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Diagnostic morphology: biophysical indicators for iron-driven inflammatory diseases

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Most non-communicable diseases involve inflammatory changes in one or more vascular systems, and there is considerable evidence that unliganded iron plays major roles in this. Most studies concentrate on biochemical changes, but there are important biophysical correlates. Here we summarize recent microscopy-based observations to the effect that iron can have major effects on erythrocyte morphology, on erythrocyte deformability and on both fibrinogen polymerization and the consequent structure of the fibrin clots formed, each of which contributes significantly and negatively to such diseases. We highlight in particular type 2 diabetes mellitus, ischemic thrombotic stroke, systemic lupus erythematosus, hereditary hemochromatosis and Alzheimer's disease, while recognizing that many other diseases have co-morbidities (and similar causes). Inflammatory biomarkers such as ferritin and fibrinogen are themselves inflammatory, creating a positive feedback that exacerbates disease progression. The biophysical correlates we describe may provide novel, inexpensive and useful biomarkers of the therapeutic benefits of successful treatments.

Insight, innovation, integration

The Biological Insight of this manuscript is that morphological changes in both erythrocytes and fibrin clot structures are a significant accompaniment to a variety of diseases. The Technological Innovation is the use of advanced microscopy techniques (including atomic force microscopy) to measure these changes. The Benefit of Integration comes from the facts that (i) these readouts are at a much higher physiological or phenotypic level than the biochemical markers such as cytokines usually measured during inflammation, and (ii) by seeing their commonality across a range of inflammatory diseases we recognize this as genuinely Integrative Biology.

Introduction

Vascular and inflammatory diseases represent one of the major present medical burdens, and cardiovascular diseases (CVDs) are the number one cause of death globally.[1–6] In 2008, the number of CVD deaths represented 30% of global deaths.[1] CVDs are accompanied (and almost certainly partly caused) by various kinds of oxidative stress, and specific research studies have shown that oxidative stress is associated with the pathogenesis of diabetes, obesity, cancer, ageing, inflammation, neurodegenerative disorders, hypertension, apoptosis, cardiovascular diseases, heart failure, and so on (*e.g.* ref. 7–22). As their name implies, the various vascular diseases share a variety of properties, including raised levels of biochemical markers such as C-reactive protein,[23–25] fibrinogen[26] and leukocyte count,[27] as well as many small molecules characteristic of exposure to 'reactive oxygen species'.[28,29] Also raised are various cytokines such as tumor necrosis factor alpha (TNFα) and interleukin-6 (IL-6) that might serve as early indicators of vascular dysfunction,[30–32] and (most pertinently here) the iron storage protein ferritin.[33–36] The enzyme heme oxygenase-1 (HO-1) catalyzes the degradation of heme, and is the major source of free iron released from heme metabolism.[37–39] HO-1 may also be an important marker of inflammation, as upregulation of HO-1 occurs in a variety of situations that involve red blood cell lyses and release of heme; an example of this is in biomaterial–blood interaction during mechanical circulatory support.[40] HO-1 activity is also increased systemically in inflammatory disorders like diabetes mellitus,[41] rheumatoid arthritis[42] and also pulmonary embolism.[43] Markers like, *e.g.*, HO-1 and cytokines, as well as iron-related inflammatory markers like ferritin, are therefore important from a biochemical standpoint. However, the scope of the current manuscript is to focus on the biophysical properties of RBCs and fibrin.

Although erythrocytes or red blood cells (RBCs) do not contain a nucleus or mitochondria,[44,45] they are surprisingly complex

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and are probably the most studied cells in medical history.[46] The dynamics of RBCs represent one of the most important aspects of the cardiovascular system[47] and fundamental to this is RBC hemorheology, aggregation capabilities, deformability and viscosity.[46,48–53] Bound up with this is the entire (intrinsic and extrinsic) clotting cascade,[54,55] ending with the thrombin-catalysed formation of fibrin from fibrinogen.[56] However, a cell-based model of coagulation is also now thought to be a more inclusive model to adopt when we study coagulation. This model incorporates the vital role of cells in coagulation processes,[57] and is considered to correct deficiencies of the older largely cell-free cascade models.[58–62] Fig. 1 shows the traditional intrinsic and extrinsic pathways (waterfall pathway), as well as the cell-based pathway.

RBCs are not only heme-iron-carrying sacks that facilitate the transport of respiratory gases, but they also play a variety of other roles:

- RBCs play a fundamental role in hemostasis and homeostasis, assuring our general well-being.[63–65] Hemostasis and thrombus formation are integrally related to blood rheology and blood flow; furthermore,
- RBCs help in bringing platelets to the surface of an injured vessel wall, by random collisions between themselves and platelets, which allows platelets to move across flow streamlines in a form of "enhanced diffusion".[63]
- RBCs have a role in binding inflammatory mediators to surface receptors.[66,67]
- RBCs play a fundamental role in the inflammatory process including having a changed deformability, rheology or sedimentation rate.[53,65,67–80]

Central to cardiovascular health is the optimal functioning of RBCs, and central to a variety of vascular and degenerative diseases is the presence of poorly liganded iron[81–83] some of which comes from erythrocytes, and which leads to a variety of morphological and phenotypic manifestations. Therefore in this review, we will be taking an integrative approach that focuses on the use of biophysical properties of RBCs and fibrin(ogen) as diagnostic indicators of health. We shall be arguing that predictive, diagnostic morphology using RBCs may give us additional phenotypic information regarding the disease states and progression and that a "simple" finger prick might give us precise information regarding the health status of the individual. This manuscript is structured as shown in Fig. 2 (denoting the benefits[84] of an overview figure of this type). Overall, we see the role of poorly liganded iron, largely released from ferritin, as having multiple effects on the morphology of both fibrin and erythrocytes, all of which contribute (negatively) to a variety of inflammatory vascular diseases (discussed later in the manuscript).

Definitions and a brief overview

The following terms will be used in this review:

The hematocrit: is also known as packed cell volume (PCV) or erythrocyte volume fraction (EVF). This is the volume of blood that contains the RBCs when packed by centrifugation, and is typically expressed as a fraction or % of blood. The hematocrit % may give us important information regarding the inflammatory state of an individual.[79,85,86] A changed hematocrit (lower or higher values) is significantly correlated with higher cardiovascular risk[87–89] and with inflammatory conditions like rheumatoid arthritis.[89,90] Table 1 shows some inflammatory diseases and associated hematocrit and hemoglobin levels.

Hemorheology: is the study of blood flow or flow properties of blood in a vessel, with an emphasis on the behavior of RBCs as they interact with each other and as quantifiable biophysical patterns emerge from the interactions of the cells in the vascular system.[48,49] These rheological properties are influenced by pathophysiological processes, thereby increasing the clinical relevance of blood rheology information.[103] The oxygen flow depends,[104]

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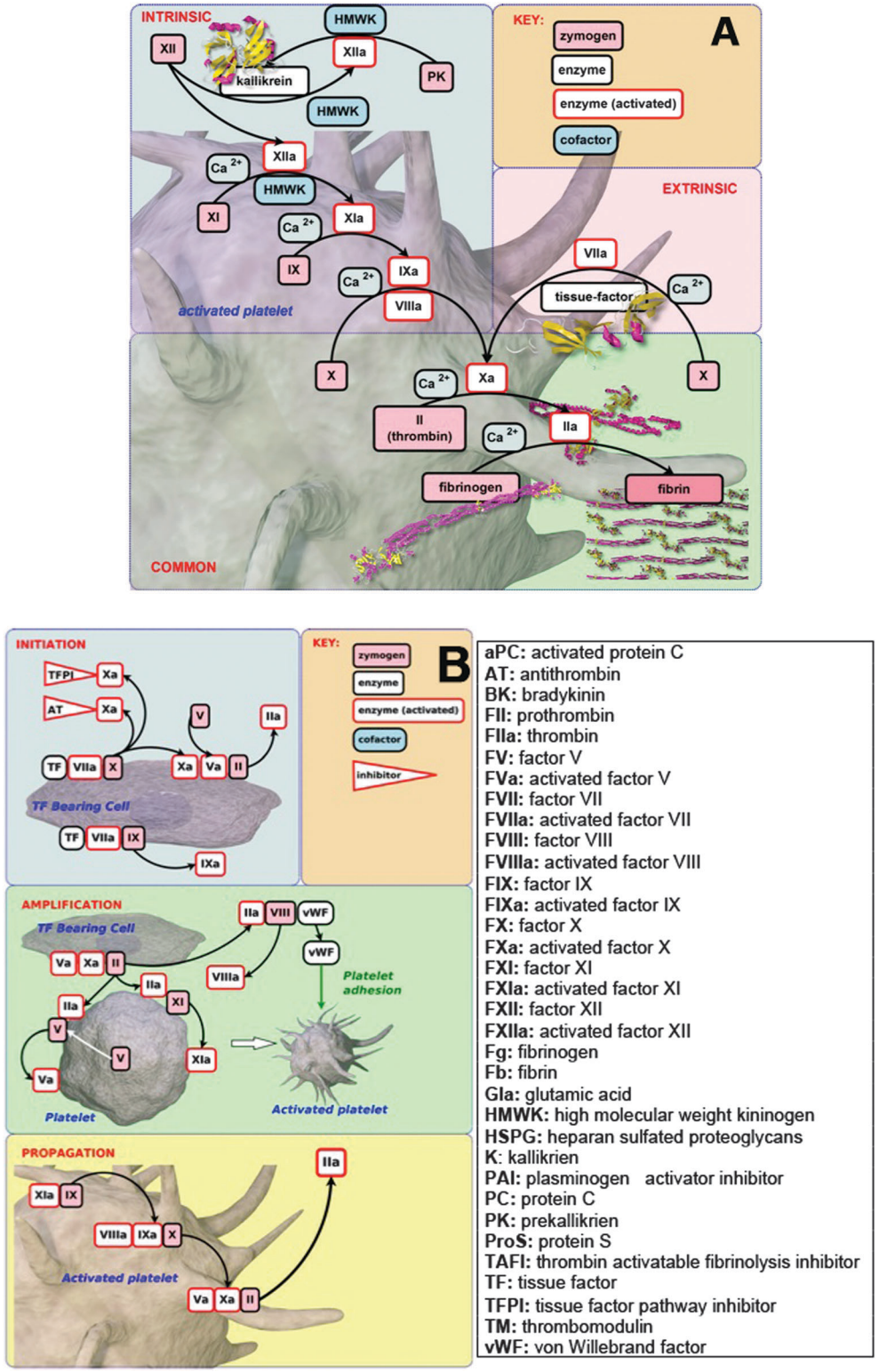

Fig. 1 (A) Waterfall (traditional intrinsic and extrinsic pathways). (B) The cell-based coagulation pathway. The pictures are broadly based on ref. 58 and were rendered by Dr Steve O'Hagan.

amongst other things, on the erythrocyte-to-blood volume ratio, which is represented by the hematocrit.[105,106] The flow properties of blood represent a major field on its own,[107–109] and for the scope of this paper, we will not discuss in much detail the rheological properties and patterns, although they follow from the various changes in cellular morphology and fibrin that we describe, and we recognize that they play a prominent role in all RBC properties discussed in the present paper.

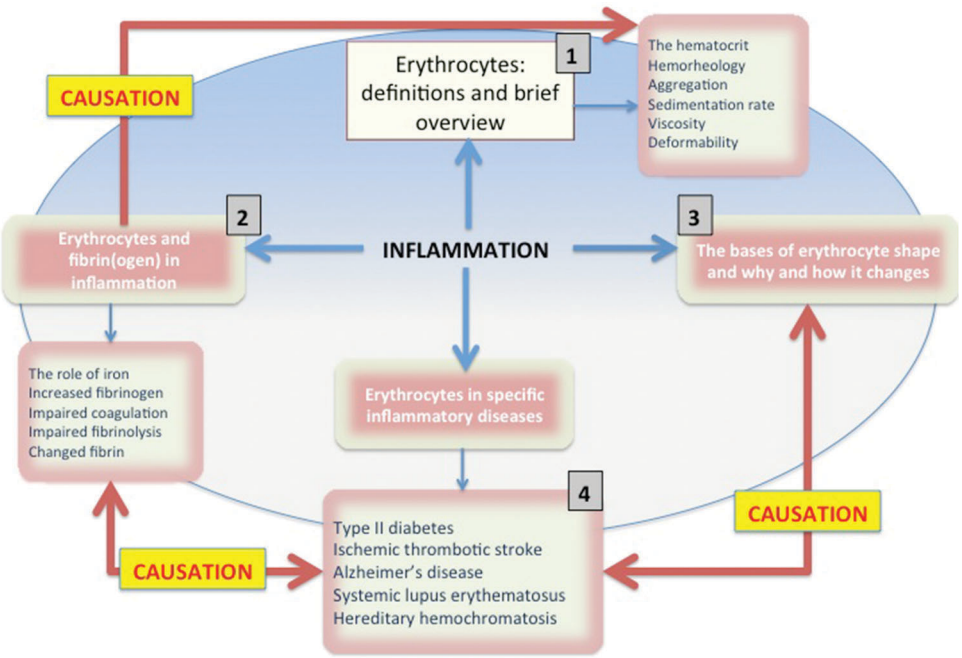

Fig. 2 An overview figure summarizing the contents of this manuscript.

Table 1 Some disease types and their hematocrit and hemoglobin counts (normal values of Hb: >13 g dl^{-1} for men; >12 g dl^{-1} for women; normal range for hematocrit: males: 40.7–50.3%; females: 36.1–44.3%)

Disease	Hematocrit
Coronary heart disease	High hematocrit[49]
Diabetes	High hematocrit[91,92] Low hematocrit[93]
Hypertension	High hematocrit[94]
Lupus erythematosus	Low hematocrit[95] Low Hb and low hematocrit[96]
Osteoarthritis	Low hematocrit and low Hb[97]
Pre-diabetes	High hematocrit[98]
Rheumatoid arthritis	Low Hb[99] Low Hb and hematocrit[100]
Stroke	Low hematocrit[101]
Venous thrombosis in women (recurring)	High hematocrit[102]

Aggregation and sedimentation rate: aggregation[110] can be defined as the reversible clumping of RBCs under low shear forces, while erythrocyte sedimentation rate (ESR) is based on the extent to which RBCs sediment, typically in 1 hour, and is expressed to the nearest mm in the first hour.[111] Both aggregation and ESR are changed (typically increased) in disease, and are therefore known to be an indicator of the presence of disease (*e.g.* ref. 49 and 112–116). When inflammation is present, RBCs tend to stick to each other more readily and the RBCs form rows or stacks, where they fit into each other, due to their

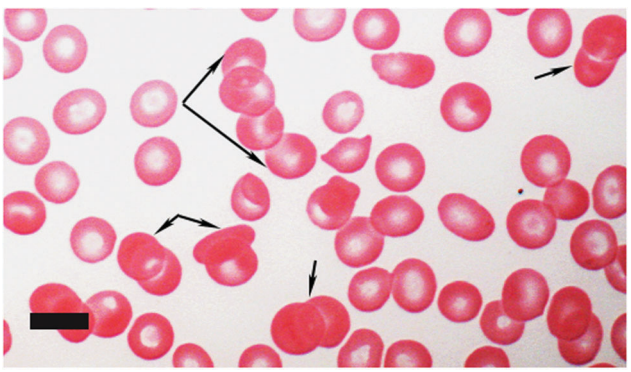

Fig. 3 Rouleau formation (black arrows) in RBCs as seen with light microscopy (40× magnification); scale = 10 μm.

discoid shape; this is known as rouleau formation (Fig. 3). Both aggregation and ESR can influence rouleau formation,[117] as can the presence of serum proteins.[118] The most important plasma protein assisting in the creation of the rouleau is fibrinogen.[119,120] A healthy plasma fibrinogen level is 2 to 4 mg mL^{-1},[121–123] while an increased fibrinogen level will cause an increase in rouleau formation.[50,51] Fibrinogen levels also influence the ESR[124] and (by virtue of their sticking to RBCs and making them 'heavier') increases in fibrinogen will result in an increased sedimentation rate.

Viscosity: closely linked to aggregation[125] is blood viscosity. RBC aggregation is considered to be the main determinant of blood viscosity (at low shear rate),[49,51] but fibrinogen contributes significantly to plasma viscosity. The viscosity of a fluid represents the friction between a moving fluid and a stationary wall.[48,126] The friction between the endothelial layer and the cellular components of blood will therefore be higher if there is an

increased blood viscosity. It is well known that hemostatic–thrombotic mechanisms are influenced by hemodynamic factors[127] and also that viscosity regulates how oxygen is transported to tissues and organs, and cardiovascular risks are also well-correlated with an increased viscosity.[48,49,85,93,98,127–129]

Deformability: RBC deformability is the ability to change shape, but to return to the original shape, which in the case of RBCs is a discoid shape. Deformability is an important determinant of blood viscosity.[46] This deformability is typically needed when the cells squeeze through narrow blood vessels and the RBC shape change occurs because of the blood flow resistance in the vascular system.[80] Therefore, deformability of RBCs, while they remain viable, is an important mechanical property of cells.[46] A reduced deformability is known to be present in conditions such as stroke;[130,131] it is linked directly to the RBC membrane properties including stiffness, rigidity and elasticity.[73,129,132–136] One of the important properties used to look at membrane stiffness (not only restricted to RBCs) is the Young's modulus, which is a very particular measure of stiffness and elasticity.[137,138] This can be measured using atomic force microscopy (AFM) technology, and is a measure of the stiffness of an elastic material,[139] and can generally be defined as stress divided by the corresponding strain, with greater values indicating increased stiffness or decreased deformability.[140] The method involves measuring the force as a probe is pushed (without membrane penetration) into the surface of and retracted from the sample in question. As each force curve's data can also be stored individually, it is possible to obtain quantitative measurements of the Young's modulus by fitting the slope of any force–distance curve of the image to an appropriate model.[141–143] It is therefore a very informative method that reflects the deformability of RBCs.[140]

All of the above properties will reflect and be reflected in the dynamics of RBC function and their capability to deform naturally under shear forces. During inflammation, RBCs have a changed shape (losing their biconcave shape) (see below), as well as inadequate flow properties, and this is thought to have major influences on the development of cardiovascular disease.[47] As we shall see, in addition to causing the biochemical changes alluded to above, inflammation also (and partly thereby) affects the mechanical properties of the RBC, the intrinsic cellular properties of the RBC membrane as well as the interactions of the RBCs with fibrin.[72,144–147] The next paragraphs will discuss the role of RBCs and fibrinogen in inflammation.

Red blood cells and fibrin(ogen) in inflammation

Inflammation is both a biological response and a pathologic process consisting of a dynamic complex cascade of events; central to this response is fibrin(ogen) and the interactions of RBCs. We first look at the role of fibrin(ogen) in inflammation. Fibrinogen, a high molecular weight (340 kDa) clottable protein of human blood, plays a major role in hemostasis and thrombosis,[148] by serving as a precursor of fibrin. Qualitative abnormalities of fibrinogen, also termed acquired dysfibrinogenemia, have been demonstrated in several disease states, mostly related to a prothrombotic tendency.[149] Increased fibrinogen levels are well-known in the inflammatory response,[86,87,150] and it is also well-known that increased fibrinogen is associated with cardiovascular events.[151–155] Therefore, it follows that long before a thrombotic event, fibrinogen levels are increased in the inflammatory patient.[152] Fibrin clot formation is part of the natural process of wound healing and tissue repair, and normally this is completed by removal of the clotted fibrin. Thus here we also have to look at fibrinolysis, as it is also known that fibrinolysis is impaired in inflammation and cardiovascular events.[56,156–160] During a thrombotic event, the increased levels of fibrinogen, as well as other parameters (*e.g.* increased oxidative stress and iron levels – discussed later), cause abnormal fibrin fiber formation, visible as a denser coagulated mass or dense matted deposits (DMDs) (Fig. 4), and the resulting coagulum causes blood cells to change shape and to be trapped in the abnormal mesh.[161] Therefore, the combination of a proneness to form a tighter fibrin network and impaired fibrinolysis is a feature of conditions like ischemic stroke,[162] diabetes type II[163,164] and numerous inflammatory diseases[165–167] (see Table 2). This tighter clot is therefore due to a general "hypercoagulability" also known as a prothrombotic state, and an increased fibrinogen level is associated with hypercoagulability.[168] Almost all inflammatory conditions are characterized by a hypercoagulable state, and this state may result in thrombotic events including thrombotic ischemic stroke.[169] Hypercoagulability is also present in diabetes type II, rheumatoid arthritis, heart conditions and other inflammatory conditions.[154,155,170–174] In other words, changed fibrinogen (whether it is increased fibrinogen levels or oxidative stress, *etc.*) is associated with hypercoagulability and results in a changed clotting profile.[175] See Table 3 for diseases where increased fibrinogen and hypercoagulability are prevalent. There is evidence that fibrinogen synthesis in the liver is stimulated by the inflammatory cytokine IL-6, and that it can thus be lowered by an appropriate inhibitor.[176,177] However, regardless of the concentration of fibrinogen, the nature of the clot that forms is particularly what matters, and Fig. 4 shows the terminal part of the typical cascade that results in the formation of either healthy or anomalous, diseased fibrin.

We have previously shown visually how fibrin, generated during coagulation, is changed markedly in inflammatory conditions, *e.g.* stroke[185,186,191,251] (Table 3 and Fig. 5A and B). Here, instead of a typical netted appearance seen in healthy individuals, the fibers form DMDs, with the individual fibers having a very much smaller diameter than that of normal fibrin fibers.[250] Importantly, these DMDs may be the cause of an enhanced prevalence of thrombotic events.[167] DMDs therefore reflect a hypercoagulability profile. Closely linked to this hypercoagulability of fibrin is the involvement of RBCs.[147,180] When fibrin clots abnormally, RBCs are entrapped more tightly inside the clot[72] (see Fig. 5B – RBC from a diabetic patient entrapped in fibrin). Previously, we have also found that fibrin fiber diameter is statistically significantly changed during thrombotic stroke where the fiber diameter becomes smaller and the fibers become netted.[250] Typically there are thick and thin fibers in a healthy fibrin clot, with the thick fibers the prevailing fibers.[250]

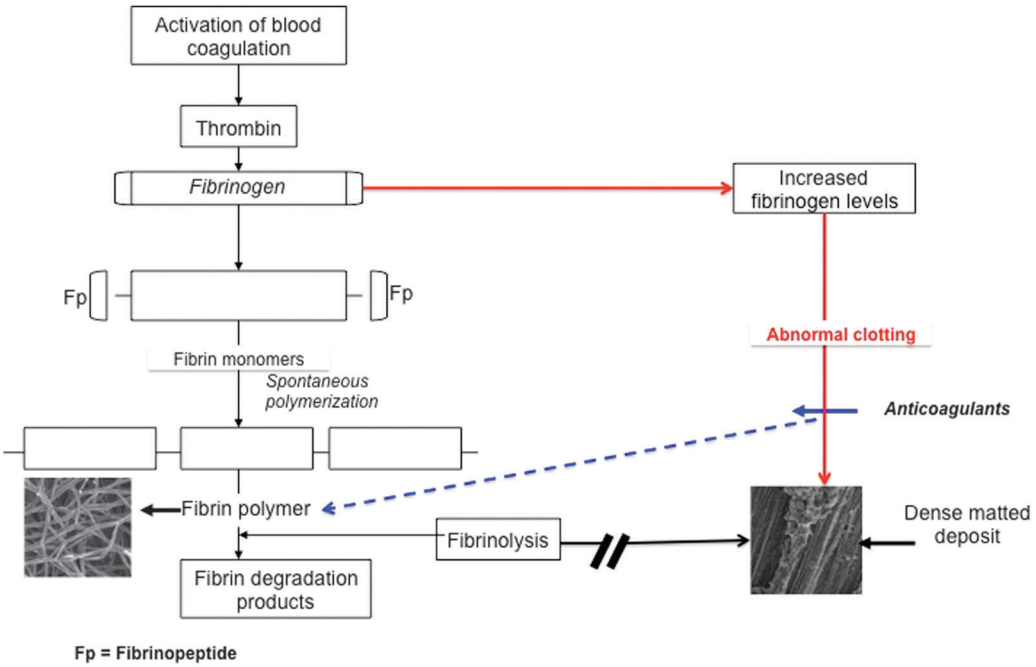

Fig. 4 Activation of blood coagulation under normal conditions and where abnormal fibrinogen is present. During normal coagulation, thrombin acts on fibrinogen to form fibrin polymer fibers, and these fibers will dissociate easily under fibrinolytic conditions. When there is an increased fibrinogen level, abnormal clotting takes place that forms a dense matted deposit, resistant to typical fibrinolysis. However, in the presence of anticoagulants, the fibrin polymerized to again form a typical net, as seen in normal coagulation – anticoagulants, *e.g.* warfarin[390] and aspirin,[391] are known to decrease fibrinogen levels. Adjusted from Lipinski and Pretorius.[167]

Table 2 Diseases where atypical fibrin fiber formation or an altered fibrin structure is prevalent – references particularly refer to a changed fibrin fiber structure as suggested by ultrastructure, scanning or transmission electron microscopy or laser-scanning confocal microscopy or clot lysis time

Disease	Representative references
Alzheimer's disease	140, 178, 179
Antiphospholipid syndrome	180
Arterial and venous thromboembolic diseases	56
Chronic obstructive pulmonary disorder	181
Coronary heart disease	182, 183
Diabetes type II	147, 184–188
Hereditary hemochromatosis	145
Lupus erythematosus	146, 189
Rheumatoid diseases	190–192
Thromboembolic pulmonary hypertension-associated dysfibrinogenemias	193
Thrombotic stroke and thrombotic disease	72, 76, 144, 159–161, 194–198

Table 3 Selected diseases where increased fibrinogen and hypercoagulability are known to be present

Disease	Representative references
Antiphospholipid syndrome	199–202
Alzheimer's disease	172, 203
Atherosclerosis	204–206
Cancer	207–210
Chronic obstructive pulmonary disorder	26, 86, 176, 177, 181, 211–230
Diabetes type II	93, 98, 152, 163, 231–233
Heart disease and cardiovascular risk	234–236
Lupus erythematosus	189, 201, 237–240
Metabolic syndrome	156, 232, 241–243
Rheumatoid diseases	155, 244–246
Thrombotic stroke/thrombosis	76, 148, 159, 160, 162, 194, 195, 247–250

To understand how fibrin is packaged in health and disease, we need to look at the structures of both fibrinogen and the fibrin clot.[56,181,197,198,252] Fibrinogen is a 340 kDa plasma glycoprotein consisting of two sets of three polypeptides (Aα, Bβ and γ) connected by 29 disulphide bonds[253,254] that assemble to form more than 20 distinct independently folded domains.[255] Thus, fibrinogen is a fibrous protein 45 nm in length with globular regions at each end and in the middle,[256]

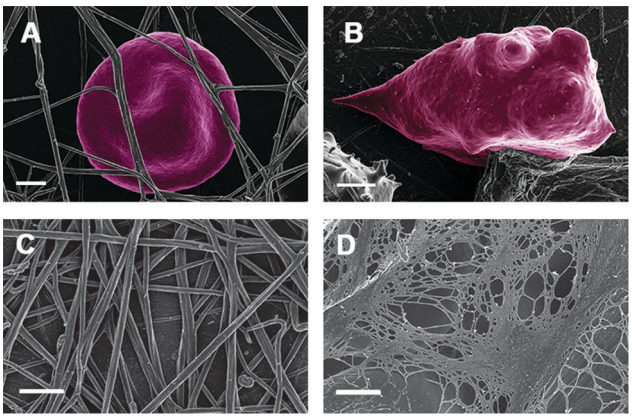

Fig. 5 (A) RBC of a healthy individual where the cells keep a discoid shape when whole blood is coagulated with the addition of thrombin; (B) RBC of a diabetic patient where the RBC is entrapped in atypical fibrin fibers; (C) healthy fibrin network; (D) fibrin network in thrombotic stroke. Scale = 1 μm.

and is composed of 2 Aα, 2 Bβ, and 2 γ-chains, arranged in a dimer of bilateral symmetry.[257] It has a central E-region and D-regions, which are connected with the E-region by a coiled coil segment, which is composed of the β- and γ-chain C-termini, and the α-chain C-termini fold back on the coiled coil and interact with the E-region[56] (see Fig. 6 – redrawn from ref. 56).

When a fibrin net is formed in a healthy individual, thrombin catalyzes the hydrolytic removal of fibrinopeptides Aα and Bβ from fibrinogen, converting the molecule to fibrin, revealing binding sites at its central domain that interact with complementary sites at the end domains of other fibrin molecules.[258] During the conversion of fibrinogen to fibrin, the αC domains dissociate from the central region, which allows them to interact intermolecularly, and this adds protofibrils to a fiber. The αC domain interactions appear to be for the enhancement of lateral aggregation during fibrin polymerization.[256]

These noncovalent interactions cause fibrin monomers to assemble in a half-staggered manner into two-stranded protofibrils.[259] Upon growing to sufficient length, the protofibrils aggregate laterally to form fibers that branch into a three-dimensional network.[260] After activation with thrombin, a pair of binding sites comprising Gly-Pro-Arg is exposed in the central nodule and combines with its complementary binding site α in the outer nodule of other molecules.[260] The Aα group of Aα Gly-1 is juxtaposed between γ Asp-364 and γ Asp-330, and the guanidino group of Aα Arg-3 between the carboxyl group of γ Asp-364 and γ Gln-329 in the α site.[260] Half molecule-staggered, double-stranded protofibrils are thus formed. Upon support of two adjacent D domains on the same strand, D–D self-association takes place involving Arg-275, Tyr-280, and Ser-300 of the γ-chain on the surface of the abutting two D domains. Thereafter, carboxy-terminal regions of the Aα-chains are released and interact with those of other protofibrils leading to the formation of thick fibrin bundles and networks,[260] typically seen when a healthy fibrin fiber net forms[259] (Fig. 5C) *versus* fibrin nets in thrombotic stroke (Fig. 5D). Yang and coworkers in 2000 mentioned that whether mature fibers are thick or thin depends on the relative contributions of different kinds of protofibril additions/interactions during fibrin assembly proteolyzed by thrombin.[261]

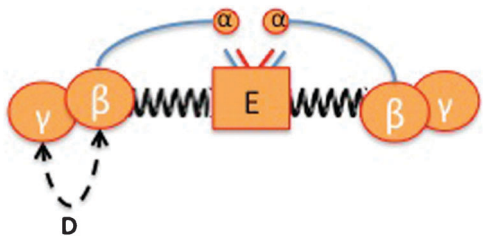

Fig. 6 Schematic representation of fibrinogen, redrawn from ref. 56. Fibrinogen consists of 2 Aα, 2 Bβ, and 2 γ-chains, with a central E-region and D-regions, which are connected with the E-region by a coiled coil segment, which is composed of the β- and γ-chain C-termini, and the α-chain C-termini fold back on the coiled coil and interact with the E-region. Fibrinopeptides A (red) and B (blue) are released by the action of thrombin, and this initiates polymerization of the fibrin protofibrils.

In the conversion from fibrinogen to fibrin there is therefore a series of large-scale conformational changes, and we have noted that the conversion of fibrinogen into the fibrin meshwork is changed significantly during stroke[250] and other inflammatory conditions (*e.g.* diabetes[185]) where altered fibrin and fibrinogen are present (see Table 2). This changed packaging was also noted in thrombotic diseases, where there are abnormal plasma levels of γA/γ′ fibrinogen.[253] Elevated levels have been associated with coronary artery disease[262] and stroke.[263] Also, the αC region is the origin of low modulus (1–10 MPa range), high extensibility, and strain stiffening in fibrin fibers.[254] Nitration of fibrinogen (caused by the initial reaction of the hydroxyl radical with nitric oxide[81,82]) is a prothrombotic risk factor.[264,265]

Pathophysiological fibrin(ogen) not only causes abnormal clotting and hypercoagulability, but also affects RBC rheology; therefore, it is appropriate to return to RBCs, and look at what happens to the RBCs during inflammation, specifically under the influence of this altered fibrinogen profile. Various epidemiological studies have shown the associations between inflammation and blood rheology, viscosity, haematocrit, and RBC aggregation and inflammation.[49,52,85,266–268] For a quick overview see Fig. 7, which shows the interactions between RBCs, inflammation, a prothrombotic state, and increased plasma fibrinogen levels, all causing pathorheology.

Under normal physiological conditions, RBCs are able to pass through narrow capillaries only as single cells rather than as aggregates.[50] The protein fibrinogen is an important determinant of RBC aggregation, with an almost linear relationship between aggregate size and plasma fibrinogen concentration.[85] Fibrinogen molecules adhere readily to the membrane surface of RBCs,[269] encouraging erythrocyte aggregation.[48,270,271] Therefore, it is not surprising that an increase in aggregation (under the control of increased fibrinogen levels in the plasma) is a well-known clinical manifestation in acute circulatory failure as well as acute coronary syndromes and stroke.[75,85,103,131,272–275] Due to fibrinogen attachment to the RBC membrane, not only aggregation is influenced, but also RBC viscosity. This increased viscosity will ultimately cause the RBCs to struggle to pass through narrow capillaries.[85] This increased viscosity is seen in

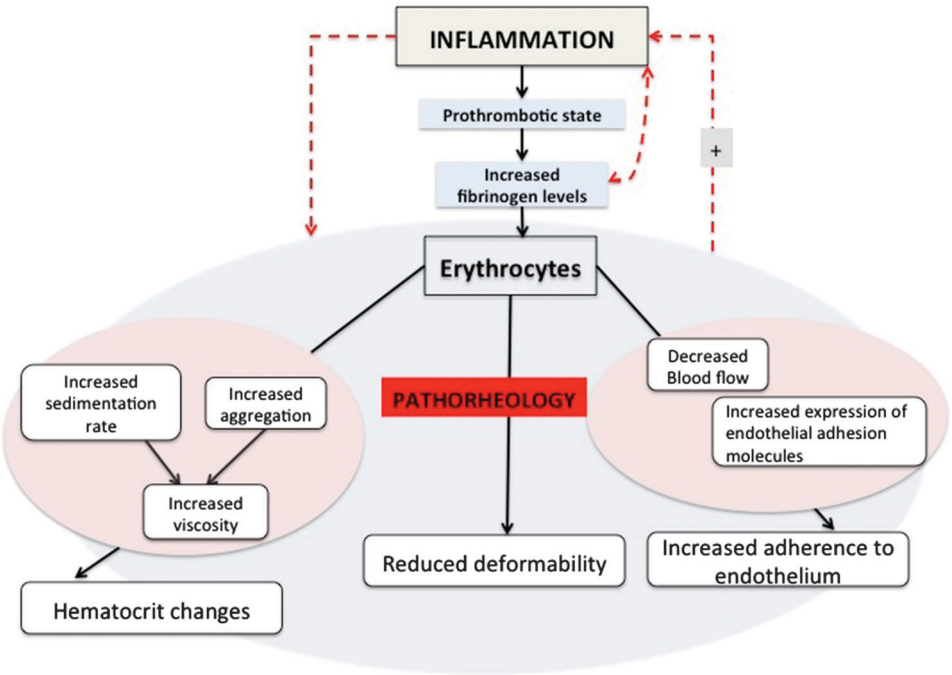

Fig. 7 Pathorheology due to inflammation, and how feedback causes an "inflammatory loop". Inflammation causes a prothrombotic state, which is associated with increased fibrinogen levels. Increased fibrinogen levels perpetuated inflammation. These processes cause pathorheology that involves major changes in RBCs (erythrocytes) – represented in the blue ellipse.

several clinical states.[49,51,98,127,128,133,276,277] It is thus appropriate to say that both RBC aggregation and fibrinogen interactions lead to a changed viscosity, and that this also plays an important role in optimal RBC functioning. It is therefore not surprising that increased blood viscosity and an increased fibrin concentration correlate with, and are strong predictors of, cardiovascular diseases and are important factors in the development of atherosclerosis.[85,276,278]

From the above paragraphs, it is clear that aggregation and viscosity are closely associated with (and indeed significantly due to) a changed fibrinogen profile, and that these parameters will ultimately influence flow dynamics.[279] Flow dynamics or rheology is closely associated with RBC shape and deformability, and during inflammation, we see reduced RBC deformability.[80]

The question that now arises is what might be the cause of the changed fibrin(ogen) cross-linking, noted in the above paragraphs. One of the major culprits, albeit not the only one, is the presence of iron and its role in the production of hydroxyl radicals (HR).[252,280–282] We have recently hypothesized that fibrinogen, as well as plasma proteins (in healthy individuals), can be converted into an insoluble, fibrin-like polymer by a non-enzymatic action of HR, following the addition of physiological levels of ferric iron.[161] Electrostatic interactions of iron with fibrinogen and fibrin are also likely responsible, and would be expected to inhibit Arg-Glu binding, for instance. Similar phenomena are seen in diseases of 'natural' iron overload such as hereditary hemochromatosis.[145,283–289] In inflammation, poorly liganded iron levels, and particularly serum ferritin levels, where the ferritin molecules have lost most of their iron en route from tissues, are increased in patients with diabetes type II, stroke, Alzheimer's disease, Parkinson's disease and many other inflammatory conditions.[34,81,82,171,290–296]

We have also recently shown that iron chelating and/or hydroxyl trapping agents are able to reverse the formation of this aberrant fibrin clot with its resultant excessive trapping of RBCs, even in the presence of iron overload.[140,145] The possibility of the reversal of the changed fibrin clotting is consistent with the view that the aberrant fibrin morphology resulting in trapped RBCs in the presence of increased iron (particularly serum ferritin) is caused, at least in part, by unliganded ('free') iron, whether derived directly *via* raised ferritin levels or otherwise, and that lowering it or affecting the consequences of its action may be of therapeutic benefit.

We can summarize the information from the previous paragraphs as follows:

In inflammatory conditions we see

- increased iron levels and, particularly, increased serum ferritin levels
- enhanced RBC aggregation and blood viscosity
- hypercoagulability, implying a changed fibrin(ogen) profile
- trapping of RBCs in abnormally clotted fibrin.

The trapping of RBCs during a prothrombotic state, associated with increased iron levels in diabetes, is illustrated in Fig. 8.

RBCs undergo a shape change during inflammation (which may be due to increased iron levels),[147,297] associated with a change in the hematocrit, aggregation and viscosity of RBCs and an abnormal clotting profile. It is known that RBCs are characterized by a shape change in many disease states.[46,73,266,298–300] Therefore, the next paragraphs will look more closely at RBC deformability properties and how and why they may change during the

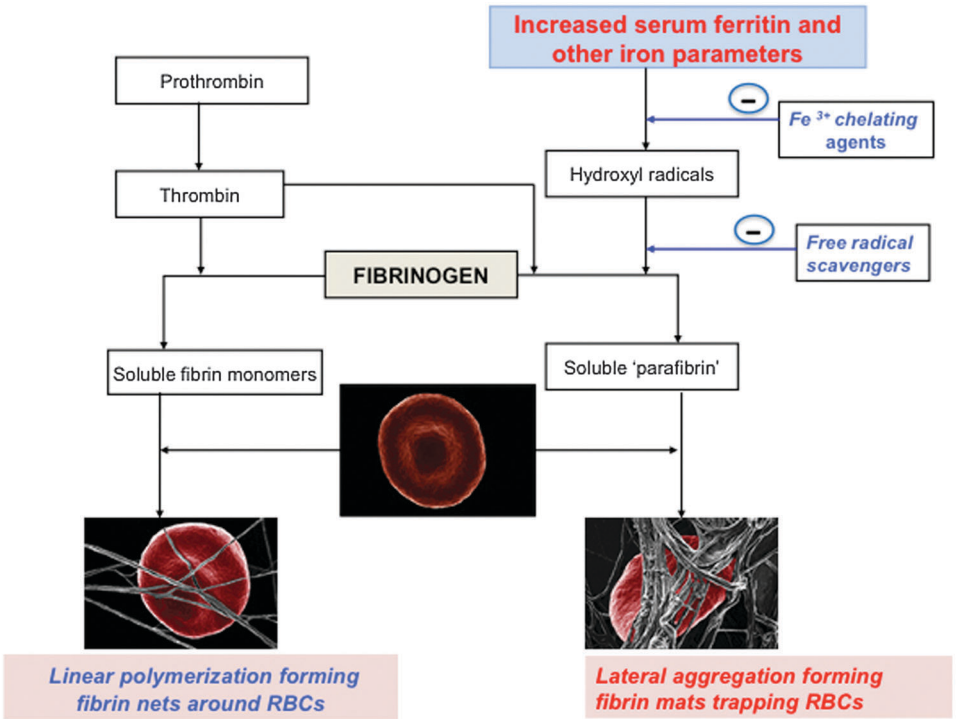

Fig. 8 Trapping of RBCs in aberrant fibrin in diabetes due to increased serum ferritin and/or iron. Adjusted from Lipinski and Pretorius.[392]

inflammatory process. Understanding the mechanics of RBC deformability under different pathophysiological conditions will lead to a better understanding of disease.

The bases of RBC shape and how and why it changes

Baskurt and Meiselman reviewed the role of RBC deformability in two comprehensive articles; they point out that RBCs are highly deformable, and that this physical property contributes significantly to aid blood flow, both under bulk flow conditions and in the microcirculation.[85,301] They also argued that the ability of RBCs to undergo reversible aggregation is an important determinant of viscosity, because the size of RBC aggregates is inversely proportional to the magnitude of shear forces; the aggregates are dispersed with increasing shear forces, then reform under low-flow or static conditions.[85] Recently, Dupire and co-workers showed that when RBCs 'flip' during flow, their orientation is determined by the shear rate. Under normal conditions, the cells have a ''rolling'' motion, and this motion permits the cells to avoid energetically costly deformations; it is a true signature of the cytoskeleton elasticity.[299] The authors also showed that the biconcave cell shape is highly stable under moderate shear stresses. However, changes in the properties and associations of membrane skeletal proteins, the ratio of RBC membrane surface area to cell volume, cell morphology, and cytoplasmic viscosity all govern RBC deformability. It is therefore not surprising that RBC deformability is significantly altered by various pathophysiological conditions, and the alterations in RBC deformability in turn influence pathophysiology.[302] Although the deformation of the RBC membrane is highly complex, it has three fundamental deformation modes – area expansion, shear, and bend of the membrane[134] – and this extraordinary combination of membrane properties allows the RBC to undergo extensive deformation without cell fragmentation.[136] We can therefore assume that when we have a changed RBC deformability, this will result in a changed erythrocyte physiology that can have a major adverse impact on blood flow. This will ultimately impact the homeostasis and hemostasis of the individual. However, to understand deformability, we need to look at the RBC membrane structure, and RBC's deformability may be a useful mechanical parameter for predicting the prognosis and for monitoring of patients.[303]

The membrane structure of RBCs consists of three layers:[134]

- an external carbohydrate-rich layer;
- the phospholipid bilayer of 4–5 nm thickness, embedded with transmembrane proteins;
- a 2-D triangular mesh-like spectrin–actin cytoskeleton network anchored to the phospholipid bilayer and ankyrin proteins. (Spectrin is a cytoskeletal protein that forms a hexagonal network that lines the intracellular side of the plasma membrane and interacts with short actin filaments that act as junctional complexes.)

The overlying asymmetric phospholipid bilayer membrane is supported by the underlying spectrin–actin cytoskeletal complex and it forms a simple hexagonal geometric matrix. Spectrins are flexible rods 0.2 microns in length with actin-binding sites at each end.[304] Spectrins are assembled from α and β subunits, each comprised primarily of multiple copies of

a 106-amino acid repeat. Spectrin α and β subunits are assembled antiparallel and side-to-side into heterodimers, which in turn are associated head-to-head to form tetramers.[304] The principal proteins at the spectrin–actin junction are protein 4.1, adducin, tropomyosin, tropomodulin, and dematin. Spectrin is coupled to the inner surface of the RBC membrane primarily through association with ankyrin and the transmembrane proteins band 3 and 4.1.[305,306] Band 3 is an abundant RBC integral membrane protein which has a membrane-spanning domain that catalyzes anion exchange and a cytoplasmic domain that binds proteins and thereby regulates the structure and function of the RBCs.[80]

Ankyrin proteins are adaptor proteins that mediate the attachment of the spectrin mesh to the integral membrane proteins, while band 3 is a transport protein responsible for mediating the exchange of chloride for bicarbonate across the membrane,[307] and it is thus a carrier-mediated transport protein. Band 4.1 (also known as Beatty's protein) is an important structural element of the membrane skeleton and it regulates mechanical stability and deformability by stabilizing the spectrin–actin interactions by interacting with the spectrin and actin filaments to form the bulk of the membrane skeleton.[308] The interaction of ankyrin and spectrin yields the major anchor between the membrane skeleton and the lipid bilayer and its optimal intact architecture is critical for RBC deformability and stability.[309] Ankyrins therefore organize and stabilize protein networks in collaboration with other proteins including cytoskeletal proteins, cell adhesion molecules and large structural proteins.[310] Cytoskeletal proteins such as β-spectrin contribute to the stability of the ankyrin protein network.[310]

Reduced deformability of RBCs is also an important feature in inflammation, also mediated by band 3, as well as by nitric oxide and ROS.[80] ROS can lead to protein degradation in RBCs and in particular degradation of membrane proteins such as band 3 and spectrin,[311] and in inflammation, a higher RBC band 3:α-spectrin ratio was associated with decreased RBC deformability. Importantly, RBC deformability was found to depend on the band 3 phosphorylation state.[312] The plasma membrane together with its cytoskeletal support is therefore responsible for the maintenance of the shape and stability of the RBC and also for allowing extensive deformations when needed.[184,305,313,314]

Modifications of the lipid composition and the asymmetry of the bilayer have also been shown to affect the overall shape of the RBC and also the cell's deformability, impact on particularly the erythrocyte membrane integrity, when encountering shear stresses.[315] Changes in the shape, mechanical characteristics or the integrity of RBCs have severe implications for the functionality of the cell, as can be seen in several dysfunctional states of the RBC, whether environmentally induced or due to hereditary defects or diseased states.[184,313,316]

Membrane roughness is another measure of interest. More specifically, it has been suggested that the cell-membrane skeleton integrity measured as surface roughness is well correlated with the functional status of the cell[317] with a decrease of the membrane roughness seen in cells from diseased or ageing individuals.[306,313,316,318–325]

Roughness is simply measured by R_{rms}, which is defined as follows:

$$R_{rms} = \sqrt{\sum_{i}^{N} \frac{(z_i - z_m)^2}{(N-1)}}$$

N is the total number of data points, z_i is the height of the ith point and z_m is the mean height, as measured by atomic force microscopy (AFM).[306]

What do we know about red blood cells in specific inflammatory diseases?

As mentioned in the previous paragraphs, fibrin and RBC deformability play important roles in hypercoagulability. One of the reasons for this may be due to unliganded iron, particularly that derived from serum ferritin. We have shown previously that in the presence of increased iron levels (particularly coupled to serum ferritin levels), RBCs lose their typical spherical shape. This was also seen in a number of inflammatory conditions like diabetes type II, stroke[72,144,147] and Alzheimer's disease.[140] The following sections discuss these changes in selected inflammatory conditions. This said, we are aware of a plethora of other diseases and cardiovascular risk factors that might influence RBCs, including type 1 diabetes, cigarette smoking, arterial hypertension, familial history of atherosclerosis, and even iron depletion (including iron deregulation in various types of anemia-related conditions). It is also well known that various drugs at appropriate concentrations can effect alterations in the structure of RBC membranes.[300,326,327] However, in the next sections, we will be focusing specifically on RBCs in inflammatory conditions, without drug-mediated interactions, and in the presence of increased iron. The conditions that will be discussed in the following paragraphs are type 2 diabetes, ischemic thrombotic stroke, Alzheimer's disease, systemic lupus erythematosus and hereditary hemochromatosis.

Type 2 diabetes mellitus

Diabetes mellitus is one of the most debilitating conditions that our society suffers from, and is regarded as a cardiovascular risk factor, as well as a cardiovascular disease, due to its ability to progress to a stage of cardiovascular co-morbidity.[328] Furthermore, diabetes is associated with oxidative stress and particularly the RBCs are vulnerable to this oxidative stress.[328] We also know that diabetes is associated with elevated serum ferritin levels.[171,293,329–332] We also know that increased iron levels cause oxidative stress.[81,82,295,333,334] RBCs are one of the cell types that are particularly vulnerable to oxidative stress found in this condition,[232] and oxidative stress is known to cause eryptosis.[335] Eryptosis is a form of suicidal cell death, similar to apoptosis, and is characterized by cell shrinkage, cell membrane blebbing, and cell membrane phospholipid scrambling. RBCs remain in a hyperglycemic environment throughout their lifespan, and this affects their flow properties through alteration of deformation at the individual cell level, as well as aggregation at a collective level.[336] Recently, we have shown that RBCs in diabetes have a changed shape as well as a decreased

membrane roughness.[147,167,184] Atomic force microscopy (AFM) was used to study membrane roughness, and scanning electron microscopy (SEM) was used to study RBC shape at high magnifications. RBCs in diabetes have a decreased membrane roughness, as well as an elongated shape. Measurements of surface roughness indicated alterations in the first order surface of the cell, relating to the cells' macro parameters. The roughness of the second order surface was also decreased, indicating alterations in the cytoskeletal matrix and the connections between band 3 and 4 proteins and the matrix. A decrease in roughness measurements in the third order surface indicates superficial protein structure rearrangement. The cytoskeletal proteins of RBCs from diabetic patients are heavily glycosylated and spectrin is oxidatively damaged;[73] also several lipids (free cholesterol, sphingomyelin and phosphatidylcholine) on the outer surface of the phospholipid bilayer are significantly decreased.[337,338] This directly correlates with the ultrastructural roughness results seen by the AFM of the second and third order respectively.[184] Previous studies suggest that RBC rheology is altered in diabetes type II,[85,266] and the results from the roughness and shape change support this.

Ischemic thrombotic stroke

Thrombotic stroke is one of the major cardiovascular diseases and it is also closely associated with oxidative stress[10,334] as well as an increase in fibrinogen levels and therefore hypercoagulability.[247] It is also known that iron plays an important role in the pathogenesis of cardiovascular diseases,[291,339] including stroke.[340–344] During ischemic stroke, erythrocytes undergo oxidative and proteolytic changes resulting in a changed cellular rheology and inflammatory processes. RBC changes have also been noted in thrombotic stroke and this includes sedimentation rate changes,[112,115] changes of erythrocyte aggregation,[274] a raised blood viscosity,[345] and an impaired plasticity of the RBCs.[69,131] We have previously reported that in thrombo-embolic ischemic stroke patients, 92% of erythrocytes present in smears are abnormal as viewed by a SEM.[72,76,144] We also noted that in stroke, RBCs change shape and form close interactions with the abnormal fibrin fibers typically associated with the diseased clot.[72] In blood smears of each of more than 60 stroke patients viewed by an SEM, RBCs have a changed form showing membrane pseudopodia-like extensions and some produce elongated membrane extensions that might be associated and form interactions with the fibrin fibers during pathological clot formation.

Alzheimer's disease

Alzheimer's disease (AD) is a devastating neurodegenerative dementing illness, also closely associated with oxidative stress.[346–348] Although the disease is typically seen as primarily affecting the brain, there is increasing evidence that vascular components, including RBCs and fibrinogen, play a fundamental role in the progression of the condition.[349–351] It is well-known that iron also plays a fundamental role in the progression of the disease.[81,82,290,291,296,329,352–358]

Increased RBC sedimentation and/or their aggregation have been observed in degenerative diseases such as atherosclerosis and inflammation, which are known to be associated with AD.[115,359] It is thought that the abnormalities in RBCs and their flow contribute to AD by obstructing oxygen delivery to the brain.[70,360,361] It is well-known that an oxygen deficit causes hypoxia and that this may lead to chronic inflammation.[362,363] Mohanty and co-workers in 2010 noted that 15% of RBCs in AD patients were elongated, and that there were alterations in the RBC membrane architecture.[364] There are also known interactions between RBCs and beta-amyloid and between fibrin and beta-amyloid[365,366] in AD patients and that these interactions may accelerate neurovascular damage.[367] We have recently shown that AD individuals with increased iron levels possess a changed RBC morphology. This was shown with light microscopy, SEM, AFM and confocal microscopy.[140] We have also shown that this subgroup of AD patients have a decreased RBC membrane elasticity, measured using force–distance curves, and that the axial ratios and shape of the RBCs differed markedly from those of healthy individuals. A changed RBC membrane deformability may therefore play an important role in AD pathology.

Systemic lupus erythematosus

Multisystem autoimmune rheumatic diseases of which systemic lupus erythematosus (SLE) forms a part are heterogeneous rare disorders associated with substantial morbidity and mortality,[368] and patients present a high prevalence of thrombotic and arteriosclerotic disease[239] as well as chronic inflammation.[369,370] SLE is also associated with an increase in serum ferritin levels.[289,371–374] Despite decades of extensive work on the understanding of the etiopathogenesis of SLE, few biomarkers have been validated and widely accepted for this disease.[375,376] Patients with SLE have enhanced RBC aggregation,[377] microparticle formation,[378] and a change in rheology.[240,379,380] Very little is known about the deformability of RBCs in this condition; however, we have recently shown that lupoid erythrocytes are fused and clumped.[146] This was suggested to be associated with distinct inflammatory ultrastructural changes found in the condition, which include platelet blebbing, generation of platelet-derived microparticles[146,190] and the spontaneous formation of massive fibrin networks. It was concluded[345] that the concerted actions of platelets and RBCs, as well as white blood cells caught in the inflammatory fibrin network, predispose to pro-thrombotic states in patients with SLE.

Hereditary hemochromatosis

Hereditary hemochromatosis (HH) is now probably the most well-known genetic iron overload disease.[109,381–383] RBC membranes in HH display an aggregation and enlargement of intramembrane particles in comparison with structures seen in membranes from healthy donors.[384] It is also known that the mean values of hemoglobin (Hb) in HH are raised.[385,386] Recently we have shown that in genetically typed HH individuals with increased iron levels, as well as in individuals without the iron overload mutations but with hyperferritinemia, RBCs have a changed ultrastructure.[145] In iron overload conditions, the sufferers are more prone to thrombotic diseases, mainly due to the presence of unliganded iron, and thereby the increased

oxidative stress.[387,388] Very striking differences were observed, in that the erythrocytes from HH and HF individuals were distorted and had a much greater axial ratio compared to that accompanying the discoid appearance seen in the normal samples.[145]

Concluding remarks

In inflammatory conditions, we see a number of changes in the biophysical properties of red blood cells, including in their hematocrit, aggregation, sedimentation rate and deformability. Closely associated with this is an increased or changed fibrin profile associated with hypercoagulability. The literature also suggests that inflammation is associated with increased serum ferritin levels. In diabetes, AD, HH as well as stroke and SLE, all of these are involved and interlinked with each other. However, what seems to be clear is that iron and in particular serum ferritin levels seem to underlie or correlate with all the rest of the parameters, in that when high serum ferritin levels are present in inflammatory conditions we find hypercoagulability and fibrin changes, as well as increased RBC aggregation and sedimentation. We have also shown that unliganded iron changes RBC shape. All of the above suggests strongly that morphological techniques may play a fundamental, and comparatively inexpensive, phenotypic role[389] in providing a quick overview of the general health of an individual. However, very few studies focus on the use of predictive RBC morphology under inflammatory conditions. RBCs are extremely adaptive cells due to their capability to deform quickly under shear forces. Predictive RBC morphology may therefore give us important insights regarding the health status of an individual, and may be used together with traditional biomarkers in inflammatory conditions. The efficacy of treatment regimes on the integrity, shape, elasticity and roughness (and therefore the general health status) of RBCs may be tracked using predictive morphology, as the health status of RBCs is crucial to the overall wellness of individuals with inflammatory conditions.

Ethical clearance and consent

Ethical clearance was obtained from the Health Sciences Ethical Committee from the University of Pretoria and informed consent was obtained from all individuals who donated blood samples.

Conflict of interest statement

The authors have declared that no competing interests exist.

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