The simultaneous occurrence of both hypercoagulability and hypofibrinolysis in blood and serum during systemic inflammation, and the roles of iron and fibrin(ogen)

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Although the two phenomena are usually studied separately, we summarise a considerable body of literature to the effect that a great many diseases involve (or are accompanied by) both an increased tendency for blood to clot (hypercoagulability) and the resistance of the clots so formed (hypofibrinolysis) to the typical, 'healthy' or physiological lysis. We concentrate here on the terminal stages of fibrin formation from fibrinogen, as catalysed by thrombin. Hypercoagulability goes hand in hand with inflammation, and is strongly influenced by the fibrinogen concentration (and *vice versa*); this can be mediated *via* interleukin-6. Poorly liganded iron is a significant feature of inflammatory diseases, and hypofibrinolysis may change as a result of changes in the structure and morphology of the clot, which may be mimicked *in vitro*, and may be caused *in vivo*, by the presence of unliganded iron interacting with fibrin(ogen) during clot formation. Many of these phenomena are probably caused by electrostatic changes in the iron–fibrinogen system, though hydroxyl radical ($OH^{\bullet}$) formation can also contribute under both acute and (more especially) chronic conditions. Many substances are known to affect the nature of fibrin polymerised from fibrinogen, such that this might be seen as a kind of bellwether for human or plasma health. Overall, our analysis demonstrates the commonalities underpinning a variety of pathologies as seen in both hypercoagulability and hypofibrinolysis, and offers opportunities for both diagnostics and therapies.

Insight, innovation, integration

The Biological Insight of this manuscript is that although they are usually studied separately (and thus typically described in separate papers) there is a considerable body of literature showing that both hypercoagulability and hypofibrinolysis are present in a large number of inflammatory, vascular diseases. This implies that they share common causes. Similarly, although these names reflect the changed kinetics of clot formation and lysis, there is also considerable evidence that the resistance to fibrinolysis comes because the nature (make-up and morphology) of the clot differs in these states. One established cause comes from the interaction of unliganded iron with fibrinogen. The Technological Innovation is the use of advanced microscopy techniques (including atomic force microscopy) to measure these changes. The Benefit of Integration comes (i) from bringing together these two separate readouts with a suggested major cause, *viz.* iron dysregulation, and (ii) by showing their commonality across a range of inflammatory vascular diseases we demonstrate this as Integrative Biology.

Introduction

Although the formation of blood clots and their subsequent removal are typical responses to the occurrence of damage in blood vessels,[1,2] and this may also be part of the innate immune system,[3–7] a common cause of thrombosis or thromboembolism (the formation of a blood clot that obstructs blood flow) is an excessive tendency toward blood clotting known[8] as hypercoagulability.

Thromboembolic diseases are caused when a blood vessel is obstructed by a blood clot (embolus), whether formed locally or by one that has been carried in the bloodstream from the site of its formation. Thromboembolic diseases include both venous thromboembolism and arterial thrombosis, and these are treated differently (both intellectually and therapeutically) (*e.g.* ref. 9–13).

Thromboembolic events are responsible for approximately 80% of human strokes,[14] specifically a type of stroke caused by

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the interruption of blood flow to a part of the brain due to the slow formation of a blood clot along the lumen of an artery. Cryptogenic ischaemic strokes are now thought to comprise about 25% of all ischaemic strokes and there is evidence that most cryptogenic strokes are thromboembolic.[15] The thrombus is thought to originate from any of several well-established potential embolic sources, including minor-risk or covert cardiac sources, veins *via* paradoxical embolism, and non-occlusive atherosclerotic plaques in the aortic arch, cervical, or cerebral arteries.[15] However, the most significant cause of thromboembolic stroke is considered to be due to a complication of atrial fibrillation (fibrillation of the muscles of the atria of the heart causing arrhythmia).[16,17]

Sudden occlusion of a cerebral blood vessel by a thrombus or embolism initiates a complex process of events that include excitotoxicity, oxidative stress, microvascular injury, blood brain barrier dysfunction and postischemic inflammation that ultimately leads to cell death.[18] Also, most pertinently, thromboembolic events are associated with a hypercoagulable state.[19,20]

A great many 'risk' factors (including both genetic[21–28] and environmental or 'lifestyle', as well as those accompanying medical interventions[29–32]) can contribute to this hypercoagulability,[33–38] and understanding these (Fig. 1) and how they interact probably makes this a problem that is thus best considered using the tools and ideas (*e.g.* ref. 39–43) of systems biology. While many diseases of haemostasis also involve a tendency to haemorrhage and/or inadequate clot formation, our focus will be on those coagulopathies where the rate and/or nature of the clot formation are greater, and its relative resistance to fibrinolysis is less, than optimal. An overview of the manuscript is given in Fig. 2.

Relationship between chronic inflammation and hypercoagulability

While a precise definition of chronic inflammation is slightly elusive, and is usually based around the observation of changes in various ('inflammatory') cytokines (*e.g.* ref. 44–46), there is abundant evidence that one of its hallmarks (or at least an extremely common co-existence in many circumstances) is a hypercoagulable state of some kind (*e.g.* ref. 10, 47–79).

Consequently, as we shall see, the long lists of inflammatory diseases that are additionally associated with iron dysregulation (*e.g.* ref. 80–85) are also those in which a hypercoagulable and/or hypofibrinolytic state may be observed.

The healthy clotting cascade, mainly focussing on the thrombin pathway and on factor XIII crosslinking

During healthy (normal) blood clotting, there are both 'intrinsic' and 'extrinsic' pathways (reviewed *e.g.* in ref. 86–88), but both converge on terminal steps in which prothrombin is converted

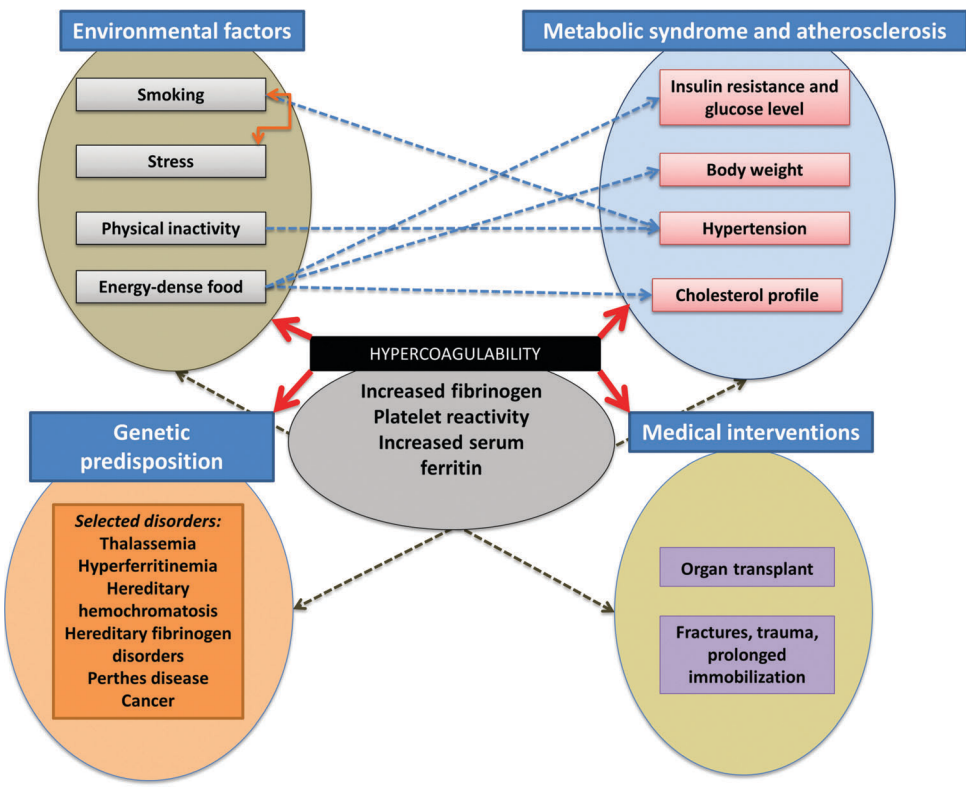

Fig. 1 Some risk factors, associated with hypercoagulability. These include environmental factors, genetic predisposition, some lifestyle factors that an individual may have control over, potentially causing metabolic syndrome and atherosclerosis, as well as medical interventions.

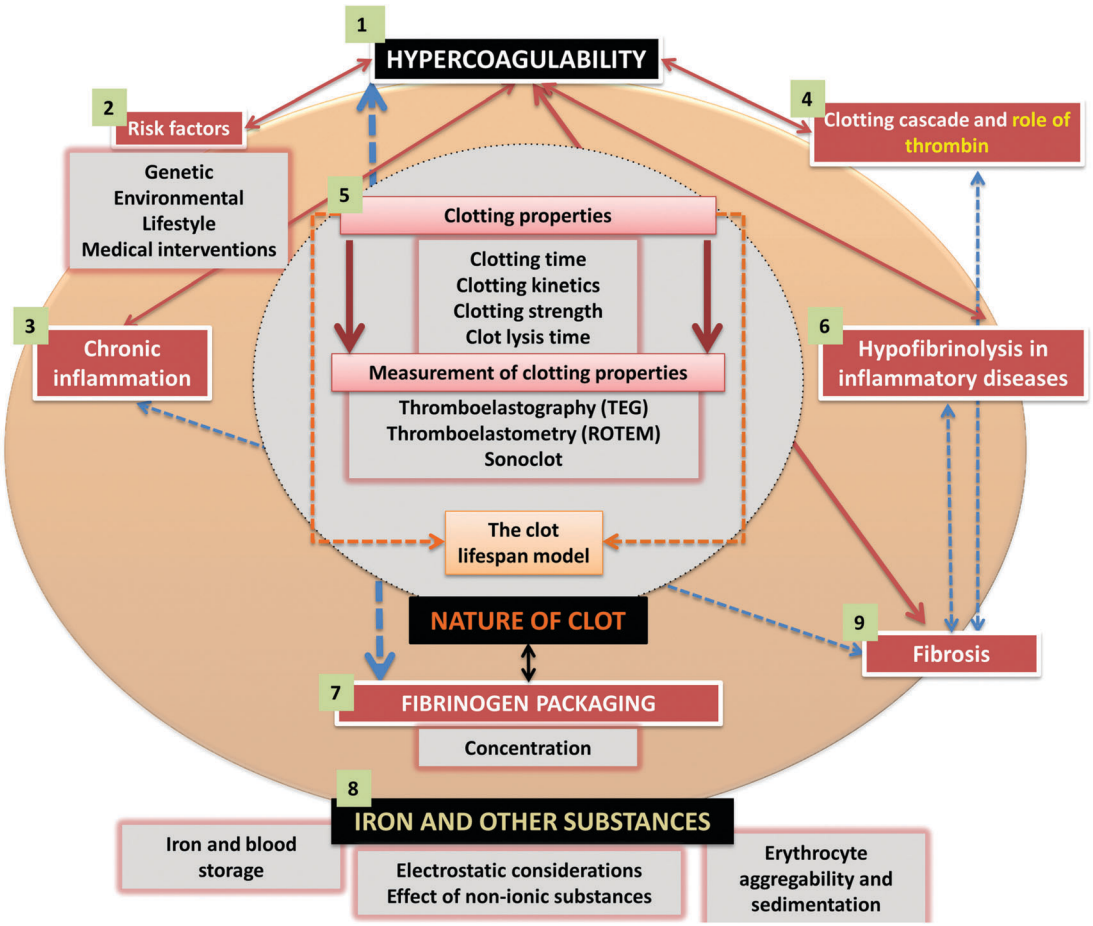

Fig. 2 An overview of the manuscript. The first focus of the manuscript is hypercoagulability (1). We discuss risk factors (2) and chronic inflammation (3), and how the clotting cascade (4) is changed during hypercoagulability. We also focus on clotting properties that are measurable using the current leading methods of thromboelastography (TEG), thromboelastometry (ROTEM) and Sonoclot technology (5). We show how this provides insight into hypofibrinolysis associated with inflammation (6) and how fibrinogen packaging (7) is changed during inflammation. Here we also discuss various substances and molecules (8) that may influence clot structure and also the relationship between hypercoagulability and fibrosis (9).

to thrombin followed by the thrombin-catalysed conversion of individual molecules of the soluble protein fibrinogen, with the release of two fibrinopeptides, to an insoluble, multimolecular complex of these molecules in the form of fibrin, the chief component of the blood clot. Thrombin also activates factor XIII to produce factor XIIIa, which catalyses intermolecular crosslinks between fibrin fibrils to stabilise the clot so formed (*e.g.* ref. 89–91).

A useful series of systems biology clotting models has been produced by Duffull and colleagues (*e.g.* ref. 92–96). Those of Wajima *et al.*[93] are available at the Biomodels database,[97–99] and are most easily accessed in SBML[100,101] form *via* the useful summary and commentary produced by Michael Schubert at http://www.ebi.ac.uk/biomodels-main/static-pages.do?page=ModelMonth%2F2011-07. Biomodels 338, 339 and 340 pertain at the Biomodels database. Biomodel 338 mimics the extrinsic pathway, where deficiencies affect the prothrombin time (PT), while Biomodel 339 includes the intrinsic pathway that allows simulation and calculations that mimic the results of the activated partial thromboplastin test (aPTT). (Biomodel 340 is concerned with the effects of warfarin.)

Although most modern models of coagulation incorporate the role of cells,[1,87,102,103] we shall concentrate here on the stripped-down terminal elements of the extrinsic (and common) pathway, where thrombin plays a crucial role in initiating the polymerisation of fibrinogen. These terminal processes, that are our focus here, are shown in Fig. 3, along with the normal (as seen in healthy individuals) degradation pathway of the fibrin *via* (fibrino)lysis catalysed by plasmin formed from the tissue plasminogen activator-catalysed activation of plasminogen.

The viscoelastic measurement of clotting properties *ex vivo*: thromboelastography, thromboelastometry, Sonoclot and others

The importance of understanding clot formation and degradation has led to the development of a considerable variety of assays for these processes (*e.g.* ref. 104–108). Most of these

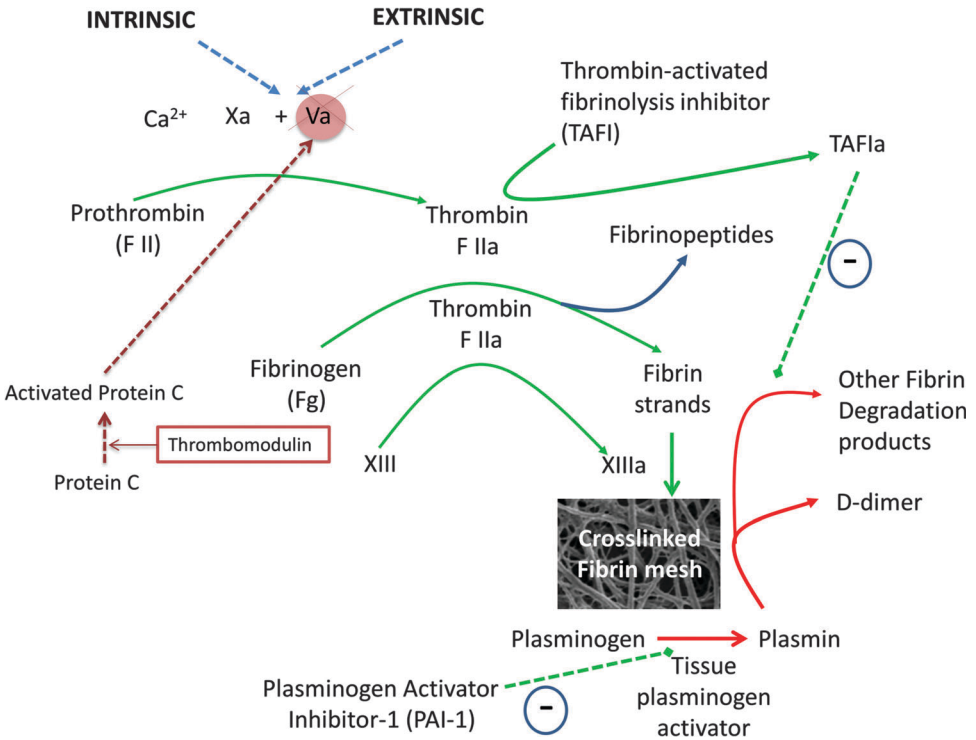

Fig. 3 The latter stages of the various coagulation pathways, showing places where thrombin plays a crucial role in initiating the polymerisation of fibrinogen.

methods are either optical or rheological/viscoelastometric in nature, and a number of commercial instruments exist. Some use plasma rather than whole blood, but plasma-based coagulation tests like prothrombin time (PT) and activated partial thromboplastin time (aPTT) have been seen as not entirely appropriate for monitoring coagulopathies,[109] with a move to analyse whole blood and using methods that determine the viscoelastic properties directly. Nonetheless, PT and aPTT are pre-eminent in the clinic.

Viscoelastic haemostatic methods[110] include thromboelastography (TEG),[109,111–114] thromboelastometry (ROTEM)[112,114–116] and the Sonoclot[117–119] instruments. Some comparisons have been made,[106,109,112–114,120–124] to the effect that results can be reasonably comparable in some circumstances but for detailed studies of specific effects it is probably wise to standardise on a particular instrument or technique.

Thromboelastography, thromboelastometry and Sonoclot instruments

In a typical thromboelastograph (a modern version is the Haemoscope) or rotational thermoelastometer (ROTEM), clotting is initiated and a trace generated where the ordinate represents the viscosity and the abscissa time. The differences are mainly as to whether a pin or the cup containing the blood is what is moved, and terminologies differ slightly according to Table 1 (based on ref. 106). Fig. 4 shows a schematic representation of a typical TEG and ROTEM readout (A), as well as viscoelastic haemostatic assay (VHA) tracings of a typical healthy trace (B)

Table 1 Terminologies of the variables measured in the TEG, ROTEM and Sonoclot instruments. Based on ref. 106, 109 and 125

Parameter	TEG	ROTEM	Sonoclot
Clot time			
Period to 2 mm amplitude	*R* (reaction time, min)	CT (clotting time, s)	ACT (activated clotting time, s)
Clot kinetics			
Period from 2–20 mm amplitude	*K* (kinetics) α (slope between *R* and *K*)	CFT (clot formation time) α (slope of tangent at 2 mm amplitude)	CR (clot rate, U min^{-1}) TP (time to peak, min)
Clot strength			
Maximum strength	MA (max amplitude)	MCF (max clot firmness)	PA (peak amplitude, U)
Clot elasticity	G	MCE (max clot elasticity)	
Clot lysis			
Lysis (at fixed time)	Ly30, Ly60 (amplitude reduction 3/60 min after MA)	CL30, CL60 (amplitude reduction 30/60 min after MCF)	

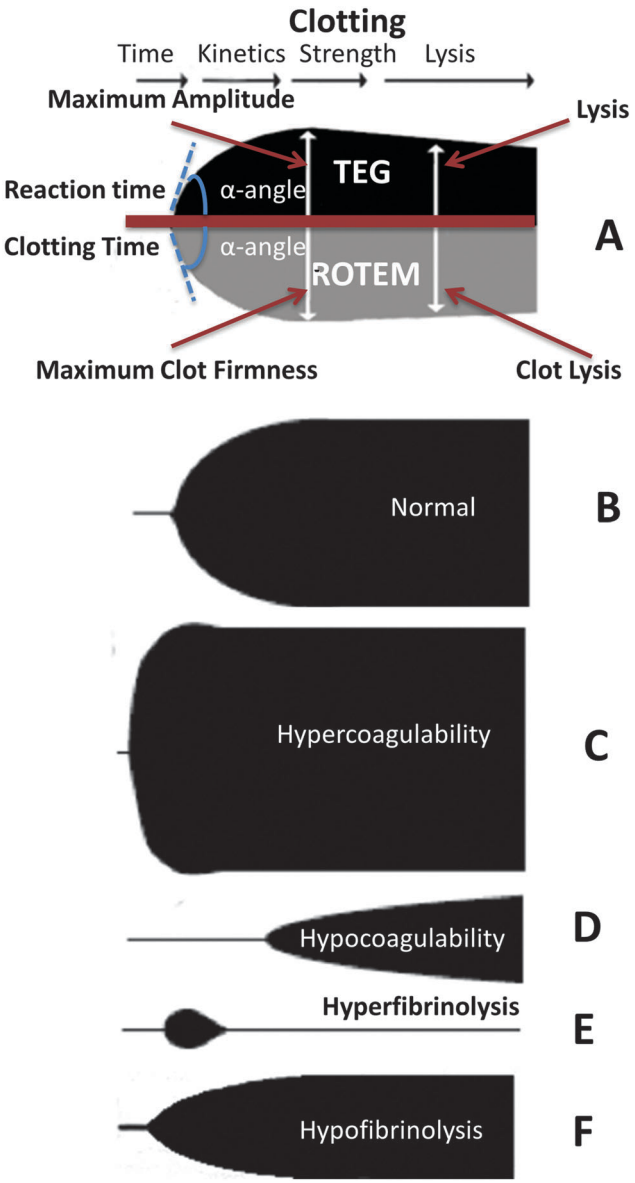

Fig. 4 (A) Diagram comparing thromboelastography (TEG) with thromboelastometry (ROTEM), showing clotting/reaction parameters, including time, kinetics, strength and lysis of the clot. (B to F) Schematic representation of various viscoelastic haemostatic assay (VHA) tracings: (B) normal (healthy); (C) hypercoagulability; (D) hypocoagulability; (E) hyperfibrinolysis; and hypofibrinolysis (F). This diagram is based on a version in ref. 109.

versus hypercoagulability (C), hypocoagulability (D), hyperfibrinolysis (E) and hypofibrinolysis (F).

A slightly different principle is embodied in the Sonoclot instruments, in that the viscoelastic probe vibrates vertically,[118] and the shape of the trace is somewhat different, producing a 'signature' with various peaks[106,125–127] (Fig. 5).

We compare typical reference values for clotting time, clot kinetics and clot strength for the TEG, ROTEM and Sonoclot in Table 2.

The clot lifespan model

A specific TEG assay that focuses rather more on the clot itself has been developed by Nielsen and his colleagues, and is

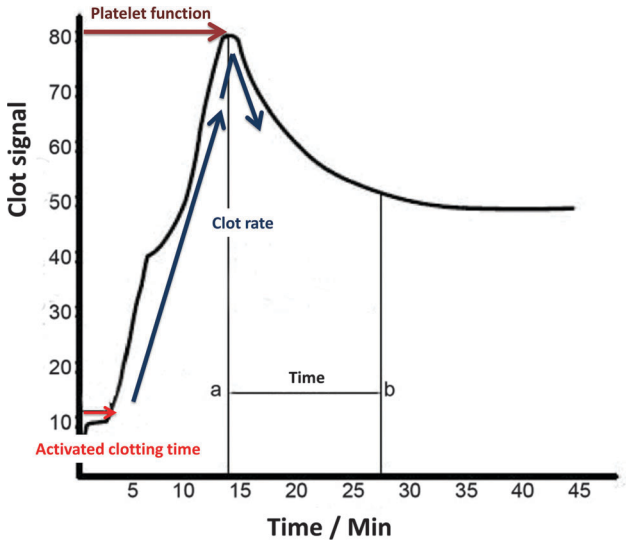

Fig. 5 A typical Sonoclot signature, adjusted from ref. 106 and 507.

known as the clot lifespan model[129–132] (Fig. 6). It purposely combines a standardized clotting stimulus such as the tissue factor with a fibrinolytic stimulus such as the tissue type plasminogen activator to assess clot growth and disintegration *via* changes in clot resistance under the same incubation conditions. Its attraction from the present perspective is that it can assess both hypercoagulability and hypofibrinolysis in the same samples while admitting a reasonably swift assay time (~30 min). In contrast to conventional TEG parameters, it also has the advantage of measuring the functionally relevant[133,134] clot resistance directly.[135]

We also note a number of approaches to the development of coagulometry based on the measurement of the passive electrical properties of the sample,[136–142] a strategy that we ourselves have found useful,[143] with radio-frequency measurements of cellular properties having a particular value.[144–147] Finally, fibrin scatters light much more than does fibrinogen such that turbidimetry is often used to detect its formation (*e.g.* ref. 148–153), and a number of more specialist coagulometric instruments based on optical (*e.g.* ref. 154) or other[155–161] properties have been proposed (see Table 2).

The relationship between hypercoagulability and hypofibrinolysis in selected inflammatory diseases

Hypercoagulability refers to the likelihood of or tendency towards making a clot, while hypofibrinolysis refers to a decreased tendency to remove it. Clearly either or both could be seen as coagulopathies. This said, and although they are usually studied separately, the common co-occurrence (*e.g.* Table 3 and ref. 162) of hypercoagulability and hypofibrinolysis can be interpreted to mean that both can have the same cause. Similarly, although the distinction (and their naming) reflects the changed kinetics of clot formation and lysis, there is also considerable evidence that the thermodynamics, *i.e.* properties related to the nature (make-up and morphology) of the clot differs in these states. (We note that sometimes

Table 2 Typical references values for clotting parameters of healthy blood and plasma as assessed using three different instruments. Based on ref. 106, 125 and 128. Note that the Sonoclot range varies considerably depending on whether activation includes glass beads

Parameter	TEG	ROTEM	Sonoclot
Clotting time	Whole blood 4–8 min Citrated kaolin 3–8 min	Citrated intrinsic 137–246 s, extrinsic 42–74 s	137–353 s
Clot kinetics	Whole blood 1–4 min Citrated kaolin 1–3 min	Citrated intrinsic 40–100 s, extrinsic 46–148 s	CR 7.6–36 U min^{-1} TP 5.2–15.2 min
Clot strengthening	Whole blood α 47°–74° Citrated kaolin α 55°–78°	Citrated intrinsic 71°–82°, extrinsic 63°–81°	
Maximum strength	Whole blood 55–73 mm Citrated kaolin 51–69 mm	Citrated intrinsic 52–72 mm, extrinsic 49–71 mm	PA 83–108 U

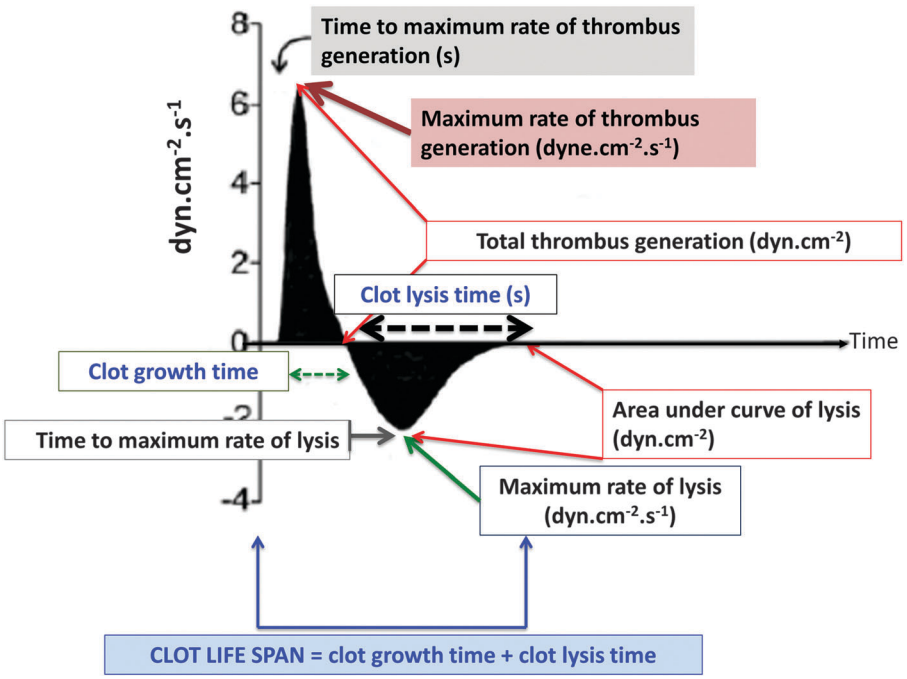

Fig. 6 The clot lifespan model developed by Nielsen and colleagues.[129–132]

Table 3 The co-occurrence of hypercoagulation and hypofibrinolysis in some selected diseases

Disease	Some references showing blood hypercoagulability	Some references showing reduced clot permeability or decreased susceptibility of clot to (fibrino)lysis
Alzheimer's disease	58 and 164 (but contrast[165])	166–171
Cancer	23, 172–186	23, 187 and 188
Cardiovascular disease	72, 73, 79, 189–198	25, 162, 191, 192, 199–211
Chronic obstructive pulmonary disease	212–214	215
Cushing's syndrome	216–222	216, 217 and 220
Deep vein thrombosis	7, 188, 223 and 224	162, 188, 223, 225 and 226
Diabetes mellitus, type 2	51, 227–233	34, 206, 228, 230, 232, 234–241
Heart failure	61, 242 and 243	244
Inflammatory bowel disease	59, 70, 245–252	246, 253–256
Metabolic syndrome	257–260	25 and 258
Pre-eclampsia	261–265	264 and 266
Pulmonary embolism	153	188, 224 and 267
Retinal vein occlusion	268	162, 268 and 269
Rheumatoid arthritis	270–276	273 and 274
Sepsis	277–284	277–280 and 284
Stroke	56, 194, 195, 285–291	63, 152, 162, 292–299
Systemic lupus erythematosus	300 and 301	266, 302 and 303
Thromboembolism	304–307	307–310
Transient ischaemic attacks	56, 290, 291 and 311	63 and 295
Trauma	307, 312–319	315, 320–322

fibrinolysis is assessed *via* D-dimer formation,[163] but that when this is increased one cannot normally tell whether that is due to an increase in fibrinolysis or simply because there had previously been hypercoagulation.)

Note that in the case of β-thalassaemia there is a particularly noticeable amount of hypercoagulation[323–335] but that fibrinolysis seems to be enhanced,[324,336,337] albeit not by enough to overcome the hypercoagulation. The same seems to be true of psoriasis[66,338–340] and of asthma.[341–345] Interestingly, we have been unable to find any direct literature evidence for or against the role of hypercoagulability and/or hypofibrinolysis in the case of hereditary haemochromatosis, despite the fact that it is a well-known iron overload disease.[346] DIC may be regarded as a contributor to a variety of syndromes, *e.g.* sepsis[347] and multiple organ failure.[78] There are also considerable changes in the coagulation variables during both healthy and compromised pregnancy.[348–351] Finally, although we have listed the various diseases separately, there are a considerable number of comorbidities, such as IBD/cardiovascular events[197] or chronic infection/multiple effects,[352] as expected for multiple diseases with a broadly common cause.[82]

The nature of the clot and clot lysis time

In many ways, fibrinogen polymerisation following thrombin activation is a remarkable process (Fig. 7, redrawn below from ref. 208), initially involving the release of two fibrinopeptides that cause lateral and end-to-end interactions *via* 'knobs' and 'holes', ultimately strengthened *via* the inter-strand covalent cross-linkages introduced later by factor XIII. Our interest is focussed on what determines the sizes, and especially the diameter, of individual fibrin fibres, since these may be observed under the electron microscope (*e.g.* ref. 171, 294, 297, 345, 353–360 and see later), and indeed the optical microscope,[361,362] directly. An individual fibrinogen molecule is an elongated coiled coil, with a length and diameter of approximately 45 and 5 nm, respectively.[363,364] However, the diameter of 'typical' healthy fibrin fibres is of the other of 80–90 nm[296,365] or more (as in Fig. 8). Thus to create every element of a fibre's width, even at the lower 'normal' diameter, requires the self-organisation of some 260 fibres (given that the formula for close-packing circles of diameter d to make, or fit into, a bigger circle of diameter D is $N = 0.9069 \times D^2/d^2$). Our interest here lies in the recognition that variation in the kinetics or accretion (rate) of individual fibrinogen molecules and proto-fibrils changes not only the amount of a clot but the nature, diameter and architecture of the fibres of which it is formed, with considerable functional consequences for the performance (and ease of removal) of the clot itself. Thus in stroke, for instance, the fibrin fibres have an average (modal) diameter of some 35 nm,[296] *i.e.* less than one half of that of healthy fibrin, evidently involving less than one quarter of the fibrinogen molecules per unit length. It is obviously of interest

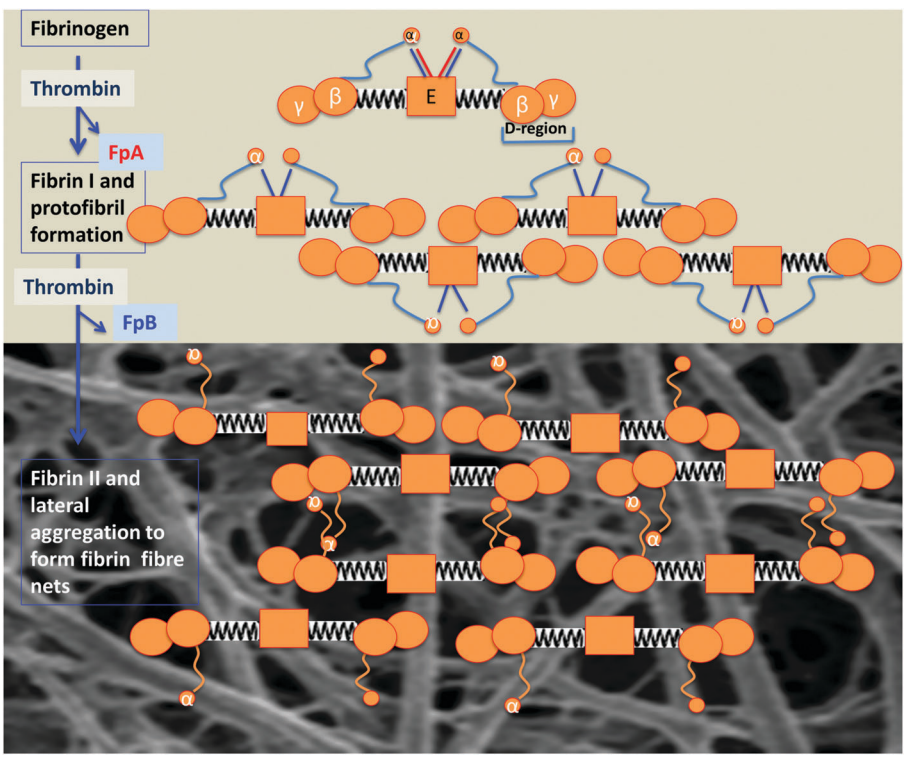

Fig. 7 Fibrinogen polymerisation following thrombin activation, indicating how the molecular structure of the fibrinogen changes and self-assembles to form macroscopic fibrin fibre nets, following the removal of fibrinopeptides A and B under the action of thrombin. Fibrinogen consists of 2 Aα, 2 Bβ, and 2 γ-chains, with a central E-region and D-regions, which are connected with an E-region by a coiled coil segment, composed of the β- and γ-chain C-termini; the α-chain C-termini fold back on the coiled coil and interact with the E-region. Fibrinopeptides A and B are released by the action of thrombin, and this initiates the polymerization of the fibrin protofibrils (redrawn from ref. 208).

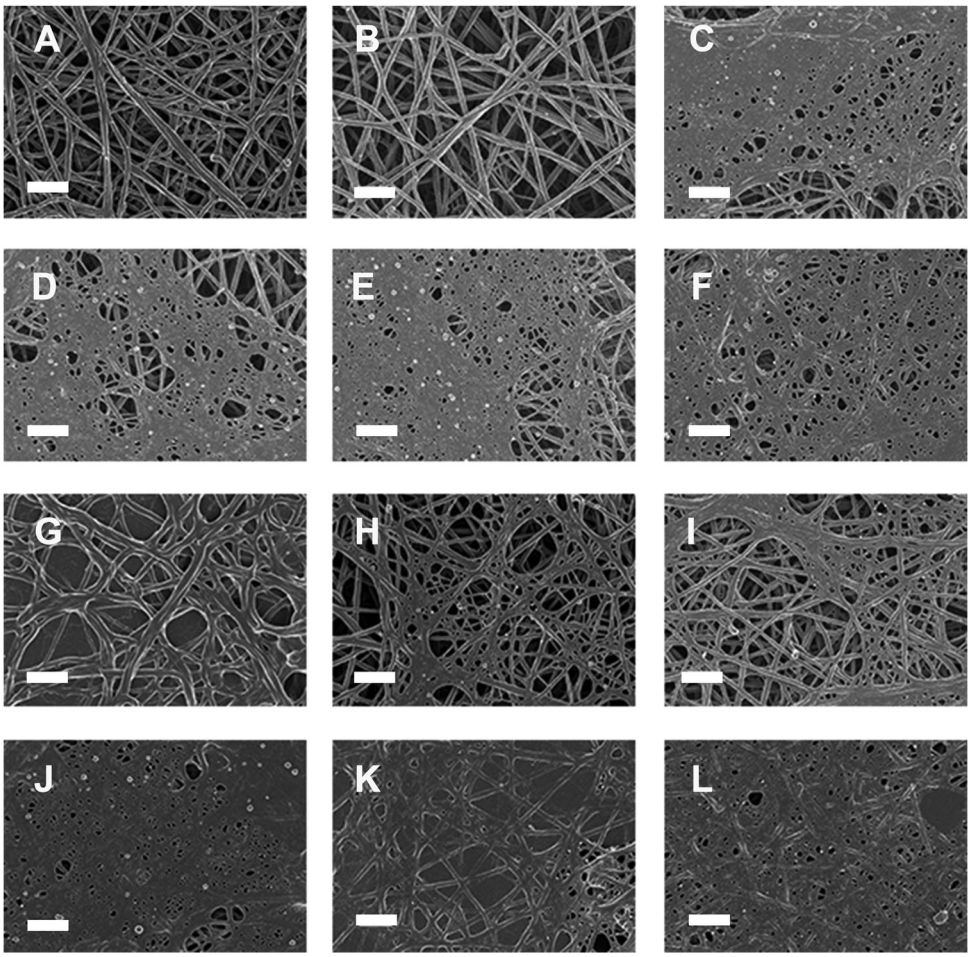

Fig. 8 Representative micrographs of platelet rich plasma (PRP) from 6 healthy individuals whose iron levels (iron, transferrin, % saturation and serum ferritin) were within the healthy ranges. (A) PRP + thrombin; (B) PRP + thiourea + thrombin; (C) PRP + phytic acid + thrombin; (D) PRP + $FeCl_3$ + thrombin; (E) PRP + $AlCl_3$ + thrombin; (F) PRP + YCl_3 + thrombin; (G) PRP + $FeCl_3$ + thiourea + thrombin; (H) PRP + $AlCl_3$ + thiourea + thrombin; (I) PRP + YCl_3 + thiourea + thrombin; (J) PRP + $FeCl_3$ + phytic acid + thrombin; (K) PRP + $AlCl_3$ + phytic acid + thrombin; and (L) PRP + YCl_3 + phytic acid + thrombin. Scale bar = 1 μm. See also Table 5 and text.

to seek to understand what kinds of changes can effect such dramatic differences. In principle there can be at least three types: (i) changes in the amount of fibrinogen, (ii) changes in the amino acid sequence of fibrinogen, and (iii) changes in other substances in blood that interact with fibrinogen and affect its polymerisation. All three are known to occur.

Effects of fibrinogen concentration on the nature of the clot

Fibrinogen itself is a well-known inflammatory marker, and as inflammatory markers, fibrinogen levels are themselves of course also associated with a variety of diseases (see *e.g.* ref. 88, 366–376), and fibrin clot properties also vary in this way with various diseases (*e.g.* ref. 191 and 377). The nature of the clot structure also depends on a complex interplay of entropic and enthalpic mechanisms accompanying structural changes and that underpin the nonlinear mechanical response in fibrin networks undergoing compressive deformation.[378]

On simple kinetic grounds alone, changes in the fibrinogen concentration might be expected to have significant effects on the nature of the clot, and this is indeed observed,[34,113,126,238,292,379] with, for instance, higher fibrinogen concentrations leading to faster clotting times and hence[148] smaller clot pores.[149,380,381]

In addition to changes in fibrinogen levels, one might suppose that changes in the fibrinogen molecule itself would have substantial effects, and this too is found. In one very nice study, a common variant BβArg448Lys in the C-terminal region of the fibrinogen beta-chain had major effects on the nature of the clot,[192] and the fact that this was also true for recombinant protein implied that the changes were due to interactions with substances in the blood itself.

The role of iron ions and other substances in affecting fibrinogen and clot formation

Fibrin(ogen) and clot formation is affected by a plethora of circulating plasma molecules, including collagen, fibronectin,

Table 4 Selected plasma molecules that influence fibrin(ogen) and clot formation

Molecules	Selected references
Calcium ions bound both strongly and weakly to fibrin(ogen) have been localized, but this binding and interactions are only beginning to be discovered.	380, 392, 398 and 399
Fibrinogen/fibrin can bind to native collagen type I (Col I) and may use the Col I fiber network as a base to provide a functional interface matrix that connects cells to the Col I.	394
Fibroblast growth factor-2 (FGF-2) is a critical growth factor in normal and malignant cell proliferation and tumor-associated angiogenesis. Fibrinogen and fibrin bind to FGF-2 high affinity to fibrin(ogen) and modulate FGF-2 functions and this binding plays an important role in augmented angiogenesis.	386 and 387
Fibronectin may also affect clot properties, as it binds extracellular matrix components such as collagen and fibrin, and sometimes serves as a general cell adhesion molecule. Along with fibrin, plasma fibronectin is deposited at the site of injury, forming a blood clot that stops bleeding and protects the underlying tissue. An increase in fibronectin concentration results in thinner and denser fibers in the fibrin matrices as it is covalently and non-covalently bound to fibrin matrices.	393
Fibulins are a family of extracellular matrix and blood proteins presently having two members designated as fibulin-1 and -2 and fibulin-1 can bind to fibrinogen; it was also found to be thrombi associated with human atherectomy specimens.	382
Similarly, the inflammatory mediator IL-1beta binds with high affinity to fibrin(ogen) and demonstrates increased activity in the bound form; and compared with free form, fibrinogen-bound IL-1beta stimulated increased activation of endothelial cell nuclear factor kappaB (NF-kappaB), monocyte chemo-attractant protein-1 (MCP-1) secretion, and nitric oxide (NO) synthesis.	384
There is also evidence that elevated lipoprotein(a) (Lp(a)) levels are associated with dense fibrin clots, reduced clot permeability and prolonged lysis time.	395
Low serum albumin was a modest marker of increased VTE risk.	388 and 390
Thrombospondins are proteins with antiangiogenic abilities and is a major platelet glycoprotein, which is released from platelets during blood coagulation and co-polymerizes with fibrin during blood coagulation and may be an important modulator of the clot structure.	400–402
von Willebrand factor-binding protein secreted by *Staphylococcus aureus* activates host prothrombin and form fibrin cables, thereby promoting the establishment of infectious lesions.	391
Zinc ions bind to the negatively charged fibrinogen, decrease thrombin's activity, but accelerate fibrin polymerisation, forming larger fibres.	383, 403–405

lipoprotein, albumin, thrombospondin, von Willebrand factor, fibulin, fibroblast growth factor-2, interleukin-1, and calcium, zinc and iron ions (see Table 4).[382–395]

A number of clot properties can vary under the influence of various molecules mentioned in the previous paragraph. These molecules may interact with fibrinogen and change the mechanical properties of clots, which are essential to the effective function of fibrin. Clot properties that may vary include the pore size, fibrin diameter, rigidity, ease of fibrinolysis, and so on[208,396] (and some of these are correlated[361,397]).

Important to this review is that fibrinogen directly recognizes iron ion, the PPIX ring and metal ions complexed with the hemin (iron–protoporphyrin IX: PPIX) ring.[389]

Electrostatic considerations

The pI of fibrinogen is 5.5, *i.e.* it is negatively charged at neutral pH (as are the surfaces of erythrocytes). As one might expect, changes in ionic strength decrease the clot pore size.[149,380,381] As one would also expect from Debye–Hückel theory, it binds divalent cations such as Ca^{2+} [380,392,398,399] and Zn^{2+} [404]; the trivalent iron ion is especially effective.[389,406] It has also been suggested that added ferric iron can carry out Fenton chemistry by reacting with peroxide that may be formed in a variety of ways.[171,353,359,407,408] Thus both electrostatic phenomena and hydroxyl radical formation may modify the fibrinogen structure in such a way that its kinetics of polymerisation, and the structure of the fibrin products, is altered substantially.

The action of phytic acid and thiourea in the presence of Fe^{3+}, Al^{3+} and Y^{3+}

Phytic acid (myoinositol hexaphosphate) has 12 exchangeable protons, giving it a strong ability to complex with multivalent cations[409] (and indeed positively charged proteins). Trivalent ions are of special interest from an electrostatics point of view, and can be chosen to have very different chemistries and solvent interactions[410] such that if their behaviour is similar it is likely their electrostatic rather than chemical properties that underpin this.[411] Phytic acid, therefore, has the potential to remove metal ions such as Fe^{3+}, Al^{3+} and Y^{3+} in the form of a metal–phytate complex, but not to serve as a hydroxyl radical trapper.[412] By contrast, thiourea is not thought to be a metal chelator, but traps hydroxyl radicals (*e.g.* ref. 413). To this end, we studied the effects of these substances on fibrinogen polymerisation.

$AlCl_3$, $FeCl_3$ and YCl_3 (final concentration 15 μM) were mixed with platelet rich plasma (PRP) from 6 healthy individuals prepared from blood drawn in citrate tubes. Full iron analysis showed that their basal iron levels (iron, serum ferritin transferrin and % saturation) were within the normal ranges as specified for

Table 5 Sample preparation for and statistical analysis (to nearest nm) of Fig. 8. Where present, final concentrations of $FeCl_3$, $AlCl_3$ and YCl_3 were 15 μM, and of thiourea and phytic acid 30 μM

Components and (figure letter in parentheses)	Mean fibre diameter (nm) (n = 50) ± SD
10 μL PRP + 5 μL thrombin (8A)	105 ± 3
10 μL PRP + 5 μL thiourea + 5 μL thrombin (8B)	117 ± 4
10 μL PRP + 5 μL phytic acid + 5 μL thrombin (8C)	46 ± 2
10 μL PRP + 5 μL $FeCl_3$ + 5 μL thrombin (8D)	45 ± 3
10 μL PRP + 5 μL $AlCl_3$ + 5 μL thiourea (8E)	39 ± 1
10 μL PRP + 5 μL YCl_3 + 5 μL thrombin (8F)	50 ± 2
10 μL PRP + 5 μL $FeCl_3$ + 5 μL thiourea + 5 μL thrombin (8G)	146 ± 4
10 μL PRP + 5 μL $AlCl_3$ + 5 μL thiourea + 5 μL thrombin (8H)	66 ± 5
10 μL PRP + 5 μL YCl_3 + 5 μL thiourea + 5 μL thrombin (8I)	69 ± 5
10 μL PRP + 5 μL $FeCl_3$ + 5 μL phytic acid + 5 μL thrombin (8J)	47 ± 3
10 μL PRP + 5 μL $AlCl_3$ + 5 μL phytic acid + 5 μL thrombin (8K)	184 ± 8
10 μL PRP + 5 μL YCl_3 + 5 μL phytic acid + 5 μL thrombin (8L)	65 ± 4

healthy individuals. Thrombin with and without either phytic acid or thiourea (final concentrations 30 μM) was added to PRP (pH: 7.38 (physiological pH)). See Table 5 for sample preparation and the mean diameter in nm ± SD. Fibrin fibres were prepared for SEM according to methods published previously.[359,360]

SEM analysis of healthy fibrin fibre morphology (Fig. 8A) shows individual fibres that form a typical netlike structure. When thiourea is added to healthy PRP (Fig. 8B), no changes in fibrin fibre structure are noted; however, in the presence of phytic acid, instead of a healthy fibre structure, a dense netted layer (Fig. 8C) is formed. This netted appearance of the fibrin fibres was also noted with the addition of $FeCl_3$, $AlCl_3$ and YCl_3 to healthy PRP (Fig. 8D to F) (final concentration of these substances in PRP was 15 μM), while the fibre diameters were approximately halved (as also seen in stroke[296]). When thiourea was added to a final concentration of 30 μM to PRP and $FeCl_3$, $AlCl_3$ and YCl_3, respectively (Fig. 8G to I), the fibrin structure was either protected or reverted back to a morphology more similar to that of healthy PRP. When phytic acid was added to a final concentration of 30 μM to PRP and $FeCl_3$, $AlCl_3$ and YCl_3, respectively, the fibrin structure changed to a thick, matted layer (Fig. 8J to L). This structure was denser than when only phytic acid was added to PRP (Fig. 8C) or when just $FeCl_3$, $AlCl_3$ or YCl_3 were added to PRP (Fig. 8D to F).

The above observations suggest that thiourea added to PRP does not have an electrostatic effect (as would indeed be expected in that it is uncharged). However, the addition of phytic acid to PRP (Fig. 8C) and $AlCl_3$ (Fig. 8E) might suggest a purely electrostatic effect of both excessive cations and anions. The effects of PRP with $FeCl_3$ and thiourea (Fig. 8G) could be interpreted as showing that at least in part the changes are due to $OH^{\bullet}$ – but that is on the basis of thiourea not chelating. This suggests that the structural changes in the fibres that take place when $FeCl_3$ is added to PRP and thrombin (Fig. 8D) might be contingent upon both an electrostatic (mainly) and an $OH^{\bullet}$ (partly) effect.

Interestingly, it seems as if thiourea also stops the effect that $AlCl_3$ (Al^{3+}) has on the structure of the fibres (Fig. 8H). Al^{3+} does not on its own effect Fenton chemistry; however, under conditions where there might be iron present (as will be the case in PRP of healthy individuals) it can actually contribute to Fenton chemistry.[414,415]

Overall, these observations (and literature) are consistent with the view that a great many substances can have substantial effects on the rate of fibrinogen polymerisation and on the nature and structure of the fibres and clots formed. In some senses, fibrin structure might be considered to be a kind of bellwether of plasma health.

Effects of non-ionic substances

The nature of the clot formed upon thrombin activation can also be modulated by a variety of other substances[416] besides trivalent ions. One such substance is glucose – and in diabetes the changes are more likely due to glycation of fibrinogen.[236,416–418] Hormonal fluctuations also influence fibrin fibre morphology. Swanepoel and co-workers in 2014 were the first to show that oestrogen changes the ultrastructure of fibrin fibres.[419,420] They found that the external and internal structure of the fibrin strands differed throughout the menstrual cycle. These changes coincided with the normal oestrogen peaks associated with the menstrual cycle. An increase in oestrogen during specifically the pre-ovulatory phase of the menstrual cycle resulted in a granular appearance of the fibrin network on high magnification. During pregnancy, which is associated with even more elevated oestrogen levels, the same granular appearance was typical, with an additional increase in dense matted deposits. These morphological changes persisted till at least 8 weeks post-partum. Oestrogen therefore may play a significant part in the hypercoagulable state associated with pregnancy.

The many oxidative and nitrosative stresses leading to protein modifications and their role in diseases are given *e.g.* by Dalle-Donne and colleagues.[421–424] Unsurprisingly, substances that can react with fibrinogen directly can also have significant effects on clotting; indeed, aspirin affects the nature of the clot directly *via* acetylation,[150] while peroxynitrite can inhibit fibrinolysis significantly,[425] and fibrinogen nitration is pro-thrombotic.[426,427]

Iron and blood storage

Although not the entire focus of this article, we note that it is becoming increasingly recognised that as blood ages during storage it has a tendency to release iron and that this may give it properties that are suboptimal.[428–438] As we have shown in the

previous paragraphs, free iron can have a substantial role in determining the outcome of the clotting process, and this will need to be borne in mind as blood ages. The same is likely true for blood that ages in individuals.[439,440]

Inflammation, fibrinogen and red cell aggregability as assessed *via* the erythrocyte sedimentation rate and other means

As indicated, inflammation, fibrinogen levels and hypercoagulability are intimately connected. In a similar vein, fibrinogen levels are one of the strongest contributors to red cell aggregability and aggregation,[441] and thence to the erythrocyte sedimentation rate, another classical indicator of inflammation.[442–448] It should be noted that the difference between RBC aggregation and RBC aggregability is that the latter describes an intrinsic RBC property and the former describes the effect that results from this intrinsic cell property together with other factors (*e.g.* plasma proteins, temperature, pH, shear rate, blood flow), resulting in RBC aggregation. In this regard it is accepted that fibrinogen increases RBC aggregation, not aggregability.

This can have a significant diagnostic and indeed prognostic value (*e.g.* ref. 449–454). Here, the Laser-Assisted Optical Rotational Red Cell Analyzer (LORRCA)[455–458] (marketed by Mechatronics) provides a convenient measurement, while atomic force microscopy assesses deformability at the level of the single cell.[459–463]

Similar comments apply to the changes in red cell distribution width also commonly seen in inflammatory diseases (*e.g.* ref. 464–474).

Cytokines linking inflammation, hypercoagulability and hypofibrinolysis

Given the considerable evidence noted above for the co-occurrence of inflammation, hypercoagulability and hypofibrinolysis, one might note in general terms that the relationship between two variables A and B that co-vary can take at least four forms: A affects B, B affects A, both A and B affect each other, and D affects both A and B. More complex variants such as A affects D and D affects B are not considered; these are known as 'indirect effects', and have proved problematic in studies of covariance (*e.g.* in protein folding based on phylogenetics,[475] although considerable progress is being made in disentangling them[476–480]).

When looking for the potential biochemical changes or co-occurrences that might serve to link coagulation and inflammation, the chief players seem to be IL-1β[481,482] and, in particular, IL-6,[61,65,338,481–483] that seems to be involved in causing an

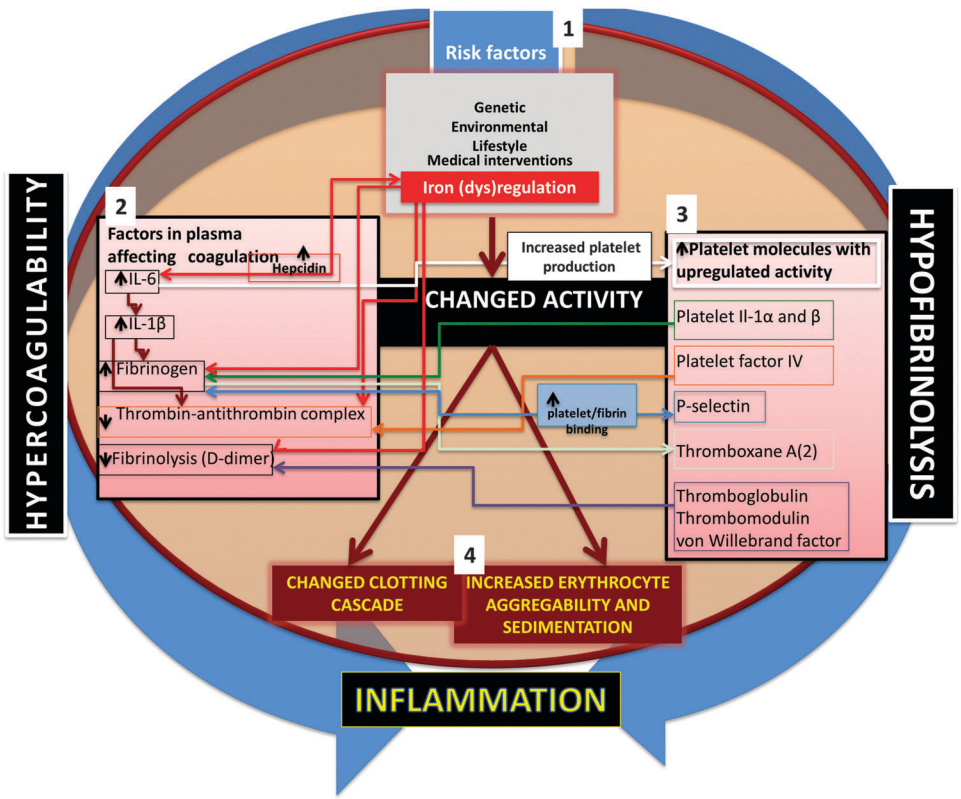

Fig. 9 Hypercoagulation, hypofibrinolysis and inflammation: a brief overview of the roles of plasma and platelet factors. Risk factors (1) cause a changed coagulation factor profile in plasma (2), as well as an upregulation activity of platelet products (3) which feeds back to affect each coagulation (indicated by arrows between 2 and 3). This results in a changed clotting profile and increased erythrocyte aggregability and sedimentation rate (4) that may be observed macroscopically.

iterative cycle of coagulability and inflammation. One important mechanism involving the (IL-1-mediated) activation of IL-6 that in turn induces fibrinogen synthesis by hepatocytes[484–486] and other cells[482,487–490] is especially pertinent. IL-6 is also involved in iron (dys)regulation, in particular by its ability to induce hepcidin biosynthesis (*e.g.* ref. 491–499). An overview of the role of these cytokines is shown in Fig. 9.

Relationship between inflammation and fibrosis

Fibrosis refers to the laying down of pathological variants of a variety of proteins in an insoluble form,[500] and also occurs in response to inflammation. Although we are concentrating here on events in blood, it is not a coincidence that 'fibrosis' shares the same etymological root as fibrin(oge)n. Most pertinently, fibrosis can be seen under the same hypercoagulable conditions as discussed here,[501–506] and may be considered to have a common cause (and, potentially, cure).

Concluding remarks

In the spirit of Integrative Biology, we have brought together a considerable body of literature on the effect that many diseases share a state of both hypercoagulability and hypofibrinolysis, and that part of this is due to the fact that they also share common causes in the form of inflammation and increased levels of substances such as inflammatory cytokines, fibrinogen and iron. Measuring these effects in blood or plasma may provide some useful and convenient approaches to diagnostics, and minimising them may provide some innovative system approaches to innovative therapies, to the benefit of all. Morphological methods cannot study changes or variations or conformational flexibility and variability of the specific forms (*e.g.* monomeric form) of the fibrin molecule, although this might indeed impact structural differences and polymerization characteristics. Therefore, ultrastructural investigation is proposed as a complementary methodology, together with methods described in this manuscript, to truly support an integrative approach to study clot formation (hypercoagulability) and hypofibrinolysis.

Acknowledgements

We thank the Biotechnology and Biological Sciences Research Council (BB/L025752/1) for supporting this collaboration. Janette Bester and Natasha Vermeulen prepared the samples for scanning electron microscopy.

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