanchez FA, Savalia NB, Duran RG, Lal BK, Boric MP, and Duran WN.** Functional significance of differential eNOS translocation. *American journal of physiology Heart and circulatory physiology* 291: H1058-1064, 2006.
45. **Shiga T, Sekiya M, Maeda N, Kon K, and Okazaki M.** Cell age-dependent changes in deformability and calcium accumulation of human erythrocytes. *Biochimica et biophysica acta* 814: 289-299, 1985.
46. **Shutova MS, and Svitkina TM.** Common and Specific Functions of Nonmuscle Myosin II Paralogs in Cells. *Biochemistry (Mosc)* 83: 1459-1468, 2018.
47. **Simchon S, Jan KM, and Chien S.** Influence of reduced red cell deformability on regional blood flow. *Am J Physiol* 253: H898-903, 1987.
48. **Simmonds MJ, Sabapathy S, Gass GC, Marshall-Gradisnik SM, Haseler LJ, Christy RM, and Minahan CL.** Heart rate variability is related to impaired haemorheology in older women with type 2 diabetes. *Clinical hemorheology and microcirculation* 46: 57-68, 2010.

49. **Smith AS, Nowak RB, Zhou S, Giannetto M, Gokhin DS, Papoin J, Ghiran IC, Blanc L, Wan J, and Fowler VM.** Myosin IIA interacts with the spectrin-actin membrane skeleton to control red blood cell membrane curvature and deformability. *Proc Natl Acad Sci U S A* 115: E4377-E4385, 2018.
50. **Stocker JW, De Franceschi L, McNaughton-Smith GA, Corrocher R, Beuzard Y, and Brugnara C.** ICA-17043, a novel Gardos channel blocker, prevents sickled red blood cell dehydration in vitro and in vivo in SAD mice. *Blood* 101: 2412-2418, 2003.
51. **Suhr F, Brenig J, Muller R, Behrens H, Bloch W, and Grau M.** Moderate exercise promotes human RBC-NOS activity, NO production and deformability through Akt kinase pathway. *PloS one* 7: e45982, 2012.
52. **Takakuwa Y, Ishibashi T, and Mohandas N.** Regulation of red cell membrane deformability and stability by skeletal protein network. *Biorheology* 27: 357-365, 1990.
53. **Tomaiuolo G.** Biomechanical properties of red blood cells in health and disease towards microfluidics. *Biomicrofluidics* 8: 051501, 2014.
54. **Ulker P, Yaras N, Yalcin O, Celik-Ozenci C, Johnson PC, Meiselman HJ, and Baskurt OK.** Shear stress activation of nitric oxide synthase and increased nitric oxide levels in human red blood cells. *Nitric oxide : biology and chemistry* 24: 184-191, 2011.
55. **von Tempelhoff GF, Schelkunov O, Demirhan A, Tsikouras P, Rath W, Velten E, and Csorba R.** Correlation between blood rheological properties and red blood cell indices(MCH, MCV, MCHC) in healthy women. *Clinical hemorheology and microcirculation* 62: 45-54, 2016.
56. **Wang CH, and Popel AS.** Effect of red blood cell shape on oxygen transport in capillaries. *Math Biosci* 116: 89-110, 1993.
57. **Weed RI, LaCelle PL, and Merrill EW.** Metabolic dependence of red cell deformability. *J Clin Invest* 48: 795-809, 1969.
58. **Yoon C, Choi C, Stapleton S, Mirabella T, Howes C, Dong L, King J, Yang J, Oberai A, Eyckmans J, and Chen CS.** Myosin IIA-mediated forces regulate multicellular integrity during vascular sprouting. *Mol Biol Cell* 30: 1974-1984, 2019.

483 **FIGURE LEGENDS**

484 *Figure 1:* The physical properties classically known to facilitate the remarkable capacity of
485 RBC to reversibly deform.

486

487 *Figure 2:* The molecular composition of the cytoskeletal component in the RBC membrane,
488 that resides under the lipid bilayer; α - and β -spectrins self-associate head-to-head, while each
489 dimer is anchored to the lipid bilayer *via* either ankyrin or actin-4.1 complexes. Myosin IIA
490 connects actin molecules within the junctional complexes and thus stabilises the bi-concave
491 shape of the RBC.

492

493 *Figure 3:* A synthesis of the observed regulatory pathways involving nitric oxide (NO)
494 generation and calcium ion-movement (Ca^{2+}) within RBC yields a complex intracellular
495 signaling network. Mechanical stimulation *via* exposure to fluid shear in blood promotes
496 Ca^{2+} -influx *via* the mechanosensitive piezo1-channel (1). Complexation of Ca^{2+} with the
497 carrier protein calmodulin also occurs, which then collectively bind RBC-NOS (2). RBC-
498 NOS is subsequently activated, producing NO which appears to bind to α - and β -spectrins in
499 close proximity *via* S-nitrosylation of free cysteine residues, leading to increased flexibility
500 of the cytoskeleton and improved cellular deformability (3). Activation of the Gárdos channel
501 occurs in response to sustained Ca^{2+} -influx (4), which facilitates export of potassium ions
502 (K^+) and leads to a loss of intracellular fluid (5). Simultaneously, Ca^{2+} is slowly transported
503 out of the RBC *via* the plasma membrane Ca^{2+} -ATPase (6).

504

Cytosolic haemoglobin
concentration dictates
intracellular viscosity

Bi-concavity facilitates
capacity to deform *via*
high surface area to
volume ratio

Viscoelastic membrane comprised of lipid
bilayer and flexible membrane skeleton

Spectrin – Ankyrin – Band3 linkage