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# Healing through the lens of immunothrombosis: Biology-inspired, evolution-tailored, and human-engineered biomimetic therapies

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ABSTRACT

Evolution, from invertebrates to mammals, has yielded and shaped immunoclotting as a defense and repair response against trauma and infection. This mosaic of immediate and local wound-sealing and pathogen-killing mechanisms results in survival, restoration of homeostasis, and tissue repair. In mammals, immunoclotting has been complemented with the neuroendocrine system, platelets, and contact system among other embellishments, adding layers of complexity through interconnecting blood-born proteolytic cascades, blood cells, and the neuroendocrine system. In doing so, immunothrombosis endows humans with survival advantages, but entails vulnerabilities in the current unprecedented and increasingly challenging environment. Immunothrombosis and tissue repair appear to go hand in hand with common mechanisms mediating both processes, a fact that is underlined by recent advances that are deciphering the mechanisms of the repair process and of the biochemical pathways that underpins coagulation, hemostasis and thrombosis. This review is intended to frame both the universal aspects of tissue repair and the therapeutic use of autologous fibrin matrix as a biology-as-a-drug approach in the context of the evolutionary changes in coagulation and hemostasis. In addition, we will try to shed some light on the molecular mechanisms underlying the use of the autologous fibrin matrix as a biology-inspired, evolution-tailored, and human-engineered biomimetic therapy.

## 1. Introduction

Every living being, from single-celled organisms to complex multicellular and multi-system animals including vertebrates, possesses a variety of physical and chemical barriers, and a vast repertoire of physiological responses in order to secure integrity, survival, and homeostasis, processes often grouped as the immune or host defense system [1–3]. Invertebrates and vertebrates, including mammals, have evolved a cluster of immediate and local responses known as immunoclotting and immunothrombosis respectively that function in a wound-sealing, microbial-killing and -clearing, and homeostasis-restoring manner resulting in tissue repair [4–9]. Pivotal players of immunothrombosis are blood circulating and tissue stationary and mobile resident cells, and the intravascular innate immune system [8,10,11]. The plasma proteins of the humoral intravascular innate immune cascade systems such as prothrombin-thrombin [12–14], fibrin (ogen) [15,16], FXII [17–20] and complement (C3, C5, and their active fragments C3a, C5a anaphylatoxins) [21–23] together with the payload released by activated platelets play a major role in tissue repair and remodelling [12,16,22,24,25]. Pathological changes in the interplay between the intravascular serine protease cascade systems and circulating blood cells as well as vascular and tissue cells, however, are involved in the pathogenesis of several systemic conditions such as sepsis, hemorrhagic shock, systemic inflammation, liver fibrosis, or rheumatic syndromes [8,11,26–28]. Recent work deciphering the roles of blood cells and of the biochemical intravascular innate immune machinery, specifically coagulation and hemostasis, has led to filter out platelets and coagulation proteins that create an autologous fibrin matrix (AFM). These blood-derived products have a healing potential as a local biology-as-a-drug approach, to repair musculoskeletal tissues, skin and corneal ulcers and burns with many other uses [11,29–31].

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**Abbreviations**

| | |
|---|---|
| AFM | Autologous fibrin matrix |
| APC | Activated Protein C |
| BK | Bradykinin |
| CNS | Central nervous system |
| CP | Classical pathway |
| CRP | C-reactive protein |
| CS | Contact system |
| DAMPs | Damage-associated molecular patterns |
| DC | Dendritic cell |
| DCO | Damage control orthopaedics |
| DC-SGN | Dendritic cell-specific intracellular adhesion molecule-3-grabbing non-integrin |
| ECs | Endothelial cells |
| ECM | Extracellular matrix |
| EPCR | Endothelial protein C receptor |
| FGF | Fibroblast growth factor |
| FXII | Factor XII |
| GAG | Glycosaminoglycans |
| GFs | Growth factors |
| HA | Hyaluronic acid |
| HGF | Hepatocyte growth factor |
| HMWK | High-molecular-weight kininogen |
| HPA | Hypothalamic-pituitary-adrenal |
| HUVEC | Human umbilical vein ECs |
| IGF-1 | Insulin-like growth factor 1 |
| IL | Interleukin |
| KKS | Kallikrein-kinin system |
| LP | Lectin pathway |
| LPS | Lipopolysaccharide |
| LXA4 | Lipoxin A4 |
| MAMPs | Microbe-associated molecular patterns |
| MASP-1 | Mannose-associated serine protease 1 |
| MBL | Mannose-binding lectin |
| MEP cell | Megakaryocyte/erythrocyte progenitor cell |
| MIF | macrophage migration inhibitory factor |
| MK | Megakaryocytes |
| MMP-2 | Matrix metalloproteinase-2 |
| MODS | Multiple organ dysfunction syndrome |
| Mya | Million years ago |
| NET | Neutrophil extracellular traps |
| NF-κB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NLRs | Nod-like receptors |
| NO | Nitric oxide |
| PAMPs | Pathogen-associated molecular patterns |
| PDGF | Platelet-derived growth factor |
| $PGE_2$ | Prostaglandin $E_2$ |
| $PGI_2$ | Prostacyclin $I_2$ |
| Piezo2 | Piezo ion channel 2 |
| PK | Prekallikrein |
| PMP | Platelet microparticles |
| PPO | Prophenoloxidase system |
| PRP | Platelet-rich plasma |
| PRRs | Pattern recognition receptors |
| RAGE | Receptors for advanced glycation end products |
| ROS | Reactive oxygen species |
| SASP | Senescence-associated secretory program |
| SDF-1 | Stromal cell-derived factor 1 |
| SIRS | Systemic inflammatory response syndrome |
| SMA | Smooth muscle actin |
| SMAD2 | Mothers against decapentaplegic homolog 2 |
| SPMs | Specialized pro-resolving mediators |
| STAT3 | Signal transducer and activator of transcription 3 |
| TFPI | Tissue factor pathway inhibitor |
| TFs | Transcription factors |
| TGF-β | Transforming growth factor beta |
| Th2 | T helper 2 cells |
| TLR | Toll-like receptor |
| TM | Thrombomodulin |
| TRP | Transient receptor potential channels |
| VEGF | Vascular endothelial growth factor |
| VVEC | Vasa vasorum ECs |
| VWF | von Willebrand factor |

This review is intended to frame both the universal aspects of tissue repair as a byproduct of the mechanisms underlying the host defense system and AFM. In addition, we will try to shed some light on the biological paradox of host defense mechanisms (immunothrombosis) as local tissue repair enhancer on the one hand while generating systemic pathogenic processes on the other. We will do so by linking an evolutionary and a clinical-therapeutic perspective, with an emphasis on the coagulation system, platelets and hemostasis.

## 2. Evolutionary perspective of intravascular innate immune cascade systems

Mammalian intravascular innate immune cascade systems (complement, coagulation, contact, and fibrinolysis systems) were not generated from scratch nor were they assembled all at once but, like mammalian cell types and their biochemical pathways, they derived from simpler versions originating in non-mammalian vertebrates and invertebrates [32–35]. At the molecular and cellular level, they evolved from precursor genes, proteins, and cell types that originally served other functions [32–34,36–38].

### *2.1. Innate immunity and coagulation: partners throughout evolution*

To cope with trauma- and infection-induced disruption of physicochemical and anatomical barriers, invertebrates evolved a vast repertoire of immediate and local cell-and/or humoral-based responses, with dual roles in immunity and clotting. These responses consist of wound-sealing and pathogen-killing, resulting in restoration of homeostasis and wound healing [6,10,39–42]. Thus, in marine invertebrates like sea urchins, with low protein content in their plasma, the only type of cell present in the coelomic fluid, the coelomocyte, aggregates upon injury or in the presence of foreign substances to form a cellular clot mediated by amassin, a plasma protein whose multimers attach the coelomocytes to each other and ending up by sealing the wound [4]. However, in more highly evolved invertebrates like the american horseshoe crab (*Limulus polyphemus*), clotting defense responses involve cell and humoral components and are initiated by the hemocyte (also known as amoebocyte) that detects non-self molecules (PAMPs) such as lipopolysaccharide (LPS) through pattern recognition receptors (PRRs), leading to hemocyte activation [4–6,9]. This activation triggers hemocyte aggregation, hemocyte antibacterial- and hemolymph clotting-factor release, including coagulase and coagulogen, the latter rapidly transformed into the gel coagulin, and the activation of the primordial complement system [4,6,9,43]. These responses are the basis of hemolymph clotting, clearance-killing of pathogens and foreign bodies, and wound healing with dual defense and repair roles of immunoclotting [4,6,9,44]. In insect, immunoclotting evolved as a locally operative mechanism, even generating microclots to entrap bacteria, with a very low risk for

thrombosis due to the open circulatory system of invertebrates [6,43,45], but also as an integral part of the healing-regeneration process [6,46]. Mammals instead, have coupled inflammation and coagulation through a combination of cooperative host defense components and strategies, whose pivotal players are circulating blood cells (polymorphonuclear cells, monocytes, lymphocytes and platelets), tissue stationary and mobile resident cells (dendritic and mast cells, nociceptors, endothelial cells, macrophages and fibroblasts), and intravascular innate immune cascade systems (complement, coagulation, contact, and fibrinolysis systems) [3,8,10,11].

In the evolutionary journey of the vertebrate coagulation and hemostasis system (Fig. 1), the first vertebrate clotting component to emerge was a thrombin-like protein. The thrombin precursor diverged from the preexisting complement and mannan-binding protein associated serine proteases (C1r, C1s, and MASP-1, MASP-2, MASP-3) already present in deuterostomes 710–780 million years ago (Mya), that were already operating as a primordial complement system (C3, Bf, and MASP genes) [47–51]. These trypsin-like serine proteases in turn had derived by gene duplication, exon shuffling, and simple mutation, from the chymotrypsin-like serine protease domains with trypsin as the origin gene [34,51], leading to amino acid replacement [35], similar to the origin of thrombin and other vitamin K- dependent serine protease clotting factors (FVII, FIX, and FX) later on [7,33–35,52].

The aforementioned variations endowed the complement, coagulation, and fibrinolysis systems with complex modular allosteric serine proteases, sharing some domains and thereby generating intrinsic chemical affinities with one another or with other protein substrates or cofactors, being especially useful in creating local, cooperative, molecular networks as infection and injury recognition molecules [7,11,34,35,64]. The evolved genes and encoded proteins conferred to their bearers advantages for host survival, but also generated systemic vulnerabilities, as we will see in section 4 [3,11,28,35]. Accordingly, the allosteric and catalytic serine protease thrombin plays multiple roles as regulator of inflammation and in tissue repair, and is an example of pleiotropism that directly cross-links immunity and coagulation with the repair process as we will describe further in section 3.1 [13,39,65].

The second and essential substrate of vertebrate blood clots, fibrinogen, is not present in invertebrates [35], although they carried the raw material in the form of fibrinogen-like loci encoding fibrinogen-domain-containing proteins with no coagulation but rather an immune-parasite defense function [66]; an example is the urochordate sea squirt, that occupies a critical intermediary position between invertebrates and vertebrates (Fig. 1), and possesses three genes that encode a protofibrinogen with almost all the features of vertebrate fibrinogen [35]. In any event, the prototype of the coagulation cascade derived from these molecules in urochordates would not be effective in hemostasis but rather an immune enhancer with hemolymph cell agglutination as a cell-based wall-off mechanism simultaneously acting as a prophylactic mechanism in trapping soluble parasite-derived molecules, and in killing bacteria [35,67,68].

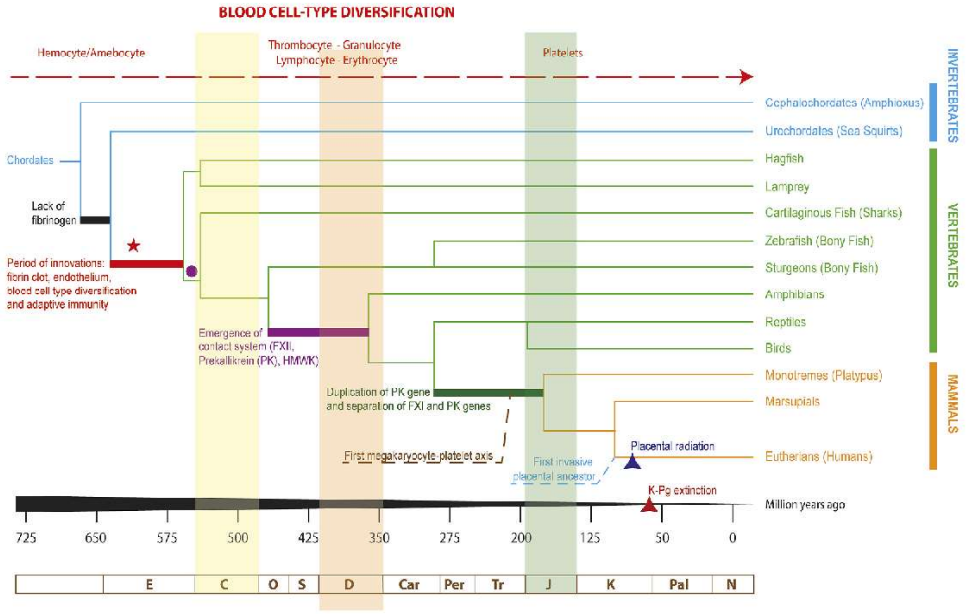


**Fig. 1. An overview of some of the main events in the evolution of the vertebrate blood coagulation system.** Phylogeny and the approximate times of evolution (as determined by fossil records and molecular clock framework analysis) of various chordates, highlighting significant evolutionary events and innovations in the emergence of the human coagulation and hemostatic systems. The red star and purple dot represent the points in evolution where the first and second whole-genome duplication events have been proposed to occur. The red bold line represents a period of innovations. This period spans the time that followed the divergence of urochordates and the lineage giving rise to the earliest jawless vertebrate (hagfish and lampreys, more than 500 Mya), which are marine animals that possess a simple coagulation version involving prothrombin, tissue factor, fibrinogen, FVII and X though they lack genes for coagulation factors VIII, and IX, and the entire contact system [51,53]. This very period of time of great inventions witnessed the emergence of a true endothelium, the blood cell-type diversification, and the adaptive immune system, and broadly coincides with the onset of Cambrian radiation (540 Mya) [54]. The purple bold line indicates the emergence of the contact system (FXII, HMWK, and PK), that is associated, somewhere along the Devonian period around 400-390 Mya, with the water-to-land transition of vertebrates, and with the appearance of first amphibians, and then reptiles [55]. The origin of the MK-platelet axis remains to be determined but has been suggested to occur in the 200-160 Mya window [56,57]. The blue triangle represents the time in evolution that includes the placental radiation of mammals, including rodentia and primates has been hypothesized to occur [58,59]. The red triangle represents the Cretaceous-Paleogene (K-Pg) mass extinction event, 66 Mya, where among the existing mammals only borrowing small animals survived (E Ediacaran, C cambrian, O ordovician, S silurian, D devonian, Car carboniferous, Per permian, Tr triassic, J jurassic, K cretaceous, Pal paleogene, N neogene) [35,55,56,58–62]. Adapted from Ref. [63] with permission. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Notwithstanding the superficial resemblances and functional common trends between invertebrate hemocyte aggregation, hemocyte clotting factor release, and hemolymph jelling on the one hand, and mammalian platelet aggregation and fibrin clotting on the other, there is little similarity between the primary structure of proteins involved in the invertebrate coagulation reactions (amassin, coagulogen, vitellogenin), and proteins necessary for the thrombin-catalyzed conversion of fibrinogen to fibrin in vertebrates (Prothrombin, fibrinogen, FVII); the similarities being an example of convergence in function [6,35,44,57,69]. Analysis of gene organization, the toolbox of protein domains, molecular cloning data, and comparative sequencing, supports the concept that the simplest version (genes and proteins for generating the thrombin-catalyzed conversion of fibrinogen to fibrin) of the complex vertebrate blood coagulation network evolved independently and was assembled more than 500 Mya, over a period of a 50–100 million year window (Fig. 1) [35,54,70,71]. Nevertheless, genetic variations (in the form of gene duplication, exon shuffling, or simple mutation) predate protein novelties and depend on environmental changes to be turned into biological innovations [57]. The relatively short window of time that followed the divergence of protochordates (cephalochordates and urochordates) and the lineage giving rise to earliest vertebrates known as jawless fish (agnathans similar to today's hagfish and lampreys) [35,36,53,70,71] also witnessed the emergence of a closed and increasingly pressurized circulatory system, blood cell specialization, the endothelium, and the adaptive immune system (Fig. 1) [35,54,70]. From the simplest version of the jawless coagulation system with tissue factor, prothrombin and fibrinogen as the starting point, to the sophisticated and embellished mammal coagulation system, the clotting machinery evolved in distinct stages [35], from downstream onwards by integrating enzymes upstream [72]. This is the case of the emergence and integration of the inflammatory arm of the contact system (CS) into the older tissue factor-triggered extrinsic coagulation pathway [35,52,73] (Fig. 1). Made up of serine proteases factor XII (FXII) and prekallikrein (PK), and the multidomain glycoprotein high-molecular-weight kininogen (HMWK), the kallikrein-kinin system (KKS) initially evolved as an inflammatory pathway by domain acquisition, chromosomal segment duplication, and exon shuffling parallel to the still independent of fibrin clot formation cascade [52,74]. Although present in all vertebrates, the non-protease HMWK expanded from lampreys to humans by domain acquisition and shuffling [35,52] as confirmed by a recent research that identified the presence of the PK gene (klkb1) and a simple version of the HMWK gene (kng1) in the coelacanth and lungfish, two lobe-finned fish ancestral to tetrapods [52,74]. On the other hand, the serine protease FXII, arose through duplication of a hepatocyte growth factor activator (HGFA) containing chromosomal segment [73–75]. The FXII gene (f12) is present in lungfish, amphibians, reptiles, and mammals but not cartilaginous or ray-finned fish, whereas the FXI gene (f11) is present in platypus and opossum, but not in amphibians, reptiles, or birds [74] somehow suggesting that the duplication of the PK gene (klkb1) giving rise to FXI gene (f11) occurred late in vertebrate evolution in a proto-mammalian ancestor (Fig. 1) [74]. Importantly, new research reveals that kallikrein (PK) may directly activate FIX [74,76] as does its parent molecule FXI, resulting in thrombin generation and fibrin formation independently of FXII and FXI [77] which suggest that the merger of inflammatory and hemostatic arms of CS contact/kallikrein system with the fibrin formation pathway might have occurred with the emergence of tetrapods [76,77] in the water-to-land transition of vertebrates around 400-390 Mya [35,52,55,74,75]. Overall, these new data confirm that the chain of genetic events necessary to lead to biological innovations with change in function predate and never come about during the great transitions that change in function are associated with (see Fig. 1 and section 3.2) [57].

### 2.2. Intravascular innate immune cascades, endothelium, and the circulatory system: a solution to multi-systemic organisms

Multicellularity and the progressive yet transitional spatially organized and regulated division of labor through cell type diversification involved segregation and divergence of function in the sister cell types [78–80]. These processes allowed primitive metazoans to perform different cellular functions at the same time, a significant evolutionary leap in sharp contrast with protozoans in which just one cell performed all of the physiological functions [1,2,78] yet with a complex life cycle with multiple temporarily regulated cell states [78]. Multicellularity however inexorably led animals to grow larger body size, and size matters. Both the earliest single-celled animals and today's 100-metric ton blue whale ultimately depend on diffusion to supply oxygen and nutrients to, and remove carbon dioxide and metabolic wastes from each cell in the body [54,81]. Moreover, growing bigger increased metabolic rates and generated large diffusion distances compromising the aforementioned functions (diffusion of oxygen, supply of nutrients, and elimination of metabolic wastes) [54,81]. In this evolutionary landscape of animals, cardiocirculatory systems have evolved to provide every cell in the body with a bulk flow delivery of gases and nutrients, and elimination of wastes to meet the metabolic requirements [54,81]. In this context, the proteolytic serine protease cascades underlying the immunoclotting defense system, which in invertebrates such as arthropods operates locally and immediately [6], faced new challenges imposed by the greater sophistication and the increasingly large bodies of many invertebrates in the Ediacaran (E 600 Mya), and in the Cambrian diversification (C 543 Mya) leading to emergence of vertebrates (Fig. 1) [82]. Initially, in lower vertebrates, the novel endothelial cells that lined an emerging closed and increasingly pressurized circulatory system together with thrombocytes were keeping the already irreplaceable commodity containing the precious red cells, namely the blood, in a fluid phase [35,54]. But this was not enough: the newly generated vascular and interstitial space had to be free from noxious biotic and abiotic particles [32,83], a challenge that in primitive vertebrates prompted blood cell type diversification, including the emergence not only of thrombocytes but of neutrophils, lymphocytes and other blood cells [84–86]. But by then, the diversification of soluble blood proteins of the complement and coagulation system was already underway and increasingly complex [32,34]. To understand this complexity, consider that humans possess approximately 100,000 km of vessels lined with 1.3 trillion of endothelial cells, covering a surface area of 4000 $m^2$–7000 $m^2$ [45,87,88], and the elbow to elbow time for an individual red blood cell has been estimated to be less than 30 s [35,83].

The challenge of integrating immunothrombosis can be interpreted as follows. Firstly, by conferring a significant survival advantage to the bearers, selection pressure conserved the local, immediate, and vigorous immunoclotting response that involved endothelial cells, thrombocytes, and negatively charged phospholipid membrane-mediated and $Ca^{+2}$-dependent activation of blood-borne proteolytic cascades [7,28,35,89]. In this way, proteolytic cascades maintained a compartmentalized defense response simultaneously limiting blood loss and avoiding both the replication and access of pathogens within a closed, endothelial cell-lined circulatory system [28,83,90]. Today, this is also so for lingering focal infections and/or the myriad breaches inflicted on vertebrate skin and organs such as lungs and gut by parasites, where immunothrombosis, aids both in killing intruders and in repairing the breaches, thereby preventing the infection from being systemic [7,40].

Secondly, the newly generated vascular and interstitial space had to be kept free from noxious biotic and abiotic particles as an additional survival requirement. This need likely exerted a strong selective pressure on the complement and coagulation systems as well as on blood cell type diversification, establishing an intense crosstalk among them [32,83]. Accordingly, the multi-arm mammalian complement system evolved in several steps through more than 1200 million years [32,47,48,50,91]. Starting as an intracellular C3-like multi-task protein with

metabolic, cell survival, and opsonic immune functions in single-celled organisms such as choanoflagellates (the precursors of sponges), the complement system of vertebrates evolved as a multicomponent network of foreign and altered host cell detectors, mediators, and effectors from trypsin as the precursor gene [32,34,38,51,91]. In doing so, the complement system, as well as other blood cascade systems, took on a novel role of guardian of the intravascular homeostasis by protecting and keeping the intracellular, interstitial, and intravascular space free from biotic, abiotic, and altered and non self cells [32,47,48,50] thereby operating as a purging system [7,32,35,83,91].

Similar to blood cascade systems, blood cell types of mammals evolved from less specialized precursor cell types that originally were multifunctional, performing several steps of an ancient innate immune response as well as serving other digestive, metabolic, and homeostatic roles [9,79,84,92]. Despite this understanding, the overall picture and the precise evolutionary history of the astounding emergence and diversification of vertebrate blood cell types remain unresolved. Endothelial cells, erythrocytes, thrombocytes, granulocytes and monocytes, and lymphocytes all emerged following the divergence of protochordates and the appearance of the earliest vertebrates (period of innovations, see Fig. 1) [9,35,54,71,93–95]. An interesting evolution/developmental hypothesis in biology that articulates phylogenetic and ontogenetic approaches suggests that all the cell lineages that make up the cell phenotypes of the vertebral vascular system including endothelial cells, pericytes, and blood circulating cells, share a common phylogenetic and ontogenic ancestor. This could be the case of a free floating coelomic cell originated from a coelomic wall of the invertebrate hemal system, termed the hemocyte [94,96,97]. Significantly, endothelial cell heterogeneity has been conserved from the most basal vertebrate (Hagfish) to mammals [54], complementing the conserved adherent, migratory and secretory functions of their invertebrate coelomic amoebocyte ancestor with the new epithelial phenotype of vertebrate endothelial cells [54,70,94,97]. Accordingly, these functions of the mammalian endothelial cell as exploratory and migratory cells are at the core of angiogenesis and repair function. For example, the mobilization of bone marrow endothelial progenitor cells to the circulation in response to vascular endothelial growth factor (VEGF), and their accumulation within the damaged tissue [94,97]. Moreover, under physiological conditions, mammalian endothelial cell secretory function contributes to the blood fluidity by promoting anticoagulant properties and counteracting platelet activation. They do so by expressing a large range of proteins and metabolites including but not limited to thrombomodulin (TM), tissue factor pathway inhibitor (TFPI), endothelial protein C receptor (EPCR), prostaglandin $E_2$ ($PGE_2$) and prostacyclin $I_2$ ($PGI_2$), and nitric oxide (NO), all acting as brakes. In this way they provide the luminal surface of the endothelium with anticoagulant antithrombotic, and anti-inflammatory properties. Noteworthy is their absence from artificial surfaces of medical devices in contact with blood [87,98–100].

Similar to the origin of endothelial cells, much of our knowledge about the cell type diversification of the myeloid lineage remains poorly understood. Sponges, one of the most basal multicellular metazoans, possess cells termed archaeocytes that bear structural and functional similarities with blood stem cells in vertebrates, and which serve as a stem cells that generate other cell types (gametes, sclerocytes) [84]. Similarly, interstitial cells in the phyla cnidarians or the neoblasts in platyhelminths (flatworms) perform stem cell functions [84]. On the other hand, molluscs and arthropods possess a hemolymph fluid with a variety of hemocytes (plasmatocytes or monocytes, granulocytes, and eleocytes) whose precursor is the immature hemolymph prohemocyte with multipotent capabilities, although in terrestrial arthropods hemocytes are interconvertible, unlike blood cells of vertebrates [85]. Invertebrate haemocytes store metabolic waste products, distribute nutrients, encapsulate and phagocytose eggs from parasites, and some contain densely packed granules similar to vertebrate neutrophils, eosinophils and basophils [9,84]. While plasmatocytes can best be compared with vertebrate monocyte/macrophages, coagulocytes may represent a specialized type of plasmatocyte-bearing granules that store hemolymph clotting and antibacterial factors similarly to vertebrate thrombocytes and mammalian platelets [6,9,84]. In addition, invertebrate granulocytes share some of their cargo with vertebrate neutrophils, eosinophils and basophils, with granules packed with enzyme-filled lysosomes [6,9,84]. These examples of circulating cell phenotypic diversity at the beginning of metazoan evolution reflect that the ancestral myeloid cells had already a high degree of phenotypic complexity, which was partially underpinned on a specific and independent genetic core regulatory complex that enabled and maintained the distinct gene expression program of a cell type within the organism [80]. Therefore, blood cell type diversification and specialization in vertebrates and mammals did not necessarily add complexity to an individual cell type but rather, and through segregation and divergence of primary and accessory or supportive functions, led to the emergence of many specialized cell types by partitioning and modifying the already existing functions of ancestral cell type into the new sister cell types [79,80,92]. Examples supporting this paradigm may be the professional macrophages [92], the red blood cells [95], or even the megakaryocytes (MKs) [101] of mammals where vertebrate thrombocytes are represented as anucleated platelets in mammals [101], and the nucleated erythrocytes of vertebrates undergo deletion of nucleus and organelles. Moreover, and indirectly supporting the hypothesis of the coelomic hemocyte as the common cell type ancestor of blood cells, erythrocytes are emerging as modulators of innate immunity in birds, amphibians, fishes, and mammals [95]. Their CD35 membrane receptor binds opsonized particles and transports them to liver and spleen where carried particles are removed [83]. At this point we must emphasize however that the developmental and evolutionary lineage of cell types are not necessarily the same, which could be the case of erythrocytes and megakaryocytes [101].

### 2.3. *The multicompetent platelet: the last newcomer in the immune continuum of mammals?*

Another layer of complexity in this evolutionary journey of coagulation and hemostasis (Fig. 1) in the context of the closed circulatory system of mammals is the emergence of platelets, as only mammals possess a megakaryocyte/platelet axis [9]. Despite remarkable phenotypic similarities including bactericidal, phagocytic, migratory, chemotactic, and hemostatic functions between invertebrate hemocytes, non-mammalian vertebrate nucleated thrombocyte and mammalian platelets [7,9], cell type homology is essentially only to be drawn between hemocytes, thrombocytes, and megakaryocytes [101] as only nucleated cells are considered units of evolution [9,80]. The blood cell type with primary hemostatic function in vertebrates is the thrombocyte that in non-mammalian vertebrates is nucleated, as is the erythrocyte, both derived from a thrombocyte-erythroid progenitor and sharing features of a nucleated, diploid oval-shaped phenotype [101]. Similarly, the mammalian unique enucleated erythrocytes and the polyploid megakaryocytes from which derive the cell fragments termed platelets, are generated from a common bipotent myeloid megakaryocyte/erythrocyte progenitor (MEP) cell [101,102].

Recent studies suggest that selective forces might have favored a more rapid and robust local immunothrombosis over systemic thrombosis risk in mammals. These consist of many survival advantages and trade-offs among defense mechanisms and hemodynamic consequences of the coagulation system and hemostasis [7,14,37,103,104]. A feature is the hemodynamic advantage of platelets that stems from their small size, which endows them with biophysically optimized features to act as the first of the circulating cells following vascular injury or pathogen aggression [105,106]. Given the diameter of capillaries, avian thrombocytes, and mammalian platelets (10–50 μm, 6–7 μm, and 1–3 μm respectively), platelets are optimized to fulfill their function under shear in flowing blood, aggregating to provide thrombus formation and then

promoting fibrin formation leading to stabilization of the initial hemostatic plug. The generation of resistant arterial plugs optimizes hemostasis in the high-pressure, high-flow conditions of mammalian arterial systems [16,103]. Furthermore, anucleated platelets possess a high surface-to-volume ratio, and a membrane with, for example, the αIIbβ3 integrin present at 50000–80000 copies per platelet. This feature renders them highly reactive, a key property to survey and repair any endothelial breach [107], which exposes collagen from the subendothelial matrix and binds to circulating von Willebrand factor (VWF) [87]. Once platelets sense endothelial damage they initially adhere through platelet surface GPIbα receptors to the A1 domain of VWF glycoprotein, which through the exposed A3 domain has already bound to the exposed subendothelial collagen I and III fibers [37]. Transient attachment is stabilized through α2β1 and GPVI [37,108,109] with platelet activation leading to platelet aggregation with principally fibrinogen, but also fibronectin, vitronectin, VWF and thrombospondin participating in the formation of platelet to platelet bridges [110]. Importantly, platelets activated by the tissue factor-induced thrombin offer their membrane surface as a gathering point for the zymogen activations involved in local generation of more thrombin, in the generation of fibrin and in controlling inflammation [35,111].

Significantly, in a quiescent manner, approximately 750 billion platelets patrol with manoeuvrability, massively occupying the external border near the vessel wall in flowing blood, a phenomenon known as margination [105,112,113]. In doing so, they survey and perform reparative labor on the vasculature, thereby acting as the first responders to endothelial disruption of multiple origins [105,106]. Roughly 100 billion new platelets (10% of the platelet count) released daily by megakaryocytes are used up in this maintenance work that, together with more than twenty plasma proteins that constitute the basis of the coagulation system and more than fifty plasma soluble and cell receptor proteins of the complement system, and endothelial-expressed and secreted thrombomodulin (TM), tissue factor pathway inhibitor (TFPI), endothelial protein C receptor (EPCR), prostacyclin ($PGI_2$), and nitric oxide (NO), keep the intravascular space and blood both sterile and the later in a fluid state [11,87,114,115].

Platelets, besides the release of platelet microbicidal peptides (thrombocidins and kinocidins) [116,117] and through toll-like receptor (TLR) 2 and 4, receptor for advanced glycation end products (RAGE), and dendritic cell-specific intracellular adhesion molecule-3-grabbing non-integrin (DC-SGN), also sense and recognize damage-associated molecular patterns (DAMPs) and PAMPs, thereby contributing to tagging and eliminating pathogens [104–106,118].

Furthermore, in the case of injuries and pathogen invasion [9,102,119], there may be an explosive fragmentation of MK generating thousands of platelets on demand [14,101,102]. Thrombin-mediated activation of IL-1α feeds back to hemostasis by adding an extra boost of platelets but also supports local immunothrombosis and wound healing, a fact that again links immunothrombosis and healing [14,119]. In fact platelets can be considered as an innate immune and repair effector of MKs as secretory cells [102,111]. Finally, Martin and Wagner [120] suggested another significant biological advantage stemming from a compartmentalized, robust megakaryocyte/platelet-mediated hemostatic response, namely, to facilitate embryo implantation by allowing an invasive placentation and to avoid mortal hemorrhage during childbirth of eutherian mammals (Fig. 1). However, the origin of the megakaryocyte/platelet axis has been suggested to have occurred around 166 Mya [9,56], a date that goes further back compared to the appearance of eutherian mammals roughly 100 Mya [58,120] (Fig. 1). Moreover, comparisons of the hemostatic systems of non-placental (egg-laying monotremes, and marsupials) and placental mammals, do not show noticeable differences in megakaryocytes and platelets [9]. But these facts do not preclude Martin and Wagner's [120] suggested selective advantage (Fig. 1).

Overall, the series of events that eventually gave rise to the emergence of the megakaryocyte/platelet axis remain a puzzle [9] as is the polyploid feature of megakaryocytes. Some authors point to megakaryocyte's enormous genome as a means to support such massive synthetic activity that leads to a daily release of 100 billion new released platelets that carry a rich repertoire of messenger RNAs, microRNAs and even transcription factors. These endow platelets with a translational activity, and a copious amount of growth factors, adhesion molecules, and cytokines that contribute to their multifaceted roles in immunothrombosis and tissue repair [111,113,121,122].

## 3. Defense and repair: two sides of the same coin

Defense and repair functions are evolutionarily linked to the invertebrate and vertebrate responses to environmental challenges, and grouped as immunoclotting [3,9]. This response consists of both immediate and local wound-sealing and pathogen-killing mechanisms to restore homeostasis and effect tissue repair (Fig. 2) [3,9,35,69]. Despite

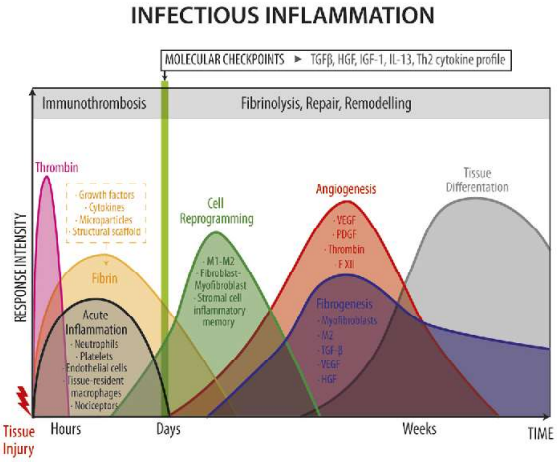


**Fig. 2. Immunothrombosis: a mosaic of defense mechanisms assembled, selected, and evolved to act locally.** Immunothrombosis is a highly conserved local and multitask cluster of defense responses made up of hemostasis and coagulation, the sensory nervous and innate immune systems, and fibrogenesis, whose key cell players are circulating blood cells (polymorphonuclear cells, monocytes, and platelets), and tissue stationary and mobile resident cells (nociceptor neurons, endothelial cells, macrophages, and fibroblasts) [8,10,11,27,28,39,89,90]. Tissue injury- and infection-derived DAMPs and PAMPs activate blood circulating cells, tissue stationary and mobile resident cells, and intravascular innate immune cascade systems [8,10,11]. Tissue factor expressed on damaged tissue, and activated FXIIa trigger the generation of thrombin which leads to stopping bleeding through the fibrin clot and platelet plug, and together with the activation of neutrophils, immunothrombosis will result in the containment, destruction, and expulsion of pathogens including metazoan parasites, as well as abiotic molecules [8,41,106]. After this early hemostatic-inflammatory process curtails bleeding and pathogen invasion, molecular checkpoints stemmed from local necrotic, hypoxic and acidic microenvironment including cytokines such as IL-6 and IL-13, and transcription and growth factors from platelets, nociceptors, leukocytes, and macrophages (TGF-β, PDGF, VEGF, IGF-1, specialized pro-resolving mediators such as $LXA_4$ and maresins) [3,7,27,126–128] will shut down inflammation, switching the process from killing and destroying mode to healing mode through cell reprogramming, angiogenesis, and fibrogenesis. Examples of the induced transient repair cell phenotypes through adaptive cellular reprogramming of adult differentiated cells [92,127,129,130] are fibroblast/myofibroblast differentiation, myelin Schwann cell/repair Bungner cell transdifferentiation, and macrophage M1, M2, M3, M4 and neutrophil polarization [92,127,129–132]. In addition, the proliferation of endothelial cells together with fibroblast/myofibroblast differentiation will lead to angiogenesis and fibrogenesis respectively [98,129,133–135], two key processes in tissue healing. Adapted from Ref. [136] with permission.

the highly conserved function of immunoclotting, however, the molecular and cellular components underlying this multistaged process differ significantly among diverse phyla [9,35,43,123–125].

### 3.1. Tissue repair process: past and priorities with precision but not perfection

In arthropods like butterflies, clot formation initiates with coagulocyte aggregation and degranulation, and activation of the prophenoloxidase system (PPO); the result is a hard clot that will attract plasmocytes late in clot formation. These events enable epidermal regeneration that grows across the wound and replaces the scab, and whose final outcome depends on the presence-absence of microbial invaders and the nature of foreign antigens [6].

Similarly, in insects like drosophila, wound healing relies mainly on plasma proteins such as fondue and hemocyte-released hemolectin, the latter a VWF domain-bearing protein that promotes hemolymph coagulation serving the formed clot as a defense against nematodes as well as a scaffold for the repair process [43,46]. In deers instead, the regeneration of antlers, a muscle and joint-free bony cranial appendage of approximately ten kg is completed in 55–60 days (up to 28 rounds in a life cycle) [137]. The process starts with bleeding on the cast plane of a pedicle stump immediately after casting of the antler, followed by a significant proliferation and differentiation of a single stem cell coming from the pedicle periostium, a process similar to human bone fracture healing [137]. Besides playing a central role in blood coagulation and NO release from ECs, thrombin and proteolytically inactive thrombin-derived peptides exhibit at low concentration anti-inflammatory, antiapoptotic, and growth factor-like activity by stimulating fibroblasts and endothelial cells similarly to activated Protein C (APC), and thrombin itself appears to be necessary for limb, heart, and lens regeneration in the salamander [12,65]. Moreover, thrombin activates the HGFA-HGF-MET signaling pathway [138], platelets through the cleavage and activation of the receptors PAR-1 and PAR-4 [109], and the proIL-1α, the latter favoring thrombopoiesis and wound repair after acute platelet loss and injury [14].

But mammals have evolved as well a Th2 response to deal with lingering focal infections and/or the myriad breaches inflicted by parasites on the epithelial layer of organs such as skin, lung and gut [41,133,134]. This is a local and immediate response which overlaps with the immunothrombotic response. Immunothrombosis in physiological conditions occurs only on demand and is dictated by microenvironmental cues coming from tissue injured. These include hypoxia, acidic pH, necrotic cell- and microbial-derived molecular signals (DAMPs, PAMPs), cytokines such as IL-6, and transcription (TFs) and growth factors (GFs) [3,7,27,127]. Furthermore, immunothrombosis shares several molecules with the senescence-associated secretory program (SASP) such as transforming growth factor beta (TGF-β), IL-6, VEGF, and Insulin-like growth factor 1 (IGF-1) [139,140]. Also to be considered are tissue injury-derived biochemical signals [127,141] such as subendothelial collagen, VWF deposited on collagen, and tissue factor that combine to activate the coagulation and hemostasis as well as the complement system [11,29,41,89]. In addition, other stimuli including but not limited to TGF-β, VEGF, NFκB, STAT3, SMAD2, will induce transient repair cell phenotypes through adaptive cellular reprogramming of adult differentiated cells [92,127,129,130,140] (Fig. 2). Some significant examples of this cell plasticity are fibroblast/myofibroblast differentiation, myelin Schwann cell/repair Bungner cell transdifferentiation, and macrophage M1, M2, M3, M4 and neutrophil polarization [92,127,129–132]. Both immunothrombosis and cell reprogramming also appear to be crucial to culminate in the tissue repair process [127,130,132,140,141] (Fig. 2). The early hemostatic-inflammatory period not only stops bleeding through the fibrin clot and platelet plug but also mediates the containment, destruction, and expulsion of pathogens and metazoan parasites, through Th2 lymphocytes. These cells will release IL-13, a cytokine that polarizes macrophages toward a profibrotic and reparative M2 phenotype thereby bridging the gap between sealing-killing and healing [41,133,134] (Fig. 2). In the absence of pathogen, as under sterile inflammatory conditions (severe trauma, ischemia, ischemia-reperfusion, intravascular artificial medical devices), activated intravascular serine protease cascades, platelets and endothelial cells together with polarized M2 macrophages release TGF-β, PDGF, VEGF, LXA4, IL-6, IL-13 and IGF-1 that operate as molecular checkpoints. In doing so, they rapidly shut down early inflammation including neutrophil infiltration and dendritic cell (DC) activation, switching the process from an inflammatory to a healing mode through the macrophage M1 to M2 phenotypic switch, differentiation of fibroblasts into myofibroblasts, and the proliferation of endothelial cells, leading to fibrogenesis and angiogenesis respectively [98,129,133–135,140,142] (Figs. 2 and 3).

This early time window appears to be determinant since the continued presence of neutrophils (necessary in infectious inflammation, but dispensable in sterile inflammation) may delay the healing process and generates fibrosis [17,23,125,140,142,148,152]. As primary effector cells in tissue repair and fibrosis together with macrophage M2, myofibroblasts synthesize and deposit extracellular matrix (ECM) components including fibrillar collagen types I to IV, fibronectin and release reactive oxygen species (ROS), thereby bridging the injury gap and replacing the transient fibrin scaffold [129]. Therefore, immunothrombosis as a multicomponent response has built in redundancy and robustness where signaling versatile proteins including thrombin and thrombin-derived peptides, FXII, VEGF, TGF-β, HGF, IGF-1, and PDGF among others, and some transcription factors rather than cells *per se*, are used interchangeably *in vivo* in a complex non-lineal manner during cell information transfer principle in the regulatory pathways which operate locally during defense and tissue repair [12,92,106,134,140,153]. It is highly likely that selection exerted a strong pressure on mechanisms underlying the early phases of immunothrombosis to operate quickly with biological precision by linking immunity and coagulation as a key effective survival factor. Evolution has left the immuno-reparative function of fibrogenesis and angiogenesis as open and condition-sensitive processes aimed at functional recovery rather than at structural perfection [3,39,41,134,154].

### 3.2. Tissue repair as a byproduct of immunothrombosis

The immunoreparative role of immunothrombosis relies on biomolecules including thrombin, fibrin (ogen), growth factors, cytokines, and microparticles primarily originating from plasma, activated platelets, and tissue resident macrophages, all of which are tissue repair enhancers [12,16,22,106,140]. In this respect, tissue healing might be conceptualized as a byproduct of immunothrombosis. And activated plasma-born intravascular innate immune cascade systems together with polymorphonuclear cells, monocytes, and platelets prevent the hemorrhaging and pathogen invasion caused by trauma- and infection-induced damage. Moreover, interaction with resident tissue cells including nociceptors, endothelial cells, macrophages, and fibroblasts will favor tissue repair [10,39,41,65,154] (Figs. 2–4). Although injury, defense, and repair appear to go hand in hand, tissue healing is developed in a manner that is not pre-specified by any genetic program, and it is dictated by microenvironmental cues stemmed from cell- and soluble factor-mediated circuits of immunothrombosis, as well as from pathogen and commensal microorganism products [92,135,140,147,148]. This is the case of fibrogenesis as a physiological process with a continuum spectrum that might be transformed into a pathological fibrotic condition [42,129,140,141], thus the repair process will not resolve with a unitary outcome (Fig. 2) [140,155]. For instance, the coexistence of commensals or pathogenic bacteria and their products (microbe-associated molecular pattern (MAMPs) and PAMPs) [147,148] or ECM-molecule debris (DAMPs) stemmed from tissue damage (sterile inflammation), elicit a persistent profibrotic M2 macrophage phenotype and the release of PDGF, IL-6, IL-13, and TGF-β [42,129,135].

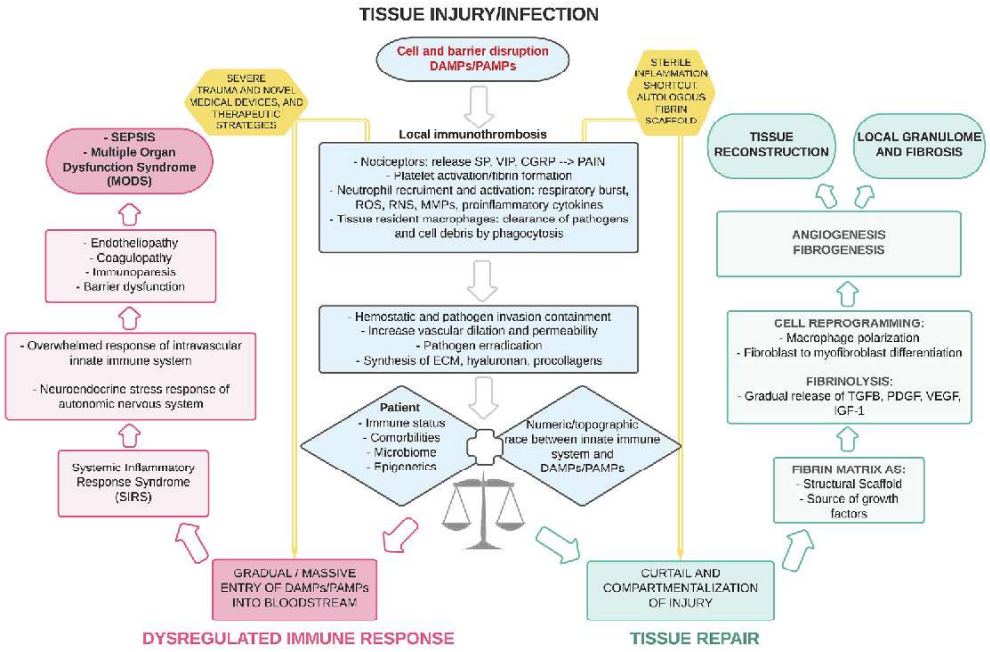


**Fig. 3. Immunothrombosis: Challenges and therapeutic opportunities.** By coupling inflammation and coagulation, immunothrombosis operates as a cooperative and amplifying strategy to cope with trauma and infection. The activation of nociceptors, endothelial cells, fibroblasts, tissue resident macrophages, circulating platelets and neutrophils, and plasma innate immune cascade systems [8,10,11] leads to curtail and compartmentalize the damage through wound-sealing, clearance-killing of pathogen and foreign bodies, and synthesis of ECM, thereby closing the damaged gap and giving way to tissue repair (see section 3). But, when the local inflammatory response is not resolved timely, often a persistent chronic inflammation leads to the formation of a nonfunctional secondary angioneurofibrotic scarring (local granulome and fibrosis) [129,143–145]. Autologous fibrin matrix may act as a shortcut therapeutic strategy in musculoskeletal sterile inflammatory pathologies by dampening inflammation and enhancing tissue repair and reducing pain [30,126,146]. However, when the triggering emergency cannot be handled in a tissue autonomous manner (due to the patient status, a severe polytrauma, and/or the use of cutting-edge medical strategies), the numeric and topographic race between DAMPs and PAMPs, and the well developed local checks including intravascular innate immune cascade systems tips the balance towards a gradual or massive entry of biotic and abiotic molecules into the bloodstream [147,148]. In this new either sterile-inflammatory or infectious-inflammatory context, the complement cascade, the coagulation-contact and fibrinolytic systems may be locally and systemically dysregulated, thereby causing a SIRS [27,149,150]. Moreover, the neuroendocrine stress response mediated by the activation of the autonomic nervous system interacts with the endothelium and the innate immune system, resulting in a barrier dysfunction that may lead to an endotheliopathy, coagulopathy, immunoparesis, and the breakdown of protective cell barrier of the gut. Frequently, the non-lineal dynamic response of systemic immunothrombosis operates as a feedback loop with several arms leading to MODS and sepsis (see section 4) [27,151].

With dual fibrogenic and anti-inflammatory roles, TGF-β influences the number, activity, and/or life span of myofibroblasts and the generation of a fibrotic process [42,129]. Accordingly, sterile well-performed surgical techniques generate little or no inflammation and scar [42], whereas an excess of trauma- and surgery-derived DAMPs induces immune activation with local and systemic detrimental consequences. This has given rise to the concept of damage-control orthopaedic (DCO) surgery and the use of minimally invasive surgical approaches (Fig. 5) [27].

Supporting this idea, several studies in germ-free Swiss mice have shown that fetal skin wound healing and skin wound healing were accelerated, scarless, and associated with a reduced presence of neutrophils and increased content of anti-inflammatory M2 macrophages [125,148,152]. Similarly, early phases of wound healing on mice lacking the complement C3 component are accelerated, which is associated with a lessening mode of the inflammation intensity, significant reduction of neutrophils, important increase of mast cells, and accelerated angiogenesis at the injured sites [23].

Last but not least, FXII is an important plasma zymogen whose FXII/uPAR/pAkt 2 axis may drive the neutrophil-driven inflammation by promoting neutrophil trafficking and neutrophil extracellular trap (NET) formation resulting in impaired wound healing in a model of sterile inflammation, even though it favors further plasma FXIIa generation and blood coagulation [17]. In addition, FXII activation accelerates and strengthens fibrin-clotting formation in the presence of inert soil particles as a proxy of contaminated wounds in the plasma of terrestrial mammals like mice and humans but not in cetaceans (whales, dolphins) which lack of PK and possess an inactive pseudogene FXII, and in birds which have lost the FXII gene [18]. Moreover, a recent study showed that the fibrin biofilm formed in air-liquid interface in human and mice plasma, and in a murine dermal injury model, covers the formed blood clots and protects from bacterial proliferation and dissemination [160]. Overall, and taking into account these three studies and the aforementioned considerations, it is reasonable to hypothesize that FXII is necessary *in vivo* mainly to reinforce clotting in infectious conditions. Accordingly, recent research indicates that FXII operates as a pattern recognition molecule in infectious epithelial wounds by binding to both microbial walls or their products [19,161], and to infectious proxies such as soil particles, shortening the fibrin formation, attracting and promoting neutrophil activities and NETosis, as well as generating local bradykinin-mediated vasodilation [17–19,160,161]. In doing so, FXII may contribute to quickly compartmentalize and sterilize the wound thereby limiting the systemic spread of biotic elements in terrestrial animals [17,160], which are in contact with dirt-soil particles [18,162], as well as the systemic spread of small abiotic nanoparticles that escape from the multiarmed complement system [11,143], thereby promoting survival. Therefore, this FXII-mediated function in epithelial wounds (and likely in the epithelium of lungs and gut) might have played a strong selection pressure in animals in their transition from water to land. In fact, intertidal, mud,

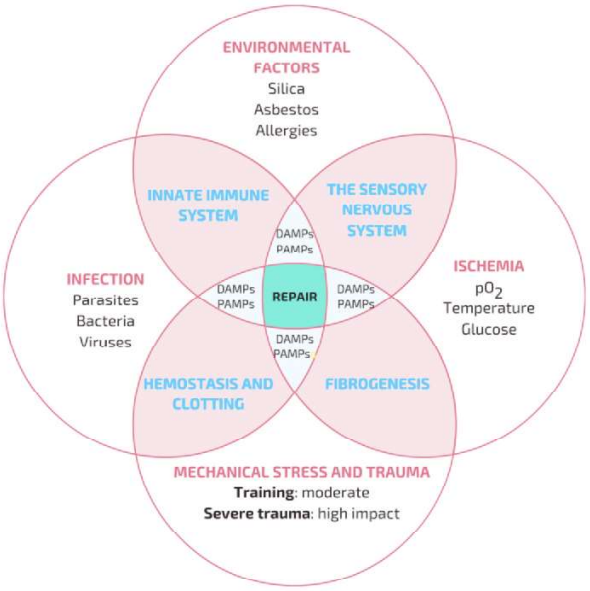


**Fig. 4. Tissue repair as a byproduct of the biological defense process.** The activation of the host biological defense processes and tissue repair appear to go hand in hand with mutual mechanisms mediating both immunothrombosis and healing. Several disruptors including infectious agents, environmental factors, ischemia, and mechanical stress may activate one or several arms of the host defense responses in an attempt to curtail the damage. In this context, the restoration of injured tissue might be conceptualized as a byproduct of the activated intravascular innate immune cascade systems, and tissue stationary and migratory cells including nociceptors, endothelial cells, fibroblasts, and resident macrophages [10,39,65,104,154]. Adapted from Ref. [30] with permission.

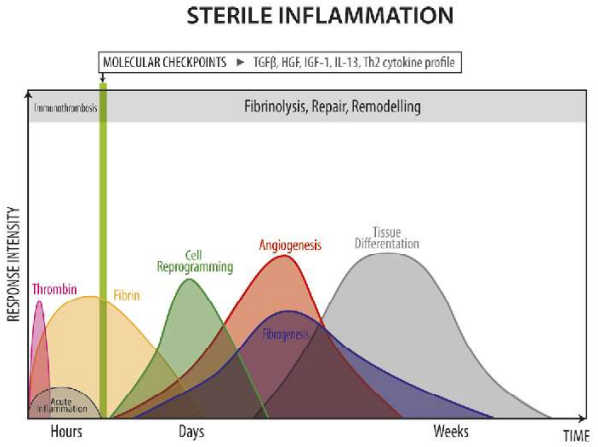


**Fig. 5. Autologous fibrin scaffold: Mimicking the immunoreparative role of local thromboinflammation in sterile inflammatory conditions.** This model is a representation of both the activation of host biological systems in sterile inflammatory conditions, and the scientific rationale behind the use of the autologous fibrin scaffold. The disruption of structural integrity of vascularized mammalian tissues by noxious agents, and affecting tissues such as skin, the central and peripheral nervous system, or musculoskeletal tissues, elicits the activation of platelets and nociceptor neurons, the formation of a fibrin clot, and the recruitment of neutrophils and monocytes to the affected site as a first stop-and-go check point to quickly stop bleeding, destroy bacteria, and avoid noxious stimuli created by behavioral changes [10,89]. However, in the absence of pathogen or their products, as in a sterile inflammation setting, molecular checkpoints rapidly shorten and dampen the early inflammatory window, switching the process from a killing and destroying mode to one that promotes healing [42,98,129,133–135] as suggested from how regenerative species react to injury [125], and from fetal scarless wound healing [156–158] (Fig.. 2 and 3). Early inflammation has been suggested to be an important therapeutic window, and its dampening might drastically reduce the fibrotic outcome of tissue injury in a cell-circuit framework for tissue repair and fibrosis [140]. Accordingly, in sterile inflammation defense responses are shorter and less intense than in infectious immunothrombosis (Fig. 2). Importantly, platelets may play an important role in the early resolution of sterile inflammation since they are a significant source of SPMs including maresins, resolvins, and LXA4 [126,128], the latter an endogenous arachidonic acid-derived mediator that has been reported to counter-regulate inflammatory processes and promote resolution responses in Achilles tendinopathic stromal cells [159]. Moreover, studies using leukocyte-free autologous fibrin matrix in musculoskeletal tissues, skin and corneal injuries reported a shorter repair time of tissue damage, a significant pain reduction, and expedite recovery function compared with the control group [24,30].

and dirty soil environments are abundant in white clay with silica-rich compounds like kaolin and Celite (key cofactors to activate FXII) [18, 163]. This microenvironment prevailed for the first amphibians, and latter for reptiles and other terrestrial vertebrates, including mammals [164]. In fact, marine mammals possess an inactive pseudogene FXII, and many fish lack FXII as do aerial animals like birds that have lost the FXII gene [73,74]. On the other hand, in the current cutting edge medical therapies, FXII as key zymogen of the contact system may impair wound healing in a model of sterile inflammation [17], as well as induce immunothrombosis on medical devices, both events to be considered as a trade-off of the contact system's activation [11,17,42].

## 4. Living with the past: emerging tradeoffs of intravascular innate immune system

From an evolutionary point of view, the integration of immunoclotting into an increasingly complex mammal bodies generated much interplay among the blood-born proteolytic cascades, blood cell types, and the neuroendocrine system changes that endowed enormous survival advantages. However, in our modern world, with developed cutting edge therapeutic strategies and technologies, this functional cross talk entails tradeoffs in a complex nonlinear manner [27,28,149, 165–167]. This is the current case of the exposure of blood to unscheduled and never anticipated medical devices and procedures, sedentary and prothrombotic life-style, and highly polluted environments so unlike those on which the integrated systems were evolved, tuned, and operated. The result has been increasing vulnerabilities at unprecedented levels in the form of new diseases, from thrombo-embolic events and ischemia/reperfusion injuries to systemic inflammatory syndrome and sepsis [11,27,106,167,168]. Sepsis, a deleterious, non-resolving inflammatory host response to infection, occurs in 1.5 million Americans annually and it causes more than 250000 deaths each year in the United States alone [169,170]. Moreover, the final common end-point of these infectious (sepsis) or severe sterile challenges (such as polytrauma, hemorrhagic shock, massive and repetitive blood transfusions, use of extracorporeal circulatory devices, organ and tissue transplantations, and intravascular artificial medical devices) is the increasingly seen multiple organ dysfunction syndrome (MODS) (Fig. 3) [27,149,171].

### *4.1. Immunothrombosis: a mosaic of defense mechanisms assembled to act locally*

Immunothrombosis in humans is a multilayered nonlinear response where the local cell-to-cell and extracellular matrix-to-cell short range signaling pathways represented by complement, coagulation and contact systems, and fibrinolysis, have been complemented with and integrated into the long-range neuroendocrine-immune signaling pathways

[10,149,165,172,173]. Pathogen- and damage-recognition molecules including Toll-like receptors (TLRs), Nod-like receptors (NLRs), receptors for advanced glycation end products (RAGE), FXII, FVII, HMWK, C1q, Mannose-binding lectin (MBL), Ficolins, Properdin, C-reactive protein (CRP) are multidomain molecules present intracellularly, in the fluid phase and/or embedded in membranes of blood cells, endothelial cells, and nociceptors [11,174–176]. The serine proteases of the complement, coagulation and contact, and fibrinolysis system, whose protein domains possess intrinsic affinities, have created molecular networks that operate locally and extremely fast both in positive and amplifying or in negative and containment feedback loops. This is the case of the thromboinflammatory response to microbial pathogens, hemorrhagic shock, ischemia-reperfusion injuries, massive and repetitive blood transfusions, extracorporeal circulation, organ and tissue transplantations, intravascular artificial medical devices and massive trauma and burning injuries [11,27,28,149,171]. In order to compartmentalize the damage [3,7,33–35,177], blood circulating cells, nociceptor neurons, endothelial cells [166,178], tissue resident macrophages, dendritic and mast cells, and stromal fibroblasts are major effectors and players with dual roles in local neuroimmune surveillance and systemic body hemodynamic and organ homeostasis [166,178].

On the one hand, sensory neurons (Fig. 4) form networks within the primary physical barriers, namely, the different epithelial layers of the skin and mucosal surfaces of the respiratory and gastrointestinal tract, as well as joints and muscles. These nociceptor terminal branches form a receptive field which may include vascular endothelial and smooth muscle cells, tissue resident macrophages, and dendritic and mast cells among other immunocompetent cells [10,179,180]. The branches are endowed with receptors at the axon endings (transient receptor potential (TRP) ion channels VI (TRPVI) and subfamily M member 8 (TRPM8), piezo ion channel 2 (Piezo2), several TLRs, and receptors for TNF, IL-1β). As a result, nociceptors sense the noxious stimuli and generate action potentials that travel in an orthodromic fashion to neuronal bodies toward the central nervous system (CNS) but when action potentials reach branch points they are diverted to other peripheral endings of the same neuron in an antidromic manner [10,179,180]. This mechanism, known as axon reflex, together with the activated platelets results in the local release of histamine, serotonin, ATP, $Ca^{+2}$, substance P, calcitonin gene-related peptide, and matrix metalloproteinases, concomitantly with the activation of coagulation and hemostasis pathways. This leads to vasodilation and increased permeability of local vessels that allow the passage of plasma and leukocytes into the damaged tissue parenchyma [10,87,129,181]. Sensory neurons not only mediate in the local but also in the remote responses to injury and infection by local and systemic immunomodulatory axon reflexes that are at the origin of neurogenic inflammation and the anti-inflammatory response [10,27,178].

In contrast, endothelial cells in their quiescent state, offer their stationary yet dynamic luminal membrane in constant contact with circulating blood as an anti-thrombotic and complement-regulatory surface thanks to a layer of proteoglycans and heparan sulfate molecules covering the endothelium known as the glycocalyx. However, the opposing endothelial membrane in contact with the basement membrane is in close vicinity with nociceptor neurons, tissue resident macrophages, dendritic and mast cells, among other tissue resident cells [10,98,179]. Therefore sandwiched between the local intravascular innate immune system with its humoral and cellular arms and the systemic neuro-immuno-endocrine system, the heterogeneous endothelium is the interface between the environment and the internal milieu, acting as a gathering point, barrier, and target of host defense mechanisms [35,98]. The evolution-tailored hierarchical interplay of serine protease proteins of the complement and coagulation cascades and the contact system with immune and endothelial cells, and neurons, with their positive and negative molecular feedback mechanisms and neural axon reflexes, have evolved to operate extremely fast and precisely providing significant survival advantages. This is the case when local challenges are minor enough to be solved by the house keeping activity primarily carried out by resident macrophages and helped by ECs, platelets and stromal fibroblasts, the later using their inflammatory memory [3,27,182–184]. However, when the triggering processes, cannot be handled in a tissue autonomous manner, the insufficient local containment response gives way with increasing amounts of DAMPs and PAMPs gaining access to intravascular space. Importantly, and in its role of a purging system, the collaborative crosstalk between blood complement, coagulation and contact systems, with tissue stationary fibroblasts, dendritic and mast cells, tissue resident and migratory macrophages, endothelial cells and circulating polymorphonuclear cells, monocytes, and platelets becomes overwhelmed and dysregulated (Fig. 3) [7,11,177]. This new scenario may lead to a systemic, amplifying inflammatory syndrome as a zealous response and vicious cycle of intravascular innate immune cascades, endothelium, and blood cells, yet revealing the costs and vulnerabilities of host defense systems (Fig. 3) [3,11,27,90].

### 4.2. *Lifestyle and medical novelties with roles in emerging tradeoffs*

This is the case of major trauma and massive burns, hemorrhagic shock, ischemia-reperfusion injuries, sepsis or when patients in intensive care units, react badly to massive and repetitive blood transfusions, organ and tissue transplantations, and the use of blood-contacting medical devices including extracorporeal circulation [11,27,28,149,171,185]. Those patients, who would previously have died, now survive, but the danger is not finished once the "*talismanic*" values of the physiological homeostasis, including blood pressure and red blood cell count among other values, have returned to normal levels [7,27,28,149,166,169,186]. The physiological systems of these "*new patients*" may usher in systemic inflammatory syndrome and sepsis, frequently leading to MODS [27,149,166,169,187]. These novel complex-to-treat medical conditions bring out biological tradeoffs that our organism tolerated in other environments due to the major benefits conferred by the interconnectedness of immunothrombosis and neuroendocrine system [3,11,35,143]. In fact, the dysregulated nonlinear host response of patients suffering from systemic inflammatory syndrome and MODS (Fig. 3), makes it enormously difficult to therapeutically rebuild the broken physiological cardiorespiratory, digestive and renal networks in those patients suffering from MODS [27,149,165,188] as well as to rebalance the intravascular innate immune system due to the evolutionary molecular interconnectedness.

Our modern society has also brought vulnerabilities in the form of new diseases, from thrombotic events and ischemia/reperfusion injuries to autoimmune conditions and chronic inflammation and cancer [11,27,143,167,168,189]. This is the case with a sedentary and prothrombotic life-style, the exposure of blood and other body fluids to medical devices, procedures, and novel and toxic environmental nanoparticles stemming from food supplements, household materials, dental applications, cosmetics, vehicle exhaust and mechanical wear of the brakes ($\alpha Fe_2O_3$) or from smoke (cigarettes, environmentally-caused wild fires, cooking). For example, small nanoparticles of $TiO_2$ (present in paint, toothpaste, sunshield and cosmetics) are too small to activate the classical complement pathway through the multi-armed recognition proteins of complement (C1q, ficolins) and get rid of them in its role of purging system [143,190]. In contrast, the pattern-recognition protein FXII binds one to one with $TiO_2$ nanoparticles as well as with other particles of similar size and may mount an inflammatory and bradykinin-mediated inflammatory and angiogenic response that might aggravate chronic inflammatory pathologies [143,190]. Therefore, we should take into account our novel life-style model and environmental innovations as a source of elements that render our biology not imperfect or imprecise but unpredictable for some current human therapeutic purposes instead of blaming the blood-born proteolytic cascades, blood cell types, and neuroendocrine system for operating and fulfilling the roles for which they were crafted and tuned very precisely [3,10,11,35,124,143,167].

### 5. From tissue repair as byproduct to fibrin matrix as biology-as-a-drug approach

Blood is a multitask, fluid, connective tissue that besides coordinating and communicating between nonadjacent tissues, its circulating proteins and cells influence the repair and regenerative capacity of multiple tissues and organs having always been present in the equation of healing therapies [191–193]. Several lines of evidence derived either from systemic or local stem cell niche therapies, and represented by parabiosis or microfractures and tendon scarifications, respectively, support the concept that factors derived from platelets or plasmatic proteins are candidates for mammalian tissue rejuvenation and healing [24,192,193].

Autologous fibrin matrix (AFM) is emerging as a local biology as a delivery system of GFs to expedite repair of sterile inflammatory injuries such as osteoarthritis, tendinopathies, cartilage injuries, peripheral neuropathies, intervertebral disc degeneration, skin burns and ulcers, corneal ulcers, and dry eyes among other conditions [24,29,30,

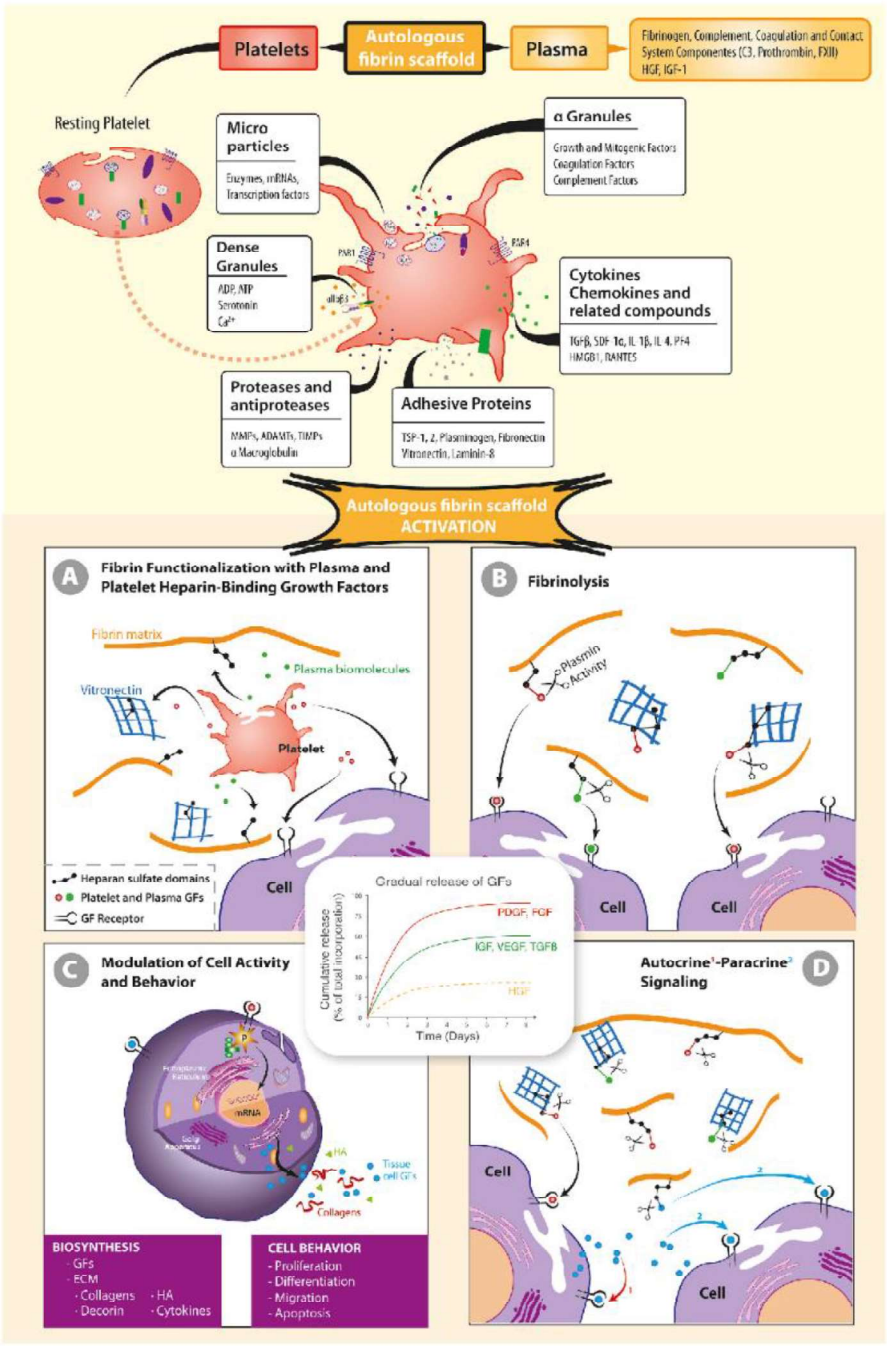


**Fig. 6. Autologous fibrin scaffolds as a local biology-as-a-delivery system of growth factors (GFs) to enhance repair of sterile inflammatory injuries.** Autologous fibrin matrix is a liquid-to-gel dynamic carrier of biological mediators for tissue repair. When the fibrin scaffold is formed, the three-dimensional matrix traps many of the plasma and platelet-released growth factors and cytokines. Several of these GFs are bound temporarily to heparin sulfate proteoglycan domains of fibrin matrix components (in a yuxtacrine or matricrine mode) (A). After its application to injured sites, the fibrinolysis mediated by plasmin that is generated through both the activation of plasminogen by a tissue plasminogen activator [87,219]. Immune and mesenchymal cells migrate into the clot and accompany fibrin degradation thereby matching the speed of the ingrowing repair tissue [220–223] (B). GFs bind to *trans*-membrane receptors present on the surface of target cells, thereby activating intracellular pathways that convey the signal to the nucleus, to eventually induce a wide range of genes that will translate into changes in cell survival, proliferation, migration, differentiation, transdifferentiation, and maturation (C). These effects include the synthesis and secretion of a new ste of GFs and cytokines, which also interact with their receptors in a diffusible manner (autocrine and paracrine pathways), the synthesis of ECM components such as collagens, decorin, hyaluronic acid and GAG, and also modify cell survival, proliferation, differentiation, and migration (C) [30]. Moreover, some GFs in a diffusion mode (autocrine and paracrine) will directly reach their cognate cell-surface receptor, thereby exerting an immediate cell-biosynthetic and cell-behaviour modification (D). Adapted from Ref. [224] with permission.

194].

The therapeutic potential of this liquid-to-gel dynamic scaffold relies on the biochemical machinery of coagulation and platelets, including but not limited to thrombin [12,13], fibrin [15,16], FXII, and the payload released by activated platelets from growth factors and cytokines to microparticles (Figs. 6 and 7) [16,24,29–31].

### 5.1. A brief history of the development of autologous fibrin scaffolds

The recent history of AFM began in the early twentieth century with the use of fibrin and its derivatives as hemostatic agents in surgery [195]. Later, Young and Medawar described the use of fibrinogen-fibrin in the suture of peripheral nerves in animals [196]. It was in 1944 when Cronkite first described the use of fibrinogen and thrombin as a biological glue in skin transplants [197]. However, the preparations used so far lacked the necessary strength and were not very stable over time. Already in the seventies, Matras and colleagues continued with the development of fibrin sealants, specifically trying to increase the adhesive and healing properties of fibrin by the concentration of fibrinogen by chemical and cryoprecipitation methods [198]. A disadvantage of these methods was that they present a risk of disease transmission and the possibility of generating allergic reactions due to the use of bovine thrombin. More recently, Tayapongsak et al. used autologous fibrin clinically as an adjuvant in maxillofacial surgery [199]. These early concepts of combining the properties of two products, fibrin sealants and platelet growth factors, led to the development of AFM technologies. Following these principles, Marx [200] developed in 1998 a method to produce platelet-rich plasma (PRP) from a density gradient separator and applied it successfully in maxillofacial surgery, however the great disadvantage of this technique was the large amount of patient blood (400–450 mL) required. In the same decade, Anitua described the use of autologous PRP, but starting from small volumes of blood (approximately 40 mL) and activating with calcium chloride instead of using bovine thrombin [201]. After the initial development in the field of oral and maxillofacial surgery, AFM have been used in various medical specialties, such as traumatology, orthopaedic surgery and sports medicine, dermatology, ophthalmology, reproductive medicine, nerve regeneration, general surgery and wound healing, among others [30, 202–205].

In addition, AFMs have been used alone or as an adjuvant in combination with different materials in order to increase their regenerative power, such as hyaluronic acid, bone, fat or dermal substitutes [206, 207].

New non-clinical applications include the use of PRP as a culture medium supplement for *ex vivo* expansion of stem cells [208] or as a patient-specific bio-ink for tissue engineering [209]. Finally, platelets in the AFM are likely to have an increasingly important role in the development of new drug delivery approaches. As we have discussed so far, these small acellular fragments derived from megakaryocytes act as key players not only in hemostasis and thrombosis but also in multiple roles such as angiogenesis, inflammation and immunity. Their particular structure linked to these functions enables to exploit their full potential as a tool in the development of new approaches of biomedical engineering. Recently developed platelet-mimetic nanoparticles, nanocoatings and nanofibers can enable the targeted delivery of therapeutic proteins to struggle against cancer, help in vascular injury or fight off infections [210–212].

### 5.2. The scientific rationale behind autologous fibrin scaffolds

Autologous fibrin scaffolds and related products derived from platelet-rich plasma (PRP), are a by-products of nature's own healing systems that involve blood anticoagulation and mild centrifugation steps followed for fibrin formation by restoring thrombin production. Initially this involves the separation of a fluid phase plasma where platelets, white cells, and red cells are suspended in different concentrations depending on the centrifugation and fractionation methods employed [29,30,213]. The *ex vivo* activation of PRP by adding CaCl2, at low concentrations to restore calcium homeostasis [214,215] generates low but yet efficient concentration of autologous thrombin [216]. This results in a slow, gradual, and dynamic liquid-to gel fibrin scaffold, via the already activated intrinsic pathway of coagulation downstream of FX

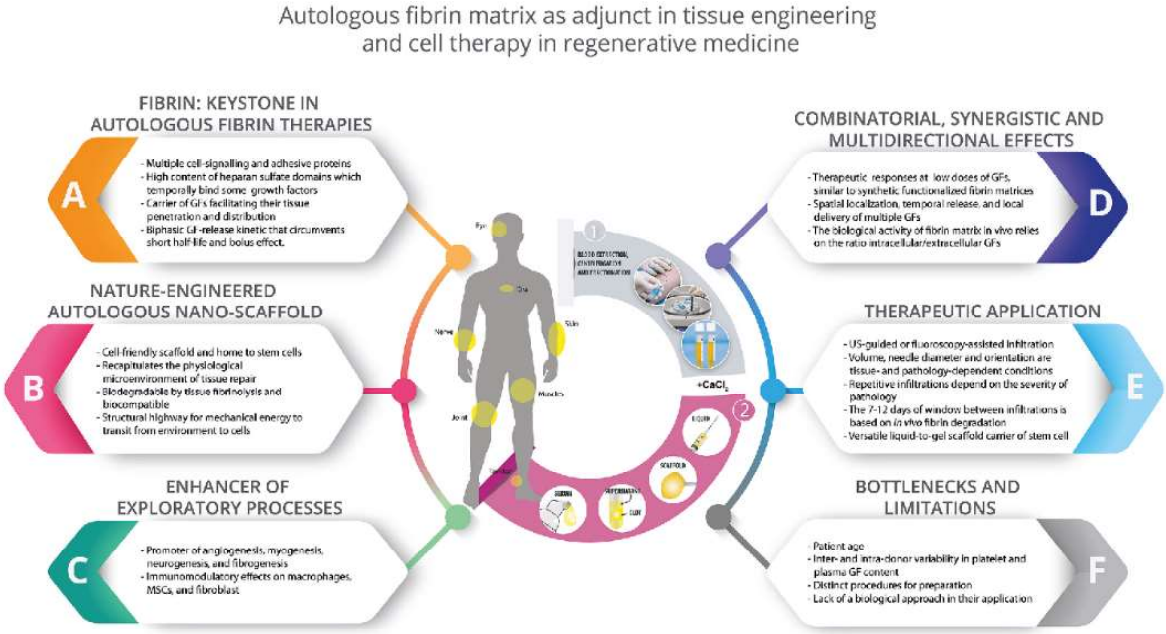


**Fig. 7. Scheme summarizing chief roles played by the fibrin as a center piece in tissue repair when autologous fibrin scaffolds are applied into injured area** [160,194,225,226]. Adapted from Ref. [224] with permission.

[162,217]. Moreover, the generated native thrombin simultaneously activates platelets and their degranulation allows the release of a panoply of biologically active proteins and metabolites; the polymerization of plasma fibrinogen into a progressively insoluble fibrin matrix provides the functional unit for tissue repair [29,31,218].

The scientific rationale behind AFM as a tissue repair enhancer in sterile inflammatory injuries is essentially provided by GFs and biomolecules stemmed from platelets and plasma, the fibrin matrix, and the interaction of native GFs with both the newly generating ECM and tissue cells where the liquid-dynamic matrix is injected or applied [29,31,221] (Fig. 6). The architectural keystone of the fibrin matrix is the high content of heparin sulfate domains that transiently bind growth factors protecting them from proteolysis thereby extending their short half-life, and placing them in the vicinity of cells facilitating tissue penetration, spatial localization, and *in situ* delivery of tissue enhancer biomolecules [30,194]. Ample evidence indicates that beside the antimicrobial and regenerative properties of the fibrin matrix and the biofilm itself [16,136,160,227,228], the biomolecules contained within the matrix including thrombin [12–14], fibrin (ogen) [15,16], complement C3a, C5a fragments [22,23] together with growth factors, cytokines, contact system zymogen FXII, and microparticles either released by activated platelets or derived from plasma, play a significant role as tissue repair enhancers [12,16,20,22,30]. In addition, platelet-released effector molecules influence the resolution of inflammation since they are a significant source of specialized pro-resolving mediators (SPMs) including maresins, resolvins and Lipoxin A4 (LXA4) [126,128], and therefore may contribute to shorten the early inflammatory process in sterile inflammatory context (Fig. 5). In doing so, fibrin matrix partially mimics the injured ECM by acting as a depot of regulatory factors (Figs. 6 and 7) [222,229–231].

AFM is injected as a liquid dynamic scaffold in the time window of 1–5 min when the fibrin matrix is still liquid macroscopically but undergoing a microscopic gelling process, which is marked by the gel point or clotting time, meaning the change from liquid to solid undergone by the matrix when 15–20% of the fibrinogen has been incorporated into the gel by branching points (approximately 4–5 min) [232]. The liquid-to gel dynamic scaffold extensively permeates through areas that surround the injection site, and anchors by way of the activated platelets conveyed by the fibrin clot to the collagen and other ECM proteins exposed in damaged tissue margins; the result is a 3-D fibrin-extracellular matrix-like malleable structure [30,87] (Fig. 7). In doing so, the injected fibrin bridges the gap of injured areas where tissue plasmin will initiate the fibrin biodegradation by tissue fibrinolysis, and during the next 7–10 days will be releasing both immediately and in a gradual and delayed manner the growth factors trapped during the fibrin formation, thereby operating as a biomimetic biphasic GF delivery system (Fig. 6) [16,30].

Several biological features might partially account for the safety and efficiency of fibrin matrix as a GF delivery system [30] (Figs. 6 and 7). The first is the autologous fibrin scaffold-mediated paracrine effect exerted on several cell phenotypes that induces the synthesis of additional amounts of HGF, VEGF and ECM compounds including hyaluronic acid that will amplify the initial cell response [233–236]. The second is the angiogenic effects of FXII, and thrombin, the latter being anti-inflammatory at low concentrations (Fig. 6) [12,13,20]. The third is that microparticles shed by platelets (PMPs) and present in the plasma may contribute to the resolution of inflammation [237–240], exert an immunosuppressive [237] and anti inflammatory [237,241,242] effect by the immediate release of TGF-β, as well as providing an angiogenic and neurogenic effect [243–245]. The fourth is the locally limited spatial and temporal scale to which autologous fibrin matrix is exposed to cells by generating chemotactic GF gradients of differing durations (Fig. 7) [246,247]. Finally, the low amounts of growth factors released by this matrix while increasing the sensitivity of cells to changes at nanoscale level will also facilitate their fast clearance of them resulting in a rapid restoration of growth factor homeostatic conditions [30,248,249].

### 5.3. *Fibrin matrix: a complex nonlinear therapeutic growth factor system*

Initially, the use of AFM raised concerns about generating fibrosis and aberrant neovascularization due to the significant presence of TGF-β1 and VEGF, two master switches regulating fibrogenesis and angiogenesis respectively [129,250,251]. Experimental and clinical evidence coming from the application of AFM or its supernatant, however, indicate that biomolecules trapped within the fibrin matrix operate, with cell-instructive functions, in a complex nonlinear dynamic manner during tissue healing process [30]. Accordingly, GFs such as TGF-β, VEGF, HGF, IGF-1, stromal cell-derived factor 1 (SDF-1), and PDGF among others, generate biological information circuits whose redundancy and easily changeable regulatory linkage at the molecular level influence and accommodate fibrogenesis and angiogenesis [3,39,41,134,154], two key processes aimed at functional healing rather than at tissue structural perfection [3,39,41,134,154].

For example, the synthesis of ECM elements such as collagen type I [234–236,252–254], decorin and fibronectin [253,254], and hyaluronic acid (HA) [236] from healthy human tendon, skin, and synovial fibroblasts, primary human gingival fibroblasts, and tendinopathic cells cultured with fibrin matrix supernatant is the same regardless of whether low or high concentration of TGF-β1, PDGF, and VEGF are present [234–236,252–254]. In addition, primary human keratocytes and conjuctival fibroblast cultures stimulated with AFM supernatant whose concentration of TGF-β1 was 2.5 ng/mL did not induce any myofibroblast differentiation whereas when stimulated with 2.5 ng/mL of TGF β1 alone they showed a significant increase in the number of myofibroblasts [255]. Similarly, primary human gingival fibroblasts cultured with 6 or 10 ng/mL of TGF-β1 in the AFM supernatant did not affect the fibroblast myodifferentiation rate [256]. Significantly, in primary human keratocyte, conjuctival fibroblast, and gingival fibroblast cultures, the concurrent presence of TGF-β1 and AFM supernatant prevented the TGF-β1-stimulated fibroblast myodifferentiation with less than 0.2% α-smooth muscle actin (SMA) positive cells present in these cell cultures [255,256]. Likewise, for TGF-β1-pretreated primary human keratocyte, conjuctival fibroblast, and gingival fibroblast cultures there was a significant reduction in the number of α-SMA positive cells to below 12% when AFM supernatant was added [255,256]. These outcomes are consistent with a HGF-reduced α-SMA and type III collagen expression and elevated matrix metalloproteinase-2 (MMP-2) expression on TGF-β1 treated primary Achilles tendon fibroblasts [257]. Finally, results from healthy tendon and primary human gingival fibroblasts cultures indicate that the paracrine secretion of VEGF and HGF is independent of the low or high concentration of TGF-β1, PDGF, VEGF conveyed by fibrin matrix supernatant or fibrin matrices whose HGF and IGF-1 is similar [233,234,236].

*In vivo* studies stemmed from corneal wound healing in mice, rabbits [258,259] and human ocular surface pathologies (neurotrophic keratitis and Sjögren syndrome) where the opacification of the cornea by fibrotic scar formation leads to loss of visual acuity [259–265] corroborate that AFM or its supernatant operate as a complex nonlinear therapeutic growth factor provider with anti-fibrotic and tissue-specific angiogenic outcomes. The application of a fibrin-free eye drops formulation (with a similar composition to fibrin matrix supernatant) in either a photorefractive keratectomy mouse model or a cornea wounded rabbit model induced a faster re-epithelization and a mature and specialized corneal epithelium with a very low presence of α-SMA expressing cells and no haze formation, significantly reducing an eventual scar formation [258,259,266]. Importantly, several human studies have evaluated the biological and clinical effects of the fibrin-free eye drops formulation on persistent corneal epithelial defects and ocular surface pathologies (neurotrophic keratitis, Sjögren syndrome and autoimmune dry eye, evaporative dry eye, ocular rosacea, traumatic glaucoma, herpes and adenovirus keratitis) [260–265]. Results show a significant reduction of

pain, dryness, photophobia, and blurred vision, associated with high rates of corneal defect/ulcer resolution, and importantly, with no angiogenesis and scarring on the damaged ocular surfaces [260–265]. Successful corneal healing, an improvement or maintenance of visual acuity, a reduction of inflammation, and an antifibrotic effect in t complex ocular surface surgeries have also been reported using a fibrin membrane formed from autologous plasma rich in growth factors [202, 267]. Overall, the analysis of data resulting from these *in vitro* and *in vivo* research suggests that the presence of TGF-β1, a hallmark of fibrogenesis and potentially profibrotic cytokine [129,268] within AFM is counteracted by the simultaneous presence of HGF, IGF-1, fibroblast growth factor (FGF), VEGF, and PDGF as well as by PMPs concomitantly conveyed by AFM or its supernatant (eye drops).

Several mechanisms have been suggested to account for this complex nonlinear dynamic of GFs within AFM on fibrogenesis. HGF, a multidomain protein with pleiotropic and multipurpose functions, has been reported to attenuate decapentaplejic (SMAD) nuclear translocation [269] on renal fibroblasts stimulated by TGF-β1 leading to an antifibrotic effect as a downstream effector of the peroxisome proliferator-activated receptor-γ (PPAR-γ) [269], and to expedite re-epithelialization in skin wound healing by dedifferentiation of epidermal cells through the B1Integrin/ILK pathway [270]. Moreover, in primary tendon fibroblasts the TGF-β1 induced myofibroblast differentiation is inhibited by HGF through the activation of AMPK signaling [271] or by activating the inhibitory protein SMAD7 leading to the inhibition of TGF-β1 signaling pathways [272–274]. Furthermore, HGF promotes myofibroblast apoptosis [275] and matrix degradation by increasing collagenase expression (MMP-1 MMP-2 and MMP-3) and decreasing their inhibitor TIMP-1 expression [257,276]. Accordingly, HGF reverses fibroproliferative disorders such as hypertrophic scars [277] and keloids [278]. Another significant morphogen within AFM is IGF I which may contribute to the non scarring cornea healing by inhibiting the TGF-β/SMAD pathway of fibrosis in human keratocytes *in vitro* [279]. In addition, IGF-1 is known to accelerate muscle and nerve regeneration by promoting a balance between inflammation and fibrogenesis through the suppression and activity of macrophage migration inhibitory factor (MIF) and NFκB, both implicated in macrophage type I polarization leading to non-resolving inflammation and fibrosis [280–282]. Supporting the aforementioned results, the treatment with IGF-1 of injured muscles in an IGF-IR ± heterozygous mouse model, inhibited TGF-β-stimulated Smad3 phosphorylation and increased the *p*-Akt and Smad3 interaction in myoblasts. In doing so, IGF-1 interrupts TGF-β signaling leading to a stimulation of myogenesis and preventing fibrosis [283–285]. However, a decrease in IGF-I has been associated with inflammation and fibrosis [285] in patients with non-alcoholic fatty liver disease, while an increased expression of IGF-I limits fibrosis through an antiapoptotic, anti-inflammatory, and antioxidant effect [285–287]. Another growth factor within AFM, namely FGF, is a further potential candidate to attenuate the fibrotic effect of TGFβ-1 by triggering the dedifferentiation of myofibroblasts and favouring the fibroblast phenotype [288,289].

Angiogenesis is a multistep process driven by the spatial and temporal cooperative interplay of endothelial cells (ECs), pericytes, macrophages, and fibroblasts and angiogenic factors including, VEGF, HGF, IGF-1, TGF-β1, PDGF, SDF-1,FXII, and Ang 1, and PMPs. It is another example of the complex nonlinear effect of AFM in promoting tissue-specific angiogenic outcomes [233,234,244,290–301]. For example, the *in situ* application of VEGF/PDGF [302,303], FGF2/PDGF [304,305] or VEGF/IGF-1 [292,304] in a hindlimb ischemia mouse model promotes a therapeutic angiogenesis indicating that a combination of angiogenic factors rather than a single growth factor is more effective in generating a mature functional vascular network [306]. Moreover, a bolus application of VEGF and IGF-1 did not show benefits in terms of vascularization and perfusion [292], whereas the sustained delivery of the same GFs within an alginate gel stimulated angiogenesis and perfusion, enhanced neuromuscular junction and muscular regeneration, and reduced fibrosis [292]. Consistent with these results, AFM supernatant and platelet-derived extract promote the proliferation of primary human umbilical vein ECs (HUVEC) and pulmonary artery vasa vasorum EC (VVEC), and induce the formation of new capillaries in the *in vivo* implanted matrigel plugs as indicated by a double immunohistochemical staining for CD31/α-SMA and for CD31/PECAM-1 respectively [307,308]. Importantly, the absence of TGF-β does not affect *in vivo* angiogenesis as demonstrated in patients with defective α-granule biogenesis in megakaryocytes, linked to a Gray platelet syndrome in humans or TGF-β neutralization with antibodies [268,309]. Significantly, TGF-β1, HGF, IGF-1, and FXII are GFs that are present in a latent form and whose cell signaling requires proteolytic activation, a fact that contribute to why AFM acts with a complex nonlinear dynamic.

Significantly, and though indirect evidence in support of AFM as complex nonlinear therapeutic growth factor provider, human therapeutic clinical doses of autologous GFs conveyed by AFM for an absolute larger skin, cartilage, nerve, and muscle injured area are significantly lower compared with the single or combinatorial recombinant GF application used for structure-modifying regenerative purposes in animal and human research [30,221,292,310–316]. Several factors might account for this observation [30]. The first is the paracrine effect exerted on several cell phenotypes that adds an amount of HGF, VEGF and ECM compounds thereby amplifying the initial response [233–235]. The second is the angiogenic effect of FXII and thrombin, the latter being anti-inflammatory at a low concentration [13,20]. The third is that platelet and plasma microparticles provide an additional source of TGF-β and other biomolecules with anti-inflammatory, immunomodulatory, angiogenic, and neurogenic effects [243–245]. The fourth is that fibrin operates as a carrier of GFs to the close vicinity of target cells somehow filling the spatial gap in the damage area. This fact is key as GFs are biochemical signals that operate for limited length and time scales in an intracrine, autocrine and paracrine modes diffusing over distances of just a few micrometers. Thus, the intensity of the signal decays more than linearly with the distance from the source roughly $1/r^2$, being in 3-D even more rapid [249].

Overall, such *in vitro*, *in vivo*, and clinical studies suggest that these blood-derived products operate in a complex nonlinear dynamic, with a robust antifibrotic and tissue-specific angiogenesis effects as emergent properties that cannot be predicted by the concentrations of TGF-β1, VEGF, HGF, or IGF-1 on their own, and where the simultaneous presence of several growth factors appears to be pivotal for the therapeutic effects [30,165,317].

### *5.4. Technical ins and outs of the use of the autologous fibrin matrix with biological repercussions*

There exist several pitfalls derived from the many preparation methods (type of anticoagulant, activation or not, centrifugation speed to separate platelets from other blood cells, pH), which in conjunction with the poor standardization in the way that it is applied can lead to misleading conclusions regarding their clinical efficacy derived from the very diverse biological composition and effects [224]. Importantly, this localized therapy does not appear to entail any thrombo-inflammatory reaction [315,318,319] as might be expected by both the activation of the contact system and the interplay between intravascular innate immune cascade systems [8,11,224].

The uncoupling of thrombotic and inflammatory responses of this biologic together with the antifibrotic, antialgic, and immunomodulatory actions are the hallmark of AFM [30]. Although the mechanisms that drive the *ex vivo* uncoupling of FXII-driven thrombosis and inflammation remains to be determined, several factors might account for this dissociation [320]. Among them, is the absence of leukocytes, the *ex vivo* activation of the intrinsic coagulation pathway with calcium chloride ($CaCl_2$), and the citrate-dependent inhibition of activation of the complement system [11,16,30]. The incorporation of leukocytes (and inevitably erythrocytes) into the fibrin matrix, primarily

neutrophils, promotes local pathophysiological reactions such as inflammation [321], a fast and early ECM and fibrin degradation, and proinflammatory cytokine release [322]. In *in vitro* and *in vivo* experimental models, leukocytes in the fibrin matrix through their incomplete separation when preparing PRP will allow their interaction with already primed and inflamed stromal fibroblasts, trigger fibroblast inflammatory memory and favor the release of pro-inflammatory cytokines [182,183], a fact that is not observed when the PRP preparations are leukocyte free [254,323–327]. Moreover, there may be other detrimental effects stemmed from an erythrocyte-derived heme-iron induction of the pro-inflammatory macrophage phenotypic switch [182,328–331] that together with neutrophil activities and NETosis may operate as non-resolving inflammation and profibrotic agents respectively [17,140,159,332,333]. This can even exacerbate the inflammation-driven pathology that the applied fibrin based network is intended to treat [334,335]. Importantly, *ex vivo* activation of PRP with $CaCl_2$, yields low concentrations of thrombin, which itself exhibits growth factor-like fibroblast and endothelial cell proliferation, migration, antiapoptotic, and inflammatory modulation activity. In contrast, alternative modes of PRP activation can generate high doses of thrombin, which would then also operate as an inflammatory mediator to recruits monocytes, activates the NF-κB of endothelial cells, and triggers the release of cytokines from mast cells [13,216]. The *ex vivo* activation of PRP, in the absence of leukocytes, will circumvent the NF-κB-dependent inflammatory gene expression and the generation of proinflammatory cytokines by monocytes [336]. Not to be forgotten is that, when directly injected non-activated PRP, platelets may activate the complement system, as well as promote the formation of platelet-leukocyte complexes and neutrophil extracellular traps (NETs) *in situ* [33,336,337]. The growth factor HGF is secreted as an inactive precursor (proHGF) and its proteolytic activation by HGFA is promoted by thrombin and kallikrein enzymes, thereby producing pleiotropic HGF regenerative activities in damaged areas provided that the coagulation cascade has been activated [138,338]. Exogenous activation with commercially available bovine thrombin runs the risk of adverse effects, which include immune reactions, thrombosis, and hemorrhage [339]. The use of citrate as anticoagulant has a significant biological impact on PRP dynamics as this anticoagulant inhibits both coagulation and complement activation by chelating and removing ion $Ca^{2+}$ from plasma but not FXII and therefore the inflammatory arm of the contact system [217], which may contribute to the uncoupling between thrombotic and inflammatory responses of this biologic. In fact, while the addition of $CaCl_2$ initiates the intrinsic arm of the coagulation cascade, the classical (CP) and lectin (LP) pathways of complement continue to be disturbed [11]; citrate is a potent complement inhibitor already at a low 0.25 mM concentration and reduces the granulocyte activation [217,340]. Significantly, since sodium citrate does not inhibit the activation of FXII there is a generation of bradykinin (BK) [217]. This major proinflammatory mediator not only recruits neutrophils to the injured site and activates resident macrophages [341] *in vivo* it also induces a second-generation of mediators such as nitric oxide, prostaglandins, and leukotrienes [320,342]. However, and due to the very short half-life of the BK in plasma and serum (<30 s) [343], by the time the fractionated plasma is first activated *ex vivo* and then injected as an AFM (at least 10–15 min after the activation of the inflammatory arm of the contact system), most of generated BK has been degraded by plasma kininases and by blood clotting enzymes [343,344]. This physiological BK degradation in AFM is indirectly confirmed by the absence of pain or inflammation at the site of the autologous fibrin scaffold injection [315,319,345]. Last but not least, the use of citrate as anticoagulant does not modify the growth factor kinetic nor the major biological properties of AFM [231].

## 6. Future perspectives

Evolution, from invertebrates to mammals, has yielded and shaped immunothrombosis as a highly conserved defense and repair response. The constitutive molecular pathways are a goldmine from where to learn to design and optimize new bioengineering and synthetic biologically-based therapies. Tissue repair as a by-product of the processes underlying immunothrombosis highlights potential therapeutic interventions to enhance the repair of musculoskeletal injuries associated with chronic inflammation. This is the case for blood-derived autologous fibrin scaffolds that have a significant repair and regenerative potential and which harness the healing properties of molecular and cellular components of coagulation and hemostasis. Nonetheless, further work is required to better address and overcome some of the challenges that have arisen from its use, the myriad of methods for AFM preparation often with incomplete characterization and with poor standardization in the way they are applied. Moreover, the precise molecular mechanisms underlying this versatile, biology-inspired, evolution-tailored, and human-engineered biomimetic therapy remain insufficiently understood, as is the paradox of host defense mechanisms acting as local tissue repair enhancers in man while in other situations generating systemic pathogenic processes.

## 7. Conclusions

1. Evolution from invertebrates to mammals has yielded and shaped a mosaic of mechanisms with roles in inflammation, clotting, and healing, that has led to consider tissue healing being considered as a byproduct of the mechanisms underlying immunothrombosis.
2. Despite the superficial resemblances and common functional trends between invertebrate immunoclotting and mammalian immunothrombosis, the complex vertebrate blood coagulation network emerged independently, together with a closed circulatory system, blood cell specialization, the endothelium, and the adaptive immune system, more than 500 Mya, over a period of a 50–100 million year window.
3. The emergence of multi-systemic organisms, through gene and whole genome duplication, exon shuffling, and mutations, has led to sophisticated host defenses by the progressive addition of complexity in the form of the contact system, the embellishment of the neuroendocrine system, blood cell type diversification and endothelium development, the expansion of the proteins of the complement pathway, and the emergence of the megakaryocyte-platelet axis among others.
4. The systemic interplay among blood proteolytic cascades, blood cell types and endothelium, and neuroendocrine system endowed their bearers with survival advantages. But in the current world, that has provided unprecedented cutting-edge therapies, the trade off is that disruption of physiological homeostasis has led to thrombotic and inflammatory diseases largely influenced by lifestyle and posing a heavy economic burden.
5. Harnessing the healing potential of fibrin matrix, an autologous liquid-to-gel scaffold operates as a local and inexpensive shortcut therapeutic strategy in sterile inflammatory conditions by mimicking the immunoreparative role of growth factors and other biomolecules trapped in the fibrin fabric. Once applied to damaged area, fibrinolysis will gradually release its cargo, including growth factors and other biomolecules with antialgic, anti-inflammatory, and trophic effects.
6. This versatile, biology-inspired, evolution-tailored, and human-engineered biomimetic therapy is in its naissance, and offers much hope for the future particularly as it is well adapted for third world countries.

### Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The authors declare that E.A. is the Scientific Director of and S.P. and R.

P. are scientists at BTI Biotechnology Institute, a biomedical company that investigates in the fields of regenerative medicine and PRGF-Endoret technology. The rest of the authors state that that they have no conflicts of interest that are relevant to the content of this article.

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