ROLE OF COAGULATION PATHWAYS IN NEUROLOGICAL DISEASES

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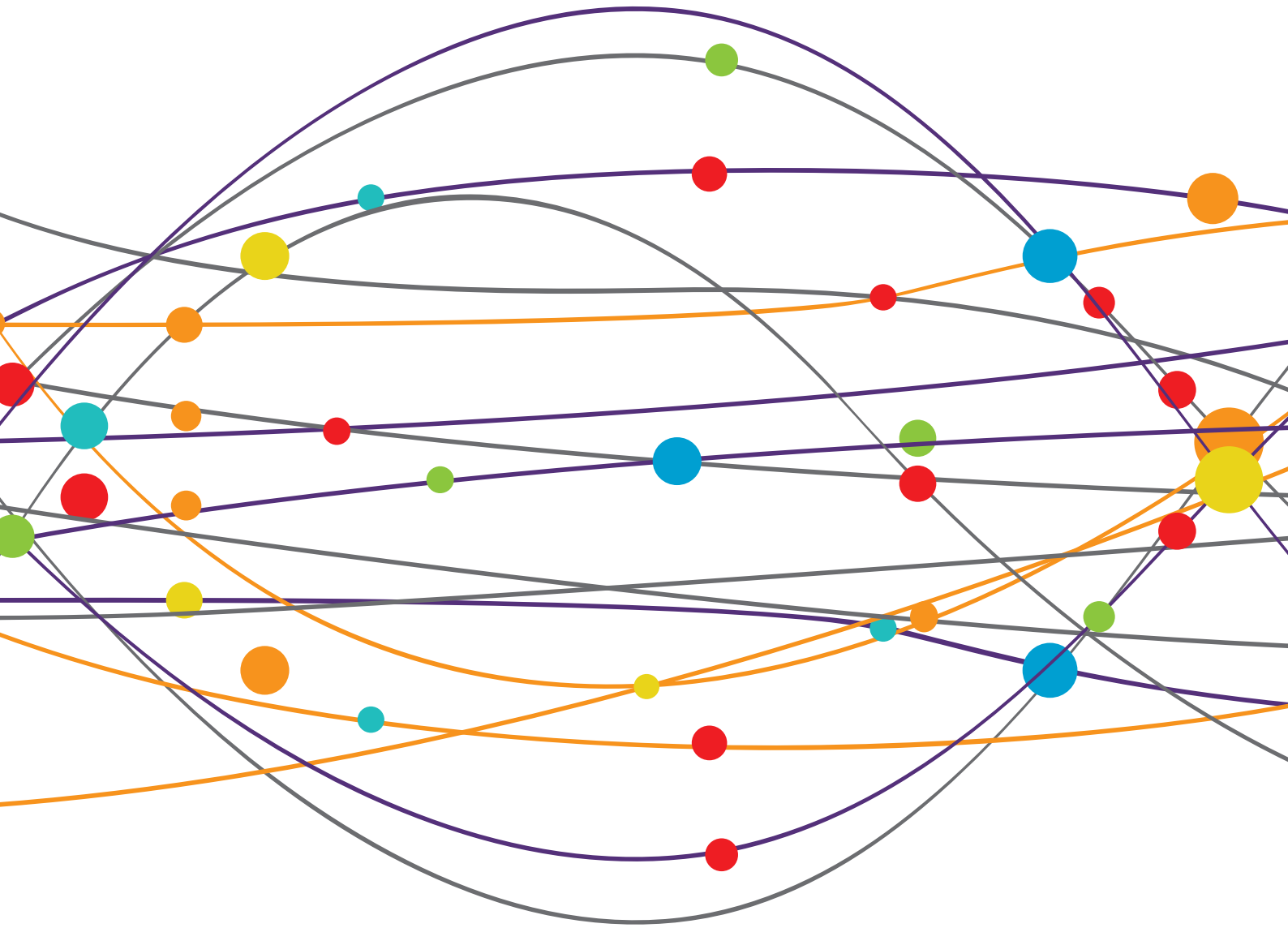
PUBLISHED IN: Frontiers in Neurology and Frontiers in Immunology



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ISSN 1664-8714
ISBN 978-2-88963-166-7
DOI 10.3389/978-2-88963-166-7

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ROLE OF COAGULATION PATHWAYS IN NEUROLOGICAL DISEASES

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Citation: Koudriavtseva, T., Lorenzano, S., Inglese, M., Plantone, D., eds. (2019). Role of Coagulation Pathways in Neurological Diseases. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88963-166-7

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frontiers in Neurology

EDITORIAL
published: 23 July 2019
doi: 10.3389/fneur.2019.00791

Editorial: Role of Coagulation Pathways in Neurological Diseases

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Keywords: neurological diseases, coagulation, innate immunity, neuroinflammation, neurodegeneration

Editorial on the Research Topic

Role of Coagulation Pathways in Neurological Diseases

There is a growing evidence that abnormalities of coagulation pathways are involved in the pathogenesis of several neurological diseases in tight correlation with both neuroinflammation and neurodegeneration. The concept of *thrombo-inflammation* was first introduced in vascular diseases of central nervous system (CNS) (1) closely related to a more general entity of *immunothrombosis*, i.e., formation of thrombi inside microvessels by innate immune cells and specific thrombosis-related molecules, having major physiological role in immune defense rather than in haemostasis (2).

This Research Topic gathers different contributions that added new information on the involvement of both coagulation factors and innate immune components in the pathogenesis of human neurological diseases with the greatest share from the studies on multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE), its animal model.

Plantone et al. focused on the key role of both coagulation and vascular thrombosis in the pathophysiology of MS. The observation of a close concordance between perivascular fibrin(ogen) deposition and the occurrence of clinical signs in EAE has led to numerous studies to investigate the role of thrombin and fibrin(ogen). Most findings supported that blood-brain barrier (BBB) breakdown, presence of active plaques, and disease exacerbation in both humans and animal models are conditions characterized by an increased coagulation activity.

Furthermore, Ziliotto et al. pointed out that increased BBB permeability leads to the irruption into the CNS of blood components including coagulation factors. Their cytotoxic deposition with the activation of microglia, resident innate immune cells, already in pre-demyelinating lesion stage in EAE and MS, cause inflammatory response and immune activation sustaining neurodegenerative events in MS. In particular, among the coagulation factors, FXII could act as an autoimmunity mediator due to its deposition near dentritic cells positive for CD87.

In their research, Ziliotto et al. investigated multiple FXII-related variables, including either its circulating levels, pro-coagulant function, ratio values or variation over time, in 74 MS patients and 49 healthy subjects. They found in MS patients an increased FXII plasma level, a significant difference over time for FXII procoagulant activity and reduced function within the intrinsic

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Edited and reviewed by:
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Specialty section:
This article was submitted to Multiple Sclerosis and Neuroimmunology, a section of the journal Frontiers in Neurology

Received: *02 May 2019*
Accepted: *09 July 2019*
Published: *23 July 2019*

Citation:
Lorenzano S, Inglese M and Koudriavtseva T (2019) Editorial: Role of Coagulation Pathways in Neurological Diseases. Front. Neurol. 10:791. doi: 10.3389/fneur.2019.00791

coagulation pathway, which supports investigation of FXII contribution to disease phenotype and progression.

Interestingly, the role of the coagulation process entangled with other pathogenic pathways in MS (i.e., a crosstalk between coagulation, inflammation, and immune system), was reinforced by over-connectivity between genome-wide associations MS data and a network of coagulation pathways studied by La Starza et al.. Moreover, genes coding for cluster of differentiation 40 (CD40), especially operative in B lymphocytes, and plasminogen activator urokinase (PLAU) shared both networks, pointing to an integral interplay between coagulation cascade and one of main pathogenic immune effectors.

The involvement of coagulation factors, especially factor XII, fibrinogen and thrombin, beyond their traditional roles in haemostasis, in the development of inflammatory diseases like MS, rheumatoid arthritis and colitis was again the focus of the systematic review by Göbel et al. who highlighted the molecular mechanisms underlying the balance between haemostasis and thrombosis, and between protection from infection and extensive inflammation.

The double nature, thrombotic and immunologic, is also evident in other specific neurologic condition such as the antiphospholipid syndrome (APS) and in the therapeutic strategy adopted for this disorder as discussed in the review by Fleetwood et al.. APS is an autoimmune antibody-mediated condition characterized by thrombotic events and/or pregnancy morbidity in association with persistent positivity to antiphospholipid antibodies. The CNS is frequently affected, as intracranial vessels are the most frequent site of arterial pathology. Nevertheless, ischemic injury is not always sufficient to explain clinical features of the syndrome and immune-mediated damage has been advocated.

Festoff and Citron reviewed available evidence on the role of coagulation cascade activation, in particular of thrombin signaling, in neurodegeneration and in the potential development of effective therapeutic approaches for ALS and traumatic brain injury. Different elements and regulators of the coagulation pathway have significant impact in these conditions and each of these molecules are entangled in choices dependent upon specific signaling pathways in play. For example, the particular cleavage of protease activated receptor 1 (PAR1) by thrombin versus activated protein C will have downstream effects through coupled factors to result in toxicity or neuroprotection.

Thrombin and its PAR1 are potentially important also in peripheral nerve inflammatory diseases as it has been addressed by Shavit-Stein et al. who studied the role of these factors in rat experimental autoimmune neuritis (EAN), a model of the human Guillain-Barre syndrome. The authors showed that thrombin activity in the sciatic nerve was elevated in EAN compared to control sham rats. Furthermore, treatment with non-selective thrombin inhibitors significantly inhibited specific thrombin activity in EAN rats' sciatic and improved clinical scores compared to the untreated EAN rats with normalization of proximal amplitude observed in nerve conduction studies.

The emerging role of coagulation in infectious diseases such as Lyme neuroborreliosis (LB), the most common tick-borne disease involving nervous system caused by the spirochete Borrelia, has been investigated by Di Domenico et al.. In fact, invasive forms of B. burgdorferi are known to expresses multiple plasminogen-binding surface proteins that likely assist pathogen dissemination through host tissues. During the course of the infection, bacteria migrate through the host tissues altering the coagulation and fibrinolysis pathways and the immune response, reaching the CNS within 2 weeks after the bite of an infected tick.

The importance of coagulation system in the management of neurological diseases, particularly in elderly, is also evident by the potential risk associated with the increasing prescription of the new direct oral anticoagulants (DOACs), namely apixaban, dabigatran, edoxaban, and rivaroxaban, in patients with epilepsy taking concomitant antiepileptic drugs (AEDs). As a result, potential interactions may cause an increased risk of DOAC-related bleeding or a reduced antithrombotic efficacy. This issue was evaluated by Galgani et al. who found that there are only few case reports describing such interactions and, therefore, limited evidence is available.

An indirect role of the coagulation system in neurocognitive disorders has been assessed by Alisi et al. who reviewed recent evidence on the emerging involvement of vitamin K, whose biological activity in blood coagulation has been thoroughly explored, even in brain cells development and survival and, hence, in brain functions. In particular, vitamin K seems to have an antiapoptotic and anti-inflammatory effect mediated by the activation of Growth Arrest Specific Gene 6 and Protein S and to be involved in sphingolipids metabolism, a class of lipids that participate in the proliferation, differentiation and survival of brain cells. Vitamin K antagonists, used worldwide as oral anticoagulants, may have a negative influence on cognitive domains such as visual memory, verbal fluency and brain volume.

All these contributions indicate that the study of coagulation pathways in neurological diseases would lead to a greater understanding of their pathophysiology and a more appropriate therapeutic approach. We hope that this Research Topic will help the reader to find a useful reference for the state of the art in this emerging research field and, in particular, both researchers and clinicians to face their challenges with a more complete pathogenic approach since the role of innate immunity and of its effector coagulation factors is very relevant in both health and pathology.

AUTHOR CONTRIBUTIONS

SL, MI, and TK all contributed equally to the literature research and writing.

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Conflict of Interest Statement: MI has received research grants from NIH, NMSS, FISM, and Teva Neuroscience; TK has received research grant from the Italian Ministry of Health.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neurology

REVIEW
published: 24 April 2019
doi: 10.3389/fneur.2019.00409

Coagulation Pathways in Neurological Diseases: Multiple Sclerosis

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Specialty section:
This article was submitted to Multiple Sclerosis and Neuroimmunology, a section of the journal Frontiers in Neurology

Received: *10 September 2018*
Accepted: *04 April 2019*
Published: *24 April 2019*

Citation:
Ziliotto N, Bernardi F, Jakimovski D and Zivadinov R (2019) Coagulation Pathways in Neurological Diseases: Multiple Sclerosis. Front. Neurol. 10:409. doi: 10.3389/fneur.2019.00409

Significant progress has been made in understanding the complex interactions between the coagulation system and inflammation and autoimmunity. Increased blood-brain-barrier (BBB) permeability, a key event in the pathophysiology of multiple sclerosis (MS), leads to the irruption into the central nervous system of blood components that include virtually all coagulation/hemostasis factors. Besides their cytotoxic deposition and role as a possible trigger of the coagulation cascade, hemostasis components cause inflammatory response and immune activation, sustaining neurodegenerative events in MS. Early studies showing the contribution of altered hemostasis in the complex pathophysiology of MS have been strengthened by recent studies using methodologies that permitted deeper investigation. Fibrin(ogen), an abundant protein in plasma, has been identified as a key contributor to neuroinflammation. Perturbed fibrinolysis was found to be a hallmark of progressive MS with abundant cortical fibrin(ogen) deposition. The immune-modulatory function of the intrinsic coagulation pathway still remains to be elucidated in MS. New molecular details in key hemostasis components participating in MS pathophysiology, and particularly involved in inflammatory and immune responses, could favor the development of novel therapeutic targets to ameliorate the evolution of MS. This review article introduces essential information on coagulation factors, inhibitors, and the fibrinolytic pathway, and highlights key aspects of their involvement in the immune system and inflammatory response. It discusses how hemostasis components are (dys)regulated in MS, and summarizes histopathological post-mortem human brain evidence, as well as cerebrospinal fluid, plasma, and serum studies of hemostasis and fibrinolytic pathways in MS. Studies of disease-modifying treatments as potential modifiers of coagulation factor levels, and case reports of autoimmunity affecting hemostasis in MS are also discussed.

Keywords: multiple sclerosis, coagulation, extrinsic pathway, intrinsic pathway, coagulation inhibitors, fibrinolytic pathway

INTRODUCTION

The complex physiological process of hemostasis involves several pathways in which procoagulant and anticoagulant forces are maintained in a constant equilibrium by autoregulation. In fact, hemostasis allows the vascular wall to provide anticoagulant blood containment until damage causes significant activation of coagulation, the confined formation of blood clot with hemorrhage cessation, and removal of that clot after the restoration of vascular integrity (1).

Increased blood-brain-barrier (BBB) permeability is a feature of several neurological diseases, and one of the first events that characterizes multiple sclerosis (MS) pathogenesis (2–5), leading to the irruption of coagulation/hemostasis factors into the central nervous system (CNS) (6). This, in turn, potentially triggers leakage of hemostasis components into the brain parenchyma, which potentially triggers the coagulation cascade. Besides their cytotoxic deposition, hemostasis components cause an inflammatory response and immune activation, sustaining neurodegenerative processes in MS (**Figure 1**) (6–12). Coagulation and inflammation are characterized by multiple links, and coagulation proteins and their fragments may promote neurodegeneration (12, 13). Preclinical models provide (albeit with some limitations) an informative means to investigate the pathophysiology of human diseases, and those mimicking MS have received attention in the last 3 decades. In particular, an increasing number of studies, largely based on animal models (14), have provided insights into the tight relationship among vasculature alterations, neuroinflammation, neuroimmunology, and neurodegeneration. Nevertheless, they only partially contribute to the relation between hemostasis components and experimental evidence in MS patients. This review article focuses on coagulation pathways in MS patients and related animal models. Current knowledge of how coagulation factors, coagulation inhibitors, and components of the fibrinolytic pathway are (dys)regulated in MS patients is reviewed and missing or inconsistent information is highlighted to guide future research.

COAGULATION CASCADE ESSENTIALS

Before exploring the contribution of coagulation components in the pathophysiology of MS, it is important to consider the basic physiology of coagulation. Coagulation occurs as a complex network of overlapping reactions tightly localized on specific cell surfaces. It is often still represented as a one-way Y-shaped model as proposed in the 1960s (15, 16). Although an oversimplification, this model posits two distinct pathways, so-called "extrinsic" and "intrinsic," that converged into a "common" one. Here, the interactions of inactive procoagulant mediators enable a sequential cascade of proteolytic events leading to their activation and the final fibrin and blood clot formation. The extrinsic pathway was so named because it requires an external factor (from the extravascular tissue), while the intrinsic pathway includes factors that are already present in the blood. In contrast to this commonly cited model, in the actual *in-vivo* process, extrinsic, and intrinsic pathways do not work independently and the pro-coagulant mediators, once activated, support the exponential amplification and propagation of the system with several interactions and feedback loops (17, 18). Although the activity in plasma of pro-coagulant factors of extrinsic and intrinsic pathways can be measured separately using clinically available coagulation tests such as partial thromboplastin time (PT) and activated partial thromboplastin time (aPTT), respectively (19), these laboratory tests do not accurately reflect the *in-vivo* situation (17). In fact, they force the system into a controlled condition on platelet-poor plasma through the exogenous supply of reagents (tissue factor/thromboplastin, phospholipids, calcium, and micronized silica) to assess the activity level of a certain factor.

In order to form a blood clot *in-vivo*, platelets and coagulation factors must communicate and support each other (20). Tissue factor (TF), the main trigger of the process responsible for the initial acceleration of cascade activation, is kept hidden on subendothelial cells until vascular damage exposes it (18). Once exposed, it promotes the activation of platelets and their recruitment into the clot (21). In turn, platelets mediate pro-coagulant functions through the release of additional coagulation factors and by the release of negatively charged phospholipids that are required cofactors for the proteolytic reactions of coagulation factors (22).

The procoagulant mediators that initiate, amplify, and propagate this cascade exist as proenzymes (also known as zymogens) in the blood (22). Under normal conditions, a basal activation of coagulation factors takes place, but it leads to an "idling" coagulation (18), which does not escalate to full clot formation. This occurs because the biochemical reactions are several orders of magnitude less efficient without the procoagulant mediators.

In summary, (1) fibrin can be produced only as a result of the complex interplay of coagulation factors, and (2) to productively trigger coagulation, cell surface exposure is necessary (TF-bearing membranes and platelets).

Based on the above, one of the main questions relevant to MS is how the coagulation cascade is triggered in the CNS.

Extrinsic Coagulation Activation and Implication for Damage Within the CNS

The initiation of the "extrinsic" coagulation pathway requires "extravascular" TF, also known as Factor (F)III, thromboplastin, or CD142. It is important to note that the names of coagulation factors, identified with roman numerals, reflect the order in which they were discovered and not the biological order of the "sequential cascade" of proteolytic events. TF is highly expressed on the surfaces of medial and adventitial cells, acting as the trigger for arresting bleeding under damaging circumstances (1).

Surprisingly, low levels of TF in an inactive configuration (cryptic state) may be found on endothelial cells and blood cells including platelets, lymphocytes, monocytes, macrophages, granulocytes, and neutrophils (23–25). Additionally, TF has been found circulating in TF-bearing microparticles that are released from cells or as a soluble protein generated by alternative splicing of TF mRNA (26). Overall induction of

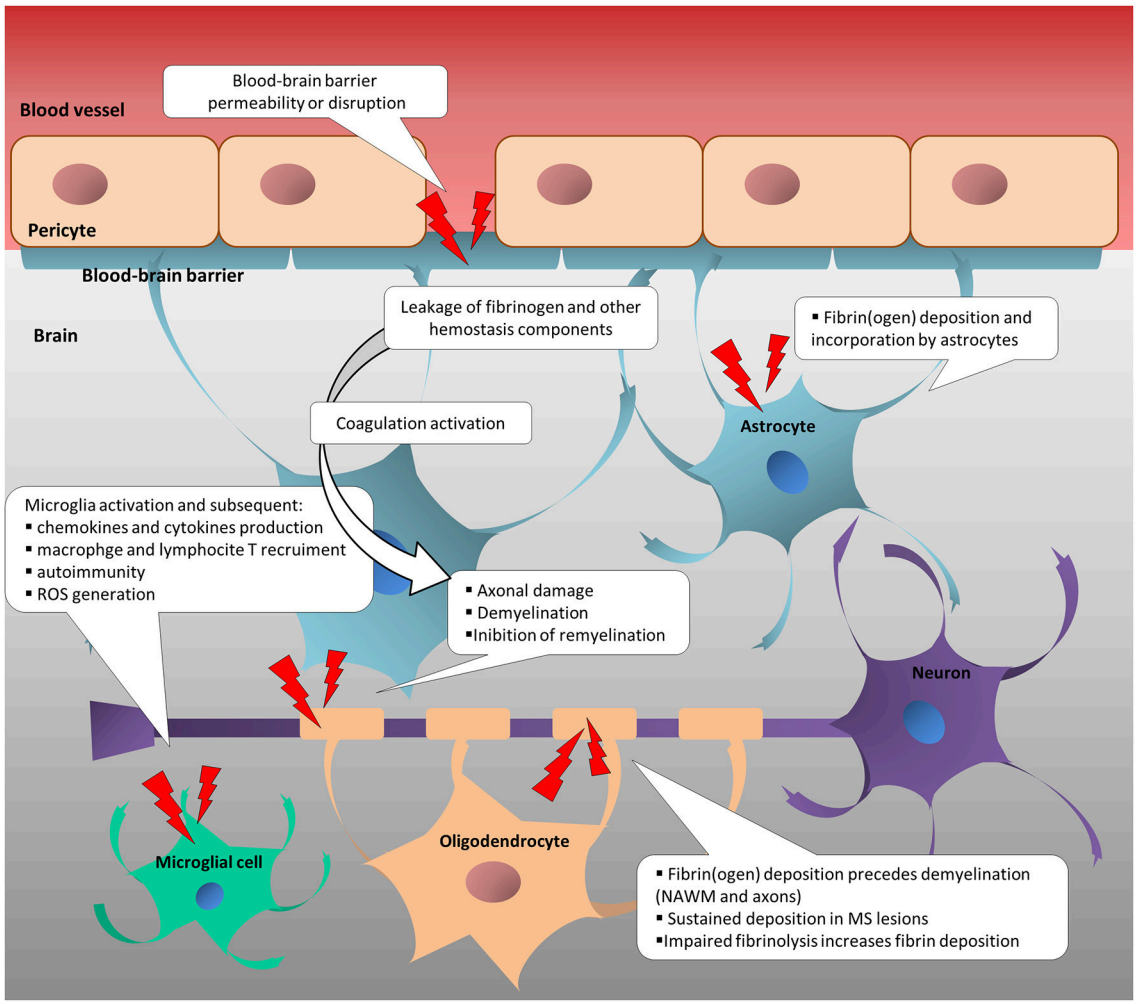

FIGURE 1 | Change in neurovascular interface is involved in inflammatory, immune and neurodegenerative responses in multiple sclerosis. Disruption or increased permeability of blood-brain barrier cause the leakage of hemostasis components into the brain parenchyma, which triggers the coagulation cascade. In turn, hemostasis components foster the inflammatory response and the immune activation, sustaining neurodegenerative events in MS. NAWM, normal-appearing white matter; ROS, reactive oxygen species.

soluble TF is stimulated during sepsis in response to bacteria, or during various chemokine- and cytokine-induced inflammatory states (27).

It has been suggested that decryption, which leads to the procoagulant activity of circulating TF, may depend on different mechanisms including a change in phospholipid environment, TF oxidation/reduction modifications, and TF dimerization (23, 28–30). Circulating microparticles may contribute to the formation of micro-thrombi (31). This has been suggested as one of the physiological defense strategies against bacteria, promoting so-called immunothrombosis in which the coagulation traps the pathogens, thereby preventing its spreading, and supporting the immune response (32). Uncontrolled activation of immunothrombosis, related to sepsis, cancer, or inflammatory states causes pathological conditions with undesired intravascular clotting contributing to pro-thrombotic risk (33).

Given these observations, the tight relation between coagulation, inflammation, and immunity can already be appreciated in the vascular compartment alone. However, prominent expression of TF is also known to occur in the human brain (34, 35), and studies in mice have demonstrated that astrocytes are the primary cellular source of TF, suggesting their role in cerebral hemostasis (36). The breakdown of BBB that characterizes the MS disease process exposes the TF of astrocytes, which can promote activation of the coagulation cascade. The cascade in turn requires activated membranes to support biochemical reactions, canonically provided by platelets (**Figure 2**) (21).

For the sake of simplicity, we have chosen to omit reporting some of the intermediate cleaved forms of clotting factors, as well as their isoforms. Transmembrane TF of astrocytes binds its ligand FVII, activating and allosterically modifying it to form a mature active binary complex (TF:FVIIa). The TF:FVIIa

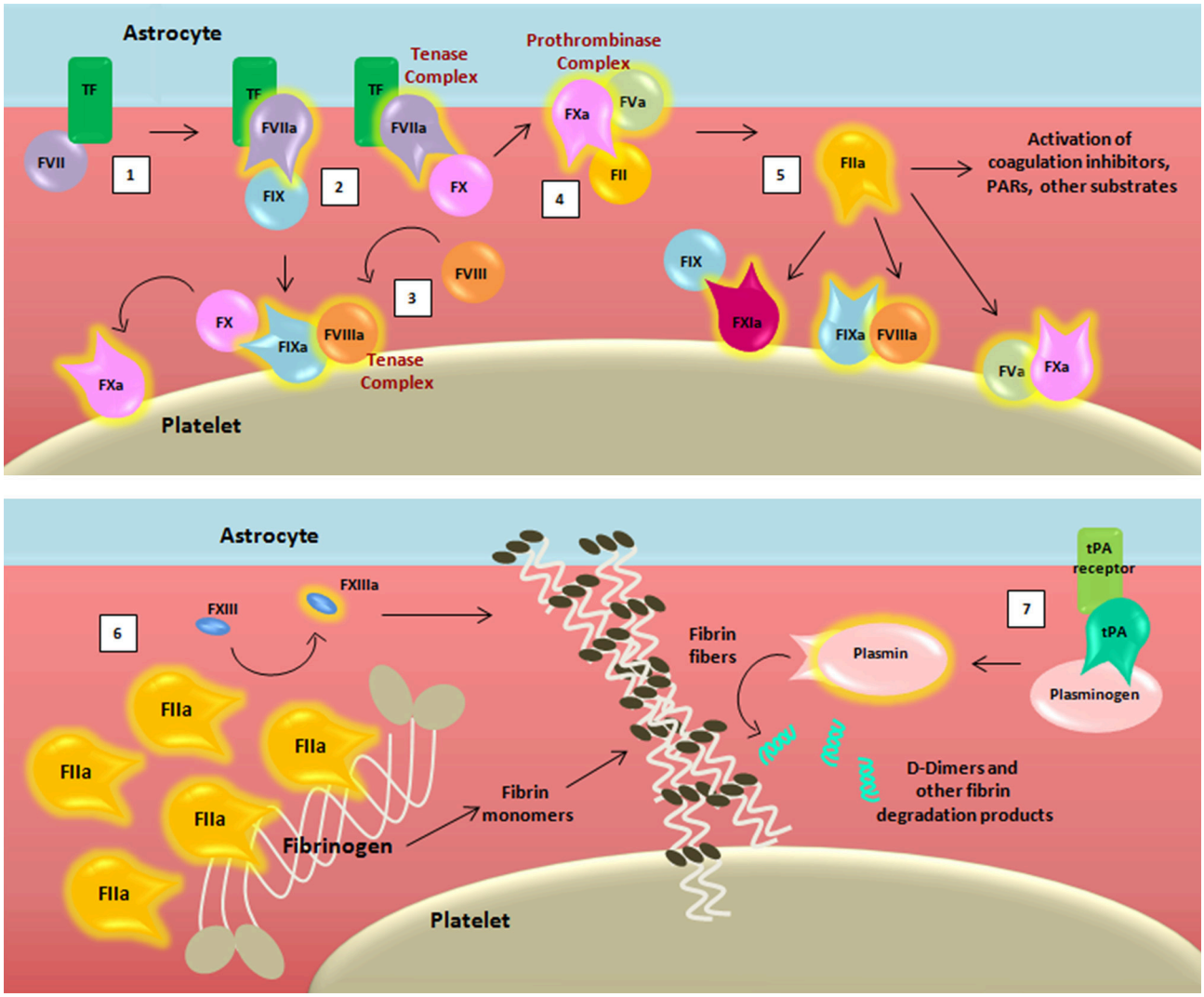

FIGURE 2 | Schematic representation of the coagulation cascade and fibrinolytic pathway after blood-brain barrier damage. The coagulation cascade is activated (1) when the TF binds to its ligand, (activated) factor (F)VII, thus forming, together with membranes, a mature active binary complex (TF:FVIIa). The TF:FVIIa complex allows to cleave and activate on one side FIX and on another FX (2). TF:FVIIa:FXa is able to activate the cofactor FVIII (3) which forms a complex with the FIXa (FIXa:FVIIIa) providing a feedback loop for FX activation. The assembly FXa:FVa, converts prothrombin (FII) into thrombin (FIIa) (4). The initial amount of thrombin exerts its proteolytic action on FXI, FV, FVIII, and other substrates (5). Then the massive thrombin generation reaches a sufficient concentration to convert fibrinogen (FI) into fibrin monomers (6). The organized three-dimensional assembly of monomers in protofibrils and fibrin fibers produces the blood clot. Cross-linking stabilization of fibrin clot requires FXIII activated (FXIIIa) by thrombin activity. Coagulation complexes depend on lipids, exemplified by the platelet membrane. The dissolution of the fibrin fibers is mediated by the fibrinolytic system (7). Tissue-type plasminogen activator (tPA) converts plasminogen into plasmin which cleaves fibrin to soluble degradation products among those the D-dimers.

complex cleaves and activates from one side FIX and from another FX, both present in the blood as zymogens (37). TF-FVIIa-nascent FXa complex activates FVIII (38). FVIIIa forms a complex with FIXa (FIXa:FVIIIa) providing a feedback loop for FX activation. Because of the ability to activate FX, both TF:FVIIa and FIXa:FVIIIa are called tenase complexes.

On the surface of TF-cells, FXa is released from TF:FVIIa and it associates with its cofactor FVa leading to the assembly of the FXa:FVa complex. The initial trace of FVa may derive from partially activated platelets (39), by proteases that are not involved in coagulation (40), or by FXa (41). The "common" pathway starts with FXa:FVa, known also as prothrombinase complex due to its ability to convert prothrombin (FII) into thrombin (42, 43) through cleavage of multiple peptide bonds, whose sequence may depend on the membrane source (44). The initially low amount of thrombin activates FV, FVIII, and FXI on platelets that become the cornerstone surface for further coagulation reactions (45–48).

After this initial sequence of events, coagulation is exponentially maximized for generation of massive amounts

of thrombin, which reaches sufficient concentration to convert fibrinogen (FI) into fibrin monomers. Therefore, thrombin promotes its own additional generation during the "propagation" phase independently from TF:FVIIa complex. Indeed, on the platelet surfaces (membranes), FVIIIa binds FIXa, and activated FX (FXa) that will form additional FXa:FVa, whereas FXIa directly activates FIX that will form additional FIXa:FVIIIa complexes. Finally, the organized three-dimensional assembly of fibrin monomers in protofibrils and fibrin fibers produces the impermeable blood clot. In the coagulation cascade, cross-linking stabilization of the fibrin clot requires activated FXIII (FXIIIa), again produced by thrombin activity (49).

An additional amplification loop by thrombin promotes platelet activation and aggregation via the cleavage and activation of proteinase-activated receptors (PARs) (1). However, activated PARs may modulate various cellular activities under different pathophysiological conditions including inflammation, apoptosis, cell migration, angiogenesis and tissue remodeling (50). Notably, hemostasis components can elicit opposite signaling responses through activation of the same PAR, as provided by *in-vitro* evidence, where PAR-1 may induce pro-inflammatory and anti-inflammatory signaling under activation by thrombin or the anticoagulant activated protein C (aPC), respectively (51, 52). It has been demonstrated that under coagulant conditions, FXa binds PARs (PAR-1 and PAR-2) at the vascular endothelial cell level, evoking the production of proinflammatory cytokines IL-6 and IL-8 (53), and the monocyte chemotactic protein-1 (54). Subsequent thrombin production reinforces the signal already started by FXa, sustaining the production of the proinflammatory cytokine IL-8 through PAR-1 (53). In addition, FXa triggers a series of Ca^{2+} oscillations (53), which may have a function in the Ca^{2+}-dependent activation of proinflammatory transcription factors (55). Moreover, FXa induces expression of adhesion molecules promoting leukocyte adhesion (54), which in turn may also be sustained by the co-localized presence of thrombin and fibrinogen (56, 57).

Based on these findings, it has been hypothesized that coagulation activation at the neurovascular interface might contribute toward eliciting and sustaining the inflammatory phenomenon characteristic of MS pathophysiology. This has been investigated to some degree, albeit insufficiently.

It has been established that some coagulation factors are expressed in the CNS, including FX and FII (58–61). However, the physiological functions related to their presence are mostly unknown. Depending on the degree of BBB damage, blood components (but not blood cells) like the high molecular weight fibrinogen as well as FV (62) can enter into the CNS, thus providing the complete repertoire of factors to trigger coagulation. Nevertheless, in order to form fibrin, a consistent amount of protein is needed, and in addition, an activated surface that sustains the coagulation process. As of now, the exact sequence of events that supports coagulation in the CNS and fibrin formation, in particular in MS patients, is inferred from the general coagulation pathway and does not take into account the specificity of astrocyte membranes.

Several findings in mice, and particularly in the experimental autoimmune encephalomyelitis (EAE) model, support the importance of coagulation factors in MS, either procoagulant in the extrinsic and intrinsic pathways, or anticoagulant. The key event in the CNS is the entry of fibrinogen, the leakage of which correlates with areas of axonal damage and has been shown to cause the undesired activation of microglia, subsequently inducing the recruitment and activation of macrophages, thus promoting inflammatory responses (6, 7). The fibrinogen enters into the CNS after BBB leakage and induces reactive oxygen species (ROS) release in microglia and its signaling via the microglial receptor $CD11b^+$ is required for development of axonal damage in EAE (6). The first EAE-related work that described the role of fibrinogen in activating microglia/macrophages through specific interaction with the $CD11b^+$/CD18 integrin receptor also showed protection either by genetic disruption of the fibrinogen region that contains the sequence for $CD11b^+$ interaction or by pharmacological blockage of this fibrinogen region with an inhibitory peptide (7). Intriguingly, treatment in this animal model with recombinant thrombin (depleted of pro-coagulant function) significantly ameliorates the pathological condition, reducing inflammatory cell infiltration, and demyelination, decreasing activation of $CD11b^+$ macrophages and reducing the accumulation of fibrin(ogen) in the CNS (63). This supports the idea that the pro-coagulant function of thrombin is involved in microglial activation (64).

Other experimental findings support the role of fibrinogen in suppressing remyelination by the inhibition of oligodendrocyte progenitor cell differentiation into myelinating oligodendrocytes (9). In the EAE marmoset model, fibrinogen was proposed to derive from the central vein in early lesions, and its deposition was found to precede demyelination and visible gadolinium enhancing lesions on MRI (65). In fact, the peak of fibrinogen deposition corresponded with the beginning of demyelination and axonal loss. Afterwards, fibrinogen was found inside microglia/macrophages, suggesting its phagocytosis. Moreover, a positive correlation between fibrinogen deposition and accumulation of microglia/macrophages and T cells was detected (65). Overall, fibrinogen leakage is one of the earliest detectable events in lesion pathogenesis. Very recent promising data in EAE mice have shown that a monoclonal antibody targeting fibrin, without interfering with the coagulant activity, avoids microglia activation, and monocyte infiltration into the CNS (66). Moreover, it decreases neurotoxicity through the inhibition of ROS production mediated by NADPH oxidase in the innate immune cells, which has been demonstrated to be fibrin induced during the neurodegenerative process (66).

Fibrinolytic Pathways

The dissolution of the fibrin clot is mediated by the fibrinolytic system (**Figure 2**), initiated by the conversion of plasminogen into active plasmin by either urokinase-type plasminogen activator (uPA) or tissue-type plasminogen activator (tPA). tPA was found to be the most abundant plasminogen activator in control brains, with antigen concentration and enzyme activity several orders of magnitude higher than those of uPA (67). Plasmin cleaves fibrin to soluble degradation products, particularly the D-dimers, which represent an indicator of

cross-linked fibrin turnover (68). Strikingly, components of the fibrinolytic system present in the CNS participate in a wealth of physiological roles (69).

tPA has been found to be involved in regulating cerebrovascular integrity (70), neuronal activity (through its action on the N-methyl-D-aspartate (NMDA)-receptor), neuronal calcium signaling, axonal regeneration, and microglial activation/inflammation (69). uPA exerts proteolytic and intracellular signaling functions by binding its receptor (urokinase plasminogen activator receptor, uPAR) on the cell surface, including microglial activation and axonal regeneration (71–73).

The activity of both tPA and uPA is regulated by specific plasminogen activator inhibitors (PAIs) of which the principal is PAI type 1 (PAI-1), a member of the serine protease inhibitor superfamily (SERPINS) (74). Tight connection of fibrinolysis with coagulation is further provided by thrombin, which enhances fibrinolysis that induces the expression and activity of tPA, and causes inactivation of PAI-1 by forming a complex with it. Interestingly, high PAI-1 expression may be induced by inflammatory cytokines in pathological conditions (75).

Experimental evidence in mice has demonstrated that PAI-1 can be released by microglia and astrocytes under inflammatory conditions, increasing microglial migration into the brain and inhibiting microglial phagocytosis (76). Accordingly, in EAE mice, the inhibition of PAI-1 has been shown to decrease axonal degeneration and demyelination (77). Conversely, tPA deficiency in EAE mice induces a more severe disease progression and CNS fibrin deposition, while uPAR depletion delays the disease onset, acting only in the initial stage by reducing the adhesion and migration of inflammatory mononuclear cells into the CNS (78). In fact, mice in the EAE model without uPAR subsequently develop chronic disease (78). Thus, data in animal models suggest that an impaired fibrinolytic pathway may be involved in both inflammatory and neurodegenerative processes of the disease.

The Eclectic Nature of Factor XII: The Crossroad Between Coagulation (Intrinsic/Contact Pathway), Inflammation, and Immunity

Recently, albeit only in an animal model, FXII was found to be involved in adaptive immune responses via uPAR (CD87)-mediated modulation of dendritic cells (DCs) (10).

The coagulation cascade may be triggered by the circulating protein FXII, also called Hageman factor (79), through its contact with negatively charged surfaces and conformational change in the catalytic domain. The contact activation system does not depend on "external" proteins to trigger the coagulation cascade and it is usually identified with the intrinsic coagulation cascade pathway (80, 81). The FXIIa-initiated intrinsic coagulation pathway proceeds through activation of FXI (FXIa) and subsequent FIX activation (FIXa) (**Figure 3**), hence reaching the common pathway (**Figure 1**). Despite its contribution to fibrin formation in coagulation assays, the role of factor FXII "*in-vivo*" has long been debated because FXII deficiency does not exhibit a clinically relevant bleeding phenotype (82). Considering that FXII is located at the crossroads of several other pathways, these features make FXII an attractive target for inhibition without concomitant bleeding complications (83, 84).

FXIIa converts prekallikrein (PK) to kallikrein (KAL) (80), starting the proinflammatory kallikrein-kinin system (**Figure 3**). KAL acts on high molecular weight kininogen (HK), releasing the active peptide bradykinin (BK), through which bradykinin receptors mediate: (1) vasodilation induced by nitric oxide formation, (2) prostacyclin release, which reduces vessel-wall exposure of TF, (3) platelet inhibition, and (4) tPA release (80). The kallikrein-kinin system is further linked to the fibrinolytic pathway by KAL, which is able to convert plasminogen to plasmin (85). Thus, from one side the kallikrein-kinin system through BK promotes inflammation and from the other, the inhibition of coagulation and promotion of fibrinolysis. In the EAE animal model, the blocking of a BK receptor (B1R), mainly expressed close to plaques, prevented the infiltration of T lymphocytes into the CNS and decreased BBB permeability (86).

Interestingly, FXIIa itself has the capacity to cleave several proteins of the complement system, driving activation of innate immunity against foreign pathogens (**Figure 3**) (87). The complement cleavage products (C3a and C5a) have also been shown to exhibit robust chemo-attractive properties to human mast cells and neutrophils, highlighting the pro-inflammatory effects of the coagulation-complement interplay (88).

An example of selective pathway activation is given by mast cells (89) that rapidly secrete granules, of which heparin is one of the major constituents, when activated. Although heparin is primarily an anticoagulant, it provides a negatively charged surface that activates FXII, thus selectively promoting the inflammatory kallikrein-kinin system and possible consequent vascular leakage and BK-driven leukocytes infiltration (89).

Another immuno-mediated mechanism able to induce FXIIa is supported by neutrophils through the release of neutrophil extracellular traps (NETs). NETs consist of negatively charged contents such as nucleic acids together with histones, and antimicrobial proteins, which are physiologically used to trap and kill bacteria during infection. On the other hand, they trigger FXIIa and, in addition, foster the recruitment and activation of platelets, thereby promoting immunothrombosis (90).

In EAE, it has been demonstrated that depletion of FXII has a protective effect, delaying disease onset and decreasing disease severity (10). Of note, no differences were found in the amount of fibrin/fibrinogen in the CNS of EAE-FXII depleted mice compared to those with the wild-type EAE phenotype. Futhermore, factor XI (directly activated by FXII) deficiency does not alter the clinical course, demyelination, cytokine levels or the immune cell infiltration in the EAE model. These results support the hypothesis that FXII does not participate through activation of the intrinsic coagulation pathway, which would imply that the FXII procoagulant activity "*per se*" is not involved in MS (10).

Platelets, Von Willebrand Factor, and ADAMTS13

Hemostasis is a complex multi-step process, involving the interaction of platelet adhesion receptors with cognate ligands such as von Willebrand Factor (vWF), collagen, and fibrin (1).

vWF is either constitutively produced or released by Weibel-Palade bodies from endothelial cells, stored platelets, and

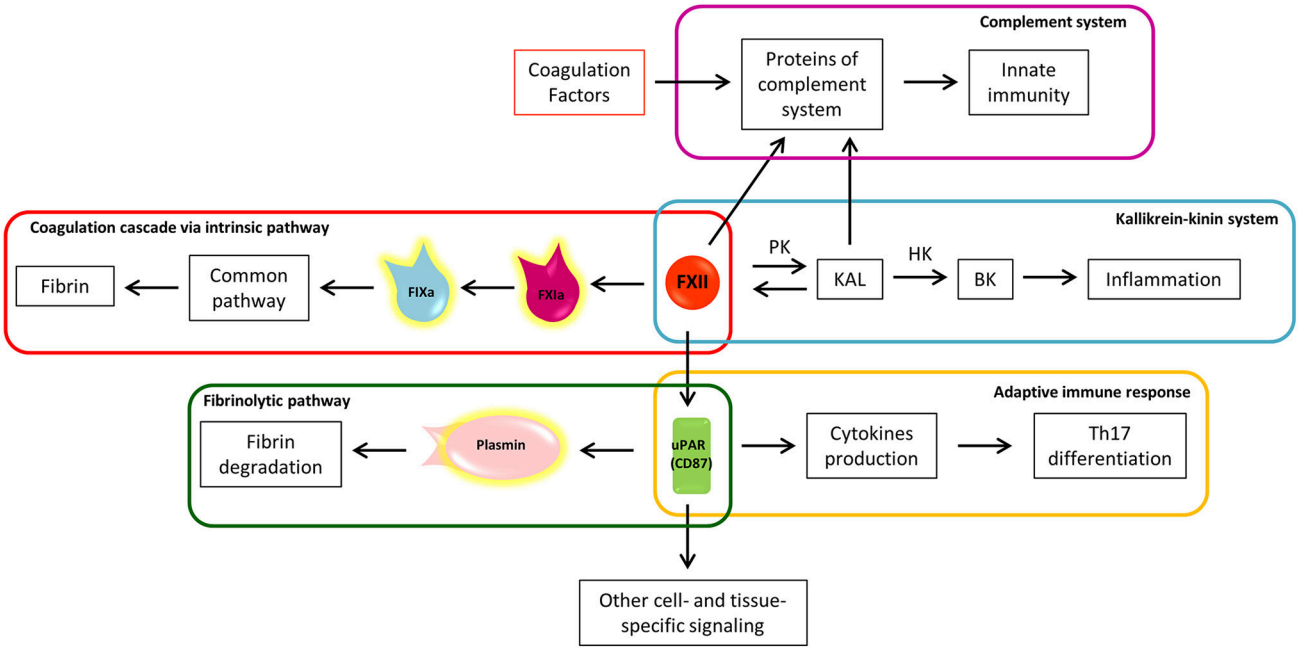

FIGURE 3 | The eclectic nature of Factor XII: the crossroad between coagulation, inflammation, and immunity. a, activated; BK, bradykinin; F, factor; HK, high molecular weight kininogen; KAL, kallikrein; PK, prekallikrein; uPAR, urokinase plasminogen activator receptor; Th17, T helper 17 lymphocytes.

subendothelial connective tissue, in an ultra-large form, a long multimeric string that is associated with FVIII molecules (91). When thrombin cleaves FVIII, it mediates its activation through extended conformational changes that additionally cause FVIII to dissociate from vWF (92). vWF serves as an adhesion surface to which platelets aggregate and form a plug. The "A Disintegrin-like And Metalloprotease with ThromboSpondin type 1 motif 13" (ADAMTS13) enzyme, a main inhibitor of hemostasis, cleaves the ultra-large vWF in vWF multimers with lower size, decreasing the propensity of vWF to support platelet adhesion and aggregation (93) (**Figure 4**). Deficiency of ADAMTS13 causes thrombotic thrombocytopenic purpura (TTP), a disease characterized by overt platelet aggregation through large vWF multimers generating microvascular thrombosis (94).

Coagulation Inhibitors

In vivo, coagulation factors are regulated by positive and negative feedback loops, the latter being provided by multiple coagulation inhibitors/anticoagulant proteins, which are also activated in a cascade-like fashion and influenced by feedback loops.

In the coagulation amplification process, the first line of inhibition is exerted by tissue factor pathway inhibitor (TFPI) (95), membrane-bound to endothelial cells (TFPIβ) as well as soluble in plasma, that is released from endothelial cells and platelets (TFPIα) (95). Circulating TFPI is mainly associated with lipoproteins, and inhibits coagulation in two distinct ways: (1) primarily, by interaction with the transient TF/FVIIa/FXa complex, and (2) by direct inhibition of free FXa. TFPI-dependent inhibition of FXa is mediated by the presence of protein S, which acts as a cofactor increasing TFPI affinity (96).

TFPIβ, which appears to be the predominant form, is anchored on the surface of the vascular endothelium, suggesting its role in the regulation of TF-mediated inflammatory responses via PARs signaling (97, 98). TFPI coagulation-independent action includes the suppression in TNF-α and IL-6 production, and an increase of anti-inflammatory IL-10 (99). Thus, TFPI may have distinct biological activities and potentially exerts a protective anti-inflammatory role in MS.

Antithrombin (AT, previously also called antithrombin III) belongs to the family of SERPINS and it inhibits several activated coagulation factors: FVIIa in complex with TF, FXa that dissociates from the TF-bearing cell (74) and, as the name suggests, thrombin via the formation of the thrombin-antithrombin (TAT) complex. Binding of cofactor heparin and heparin-like molecules are required for achieving inhibitory rates of AT.

The rising concentration of thrombin during coagulation proteolytically activates a main coagulation inhibitor, protein C, by binding the membrane protein thrombomodulin (TM), which is expressed on endothelial cells together with the endothelial protein C receptor (EPCR) (51). As with other receptors involved in hemostasis, both TM and EPCR can be cleaved from the cell surface in response to endothelial damage (100, 101). Activated Protein C (aPC) associates with its cofactor protein S, and the aPC/protein S complex proteolytically attacks FVa and FVIIIa, which are mostly membrane-bound, thus suppressing tenase and prothrombinase complexes (51). Of note, aPC may inactivate FVa when the thrombin-generating surface is provided by endothelial cells, but not from platelets (102). aPC may remain associated with EPCR and interact with

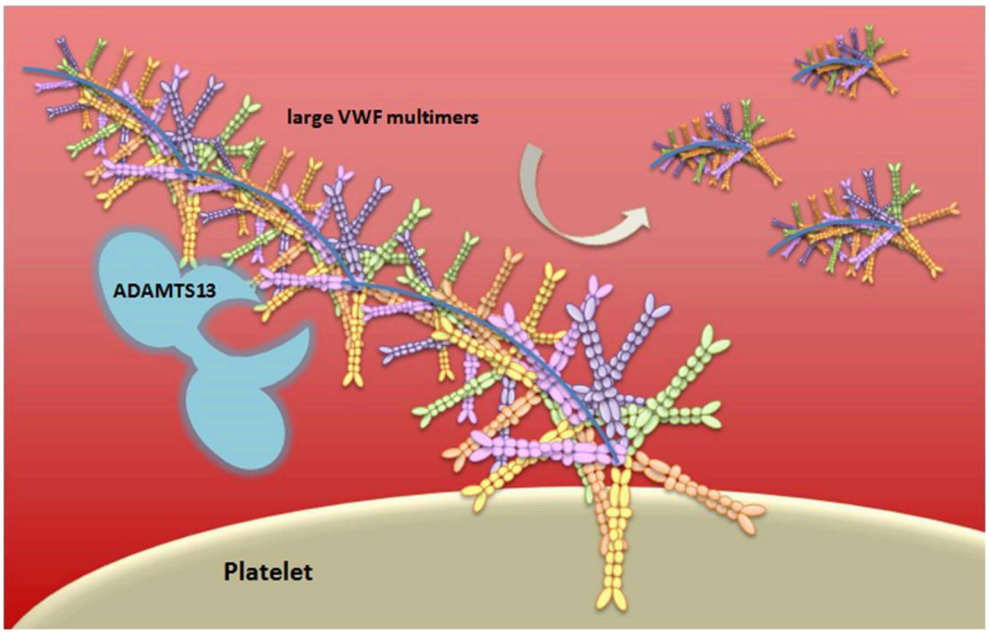

FIGURE 4 | Schematic representation of vWF multimer size regulation by ADAMTS13. von Willebrand Factor (vWF) is stored in the Weibel-Palade bodies of endothelial cells or in the α-granules of platelets and it is released in an ultra-large form, a long multimeric string. The vWF serves as an adhesion surface to which platelets adhere and aggregate, and form a plug. The regulation of platelets adhesion depends upon cleavage of vWF in different size of multimeric string by ADAMTS13.

PARs, exerting antiapoptotic and anti-inflammatory actions as signaling molecule (51), thus providing a tight link between (anti)coagulation and (anti)inflammation.

It has been observed in EAE that the administration of recombinant aPC and mutant forms of aPC with either the anticoagulant function alone or the signaling function alone reduces disease severity. This provides evidence that both of aPC's anticoagulant and signaling functions are required to improve the disease condition (103). EAE mice with a TM gene mutation that disrupts the TM-dependent activation of protein C ($TM^{Pro/Pro}$) have perturbed myelination and mitochondrial functioning, resulting in increased ROS production and aggravated EAE pathology. Administration of aPC or TM provided relief in $TM^{Pro/Pro}$ EAE mice (104, 105). Given these results, the role of the protein C system in amelioration of the disease is worth further consideration.

Other noteworthy inhibitors from the SERPINS family are heparin cofactor II (HCII), C1 inhibitor (C1INH) and protein C inhibitor (PCI). The inhibitory role of SERPINS is modulated by binding to cofactors, especially glycosaminoglycans like heparin, which present on cell surfaces and on the extracellular matrix (74, 106). HCII acts similarly to AT in the negative regulation of thrombin (107). C1INH is the most powerful FXIIa inhibitor (108). PCI inhibits anti-coagulant aPC and thrombin-TM complex but also the pro-coagulant thrombin, FXa, FXIa and FVIIa-TF complex. It also inhibits the fibrinolytic pathway by inhibiting uPA and tPA (74).

Overall, pro-coagulant, anti-coagulant, and fibrinolytic pathways are responsible for maintaining the hemostasis balance under physiological conditions. Significant deviation from these pathways would result in hypercoagulability leading to life-threatening thrombotic or, alternatively, to acquired/inherited bleeding diseases (e.g., hemophilias). Based on this, the role of coagulation (im)balance in MS patients is further reviewed below.

COAGULATION AND HEMOSTASIS FINDINGS IN MULTIPLE SCLEROSIS PATIENTS

Fibrin(ogen) Brain Deposition

Direct studies of histological brain samples, aimed at addressing fibrin deposition and alteration of the fibrinolytic pathway, began in the 1980s (**Tables 1**, **2**). Nowadays, it is well known that one of the key events in the pathophysiology of MS is BBB breakdown, which leads to the entry of several neurotoxic blood-derived proteins (**Figure 1**) (119). Thanks to these histological studies, fibrinogen, an abundant protein in plasma, has been identified as a contributor to neuroinflammation in the CNS (11, 120). However, since most of the antibodies used across these studies were unable to distinguish fibrin from fibrinogen, the term "fibrin(ogen)" is likely more appropriate. The properties of fibrin favor the formation of oligomers and protofibrils, which aggregate laterally to make fibers, and ultimately branch to yield a three-dimensional network of insoluble fibrin (121). The detection in tissues of insoluble fibrin (fibrin deposition) by antibodies is therefore enhanced as compared with the detection of fibrinogen.

The relation between hemorrhage and demyelinating plaques was first considered by an early case report of 2 MS patients who developed CNS hemorrhage. It was suggested that the demyelinating event could contribute and set the stage for focal hemorrhages (122). However, over the course of the following years, the leakage of blood protein fibrinogen into the brain parenchyma was established as a potential marker of BBB damage (2, 113), and as a contributor to neurodegenerative events. In initial reports, the presence of heavy extracellular fibrinogen was detected in demyelinated centers of acute MS plaques (109) as well as in most of the examined inactive plaques, particularly close to astrocytes (110). Moreover, the fibrinogen within the plaques was found to overlap with macrophages and axons, and even extended into the surrounding normal-appearing brain tissue. Nevertheless, fibrinogen did not co-localize with the enlarged astrocytes outside the plaques (110). Moreover, it was shown that fibrinogen leakage gradually increased through the progression of MS lesions, reaching the highest levels within the central parenchyma of those plaques with the greatest degree of activity (111). Interestingly, fibrinogen co-localized with areas of activated microglia in MS lesions (111).

Confocal microscopy confirmed the presence of extravascular fibrinogen in active MS lesions, most commonly with a distinct perivascular distribution, and in a few cases widely distributed throughout the parenchyma (112). Association of such leakage with areas of microglial activation was found to be consistent with increased tight junction abnormality in the same areas (112). Confocal microscopy was also extensively used to confirm the perivascular distribution of the fibrinogen leakage and demonstrate varying fibrinogen levels within MS lesions (113). Hence, the severity of altered tight junctions was associated with BBB dysfunction, which in turn was proportional to the increase in fibrinogen leakage reaching particularly high levels in active lesions (113). A threshold of tight junction injury might be required before significant and visible BBB leakage of the large, high-molecular-weight protein fibrinogen (113), which could explain the lack of fibrinogen detection close to vessels with a lower degree of tight junction abnormality.

Postmortem magnetic resonance imaging (MRI) was also applied to detect both diffuse and focal brain abnormalities, allowing targeted histopathological examination of MS lesions (2). BBB disruption was detected by increased immunopositivity for fibrinogen in the brain parenchyma as described by previous studies (109–113). Fibrinogen leakage was found in both active and chronic MS lesions, co-localizing with astrocytic processes and occasionally with axonal processes (as demonstrated by neurofilament immunoreactivity), which suggested that astrocytic and neuronal processes may bind or incorporate extravasated fibrinogen. Moreover, fibrinogen was not limited only to demyelinating lesions, but it was seen in both reactive lesions characterized by small clusters of microglial cells without apparent loss of myelin with a variable degree of edema, and in areas with diffusely abnormal white matter (WM) (2). Nevertheless, the presence of fibrinogen was more extensive in chronic active and inactive lesions when compared to reactive lesions (2).

A more recent analysis of chronic MS lesions revealed that fibrinogen extravasation was present in chronic active lesions close to the blood vessels, but not in the chronic inactive ones (65). It was also shown that fibrin deposition might occur early in MS and precede demyelination (11), since the "pre-demyelinating" areas of activated microglia hosted fibrin precipitates within the extracellular space of the lesions (11). The high precipitation of fibrin on the surface of microglia was suggested to be the driving force for microglial activation according to its detection in focal plaques of microglial activation with features of hypoxia-like damage but in the absence of demyelination (11). Thus, changes in the NAWM precede the formation of inflammatory demyelinating plaques, in particular in those exhibiting a pattern of hypoxia-like demyelination. Such changes were suggested to settle the inflammatory response and infiltration of T-cells, B-cells, and macrophages in the brain tissue, leading to the formation of the classic inflammatory demyelinating plaque detected by MRI (11). This is in agreement with recent findings showing that fibrin can mediate microglial activation and oxidative stress with ROS production, contributing to local neurodegenerative events (66). Finally, fibrin(ogen) was reported in the cortex of progressive (P-MS) cases. Extracellular fibrin(ogen) deposition was mostly found in the deeper cortical layers (layers 5 and 6 vs. layer 2). In contrast, its co-localization within neuritic and astrocytic processes was predominantly in the superficial cortical layers (12). The presence of intracellular fibrin(ogen) has been suggested to occur by direct synthesis of those cells or to be mediated by retrograde transport in damaged axons exposed to increasing amounts of protein. Overall, severe fibrin(ogen) deposition was detected in areas of significantly reduced neuronal density and particularly appeared to affect the loss of layer 5 projection neurons (12). No relationships were observed between the presence of fibrin(ogen) and microglial/macrophage density. Of note, the deposition of other proteins, such as albumin, remains controversial because of their inability to be converted into an insoluble matrix as fibrinogen does to fibrin, precluding accurate assessment.

In summary, these data point toward the role that fibrinogen has on sustaining the pathogenesis of MS lesions following its entrance into the CNS. In particular, its conversion into fibrin seems to trigger the activation of microglia and to support inflammation and the consequent development of demyelinating lesions.

Histological Evidence for an Altered Fibrinolytic Pathway in Multiple Sclerosis CNS

Besides fibrin(ogen), several studies have focused on the fibrinolytic pathway (**Table 2**), and the capacity of MS lesions to break down fibrin. Initial findings were provided by histochemical techniques, showing that the amount of fibrinolytic activity was comparable between active lesions and inactive ones (115). The fibrinolytic zones in MS brains appeared to originate from areas around vessels or capillaries, and the presence of lymphocytic infiltrates, gliosis, or macrophages did not change the localization and degree of fibrinolysis. Moreover,

TABLE 1 | Histopathological evidence of hemostasis components in multiple sclerosis.

Hemostasis components	Main findings (patient sample size/methodology)	References
COAGULATION		
FXII	Deposition nearby dendritic cells positive for uPAR.	(10)
Fibrinogen	Presence within demyelinated centers (23 acute MS plaques).	(109)
	Detected in 19 inactive plaques, co-localize with astrocytes (32 inactive plaques).	(110)
	Perivascular detection in type I, II and V lesions. Leakage within central plaques parenchyma (immunohistochemistry on 155 MS lesions from 13 early cases of MS).	(111)
	Extravascular staining with perivascular distribution in association with microglial activation (active MS lesions analyzed by confocal microscopy).	(112)
	Perivascular distribution of leakage and differential degree of deposition in WM. Co-localization with astrocytes. Correlation with the grade of tight junctions' abnormality (2,198 MS and 1,062 control vessels analyzed by confocal microscopy).	(113)
	Leakage both in active and chronic lesions; reactivity also in NAWM and in WM. Co-localization with astrocytes and neuronal process (postmortem MRI on MS lesions).	(2)
	Extravasation close to the blood vessels only in chronic active lesions (4 chronic active lesions and 5 chronic inactive lesions from 4 MS brains).	(65)
Fibrin(ogen)	Extracellular deposition predominantly located in layers 5 and 6 of the cortex in MS. Intracellular deposition detected in neurons and astrocytes (immunohistochemistry on the cortex of 47 progressive MS and 10 controls).	(12)
Fibrin	Staining overlap with macrophages and axons, and extended into NAWM (32 inactive plaques).	(110)
	Deposition in areas of activated microglia (immunohistochemistry on 155 MS lesions from 13 early cases of MS).	(111)
	Deposition occurs in pre-demyelinating areas of activated microglia.	(11)
INHIBITORS		
Protein C inhibitor	Detected in chronic active plaques (Mass spectrometry MS plaques).	(103)
C1INH	Detected in MS plaques.	(114)

C1INH, C1 inhibitor; F, factor; NAWM, normal-appearing white matter; MS, multiple sclerosis; uPAR, urokinase plasminogen activator receptor; WM, white matter.

TABLE 2 | Histopathological evidence of fibrinolytic pathway components in multiple sclerosis.

Fibrinolytic components	Main findings (patient sample size/methodology)	References
Fibrinolysis	Higher fibrinolytic activity in plaques than adjacent NAWM.	(115)
tPA	Staining for infiltrated mononuclear cells in MS lesions and WM. Strong positivity of foamy macrophages in areas of demyelination and decline in chronic lesions.	(116)
	Co-localization with non-phosphorylated neurofilament and fibrin deposition in demyelinated axons.	(67)
	Decreased tPA activity in acute MS lesions. Decreased fibrinolytic activity in demyelinating MS plaques due to tPA/PAI-1 complex.	(117)
tPA receptors	Localization on macrophages, astrocytes. Increased in MS lesions compared to NAWM.	(118)
uPA, uPAR	Detected in acute MS lesions, expressed by mononuclear cells in perivascular cuffs and to macrophages in the lesion parenchyma. uPAR additionally detected in NAWM.	(67)
D-dimers	Localization on foamy macrophages and demyelinating axons.	(117)
PAI-1	Detected in acute MS lesions, expressed by mononuclear cells in perivascular cuffs and to macrophages in the lesion parenchyma.	(67)
	Up-regulation in progressive MS cortex but without an efficient fibrin degradation (immunohistochemistry on the cortex of 47 progressive MS and 10 controls).	(12)

NAWM, normal-appearing white matter; MS, multiple sclerosis; PAI-1, plasminogen activator inhibitor 1; tPA, tissue-type plasminogen activator; uPAR, urokinase plasminogen activator receptor; WM, white matter.

the NAWM from MS patients was not more fibrinolitically active than that of the controls, but plaques showed more fibrinolytic activity compared to adjacent NAWM (115), suggesting to an attempt to combat fibrin. Subsequently, positive infiltrating mononuclear cells stained for tPA were observed in MS lesions, particularly within active ones (116). This pattern converted into a strong positivity of foamy macrophages in areas of demyelination and declined in chronic lesions. Similarly, PAI-1 expression paralleled that of tPA on foamy macrophages (116). The disappearance of immunoreactivity for tPA in chronic MS plaques also supported the role of impaired fibrinolysis as a contributing event to the inflammatory stage of demyelination mediated by fibrin. In fact, the increased expression of tPA on mononuclear cells in perivascular cuffs was suggested to be one of the earliest detectable signs of inflammation in MS. tPA might trigger the matrix metalloproteinase (MMP) cascade

and thus facilitate entry of leukocytes into the CNS (116). It is important to note, though, that another study provided partially discordant data: although quantitatively decreased in MS lesions, it found that tPA was co-localizeed with non-phosphorylated neurofilament and fibrin(ogen) deposits on demyelinated axons (67). On the other hand, highly significant increases in uPA, uPAR, and PAI-1 were detected in acute MS lesions and uPAR in NAWM when compared to control tissue. These three proteins were immunolocalized with mononuclear cells in perivascular cuffs and with macrophages in the lesion parenchyma. The significant increase in the uPAR complex was thought to be a trigger for focal plasmin generation and for cellular infiltration, cooperating with MMP activity in the opening of the BBB (67).

Further investigations provided evidence for the lowest fibrinolytic activity within acute lesions, which was due to the formation of tPA/PAI-1 complex (117), in turn contributing to fibrin accumulation. Nevertheless, D-dimers and fibrin degradation products were mostly localized at the neurovascular interface and on foamy macrophages and axons during the chronic inflammatory stage of lesions (117). In addition, increased PAI-1 synthesis leading to defective fibrinolysis appeared to develop before lesion formation (117). However, during lesion progression, an increase in lower molecular weight PAI-1 peptides was detected, as a result of PAI-1 intracellular degradation mediated by macrophages (117).

Plasma membrane tPA receptors, which may concentrate proteolytic activity on the cell surface and in turn locally enhance the fibrinolytic response, were immunolocalized in acute MS lesions on macrophages and astrocytes (118) and increased in MS lesions when compared to NAWM samples. Furthermore, a tPA receptor was found on neuronal cells within the cortex. However, the limited availability of tPA, bound to PAI-1, reduces the production of plasmin, which further decreases the fibrinolytic activity in active MS lesions and increases axonal fibrin deposition and neurodegeneration (118). Indeed, perturbed fibrinolysis was found to be a hallmark of P-MS cases with abundant cortical fibrin(ogen) deposition (12). Overall, significant upregulation of PAI-1 in the cortex, where fibrin deposition was most severe, points toward dysregulated fibrin clearance that allows for its pathological accumulation in the later stages of MS (12).

Detection of Protein C Inhibitor (PCI), C1INH, and FXII in Multiple Sclerosis Plaques

An early biochemical study based on isolation of brain capillaries from human brain samples close to MS lesions showed positive staining for FVIII (123). Further insights into coagulation components and inhibitors in MS lesions have been provided by lesion-specific proteomic profiling (103), which detected TF in particular. This is to a certain extent expected in relation to the abundance of this protein in perivascular spaces, whereas PCI is only found in chronic active lesions. PCI, which inhibits aPC, seems to accumulate within these lesions secondary to the disruption of the BBB during neuroinflammation. The combined presence of TF and PCI suggests pro-inflammatory thrombin formation and suppression of the PC pathway, supporting a mechanism involved in MS lesion formation that suppresses the action of coagulation inhibitors in the presence of coagulation activation (103). Further evidence for the intricate connection between coagulation, inflammation, and immunity was provided by the positive reactivity of MS lesions for proteins of the complement system, and regulators as C1INH. Taken together, these findings point toward continuing local complement synthesis, activation, and regulation despite the absence of evidence of ongoing inflammation (114). Interestingly, deposition of FXII, which is inhibited by C1INH and might support autoimmunity, was detected in the histological analysis of CNS tissue from MS patients nearby DCs positive for CD87 (uPAR) (10).

Overall, impaired fibrinolysis seems to reinforce fibrin(ogen)-associated damage in MS. Impaired inhibition of coagulation, and the contribution of coagulation factors through inflammatory and autoimmunity pathways in CNS therefore deserves further investigation.

Historical Perspective of Hemostasis Abnormalities and Circulating Hemostasis Component Levels in Multiple Sclerosis

The first description of hemostasis abnormalities in MS was provided by Putnam (124) who reported the presence of definite thrombi in half of the analyzed MS cases (9/17). Thrombi were described as the frequent occurrence of perivascular hemorrhages within acute lesions and as a vascular obstruction in chronic lesions. Therefore, the primary abnormality of MS was suggested to reside in the alteration of the blood clotting mechanism (124), and as a consequence of this hypothesis, 43 MS cases were treated with dicoumarine for a timeframe between 6 months and 4 years (125). Despite the side effects, Putnam and colleagues concluded that anticoagulant treatment reduced relapses in the relapsing-remitting (RR) form of MS while the course of chronic progressive disease was not affected (125). Soon after, Putnam interpreted venous thrombosis as a possible pathognomonic process in MS, while others reported increased capillary fragility (126) and subcutaneous hemorrhages (127).

Later on in 1955, Persson reported increased levels of plasma fibrinogen in MS patients during relapse exacerbations, which were not related to thrombus formation (128). By that time, it was already known that fibrinogen levels were higher than in controls in the majority of chronic and degenerative diseases, thus laying the foundations for later discoveries of fibrinogen levels as a marker of inflammation (128, 129). A few years later, another study investigated blood coagulation in 33 MS patients and corroborated previous findings, showing no tendency toward increased blood coagulability (130). Overall, in the majority of investigated patients fibrinogen levels were within the normal range, despite wide variations that were not associated with the stage of the disease (130). A subsequent study in 10 MS patients explored both blood and cerebrospinal fluid (CSF) and revealed that neither had thromboplastin activity, nor significant abnormalities in blood platelet, coagulation factors, serum platelet-like activity nor fibrinogen levels (131). Despite

the lack of abnormal findings, increased capillary fragility was reported (131).

The "antithrombic" activity of normal and pathological CSF was later discovered in 1961 (132). With the exception of larger proteins like fibrinogen and FV, further studies demonstrated the presence of coagulation proteins in the CSF (133) and corroborated findings that degradation products of fibrin were present under pathological conditions (62).

The discrepancy in results regarding coagulant balance of that epoch needs to be interpreted in light of possible unstandardized examination techniques. However, after 80 years from the first report on altered coagulation in MS (124), findings on this research topic in MS are still controversial. This is probably also due to the inclusion of small cohorts of patients and the sporadic analysis of circulating levels of certain hemostasis components, which prevents the consolidation of conclusions and clearly highlights the need for larger, more well-controlled additional investigations. Evidence regarding CSF, plasma and serum levels of hemostasis components is summarized in **Table 3**. Of note, levels are often used to refer to either protein concentration or activity of a protein without a clear distinction. However, concentration levels provide information that is independent of the protein's ability to be intrinsically functional and do not depend on activatory or inhibitory molecules. Similarly, testing the functional activity does not provide direct information about its protein concentration but integrates the influence of activators or inhibitors. During a PT or aPTT assay, information about clotting time is obtained, providing the overall functionality of the system. When alteration in clotting time is observed, it is possible to supplement the assay using a plasma depleted of a specific coagulation component (thought to be the cause of the alteration) in order to assess the specific functional activity of that component. Since ethylenediamine-tetra-acetic acid (EDTA) removes calcium from the sample, which is needed for blood clotting, plasma collected in EDTA is not an appropriate sample to test PT and aPTT.

Considering the tight relation between coagulation factors and immune response discussed earlier, it is intriguing to speculate that the clinical manifestation of MS could also be related to increased pro-coagulant activity. No significant differences have been reported for PT or aPTT times in the plasma of MS patients (140) nor for fibrinogen concentration in either CSF or blood (140, 149). However, the analysis of the CSF proteomic profiles in patients, collected in different phases of their clinical course, showed significantly lower fibrinogen concentration in clinically isolated syndrome compared to PMS patients (134). Moreover, increased fibrinogen beta chain concentration was detected in CSF samples from two fulminant MS cases by mass spectrometry (150). A relationship with the activity of the disease was also reported in a recent investigation where high fibrinogen levels were detected in plasma in a substantial proportion (17/58) of patients, particularly in those with active lesions on MRI (141). Taken together, these studies support the role of fibrinogen as a contributor of neuroinflammation and neurodegenerative processes in the CNS following BBB damage.

In a more comprehensive investigation, the activity of PC, FII, FX, FXI and FXII, and propensity of fibrinogen to clot was determined in plasma samples of MS patients with different clinical phenotypes compared to healthy individuals (136). Increased activity of FII:C and FX:C was detected in RRMS and SPMS patients when compared to controls (136). These experimental findings suggest an increase in thrombin activity and its generation through FX activity, which by definition is part of the prothrombinase complex, in MS patients. However, these increased activities do not seem to be balanced by increased activity of PC, a key inhibitor (136). Similarly, plasma AT activity was reported to show no differences in MS patients or associations with periods of relapses or remissions (143).

Evaluation of coagulation activity by thrombin generation assay, a more sensitive and flexible method that accurately reflects the initiation, propagation, and termination phases of coagulation (151, 152), showed enhanced thrombin generation in RRMS patients compared to PPMS and controls, pointing to a prothrombotic state within the RRMS phenotype (139).

Elevated FXII activity was found in RRMS and SPMS compared to controls, and greater activity levels were associated with higher occurrence of relapses and shorter relapse-free periods, independently from the use of immune modulatory therapy (10). Another recent study that evaluated the ratio of FXII activity and the amount of circulating protein found increase activity of FII and FX was protein levels and reduced function within the intrinsic coagulation pathway (137). Notably, intrinsic thrombin generation did not result in the detection of prothrombotic features in the evaluated PMS patients (137). These data underline the importance of evaluating the activity of both antigen and coagulation factors. Furthermore, the contribution of FXII in MS may be independent of its coagulant property and the protein may potentially be hijacked to participate in other FXII-mediated pathways. Immune-modulatory function in relation to, or its possible independence, from coagulation activity, particularly for FXII, still remains to be elucidated in MS. On the other hand, in evaluating the activity of cellular components of coagulation, unstimulated and stimulated monocytes were not found to differ in MS and controls with respect to expression of cell surface TF or production and secretion of TF (153), which does not support the presence of pro-thrombotic components on cell surfaces. However, higher TFPI levels in P-MS patients compared to RR-MS patients and controls were recently reported (138). Taking into account that TFPI is the first line of inhibition in the coagulation cascade, this could merit further investigation.

Analysis in pre-symptomatic and post-symptomatic MS pooled serum detected proteomic changes for factors involved in the complement and coagulation pathways, with a particular decrease in MS of FX, FII, and C1INH (145). Because the serum is isolated after coagulation, these results might be interpreted as residual coagulation factors remaining after conversion of fibrinogen into fibrin, the last step of the pathway. Concerning the complement protein, lower C1INH in patients is of interest in light of its inhibitory activity against FXII through its recruitment in the CNS. Although depositions of FXII and C1INH have been reported, the demonstration of their co-localization in MS brain tissue is still needed (10, 123).

It is worth mentioning that a few studies have investigated platelet stickiness in MS (154–156). Nevertheless, platelets

TABLE 3 | CSF, plasma, and serum evidence of altered hemostasis components in multiple sclerosis.

Hemostasis factors, inhibitors, and receptors	Main findings (patient sample size/methodology)	References
CSF		
Fibrinogen	Lower levels in CIS vs. PMS (proteomic profile by mass spectrometer in 24 CIS, 16 RRMS, 11 PMS).	(134)
TM	Higher levels in OIND vs. SPMS. Ninety percent of TM in CSF is related to intrathecal synthesis (17 relapse, 11 remission, 11 SPMS, 19 OND, 15 OIND).	(135)
PLASMA		
FII, FX, Fibrinogen, PC, FII, FX, FXI	Higher FII:c and FX:c in RRMS and SPMS vs. controls. No differences in activity of Fibrinogen, FXI and PC (PT in citrate plasma: 116 RRMS, 10 PPMS, 73 SPMS, 20 controls).	(136)
FXII	Higher FXII:c in RRMS and SPMS vs. controls. Higher activity correlates with higher occurrence of relapses and shorter relapse-free period (aPPT in citrate plasma: 138 RRMS, 13 PPMS, 90 SPMS, 19 CIS, 130 controls).	(10)
	Increased of FXII protein concentration levels and reduced function in MS (aPTT and ELISA on citrate plasma: 12 RRMS, 34 SPMS, 28 PPMS, 49 controls). Intrinsic thrombin generation in 10 PMS, 10 controls.	(137)
FXII, ADAMTS13, HCII, TFPI, TM	Lower ADAMTS13 levels in MS vs. controls. Higher TFPI levels in PMS vs. RRMS and vs. controls. No differences in FXII and HCII (ELISA on plasma EDTA: 85 RRMS, 53 PMS, 42 controls).	(138)
FII	Prothrombotic state in RRMS (thrombin generation on citrate plasma: 15 RRMS, 15 PPMS, 19 controls).	(139)
Fibrinogen	No differences in fibrinogen levels, PT and aPTT times (42 RRMS and 31 controls).	(140)
	High levels, particularly associated with active lesions on MRI (17 out 58: 45 CIS, 12 RRMS, 1 PMS).	(141)
vWF, TM	Higher vWF activity in active MS. No differences in TM protein concentration (26 RRMS, 35 controls).	(142)
AT	No differences in AT:c (37 RRMS, 32 SPMS, 34 controls).	(143)
EPCR	Trend for higher levels in MS (63 MS, 20 controls).	(144)
SERUM		
FX, Prothrombin, C1INH, FXIII, Plasminogen	Reduction of FX, prothrombin and C1INH levels in pre- and post-symptomatic MS serum. Reduction in FXIII and plasminogen in post-symptomatic MS (Mass spectrometry (pooled serum of 100 MS vs. pooled serum of 100 controls).	(145)
TM	Higher levels in MS during exacerbation vs. remission state, OND, and controls (17 acute relapse, 9 PMS, 13 HAM, 10 non-HAM, 10 OND, 20 controls).	(146)
	Higher levels in OIND vs SPMS (17 relapse, 11 remission, 11 SPMS, 19 OND, 15 OIND).	(135)
TM, aPC	No differences (100 RRMS, 22 SPMS, 122 controls).	(147)
vWF	No difference (9 RRMS, 9 SPMS, 10 PPMS).	(148)

ADAMTS13, A disintegrin-like and metalloprotease with thrombospondin type 1 motif 13; AT, antithrombin; aPC, activated protein C; aPTT, activated partial thromboplastin time; C1INH, C1 inhibitor; CIS, clinically isolated syndrome; CSF, cerebrospinal fluid; :c, activity; EDTA, ethylenediamine-tetra-acetic acid; ELISA, enzyme-linked immunosorbent assay; EPCR, endothelial protein C receptor; F, factor; FII, thrombin; HAM, HTLV-1-associated myelopathy; HCII, heparin cofactor II; OIND, other inflammatory neurological disorders; OND, other neurological diseases; PC, protein C; PMS, progressive multiple sclerosis; PPMS, Primary Progressive Multiple Sclerosis; PT, prothrombin time; RRMS, relapsing-remitting multiple sclerosis; SPMS, secondary progressive multiple sclerosis; TFPI, tissue factor pathway inhibitor; TM, thrombomodulin; vWF, von Willebrand Factor.

adhesion and aggregation are supported by vWF activity, which was found to be higher in patients with active MS than in controls, and significantly decreased after immunosuppressive treatment (142). The authors suggested that vWF could serve as a marker for evaluating BBB breakdown resulting from endothelial damage in MS, in line with the idea of hemostasis activation at the neurovascular interface after injury. Interestingly, decreased ADAMTS13, which exerts an inhibitory function on vWF activity, was detected in MS patients and in particular in those with cerebral microbleeds (138, 157). These data should motivate future investigations in MS patients with focal extravascular leakage of blood components, measured as cerebral microbleeds, to potentially identify as of yet unknown molecular component(s) driving hemostasis alteration in MS patients.

In addition to vWF release as consequence of endothelial damage, the shedding of membrane proteins could be promoted. In this context, soluble TM has been the most investigated protein in MS (135, 138, 142, 146, 147). Studies on larger cohorts of MS patients concluded that TM concentration levels do not differ in plasma or in serum (138, 147). However, circulating TM appeared to increase in MS during exacerbation compared to the remission, reaching higher concentration levels in patients with acute relapse (146). An intriguing hypothesis was also offered by the authors of a study who speculated that 90% of TM in CSF is of intrathecal synthesis, with higher TM production during relapses (135). Based on this hypothesis, TM should be further explored in association to the relapsing and remitting states of patients.

Correlations between peripheral plasma levels of FXII, TFPI, ADAMTS13, HCII, and TM with MRI measures, indicative of severity of inflammatory and neurodegenerative tissue injury, were recently investigated for the first time (138). Several correlations were detected in MS patients: (1) higher FXII levels with lower ventricular and higher deep gray matter (DGM) volumes, (2) higher HCII levels with lower brain and cortical volumes and higher ventricular volume, (3) higher TFPI levels with lower DGM volume. However, after correction for multiple comparisons, no significant relationships between hemostasis component levels and MRI measures

remained significant. Nevertheless, in light of the fact that glycosaminoglycans are cofactors of HCII, and considering their role in the inflammatory process (158), as well as in normal CNS functioning or pathological conditions [MS included, as reviewed in reference (106)], the study of HCII involvement in MS deserves further investigation.

Overall, the discordant data on prothrombotic features in MS patients in the limited number of studies point toward altered pro-coagulant status during the more active phase of the disease. Patient prothrombotic heterogeneity could be approached through stratification according to coagulation balance in order to prospectively evaluate the impact of coagulation differences on disease evolution.

Plasma, Serum, and Cerebrospinal Fluid Levels of Fibrinolytic Pathway Components

Early evidence of increased fibrinolytic activity, which again pointed toward an altered coagulation system in MS (159), and evidence of fibrin degradation products in the CSF of MS patients (160) paved the way for further studies investigating the proteins of the fibrinolytic pathway (**Table 4**).

In the CSF, higher tPA activity was found in MS patients (163) coupled with evidence of very low uPA activity (163). Increased total PAI-1 concentration was found in MS patients and a significant inverse relationship between PAI-1 levels in CSF and plasma was observed (161). Very high PAI-1 levels were observed during relapses (reaching values 6 times greater than controls), and follow-up investigation showed 2-fold decreased values even 1-2 months after the relapses. However, a correlation between PAI-1 and tPA plasma levels was not observed (162).

Recently, higher PAI-1 plasma levels in MS patients were reported when compared to controls (138). Moreover, in MS patients, but not in healthy controls, a positive association between PAI-1 and FXII concentrations and a negative association between PAI-1 and HCII concentrations were found. Accordingly, if thrombin is inhibited, there is no fibrin formation, hence there is no fibrinolysis to block. On the other hand, FXII stimulates fibrinolysis through uPAR (**Figure 2**), and if tPA increases, an inhibition target exists for PAI-1.

Again in plasma, levels of D-dimer, tPA, and PAI-1 did not differ between patients and controls in one study (164), but significantly higher D-dimer levels were found in another investigation (140).

Overall, data regarding increased PAI-1 antigen levels supports the notion that impaired fibrinolysis sustains the ongoing neuroinflammatory (particularly during relapse) and neurodegenerative events in the brain as evidenced by histological studies. Considering the few and discordant studies on tPA and D-dimers, further investigation is needed, which would provide a more comprehensive view of fibrinolysis in relation to the MS disease course.

Effect of Disease-Modifying Treatments on Coagulation Pathways

Disease-modifying treatments (DMTs) are potential modifiers of coagulation factor levels. However, few studies are available on this topic. MS patients treated with steroids showed lower plasminogen and fibrinogen levels (165). Additionally, increased fibrinolytic activity has been observed in treated MS patients. At the time, these abnormalities were considered to be a consequence of a non-specific activation of coagulation in a setting of chronic immunological disease (165). However, because of the aforementioned role of fibrinogen and potentially decreased fibrinolysis in MS, these data are of interest and deserve additional investigation.

Another study investigated patients that developed progressive multifocal leukoencephalopathy (PML) under natalizumab treatment (pre-PML) (166). PAI-2, uPA, uPAR, TFPI, and TM were among the top differentially expressed genes in peripheral blood mononuclear cells collected at baseline and during PML. These genes were significantly down-regulated at baseline in pre-PML patients compared to the group that did not develop PML, providing evidence for a potential role of natalizumab as a modifier of hemostasis component levels. Although levels of serum proteins encoded by the differentially expressed genes did not show significant differences (166), their evaluation in plasma would be potentially informative.

Because glucocorticoids induce procoagulant reactions, the effect of high-dose intravenous methylprednisolone on fibrinogen, FVIII activity, vWF protein concentration, TAT, prothrombin fragments1+2 (F1+2), tPA protein concentration, PAI-1 activity and plasmin-antiplasmin complexes (PAP) was investigated using a prophylactic low dose of low molecular weight heparin (167). Whereas, the fibrinogen levels significantly decreased, FVIII activity and vWF protein concentration significantly increased in the absence of evidence for fibrinolytic system activation or suppression (167). At high-dose methylprednisolone, 5 out of 188 MS patients developed venous thrombosis, which led the authors to speculate on the synergistic effect between the treatment and MS immunopathology (168), which could predispose patients to prothrombotic risk.

In RRMS patients under glatiramer acetate (GA) treatment, TM levels were significantly increased compared to the respective drug-free group and healthy controls, regardless of the presence of current relapse. The authors speculated about a GA-induced mechanism of neuroprotection potentially leading to the generation of aPC (144).

A recent study on this topic evaluated EDTA plasma levels of a number of coagulation inhibitors (TFPI, ADAMTS13, HCII, TM, and PAI-1) according to DMT status, particularly in patients being treated with interferon-beta (IFN-b) or GA, each used in 1/3 of the patients. The high overall variability of levels in patients, and particularly those of PAI-1, could have prevented the detection of significant differences in the circulating levels of these proteins, including TM (138).

Considering the heterogeneity of coagulation balance in MS patients and the few studies that evaluated the effect of DMTs on coagulation, prospective investigation would be of great help to develop a better understanding drug–hemostasis interactions.

TABLE 4 | CSF, plasma, and serum evidence of fibrinolytic pathway components in multiple sclerosis.

Hemostasis components	Main findings	References
PAI-1	Higher levels in MS vs. controls. PAI-1 concentration has a reverse relationship of tPA:c (ELISA in CSF and plasma EDTA of 19 MS, OND, controls).	(161)
	Higher levels in MS vs. controls (ELISA on plasma EDTA: 85 RRMS, 53 PMS, 42 controls).	(138)
PAI-1, tPA	High levels of PAI-1 during relapses. No differences for tPA. No correlation between PAI-1 CSF and plasma levels (Plasma of 12 active RRMS, 12 stable RRMS, 10 controls).	(162)
tPA	Higher activity in MS (CSF of 7 MS, 9 leukemia, 21 encephalitis, 20 controls)	(163)
PAI-1, tPA, D-dimer	No differences (Plasma of 90 MS, 250 glioma patients 270 controls).	(164)
D-dimer	Higher levels in MS (ELFA on plasma of 42 RRMS, 31 controls).	(140)

CSF, cerebrospinal fluid; :c, activity; EDTA, ethylenediamine-tetra-acetic acid; ELISA, enzyme-linked immunosorbent assay; ELFA, Enzyme Linked Fluorescent Assay; OND, other neurological disorders; PAI-1, plasminogen activator inhibitor 1; PMS, progressive multiple sclerosis; RRMS, relapsing-remitting multiple sclerosis; tPA, tissue-type plasminogen activator.

Case Reports of Autoimmunity Affecting Hemostasis in Multiple Sclerosis

Unfortunately, very few cohort studies addressed whether or not coagulation imbalance was supported by the immune activity. Higher frequency of antiphospholipid antibodies, belonging to the IgM family, were observed in MS patients during exacerbation (10 out of 17 patients, 2–4 fold increase) compared to remission. Of note, a significant correlation between contrast-enhancing lesions and antibodies against FVII was found (169).

Case reports of autoimmunity affecting hemostasis components in MS shold be considered in light of the acquired dysregulation of coagulation, with a focus on those components that are mainly targeted and that may contribute to clinical worsening. Interestingly, a few cases have been reported with TTP episodes (170) and acquired ADAMTS13 deficiency in the context of IFN-b treatment for MS (171, 172). Despite the limitation of their low number, these reports highlighted acquired deficiency induced by auto-antibodies against ADAMTS13. Notably, lower levels of ADAMTS13 have been reported in MS compared to control subjects (138, 157). Additionally, several MS patients who received alemtuzumab treatment developed autoimmune TTP (173, 174).

Analogously, but on the other side of the spectrum, anticoagulant FVIII inhibitors may arise in autoimmune diseases, during and after pregnancy, and during drug therapy including IFN-alpha (used to treat leukemia and blood disorders such as TTP) with the outcome of acquired severe hemophilia. The first case report of an MS patient who developed hemorrhagic disorder was described as a rare case of antibody development against FIX and FVIII (175). A second case of acquired FVIII inhibitor was later described (176), and an additional case was reported in an MS patient after IFN-b treatment (177). Acquired hemophilia has also been described as an extremely rare complication in patients treated with alemtuzumab. Other case reports in the literature include two sisters with MS who had a quantitative deficit of factor VIII-vWF complex (178). These phenomena could be mediated by secondary B cell-mediated autoimmune complications leading to inhibitory autoantibodies to coagulation FVIII (179). However, a reference study for thrombophilia reported that among the 4,311 patients with a first episode of venous thrombosis, 30 had MS with increased FVIII activity levels (180).

Imbalance of the coagulation system seems to be supported by inflammatory and immune activity (181). Taken together, these reports and the role of (auto)immunity in MS, clearly support the need for additional investigation of specific acquired autoimmunity affecting hemostasis factors, either with pro-coagulant or anti-coagulant outcomes.

CONCLUSIONS AND FUTURE DIRECTIONS

The data reviewed indicate that fibrinogen leakage due to BBB damage in MS is consistent with microglial activation, particularly once thrombin cleaves it to fibrin. Additional fibrin(ogen) deposition might occur early in MS and precede demyelination, as well as contribute to cortical pathology in the progressive stages. Although histological studies point to fibrin(ogen) as a principal contributor to neuroinflammation and neurodegeneration in MS, these processes are also supported, and may be potentiated, by decreased fibrinolysis. In particular, increased PAI-1 synthesis and decreased tPA activity in MS lesions reflect an impaired clearance of fibrin due to the formation of tPA/PAI-1 complex, further contributing to the inflammatory stage of demyelination.

Other mechanisms also contribute to this intricate pathophysiological picture. The combined presence of TF and PCI in MS lesions suggests pro-inflammatory thrombin formation and suppression of the anti-inflammatory aPC pathway. Moreover, deposition of FXII might support autoimmunity through increased expression of uPAR, which has been reported in MS lesions. At the peripheral level in MS, coagulation activity variation still remains to be elucidated, and underlying factors uncovered. As potential contributors to the heterogeneity, protein plasma levels of hemostasis factors have been sporadically investigated in MS, and some results suggest that they could serve as potential biomarkers of ongoing alterations in the CNS. Indeed, the lower ADAMTS13 levels detected in MS patients (and in particular in MS patients with cerebral microbleeds) should stimulate further investigation based on potentially enhanced vWF activity in plasma, which has been suggested as a possible marker for evaluating the endothelial damage, leading to BBB breakdown.

Additional experimentation by use of high throughput transcriptomic and proteomic techniques is needed to determine how hemostasis components contribute to or decrease inflammatory and immune responses in MS patients, and how these genes/proteins can be modulated by current DMTs in MS.

Newly acquired molecular details of how hemostasis components trigger neuroinflammation and neurodegeneration could in turn favor the development of novel therapeutic approaches to ameliorate the disease evolution, favored by the wealth of powerful inhibitors or potentiators of hemostasis used for treatment and prophylaxis in prothrombotic and hemorrhagic disorders.

AUTHOR CONTRIBUTIONS

NZ and RZ contributed to the study concept and design, critical revision of the manuscript for important intellectual content, and study supervision. DJ and FB contributed to the study concept and design, analysis and interpretation, critical revision of the manuscript for important intellectual content, and study supervision.

FUNDING

This study was funded in part by The Annette Funicello Research Fund for Neurological Diseases and internal resources of the Buffalo Neuroimaging Analysis Center. In addition, we received support from the Jacquemin Family Foundation. Research reported in this publication was also funded in part by the National Center for Advancing Translational Sciences of the National Institutes of Health under award Number UL1TR001412. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH. This study was also partially supported by the grant 1786/2012 from the strategic 2010–2012 Research Program of Emilia Romagna Region, Italy.

ACKNOWLEDGMENTS

Authors thank the TEAM Engineering S.p.A. for support of the fellowship of NZ in the Buffalo Neuroimaging Analysis Center, Department of Neurology, University at Buffalo. We also thank Niels Bergsland and Michael G. Dwyer for proof-editing the manuscript.

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Conflict of Interest Statement: RZ has received speaker honoraria and consultant fees from Genzyme-Sanofi, Novartis, Claret Medical, Celgene, and EMD Serono. He has received research support from EMD Serono, Genzyme-Sanofi, Claret Medical, Protembis, QuintilesIMS, and Novartis.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neurology

MINI REVIEW
published: 19 March 2019
doi: 10.3389/fneur.2019.00239

The Relationships Between Vitamin K and Cognition: A Review of Current Evidence

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Vitamin K is a fat-soluble nutrient discovered in 1935 and its role in blood coagulation has been thoroughly explored. In recent years, studies conducted *in vitro* and on animals highlighted vitamin K involvement in brain cells development and survival. In particular, vitamin K seems to have an antiapoptotic and anti-inflammatory effect mediated by the activation of Growth Arrest Specific Gene 6 and Protein S. Moreover, this vitamin is involved in sphingolipids metabolism, a class of lipids that participate in the proliferation, differentiation, and survival of brain cells. An altered expression in sphingolipids profile has been related to neuroinflammation and neurodegeneration. This review stems from a growing interest in the role of vitamin K in brain functions, especially in cognition, also in view of an expected increase of prevalence of Alzheimer's disease and other forms of dementia. It collects recent researches that show interesting, even though not definitive, evidence of a direct correlation between vitamin K levels and cognitive performance. Moreover, vitamin K antagonists, used worldwide as oral anticoagulants, according to recent studies may have a negative influence on cognitive domains such as visual memory, verbal fluency and brain volume. The aim of this review is to analyze the evidence of clinical studies carried out up to date on the relationship between vitamin K intake and cognitive performances. The involvement of vitamin K antagonists (VKAs) in declining cognitive performances is also addressed separately.

Keywords: vitamin K, phylloquinone, cognitive impairment, vitamin K antagonists, warfarin

OPEN ACCESS

Edited by:
Matilde Inglese, Icahn School of Medicine at Mount Sinai, United States

Reviewed by:
Marta Maschio, Istituto Nazionale del Cancro Regina Elena, Italy
Maria Maddalena Filippi, Fatebenefratelli Foundation for Health Research and Education, Italy

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Specialty section:
This article was submitted to Multiple Sclerosis and Neuroimmunology, a section of the journal Frontiers in Neurology

Received: *30 July 2018*
Accepted: *22 February 2019*
Published: *19 March 2019*

Citation:
Alisi L, Cao R, De Angelis C, Cafolla A, Caramia F, Cartocci G, Librando A and Fiorelli M (2019) The Relationships Between Vitamin K and Cognition: A Review of Current Evidence. Front. Neurol. 10:239. doi: 10.3389/fneur.2019.00239

INTRODUCTION

Vitamin K is a fat-soluble nutrient mainly found in green leafy vegetables as phylloquinone (Vitamin K1). This vitamin is widely known for its procoagulant effect. It acts as a cofactor for the enzyme that allows the activation of vitamin K-dependent factors (II, VII, IX, X, protein C, and protein S). A recent review collected studies that show its involvement in the metabolism of the central nervous system (CNS), suggesting the possibility that a vitamin K deficiency might be related to the onset of cognitive impairment (1).

These recently discovered functions, revealed that this vitamer participate in the enzymatic activation of growth-arrest specific 6 protein (Gas-6) and protein S. The first has an anti-apoptotic, mitogenic, and myelinating activity, the latter offers neuronal protection during ischemic/hypoxic injury both *in vivo* and *in vitro* (2–4). Furthermore, vitamin K is known to be an inductor

of sphingolipids synthesis. These polar lipids are an essential part of CNS cell membrane and are linked to neuronal proliferation and differentiation (1). Several studies are also investigating the correlation between cognitive impairment and the use of vitamin K antagonists (VKAs, i.e., warfarin, acenocoumarol, and fluindion) as oral anticoagulants. In fact, these Coumarin derivatives prevent the recycle of vitamin K after the ɣ-carboxylation (5).

Due to the progressive aging of global population, data indicate that cases of dementia will double between 2020 and 2040, rising up to 81 million and consequently burdening society and national health systems. For instance, Alzheimer's disease (AD) cases in the US will rise from 5.4 million up to 13.8 million by mid-century (6, 7). Hence the importance of identifying modifiable factors that could impact the course of neurodegenerative pathologies. The aim of this review is to analyze the evidence of clinical studies carried out up to date examining the hypothesis of a cognitive decline among adults with low serum levels or dietary intake of vitamin K. Moreover, studies evaluating the potential link between VKAs and cognitive functions were also included.

DEMENTIA AND COGNITIVE IMPAIRMENT

Dementia can be defined as a clinical syndrome of mental capacity characterized by a substantial global decline in cognitive function that is not attributable to altered consciousness; it consists of a combination of symptoms attributable to various causes or pathological events (8). Cognitive impairment is a definition used in this review to indicate alterations in multiple cognitive domains highlightable with standardized tests, as clinically manifest dementia is often preceded by a heterogeneous spectrum of cognitive performances (9).

It is difficult to find univocal data about the prevalence of cognitive impairment and other forms of dementia (10); some studies show that the global prevalence of dementia varies among different countries, this could be related to a large number of variables including education, mean age, socioeconomic level, lack of a comparable methodology (11). However, it can certainly be affirmed that the two most common type of dementia in Western countries are Alzheimer's disease (up to 60% of cases) and vascular dementia (up to 20% of cases). These two forms of dementia are easily mistaken one for another due to their similarities in symptomatology, pathophysiology, and risk factors (12).

The mechanism underlying Alzheimer's disease is the deposition of β-amyloid peptide (Aβ) and the neurofibrillary tangles of the microtubule binding protein tau. In particular, Aβ peptides are responsible for the massive neuronal death that defines the disease (13).

A few studies concluded that Vitamin K seems to prevent Aβ-induced apoptosis through the activation of Gas-6, showing a pro-survival effect on brain cells (14).

Regarding vascular dementia, the main causes are represented by several vascular pathologies that result in cerebral ischemia. Studies published in the last years have attributed to Protein S (activated by vitamin K) a role in improving post-ischemic cerebral blood flow (15) and potentially leading to a more favorable cognitive outcome.

VITAMIN K STRUCTURE AND FUNCTION

Vitamin K can be found as phylloquinone (the main dietary source of vitamin K) and it's also identified as menaquinones (vitamin K2) which include several vitamers of bacterial origin (2). Menaquinone-4 (MK-4) is the most represented vitamer in both human and rats' brains (16, 17).

MK-4 seems to protect against oxidative damage and inflammatory cascade activation in *in vitro* studies (18, 19). In addition, in murine models MK-4 depletion has been found correlated with worse cognitive performances (20).

Vitamin K is widely known for its role in blood coagulation as the cofactor of ɣ-glutamyl carboxylase that allows the activation of vitamin K-dependent factors such as factor II, VII, IX, X, protein C, and protein S. Vitamin K is also involved in the ɣ-carboxylation of two vitamin K-dependent proteins whose activity contributes to an adequate cerebral homeostasis, namely Gas-6 and protein S (3, 4). Moreover, vitamin K participates as a cofactor in the synthesis of sphingolipids, an important constituent of brain cells membrane (21). Several studies conducted on *in vitro* and murine models have highlighted the role of these constituents in brain metabolism. In some cases, a correlation with neurodegenerative diseases emerged that could be further examined trough human studies.

Gas-6

Gas-6 has a central role in the development and survival of nervous system. In addition, it shows an anti-apoptotic, mitogenic, and myelinating activity in neuronal and glial cells (1).

Gas-6 binds and activates the receptor tyrosine kinases of the Tyro3, Axl, and Mer (TAM) family. Axl is involved in the proliferation of numerous cell types and in the survival of gonadotropin-releasing hormone (GnRH) neurons allowing their migration from the olfactory bulb to the hypothalamus (22, 23).

Mer protects primary macrophages from oxidative stress induced-apoptosis (24).

The specific role of Tyro3 in cell survival is yet to be defined, but activities similar to Axl have been observed concerning the migration of GnRH neurons A (25–27).

An *in vitro* study revealed that recombinant Gas-6 protects hippocampal rats' neurons from apoptosis, underlining the pro-survival effect of this protein through the activation of TAM proteins (28).

Through the activation of Axl and phosphatidylinositol 3-kinase (PI3K) pathways, Gas-6 modulates oligodendrocyte survival and microglial phenotype both *in vitro* and *in vivo* (3) preventing tumor necrosis factor alpha-induced apoptosis. A study on cuprizone-induced demyelination model (adopted as a model of multiple sclerosis) revealed that the deletion of Gas-6/Axl signaling leads to prolonged neuroinflammation with axonal damage and consistent demyelination (29, 30). This immune-regulatory role links Gas-6 to autoimmune disorders,

more specifically to the pathogenesis of multiple sclerosis (MS) (31, 32).

Studies developed using murine culture of microglial cells showed that Gas-6 downregulates the expression of Interleukin-1b e induced nitric oxide synthase, thereby reducing the proinflammatory response (33). Two recent murine models using knock-out mice for Mer and Axl, demonstrated a reduced recruitment of microglial cells to neuronal sites of injury, also affecting the phagocytic activity through cytoskeleton changes (34, 35).

Furthermore, Gas-6 has shown a decrease of ß-amyloid-induced apoptosis, a hallmark of Alzheimer's disease, through the inhibition of low-voltage Ca^{2+} influx channels (14). However, a more recent study found that Gas-6 inhibits Tyro3 whose effects prevent β-amyloid deposition (36).

Protein S

Historically protein S has been known for its anticoagulant effect, but recent studies are exploring further effects, such as a possible role in inflammation, angiogenesis, and cancer (37). As well as in ameliorating post-ischemic cerebral blood flow (15).

Protein S shares with Gas-6 almost half of its amino acid structure (44%) (4), and consequently it performs part of its actions as a TAM receptor ligand. Zhu et al. showed a direct correlation between the inhibition of Tyro3/Akt signaling pathway and the hypoxic-induced apoptosis of hippocampal neurons, underlining a potential protective effect of protein S in cerebral infarct (38). In particular, protein S has a protective effect against NMDA-induced toxicity and apoptosis via the Tyro3/Akt pathway (39). This finding may suggest a possible role of protein S as an adjunct of tissue Plasminogen Activator to reduce cerebral post-ischemic toxicity (40), without increasing the risk of bleeding when administered alone in high concentration to stroke rodent models (15). An additional property of protein S seems to be the preservation of the integrity of the blood-brain barrier (BBB) as it operates as a safeguard against chronic ischemic damage and BBB-related disorders (41).

Sphingolipid Metabolism

Sphingolipids is one of the major classes of eukaryotic lipids and an essential component of cell membranes and their synthesis is known to be induced by vitamin K (21).

The sphingolipids most frequently observed in neuronal cell membranes are ceramides, sphingomyelin, cerebrosides, sulfatides, and gangliosides (42). This class of lipids seems to be a vital modulator of cell proliferation, differentiation, and survival (43). A growing amount of evidence are associating sphingolipids metabolism to the pathophysiology of CNS diseases. These polar lipids have been related to a neuroinflammatory and neurodegenerative states due to microglial activation and accumulation of amyloid precursor protein (APP) (44).

These are at the basis of the development of several pathologies like AD (43, 45), where inflammation is a consequence of microglial activation triggered by β-amyloid plaques (46).

Lastly, sphingolipids guide the process of myelination in the CNS and are themselves major components of oligodendrocyte membrane. In the serum and cerebrospinal fluid of patients affected by multiple sclerosis antibodies have been detected against myelinic sphingolipids (47, 48) along with ceramide accumulation in active lesions (49).

The Assessment of Vitamin K Status

Both biomarkers and questionnaires have been suggested to evaluate vitamin K status. Circulating phylloquinone can be measured with high performance liquid chromatography (HPLC) a method that responds to changes in dietary phylloquinone intake. Serum phylloquinone should be measured in fasting samples to better reflect overall nutritional status. Moreover, vitamin K serum levels are influenced by serum triglycerides and should be corrected accordingly. There is currently no established threshold of circulating phylloquinone that indicates insufficiency or deficiency (50).

Other circulating markers are: PIVKA-II (protein induced by vitamin K absence-II) that has shown low sensitivity to dietary variation of vitamin K; undercarboxylated fraction of osteocalcin (unOc) and desphospho-uncarboxylated matrix Gla-protein (dp-ucMGP) that are both more sensitive than PIVKA-II but still conditioned by factors such as age and the total amount of matrix Gla-proteins available (50).

Vitamin K intake can be assessed with questionnaires such as the food frequency questionnaire (FFQ) or dietary records. While being efficient in terms of costs and time and easy to implement, questionnaires rely on the recall ability and perceptions of individuals' diet and therefore may be subjected to bias (51).

Considering the lack of a single gold standard measure, and the limitations affecting the available methods, vitamin K status may be best assessed with a combination of both questionnaires and biomarkers (50).

VKAS-BRAIN RELATIONSHIP

VKAs exercise their function by blocking the activity of vitamin K oxidoreductase (VKOR) preventing the recycle of vitamin K after the ɣ-carboxylation (5). The possibility of an adverse impact of VKAs on the brain has been evident since the finding of abnormalities of the CNS in newborns exposed to warfarin or other coumarin derivatives (52). However, detailed mechanisms are yet to be comprehended. Studies on whether Gas-6 and protein S ɣ-carboxylation are impaired by VKAs in the human brain have not yet been conducted. Studies performed on murine models have shown how VKAs determine a decrease in MK-4 brain concentrations, the most represented vitamer in rats' brain (16). Nagakawa et al. identificated the enzyme responsible for the conversion of phylloquinone in MK-4 (UBIAD1) (17) and Tamadon-Nejad et al. demonstrated that despite an excess of phylloquinone in rats' brain, MK-4 brain concentrations remained low in warfarin treated rats suggesting an alteration of the MK-4 biosynthetic pathway in the presence of warfarin (53). Moreover, rats treated with VKAs showed worse performances in tests to evaluate their cognitive and behavioral functions (53, 54).

It is well known how anticoagulant use can decrease the risk of dementia by reducing the number of cerebral ischemic events in AF patients (55).

Mostaza et al. observed that in a population taking vitamin K antagonists, there was a trend toward higher warfarin prescription among patients with cognitive impairment, regardless dependency or frailty. Thus, a thorough evaluation on the association between non-vitamin K antagonists oral anticoagulants (NOACs) use and cognitive decline is crucial (56).

A recent meta-analysis found a borderline significant association between the use of NOACs and the lower risk of cognitive impairment when compared with VKAs and acetylsalicylic acid (57).

Similar findings were observed in other studies where NOAC were considered an optimal or even better alternative to warfarin, due to their lower bleeding risk and variability in anticoagulation effect (58). A previous study validated the same hypothesis: NOACs provide a better protection against atrial fibrillation-related stroke in terms of lower risk of cerebral ischemic events and new-onset dementia than those treated with warfarin (59).

VITAMIN K AND COGNITIVE DECLINE

Considering the numerous roles of Vitamin K highlighted in the previous studies, in recent years some authors have started to investigate the potential link between cognitive impairment and vitamin K.

Whether vitamin K deficiency is associated to cognitive decline is still a matter of debate today. From the literature search, we were able to include 7 human studies and all, except one (60), confirmed an association between vitamin K and cognitive function among older adults (**Table 1**).

Six studies demonstrated, in a population of 65 years and older, a direct correlation between low vitamin K dietary intake or serum concentration and deteriorated cognitive and behavioral performances. In particular, Presse et al. in 2013 published the results of a cross sectional study conducted on 320 elderly, aged 70–85, free of cognitive impairment from the NuAge study cohort (63). Phylloquinone serum concentration analysis was measured using High performance liquid chromatography (HPLC), a method already validated as an indicator of dietary phylloquinone intake over a long period of time. Circulating phylloquinone concentrations are conditioned by the blood lipid profile that needs to be assessed as well while using HPLC (67). Cognitive assessment was performed using specific tests for each cognitive domain (verbal and non-verbal episodic memory, executive functions, and speed of processing). Results showed that recruited subjects with higher serum phylloquinone performed better in verbal episodic memory, while no correlation was found with non-verbal episodic memory, executive functions, and speed of processing, underlining the role of vitamin K in memory consolidation (63). These results are supported by a previous murine model where rodents underwent a 5-days learning test in the Morris Water Maze and those fed with lower vitamin K levels required longer time to perform the task compared to those adequately fed (20).

Two studies evaluated vitamin K intake using a semi quantitative food frequency questionnaire (FFQ). They observed a less severe subjective memory complaint (65) as well as a better cognition and less behavioral disturbances (64) among geriatric patients with higher vitamin K intake. The FFQ is a 50 items questionnaire that aims to evaluate vitamin K dietary intake. Although it has been validated in elderly people (68) it may result in an underestimation when investigating patients with cognitive decline. Therefore, the FFQ may not be as representative of phylloquinone intake as its serum levels measured with HPLC in this specific circumstance.

In line with previous studies, Kiely et al. described that elderly with poor cognitive functions, evaluated with MMSE, had the lowest dietary vitamin K intake (assessed with FFQ). Similar results were observed correlating MMSE scores and phylloquinone serum levels measured with HPLC (66).

Further publications highlighted the potential role of this vitamer among patients with Alzheimer's disease-related dementia (61, 62), who were found with significant lower levels of vitamin K even after data were adjusted for energy intakes. In particular, Presse et al. reported in 2008 how vitamin K intake, evaluated with 3–5 days diet records, were notably lower in patients with early stages of AD. Partially limiting the strength of this study are the diet records used, as they show limited value for the assessment of vitamin K intake (62).

Opposite results were obtained by Van Den Heuvel et al. (60) in a middle-age sample (55–65 years) of 599 individuals, measuring vitamin K in an indirect way through levels of desphospho-uncarboxylated matrix Gla-protein (dp-ucMGP). Over the 6 years follow up, no significant association between vitamin K and cognitive decline was found. The recruitment criterion of a different age group may be the reason why contrasting results were observed, since the brain might be susceptible to nutrient deficiency in different ways at different times in life (20). Moreover, as stated by the authors themselves, dp-ucMGP may not be the most suitable marker of vitamin K levels in the brain (60).

An emerging issue is how the use of VKAs could influence brain metabolism and this topic is analyzed separately. The few papers published until now point out, to a limited extent, a potential correlation between the use of VKAs and both cognitive decline and brain focal atrophies (**Table 2**).

Ferland et al. observed in a large cohort study a significant decrease in visual memory and verbal fluency among patients treated with VKAs when compared to individuals under no blood-thinning treatment, but no association was observed between the global cognitive function and VKAs use over at 10 years follow up (70).

Brangier et al. analyzed the brain volume, using 3 or 1.5 Tesla MRI, of 54 subjects (18 under VKAs treatment and 36 matched controls) and found a significant inverse correlation between the duration of drug exposure and gray/white matter volume in the right frontal inferior operculum, right precuneus, and left middle frontal gyrus (71). The same author found an important decline in executive functions (assessed with frontal assessment battery) among geriatric patients treated with VKAs over at 24 months follow up. It's worth noticing how, in the same study, decline in Mini mental state examination (MMSE) scores, used as an assessment of cognitive performance, was not found significantly associated with VKAs use over the same period of time (72).

TABLE 1 | Studies on vitamin K concentration and cognitive performances.

References	Participants	Design	Vitamin K determination	Cognitive evaluation	Main results	Strenghts	Limitations
Sato et al. (61)	100 demented women (mean age 81), 100 cognitively normal female controls (mean age 81)	Cross Sectional	Standardized HPLC[a] procedures	MMSE[b]	Patients with severe dementia found to have a lower Vitamin K serum concentration	Reliable methodology for serum levels of vitamin K	No follow-up, relatively small sample size
Presse et al. (62)	31 AD[c] patients and 31 healthy controls (mean age 78)	Cross Sectional	Vitamin K intake measured using 3- or 4-days diet records	MMSE[b]	9 patients with AD[c] found to have a lower intake of vitamin K	Accuracy of food records ensured with a dietitian and the help of caregivers	Dietary intake data of limited value for assessing vitamin K status, small sample size, potential over selection of patients
Presse et al. (63)	320 elderly (mean age 76) from Québec Longitudinal NuAge Study	Cross Sectional	Standardized HPLC[a] procedures	MMMSE[d], 6 tests covering 4 cognitive domains	Positive association between higher vitamin K intake and verbal episodic memory	Comprehensive cognitive battery assessing 4 cognitive domains, large sample size, reliable methodology for serum levels of vitamin K	No follow-up, potential confounders (such as ApoE genotype) potential over selection of patients
Van der Heuvel et al. (60)	599 participants (mean age 60) from the Longitudinal Aging Study Amsterdam	Prospective (follow up: 6 years)	dp-ucMGP[e]	Alphabet coding task, Auditory verbal learning test, Raven's colored progressive matrices	No association found between dp-ucMPG[e] and cognitive functions	Inclusion of a range of cognitive tests sensitive to aging measured over 6 years follow-up	Indirect measure of vitamin K status (dp-ucMGP[e]), only a single measurement of Vitamin K status was available
Chouet et al. (64)	192 patients (mean age 83) recruited from the Cognition and LIPophilic vitamins study	Cross Sectional	FFQ[f]	MMSE[b], FBRS[g]	Positive association between vitamin K intake and MMSE[b] and FBRS[g] scores	Assessment of the dietary vitamin K intake over 12 months, assessment of both cognitive and behavioral outcomes	FFQ[g] validated on Canadian population (tends to overestimate intakes), poor estimation of Vitamin K intake in patients with cognitive disorders
Soutif-Veillon et al. (65)	160 patients (mean age 82) recruited from the Cognition and LIPophilic vitamins study	Cross Sectional	FFQ[f]	MAC-Q[h]	Positive association between vitamin K intake and memory complaint questionnaire score	Assessment of the dietary phylloquinone intake over 12 months	Use of MAC-Q[h] tool may have overestimated the prevalence of subjective memory complaint, poor estimation of vitamin K dietary intake in patients with cognitive disorders
Kiely et al. (66)	156 elderly (mean age 78) from ELDERMET cohort	Cross Sectional	FFQ[f] and HPLC[a]	MMSE[b]	Serum and dietary phylloquinone were significant and independent predictors of good cognitive function	Simultaneous measurement of both dietary and serum phylloquinone	Self-selected, motivated cohort, with potentially better diet, education, and lifestyle than general population

[a]*High Performance Liquid Cromatography.* [b]*Mini-Mental State Examination.* [c]*Alzheimer's Disease.* [d]*Modified Mini-Mental State Examination.* [e]*Desphospho-uncarboxylated matrix Gla protein.* [f]*Food Frequency Questionnaire.* [g]*Frontotemporal Behavioral Rating Scale.* [h]*Memory Complaint Questionnaire.*

TABLE 2 | Studies on VKAs and cognitive performances.

References	Participants	Design	Cognitive evaluation	Main results	Strenghts	Limitations
Annweiler et al. (69)	267 patients (mean age 83) hospitalized or seen in consultation from WARHOL[a] study	Cross Sectional	MMSE[b]	Fluindione is positively associated to lower MMSE[b] score	Standardized collection of data, detailed description of the participants' characteristics	Restricted study cohort, cognitive impairment assessed using only MMSE[b] score, no serum vitamin K concentration evaluated, association between cognitive impairment and the use of VKAs[c] accounted neither for the length of treatment nor for INR[d] history
Ferland et al. (70)	7,133 nondemented community-dwellers (mean age 73)	Prospective (follow up: 10 years)	IST[e], BVRT[f], MMSE[b]	VKAs[c] usage is positively associated to lower IST[e] and BVRT[f] scores, no association was found between vitamin K and MMSE[b]	Large population cohort, follow-up of 10 years	No dietary Vitamin K intake or serum levels evaluated, no detailed information of VKAs[c] treatment (doses, length), limited number of cognitive tests available at each follow-up
Brangier et al. (71)	18 VKAs[c] and 36 community-dwellers from GAIT[g] cohort (mean age 76)	Cross Sectional	None (MRI scans for brain volumery)	The duration of exposure to VKAs[c] correlated with focal brain atrophy	Use of VBM[h] approach to locate focal atrophies, standardized collection of data from a single research center	Geriatric study cohort, use of two different MRIs (1,5T and 3T), no information provided by VBM[h] analysis on the potential mechanisms causing atrophies, segmentation, and normalization defect in VBM[h] analysis
Brangier et al. (72)	378 geriatric outpatients (46 VKAs[c] users) from MERE[i] study (mean age 82)	Prospective (follow up: 24 months)	MMSE[b], FAB[j]	Use of VKAs[c] was associated with lower FAB[j] scores at baseline, and with more significant worsening after 24 months. No significant association between MMSE[b] scores and VKAs[c] use.	Longitudinal prospective design with an intermediate mid-term evaluation and a final long-term evaluation, standardized collection of data from a single research center	Geriatric study cohort, incomplete follow-up at 12 and 24 months, no detailed information of VKAs[c] treatment (length, history of INR[d]), no dietary vitamin K intake or serum levels evaluated

[a]Who is At Risk of Hypovitaminosis in Older study. [b]Mini-Mental State Examination. [c]Vitamin K Antagonists. [d]International Normalized Ratio. [e]Isaac Set Test. [f]Benton Visual Retention Test. [g]Gait and Alzheimer Interactions Tracking. [h]Voxel Based Morphometry. [i]Alzheimer's Disease and Related Disorders' study. [j]Frontal Assessment Battery.

Lastly, Annweiler et al. in 2015 reported a 15% higher risk of cognitive impairment in patients treated with fluindione which is used as an anticoagulant drug. The positive association (not observed in patients treated with warfarin or acenocoumarol) remained statistically significant even after adjustment for covariables (69).

Clinical studies have suggested that VKAs use does not affect vitamin K plasma concentration (73) and involved molecular patterns that might lead to cognitive impairment in humans using VKAs are yet to be comprehended.

When implying an involvement of VKAs in cognitive impairment it must be considered that patients are under treatment for pathologies like atrial fibrillation, that can possibly influence, to some level, mental deterioration (74).

CONCLUSIONS

The present review stems from a growing interest in the role of vitamin K in brain function, especially in cognition. It collected recent contributions to the topic, showing interesting, even though not definitive, evidence of direct correlation between vitamin K levels and cognitive performance. Moreover, VKAs might influence negatively some cognitive domains such as visual memory and verbal fluency and the brain volume. Only a small number of publications were based on studies performed on humans, limiting the amount of papers included. These studies were heterogeneous in several ways: study design, markers used to measure vitamin K levels, method used to assess cognitive performance and age of patients included in the studies. Further evidence should be gathered using more standardized methodology to foster comparability of results.

The paucity of published papers suggests the need of a more thorough investigation from the scientific community, using randomized trials with large samples to confirm the hypothesis that low vitamin K can be associated to cognitive decline. A standardized methodology for both cognitive evaluation and vitamin K dietary intake and serum concentrations must be adopted in order to develop more comparable and reliable data.

Due to the large number of individuals treated with VKAs, a large prospective study is possible and could be crucial to elucidate the influence of these drugs on vitamin K serum levels and consequently on cognitive decline.

In conclusion, considering the growing social and economic burden linked to the increasing number of patients suffering from cognitive impairment and dementia, further researches on this topic can prove to be beneficial and applicable results can be expected.

AUTHOR CONTRIBUTIONS

MF and LA conceived the original idea. LA, CD, and RC organized the database and wrote the various sections of this review. AC, MF, FC, AL, and GC supervised and made sure that this work was accurate and formally correct. All authors reviewed the manuscript before the submission.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neurology

MINI REVIEW
published: 14 February 2019
doi: 10.3389/fneur.2019.00095

Genome-Wide Multiple Sclerosis Association Data and Coagulation

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The emerging concept of a crosstalk between hemostasis, inflammation, and immune system prompt recent works on coagulation cascade in multiple sclerosis (MS). Studies on MS pathology identified several coagulation factors since the beginning of the disease pathophysiology: fibrin deposition with breakdown of blood brain barrier, and coagulation factors within active plaques may exert pathogenic role, especially through the innate immune system. Studies on circulating coagulation factors showed complex imbalance involving several components of hemostasis cascade (thrombin, factor X, factor XII). To analyze the role of the coagulation process in connection with other pathogenic pathways, we implemented a systematic matching of genome-wide association studies (GWAS) data with an informative and unbiased network of coagulation pathways. Using MetaCore (version 6.35 build 69300, 2018) we analyzed the connectivity (i.e., direct and indirect interactions among two networks) between the network of the coagulation process and the network resulting from feeding into MetaCore the MS GWAS data. The two networks presented a remarkable over-connectivity: 958 connections vs. 561 expected by chance; z-score = 17.39; p-value < 0.00001. Moreover, genes coding for cluster of differentiation 40 (CD40) and plasminogen activator, urokinase (PLAU) shared both networks, pointed to an integral interplay between coagulation cascade and main pathogenic immune effectors. In fact, CD40 pathways is especially operative in B cells, that are currently a major therapeutic target in MS field. The potential interaction of PLAU with a signal of paramount importance for B cell pathogenicity, such as CD40, suggest new lines of research and pave the way to implement new therapeutic targets.

Keywords: multiple sclerosis, genome-wide association studies, cluster of differentiation 40, plasminogen activator, urokinase gene, connectivity analysis

OPEN ACCESS

Edited by:
Matilde Inglese, Icahn School of Medicine at Mount Sinai, United States

Reviewed by:
Kerstin Göbel, Universität Münster, Germany
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Specialty section:
This article was submitted to Multiple Sclerosis and Neuroimmunology, a section of the journal Frontiers in Neurology

Received: *30 September 2018*
Accepted: *24 January 2019*
Published: *14 February 2019*

Citation:
La Starza S, Ferraldeschi M, Buscarinu MC, Romano S, Fornasiero A, Mechelli R, Umeton R, Ristori G and Salvetti M (2019) Genome-Wide Multiple Sclerosis Association Data and Coagulation. Front. Neurol. 10:95. doi: 10.3389/fneur.2019.00095

INTRODUCTION

Recent studies focused on the role of coagulation cascade in neuroinflammation and neurodegenerative disease, considering new suggestions on a crosstalk between hemostasis, inflammation and immune system (1). The majority of these studies regarded multiple sclerosis (MS), but others demonstrated a dysregulation of several proteins of the coagulation cascade in many other central nervous system (CNS) diseases: traumatic brain and spinal cord injury, Parkinson disease, amyotrophic lateral sclerosis, Huntington disease and Alzheimer dementia (2–6).

A recent review discussed the role of fibrinogen in some neurological diseases, with an emphasis on the cellular targets and the fibrinogen-induced signal transduction pathways in the CNS: fibrinogen has a pleiotropic role in the activation of inflammation and pathologies that share, as common change, the increased blood-brain barrier (BBB) permeability. This produces the extravasation of plasma proteins that are undetectable in a healthy CNS, but abundantly deposited in many neurological conditions, whereby they mediate both pathological inflammation and tissue repair (7).

In MS the BBB breakdown and activation of the innate immune system appears to be an early event in the diseases development, that may precede the clinical onset. Different studies showed that fibrin deposition is a leading feature of MS pathology and it is presents all over the disease course (7). Fibrinogen can directly activate microglia cells *in vitro* and increase their phagocytic ability by binding to the integrin receptor CD11b/CD18, which is specifically expressed in the CNS (8). Participation of the coagulation cascade to the neuropathology of MS was strongly suggested by a proteomic analysis on laser-micro dissected, post-mortem brain lesions. Comparative proteomic profiles identified tissue factor and protein C inhibitor within chronic active plaque samples. *In vivo* experiments with antagonists of the coagulation factors identified (hirudin or recombinant activated protein C) were capable of ameliorating animal models of MS and suppressing pathogenic immune effectors, confirming the impact of dysregulated coagulation factors on demyelinating processes and suggesting potential therapeutic targets (9).

Another approach focused on the study of circulating coagulation factors, as possible biomarkers and targets of treatment tactics in MS pathogenic process. Gobel et al. (10) studied different neurological diseases (all the forms of MS, neuro myelitis optica spectrum disorders, other inflammatory neurological diseases, and non-inflammatory neurological conditions) compared to healthy status. The plasma levels of different coagulation proteins measured and the results demonstrated significantly higher levels of prothrombin and factor X in MS patients, without significant changes in the other conditions. Thrombin produces different inflammatory responses, including platelet activation, vasodilatation, leukocyte attraction, production of cytokine, and chemokine (IL-1, IL-6, TNFα) (11). These effects in CNS are also dependent on thrombin concentration: at low-to-moderate concentrations, it protects hippocampal neurons and astrocytes from insults, while at higher concentrations thrombin induces cell death (12, 13). Another coagulation factor that proved to be somehow involved in MS pathogenic process was factor XII (FXII). Increased FXII levels and reduced function within the intrinsic coagulation pathway were evident in people with MS (14); Gobel et al. found high levels of FXII activity in the plasma of MS patients during relapse, and immune activating effects mediated by interactions between FXII and dendritic cells in a CD87-dependent manner (15).

The above studies [with the prominent exception of the proteomic analysis by Han et al. (9)] were planned with a hypothesis-driven approach focusing on single factors of coagulation cascade. The coming of genome-wide association studies (GWAS) data would allow unbiased approaches capable of disclosing a more extensive landscape of coagulation process involvement in MS pathogenesis. GWAS results are derived from population-based association studies, comparing disease cases and controls for common genetic variants, that have variable frequencies in the general population. Each common variants (signaled by a single nucleotide polymorphism) explain a small fraction of the risk/protection in a population. The overall MS genetic risk is multifaceted: many common variants of small effect spread throughout the genome, loci of stronger effects lying in the human leukocyte antigen (HLA) haplotype, that had been associated to disease risk since eighties, as well as recently described low-frequency and rare-coding variants all contribute to the complex genetic architecture of MS (16).

GWAS STUDIES AND COAGULATION

GWAS studies encompassing the last decade have identified more than 200 MS-associated loci across the human genome (17). Technological advances, adequate increase of sample size, and improved statistical approaches have all contributed to a substantial progress in the definition of the complex genetic architecture of MS. This prompted a significant extension of the view on MS genetics, that was essentially limited to the role of human histocompatibility haplo types until 15 years ago. At least two challenges remain: (i) the definition of a comprehensive etiological model, with the need of better understanding both the plausibly causal effects in altering disease risk for many of the susceptibility gene regions identified, and the impact of non-genetic factors, as demonstrated, among others, by twin studies (18, 19); (ii) the clinical translation of genomic data, that may exploit the relevance of pathogenic pathways, for which therapeutics is already available in clinical practice, or may drive the discovery of new druggable targets.

One potentially informative approach to deal with these issues includes bioinformatics attempts capable of extracting from GWAS data the biological consequences and the functional implications of individual disease-associated variants. Our group implemented analyses aimed at clarifying the interplay between diseases-associated genomic regions and presumed

Abbreviations: GWAS, genome-wide association studies; PLAU, plasminogen activator, urokinase.

causal environmental factors (20–22). Another bioinformatics reworking allows to explore the reciprocal interactions of pathways resulting from GWAS data, to disclose unknown networks and to focus on previously under estimated pathways in MS etiology.

By applying the latter approach we used bioinformatics tools to analyze the role of the coagulation cascade in connection with other biological pathways contributing to the complex disease pathogenesis. Using MetaCore (version 6.35 build 69300, 2018) we analyzed the connectivity (i.e., direct and indirect interactions among two networks) between the network of the coagulation process (a standard map in MetaCore, presented in **Figure 1**, that includes 94 components) and the network resulting from feeding into MetaCore the MS GWAS data. In particular we considered genes that were reported in 19 MS GWAS studies (23–41) filed in the GWAS Catalog (https://www.ebi.ac.uk/gwas); such list (**Supplementary Table 1**) contains 398 genes, that were either reported as associated to MS in the aforementioned studies, or that were originally reported as hits on non-well specified regions, later mapped to better characterized regions and genes. The connectivity analysis in MetaCore takes place in two steps: first, the genes that are shared by the two networks (i.e., elements that appear in both the coagulation process network and the MS GWAS network) are identified; second, every element in each network is enriched with its interactors. A statistics is then computed counting how many interactions are observed among the two enriched networks, comparing this number to what would be expected by chance. MetaCore connectivity analysis showed the following results: the coagulation process network and the MS GWAS network presented a remarkable over-connectivity, showing 958 connections (561 were expected by chance) that lead to z-score of 17.39 and p-value < 0.00001; genes coding for cluster of differentiation 40 (CD40) and plasminogen activator, urokinase (PLAU) appeared both in the coagulation process network and the MS GWAS network (**Figure 2**).

These analyses on one hand confirm that the coagulation cascade may have an impact on MS development, as already reported (see above), on the other hand fail to detect main coagulation components previously indicated by experimental studies. This limitation may pertain the analyses based on GWAS studies in general, which incorporate huge number of gene variants and several levels of possible functional complexities. Specifically, PLAU pathway has already been scrutinized for its role in the activation of matrix metallopeptidase9, that has in turn been associated with BBB breakdown, a crucial event in MS development (42). However, the network sharing by PLAU and CD40 pathways, resulting from our analysis, points to a more integral interplay between coagulation cascade and immune effectors, that are currently the main focus of research on MS etiopathogenesis and therapy. CD40 pathways is especially operative in B cells, being the typical signal mediating help by T cells (through CD40 ligand) on cognate B lymphocytes for antibody production and other important functions, such as antigen presenting cells and cells modulating the immune response. Recent studies show indeed that MS-associated genetic variants alter the expression of co-stimulatory molecule, including CD40 in B cells, as well as the level of steering cytokines such as interleukin-10, which is considered to have an immunoregulatory function downstream of CD40 (43). Moreover, the CD40-CD40 ligand dyadis intensively investigated for its essential role in the development of MS, with the aim of targeting it therapeutically and antagonize neuroinflammation (44).

The role of CD40 pathway in MS development refers to the more general topic of the role of B cells in neuroinflammation. Our recent works suggest that B lymphocytes, in an activated and pro-survival status, contribute to MS development with functions other than antibody-production (45). Indeed, B lymphocytes are professional antigen-presenting cells for autoreactive T cells (43, 46), as well as potent producers of steering cytokines and other immune effectors influencing both pathogenic (lymphotoxin, tumor necrosis factor, granulocyte macrophage-colony stimulating factor, and metallo-peptidases) and protective (interleukin 10) milieus in neuroinflammation (47–49). Accordingly, CD20-targeted monoclonal antibodies, that deplete B cells in their earlier stages of development, turned out to be highly and consistently effective in tackling the disease development (50, 51). Hence, the finding that PLAU pathway may potentially interact with a signal of paramount importance for B cell pathogenicity, such as CD40, may open new perspectives for translational research. Along this line, the protease activity of microglial cells activated by urokinase plasminogen activator coupled with its receptor seems very important for their pathogenic role in MS (52) and, notably, this pathogenic role is increasingly recognized also in a very recent GWAS study on MS.

CONCLUSION

The case of the relationship between coagulation pathway and MS molecular model may teach us how fruitful a bioinformatics reworking of GWAS data may be. In particular bioinformatics approaches that match GWAS data with other biological repositories of unbiased comprehensive records may shed light on the functional relevance of common diseases-associated single nucleotide polymorphism: each genetic variant is often located in regulatory genomic regions, and may be active in different ways in diverse tissues, making it very difficult to encompass a detailed understanding of the underpinning pathobiology.

Future works based on connectivity analyses may inform a number of questions that are still open in the context of MS heritability: the degree of epistasis and interaction with non-genetic causative factors; the existence of genetic interactors determining disease forms, clinical course, and response to diseases modifying therapies; the predictivity of endophenotypes, in particular the imaging data, that often segregate on a familiar basis. Moreover, the discovery of "clinically actionable genes" may represent a timely task in the current landscape of MS therapeutics.

Many new diseases modifying therapies, already available in clinical practice, show superior effectiveness compared to the treatments that were in place only a decade ago. The "cost" is the safety profile, being at least suboptimal. Approaches based on drugs targeting PLAU system, that have successfully been used to ameliorate CNS inflammation (53, 54), may be potential resources, with good therapeutic index and synergic action

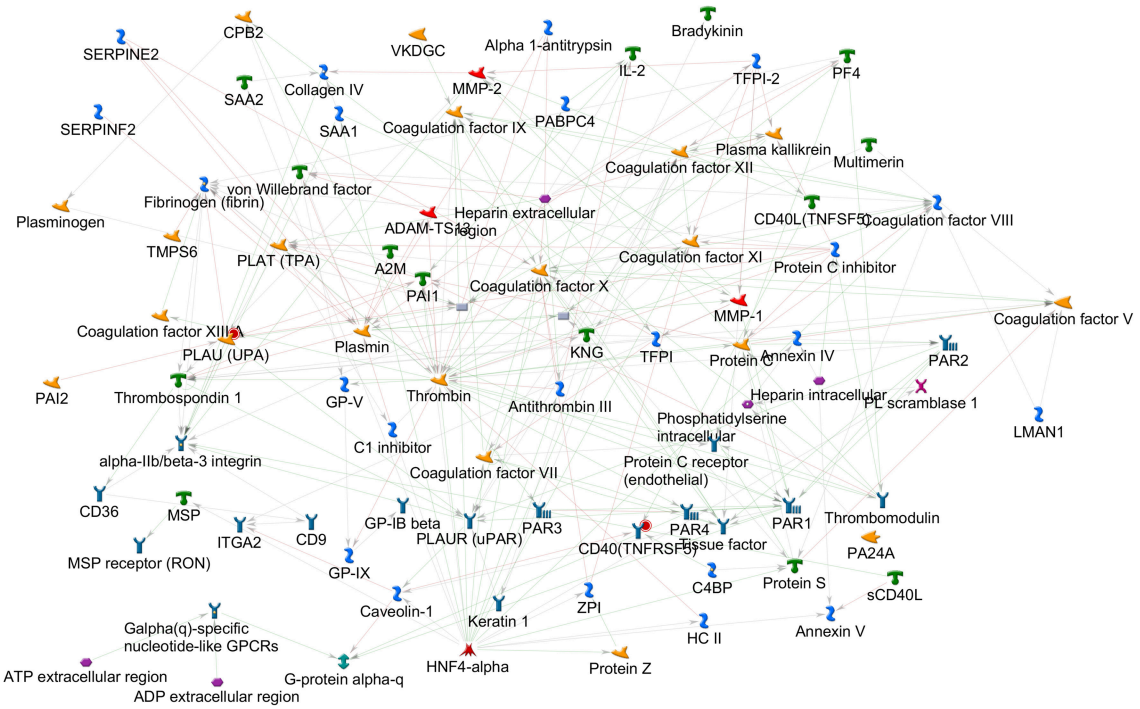

FIGURE 1 | Coagulation process map, as available in MetaCore 2018. The elements on this map, together with their interactors, were used for the connectivity analysis.

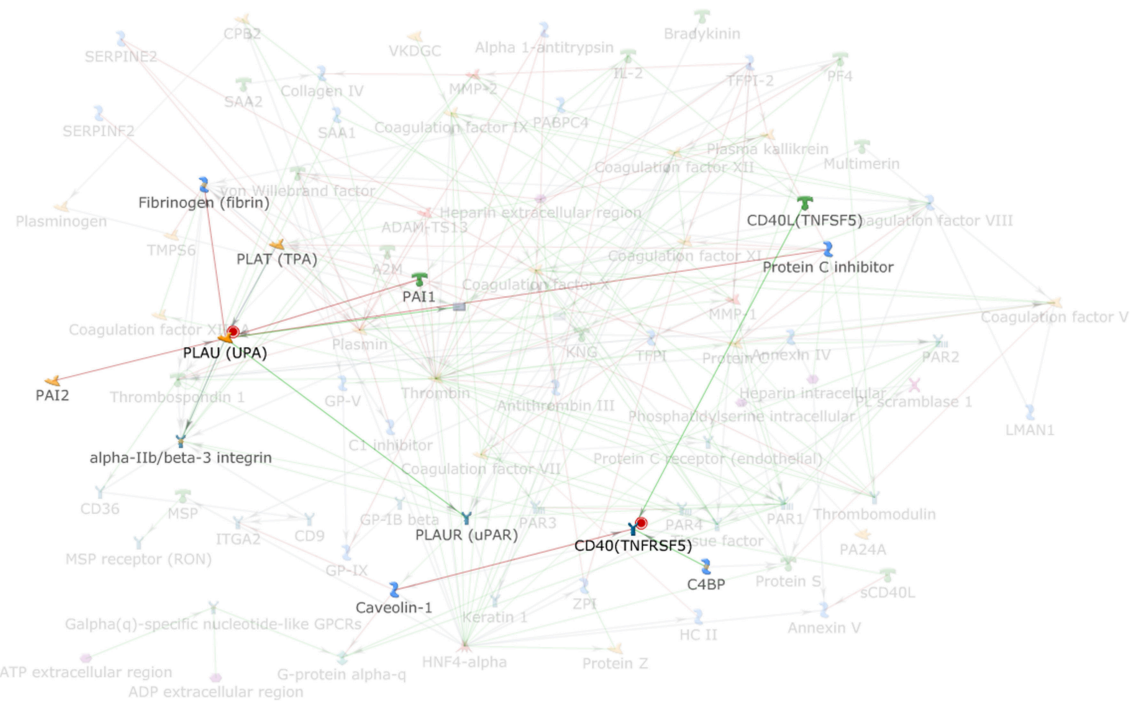

FIGURE 2 | Highlight of the elements (PLAU and CD40) that are shared among the coagulation process map and the MS GWAS gene list; PLAU and CD40 interactors are also highlighted.

with currently available immune-modulators, potentially to be exploited in combination schemes.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2019.00095/full#supplementary-material

Supplementary Table 1 | List of genes that were gathered from the GWAS Catalog as associated with MS and that were used for this work.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neurology

REVIEW
published: 05 February 2019
doi: 10.3389/fneur.2019.00059

Thrombin and the *Coag-Inflammatory Nexus* in Neurotrauma, ALS, and Other Neurodegenerative Disorders

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This review details our current understanding of thrombin signaling in neurodegeneration, with a focus on amyotrophic lateral sclerosis (ALS, Lou Gehrig's disease) as well as future directions to be pursued. The key factors are multifunctional and involved in regulatory pathways, namely innate immune and the coagulation cascade activation, that are essential for normal nervous system function and health. These two major host defense systems have a long history in evolution and include elements and regulators of the coagulation pathway that have significant impacts on both the peripheral and central nervous system in health and disease. The clotting cascade responds to a variety of insults to the CNS including injury and infection. The blood brain barrier is affected by these responses and its compromise also contributes to these detrimental effects. Important molecules in signaling that contribute to or protect against neurodegeneration include thrombin, thrombomodulin (TM), protease activated receptor 1 (PAR1), damage associated molecular patterns (DAMPs), such as high mobility group box protein 1 (HMGB1) and those released from mitochondria (mtDAMPs). Each of these molecules are entangled in choices dependent upon specific signaling pathways in play. For example, the particular cleavage of PAR1 by thrombin vs. activated protein C (APC) will have downstream effects through coupled factors to result in toxicity or neuroprotection. Furthermore, numerous interactions influence these choices such as the interplay between HMGB1, thrombin, and TM. Our hope is that improved understanding of the ways that components of the coagulation cascade affect innate immune inflammatory responses and influence the course of neurodegeneration, especially after injury, will lead to effective therapeutic approaches for ALS, traumatic brain injury, and other neurodegenerative disorders.

Keywords: thrombin, thrombomodulin, PAR1, DAMPs, HMGB1, blood brain barrier, ALS, neurodegeneration

OPEN ACCESS

Edited by:
Tatiana Koudriavtseva, Istituto Nazionale del Cancro Regina Elena, Italy

Reviewed by:
Michele Papa, Università degli Studi della Campania Luigi Vanvitelli Caserta, Italy
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Specialty section:
This article was submitted to Multiple Sclerosis and Neuroimmunology, a section of the journal Frontiers in Neurology

Received: *09 October 2018*
Accepted: *17 January 2019*
Published: *05 February 2019*

Citation:
Festoff BW and Citron BA (2019) Thrombin and the Coag-Inflammatory Nexus in Neurotrauma, ALS, and Other Neurodegenerative Disorders. Front. Neurol. 10:59. doi: 10.3389/fneur.2019.00059

INTRODUCTION

In humans, the coagulation system or cascade was conceptualized over the past five to six decades to consist of five serine proteases (factor VII, FVII; factor IX, FIX; factor X, FX; protein C, PC and prothrombin, PT) that act with five cofactors (tissue factor, TF; factor V, FV; factor VIII, FVIII; thrombomodulin, TM; and protein S, PS) to control the generation of fibrin, which is subsequently

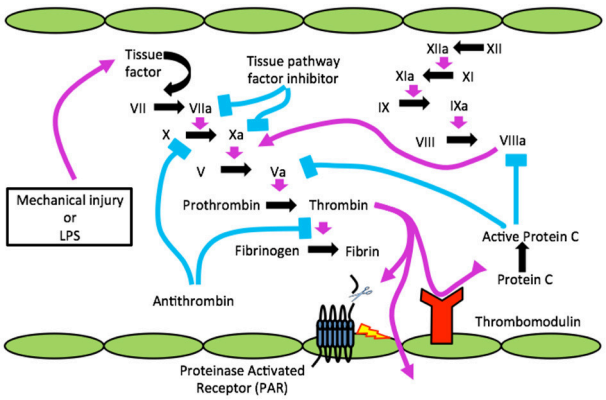

FIGURE 1 | The clotting cascade, TBI, infection (LPS) and the blood brain barrier. Cartoon shows injury or infection, lipopolysaccharide (LPS) releasing tissue factor (TF) to activate clotting resulting in active α-thrombin, which cleaves a receptor(s) known as PAR on endothelial cells (ECs) to disrupt BBB. Through disrupted BBB thrombin gains access to CNS and can cleave to activate PARs on microglia/astrocytes for neuroinflammation and neurons to form neurofibrillary tangles (NFTs).

cross-linked by Factor XIII (FXIII), a transglutaminase (1). This system is essentially conserved throughout mammalian species (schematically shown in **Figure 1**), but the system's endpoint, hemostasis, has been around for 450 million years. Hemostasis consists of three activities that are closely regulated; vasoconstriction, platelet aggregation, and clotting factor activation. Two different pathways, the *intrinsic* (contact) and *extrinsic* (TF), exist to activate clotting and the principal difference is the role of TF in the extrinsic pathway, which works very rapidly. With blood vessel damage, *inactive FVII* comes in contact with TF, a protein on the endothelial cell (EC), and activates it to a protease (2). Activated Factor VII then proteolytically activates *FX* that then binds *activated FV* to form *prothrombinase*. So, recapping, TF release is very rapid and generated by damaged blood vessels and surrounding tissues, which is especially high in brain, and initiates the extrinsic pathway.

Since endothelial cell damage is the principal mechanism for clotting factor activation via TF generation it invariably occurs with systemic microbial infection, as in sepsis, where the innate immune system is activated (2). Indicating sepsis in **Figure 1** is bacterial LPS (lipopolysaccharide or endotoxin). It was subsequently found that trauma, a sterile injury, also produces TF-generated coagulation (**Figure 1**) (3).

The clotting system is involved in host defense, and arose with and is linked to innate immunity or inflammation at very early evolutionary stages. TF is the key actor and common generator providing the critical *nexus* between these two major host defense systems (4). TF belongs to the cytokine receptor superfamily and is a type I integral membrane glycoprotein (5). Thrombin, the ultimate serine protease in the cascade, is the key downstream product of TF-initiated coagulation. Not only does it play a central role in hemostasis but more recent studies have revealed its fundamental and intense proinflammatory effects (6). These latter attributes of thrombin, just as its role in causing platelet aggregation, were subsequently ascribed to its non-coagulation actions as a ligand for cell-surface receptors, now known as protease-activated receptors (PARs) (7–9).

Although these thrombin-mediated, PAR-activated cellular effects involve thrombin's roles in cell proliferation and modulation, cytoprotection and apoptosis, its role as a proinflammatory mediator is key that further brings together coagulation and inflammation—the *coag-inflamm nexus*. Furthermore, it incorporates innate immune pathways such as toll-like receptors (TLRs) and complement, exosomes/microparticles (MPs) into this nexus. With cellular activation thrombin also recruits other systems to provide a balance for this coag-inflammatory pressure, and this includes the *protein C (PC)–thrombomodulin (TM)* natural anticoagulant/anti-inflammatory machinery along with activation and monitoring of the fibrinolytic system.

In the 1980's a few studies began to explore the direct effects of thrombin on cultured neural cells (10–13). Those initial reports ushered in a number of successive studies of thrombin, the coagulation and fibrinolytic cascades, TM, PARs in the CNS that continues to the present time. More recent efforts at translation of tissue culture and animal studies to neurologic diseases are now chronicled in other reports in this Frontiers in Neurology collection.

AMYOTROPHIC LATERAL SCLEROSIS (ALS) AND NEURODEGENERATIVE DISORDERS

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder exemplified clinically by muscle weakness and wasting and neuropathologically by degeneration of upper and lower motor neurons in the spinal cord, brain and brainstem (14–16). More recent evidence indicates that a number of *endophenotypes* exist for ALS beyond what was considered 30–50 years ago: the four *classic* motor neuron disorders. These are: classical ALS (upper and lower motor neuron and bulbar involvement), progressive muscular atrophy (PMA; only lower motor neuron), progressive bulbar palsy (PBP; brainstem with little if any extremity features) and primary lateral sclerosis (PLS; only upper motor) if it is actually part of the spectrum. As a distinct *nosologic* disorder ALS has been known in the medical literature since Charcot first described it 150 years ago in the late nineteenth century (17).

It is a fatal and currently enigmatic disease with death usually resulting from the inexorable progression of diaphragmatic and intercostal muscle weakness ultimately causing paralysis and respiratory failure typically within 5 years of diagnosis. The incidence of ALS has changed only slightly since the 1970 s and is ∼1.5–3 per 100,000 in Western Europe and North America with little variation. It is overwhelmingly a sporadic disease (sALS), but genetic variants exist (fALS) accounting for no more than 10% of all cases (see below), although newer information may be changing this. ALS has an estimated lifetime risk of 1 in 400, is an adult-onset illness that is rare before the age of 40

TABLE 1 | Partial history of thrombin signaling in CNS and PNS: Key processes involving neural and neuromuscular health and the thrombin signaling pathway.

Event	References
Release of acetylcholinesterase (AChE) in ALS and denervation of muscle	(19–26)
Denervation and plasminogen activators	(27–38)
Thrombin, protease nexin I, PAR1, and synapse formation/elimination	(39–52)
Thrombospondin and ALS	(53–59)
Coagulation, astrocytes, BBB and neuroinflammation	(60–64)

years increasing exponentially with age. There are no known treatments that impact progression of the disease. Until 2017, the last Food and Drug Administration (FDA) approved drug was Riluzole™, licensed in 1996 and that only extended survival of ALS patients 3 months. In May 2017 the FDA approved edaravone (Radicava™) to treat ALS patients based on a 2nd Phase 3 study after the first was negative (18). As the authors wrote: the drug "…..showed efficacy in a small subset of people with ALS who met criteria identified in *post-hoc* analysis of a previous phase 3 study, showing a significantly smaller decline of ALSFRS-R score compared with placebo."

As discussed in detail below, our laboratory at the Kansas City VA Medical Center began studies of the coagulation system in ALS in the 1980's (see **Table 1**).

GENETICS AND ALS: fAMILIAL ALS (fALS)

Although it was considered a sporadic illness beginning in the 1990 s interest in the ∼5–10% of ALS cases that had family history began. Identification of mutations in the superoxide dismutase 1 gene (*SOD1*) was reported in 1992 (65, 66). Over the next 25 years remarkable progress in our understanding of SOD1 and fALS has occurred (67–73).

Even amongst otherwise sALS cases about 1–3% possess missense mutations in SOD1 (74) and even more, about 5–10% of sporadic ALS cases are caused by intronic expansions in *C9orf72*, the open reading frame (ORF 72) on chromosome 9 (75–77). This indicates that 1 in 20 cases of sALS and about 40% of fALS are due to *C9orf72* hexanucleotide repeats.

With SOD1 and *C9orf72* more than 20 mutated genes have now been found to be specifically associated with fALS (78) that include *TARDBP* (79–81) and *FUS* (82, 83), the *fused in sarcoma* gene on chromosome 16p11.2, that is involved with RNA processing, which together with *SOD1* and *C9orf72*, are the four most common genes involved in causing ALS clinically. TARDP encodes a protein, TAR DNA binding protein (TDP-43) that accumulates in most sALS motor neurons but not SOD1 fALS neurons (84). These genes have been numbered now as ALS1-ALS22, along with FTDALS1, FTDALS 2, FTDALS 3, and FTDALS 4 (78). Genome-wide association studies (GWAS) may be changing the role of genetics in ALS including what we now consider sALS (85–87).

The changing viewpoint results from studies of the relatively uncommon genetic cases of this enigmatic and fatal neurodegenerative disorder that have revealed some fundamental clues that might uncover novel therapeutic targets. Amongst these are more recently identified *endophenotypes* beyond the classical motor sub-types. Endophenotypes are inherited traits identified using clinical or laboratory measures including electroencephalographic or electromyographic abnormalities, neurocognitive deficiencies, and other modalities that identify impairment. Until recently they have been largely used in psychiatric and psychopathology-related research. Originally conceived by Gottesman and Shields (88), they were proposed to appear not only in patients but also in their unaffected relatives. The presumption of endophenotypes is that they are more proximate to gene action than the clinical diagnoses (89, 90). In neurodegenerative diseases such as Parkinson's (PD) and Alzheimer's (AD) diseases, in addition to ALS, they might provide dual positives such as improving diagnoses and initiating therapy in preclinical stages (91–93).

ALS Endophenotypes Beyond the Motor System

ALS is now recognized as a multi-system neurodegeneration rather than a disease limited to motor neurons (94–97). Although 40 years ago if a patient was clinically diagnosed with ALS but exhibited cognitive symptoms that patient was not considered to have classical ALS and typically was removed from consideration. In fact, in the original *El Escorial* criteria (98) and El Escorial revisited (99, 100), the presence of dementia essentially ruled out ALS as diagnosis. This action was taken despite the fact that descriptions of cognitive and behavioral symptoms resembling frontotemporal dementia (FTD) in otherwise typical ALS motor phenotypes date back to the 1880 s. The neurologic giant, Arnold Pick, whose name is eponymic for a subgroup of FTD known as Pick's disease, was aware that Charcot had considered that non-motor brain regions might also be involved in the neurodegeneration of what is now known as *Maladie de Charcot* or *la sclérose latérale amyotrophique (SLA)* by francophones.

One of the first descriptions of FTD associated with ALS in the modern era was provided by the late Canadian neurologist, Arthur Hudson (101), who also described mixed types of ALS with parkinsonism as well as with dementia and other clinical features, reminiscent of the *ALS-parkinsonism-dementia* complex of Guam (102, 103). Since then increasing interest in FTD-like symptoms in ALS patients appeared and it is now thought that about 10% of patients with one of the four classic motor-neuron disorders: classical ALS, PMA, PBP, and PLS, have cognitive features.

Subsequent reviews of the ALS/FTD complex have appeared (104, 105) that now also include associations with *C9orf72* expansions (77, 96, 106, 107). In fact, the seminal discovery of a GGGGCC hexanucleotide repeat expansion (HRE) within the chromosome 9 ORF 72 (75–77, 108), has been established as cause for the most common form of ALS/FTD (107).

Given this common cause of sALS with FTD, 10% of sALS and an additional 10% of FTD, the next question, given that it has taken more than 25 years with SOD1 mutations, is just how cytotoxicity occurs with the GGGGCC (G4C2) HRE

within the C9ORF72 gene? Several recent studies have shed light on this: it has been reported that HRE RNA forms hairpin and G-quadruplex structures that bind and sequester RNA-binding proteins (RBPs). The GGGGCC are translated into specific dipeptide-repeat (DPR) proteins, and these form toxic aggregates, particularly the arginine-rich dipeptides, specifically proline-arginine (PR), that possess potent neurotoxicity forming aggregates in nuclei and nucleoli, and stress granule formation, with likely effects on translation (109).These authors used inducible pluripotent stem cells (iPSCs) to differentiate into human motor neurons (iMNs), including those from ALS patients carrying the repeat-expanded *C9ORF72*. These studies revealed that C9ORF72 in the ALS patients was *haploinsufficient*. Thus, the ALS/FTD gene had only one functional copy, causing a loss-of-function mutation. Using blood cells from healthy individuals they used gene-editing techniques to delete the *C9ORF72* or from ALS patients with the abnormal gene. They found that C9ORF72 cooperated with endosomes, was involved in vesicle trafficking and formation of lysosomes in motor neurons. When they repeated the nucleotide expansion this reduced *C9ORF72* expression, and with this process, neurodegeneration was triggered via both gain- and loss-of-function mechanisms. The former produced a buildup of glutamate receptors, causing excitotoxicity, while the latter weakened neurotoxic dipeptide repeat proteins clearance derived from the repeat expansion. This cooperative action led to neurodegeneration. These and other researchers have begun using the gene-editing tool, CRISPR, specifically the CRISPR–Cas9 system, to perform genome-wide gene-knockout screens similar to studies in cancer (110).

Frontototemporal lobar degeneration (FTLD) is the 2nd most common cause of dementia in elderly (over age 65) individuals and is actually a broad spectrum of neurological disorders. FTD is a variant of FTLD and from GWAS studies now appears to share a number of genetic as well as clinical and neuropathological features. In a recent GWAS study of more than 120,000 neurodegenerative diseases and controls unique genetic overlap between ALS and FTD spectrum diseases was found (111). Of interest, the H1 haplotype of the tau protein gene (MAPT) appeared to confer risk for ALS, as did *BNIP1*, a mitophagy-associated, proapoptotic protein.

If an *endophenotype strategy* in ALS should be implemented, as has since been undertaken in several neurodegenerative studies, it will depend both on quality and properties of a specific trait. It will be necessary to critically evaluate the trait(s) to determine if it truly can capture pre-diagnosis features of ALS/FTD. However, no consistency has yet appeared for endophenotypes or intermediate traits or even biomarkers, but some encouraging signs have appeared (112, 113). When such validated intermediate traits or biomarkers are considered, it will be necessary to forgo requiring that they be absolutely specific for ALS or FTD. Consequently, application of endophenotypes to future analyses of ALS and FTD seems more than justified.

With consideration of the ALS spectrum as a *non-cell autonomous* condition (114, 115), it brought to the picture the evidence that glial cells, including astrocytes, oligodendrocytes, and even microglia play important roles in the pathogenesis of ALS (116–118). Prior to the last decade it was widely assumed that motoneuronal cell death proceeded by cell autonomous mechanisms. However, information gained initially from using SOD1 transgenic mice and subsequently with other genetic models, the non-cell autonomous position evolved. In terms of *SOD1* more than 170 different mutations have been shown to cause fALS. When SOD1 mutations were expressed only in neurons neurodegeneration did not occur in the mice (119). But just how these mutations in SOD1 result in cytotoxicity is still unclear, despite more than 25 years of study. In fact, no consensus has emerged as to the principal mechanism for neurotoxicity or even how cells might protect themselves from it. Cleveland and colleagues proposed that ALS was just the tip of the iceberg and that non-cell autonomy will be shown to be the mode in other neurodegenerative diseases (114, 115, 120, 121).

Along these lines the multi-faceted roles of astrocytes have now become prominent for investigation in ALS (115, 118, 122–124). Discussed in more detailed below, reactive *astrocytosis* also known as astrogliosis, is a classic glial response to CNS injury and scar-forming reactive astrocytes are usually viewed as detrimental to clinical outcome (125), but not always (126). Astrocytes are also hallmarks of neurodegeneration (127) and using the ME7 prion mouse model Cunningham and colleagues showed that neurodegeneration primed astrocytes to produce exaggerated chemokine responses when stimulated with acute proinflammatory cytokines (128). The usual neuropathologic means to characterize reactive astrocytes is by using antibodies to the intermediate filament glial fibrillary acidic protein (GFAP). However, all phenotypes of astrocytes including reactive astrocytes and scar-forming astrocytes strongly express GFAP. In fact, being able to modulate extent and phenotypes of reactive astrocyte function (129) is potentially attractive as novel targets to enhance the functional outcomes after spinal cord injury (SCI) (130) or in ALS and other neurodegenerative diseases might be revealed (116, 131).

CONNECTING DOTS TO NEURODEGENERATION: NEUROINFLAMMATION, COAGULATION, BBB

Inflammatory, and Innate Immune Aspects of ALS

Reviewing numerous studies of the past two decades has divulged previously held concepts that upper and lower motor neurons were the focus of ALS disease burden have now been replaced by non-cell autonomous mechanisms. Such non-cell autonomous mechanisms, particularly neuroinflammation, may not only contribute to the disease process but may initiate it, as detailed below.

Based on several lines of evidence within the last 20 years both sALS and fALS have had numerous proinflammatory markers associated with them (132–135). More than two decades ago, Appel et al. emphasized potential autoimmunity in ALS (136–138), and several different approaches revealed that immunoglobulin G (IgG) from ALS patients' sera caused toxicity

in cultured motor neurons and in mouse models (138–141), with activation of L-type Ca^{2+}-channels.

As exception proving the rule or standing in apparent contradiction, since it was present in the context of immunodeficiency, was our earlier report documenting ALS in a young homosexual male patient in whom HTLV-III (subsequently re-named HIV) was isolated (142). This initial observation was later confirmed in more recent accounts (143–147), suggesting that ALS, if truly autoimmune, may also be associated with immune deficiency disorders such as AIDs.

By definition, neuroinflammation is inflammation of nervous tissue and is characterized by proliferation and activation of glial cells, primarily microglia, and astrocytes, as well as transmigration of circulating immune cells, including polymorphonuclear neutrophils (PMNs), monocyte/macrophages, and T lymphocytes (T-cells) into the parenchyma across the blood-brain barrier (BBB) (148–152). In addition to these cellular characteristics, neuroinflammation includes humoral features such as proinflammatory cytokine and chemokine overproduction, along with their respective receptors (151). Of relevance here are the numerous reports of neuroinflammation in both sALS and fALS including its appearance in pre-symptomatic phases in transgenic mice. However, confusion has developed from these data since both deleterious and beneficial effects have been found especially when focusing on motor neuron survival and also depending on what disease stage was examined (153, 154).

Microglia

The understanding of these complex interactions largely centers on microglia, considered the brain's resident macrophages, and their dual roles or *Janus faces*, in neurodegeneration in general and ALS in particular (107, 153). In essence, although microglial phenotypes were classified as either M1 ("classically activated") or M2 ("alternatively activated"), similar to circulating macrophages, phenotypic diversity of microglia is actually a spectrum (155). M1 microglia could be considered more proinflammatory while M2 more anti-inflammatory, possibly viewed as "deactivated" after phagocytosis of apoptotic cells. Clearly, a therapeutic strategy in ALS or in AD or PD for that matter, might be to selectively modulate microglial phenotypes, such as inhibiting or blocking M1 or enhancing M2. That may be too simple, although it is a strategy worth evaluating. However, this should not be done with pre-clinical animal models due to known differences in inflammatory responses compared to humans, but in human iPSC *ex vivo* models that incorporate elements of the blood-spinal cord barrier (BSCB)BBB/NVU along with neurons (156, 157).

Astrocytes

Of the several types of glial cells in the CNS astrocytes are the most abundant. Classically considered "supportive" cells for neurons astrocytes have recently been shown to be critical in regulating CNS immunity, but exactly how they do this is largely unknown. Astrocytes have been shown to be regionally diverse within the brain and in the spinal cord. Regions where astrocytes may be involved in regulating CNS immunity are at their "end-feet" localized to where they are contiguous with ECs of the BBB and neurovascular unit (NVU) as well as perivascular end-feet that form the *glia limitans*. All astrocytes are ramified and have processes that terminate on basal lamina impacting the perivascular compartment with their end-feet (127).

Of interest, one molecule highly concentrated in astrocytic end-feet is the gap junction protein, connexin 43 (Cx43) (158). Cx43 may have roles in the non-cell autonomous pathogenesis of sALS, implicating toxic mitochondria transferring from astrocytes to motor neurons at the BSCB (159, 160), as detailed below.

By analogy to macrophages, the M1/M2 macrophage and microglial nomenclature (161), although with caveats for its potential simplicity researchers have also applied these to reactive astrocytes (125, 127) into A1 and A2 sub-classes (162), whether caused by neuroinflammation or ischemia, respectively. As with microglial M1 and M2 sub-classes the macrophage phenotypic literature clearly indicates that these circulating immune cells display more than two polarization states (155, 163, 164). Chronic neurodegeneration also produces changes in the secretory profile of astrocytes in terms of what cytokines and chemokines are produced (128).

As M1 macrophages were considered destructive, so, too, are A1 astrocytes. Conversely, since M2 were considered reparative and protective as a macrophage or microglial phenotype, so were the A2 astrocytes. Liddelow, in the late Ben Barres' group, further showed that A1 were induced by reactive microglia (165).

INNATE IMMUNE ACTIVATION IN ALS

Over the past two decades our thinking about the brain and spinal cord as being an immunologically privileged site has changed. It was previously thought that the CNS could not mount an immune response nor process antigens. More recent studies have reversed that indicating that immune surveillance does take place in the CNS, and glial cells of all types act as immune effector cells within the CNS (149, 166). We now know, for example that the primary function of the CNS innate immune system is to provide neuroprotection against invading pathogens. However, in addition to infectious agents it is also protective for injury stimuli, and by so doing maintains CNS homeostasis.

Pattern Recognition Receptors, Pathogen-Associated Molecular Patterns in ALS

Knowledge of how membrane and intracellular receptors respond to pathogenic components dramatically increased with identification of pattern recognition receptors (PRRs) to identify pathogen-associated molecular patterns (PAMPs), the prototype for which is lipopolysaccharide (LPS) or endotoxin, from Gram-negative bacteria. The effect of LPS in the CNS is to cause *sickness behavior*, a coordinated set of adaptive behavioral changes to LPS and others (166–168) that includes: fever, anorexia, social withdrawal, lethargy, and decreased rapid-eye movement sleep

TABLE 2 | PAMPs and DAMPs: danger/damage recognition systems extrinsic and intrinsic.

Patterns	Characteristics
Pathogen-associated molecular patterns (PAMPs)	Prototype is lipopolysaccharide (LPS; endotoxin)
	Activate several pattern recognition receptors (PRRs)
	PRRs include Toll-like (TLRs) and advanced glycation end-product (RAGE) receptors
	Highly Conserved among diverse species
	MAMP (microbe-associated molecular pattern) may be more accurate
Damage-associated molecular patterns (DAMPs)	Prototype is high mobility group box protein 1 (HMGB1)
	Non-histone DNA binding, nuclear protein
	Also present in mitochondria (mtDAMPs), like TFAM
	Activated late in sepsis, necrotic and dendritic cells
	Released with trauma, necrosis, including in the CNS

(REMS). Major innate immune system PRRs such as the Toll-like receptors (TLRs) and the receptor for advanced glycosylation endproducts (RAGE) are expressed in the CNS. Most TLRs, now 15 members of the family, and RAGE, are expressed in all neural cells (167–174).

Damage-Associated Molecular Patterns (DAMPs)

The *danger-damage theory* expressed in 1994 by Polly Matzinger (175–177) changed the concept of immunology from simply detecting self vs. non-self. Her thesis was that the immune system's driving force is the *need to recognize danger and prevent destruction*. This theory evolved with publication of the proceedings of the EMBO Workshop on Innate Danger Signals and HMGB1 that took place February 2006 in Milan, Italy (organized by M. Bianchi, K. Tracey, and U. Andersson) (178). In keeping with this concept a group of endogenous molecules that signaled damage or danger were developed and were referred to as *alarmins*, a sub-category of DAMPs. Subsequent studies indicated that the PRRs recognized and responded to DAMPs in essentially the same manner as their response to PAMPs (177, 179, 180) and that the CNS also participated (181). The comparison of PAMPs and DAMPs and list of both is shown in **Table 2**.

In fact, as an example that science, certainly more in the pre-cloning era, was guilty of the *blind men describing the elephant* parable, Finnish workers had identified a protein that guided early neuroblasts to their final locations in developing mouse brain and called this protein, *amphoterin* (182, 183). Amphoterin, also called P30 protein, was subsequently found to be identical to HMGB1 (170), the prototypic alarmin/DAMP. The structure of the alarmin/DAMP HMGB1 is shown in **Figure 2A** and its known signaling in **Figure 2B**. The relationship between HMGB1 and thrombin is interesting. Both are prototypes of ancient host defense systems, inflammation and coagulation (60), but in addition, as shown in **Figure 2B**, thrombin can cleave HMGB1 at its –NH2 end and does so when bound to TM (184). Of interest, since then HMGB1 has been shown to be involved in a number of neuropathologic processes in the CNS and is also essential for brain development (181, 185).

As mentioned above PRRs, especially TLRs and RAGE, expressed by immune cells are also expressed by neural cells, particularly astrocytic and microglial, to mediate resident immune cell activation (169, 172). As described for AD and other neurodegenerative diseases (63, 148, 181, 186), DAMPs are probable candidates to partake in, and possibly initiate, ALS neurodegenerative activities. HMGB1 is over-expressed in SOD1 mutant mouse spinal cord and motor cortex and from patients with ALS (187). TLRs were also found to be over-expressed in ALS patients' spinal cords (188), as was RAGE, along with its proinflammatory ligands, including HMGB1, S100B and calgranulin (189). Furthermore, a number of groups have focused on levels of circulating soluble RAGE (sRAGE) in various diseases including diabetes mellitus, cardiovascular and neurodegenerative diseases (190, 191), recently including ALS (174, 189, 192). As opposed to sRAGE being "specific" for any of those diseases it is clear that it is implicated in their pathogenesis and contributes to our understanding of innate immunity in these conditions. Furthermore, it might be useful as therapeutic strategy in one or more of them. Additionally, sRAGE has been considered a "decoy receptor" to block the cellular membrane receptor to block RAGE-mediated signaling. In this regard, sRAGE is decreased in blood while increased in affected CNS in ALS and other neurodegenerative diseases (193).

MITOCHONDRIA, ALS, AND mtDAMPs

A key mechanism whereby motor neurons degenerate in ALS is by influence of dysfunctional mitochondria (194–196). As in PD and AD and other neurodegenerative diseases, studies in SOD1 transgenics as well as in sALS cells have been performed that show such mitochondrial defects, with an eye to novel therapeutics (197–199). Almost 20 years have elapsed since the close temporal relationship of the onset of motor neuronal degeneration with initiation of astrogliosis in the SOD1 mouse model was first identified (200). With further understanding of the non-cell autonomous, specifically astrocytic, aspect of ALS pathogenesis abnormalities in astrocyte mitochondria have been found (201–205). In particular, the demonstration that "positive" aspects of mitochondria can be shifted to neurons in transcelluar organelle transfer (159) indicates that negative or toxic aspects of astrocytic mitochondria might be transferred to motor neurons in sALS or fALS (206, 207), possibly via connexin 43 (159, 160).

Of interest, aligned with the "danger theory" is the *endosymbiotic* theory that mitochondria originated from protobacteria that entered into an endosymbiotic relationship with phagocytic, unicellular anaerobes at least a billion years ago (208), prior to the accumulation of oxygen in the atmosphere. Mitochondrial DAMPs (mtDAMPs), are protein DAMPs, coded

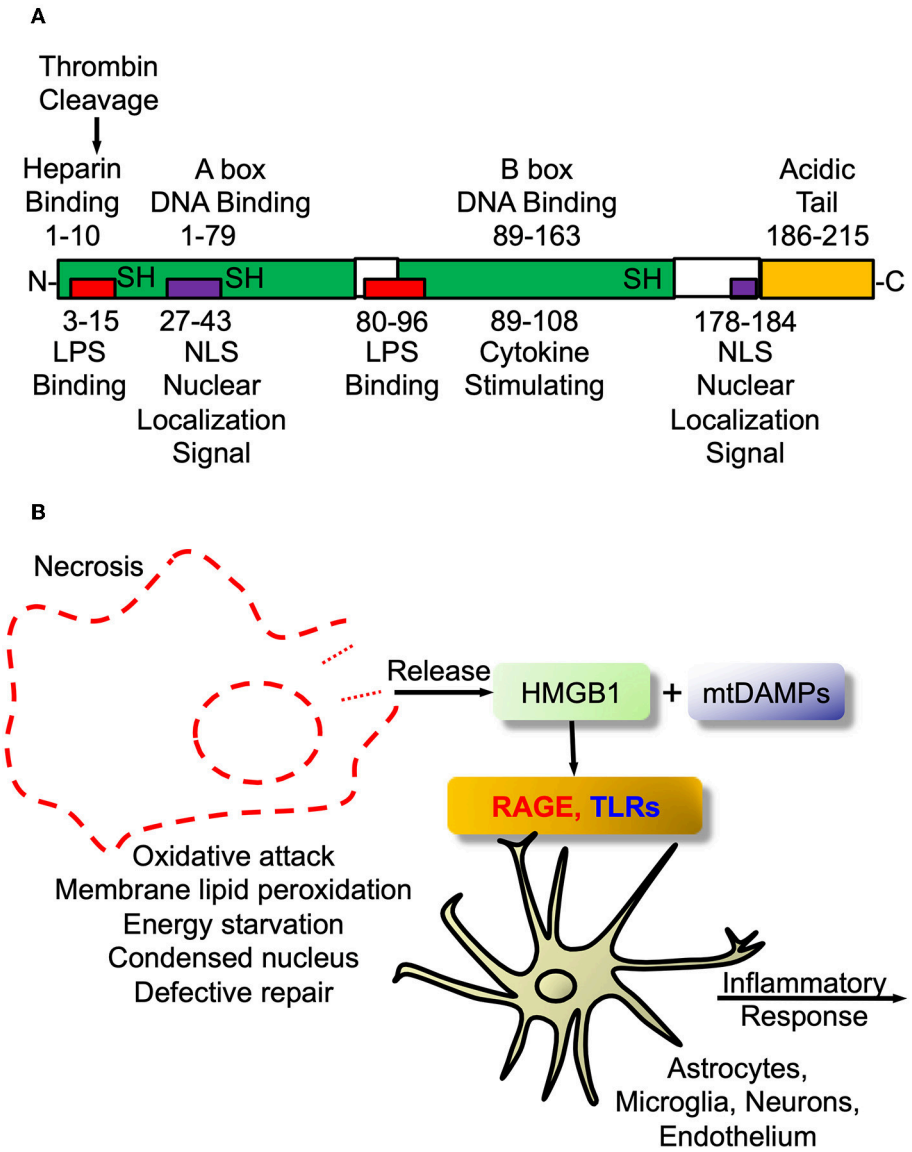

FIGURE 2 | High mobility group box protein 1 **(**HMGB1). **(A)** HMGB1 Structure. Schematic of HMGB1's two binding motifs, A-box and B-box. Also showing critical cysteines that determine whether it is proinflammatory when outside cell or DNA binding when inside nucleus. In addition, the C-terminal acidic tail appears to confer protein stability and DNA bending *in vitro*. **(B)** HMGB1 signaling. The damage-associated molecular pattern (DAMP) HMGB1 is passively released from necrotic or exploding cells such as with infection or trauma. Within the CNS it engages pattern recognition receptors (PRRs) such as toll-like receptors (TLRs2/4) and receptor for advanced glycation end products (RAGE) to initiate proinflammatory signaling as part of innate immune activation.

for by mitochondrial or nuclear genes, that when released from mitochondria are potently proinflammatory (209). Most mtDAMPs are encoded by nuclear genes that after transcription translocate from nuclei to mitochondria. These mtDAMPs are then released into the circulation with infection (sepsis), trauma and/or systemic inflammatory response syndrome (SIRS). In support of this being relevant in the CNS we showed that mtDNA, a nucleic acid mtDAMP, was potently proinflammatory for neurons and microglia (210). Of interest, PCR-amplified purified mtDNA was not proinflammatory, rather only brain isolated mtDNA in the form of nucleoids bound to transcription factor of mitochondria A (TFAM), itself a mtDAMP (211, 212), was proinflammatory (211, 212). Such studies support the prediction that mitochondrial dysfunction in neurodegeneration, and neurotrauma, is tightly linked to neuroinflammation (151, 213), especially with mtDAMP involvement in neurodegeneration (214). The potential roles of mtDAMPs in neurodegeneration are shown in **Figure 3**.

BLOOD-BRAIN BARRIER (BBB)

Just as the CNS responds to PAMPs like LPS so does it respond to DAMPs, both initiating proinflammatory signaling and for the bridging or disruption of the BBB (63, 64). The history

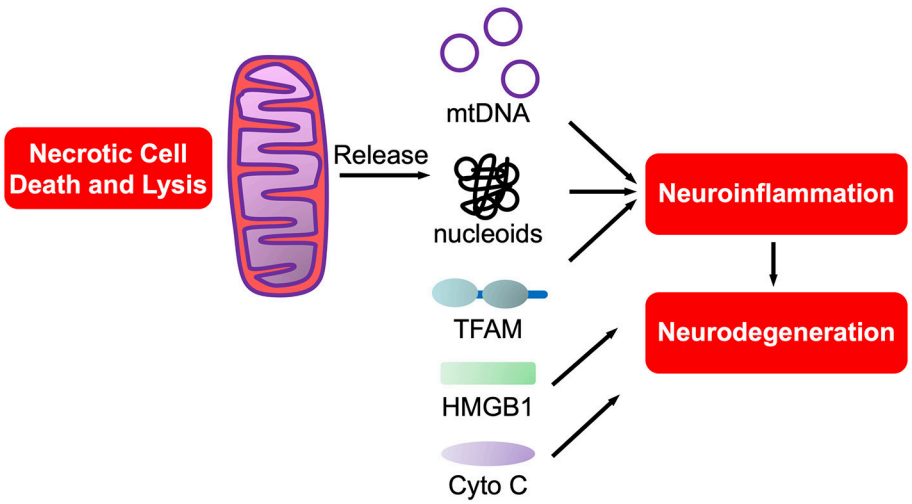

FIGURE 3 | mtDAMPs. A sub-category of DAMPs, mtDAMPs are components of mitochondria released from necrotic or exploding cells. Comprised of mtDNA bound to mitochondrial proteins such as transcription factor of mitochondria A (TFAM), a homolog of HMGB1, in nucleoids. Also includes n-formyl peptides, cytochrome C and others (not shown).

of a perceived association between ALS and BBB dysfunction actually dates back to the 1940 s when Robert Aird began a 40 year involvement with the BBB in neurologic diseases (215–219). However, it took several more decades before technology caught up with the concept that ALS was associated with BBB or blood-spinal cord barrier (BSCB) dysfunction, perhaps even at the onset of the disorder (220–231).

Evans blue extravasation from capillaries into spinal cord parenchyma was found in early symptomatic SOD1 transgenic (G93A) mice but it was uncertain whether BBB/BSCB disruption was cause or effect of motor neuron degeneration (220, 221, 223, 232, 233). More evidence suggestive of causative influences have since appeared with further studies of SOD1 transgenic mice with an eye toward therapy as well (233–235). This situation is fundamentally the same for neuroinflammation in ALS—that is, is it cause or effect? The findings that the *C9orf72* expansions are also associated with myeloid cell abnormalities and early BBB dysfunction supports the role of these processes in pathogenesis (107, 236). The concept of the BBB being both *target* of circulating coag-inflammatory molecules as well as the *source* of pro-(neuro)inflammatory mediators is shown by Festoff et al. (63).

NEUROTRAUMA AND ALS

The progression of neurodegenerative disease following neurotrauma is both anecdotal and supported by epidemiologic statistics. Dementia, including AD, and microvascular dementia (mVAD), is now considered to have increased risk following traumatic brain injury (TBI), while the specific mechanistic details are still under study. Similarly, ALS occurs at an increased risk following TBI, more so, in fact than after typical SCI. Numerous mechanisms have been suggested for this association of neurotrauma and neurodegeneration including increased interest for almost 30 years in the nexus of inflammation, BBB disruption and coagulation.

Early reviews of mechanical and other forms of spinal and CNS injury associated with the development of ALS have appeared, some positive while others were negative (237, 238). Case-control studies, however, are few and those, such as that published from Olmsted County, Minnesota by Kurland and colleagues were not supportive (239). However, more recent larger population-based case control investigations, such as the Danish study, have shown an association especially with trauma at an early age (240, 241). Even broader studies such as the European EURALS consortium study (242) are giving credence to a role for trauma in ALS pathogenesis. This large study showed that more than 2 head injuries was associated with >3-fold increased risk of ALS. Although the site of injury was not important the risk was only ∼2-fold when trauma occurred between 35 and 54 years of age. Certainly the age at first trauma might help to explain discrepancies in results of past studies of trauma and ALS.

In addition, studies of chronic traumatic encephalopathy (CTE) with repeated mild traumatic brain injury (mTBI) or concussions (243–245), indicate that there may be methods to identify, monitor and treat and/or prevent neurodegenerative disease development in the context of neurotrauma. With more thorough investigation into CTE and former professional athletes, an increased incidence of clinical ALS diagnosis has been reported. A recent study of CTE and CTE associated with ALS (CTE-ALS) confirmed that molecular changes co-existed pathologically. Specifically, these were phosphorylation of tau at threonine 175 (Thr175) and at Thr231 along with GSK3β were found in these tauopathies (246). Furthermore, similar findings were present in rats subjected to moderate TBI in a controlled cortical impact (CCI) model (246). These findings suggest that comparable underlying molecular mechanisms for abnormal tau

phosphorylation associated with CTE neuropathologic aspects may be mimicked in a rat moderate TBI model. However, they do not provide evidence for a neurotraumatic basis for sALS.

What neurotrauma does tell us for ALS is that there is a distinct relationship between trauma, BBB disruption and neuroinflammation (247–250), all potential contributing pathogenetic factors in ALS. From the BBB disruption perspective, mechanisms involved with TBI include impact-induced shear force stress that causes initial vascular injury followed by escape of proteins along with extravasation from brain to blood as well macromolecule leakage and cell transmigration from blood to brain.

COAGULATION ASPECTS OF ALS AND OTHER NEURODEGENERATIVE DISEASES

Beginning in the 1980s through the 2000's our laboratory focused its attention on thrombin, the ultimate serine protease in the coagulation cascade, its inhibitors and receptors as specific mediators of either toxic or trophic effects on the nervous system. Our studies utilized *in vitro* tissue culture to probe the effects of thrombin, and inhibitors of thrombin, on neurons and glial cells. Once the thrombin receptor, subsequently named protease-activated receptor 1 (PAR1), was identified and sequenced by Coughlin's group in the early 1990 s, our studies also involved the expression of PAR1 in parts of the nervous system, in particular, the spinal cord as well as in the brain and neuromuscular system. Our translational interest was primarily in SCI and ALS, since the initial studies in neuronal types found exquisite sensitivity of spinal cord motor neurons that lead to apoptotic motor neuronal cell death in culture by thrombin cleavage of the G-protein coupled receptor (GPCR), PAR1. We also explored PARs in AD and PD as well. A number of other groups in Switzerland, Germany, Israel, Italy, Korea, China and Japan, amongst others, as well as the U.S. also began exploring coagulation and fibrinolytic proteases and inhibitors in the nervous system, especially after publication of The Maratea Meeting Proceedings in 1990 (251).

Although the emphasis of this review is on ALS similar results and concepts have been found for other neurodegenerative diseases including AD, PD, and multiple sclerosis (MS) and numerous reviews are available (252). Of interest, until recent evidence for biased signaling (see below) through PAR1 by APC was discovered (253), the previous data indicated that high thrombin concentrations were neurotoxic and pathologic in brain while low thrombin concentrations could induce neuronal and astrocytic survival after various brain insults. Interestingly, thrombin-mediated cell death and cell survival shared initial signaling proteins (48, 254).

THROMBOMODULIN (TM) IN CNS DEVELOPMENT, NEUROTRAUMA, AND NEURODEGENERATION

TM was discovered by Esmon and Owen in the 1970 s and reported in 1982 (255, 256). This discovery came after a decade or more of research that resulted in discovery of Protein C (PC), a vitamin-K dependent factor that is activated by thrombin that results in activated protein C (APC), a serine protease. Initially, the principal role of APC was thought to be its anticoagulant function whereby it proteolytically inactivated FV and FVIII (**Figure 1**). However, this multi-molecular system, now termed the PC–TM-EPCR (endothelial PC receptor) pathway (257, 258), is a natural mechanism to regulate hemostasis and to integrate it with other host defense system such as innate immunity, inflammation, and to control cell proliferation. Since its cloning, sequencing and chromosomal localization (259), the bulk of studies on TM have also been in terms of its role as a natural anticoagulant. However, as important as this action is, the integration by TM of hemostasis and innate immunity may determine its even greater future in disease processes that affect the CNS.

Of interest, shortly after the discovery of TM a report indicated the presence of a surface marker protein in developing mouse parietal endoderm that was modulated by cAMP (260). Shortly thereafter, fetomodulin was found to be identical to TM by contemporary gene cloning techniques (261). Thus, TM or fetomodulin (FM) is present at very early developmental stages and in adults TM expression is greatest in ECs, more predominant in small, microvascular than in large vessel ECs, and was found in essentially all ECs (262). However, the first article concerning TM and CNS vasculature was negative reporting its absence in brain ECs (263). This was not correct since it was subsequently reported that bovine as well-human brain capillaries expressed TM (264, 265), again suggesting its role as a microvascular EC marker. Not surprisingly given its early developmental appearance in parietal endoderm (FM), TM is also expressed in a number of other cells including keratinocytes, osteoblasts, monocytes, neutrophils and chondrocytes, amongst others. We first found that TM was expressed in mouse brain astrocytes, where it was functionally identical to its role in ECs (266). Subsequently, TM was found to be a novel marker of injury-induced astrogliosis, and identified the involvement of thrombin-activated PAR1 (267). This finding suggested its involvement in nervous system injury, i.e., neurotrauma. The TM gene (*THBD*) is intronless and is structurally separated into five distinct domains (see **Figure 4**). Biochemically, TM is a chondroitin sulfate proteoglycan (CSPG), and consistent with its role as a CNS injury-related CSPG would be increased in the "glial scar" and assemble along with other CSPGs such as neurocan and phosphacan that are also expressed in reactive astrocytes (268).

As shown in **Figure 4** TM's additional role in regulating inflammation, apart from coagulation and thrombin's proinflammatory role by PAR1 activation, is largely encoded at its –NH2 terminal, known as the C-type lectin like domain (TM-CTLD). The CTLD is involved in a host of inflammatory diseases, as described in the treatise by Conway (258), one of the leaders in this field. A mechanism for the CTLD in these conditions was provided by the pioneering work of Maruyama's group that discovered that the TM-CTLD bound and neutralized the DAMP alarmin HMGB1 (269). This same group found that HMGB1 was upregulated in spinal cord parenchyma

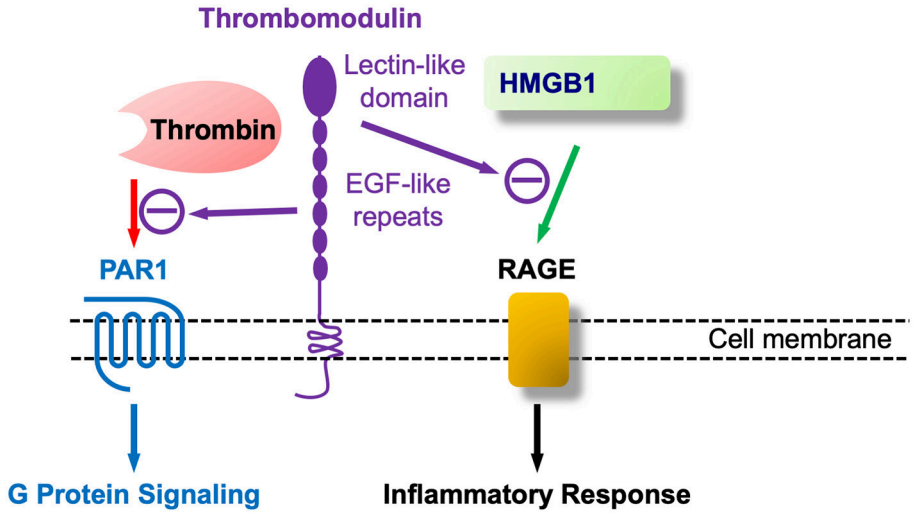

FIGURE 4 | Thrombomodulin (TM) at nexus of coagulation and inflammation (innate immunity): TM as at least a bifunctional molecule that binds coagulation (thrombin) and proinflammatory (HMGB1) agents.

following SCI (270). Beyond its sequestering and neutralization of HMGB1 the TM-CTLD also interferes with complement activation and binds to LPS/endotoxin, and, in Gram-negative bacterial infections, to the Lewis Y antigen (271). Of interest, transgenic mice lacking the NH2-terminal CTLD ($TM^{LeD/LeD}$) have heightened susceptibility to treatment with LPS (258) and should be more vulnerable to weight-drop SCI than wild type mice. The deposition of HMGB1 in the injured spinal cord was shown in rats (270), along with its release into the circulation.

The relationship between the BBB, more precisely the BSCB, and the *coag-inflammation nexus* in ALS merits comment. As mentioned above, although BBB/BSCB dysfunction in ALS was discussed as far back as the 1940 s, it took many decades and newer technology to establish its actual existence. The identification by Garbuzova-Davis and her colleagues that BSCB dysfunction occurred in both ALS patients and fALS SOD1 mice, prior to motor neuron degeneration (220, 221, 226–229), has been confirmed by other groups (222–225, 230, 231). Furthermore, there is a connection between BSCB dysfunction and the PC–TM-EPCR pathway, as shown by the amelioration of motor problems in SOD1 mice by treatment with non-proteolytic/non-anticoagulant APC analogs (224).

A simultaneous activation of the coagulation cascade after injury, as occurs in sepsis, is an ancient host response dating back very early in the evolution of eukaryotes. The contemporary clinical correlate happens in sepsis and injury where excessive thrombin activation develops in disseminated intravascular coagulation (DIC) associated with sepsis and sterile traumatic SIRS (272, 273). A phylogenetic clue into the nexus of clotting and inflammation comes from studies of the omnipresent East Coast North American horseshoe crab, *Limulus polyphemus*, with its open circulatory system containing the hemolymph, and single cell, the amoebocyte, with properties of both platelets and phagocytes. Limulus has survived for >250 million years exposed to LPS or endotoxin in the ocean from *Cyanobacteria* or blue-green algae where they have been for the past 2 billion years (274). The Limulus lysate detection kit for LPS in blood has been in use worldwide for over 30 years. Coagulopathy also commonly develops with TBI since the brain is a rich source of TF and thromboplastin (275).

It should be noted at this point that the thrombin→PAR1→BBB dysfunction pathogenetic pathway is not specific for ALS but occurs in all situations where intravascular prothrombin activation to α-thrombin exceeds its neutralization either by circulating anti-thrombin (AT) or EC-bound TM and the EPCR (276, 277). This dysfunction pathway would be applicable to AD, PD, ALS and neurodegeneration, in general, especially in those situations associated with antecedent trauma. In this regard, all PARs are expressed on ECs and brain microvascular ECs are no exception. However, PAR1 and PAR4 are also expressed on brain pericytes, which appear to be the most thrombin-sensitive perivascular cells to release membrane metalloprotease-9 (MMP-9) (278, 279). MMP-9 has been shown to cause BBB disruption by proteolyzing tight junction (TJ) proteins (280, 281).

Recombinant APC (rAPC; drotrecogin alfa, activated; Xigris™) was the first agent shown to stimulate PAR1-mediated cytoprotection approved for human use (in severe sepsis). However, it was voluntarily removed from the market by Eli Lilly in 2011. A number of studies have emphasized the cytoprotective role of APC, encompassing anti-apoptotic and anti-inflammatory activities, as well as significant stabilization of endothelial barriers including the BBB and BSCB. Most studies indicated this was mediated by PAR1 or PAR3 (282). All PARs are expressed on ECs (9, 283, 284) and brain microvascular ECs of the BBB should be no exception. The evidence that thrombin, via PAR1 activation, caused vascular leakage and disruption across various vascular barriers (285–287), including the BBB (288)

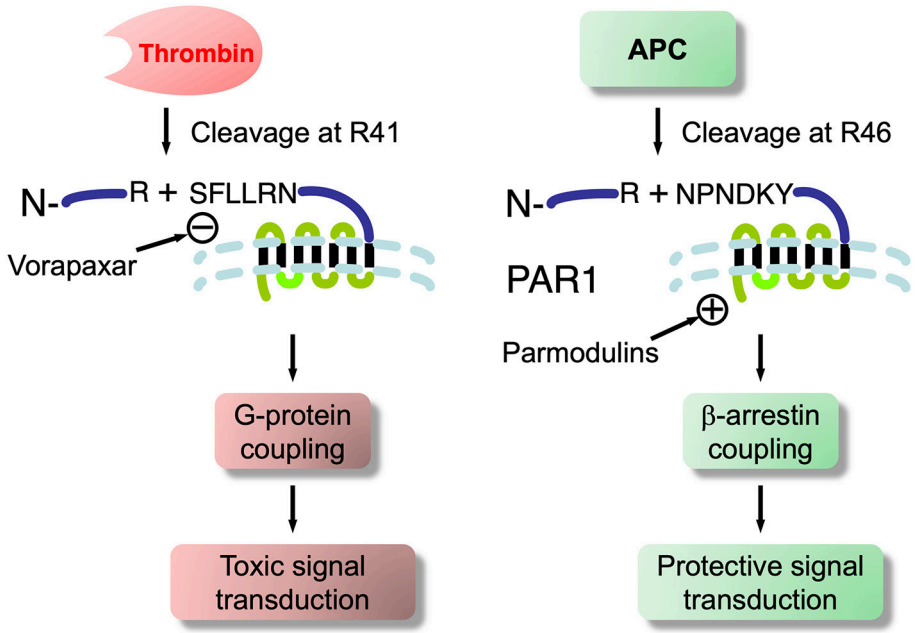

FIGURE 5 | Biased signaling at PAR1 showing thrombin and APC cleavage sites- basis for toxicity and cytoprotection at this G-protein coupled receptor. Also shows synthetic PAR1 antagonist, vorapaxar, an FDA approved therapeutic for cardiovascular conditions. It has been shown to induce endothelial injury, which is associated with BBB/BSCB dysfunction in SOD1 mice and ALS patients. Whether ALS may be triggered in susceptible individuals so treated requires evaluation. Also shown are novel parmodulins that do not have endothelial injury side effects of vorapaxar and, thus, may have therapeutic application to ALS and neurodegenerations.

while APC activation of PAR1 did the reverse, i.e., protection and prevention of leakage (253, 282), was a conundrum. However, as reviewed by Griffin et al. (282) this lead PAR/APC researchers to the notion of "biased signaling", a phenomenon found in other GPCRs, a group to which PARs belong. Biased signaling through PAR1 for thrombin and APC, as conceived by Griffin et al. is shown in **Figure 5**: thrombin cleaves PAR1 at ARG41 in the extracellular –NH2 domain, while APC does so at ARG46 (282). At PAR1 thrombin signals through the small GTPase RhoA and ERK1/2 to disrupt, while APC through RAC1, β-arrestin and P13k/Akt to protect. This puts PAR1 on BBB ECs in a very significant position and its different proteolytic ligands to destroy or save BBB function. APC is effective in compression SCI (289) and we found that recombinant TM is also neuroprotective in rat weight-drop contusion SCI (61). More recently, Noble-Haeusslein and colleagues reported that APC biased signaling through PAR1 enhanced locomotor recovery in rat SCI (290). Zlokovic and colleagues showed that treatment with non-proteolytic/non-anticoagulant APC analogs (224) improved motor functions in SOD1 mice.

PARs in ALS and Other Neurodegenerative Diseases

We found that nM thrombin concentrations induced tau neurofibrillary tangle-like aggregates (NFTs) in murine hippocampal neurons and that this required PAR activation that was followed by delayed synaptophysin reduction and apoptotic neuronal death (291). Subsequently, others showed that the initial fragmentation of tau, necessary to then cause aggregation into NFTs, was due to a thrombin-like protease (292). These authors wrote that fragmentation by a thrombin-like protease was a "prelude" to aggregation, although phosphorylation was not. HIV-associated neurodegeneration (HAND) was also shown to require thrombin and PAR1 expression in astrocytes, as subsequently reviewed (293). McGeer and colleagues then showed that thrombin, as well as prothrombin, accumulated with NFTs in the brains of AD patients (294). Additional evidence for thrombin and PAR1 in neurodegeneration was provided by others in AD (295, 296) and PD (297) and then reviewed as well (50).

Our preliminary data indicated that PARs were increased and active in several murine ALS models in which microglia express increased monocyte chemoattractant protein 1 (MCP-1) and other markers**.** In regards to neurotrauma, we found that SCI was accompanied by an early and significant upregulation of neurotoxic serine proteases, prothrombin, and PAR1 in the rat spinal cord (298). It was subsequently reported that thrombin-recruited microglia also express MCP-1 (now CCL2) and that PAR1 activation is required for this (299).

The *wobbler* mouse is a model of motor neuron disease sharing many features with ALS, including loss of spinal motor neurons, neuromuscular loss of function over time, and TDP-43 aggregates and C-terminal fragments identical to those seen in the sporadic form of ALS (300). By optimizing transcription and quantitative PCR procedures to facilitate rapid copy number determination in small RNA samples, we documented a 5-fold greater level of PAR1 mRNA in the cervical spinal cord of *wobbler* (wr/wr) compared to wild type mice (301). Then we subsequently

confirmed and extended these results showing that PAR1 mRNA was dramatically increased in spinal cord alpha motor neurons in homozygous, spontaneously mutant autosomal recessive *wr/wr* mice (302). The gene for wobbler mutation is located on mouse chromosome 11 (303) and was since shown to be a point mutation on Vps54 (vacuolar protein sorting 54) involved with the Golgi apparatus (304). Even before the gene was determined an informative marker at the wobbler locus, the glutamine synthase 1 (glns-1) pseudogene, permitted genotyping mice prior to phenotype development as previously described (305). Using this technique, we found that homozygotes expressed an 8-fold increase in PAR1 message by P8, more than 2 weeks prior to phenotype detection and this appeared primarily in motor neurons (301–303, 305, 306). These earlier studies focused attention on potential roles of coagulation proteases and PARs in the nervous system but took some time before they had generated additional interest in pursuing direct connections between them, neurotrauma, neuroinflammation and neurodegeneration.

Following our earlier reviews (47, 48, 251, 307) more recent efforts have emphasized the participation of coagulation in various neuroinflammatory diseases of the CNS (64, 308–310). Connections between tissue transglutaminase (tTG), in the same family as the clotting cascade Factor XIII, cross-linking and neuroinflammation in ALS also exist. SCI has been shown to upregulate cytokines, microglia and tTG (311). In addition, we found that SCI induced a "switch" from a GTPase function of tTG to a novel GTP-independent cross-linking isoform in the spinal cord (311). More recently, tTG has been implicated in promoting neuroinflammation in SOD1 mice (312). It would be of interest to determine whether tTG upregulation was present in ALS spinal cord and if alternative transcription to a short isoform existed.

EPILOG, AND POSSIBLY, PROLOG (TO THE NEXT PHASE)

Vorapaxar is a natural product-based orthosteric antagonist of thrombin-induced PAR1 that inhibits all signaling downstream (313). The FDA approved it for post-myocardial infarction following success in two large pivotal multi-center Phase III outcome clinical trials in patients with coronary atherothrombosis. It has a low molecular weight (590.7) and a long effective half-life (3–4 days).

Surprisingly, the FDA review of the adverse events for both Phase III clinical trials revealed an increased number of ALS diagnoses in the vorapaxar arm compared to the placebo arm (314). This adverse event was not mentioned in the publication of the results and this vorapaxar-ALS association may recall the studies we and others carried out with thrombin, PAR1, thrombospondin, TM and related components of the *coag-inflamm* system in development, neurotrauma, ALS and other neurodegenerative disorders, as described above (see **Table 1**). Since vorapaxar appears to inhibit all signaling downstream of the PAR1 GPCR it would seem that is where attention should be paid for clues to ALS pathogenesis related to it. Although it is still a relatively rare occurrence after vorapaxar we would hope that knowledge of this small but surprising ALS *signal* after vorapaxar will uncover novel therapeutic targets for this enigmatic and fatal neurodegenerative disorder and related disorders where synapse retraction may be the earliest pathophysiologic sign of the disease (22, 24–26) and where thrombin→PAR1 activation may well play a role (51, 52).

In this regard, recent development of small molecule PAR antagonists termed *parmodulins* (315, 316) are based on findings that biased signaling peptides developed around APC are cytoprotective at PAR1 and not anticoagulant (282). We hope that such research will help advance whether or not potential neuronal degeneration and/or impaired neuromuscular activity is a class-specific adverse effect after PAR antagonists (317).

AUTHOR CONTRIBUTIONS

BF conceived of the review, and was lead on the evaluation of the literature and writing. BC also contributed to the reviewing, writing, and figures.

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Conflict of Interest Statement: BF is the Founder of PHLOGISTIX LLC, a startup biotech company.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neurology

MINI REVIEW
published: 14 January 2019
doi: 10.3389/fneur.2018.01175

A Perspective of Coagulation Dysfunction in Multiple Sclerosis and in Experimental Allergic Encephalomyelitis

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A key role of both coagulation and vascular thrombosis has been reported since the first descriptions of multiple sclerosis (MS). Subsequently, the observation of a close concordance between perivascular fibrin(ogen) deposition and the occurrence of clinical signs in experimental allergic encephalomyelitis (EAE), an animal model of MS, led to numerous investigations focused on the role of thrombin and fibrin(ogen). Indeed, the activation of microglia, resident innate immune cells, occurs early after fibrinogen leakage in the pre-demyelinating lesion stage of EAE and MS. Thrombin has both neuroprotective and pro-apoptotic effects according to its concentration. After exposure to high concentrations of thrombin, astrocytes become reactive and lose their neuroprotective and supportive functions, microglia proliferate, and produce reactive oxygen species, IL-1β, and TNFα. Heparin inhibits the thrombin generation and suppresses EAE. Platelets play an important role too. Indeed, in the acute phase of the disease, they begin the inflammatory response in the central nervous system by producing of IL-1alpha and triggering and amplifying the immune response. Their depletion, on the contrary, ameliorates the course of EAE. Finally, it has been proven that the use of several anticoagulant agents can successfully improve EAE. Altogether, these studies highlight the role of the coagulation pathway in the pathophysiology of MS and suggest possible therapeutic targets that may complement existing treatments.

Keywords: coagulation, neuroinflammation, multiple sclerosis, neuromyelitis optica spectrum disorders, thrombosis

OPEN ACCESS

Edited by:
Scott S. Zamvil, University of California, San Francisco, United States

Reviewed by:
Bruno Gran, Nottingham University Hospitals NHS Trust, United Kingdom
Tatsuro Misu, Tohoku University, Japan

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Specialty section:
This article was submitted to Multiple Sclerosis and Neuroimmunology, a section of the journal Frontiers in Neurology

Received: *30 September 2018*
Accepted: *18 December 2018*
Published: *14 January 2019*

Citation:
Plantone D, Inglese M, Salvetti M and Koudriavtseva T (2019) A Perspective of Coagulation Dysfunction in Multiple Sclerosis and in Experimental Allergic Encephalomyelitis. Front. Neurol. 9:1175. doi: 10.3389/fneur.2018.01175

INTRODUCTION

Multiple sclerosis (MS) is an inflammatory demyelinating and degenerative disease of the central nervous system (CNS) characterized by neuroinflammation and neurodegeneration and affecting prevalently women (1). Most commonly, MS begins with a relapsing–remitting course with alternation of clinical relapses and remissions (2). With time, most of these cases switch to a secondary progressive phase with steady accumulation of disability. In a lower percentage of patients (about 20%) the disease is progressive from the beginning and is defined as primarily progressive form. Experimental autoimmune encephalomyelitis (EAE) is the most studied animal model of MS (3). It is possible to induce EAE in mice by immunization with spinal cord homogenates or by passive transfer of sensitized T cells.

Several recent studies have highlighted the importance of the interplay between the activation of the coagulation cascade and neuroinflammation, suggesting that coagulation factors are crucial not only for the activation of the acute hemostatic cascade, but have a broader role involving neurodegeneration and neuroinflammation (4–20).

We will review this evidence, trying to offer an overview of possible targets that may complement existing immunomodulatory therapeutic tools.

HISTORY

The role of vascular thrombosis due to increased coagulation in MS has been taken into consideration since its first description based on histopathological observations. In 1882, Ribbert postulated that MS lesion could be consequent to central vascular thrombosis due to bloodstream infection (21). This hypothesis was supported by Pierre Marie, who thought that infections have a causative role in MS through the induction of brain vascular damage and thrombosis (21). Thereafter in 1930–40s, Tracy Putnam pointed out venular thrombosis as the primary MS cause based on histologic and experimental observations (22). He observed thrombi in acute lesions with small plaques surrounding the engorged veins. He occasionally observed thrombi also in other body organs of MS patients. Moreover, Putnam found that most MS patients had a peculiar defect of the clotting mechanism, suggesting that thrombosis was not consequent to the vessel wall injury but to blood alterations such as an increase in fibrinogen.

The first studies on serum coagulation factors were conducted in small MS patient cohorts and led to conflicting results. The clotting time was found shortened (23), normal (24) or prolonged (25). There was disagreement also regarding the prothrombin time since it was similarly reported either shortened (26), prolonged, (27, 28) or normal (25, 29). Putnam (30) and Persson (31) showed an increase of fibrinogen especially during the exacerbations while other studies found it to be normal in 9 patients in active clinical progression except one (25) as well as in 33 MS patients except two patients with increased fibrinogen, of which one examined during a relapse (32).

Furthermore, thrombocytes were studied in MS with divergent results. One study reported a short clot retraction time during acute disease exacerbation (33), whereas it was found to be normal or prolonged in other works (25, 32). An elevated platelet adhesiveness and a short clot retraction time was found mainly during acute disease exacerbation (33). Fog et al. found the reduction in thrombocyte count during the disease exacerbation and its increase during the clinical improvement (34) whereas other authors found thrombocyte count normal in MS patients (24, 29, 32) or reduced in several patients in phase of disease remission (32). Persson found an increase in adhesive platelets in the prodromal phase of thrombosis, which rapidly decreased during the thrombi formation (35). Similarly, Wright et al demonstrated increased adhesiveness of platelets in acute and severe cases compared to controls suffering from other neurological disorders and reporting normal values of platelet adhesiveness (36). However, the possibility that steroid therapy could influence this adhesiveness was not excluded. Another explanation for the negative results related to platelet adhesiveness in MS patients found by Field and Caspary could be presence of edetic acid (EDTA) in the test tubes which is known to reduce platelet adhesiveness (37).

Interestingly, Putnam treated his MS patients with Dicumarol in the 1940s and concluded that only patients with acute relapses benefited from this therapy (21). All in all, the enthusiasm for anticoagulant therapy in the scientific community waned in the following years because of its doubtful effectiveness (21).

At the same time, in the 1930s the pathological MS research radically changed its own course after the development of EAE, a prototypic model of MS, through the immunization of susceptible animals with CNS components. Since then, the majority of the studies on disease pathophysiology focused on immunological mechanisms (38). While the results of genome-wide association studies (GWAS) (39) and the success of treatments based on immunological targets reinforce this hypothesis, other studies still suggest a key role for a dysfunction of coagulation, possibly linked to the ongoing inflammation, in CNS autoimmunity (9–11, 15, 40–42).

COAGULATION CASCADE SUMMARY

Coagulation is a complex process involving blood changes that lead to the formation of a blood clot (43). It is classically aimed to ensure haemostasis, an important biological process that contrasts bleeding after vessel injury. However, coagulation is activated not only by direct vascular injury but also by functional injury due to hypoxia, sepsis, malignancy, inflammation, etc (44, 45). A pathological imbalance of haemostasis may lead to intravascular thrombosis despite the coagulation process is controlled by several inhibitors limiting the clot formation. However, in certain conditions thrombosis is a physiological process called "immunothrombosis" involving an intrinsic effector mechanism of innate immunity (46). Immunothrombosis is specifically activated by either blood-borne pathogens or circulating altered-self components on a local platform consisting of fibrin, monocytes, neutrophils, and platelets contributing to pathogen recognition. Innate immune cells such as monocytes, neutrophils, dendritic cells participate actively in this process propagating fibrin formation and triggering platelet activation. This process contrasts either tissue invasion, dissemination, or survival of pathogens. The delimitation of immunothrombosis to only a restricted number of microvessels likely ensures a sufficient overall organ perfusion.

Briefly, two traditional coagulation cascade pathways, so-called intrinsic, and extrinsic, lead to the same final common pathway of factor X and thrombin ending with fibrin formation. These coagulation pathways are a series of reactions converting the inactive precursors to active ones in order to catalyze the next reaction in the cascade. Majority of clotting factors are precursors of proteolytic enzymes known as zymogens that circulate in an inactive form.

Platelets exert potent procoagulant functions via the calcium-dependent cell-surface exposure of phospholipids such as phosphatidylserine, which act as cofactors for the proteolytic reactions triggered by coagulation factors. Coagulation process, in turn, fosters platelet activation and accumulation, mainly through the protease thrombin, which promotes platelet activation by both cleavage and activation of platelet's proteinase-activated receptors (PAR). Platelets early aggregate to form a "platelet plug" to close provisionally the vessel wall injury. This platelet adhesion to subendothelial surface is reinforced by von Willebrand factor (vWF), which is a glycoprotein present in blood plasma and produced in endothelium, megakaryocytes, and subendothelial connective tissue. Activated platelets release into the plasma the contents of their granules, which activate other platelets.

In the extrinsic *tissue factor* (TF) pathway, after vessel damage blood-based coagulation factor VII links with TF, which is present in the subendothelial tissue and fibroblasts as well as in a smaller quantity in circulating form on monocytes, to form an activated complex TF-FVIIa. FVII is also activated by FXa, FIXa, FXIIa and thrombin. Under some pathologic circumstances, TF is expressed also by monocytes, neutrophils, endothelial cells, and platelets with increased levels of circulating TF-positive microparticles that amplify the process of coagulation cascade. The activated complex TF-FVIIa activates coagulation factors FIX and FX.

Intrinsic *contact activation* pathway, which mainly activates thrombin, begins with formation of the primary complex on exposed collagen by factor XII, high-molecular-weight kininogen, prekallekerin, and factor XI. Endothelial collagen is exposed only in course of endothelial damage. Factor XII convers in active FXIIa that converts FXI into activated FXIa. FXIa further activates factor IX, which acts with its cofactor FVIII to form tenase complex on a phospholipid surface and to activate factor X to FXa.

In common pathway FXa along with its cofactor FVa, tissue phospholipids, platelet phospholipids and calcium forms the prothrombinase complex, which activates prothrombin to thrombin. Thrombin activates FV and FVIII, releasing the latter from its link with vWF. Thrombin further cleaves circulating fibrinogen to insoluble fibrin and activates factor XIII, which covalently crosslinks fibrin polymers incorporated in the platelet plug. This creates a fibrin network the building block of a hemostatic plug. Thrombin has also pro-inflammatory effects exciting the PAR present on monocytes, lymphocytes, endothelium and dendritic cells. In addition, it is the most important platelet activator activating FVIII and FV and their inhibitor protein C in the presence of thrombomodulin (TM).

PLATELETS

Various studies have highlighted the contribution of blood platelets to the inflammatory process that characterizes MS. These cells may be involved also in the pathophysiology of other neurological diseases, such as Alzheimer's disease, Parkinson's disease and Huntington's disease (47). The role of blood platelets during the acute and chronic phase of inflammation is not marginal. These cells release proinflammatory mediators, display molecules on their surface with inflammatory functions and interact with endothelial cells and leukocytes (48).

Platelets release several proinflammatory mediators. Three types of secretory granules have been described in platelets: dense granules, lysosomes, and alpha-granules (48). The latter type is the most abundant. They are produced during megakaryocyte maturation and are considered crucial for platelet functions. Hundreds of soluble factors are stored in these alpha-granules, including prothrombin, tissue factor, high molecular weight kininogen, chemokines (RANTES, CXCL1, CXCL4, CXCL5, CXCL7, CXCL8, CXCL12, CCL2, macrophage inflammatory protein 1-alpha), proangiogenic and antiangiogenic proteins, growth factors, and inhibitory proteases [e.g., plasminogen activator inhibitor, alpha2-antiplasmin, antithrombin III (AT III), protein S, protease nexin-2, plasminogen, and tissue factor pathway inhibitor](48). Dense granules store ATP, GDP, ADP, 5-HT, Ca, Mg and histamine, whereas lysosomal granules contain glycohydrolases, and acid proteases (48). Interestingly, platelets synthesize and secrete matrix metalloproteinases (MMPs) as well as tissue inhibitors of MMPs (TIMPs). The main MMP in platelets is MMP-1, which is important because it activates protease activated receptor 1 (PAR-1), which, in turn, is important for platelet aggregation (48). Through the cyclooxygenase (COX) and platelet activating factor (PAF) pathways, platelets are also able to synthesize lipid mediators, including eicosanoids. PAF can also induce the production of IL-1beta in platelets (48).

Platelets express many molecules on their surface that play a role during the inflammatory response. P-selectin, which translocates from the granules to the surface during the platelet activation, can interact with leukocytes and endothelial cells through the interaction with P-selectin glycoprotein-I (PSGL-1). Platelets express also CD40L on their surface and release its soluble form during activation. The latter, (49) together with PAF (50) and MMPs (51), is crucial in order to increase the permeabilization of the blood brain barrier (BBB).

Whether platelet activation is a primary event of MS pathogenesis or it is secondary to endothelial injury is still matter of debate (52). Platelets interact with leukocytes at the endothelium of the BBB by releasing the adhesion molecule PECAM-1 that triggers leukocyte infiltration (53). Moreover, PAF disrupts endothelial BBB junctions and PAF receptors are up-regulated in MS lesions (53).

Platelets are among the first cell types to begin the inflammatory response in the CNS in the acute phase of MS immune response and may be important to trigger and amplify it by producing significant amounts of IL-1alpha (54).

Finally, activated platelets can produce large amounts of Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) that can cause damage to proteins, lipids and nucleic acids, leading to death of CNS cells. Importantly, CNS has low antioxidant defenses and the composition of myelin seems particularly vulnerable to ROS (55).

The importance of platelets in MS and in EAE has been demonstrated also in experimental settings. Platelet depletion

has been found to ameliorate the course of EAE (56). Moreover, during EAE, platelets have been demonstrated to activate in response to sialated glycolipids integrated into neuronal and astroglial lipid rafts found within the BBB (57).

Starossom et al demonstrated that glatiramer acetate (GA), a disease modifying treatment for MS, significantly inhibited thrombin-induced activation of human and mouse platelets (58). They showed that GA was able to inhibit calcium influx, upregulation of CD62P and other markers of activation and aggregation in human and mouse thrombin-activated platelets. They also found that GA significantly reduced platelet-induced upregulation of CD86 and MHC class II on macrophages, leading to a decreased platelet-mediated activation of macrophages.

THROMBIN

Thrombin is a 36-kDa serine protease, a key enzyme in the coagulation cascade formed after cleavage of its precursor, prothrombin (with a molecular weight of ∼72-kDa), by the coagulation factor Xa (59). Prothrombin contains gamma-carboxyglutamic acid, is synthesized in the liver and released into bloodstream. It can be activated by vascular injury, through limited proteolysis following upstream activation of the coagulation cascade. Serum inhibitors and its own action regulate the activity of thrombin. With its procoagulant and anticoagulant functions, the role of thrombin is pivotal in thrombosis and haemostasis. However thrombin has also hormone-like properties that can influence many cells, including platelets, lymphocytes, neurons and astrocytes (60).

Thrombin converts fibrinogen into fibrin and activates factor XIII, affecting the cross-linking of fibrin monomers to produce a stable fibrin clot. Thrombin's function is peculiar as it has both procoagulant and anticoagulant functions. The latter is mediated through binding to TM, a receptor protein expressed on the endothelial membranes, triggering a series of reactions that lead to fibrinolysis. The endothelial protein C receptor (EPCR), shed from the endothelial cells by inflammatory mediators and thrombin, increases protein C activation by the thrombin-TM complex, and inhibits leukocyte extravasation. Interestingly, TNF alpha can down-regulate EPCR, and TM (61).

Depending on the concentrations, thrombin has been demonstrated to have both neuroprotective and pro-apoptotic effects (6, 62). At low to moderate concentrations, thrombin is neuroprotective for hippocampal neurons and astrocytes that lose their star morphology and maintain their supportive role in the production of glutathione and in the reduction of glutamate (7). On the contrary, at high concentrations, thrombin is able to induce cell death (63). In fact, after exposure to high concentrations of thrombin, astrocytes become reactive and lose their neuroprotective and supportive functions, microglia proliferate and produce reactive oxygen species, IL-1β, and TNFα. Moreover, high concentrations of thrombin may produce axonal damage and retraction, intracellular calcium upregulation, and finally cell death. Furthermore, thrombin can induce BBB damage by digestion of extracellular matrix mediated by MMPs (7).

Another function that thrombin has in common with activated factor VII (FVIIa) and FXa is the activation of PARs family proteins, expressed on the surface of several tissues (preferentially PAR1 and PAR3 rather than PAR4 due to their hirudin-like motif) (64) and involved in hemostasis, phlogosis, cancer development, and embryologic differentiation (65).

There are two main thrombin inhibitors: Protease nexin 1 (PN-1) and ATIII. PN-1 is a 47 kDa serine protease inhibitor (SERPIN) that acts as a suicide substrate for thrombin and urokinase-type plasminogen activator (66). It represents the most abundant and potent endogenous brain thrombin inhibitor (67). The expression of PN-1 is high in the brain and this glycoprotein is secreted by glial cells (68) and neurons (69). ATIII is also a SERPIN normally expressed in the liver and at low levels in brain tissue (70, 71). ATIII is a non-vitamin K-dependent protease that inhibits the activity of thrombin and factors IXa and Xa. These SERPINs have been demonstrated to be highly expressed in mice with EAE. The expression of PN-1 in the brain of the mice with EAE peaks at day 8 post-immunization (during the preclinical phase), whereas ATIII peaks at day 13, when the mice experience the highest clinical score and correlate to the disease severity (6).

Significantly higher plasma levels of both prothrombin and factor X have been found in relapsing-remitting MS whereas increased levels of prothrombin have been found in secondary-progressive MS patients compared to healthy controls (9). Conversely, no significant difference was found between controls and patients with both primary-progressive MS and NMOSD. Interestingly, relapse free time negatively correlated with level of either prothrombin, factor XII, or factor X indicating disease exacerbation as a condition characterized by increased coagulation activity (9). Similarly, the speed of thrombin generation was found faster in relapsing-remitting than in primary progressive MS or healthy controls and correlated with time from clinical diagnosis likely reflecting the differential active proinflammatory state in each MS subtype (72). Dermatan sulfate and heparin inhibit the generation of thrombin activity and both have been demonstrated to be effective therapeutic agent for EAE (73–75).

Drugs available to block thrombin action include heparins, hirudins (lepirudin and bivalirudin), vitamin K antagonists and a new generation of direct thrombin inhibitors such as dabigatran and argatroban.

FIBRINOGEN

Fibrinogen is a soluble 340-kDa glycoprotein comprised of three distinct polypeptide chains: A-alpha, B-beta, and gamma produced in the liver by the hepatocytes (76). Plasma concentration of fibrinogen is 2–4 g/L, and its half-life is about 4 days (77). Fibrinogen represents an acute-phase reactant, therefore its plasma concentrations increase during inflammatory response. Thrombin cleaves off fibrinopeptides A and B from the fibrinogen molecule, exposing multiple polymerization sites, leading to the polymerization, formation of insoluble and stable fibrin clot and finally, with the involvement of circulating platelets, formation of a platelet plug (78). Platelets

bind to the C terminus of fibrinogen's gamma-chain binds, through their surface αIIbβ3 integrin receptor, facilitating the formation of a platelet plug (41).

The deposition of fibrin is frequently associated with inflammation (40) and fibrin can increase the expression of several cytokines which, in turn, modulate cell adhesion, and migration (79). The pattern of fibrin deposition in MS coincides with the areas occupied by demyelinating lesions (80), and with the areas characterized by axonal damage (81). Interestingly, fibrin deposition may precede the formation of demyelinating lesions (82–84).

Plasminogen and fibrinogen were found to be lower in MS compared to healthy controls (85). These results have been explained by a possible fibrinogen consumption and fibrin formation due to activation of coagulation cascade leading to up-regulation of fibrinolytic system with both increased plasmin's and reduced plasminogen's levels. A recent study showed in patients with both clinically isolated syndrome (CIS) and relapsing-remitting MS that a high plasma fibrinogen levels had a high specificity and specificity, but a low sensitivity for detection of active lesions on MRI during relapses supporting a role of fibrinogen on the development of MS lesions (86). A microarray study has demonstrated the presence of fibrinogen transcripts in chronic lesions of MS patients (87). Fibrinogen is able to directly activate microglia *in vitro* and increase its phagocytic ability (88). The importance of fibrinogen, especially in the early phases of MS, has been postulated and eventually demonstrated in mice with EAE, in which the leakage of fibrinogen from the BBB is crucial for microglial activation (89). The deposition of fibrin interferes with axonal regeneration (4) and pharmacologic removal of fibrin in EAE mice has been demonstrated to suppress disease development and improve the resulting disability (90, 91). Fibrinogen can bind to members of three major families of integrins, beta-1 (alpha5-beta1), beta-2 (CD11b/CD18 and CD11c/CD18), and beta-3 (alpha-v-beta3), that are expressed by leukocytes on their surface (92).

The conversion of fibrinogen to insoluble fibrin exposes the cryptic epitope γ377–395. This epitope in crucial for the binding of fibrin to the integrin receptor CD11b/CD18, expressed by microglia (41). Fibrinogen induces release of ROS in microglia and its signaling through CD11b/CD18 is necessary for the formation of perivascular microglial clusters and axonal damage in EAE (89). By the activation of CD11b/CD18 pathway, fibrinogen can stimulate the production of tissue factor (93) and Tumor Necrosis Factor (TNF) (94) by monocytes. Furthermore, the binding of fibrinogen to CD11b/CD18 can result in activation of extracellular signal-regulated kinase 1/2 (ERK1/2) or the phosphoinositide-3 kinase (PI3K) pathway, important for neutrophil survival (95). Nuclear factor kappa B (NF-kappaB) pathway is also activated by fibrinogen and results in increased production of IL-1alpha in monocytes (96).

Mice with EAE, treated with pharmacological depletion of fibrinogen showed a direct reduction of microglia activation (88, 97). An interesting transgenic mouse model (Fib-gamma$^{390-396A}$), characterized by the suppression of the interaction of fibrinogen with CD11b/CD18 was studied in order to analyse the exact role of fibrinogen in EAE. The Fib-gamma$^{390-396A}$ mice with EAE had better clinical scores, decreased inflammation, increased survival rate, and improved motor function than laboratory controls.

A new generation of inhibitors of the coagulation pathway, for example inhibiting fibrinogen binding to CD11b/CD18, with decreased haemorrhagic side effects have been proposed as future treatments of chronic inflammatory diseases, including MS (15, 41). Recently, fibrin-targeting immunotherapy with monoclonal antibody 5B8, targeted against the cryptic fibrin epitope $\gamma_{377-395}$, has been demonstrated to inhibit autoimmunity- and amyloid-driven neurotoxicity without globally suppressing innate immunity or interfering with coagulation in MS and Alzheimer's disease (42).

FIBRINOLYTIC SYSTEM AND ANTICOAGULANT PATHWAYS

Several studies have documented presence of products of the fibrinolytic system in MS. Plasminogen is a 93-kDa single chain glycoprotein with an average plasma concentration of 0.2 mg/mL (98). Tissue plasminogen activator (tPA) is a 69-kDa glycoprotein consisting of 527 or 530 amino acids, released as a single chain enzyme, with an average plasma concentration of 5–10 ng/mL (98, 99). Urokinase plasminogen activator (uPA) exists in two forms with different molecular weight: high molecular weight uPA [54 kD], and low molecular weight uPA [33 kD], with a plasma concentration of 1 ng/mL (98). The binding of uPA to its receptor (uPAR) is crucial for the activation of plasminogen to plasmin (100). Leukocytes constitutively express uPAR and the presence of soluble forms of uPAR has been associated with BBB disruption in neurological diseases (101).

Both tPA and uPA convert plasminogen to plasmin and, through a positive feedback mechanism, plasmin cleaves both tPA and uPA, transforming them from their single chain forms to the more active double-chain forms (102). Fibrin represents the major plasmin substrate and enhances plasmin generation by binding both plasminogen and tPA on its surface, increasing also the affinity between tPA and plasminogen (102). Plasmin cleaves fibrin, generating soluble degradation products.

Samples of CSF from MS patients have increased tPA activity as compared to control subjects and the increase in tPA activity correlates with the disease progression (103, 104). In mice with EAE, tPA is detected in macrophages of inflammatory cuffs in the spinal cord (105), and tPA mRNA and protein expression are upregulated, also in neurons (106, 107). Moreover, it has been shown that in MS and in EAE, the uPA, and tPA mediated activation of ubiquitous plasminogen represents a key step in the activation cascade of the four classes of matrix MMPs: collagenases, stromelysins, membrane-type MMPs, and gelatinases. MMPs contribute to the extravasation of circulating lymphocytes and monocytes, by modifying matrix components and can generate encephalitogenic peptides from myelin basic protein (107).

Neurons and microglia in the CNS express tPA (108) and it has been shown that tPA has also an interesting function in neural plasticity. In fact, tPA system has a significant role also

in brain tissue remodeling and cell migration (109, 110). tPA is secreted during axonal growth and regeneration, facilitating nerve outgrowth through a tissue matrix (111). tPA levels are reduced in mature brain, with highest levels found in the dentate gyrus and cerebellum (112). tPA mRNA expression is enhanced during cerebellar motor learning tasks in rats and this is considered as a mechanism of synaptic plasticity (113).

In contrast with the increased tPA activity in the CSF from MS patients, tPA deficient mice experienced an early and a more severe and acute EAE as compared to wild-type controls. On the contrary, uPAR deficient mice experienced a delayed and less acute EAE, with a delayed but steadily increased infiltration of inflammatory cells (114). These data highlight the complex role of tPA and uPAR in the pathogenesis of EAE by regulating fibrin deposition at sites of inflammation and cell trafficking into the CNS (114).

Protein C is a vitamin K-dependent zymogen of a serine protease. Protein C is activated by thrombin when both bind to endothelial cell TM. The endothelial protein C receptor (EPCR) also binds protein C. Activated protein C (APC) is a natural anticoagulant. With its cell membrane localizing cofactor, protein S (PS), APC binds to endothelium and activated platelet membranes and intervenes in degradation of procoagulant factor Va and VIIIa, consequently limiting further thrombin formation (115). Impaired TM-dependent aPC generation aggravates EAE disturbing myelination and mitochondrial function and increasing mitochondrial ROS (116). Soluble TM ameliorates EAE and dampened demyelination in the cuprizone-diet model. Recombinant TM ameliorated the clinical and pathological severity of EAE by suppressing plasma levels of inflammatory cytokines (117). Protein C deficiency can be inherited or acquired and causes an important predisposition to thrombosis. However, the roles of APC are not limited to coagulation. APC helps maintain endothelial cell integrity (118), inhibits leukocyte adhesion and BBB crossing (119), reduces the production of pro-inflammatory cytokines (118, 120–123) and has anti-oxidant properties (124). EPCR has structural similarities with the MHC1/CD1 family of molecules, suggesting further possible roles of the protein C pathway in regulating the immune response (118, 125).

Protein C activity was found reduced in MS patients independently from their lupus-anticoagulant activity or factor Va resistance (126). The role of APC in MS has become matter of debate (125).

ANTIPHOSPHOLIPID ANTIBODIES

In common clinical practice serum reactivity for antiphospholipid antibodies (APLs), reduction of prothrombin time or increase of both fibrinogen and D-dimer (a product of fibrin degradation) are accepted indicators of increased coagulation activity, which may indicate intravascular thrombosis. APLs have been widely studied in MS with conflicting results, in part depending on the type of antibodies used in the assays (127). Recently, most authors agree on a higher APL reactivity in MS than in healthy controls even if it is variable according to different disease forms and phases (127–130). APL positivity in MS patients is associated with a more severe clinical and MRI disease progression supporting the concept that the degree of involvement of coagulation in inflammatory-demyelinating diseases is proportional to disease severity (127, 131). Increased APL reactivity has been found in both relapsing-remitting and secondary-progressive MS compared to healthy controls with the highest APL positive rate (> 50%) during the clinical exacerbations and with its decrease a few months after relapse (132). Interestingly, among a broad different APLs only anti-prothrombin and anti-β2 glycoprotein-I antibodies were independently higher in relapse compared to both remission and secondary progressive phase (132). Furthermore, as an example of the close correlation between neurodegenerative and thrombogenic mechanisms in MS, it was showed that high total and LDL levels of cholesterol in MS patients were significantly associated with both disease duration and disability as well as with anti-annexin V positivity (133). Since hydroxychloroquine, a drug with anti-infective, anti-inflammatory and anti-thrombotic properties, protects the annexin V anticoagulant shield from disruption by antiphospholipid antibodies on phospholipid bilayers (134), annexin V has been proposed as a new attractive therapeutic target in MS (135). Ongoing clinical trials are currently testing the effect of hydroxychloroquine in slowing down the progression of clinical disability in MS (ClinicalTrials.gov identifiers NCT02913157 and NCT03109288).

ALTERATION OF THE COAGULATION PATHWAY IN NEUROMYELITIS OPTICA SPECTRUM DISORDERS

Neuromyelitis optica spectrum disorders (NMOSD) represent a more severe CNS inflammatory-demyelinating disorder than MS, characterized by optic neuritis, longitudinally extensive myelitis, and water channel aquaporin-4 autoantibody positivity (136). There are only a few studies comparing coagulation markers including APLs and thrombotic events between MS and NMOSD. A higher anticardiolipin positive rate was found in NMOSD compared to MS patients, associated with a greater ATIII activity and D-dimer level (137). Farber and co-authors reported a significantly higher association of venous thromboembolism with NMOSD than with MS, within 6 weeks of acute relapse, after its correction for influencing factors such as age, length of stay and ambulatory disability (138). These findings are not surprising since a coagulation activation is greater in so far as there is a more severe disease. Partially common pathogenetic mechanisms mediated by coagulation factors and complement, which are part of innate immunity and activate the adaptive immunity, have been supposed for both inflammatory-demyelinating and thrombotic (e.g., antiphospholipid syndrome) CNS diseases (139).

In a recent study investigating the coagulation status of NMO and MS patients, Zhang and colleagues demonstrated that fibrinogen levels were significantly higher in NMO and MS patients compared to non-inflammatory neurological

disease subjects as a control group and that there was no difference between MS and NMO. Moreover, fibrinogen levels were significantly associated with the severity of the disease (19). In another study, Göbel and colleagues showed that fibrinogen level was significantly lower in NMOSD compared to both relapsing-remitting and secondary-progressive MS, albeit NMOSD patient's number was low (10). Undoubtedly, peripheral blood measurement of coagulation factors in organ-specific diseases such as MS and NMOSD has some limitations, however their role in the pathogenesis of these disease is matter of intense debate.

MS PATHOGENIC HYPOTHESIS INVOLVING COAGULATION PATHWAYS

Although there is only a few histopathological examinations of MS samples from the acute phase of the disease (140–143), they have shown early microglia activation (pre-demyelinating lesion stage) after fibrinogen leakage through the damaged BBB (143), as well as the presence of some clotting factors in chronic active lesions identified by a proteomic approach (144).

Moreover, quantitative contrast-enhanced MRI studies found a low grade of BBB leakage in visibly non-enhancing MS lesions, distinct from a significantly greater BBB damage in visibly enhancing lesions (145). The authors showed that this low grade BBB leakage was not influenced by ongoing immunomodulatory therapies supposing a permanent structural changes of vessel walls in chronic long-standing lesions. The abnormalities in "tight" junctions (TJ) between adjacent endothelial cells, which are part of BBB, were found even in normal-appearing white matter (NAWM) (146). The TJ abnormality was not confined to microvasculature but involved the full range of vessels either in MS lesions or in NAWM by a possible effect of pro-inflammatory soluble mediators such as cytokines acting "a distance" (147). The association of fibrinogen leakage with astrocyte's processes as well as with TJ abnormality was most pronounced in active lesions. Also *ex vivo* pathological-imaging correlations using magnetization-transfer ratio and diffusion-tensor imaging showed subtle abnormalities in NAWM, closes to MS lesions and correlated with diffuse microglia activation along with impaired axonal and myelin integrity (148). Furthermore, dynamic-susceptibility enhanced T2*-weighted MRI demonstrated prolonged brain blood mean transit time and decreased cerebral blood flow in both white and gray normal-appearing matter of relapsing-remitting MS patients as well as in NAWM of patients with CIS suggesting a continuum of tissue perfusion slowdown starting from white matter and spreading to gray matter (149).

Similarly, histological studies in chronic MS have showed a small deposition of extravascular fibrin in chronic, non-active MS lesions suggesting a persistent BBB damage (82). This steady BBB dysfunction, likely due to its permanent reparative thickening, could determine a continuous low outflow of soluble mediators and inflammatory cells from blood to CNS. Inflammation in progressive MS occurs in the form of compartmentalized immune reaction behind a closed/repaired BBB leading to a formation of lymph-follicle like structures in the meninges and perivascular spaces (150). These local structures produce cytokines, chemokines, and intrathecal immunoglobulins leading to brain damage and disease progression. Compartmentalized inflammation could in part explain the incongruity between greater brain atrophy and fewer radiological inflammatory lesions in progressive MS.

Based on these observations, we could speculate that "soluble" clotting factors and pro-inflammatory mediators released from platelet's granules may in part mediated MS pathogenesis by innate immune activation and consequent adaptive immune stimulation. They pass persistently and subtly in the CNS due to a long-lasting BBB dysfunction in the course of the disease, and more strikingly during MS relapses through acute BBB damage. The big question remains: what triggers these processes and why they occur only in a subgroup of people?

In the health, CNS "immune privilege" status is determined by BBB integrity together with neurons, glia, and the extracellular matrix, which form the neurovascular unit regulating immune responses in the CNS (151). Cell-contact signals expressed by neurons and glia (as a result of neuronal cell adhesion molecules) inhibit both microglia activation and maturation of antigen-presenting cells. Additionally, neurons produce chemokines, neuropeptides, neurotransmitters, and neurotrophins acting as neuroimmunoregulatory mediators to inhibit microglia activation and limit the survival of activated lymphocytes. The impairment of these cell-contact signals due to neuronal damage depletes CNS homeostatic protective environment increasing neuroinflammation (151). This occurs physiologically in aging due to neuronal loss, genetic mutations, oxidative, or metabolic stress with endoplasmic reticulum and mitochondrial dysfunction.

Neurodegeneration seems to be closely associated with neuroinflammation not only in MS, but also in other neurodegenerative disorders due to a chronic activation of the local innate immunity, and in particular of microglia. Microglia is involved in defense against CNS infections and in cleaning of cell debris and damaged proteins, however, its excessive or prolonged activation may cause tissue damage (151). Furthermore, local innate immune activation largely determines adaptive immune response. In fact, neuroinflammation manifests not only with activation of local microglia, astrocytes, oligodendrocytes but also with a recruitment of peripheral innate immune cells such as natural killer, natural killer T cells, mast cells, granulocytes and γδ-T cells as well as of circulating lymphocytes and myeloid cells from the periphery. Activated microglia, by secreting Il-1α, TNF and C1q, induce reactive A1 astrocytes that induce the death of neurons and oligodendrocytes due to their lost ability to promote neuronal survival, outgrowth, synaptogenesis and phagocytosis in neurodegenerative disorders (152). Mutually, systemic immune activation influences the local innate immunity. Peripheral ongoing and precedent infections determine the so-called "primed" environment that increases CNS susceptibility to injury. Experimental studies showed that peripheral inflammation is associated with disease exacerbations in experimental models of either MS, stroke or other neurodegenerative diseases (151).

The same mediators and cells that are involved in neuroinflammation and neurodegeneration, have provide for CNS repair, growth and development (151). Astrocytes are the major source in the CNS of nerve growth factor and glial cell line-derived neurotrophic factor, which are secreted also by T cells. Microglia/macrophages release growth factors and cytochines stimulating axonal regeneration and oligodendrocyte-precursors maturation. However, as in other neurodegenerative diseases, in MS spontaneous regeneration or self-repair of damaged CNS tissue is inadequate compared to the extent of neuroinflammation and neurodegeneration, which co-exist in different degree depending on many factors including tissue localization, lesion formation stage, disease phase and immune system age (153). In acute and limited CNS injury, neuroinflammation could circumscribe neurodegeneration and stimulate regeneration. Conversely, chronic neuroinflammation leads to increased neurodegeneration that in turn impairs homeostatic protective environment further amplifying neuroinflammation and weakening regeneration.

Recurrent or chronic infections lead to immunothrombosis, which is activated by blood-borne pathogens and circulating damaged self-components (46), and are presumably among the causes of chronic neuroinflammation. There is a continuous crosstalk between the immune system and blood coagulation components, closely inter-correlated, and essential for an effective immune response to limit pathogen dissemination and support pathogen killing and tissue repair (45). However, over-activation of coagulation may induce thrombotic complication, excessive inflammation, and tissue damage. Infections cause the modification of proteins' structure and function by increased oxidative stress. A progressive trend of oxidation of several serum proteins including coagulation factors from remission to relapse was found in relapsing-remitting MS patients (154). Moreover, a possible role of transient virus-BBB interactions during viral infections triggering focal inflammation, BBB breakdown and demyelination in some cases of MS has been previously supposed (155). The study of gene-environment interactions showed the relevant relationship between MS genotype and Epstein Barr virus, however also other viruses may perturb the human molecular system by common and unique virus strategies (156). A Danish nationwide nested case-control study found that children with MS have more infections in the 3 years preceding MS beginning that is influenced also by their immune response to infections (157). It is known that several micro-organisms play a role in MS relapse and pathogenesis. Additionally, a recent review based on Cochrane library guidelines concluded that some micro-organisms such as Human herpesvirus 6, Chlamydia pneumoniae and Torque teno virus have contributed to making MS a chronic progressive disease, but it does not rule out the role of other pathogens in MS progression (158). Finally, by immunohistochemistry using specific antifungal antibodies, the microfoci of fungal structures in CNS tissue sections, which was also positive for bacteria, were observed in MS patients but not in controls supporting the polymicrobial infections as a possible cause of MS (159).

CONCLUSIONS

In addition to histopathological observations on early microglia and astrocyte activation after fibrinogen leakage through the damaged BBB in MS and EAE, many observational and experimental studies in MS and NMOSD showed their association either with pro-thrombotic risk factors, increased prevalence of thrombotic and vascular diseases or involvement of clotting factors as well as of complement and platelets, other components of coagulation cascade.

Taken all together, there is evidence for a role of coagulation in the pathogenesis of both MS and NMOSD. It will be important to better define the exact links between immune response and coagulation pathways dysregulation. The new challenge ahead will be to understand how this interaction converges on recently described mechanisms of neurodegeneration induced by activated microglia and reactive astrocytes. This approach may lead to improved treatment options (e.g., polytherapies), not only for demyelinating diseases but also for other neurodegenerative conditions.

AUTHOR CONTRIBUTIONS

DP, MI, MS, and TK all contributed equally to the literature research and writing.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CORRECTION
published: 12 March 2019
doi: 10.3389/fneur.2019.00210

Corrigendum: A Perspective of Coagulation Dysfunction in Multiple Sclerosis and in Experimental Allergic Encephalomyelitis

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Keywords: coagulation, neuroinflammation, multiple sclerosis, neuromyelitis optica spectrum disorders, thrombosis

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Approved by:
Frontiers in Neurology Editorial Office, Frontiers Media SA, Switzerland

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Specialty section:
This article was submitted to Multiple Sclerosis and Neuroimmunology, a section of the journal Frontiers in Neurology

Received: *15 February 2019*
Accepted: *18 February 2019*
Published: *12 March 2019*

Citation:
Plantone D, Inglese M, Salvetti M and Koudriavtseva T (2019) Corrigendum: A Perspective of Coagulation Dysfunction in Multiple Sclerosis and in Experimental Allergic Encephalomyelitis. Front. Neurol. 10:210. doi: 10.3389/fneur.2019.00210

A Corrigendum on

A Perspective of Coagulation Dysfunction in Multiple Sclerosis and in Experimental Allergic Encephalomyelitis

by Plantone, D., Inglese, M., Salvetti, M., and Koudriavtseva, T. (2019). Front. Neurol. 9:1175. doi: 10.3389/fneur.2018.01175

In the published article, there was an error regarding the affiliations for Marco Salvetti. As well as having affiliation "3," he should also have "IRCCS Istituto Neurologico Mediterraneo (INM) Neuromed, Pozzilli, Italy."

There was also an omission in the affiliation for Tatiana Koudriavtseva. Instead of "Department of Clinical Experimental Oncology, Regina Elena National Cancer Institute, Rome, Italy", it should be "Department of Clinical Experimental Oncology, IRCCS Regina Elena National Cancer Institute, Rome, Italy".

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

frontiers in Neurology

ORIGINAL RESEARCH
published: 04 January 2019
doi: 10.3389/fneur.2018.01139

Blocking Thrombin Significantly Ameliorates Experimental Autoimmune Neuritis

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Specialty section:
This article was submitted to Multiple Sclerosis and Neuroimmunology, a section of the journal Frontiers in Neurology

Received: 02 August 2018
Accepted: 10 December 2018
Published: 04 January 2019

Citation:
Shavit-Stein E, Aronovich R, Sylantiev C, Gera O, Gofrit SG, Chapman J and Dori A (2019) Blocking Thrombin Significantly Ameliorates Experimental Autoimmune Neuritis. Front. Neurol. 9:1139. doi: 10.3389/fneur.2018.01139

Thrombin and its protease-activated receptor 1 (PAR1) are potentially important in peripheral nerve inflammatory diseases. We studied the role of thrombin and PAR1 in rat experimental autoimmune neuritis (EAN), a model of the human Guillain-Barré syndrome (GBS). EAN was induced by bovine peripheral myelin with complete Freund's adjuvant (CFA). Thrombin activity in the sciatic nerves, clinical scores and rotarod performance were measured. Thrombin activity in the sciatic nerve was elevated in EAN compared to CFA control rats (sham rats) ($p \leq 0.004$). The effect of blocking the thrombin-PAR1 pathway was studied using the non-selective thrombin inhibitor N-Tosyl-Lys-chloromethylketone (TLCK), and the highly specific thrombin inhibitor N-alpha 2 naphtalenesulfonylglycyl 4 amidino-phenylalaninepiperidide (NAPAP). *In-vitro* TLCK and NAPAP significantly inhibited specific thrombin activity in EAN rats sciatics ($p<0.0001$ for both inhibitors). Treatment with TLCK 4.4 mg/kg and NAPAP 69.8 mg/kg significantly improved clinical and rotarod scores starting at day 12 and 13 post immunization (DPI12, DPI13) respectively ($p < 0.0001$) compared to the untreated EAN rats. In nerve conduction studies, distal amplitude was significantly lower in EAN compared to sham rats (0.76 ± 0.34 vs. 9.8 ± 1.2, mV, $p < 0.0001$). Nerve conduction velocity was impaired in EAN rats (23.6 ± 2.6 vs. sham 43 ± 4.5, m/s $p = 0.01$) and was normalized by TLCK (41.2 ± 7.6 m/s, $p < 0.05$). PAR1 histology of the sciatic node of Ranvier indicated significant structural damage in the EAN rats which was prevented by TLCK treatment. These results suggest the thrombin-PAR1 pathway as a possible target for future intervention in GBS.

Keywords: thrombin, experimental autoimmune neuritis, node of Ranvier, PAR1, Guillain-Barré syndrome

INTRODUCTION

The inflammatory neuropathies are a diverse group of conditions. They share common features of inflammatory damage to myelin/axons (1, 2). Guillain-Barré syndrome (GBS) is a representative of the inflammatory neuropathies. The most common variant of GBS is acute inflammatory demyelinating polyneuropathy (AIDP), characterized by a progressive and sometimes painful weakness of limbs (3). GBS has an incidence rate of 0.8–1.9 cases per 100,000 people per year

(4), and is potentially fatal. Although patients with GBS show a high recovery rate, it still causes extensive disability. 20 to 30% of the patients suffer respiratory failure (5). During the 6 months period after the onset of the disease, 20% of adult patients are still unable to walk without support (6).

The mechanisms by which nerve conduction dysfunction is generated in GBS are a topic of debate (2, 7). GBS pathophysiology includes a wide variety of immune responses. T cells are thought to participate mainly in the induction phase of the disease, whereas the progressive phase of the disease includes humoral mediated response (5). Perivascular presence of T cells can be seen in the experimental autoimmune neuritis (EAN) rat model for GBS soon after disease induction. The T cells activate monocytes to macrophages. The macrophages in turn, participate in nerve damage via inflammatory cytokines (8). B cells are thought to play a role via the creation of autoantibodies cross reactive to the lipopolysaccharide (LPS) of Campylobacter jejuni and the ganglioside GM1 (9). These autoantibodies bind nodal membrane and fix complement. They are suspected to be the cause of nodal dysfunction. They may also lead to axonal degeneration (10), and cause direct axonal damage (11). Recent evidence connects Zika infections with GBS as well, with negative anti-ganglioside antibodies (12, 13).

Current treatments of GBS include plasmapheresis and intravenous immunoglobulin (IVIG). The physiological mechanisms by which these treatments improve patients' condition are still unclear as is GBS pathophysiology itself. Despite treatment, 9–17% of GBS patients die or remain severely disabled (14). Other treatments, including adding steroids to IVIG, showed only minor short term improvement (15).

Inflammatory states are tightly related to the coagulation system, with extensive crosstalk between the two, as can be seen in the pathogenesis of vascular diseases (16, 17). This relationship was demonstrated in experimental autoimmune encephalomyelitis (EAE), the animal model for multiple sclerosis, where elevation of thrombin was shown to precede the appearance of clinical signs (18–20).

Thrombin is a key factor in the coagulation cascade and is known to participate in a wide variety of cellular and physiological processes including inflammation (21, 22), neurotrauma (23, 24), neuronal plasticity after vascular damage (25) and neuronal degeneration (26). Thrombin has a known role in central nervous system inflammatory diseases as shown in the EAE model (18, 19). Thrombin has been previously shown to have a role in the peripheral nervous system (PNS) and in pathophysiology of pain (27). We have demonstrated that the thrombin receptor protease active receptor 1 (PAR1) is localized to the nodes of Ranvier in peripheral nerves and that its activation creates a conduction block (28). These findings have suggested that excess levels of thrombin with PAR1 activation may play an important role in causing conduction failure in inflammatory diseases and thrombotic nerve diseases such as infarctions or trauma.

The aim of this study was to evaluate the effect of thrombin inhibition on clinical, histological and electrophysiological EAN parameters.

MATERIALS AND METHODS

Animals

Females Lewis rats were purchased from Harlan (Jerusalem Israel) at age of 8 weeks, and were housed at the animal facility at Tel Aviv University Medical School 1 week prior to experiment for acclimatization. The animals were kept under standard conditions of $23 \pm 1^{\circ}C$ with a 12 h light-dark cycle and access to food and water ad libitum. The animals were in weight range of 160–180gr at the beginning of the experiment.

All experiments were approved by the animal welfare committee (M-06-004) and appropriate measures to avert pain and suffering to the rats were taken.

Induction of EAN

The animals were immunized with 200 µl of inoculum containing 10 mg of bovine peripheral myelin (BPM), prepared in our laboratory from a bovine spinal cord roots, using the method of Kadlubowski (29), and 4 mg of *Mycobacterium tuberculosis* (strain H37RA; Difco, Detroit, Michigan) emulsified in 100 µl saline and 100 µl of complete Freund's adjuvant (CFA), injected into hind footpad and one subcutaneous site (flank). Control animals (sham) were injected with an inoculums containing *Mycobacterium tuberculosis* emulsified in saline and CFA. Immunization of the animals was marked as day 0, and the days that followed were marked as days post-immunization (DPI). Subjective clinical evaluation for neurological signs was performed before immunization and every 1–2 days (total of 24 time points) by an evaluator blinded to treatment. Severity of weakness was graded as follows: 0-undetected; 1-limp tail; 2-abnormal gait; 3-mild paraparesis; 4-severe paraparesis; 5-paraplegia; 6-paraplegia with forelimb involvement; 7-paraplegia with forelimb involvement and respiratory distress; and 8-moribund or dead (30).

Motor Performance

Motor performance was assessed by means of a rotarod test. The rats were pre-trained before immunization to run on the rod, which rotated at a fixed speed of 13 rounds per minute. After immunization the rats were assessed every 1–2 days (total of 24 time points). The rats were allowed to run for up to 60 sec on each trial, or until they fell off. The mean of the three consecutive trials was recorded for each rat.

Treatment Protocol for TLCK and NAPAP

N-alpha 2 naphtalenesulfonylglycyl 4 amidino-phenylalaninepiperidide (NAPAP-pefablock 76308, Fluka), N-Tosyl-Lyschloromethylketone (TLCK-616382) were purchased from Calbiochem, La Jolla, California.

The effect of treatment timing was determined based upon a preliminary study comparing 3 different treatment regimens for TLCK and NAPAP to untreated, adjuvant injected (marked sham) group and saline treated EAN (marked EAN). The specific time frames and number of animals used for the different protocols were chosen based on a previous work (31). First, an evaluation of clinical scores and rotarod scores was made in EAN versus sham rats. Follow-up was conducted in two independent repeated experiments for the sham rats (a total number of 9

animals) and four independent repeated experiments for the EAN rats (a total of 24 animals).

Second, three different protocols were compared to untreated EAN. The protocols included "early protocol" (EP) with daily injections at DPI5-15, "late protocol" (LP) with daily injections at DPI10-20, and "short protocol" (SP) with daily injections for DPI10-15. EP was started before the appearance of EAN symptoms as previously described in the literature (31, 32).

The EP experiment included 8 animals in each treatment group (NAPAP and TLCK). The SP experiment included 4 animals in each treatment group (NAPAP and TLCK). Main study was based on the preliminary study results. The LP protocol was chosen for the main *in-vivo* study, and therefore, was repeated in four independent experiments (a total number of 16 animals in the NAPAP group and 18 animals in the TLCK groups). The animals in the treatment groups were injected with 1 ml carrier solution (saline) containing either 4.4 mg/kg of TLCK (33) or 69.8 mg/kg of NAPAP intraperitoneally (IP) once daily.

Thrombin Activity

In order to measure thrombin activity in the sciatic nerve, it was dissected, washed with ice-cold phosphate buffered saline (PBS) to remove overt blood and moved to a PBS buffer. The activity was measured as previously described in detail (34, 35). Briefly, the PBS surrounding the nerve was evaluated utilizing a fluorometric assay, quantifying the cleavage of the synthetic peptide substrate N-p-Tos-Gly-L-Pro-L-Arg-7- amido-4-methylcoumarin (tos-GPR-AMC; excitation 350 nm, emission 430 nm, Sigma T-0273). Substrate (20 μl, final concentration 10 μM) was added to 140 μl of PBS containing 0.1% bovine serum albumin (BSA). The fluorescence was measured continuously for 20 min at 37°C. The hydrolysis of tos-GPR-AMC substrate was determined from the increase in fluorescence (measured on FL-600 microplate fluorescence reader, BIOTEK, excitation wavelength/bandwidth 360/20 nm; emission wavelength/bandwidth 465/20 nm). Known concentrations of purified human thrombin (Sigma T 0553; 300 units/mg protein) were used in the same assay for calibration.

Localization of PAR1 in Sciatic Nerve Slices

Sciatic nerves were dissected from rats at DPI32. Four animals were randomly selected from each group (sham, EAN, EAN treated with NAPAP and EAN treated with TLCK). 7–8 nodes-containing-fields were analyzed from each animal, adding up to a total of 30 per group. The sciatic nerves were fixed overnight in 4% PFA in 0.1 M phosphate buffer, pH 7.4, and then placed in 30% sucrose for 4 h. Frozen sagittal sections (50 μm) were then cut on a sliding cryostat and collected serially. Slides were then washed with PBS, blocked for 1 h in PBS containing 1% goat serum, 0.2% glycine and 0.1% Triton X-100, and incubated overnight with rabbit anti-PAR1 antibody (1:50) and mouse monoclonal anti-Caspr I (1:500; a generous gift of Professor Elior Peles). After subsequent washes in PBS, the slides were reacted with RRX- and Cy2-coupled secondary antibodies: Rhodamine Red-X-conjugated donkey anti-rabbit (1:400; Jackson Laboratories, Bar Harbor, ME, United States) and cy2-conjugated donkey anti-mouse (1:400; Jackson Laboratories).

Immunofluorescence slides were viewed and analyzed using a confocal ZEISS CLSM 410 microscope, using magnification of X630. Specific localization of PAR1 in sections from sciatic nerves was made using immunofluorescence, utilizing double staining for PAR1 together with specific markers of the node of Ranvier, including the paranodal axonal marker Caspr I. Normal node architecture was defined as the presence of Caspr paranodal staining, and PAR1 staining in the node between the paranodal Caspr stains (28). The data was evaluated blindly.

Electrophysiological Studies

Electrophysiological tests were performed on DPI26. The rats were anesthetized with Equitezin (4% chloral hydrate, 6% sodium pentobarbital, IP, 0.5 ml/100 g). Body temperature was maintained warm by placing the rats on a heating mat. Temperature differences were minimized by conducting the study as soon as the anesthesia had taken effect and by warming the tail with a heating lamp. Electrophysiological studies were conducted on the rats tails in order to minimize an effect of local inflammation in sciatic nerve of the EAN rats (36, 37). Furthermore, length measurements were more accurate at the tail. The tail skin was cleaned carefully with alcohol before the electrodes were placed. Recordings of responses were from the muscles of the tail using a pair of ring electrodes coated with electrode jelly and placed 50 mm distal to the base of the tail. A pair of monopolar needle electrodes were inserted to a depth of 4–5 mm to stimulate the tail nerves. Stimulation was performed 10 (distal site) and 50 (proximal site) mm proximal to the recording electrodes. A ground electrode was placed between the stimulating electrode and recording electrodes. Supra-maximal stimulation, at a range of 3–5 mA was employed, and the low and high frequency filters were set at 10 Hz and 10 kHz, respectively. The responses were displayed on a fully digital recording Keypoint apparatus (Dantec, Skovlunde, Denmark). Both proximal and distal latencies were measured using time intervals from the stimulus artifact to the first deflection from the baseline. To calculate the motor nerve conduction velocity (MNCV), the distance between the stimulation sites (40 mm) was divided by the latency difference. Amplitudes of the compound muscle action potential (CMAP) from both proximal and distal stimulations were measured from the preceding baseline to negative peak.

Splenocytes Proliferation Assay

Single-cell suspensions of freshly dissected spleens were obtained on DPI14 ($n = 4$ per each group) and assayed *ex-vivo* for their response to antigen (myelin) and immune-inducer (LPS) by a proliferation assay. Each well was seeded 2×10^4 cells in 0.2 ml of proliferation medium (RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) containing myelin (10 μg/ml) or LPS (1 μg/ml). The experiments were performed in triplicate in 96-well, flat bottom microplates (Costar, Cambridge, USA). Cultures were incubated for 72 h in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cell proliferation

was determined by a Colorimetric Bromodeoxyuridine (BrdU) Cell Proliferation kit (Roche Applied Science) following manufacturer's instructions. Briefly, 20 µl of BrdU labeling solution diluted 1:100 in culture medium was added to each well for the last 18 h of the assay. After removing the medium, cells were fixed, and anti-BrdU-peroxidase solution was added to each well. Cell cultures were incubated for 90 min at room temperature. The wells were washed and substrate solution was added. The absorbance was measured by means of microplate reader at 370 nm, using 492 nm as reference for subtraction.

Statistics

Statistical analysis of the differences between clinical scores and rotarod progression were assessed by analysis of variance (ANOVA) with repeated measures. The scores at disease peak were compared by one-way ANOVA. The use of ANOVA test with Tukey's *post-hoc* analysis for clinical scores was possible due to the high number of animals, and after normal distribution was evaluated (by D'Agostino-Pearson normality test). The difference in thrombin activity was calculated using two-way ANOVA. One-way ANOVA was used to compare the means of TLCK, NAPAP and sham in the immune biochemical and electrophysiological measures. Dunnett's test was used in ANOVA with multiple comparisons. A two-way ANOVA with Sidak test for multiple comparisons was performed for the effect of EAN and progression in time on clinical score and rotarod. Comparison of immune-histochemical images was performed using Fisher's exact test. Calculations were performed using GraphPad Prism 6.

RESULTS

Clinical Course and Thrombin Activity in the Sciatic Nerve of EAN

EAN rats showed first clinical signs at DPI12 (**Figure 1A**), including flaccid tail and slight paresis rapidly progressing to severe paraparesis. The mean clinical score of EAN rats was significantly elevated when comparing to the sham group [$F_{(13, 403)} = 28.08$, $p < 0.001$ the interaction between all-time points and the two groups was analyzed by repeated measure ANOVA]. Multiple comparisons with Sidak *post-hoc* analysis indicated that the significant increase in clinical score began starting at DPI14, and continued throughout the experiment (2.46 ± 0.35, 0.00 ± 0, mean $\pm$ SEM, compared to sham, $p < 0.01$). The mean rotarod walking time of the EAN rats was found to be significantly decreased when comparing to sham group [$F_{(12, 403)} = 11.87$, $p < 0.001$, the interaction between all-time points and the two groups was analyzed by repeated measure ANOVA]. No neurological signs were observed in the sham group during the experiment. EAN rats demonstrated decreased mean rotarod walking times indicated by multiple comparisons with Sidak *post-hoc* analysis beginning at DPI14 (**Figure 1B**, 41.42 ± 4.8 seconds, mean $\pm$ SEM, compared to sham rats, $p = 0.013$).

Thrombin activity in EAN rat sciatic nerves was found to be increased early in the clinical course of the disease, but was normalized in the progression and at the peak of disease. Thrombin activity was significantly elevated in the EAN rat sciatic nerves compared to sham rats at DPI10 prior to the appearance of clinical signs (**Figure 1C**, 182 ± 17.8 vs. 100 ± 4.85, percent of sham, $p \leq 0.005$). Thrombin levels measured in the sciatic nerves from EAN and sham rats ceased to be statistically different at DPI14.

Both the highly specific thrombin inhibitor NAPAP and the non-selective thrombin inhibitor TLCK reduced the *in-vitro* EAN excess thrombin activity to sham baseline (**Figure 1D**, 182.4 ± 17, 120.7 ± 16.5, 107.4 ± 10.7, percent of sham, respectively, $p < 0.0001$ for both inhibitors). Measurements indicate the specificity of this early thrombin activity.

Inhibition of Thrombin Activity as a Treatment for EAN

We further verified the importance of thrombin-like protease activity in the EAN nerves by performing therapeutic experiments utilizing exogenous protease inhibitors. These included the highly specific thrombin inhibitor NAPAP and the non-selective thrombin and trypsin-like serine protease inhibitor TLCK. The effects of these treatments were compared to saline treated EAN controls.

Preliminary Treatment Protocols Study

Three protocols were assessed as described in detail in the Methods section: an early pre-clinical protocol (EP), a short protocol (SP) covering the period of disease progression and a long protocol (LP) covering both the early clinical stage and peak of disease (**Figure 2**). Clinical score and rotarod scores were recorded for all TLCK and NAPAP treatment protocols. Animals were assigned to different protocol groups prior to the development of disease, but later all of them showed clinical signs of EAN.

The effect of NAPAP treatment protocols on clinical score are presented in **Figure 2A**. Following the onset of clinical deterioration, all treatment protocols were found to significantly reduced disease severity compared to EAN [$F_{(33, 484)} = 3.38$, $p < 0.001$, for the interaction between all-time points and groups, analyses using two-way ANOVA with Dunnett's correction for multiple comparisons]. At disease peak (DPI18) all NAPAP treatment protocols had a similar modest non-significant effect on clinical score relative to EAN [$F_{(3, 31)} = 1.79$, $p = 0.17$, for the comparison between different treatment groups to the EAN, using one-way ANOVA, with Dunnetts' correction for multiple comparisons]. EP and LP treatment protocols improved clinical score during the recovery phase of EAN [$F_{(9, 105)} = 2.149$, $p = 0.0316$ for the interaction between groups and days of recovery calculated using two-way ANOVA with Tukey's *post-hoc* analysis revealing the EP and LP effect]. Motor function assessed by rotarod score detected similar effects of NAPAP treatment. The treatment was not found to improve the rotarod function over the entire course of disease [$F_{(27, 387)} = 1.29$, $p = 0.16$, for interaction between all-time points and groups, **Figure 2B**].

Following the onset of clinical score deterioration (DPI14) all TLCK treatment protocols were found to be better than EAN [$F_{(39, 598)} = 11.72$, $p < 0.01$, the interaction between all-time points and the groups was analyzed by repeated measure

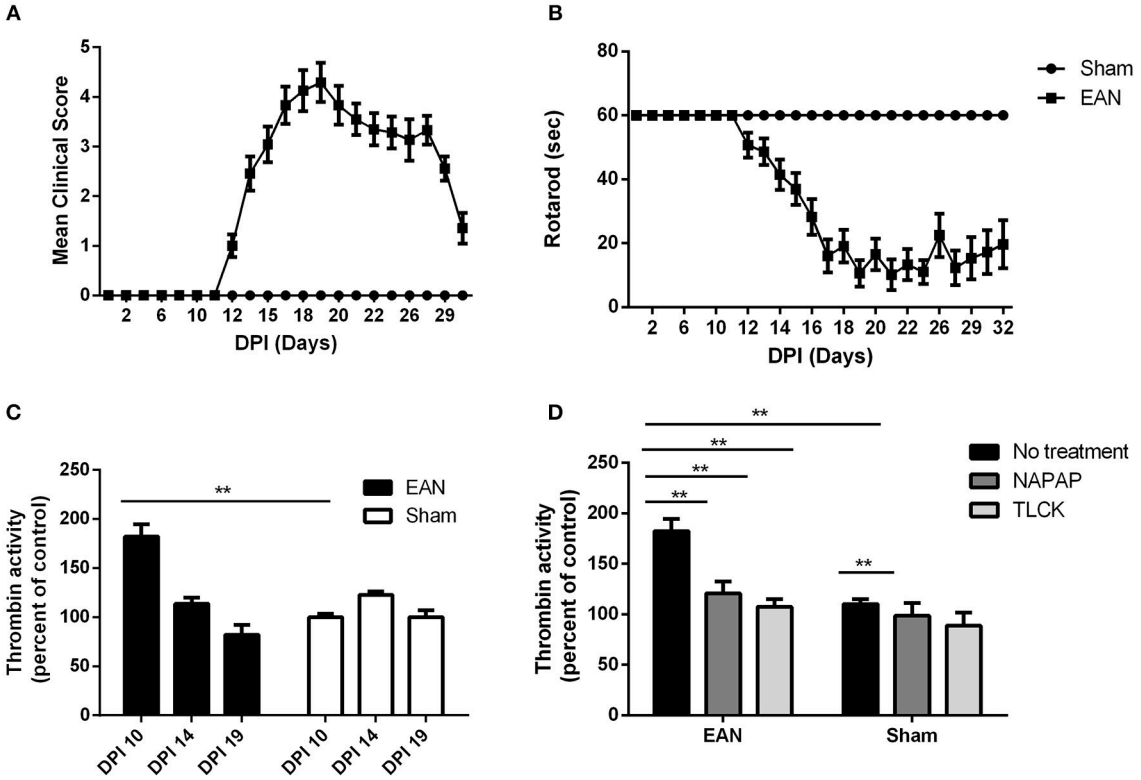

FIGURE 1 | Clinical parameters and thrombin activity. Clinical parameters were followed using clinical score and rotarod score. Thrombin activity was measured using fluorometric assay, quantifying the cleavage of the synthetic peptide substrate, after the removal of the nerve from the animals. Thrombin activity following NAPAP and TLCK administration was measured *in-vitro*. **(A)** Mean clinical score (CS) of EAN rats compare to sham. **(B)** Rotaroad score (RR) of EAN rats compare to sham. **(C)** Thrombin activity in EAN rats sciatic nerve compare to sham rats (thrombin activity in sham rats was defined as 100%). Thrombin activity was measured at DPI10, DPI14, and DPI19. Thrombin activity levels were significantly increase in DPI10 in EAN compare to sham ($p < 0.0001$). **(D)** Increased thrombin activity in sciatic nerves of EAN rats in DPI10 is inhibited *in-vitro* by NAPAP and TLCK (thrombin activity in sham rats treated with NAPAP was defined as 100%). Results are presented as mean±SEM. $^{**}p \leq 0.001$. Total number of animals in each group: A.B. CS and RR: EAN-24, sham-9. C. Thrombin activity EAN: DPI10-3, DPI14-4, DPI19-4. Sham: DPI10-3, DPI14-5, DPI19-4. D. Thrombin activity *in-vitro* EAN and sham: No treatment-3, NAPAP-3, TLCK-3. Statistical analysis used: Statistical analysis of the differences between clinical scores and rotarod progression were assessed by analysis of variance (ANOVA) with repeated measures. Sidak correction was done for multiple comparisons. The difference in thrombin activity was calculated using two-way ANOVA with Dunnett's correction for multiple comparisons.

ANOVA, with Dunnett's correction for all treatments compare to EAN]. At disease peak, (DPI17) all treatments improve clinical score [1 ± 0.4, 1.75 ± 0.16, 0.72 ± 0.13, 4.07 ± 1.47, SP, EP, LP, vs. EAN respectively, mean $\pm$ SEM, $F_{(3, 40)} = 25.04$, $p < 0.001$] and there was no significant difference between treatment protocols. During the recovery phase (DPI18-20) EP and LP treatments improved clinical score [$F_{(3, 37)} = 11.57$, $p < 0.001$, analyzed by repeated measure ANOVA, followed by Tukey's *post-hoc* analysis, **Figure 2C**]. Motor function assessed by rotarod score detected similar effects of TLCK treatment. From the beginning of motor deterioration, all treatments had a beneficial effect [$F_{(36, 552)} = 2.9$, $p < 0.001$ for the interaction between all-time points and groups, **Figure 2D**]. LP treatment was found to enhance recovery compare to EAN [$F_{(3, 37)} = 3.82$, $p = 0.017$ analyzed by repeated measure ANOVA with Tukey's correction].

Electrophysiology

NCV studies were carried out at a late phase of disease (DPI26) since there is a known 1–2 weeks delay between the disease onset and the appearance of electrophysiological changes in both EAN and GBS. Furthermore, performance of electrophysiology studies requires anesthesia, which by morbidity and mortality may have deleteriously affected the clinical measures. All EAN rats demonstrated severe pathology in electrophysiology parameters (**Figure 3**). Distal amplitude was significantly lower in EAN compared to sham rats (0.76 ± 0.34, 9.8 ± 1.2 mV, respectively, $p < 0.0001$, **Figure 3A**) and was not significantly improved with either treatment. Latency was longer in EAN compared to sham (1.43 ± 0.02, 0.54 ± 0.03 msec, $p < 0.0001$, **Figure 3B**), with some improvement with TLCK and NAPAP treatments (no statistical significance, $p = 0.27$, $p = 0.4$, respectively). The results obtained confirm a severe axonal and demyelinating neuropathy in the EAN animals compared to sham. The effects of NAPAP and TLCK treatments on the axonal component of the disease were minimal, as expressed by muscle response amplitude. Nerve conduction velocities were slower in EAN compared to sham (23.6 ± 2.16, 43 ± 3.7 m/s. **Figure 3C**, $p = 0.0214$) and were significantly improved by TLCK treatment (23.6 ± 2.65,

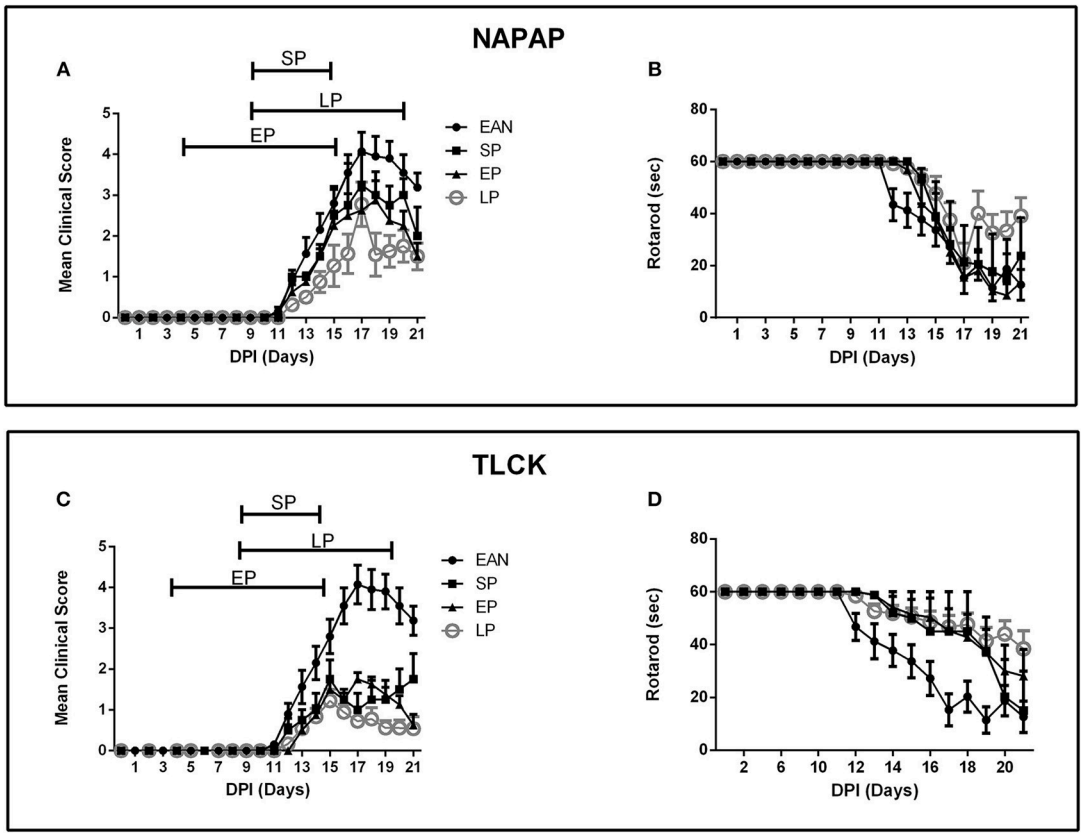

FIGURE 2 | Time course of clinical parameters in EAN rats under different treatment protocols. Comparison between 3 treatment protocols for NAPAP and TLCK in order to evaluate the most effective time frame for treatment. Treatment protocols included early protocol (EP), with daily injections at DPI5-15, late protocol (LP) with daily injections at DPI10-20, and short protocol (SP) with daily injections at DPI10-15. Each protocol was applied in both NAPAP and TLCK. Days are marked DPI following immunization day (DPI0). The bars above mean clinical score graphs mark the length of each treatment protocol. **(A)** Mean clinical scores (CS) with different NAPAP treatment protocols. **(B)** Rotarod (RR) scores with different NAPAP treatment protocols. **(C)** Mean CS with different TLCK treatment protocols. **(D)** RR scores with different TLCK treatment protocols. The late treatment protocol marked with gray circles, was the most effective. Results are presented as Mean±SEM. Total number of animals in each group: For NAPAP (CS, RR): SP-4, LP-18, EP-8, EAN-20. For TLCK (CS, RR): SP-4, LP-16, EP-8, EAN-20. Statistical analysis used: Two-way ANOVA with Tukey test for multiple comparisons was performed for the effect of EAN and progression in time on clinical score and rotarod.

41.27 ± 7.7 m/s, $p = 0.047$). Significant beneficial effects were demonstrated in the demyelination measure of nerve conduction velocity by TLCK, which is in-line with the more striking effect of TLCK on the clinical measures.

Immune-Histochemical Evaluation and Splenocyte Proliferation

Nerve sections taken from sham, EAN, EAN treated with NAPAP and TLCK were quantitatively analyzed using randomly mixed images. EAN nerve sections showed abnormal structure defined as lack of PAR1 staining as described earlier, in 25 out of 30 images compare to 1 out of 30 in sham sections ($p = 0.0001$, Fisher's exact test, **Figures 4A,B**). Sections taken from NAPAP and TLCK treated EAN showed abnormal structure in 21 out of 30 nodes and 17 out of 30 nodes ($p = 0.18$, $p = 0.02$ respectively, when compared to EAN, **Figures 4C,D**).

The beneficial effects of inhibiting thrombin on EAN neuropathy may be due to local inhibition of thrombin in the nerve or through a more general immune-suppressive effect. We tested this possibility by isolating immune cells from the spleens of EAN animal treated by TLCK or sham. The general B-cell and macrophage stimulator LPS induced splenocytes proliferation reaction increase in EAN compare to sham ($p = 0.01$, **Figure 4E**). TLCK did not affect this proliferation significantly. The specific antigen used to induce EAN in this mode, bovine spinal root myelin induced a small increase in splenocytes proliferation in EAN rats ($p = 0.37$, sham vs. EAN), and the effect of TLCK was to further increase proliferation ($p = 0.2$, sham vs. TLCK treated EAN, **Figure 4F**).

DISCUSSION

This study demonstrates specific thrombin and PAR1-related biochemical, histological and electrophysiological changes in peripheral nerves of EAN rats. The initial event is an early elevation of thrombin levels in the sciatic nerve and destruction of the PAR1-associated structures at the node of Ranvier. The functional importance of increased thrombin levels is supported

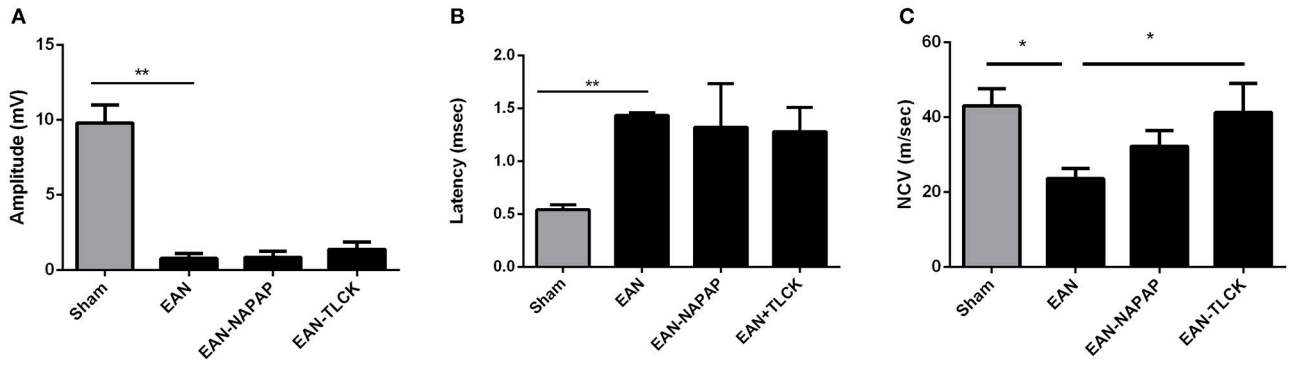

FIGURE 3 | Electrophysiological findings in sham, EAN, and treated EAN, DPI26. Electrophysiological measurements were conducted on the tail nerve. Amplitudes of distal compound muscle action potential (CMAP), distal latency and nerve conduction velocities were evaluated. **(A)** CMAP in sham, EAN, and different treatment protocols. Amplitude was significantly lower in EAN compared to sham rats. **(B)** Distal latency in sham, EAN, and different treatment protocols. Latency was significantly longer in EAN rats. **(C)** Nerve conduction velocities (NCV) in sham, EAN, and different treatment protocols. Velocity was significantly slower in EAN compare to sham rats. This was partially corrected by TLCK treatment. Results are presented as Mean±SEM. $^{*}p \leq 0.05$, $^{**}p \leq 0.001$. Number of animals in each group (amplitude, latency, NCV): Sham-3, EAN-3, EAN-NAPAP-3, EAN-TLCK-3. Statistical analysis used: One-way ANOVA with Dunnett's correction for multiple comparisons was used to compare the means of electrophysiological measures.

by the major beneficial effect of exogenous thrombin inhibitors on the severity and course of EAN. All treatments in different protocols had various degree of beneficial effect on the clinical and rotatod scores of the EAN rats. This was expressed as a lower disease peak, and as better final outcome. It is interesting to note that EP and SP treatments, which were limited to the time period prior to recovery, had an impact on recovery phase as well. This may indicate a complex series of event induced by early intervention.

The EAN rats developed the expected monophasic diseases course, characterized by a relatively rapid deterioration to paresis, followed by a gradual recovery. The EAN rats showed a significant rise in thrombin activity in the sciatic nerve. The rise in thrombin activity was an early event, noted just prior to the appearance of clinical signs on DPI10. In EAN rats, nerve thrombin activity was higher compared to sham rats and was inhibited to levels close to baseline activity using TLCK, which is a non-selective thrombin and trypsin-like proteases inhibitor. This effect was also found by using the highly specific thrombin inhibitor NAPAP. The significant inhibition caused by NAPAP supports the specific involvement of thrombin rather than other serine proteases in EAN nerve pathology. These results are thus compatible with an early increase in specific thrombin activity which is then probably countered by an increase in endogenous thrombin inhibitors, as can be seen in other reported animal models for neuroinflammation such as the EAE (18, 34).

The peak of measured excess thrombin activity is on day 10 and therefore the treatment protocols included one that preceded this point by 5 days, a short protocol for the period when this elevation occurred and a longer protocol which was initiated from the elevation of thrombin well into the recovery phase. Of these protocols, the long protocol gave significantly better results in the TLCK treatment, thus supporting a role for thrombin-like proteases from the first appearance of clinical signs into the recovery phase. The longer protocol was significantly better in the recovery phase than both other protocols, which ended on day 15. Indeed, following the cessation of treatment on day 15, both protocol groups demonstrated an exacerbation of motor deficit (as measured by rotarod), in contrast to improvement in the long treatment protocol group which were still treated. In the NAPAP group there was an early effect on the severity of disease in all 3 protocols, similar to the TLCK group. The results strongly suggest that at these early time points (DPI10-15) thrombin is specifically important in disease propagation. In contrast, in both the peak of disease and in the recovery phase, the non-selective TLCK treatment was significantly better than NAPAP in all study measures: clinical score, motor deficit, nerve conduction and histology of PAR1 staining at the node of Ranvier. These results suggest that at later stages of disease a more general trypsin and thrombin-like protease activation is important in disease progression. These results suggest that targeting thrombin and trypsin-like proteases that can affect the node of Ranvier through PAR1 is a viable approach to the therapy of GBS.

The node of Ranvier is thought to be a primary structural and functional participant in the pathophysiology of GBS and EAN. The presence of relevant antigens such as gangliosides in the nodes of Ranvier was reported before (38). We have previously found PAR1 localized to the node of Ranvier (28). Thrombin, the main PAR1 activator, was found to cause a sciatic nerve conduction block in a PAR1 mediated manner (28). It is also known that thrombin is related to neuronal inflammation and glial activation (20, 39, 40). In the present study, we found degeneration of the nodes of Ranvier containing PAR1 structure as seen by immune-histochemistry, together with a reduction of nerve conduction velocity. These findings further support the node of Ranvier as an important participant in GBS and EAN pathophysiology. The mode of action of the treatments used in the present study is hypothesized to be essentially protease inhibition and specifically thrombin inhibition since one of the substances used is a highly selective for this protease.

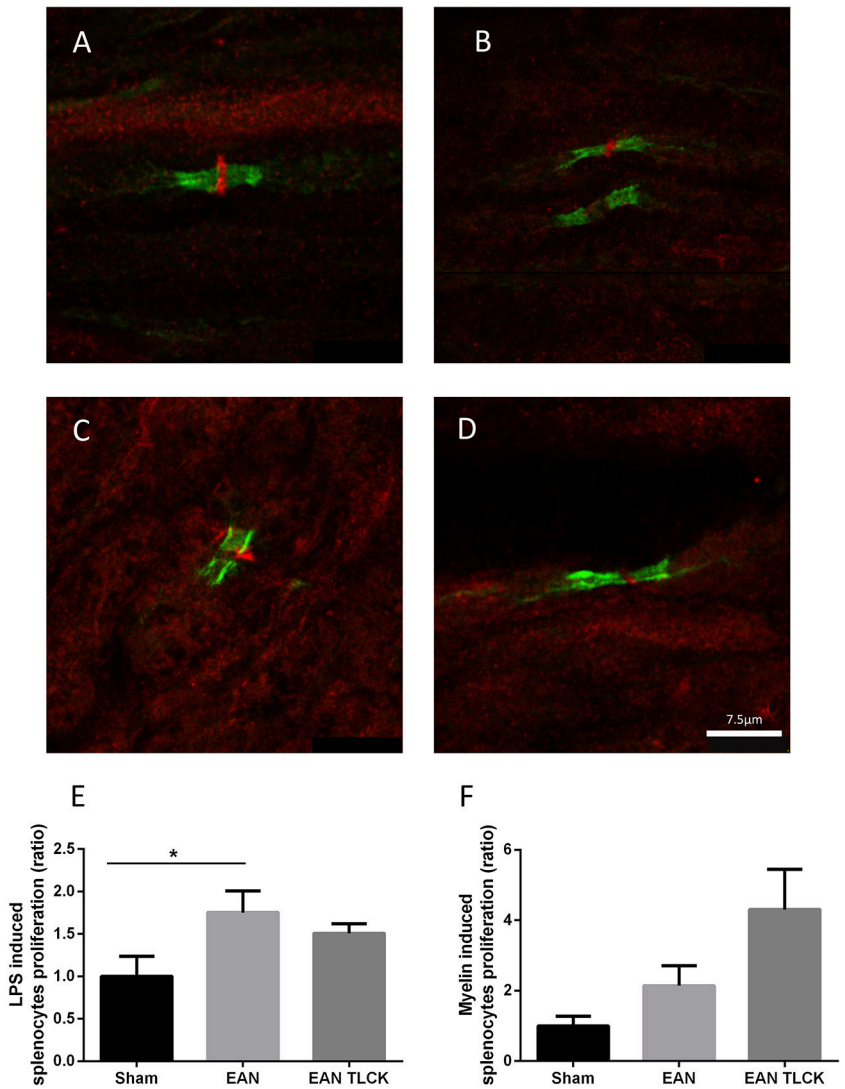

FIGURE 4 | Immune-histochemical staining of nodes of Ranvier and splenocytes proliferation assay. Representative images from EAN, sham and treated rats, taken at DPI32. Normal node architecture was defined as the presence of paranodal Caspr1 stain, and staining of PAR1 in the node between the paranodal Caspr1 stains. Sciatic nerve fibers of sham **(A)** EAN **(B)**, EAN treated with NAPAP **(C)** and EAN treated with TLCK **(D)** rats. Nodes of Ranvier taken from EAN showed abnormal lack of PAR1 stain compare to sham rats. TLCK treatment significantly improves the appearance of the nodes. Splenocytes proliferation reaction in response to LPS **(E)** and myelin **(F)** in sham, EAN and EAN treated TLCK rats. Proliferation in EAN in response to LPS was not significantly inhibited by TLCK. Results are presented as Mean±SEM. *$p < 0.05$. The sciatic nerves have been double-stained for PAR1 (red) and Caspr1 (green). Scale bar: 7.5 µm. Magnification X630. Immune-histochemistry: four animals were taken from each group (sham, EAN, EAN treated with NAPAP and EAN treated with TLCK), and 7–8 nodes of Ranvier were taken from each animal, to a total number of 30 nodes per group. Proliferation assay: four specimens were analyzed in each group. Experiments were performed in triplicate. Analysis of immune-histochemistry was done using Fisher's exact test. Analysis of proliferation assay was done using one-way ANOVA with Tukey *post-hoc* analysis.

Direct evidence is provided to substantiate that the increased thrombin activity is inhibited by both TLCK and NAPAP. The proteolytic consumption of PAR1 is demonstrated in the present results and supports this hypothesis. Thus, the hypothesis that excess activation of thrombin cause first dysfunction through the activation of PAR1, and then tissue destruction at the node of Ranvier, is substantiated by a number of findings in the present study. Further modes of action are certainly possible, and subjected to future research. Thrombin causes a secretion of interleukins from blood dendritic cells (41), it is therefore reasonable to suspect that its mechanism of action in EAN may include additional immunological effects besides its role in the node of Ranvier. In our hands, using splenocytes proliferation assay, TLCK was not found to have a significant immunological effect. There are very few studies on the modulation of immune and inflammatory cells by thrombin inhibition and this may well be systematically approached in further research.

Both node of Ranvier structure and nerve conduction function were partially restored using thrombin inhibitors. Electrophysiological studies demonstrated the expected lower

CMAP amplitude as well as slower nerve conduction velocity and longer latency in the EAN rats compared to sham. The treatment with thrombin inhibitors improved these functions achieving statistical significance in measures of nerve conduction velocity. Lack of statistical significance in other electrophysiological measures might be due to the small number of animals in each group. This number of animals (three from each group) was set due to the known high risk of mortality during this part of the experiment and the great importance in continuing monitoring the treatment effect over the study period (electrophysiology was done on DPI26). The statistical significance found in nerve conduction velocities, despite the limited number of animals, may indicates a solid therapeutic effect. Alternatively, since the mechanisms for the changes in electrophysiology remains elusive, amplitude and latency may not participate in its pathophysiology.

Current GBS treatments include IVIG (42), plasma exchange (43) and supportive care. The natural history of GBS is to eventually subside, although only about 57% of the patients show complete cure without treatment. The underlying mechanism of these treatments is partially understood as well of the disease pathophysiology itself. Our study suggests a novel treatment target not previously used for treatment of GBS, and improves our understanding of GBS pathophysiology.

Thrombin inhibitors have anticoagulation properties. Although this might be of use in some patients with the appropriate comorbidities, they carry risks as well. The non-selective thrombin inhibitor TLCK used in this study caused bleeding tendency in laboratory animals (was detected post mortem, not shown). TLCK caused more bleeding than NAPAP, perhaps due to its wide range of activities. Although NAPAP did not cause evident bleeding in this study, it is a potent thrombin inhibitor and the use of similar medication in GBS would require caution. Further research is needed in order to develop more selective thrombin inhibitors, in order to allow separation of the inflammatory and anti-coagulatory effects. The current use of these agents allow better understanding of GBM underlying mechanism, for future development of therapies.

In conclusion, thrombin and PAR1 inhibitors offer new directions in the treatment and understanding of GBS. Further research is needed in order to find more specific thrombin inhibitors and in order to understand the interplay between thrombin and the damage to nodes of Ranvier. Future structural, physiological and immunological evaluation of the changes associated with thrombin inhibition in EAN rats may shed light on the mechanism by which inhibition of PAR1 activation induces its beneficial effect.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the animal welfare committee (M-06-004). All experiments were approved by the animal welfare committee (M-06-004) and appropriate measures to avert pain and suffering to the rats were taken.

AUTHOR CONTRIBUTIONS

ES-S: planning of experiments, analysis of results, writing manuscript; RA: planning of experiments and execution; CS: execution and analysis of nerve conduction experiments; OG: writing manuscript, consultation regarding electrophysiology; SG: analysis of results, writing manuscript; JC: planning of experiments, analysis and interpretation of results; AD: consultation regarding electrophysiology, interpretation of results. All authors have approved the final manuscript. The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

FUNDING

Partly supported by a grant from the Wolfson Foundation, Israel.

ACKNOWLEDGMENTS

We thank Raphael Rosenbaum for his contribution in editing and proofreading of this manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neurology

MINI REVIEW
published: 07 December 2018
doi: 10.3389/fneur.2018.01067

Pharmacokinetic Interactions of Clinical Interest Between Direct Oral Anticoagulants and Antiepileptic Drugs

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Direct oral anticoagulants (DOACs), namely apixaban, dabigatran, edoxaban, and rivaroxaban are being increasingly prescribed among the general population, as they are considered to be associated to lower bleeding risk than classical anticoagulants, and do not require coagulation monitoring. Likewise, DOACs are increasingly concomitantly prescribed in patients with epilepsy taking, therefore, antiepileptic drugs (AEDs), above all among the elderly. As a result, potential interactions may cause an increased risk of DOAC-related bleeding or a reduced antithrombotic efficacy. The objective of the present review is to describe the pharmacokinetic interactions between AEDs and DOACs of clinical relevance. We observed that there are only few clinical reports in which such interactions have been described in patients. More data are available on the pharmacokinetics of both drugs classes which allow speculating on their potential interactions. Older AEDs, acting on cytochrome P450 isoenzymes, and especially on CYP3A4, such as phenobarbital, phenytoin, and carbamazepine are more likely to significantly reduce the anticoagulant effect of DOACs (especially rivaroxaban, apixaban, and edoxaban). Newer AEDs not affecting significantly CYP or P-gp, such as lamotrigine, or pregabalin are not likely to affect DOACs efficacy. Zonisamide and lacosamide, which do not affect significantly CYP activity *in vitro*, might have a quite safe profile, even though their effects on P-gp are not well-known, yet. Levetiracetam exerts only a potential effect on P-gp activity, and thus it might be safe, as well. In conclusion, there are only few case reports and limited evidence on interactions between DOACs and AEDs in patients. However, the overall evidence suggests that the interaction between these drug classes might be of high clinical relevance and therefore further studies in larger patients' cohorts are warranted for the future in order to better clarify their pharmacokinetic and define the most appropriate clinical behavior.

Keywords: DOACs, antiepileptics, interactions, CYP, P-gp, AEDs, dabigatran, rivaroxaban

OPEN ACCESS

Edited by:
Svetlana Lorenzano, La Sapienza University of Rome, Italy

Reviewed by:
Olagide Wagner Castro, Federal University of Alagoas, Brazil
Francesco Paladin, Ospedale SS. Giovanni e Paolo, Italy

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Specialty section:
This article was submitted to Epilepsy, a section of the journal Frontiers in Neurology

Received: *08 August 2018*
Accepted: *23 November 2018*
Published: *07 December 2018*

Citation:
Galgani A, Palleria C, Iannone LF, De Sarro G, Giorgi FS, Maschio M and Russo E (2018) Pharmacokinetic Interactions of Clinical Interest Between Direct Oral Anticoagulants and Antiepileptic Drugs. Front. Neurol. 9:1067. doi: 10.3389/fneur.2018.01067

INTRODUCTION

The *direct-acting oral anticoagulants* (DOACs), also known as *non-vitamin K oral anticoagulants* (NOACs), are five drugs acting on coagulation cascade, without the use of anti-thrombin as a mediator, subdivided in factor Xa inhibitors (apixaban, edoxaban, and rivaroxaban) and direct thrombin inhibitors (argatroban and dabigatran).

Their indication in the clinical practice is as anticoagulants for primary and secondary prevention of ischaemic stroke, in patients suffering from non-valvular atrial fibrillation (AF) (1), but also for prevention and treatment of pulmonary embolism and deep venous thrombosis (2).

Strokes and cerebrovascular diseases represent the main cause (30–40%) of symptomatic epilepsy among elderly (3) and most of these patients need a chronic treatment with antiepileptic drugs (AEDs). Therefore, it is not rare that some patients might undergo concomitant treatment AEDs-DOACs and this co-treatment could lead to pharmacological interactions with serious consequences for patient's health. In particular, AEDs causing a reduced absorption or an increase of DOAC metabolism can cause a reduced antithrombotic efficacy of these drugs; conversely, a reduced DOAC metabolism can increase significantly the risk of bleeding in these patients [(4); and see below].

The aspect of drug-drug interactions is particularly important in persons with epilepsy, since optimal seizure control is often achieved only after different treatment attempts or using AEDs polytherapy (5). Furthermore, convulsive seizures expose patients to potential traumatic injuries that can be more dangerous in patients under anticoagulant treatment. Consequently, the potential interactions between DOACs and AEDs represent a field of particular clinical interest.

Aim of this review is to provide an overview on interactions between DOACs and AEDs using clinical and pharmacokinetic data. We considered only DOACs that are currently marketed in EU countries: edoxaban, rivaroxaban, apixaban, and dabigatran.

METHODS

The articles on clinical series and case reports specifically addressing the interactions between DOACs and AEDs were selected starting from a PubMed search with the following search terms: "eslicarbazepine" or "felbamate" or "gabapentin" or "lamotrigine" or "levetiracetam" or "oxcarbazepine" or "perampanel" or "pregabalin" or "retigabine" or "rufinamide" or "stiripentol" or "tiagabine" or "topiramate" or "lacosamide" or "vigabatrin" or "zonisamide" or "phenobarbital" or "phenytoin" or "ethosuximide" or "carbamazepine" or "valproate," and "dabigatran" or "rivaroxaban" or "apixaban" or "edoxaban," with publication dates between 2005 and 2018. The Flow-Chart in **Figure 1** details the process of inclusion/exclusion of the articles.

Data on pharmacokinetics of AEDs and DOACs for this review article were collected performing a search on PubMed using the following search terms: "eslicarbazepine," "felbamate," "gabapentin," "lamotrigine," "levetiracetam," "oxcarbazepine," "perampanel," "pregabalin," "rufinamide," "stiripentol," "tiagabine," "topiramate," "lacosamide," "vigabatrin," "zonisamide," "phenobarbital," "phenytoin," "ethosuximide," "carbamazepine," or "valproate" and "CYP3A5" or "CYP2J2," or "CYP3A4" or "P-gp," or "P-glycoprotein," They were considered *in vitro* and *in vivo* experimental studies, and studies in humans from 1975 to March 2018. Similarly, it was subsequently performed another PubMed search, from 1999 to March 2018, for "dabigatran," "rivaroxaban," "apixaban," "edoxaban" and "CYP3A5," "CYP2J2," "CYP3A4," "P-gp," "P-glycoprotein." To reduce publication bias, we also searched the abstract proceedings of the international congresses by the International League Against Epilepsy (ILAE) and by the American Epilepsy Society.

The latter searches aimed at the definition of pharmacokinetic parameters and the most salient review papers, together with all product characteristics (SPCs) of the single drugs, were selected by the authors based on their experience in the field.

PHARMACOKINETIC OF DOACS

All DOACs pharmacokinetic features are summarized in **Figure 2**.

Direct Thrombin Inhibitor

Dabigatran reversibly binds the active site of thrombin and it is administered as a pro-drug, dabigatran etexilate, since it is not absorbed by gastrointestinal tract after oral intake because of its high polarity; the etexilate form is rapidly hydrolyzed by carboxyl esterases (CES) to the active compound. The intestinal absorption of dabigatran etexilate, as well as other treated DOACs, depends on Permeability glycoprotein (*P-gp*) (6). The latter, is an ATP-dependent efflux transporter located in the plasma membrane of many different cell types; it regulates the absorption of xenobiotics from the gut lumen and is involved in the hepatic and renal excretion of these substances; it is also involved in blood-brain barrier permeability to drugs (7).

Bioavailability is 6.5% after administration, the lowest of all DOACs, is probably due to Pg-p intestinal excretion and low solubility of the pro-drug considering that it is not a substrate of cytochrome P-450 system. Considered a 12 h half-life, with a maximum concentration reduced by 30% after 4–6 h, dabigatran is administered twice a day (8).

This DOAC is dialyzable considering its very low binding with plasma proteins (∼30%) and its 80% eliminated by kidneys (75% unchanged and 4% as active acyl-glucuronide metabolites), the remaining non-renal excretion is due to conjugation by uridine *diphosphate-glucuronyl-transferase* (UGT)2B15. Conjugation with activated glucuronic acid apparently represents the only metabolic modification of dabigatran (9). Food has no interaction with dabigatran, but the concurrent intake could decrease the plasma peak concentration (8).

Direct Factor Xa Inhibitors

Apixaban, edoxaban and rivaroxaban are selective inhibitors of Xa factor (FXa) by binding its active site both when free or thrombin-bound.

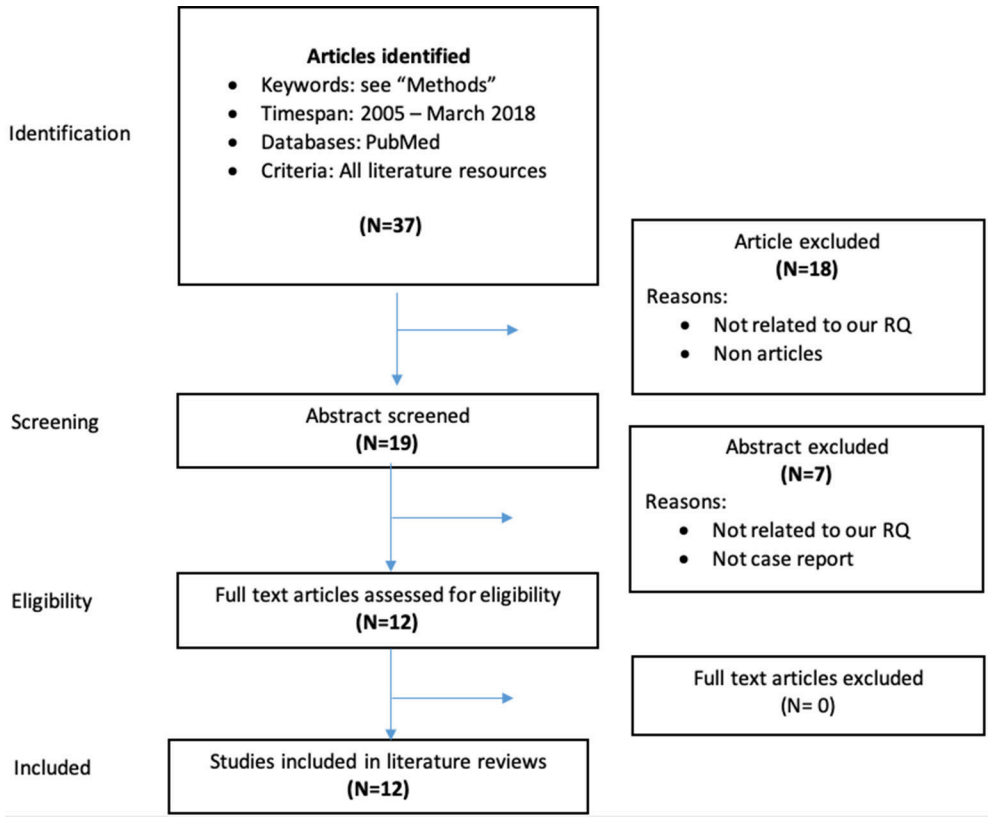

FIGURE 1 | Data collection process for case reports and clinical series on interactions between DOACs and AEDs.

Unlike dabigatran, these are not pro-drugs and have, when orally administered, an optimal and rapid absorption profile through the gastrointestinal tract that also depends on P-gp (9, 10) and this transporter also contributes to the renal excretion of rivaroxaban (11). The latter have a very high oral bioavailability (∼90% with food), compared with apixaban and edoxaban (∼ 50 and ∼62% for apixaban and edoxaban, respectively).

Apixaban and edoxaban need to be administered twice a day and have a plasma half-life of 9–14 and 9–10 h, respectively, after administration of multiple doses. On the other hands, rivaroxaban is administered once a day due to a persistence of high concentration after 24 h from oral intake. FXa inhibitors are not dialyzable and plasma protein binding is higher for rivaroxaban and apixaban (∼ 93%) compared to edoxaban (∼55%) and are excreted unchanged for 27, 33, and 50% of their bioavailable dose, respectively (12–14).

These DOACs are substrates of the cytochrome P-450 system, and especially the CYP3A4 isoform (15, 16). In particular, rivaroxaban undergoes CYP3A4/3A5- and CYP2J2-mediated oxidative metabolism (18 and 14% of the total absorbed dose, respectively) (17). Apixaban is primarily metabolized by CYP3A4/3A5 and secondly by sulpho-transferase (SULT) 1A1, while edoxaban is minimally metabolized by CYP3A4/3A5 and mainly eliminated unchanged in bile (40%) (18).

None of the FXa inhibitors have interactions with food, have been tested in pregnancy and have shown any liver toxicity but dedicated safety studies should be realized to better define DOACs drug-induced liver injury (19).

PHARMACOKINETICS OF AEDS

The most important pharmacokinetic interactions of different AEDs with each other and with other classes of drugs, involve cytochrome P450 (CYP) and, to a lesser extent, the uridine diphosphate glucuronosyltransferase (UGT) system (20). Carbamazepine, phenytoin, and phenobarbital, among first-generation agents, are inducers of several enzymes such as CYP1A2, CYP2C9, CYP2C19, and CYP3A4, but also of UGTs and epoxide hydrolase (21–23).

Lamotrigine does not interfere significantly with drug metabolizing enzymes at low dosages, but at a dose higher than 300 mg/day, it has been proven to cause a reduction of 20% of levonorgestrel serum concentration (24). Valproate is able to inhibit the activity of CYP2C9, and, to a lesser extent CYP3A4 and CYP2C19, as well as UGT1A4 and UGT2B7 (25). By contrast, valproate does not inhibit CYP2D6, CYP1A2, and CYP2E1 (23, 26, 27).

Even though newer AEDs have a limited enzyme-inducing potential compared with older—generation compounds,

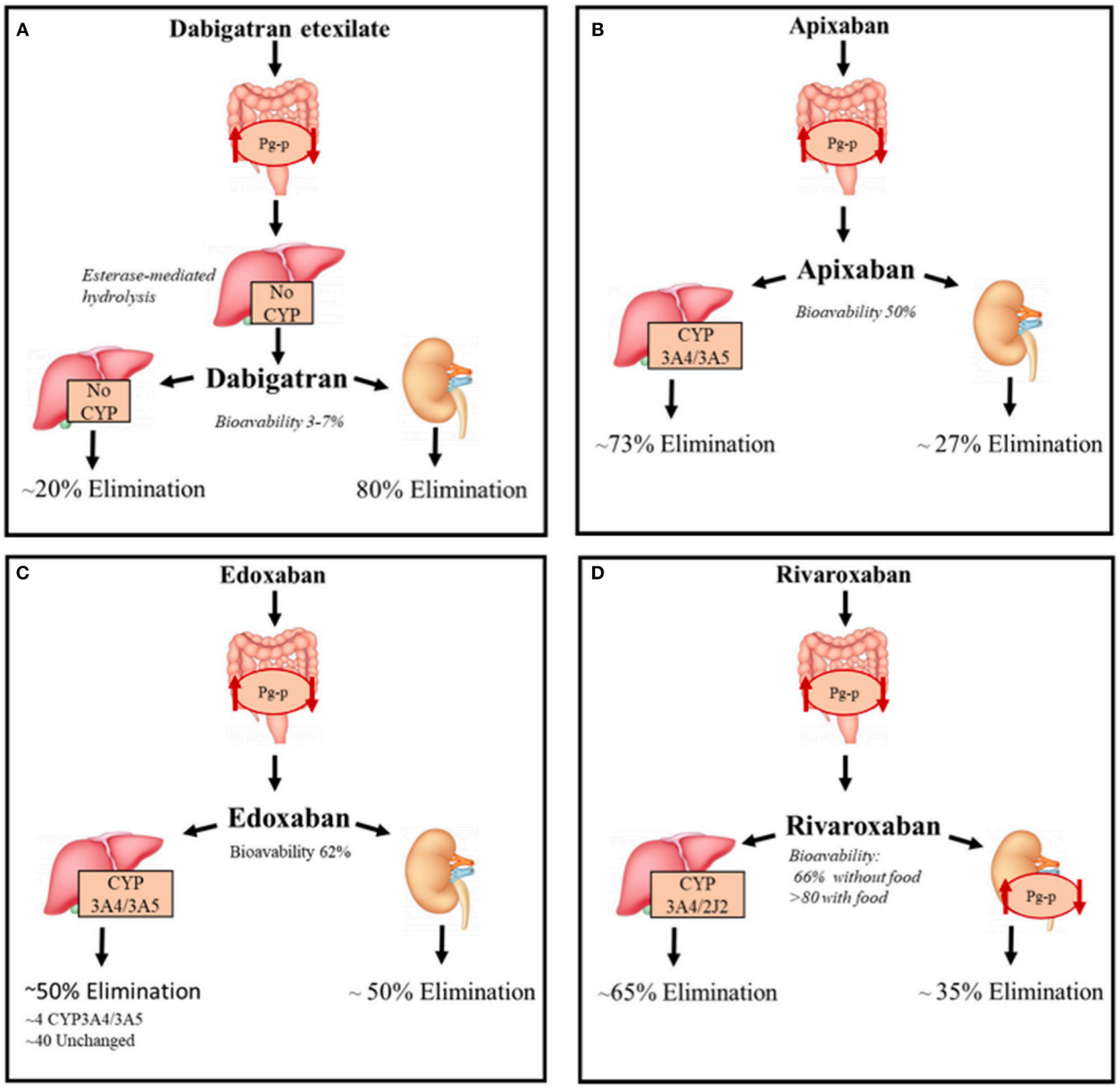

FIGURE 2 | DOACs pharmacokinetic characteristics. Summary of the pharmacokinetic characteristics of DOACs with focus on the metabolization and elimination processes. **(A)** Direct thrombin inhibitor, **(B–D)** Direct factor Xa inhibitors. *CYP, Cytochromes P450; P-gp, P-glycoprotein 1*.

some of them are involved in metabolic modifications. In particular, perampanel (at doses ≥8 mg/day), eslicarbazepine acetate, felbamate, oxcarbazepine (at doses ≥1,200 mg/day), topiramate, levetiracetam and rufinamide (at doses ≥400 mg/day) bear weaker enzyme-inducing properties and may stimulate the activity of CYP3A4 and/or some UGT isoenzymes (21, 22, 28). Furthermore, oxcarbazepine, eslicarbazepine, felbamate, and topiramate show a weak inhibitory activity on CYP2C19 (29); stiripentol, on the other hand, is a strong inhibitor of CYP3A4, CYP2D6, CYP2C19, and CYP1A2 (30).

At therapeutic doses, zonisamide inhibits *in vitro* the activity of CYP2A6, CYP2C9, CYP2C19, and CYP2E1, but does not affect significantly CYP3A4, CYP1A2, and CYP2D6 (21). No data on the induction or inhibition capacity of ethosuximide, lacosamide, gabapentin, pregabalin, and vigabatrin, on human CYP or UGT isoenzymes have been published (21).

Some AEDs affect P-gp functions; in animal studies, levetiracetam, phenytoin and phenobarbital have been shown to cause P-gp induction, as well as carbamazepine for which there are also data in humans (31–33). An *in vitro* study shows that

TABLE 1 | Interactions between AEDs and P-gp or CYP3A4/3A5 and CYP2J2 systems.

AEDs	P-gp	References	CYP3A4	CYP3A5/CYP2J2	References
Eslicarbazepine acetate	Substrate (*in vitro*)	(35)	Weak inductor (*in vitro e vivo)*	NR	(21)
Felbamate	Substrate (*in vivo)*	(36)	Weak inductor/No effects (*in vitro*)	NR	(26, 37)
Gabapentin	Not substrate	(38)	NR	NR	(21)
Lamotrigine	No effects/substrate	(39)	No effects	No effects	(40)
Levetiracetam	Inductor/substrate *(in vivo)*	(41)	Weak inductor *(in vitro)*	No effects	(42)
Oxcarbazepine	NR		Inductor *(in vivo e vitro)*	Inductor 3A5 *(in vivo e vitro)*	(43)
Perampanel	No effects	(44)	Weak inductor *(in vitro)*	Weak inductor 3A5 *(in vitro)*	(28)
Pregabalin	No effects	(45)	No effects	No effects	(46)
Rufinamide	NR		Mild induction *(in vitro)*	No effects	(47)
Stiripentol	NR		Inhibitor *(in vitro)*	No effects	(48)
Tiagabine	NR		Substrate	No effects	(49)
Topiramate	No effects/substrate	(39)	Mild inductor *(in vitro)*	No effects	(50)
Lacosamide	No effects	(51)	No effects *(in vitro)*	No effects	(52)
Vigabatrin	NR		No effects	No effects	(21)
Zonisamide	Weak inhibitor	(34)	No effects/substrate	No effects	(53)
Phenobarbital	Inductor/substrate	(54)	Inductor	No effects	(55)
Phenytoin	Inductor/substrate *(in vivo)*	(56)	Inductor/substrate *(in vivo)*	NR	(55)
Ethosuximide	NR		Substrate	NR	(26)
Carbamazepine	Inductor *(in vivo)*	(57)	Substrate/inductor *(in vitro and vivo)*	NR	(58)
Valproate	Inductor/inhibitor *(in vitro)*	(59, 60)	Inductor/weak inhibitor *(in vitro)*	NR	(25, 61)

zonisamide is a weak inhibitor of P-gp with a CI50 of 267 μmol/L (34) All the main evidence on the effects of AEDs on P-gp and CYP3A4 (obtained from *in vivo* and *in vitro* studies) are listed in **Table 1**.

The main interaction between DOACs and AEDs are related to the effects of the two classes of drugs on CYP3A4 and vice-versa and can be hypothesized by knowing their effects on these targets; for P-gp, interactions are less intuitive.

CASE REPORTS

We performed a detailed search, including Pubmed publications and abstract proceedings of the international congresses by the International League Against Epilepsy (ILAE) and by the American Epilepsy Society, of all clinical descriptions, without language limits, concerning all the AEDs and all of the DOACs in the search. Unfortunately, up to date, there are only 12 clinically relevant articles available on this topic (**Table 2**).

Phenytoin

The AED for which most of studies and interactions with DOACs have been reported is phenytoin, which bears relevant clinical interactions with dabigatran, rivaroxaban, and apixaban. In 2017, Chang et al. found that in a large cohort of 91.330 patients suffering from non-valvular AF and treated with dabigatran, rivaroxaban or apixaban, there was a higher risk of major bleeding when the patients were taking phenytoin (N-7158) for concomitant epilepsy, as compared with patients not assuming this drug with an adjusted incidence rate difference (99% CI) per 1,000 person-years of 52.31 (32.18–72.44; $p < 0.01$). However, this study bears the strong limitations of a Health Insurance database system analysis (including the lack of detailed clinical/radiological information on the single patients) (66). In the same year, Hager et al. (68) described the occurrence of a left atrial thrombus in a 70-years-old patient whit a clinical history of hypertension, persistent AF, heterozygous factor V Leiden, recurrent deep venous thrombosis (DVT), and a pulmonary embolus, in co-treatment with atenolol, betahistine, diltiazem, valsartan, phenytoin 300 mg orally QD, and dabigatran etexilate 150 mg BID.

Phenytoin can affect both the absorption and metabolism of dabigatran, suggesting that could have led to a decreased anticoagulant effect and the development of atrial thrombus. Clinical relevance of this drug interaction has not been well described; anyway, the co-administration should be avoided (68). In 2016, Wiggins et al. had hypothesized the same type of interaction. They showed undetectable serum levels of dabigatran in a 45-years-old Afro-American male patient with AF treated also with phenytoin indicating that this drug could have a significant influence on dabigatran's metabolism and that this patient was at high risk for stroke (67).

TABLE 2 | Clinical experiences on interaction between DOACs and AEDs.

AEDs	Dabigatran	Rivaroxaban	Apixaban
Eslicarbazepine acetate	/	/	/
Felbamate	/	/	/
Gabapentin	/	/	/
Lamotrigine	/	/	/
Levetiracetam	/	/	/
Oxcarbazepine	/	(62)	/
Perampanel	/	/	/
Pregabalin	/	/	/
Retigabin	/	/	/
Rufinamide	/	/	/
Stiripentol	/	/	/
Tiagabine	/	/	/
Topiramate	/	/	/
Lacosamide	/	/	/
Vigabatrin	/	/	/
Zonisamide	/	/	/
Phenobarbital	(63)	/	(64)
Phenytoin	(63) (66) (67) (68)	(65) (66)	(66)
Ethosuximide	/	/	/
Carbamazepine	(69)	(70) (71) (72)	/
Valproate	/	(73)	/

Similarly, Becerra et al. (65) observed, in the first case documenting laboratory interaction between rivaroxaban and phenytoin, that DOAC levels were considerably low in a 48-years-old woman with cerebral vein thrombosis receiving also phenytoin, a combined CYP3A4 and P- glycoprotein inducer, which might reduce rivaroxaban levels (65).

Phenobarbital

Phenobarbital may bear relevant interactions, too. In 2014, Chin et al. (63) evaluated median dose- corrected steady-state plasma dabigatran concentration (60 lg/L; range 9–279) in 52 patients (38–94 years). The dose-corrected concentration in a patient with co-administration of phenobarbital and dabigatran etixilate 110 mg BID was found 3 standard deviations below the cohort mean (concentration of 9 lg/L; dabigatran = 0.04l g/L per mg/day, z-score of the log-transformed dabigatran = −3.25). Authors hypothesized that this could occur via P-gp induction (63). In 2018, King et al. (64) reported the case of a 77-years-old patient on low-dose phenobarbital treatment for essential tremor, who was diagnosed with AF and, after dabigatran (150 mg BID) failure, she was switched to apixaban 5 mg BID.

In the following year, she suffered from two distinct episodes of cardioembolic stroke, and apixaban serum levels were lower (89 ng/mL approximately 11 h post-dose) than the expected therapeutic concentration. Furthermore, after phenobarbital discontinuation, the DOAC concentration rose to normal levels (361 ng/mL; unknown time post-dose) confirming a direct effect (64).

Carbamazepine

Carbamazepine may affect the anticoagulant efficacy of dabigatran and rivaroxaban. In 2016, Laureano et al. described 2 patients, a 53-years-old man with epilepsy and AF (CHADS2 score of 2) and a 66-years-old woman with bipolar disorder, previous pulmonary embolism, and right leg deep vein thrombosis, receiving carbamazepine and dabigatran 150 mg BID. In both patients, dabigatran serum concentrations were reduced (steady state 24 ng/mL and 20 ng/mL, respectively), effect probably due to induction of P-gp by carbamazepine (69). In 2017, Stollberger and Finsterer, described the case of a 55-years-old Caucasian male, suffering from recurrent venous thrombosis in treatment with rivaroxaban (10 mg BID) and carbamazepine (900 mg/die) for structural epilepsy with complex partial seizures and secondary generalization. He was hospitalized because of increasing pain and swelling of his right leg starting spontaneously. Sonography showed a thrombosis of the right popliteal and femoral vein and analysis of drug concentrations showed a serum-carbamazepine level in the therapeutic range while anti-Xa activity was low (<20 ng/ml) (70). Independently, in 2018, Burden et al. reported the case of a 71-years-old woman with clinical history of pulmonary embolism, subjected to the same therapy as the previous case. Presented to the Emergency department with acute onset shortness of breath, chest pain and palpitations, computed tomographic pulmonary angiography (CTPA) revealed multiple bilateral pulmonary emboli. Carbamazepine was hypothesized to be responsible of the DOAC inefficacy as the anti Xa activity was reduced in both cases (71).

Finally, Risselada et al. reported in 2013, only in Dutch language, a case of a 53-years-old man who underwent a partial knee arthroplasty and 4 days before developing a pulmonary embolism, whose symptoms started 1 day after he was switched from prophylactic dalteparin 5000 IE QD to rivaroxaban 10 mg one a day. Being the patient also in therapy with carbamazepine 600 mg BID for epilepsy, the authors of the case report hypothesized that pulmonary embolism was caused by a decrease in serum rivaroxaban levels due to the enzymatic induction of CYP3A4 by carbamazepine (72).

Valproate and Oxcarbazepine

Rivaroxaban efficacy may also be affected by valproate and oxcarbazepine. In 2014, Stollberger and Finsterer described the case of an 88-years-old female patient, taking valproate and rivaroxaban 15 mg/die together, and whose anti-Xa activity was higher than expected. Indeed, coagulation tests after 28 h rivaroxaban-intake showed INR 2.26, PT 35%, aPTT 38.3 s and anti-Factor Xa-activity 2.00 U/m.

Even after the DOAC withdrawal, it took several days before coagulation was normalized, despite the short half-life of rivaroxaban (5–9 h) (16). The authors themselves acknowledged a potential key role of the patient's poor renal function and low body-mass-index (eGFR 34–42 ml/min/1.73 m^2 and BMI = 19.95), but they could not exclude a drug interaction

between rivaroxaban and valproate (73). The potential role of oxcarbazepine as a rivaroxaban inhibitor was suggested by Serra et al. (62), which described the case of a 68 years old man, suffering from permanent AF who had been put on rivaroxaban treatment, before undergoing external electrical cardioversion. He eventually did not undergo cardioversion because he developed a left atrial thrombosis despite DOAC treatment. The authors supposed that the thrombotic event was due to the interaction between rivaroxaban and oxcarbazepine, which the patient was taking for epilepsy, and which, as a strong CYP3A4 inducer, could have reduced rivaroxaban efficacy (62).

DISCUSSION AND CONCLUSIONS

In this focused review, we summarized the clinical data available on the potential interactions existing between DOACs and AEDs. Although most of the clinical descriptions are merely anecdotal and do not allow further speculations, we can summarize that some old AEDs might modify DOACs efficacy; phenytoin, carbamazepine and phenobarbital might reduce significantly all DOACs efficacy while for oxcarbazepine and valproate, there are some data demonstrating a reduction of rivaroxaban efficacy, even though an interaction with other DOACs cannot be excluded. Finally, the interaction between phenobarbital and dabigatran has been better studied and it seems very convincing that phenobarbital reduces significantly dabigatran blood levels and efficacy. Based on their well-known enzymatic induction effects, phenytoin, carbamazepine and phenobarbital all potentially decrease the efficacy of rivaroxaban, apixaban, and edoxaban. One case report suggests that this would be the case also for dabigatran, even though this interaction was not easily predicted by knowing dabigatran CYP metabolism. This further emphasizes that specific predictions on the interactions between DOACs and AEDs are difficult, that many more clinical data are needed and that predictions based only on theoretical models might lead to wrong assumptions. However, theoretically based on the well-known effects of DOACs and AEDs on CYP and P-gp, several interactions can be hypothesized and should be kept in mind when starting a therapy with AEDs and DOACs.

Concerning newer AEDs, it can be speculated that those not affecting significantly CYP or Pg-p are not likely to affect DOACs efficacy and thus may be safer; this would be the case for lamotrigine or pregabalin. Lacosamide and zonisamide do not affect significantly CYP 3A4 activity, but their effects on Pg-p are not well-known yet. If the latter will be shown to be weak, they might be a good choice for patients on DOACs. For levetiracetam (which is otherwise considered quite neutral and "safe" in terms of pharmacokinetics interactions with many common drugs), an effect on CYP has not been shown but, since this AED may induce Pg-p activity, and is a substrate itself of this transporter, its safety in patients taking DOACs still needs to be demonstrated. Valproate and oxcarbazepine are AEDs still largely used in epileptic patients. Concerning valproate, its use is likely to affect significantly the pharmacokinetics of DOACs, as it affects significantly both CYP and Pg-p activity *in vitro*; the only case report available on valproate and rivaroxaban apparently contradicts this speculation, as it showed a reduced anti-Xa activity in 1 patient taking both drugs. However, the renal comorbidity of this patient probably played a role in these results. Oxcarbazepine, which is predicted to induce CYP activity, might also affect in a relevant way DOACs metabolism.

In conclusion, when a clinician has to choose an AED in a patient already taking DOACs, he might potentially choose among different second and third generation compounds which possess similar, significant, antiepileptic activity.

On other hand, it might be more complicated to start anticoagulants in patients with an established epilepsy that is well-controlled by old AEDs, and especially phenytoin, carbamazepine, phenobarbital or valproate. In these patients it might be risky, in terms of seizures recurrence, to modify an established AED. Such risk might be even increase when they are under anticoagulant drugs, i.e., at higher risk of major bleeding due to traumatic injury. Furthermore, previously pharmaco-resistant epileptic patients often take a combination of two or more of these AEDs at the same time, which further complicates predicting their effect on drug metabolism. In these patients, it might be still a reasonable clinical choice to use classical anticoagulants, such as warfarin, and tailoring its dosage in the single subject based on their INR values.

It is clear that population-based studies are needed to establish whether the pharmacological interactions between the two classes of drugs really represent a problem of clinical interest. Potentially, these aspects could be addressed at least in two ways. First, in order to establish the safest pharmacological combinations, it would be useful to perform cohort's studies comparing the compatibility between the most frequently administered AEDs and DOACs, starting at least with the ones which have the lower influence on CYP3A4, CYP3A5, and Pg-p (e.g., lamotrigine, pregabalin, lacosamide or levetiracetam). Another approach might consist in assessing *in vivo*, in the single patients taking both DOACs and AEDs, the efficacy of DOACs. Actually, one of the main reasons why DOACs have been developed is indeed to avoid the problem of strict coagulation monitoring, which is needed for patients using Vitamin-K antagonists. Some epileptic patients might represent one of the special populations in which such monitoring is indicated also for DOACs (which are considered, in any case safer, in terms of bleeding risk, than old anticoagulants, and thus to be preferred also in this population of subjects). Unfortunately, nowadays it is difficult to evaluate *in vivo* the antithrombotic effects of DOACs, and especially for those acting on factor Xa. These tests are not routinely available in some laboratories and their use needs a specific expertise. Moreover, mainly due to technical reasons, there are still differences in the results obtained among different laboratories and even among different specific DOACs within the same laboratory (74, 75). Hopefully, when in the future these methods (or different ones) will be more reproducible and approachable by different laboratories, it will be possible to assess in the single patient the existence of potential interactions between the DOAC and the AED(s) he/she is taking; once in the single patient such interaction has been excluded, it is likely that they would not need further evaluations (as usual for DOACs).

In conclusion, the risk of drug-drug interaction might be significant among patients taking AEDs and DOACs simultaneously, at least for some AEDs; future studies will help to better quantify this risk and to facilitate an optimal therapeutic handling of these patients.

LIMITATIONS

The main limitations of this review consist in the lack of *in vivo/clinical* studies specifically addressing interactions between most DOACs and most AEDs, and the results reported are mainly speculative based on the knowledge of their pharmacokinetics features. Concerning some AEDs, we do not even know these effects in detail, and thus, their interaction with DOACs are not even predictable yet. The few data available from case reports are not strong enough to allow drawing definitive conclusions.

AUTHOR CONTRIBUTIONS

AG, CP, LI, GD, and FG: Text writing, bibliographic research and literature analysis. MM: Text review. ER: Text review and team coordination.

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Conflict of Interest Statement: ER has received speaker fees and participated to advisory boards for Eisai. MM has received speaker fees and participated to advisory boards for Eisai and UCB Pharma.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neurology

REVIEW
published: 03 December 2018
doi: 10.3389/fneur.2018.01048

The Emerging Role of Microbial Biofilm in Lyme Neuroborreliosis

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Lyme borreliosis (LB) is the most common tick-borne disease caused by the spirochete *Borrelia burgdorferi* in North America and *Borrelia afzelii* or *Borrelia garinii* in Europe and Asia, respectively. The infection affects multiple organ systems, including the skin, joints, and the nervous system. Lyme neuroborreliosis (LNB) is the most dangerous manifestation of Lyme disease, occurring in 10–15% of infected individuals. During the course of the infection, bacteria migrate through the host tissues altering the coagulation and fibrinolysis pathways and the immune response, reaching the central nervous system (CNS) within 2 weeks after the bite of an infected tick. The early treatment with oral antimicrobials is effective in the majority of patients with LNB. Nevertheless, persistent forms of LNB are relatively common, despite targeted antibiotic therapy. It has been observed that the antibiotic resistance and the reoccurrence of Lyme disease are associated with biofilm-like aggregates in *B. burgdorferi*, *B. afzelii,* and *B. garinii*, both *in vitro* and *in vivo*, allowing *Borrelia* spp. to resist to adverse environmental conditions. Indeed, the increased tolerance to antibiotics described in the persisting forms of *Borrelia* spp., is strongly reminiscent of biofilm growing bacteria, suggesting a possible role of biofilm aggregates in the development of the different manifestations of Lyme disease including LNB.

Keywords: *Borrelia*, lyme, neuroborreliosis, biofilm, skin, erythema migrans

OPEN ACCESS

Edited by:
Matilde Inglese, Icahn School of Medicine at Mount Sinai, United States

Reviewed by:
Manola Comar, University of Trieste, Italy
Yoshiro Ohara, Kanazawa Medical University, Japan

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Specialty section:
This article was submitted to Multiple Sclerosis and Neuroimmunology, a section of the journal Frontiers in Neurology

Received: *29 September 2018*
Accepted: *19 November 2018*
Published: *03 December 2018*

Citation:
Di Domenico EG, Cavallo I, Bordignon V, D'Agosto G, Pontone M, Trento E, Gallo MT, Prignano G, Pimpinelli F, Toma L and Ensoli F (2018) The Emerging Role of Microbial Biofilm in Lyme Neuroborreliosis. Front. Neurol. 9:1048. doi: 10.3389/fneur.2018.01048

INTRODUCTION

Lyme borreliosis (LB) is the most prevalent vector-borne disease (1) caused by the spirochete *Borrelia burgdorferi*. This Gram-negative bacterium is an obligate pathogen, transmitted to different hosts by ticks in the genus *Ixodes*. LB is frequently reported in North America, Europe, in different parts of Asia, including Mongolia and China as well as in Australia and in Africa (2–4).

LB affects multiple organ systems, including the skin, eyes, joints, muscles, cardiac, and nervous system, presenting, at different stages, with a variety of clinical manifestations (5). Incubation varies from 3 to 32 days, after which a characteristic skin rash, known as erythema migrans, appears in association with fever, headache, malaise, and myalgias (stage 1) (6). After several weeks to months, in 8–15% of patients can be reported the presence of neurologic and cardiac abnormalities (stage 2). Within few weeks, in untreated patients or in case of delayed antibiotic treatment, the infection can disseminate leading to systemic inflammation (7, 8). In the last phase of LB (stage 3), patients may experience chronic monoarticular or oligoarticular arthritis, involving large joints, particularly the knee (9–11).

The most severe manifestations of LB, is Lyme neuroborreliosis (LNB), reported in 10–15% of individuals with localized erythema migrans (12, 13). The activation of the inflammatory response in LNB contributes to the pathogenesis of a broad spectrum of neurologic disorders. Different geographical distribution of *B. burgdorferi* species correlates with specific manifestation of LNB, which is more frequent in Europe than in the North America (6). The most common symptoms of LNB in Europe are painful meningoradiculitis known as Bannwarth syndrome and facial nerve palsy (14). Symptoms involving the central nervous system (CNS) are less common and their exact incidence is not known. *B. burgdorferi* infection of the CNS cause mainly encephalitis, segmental myelitis, cranial neuritis, radiculoneuritis, vasculitis, and intracranial hypertension (13, 15, 16). The clinical manifestation of the LNB may include ataxia, paraparesis, sphincter dysfunction, Parkinson-like symptoms, confusion and cognitive impairment (17, 18). Ischemic stroke is the most frequent cerebrovascular manifestation of LNB presenting in 76% of cases, followed by transient ischemic attack (11%) (19).

LNB is, in many cases, responsive to appropriate antimicrobial therapy and the clinical improvement sustained by the antibiotic treatment provide further evidence for the direct contribution of *B. burgdorferi* in disease pathogenesis. However, the chronic persistence, the frequent reoccurrence of LNB and the ability of *B. burgdorferi* to tolerate multiple cycles of antibiotic treatment is strongly suggestive for the formation of biofilm or biofilm-like protective structure (20–23). Indeed, different studies have shown that *B. burgdorferi* can switch from a motile to a stationary status, in which the cells are embedded within a biofilm matrix (22). *B. burgdorferi* biofilms have been observed both *in vitro* and in human infected skin tissues (22, 23). These structures express different mucopolysaccharides, particularly alginate, extracellular DNA and calcium, which are all typical markers of biofilm (22). The presence of biofilm may explain the low rate of *Borrelia* detection in the blood of infected patients as well as the ability of the spirochetes to evade the host immune system and resist the antibiotic therapy (21, 24–27).

This review investigates the differences in the epidemiology and clinical manifestations of LNB with particular emphasis on the pathogenetic role of *B. burgdorferi* biofilm in tissue adhesion, colonization and survival.

MATERIALS AND METHODS

The present review focuses on a systematic review of the literature to identify all published articles of LNB using online databases (PubMed, Web of Science, and Google Scholar). The reference list was updated in September 2018. There were no language restrictions; The search terms were "*Borrelia*," "*B. burgdorferi,*" "*Borrelia* biofilm," "Lyme disease," "neuroborreliosis," "LNB," "borreliosis." We reviewed titles, abstracts, case reports, and full articles to assess their relationship with the research criteria. References reported in each article were also reviewed to identify additional study not found by initial search terms.

Epidemiology of *Borrelia burgdorferi* Infection

LB is increasing worldwide with ∼300,000 new cases annually in the United States and 85,000 cases in Europe each year (28–30). Incidence of human LB in endemic areas of the United States ranges from 10 to 100 per 100,000 population with a peak of 134 per 100,000, reported in Connecticut in 2002 (4, 31, 32). The number of documented LB cases and the geographic distribution has expanded during the last two decades, from the Northeastern and North Central United States (4). LB is widespread also in Europe and the incidence for LB ranges from 20 to 80 per 100,000 in the Czech Republic, Germany, Latvia, the Netherlands, Poland, Switzerland, and Sweden, peaking to more than 100 per 100,000 in Austria, Estonia, Lithuania, and Slovenia (4, 29, 33, 34). Incidence of LB decreases southward, in Spain, France, Italy, and Greece with approximately 1 case per 100,000 (4, 29).

B. burgdorferi sensu stricto, *B. garinii*, and *B. afzelii*, are primarily responsible for human LB in different geographical regions presenting specific symptoms (34–38).

The genomes of *Borrelia* species consist of a set of circular and linear plasmids and a linear chromosome of ∼900 kb ending with DNA sequences regulated by breakage and reunion reactions (39, 40). Different isolates show a variable number of plasmids depending on the species and affected by frequent reorganization (41–47). *B. burgdorferi* B31 strain harbors 10 circulars and 12 linear plasmids while *B. afzelii* B023 and *B. garinii* CIP 103362 have 6 linear and 2 circular plasmids and 4 linear and 1 circular plasmids in Fraser et al. (41), Casjens et al. (42), and Bontemps-Gallo et al. (47).

Most of the essential genes involved in metabolism or regulation are located in the linear chromosome while only a subset of genes encoding proteins required for growth and specific virulence factors are located on plasmids (41, 48–50).

B. burgdorferi sensu stricto is the predominant causative agent of LB, Lyme arthritis, and also LNB in the United States (51). Nevertheless, consistent differences in the ability to induce LB exist between *B. burgdorferi* sensu stricto subtypes suggesting that, neurotropism is an ability present only in a restricted subtype of *Borrelia* (52, 53). Different genotypes of *B. burgdorferi* sensu stricto diverge ecologically and epidemiologically, suggesting that genotype classification is relevant to understanding the basic biology of the spirochete (54–56). Studies conducted in endemic areas of the United States revealed that patients with disseminated infection were more likely infected by the RST1 strains of *B. burgdorferi* than with RST3 strains (57, 58). Moreover, dissemination of *B. burgdorferi* to blood or cerebrospinal fluid (CSF) was mostly related to *ospC* genotypes A, B, I, or K (58–62).

The distribution and relative frequency of infection by the different genospecies of *Borrelia* sensu latu vary across European regions. *B. burgdorferi* sensu lato comprises 20 different genospecies and this diversity correlates with the large variability in the clinical manifestations observed in LB (4, 13, 63). In the northern and eastern Europe *B. afzelii* is the most prevalent species, whereas in Western European countries *B. garinii* is the most common pathogen (4, 29). *B. afzelii*, *B. garini,* and the recently identified species *B. bavariensis*

are major cause of LB and LNB in Europe (52, 64–68). The heterogeneity among *B. burgdorferi* sensu lato genospecies is linked to different geographical areas, which, in turn, correlates with the different clinical expression of human LB (69). For instance, *B. afzelii* induces prevalently skin infections, whereas *B. garinii* is in most cases neurotropic (5, 69). Other species, such as *B. lusitaniae* or *B. valaisiana*, have only occasionally been associated with human disease (70–72). In endemic areas of Europe was proposed that the variety of symptoms observed in children and adults with LNB correlated with the *B. burgdorferi sensu lato* genotype (73–75). Individuals with erythema migrans caused by *B. afzelii* and *B. garinii* showed distinct epidemiological and clinical characteristics. Indeed, erythema migrans caused by *B. garinii* were located prevalently on the trunk and less often on extremities, had shorter incubation and faster evolution, leading to frequent systemic symptoms, abnormal liver function test results than individuals with erythema migrans caused by *B. afzelii* (76, 77).

The genetic diversity observed in *B. burgdorferi*, at both inter- and intra-species level, is probably the reason for the multiple epidemiological and clinical presentation of these bacteria in humans (62, 78–80). A major role in maintaining the intraspecific genetic diversity of *B. burgdorferi* is the adaptation to multiple vertebrate hosts, which act as ecological niches for different genotypes (81, 82). Consequently, variations in the vertebrate host fitness may result in changes in the abundance of the more pathogenic species (83–85).

Host Invasion Strategies of *Borrelia burgdorferi*

Colonization, dissemination and invasion of the tick vector and mammalian host by *B. burgdorferi* requires a complex temporal and spatial regulation of borrelial genes to adapt to environmental challenges. Transition of *B. burgdorferi* from the tick midgut to the hemolymph during a blood meal is an important step for bacterial diffusion through the salivary glands to a mammalian host (86). *Borrelia* possesses a sophisticated mechanism of gene regulation based on the two-component pathways HK1/Rrp1 and Rrp2-RpoN-RpoS, which regulate metabolism, antigenic variation, chemotaxis, and adhesion in a tissue- and temporal-specific manner in both the tick vector and mammalian host (48, 87). During the blood uptake *B. burgdorferi* expresses the outer surface proteins (Osp) A and B. These proteins mediate the adherence to the tick's gut by the binding to the tick receptor of OspA (TROSPA), thus facilitating the subsequent transmission into the mammalian host (88–90). Infection of the mammalian host requires the migration of the spirochetes from the midgut to the salivary glands of the tick. After the blood uptake into the midgut of the tick, the production of OspA and OspB decrease while *ospC* is expressed in conjunction with many other genes controlled by the RpoN, RpoS, and Rrp2 system (90–96).

OspC is required to establish the early phase of *B. burgdorferi* infection in mammalian host and to promote evasion from the innate immune defenses (96–98). Different studies revealed that the OspC mutant strains are unable to establish infection in mice, suggesting a protective role of this protein against host innate defenses (96, 99–104). Nevertheless, to escape from the host immune system, the expression of *ospC* decreases within 2–3 weeks after infection in response to anti-OspC antibodies in mice (105, 106). In addition to OspC, *B. burgdorferi* hides other important immunogenic surface proteins (107). In particular, OspA, which stimulates neutrophils and a strong inflammatory response mediated by interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-6, is highly expressed in the tick gut but it is rapidly downregulated in the host (108–112). OspA-positive strains of *B. burgdorferi* penetrate the host, but are unable to establish an infection (113). Similarly, *Borrelia* strains isolated from mice 4 days after infection, were found to be OspA negative suggesting that this protein is not expressed in the early phase of the host infection (114, 115).

The expression of different proteins, including OspC, ErpP, ErpA, ErpC, and enolase is required to readily immobilize host plasminogen on spirochetal surface, facilitating efficient dissemination (116–118). Plasminogen is a glycoprotein produced by the liver and abundant in the plasma and in certain tissues (119). Conversion of plasminogen to active plasmin is promoted by proteolytic activation induced by either tissue-type plasminogen activator (tPA) and/or urokinase-type plasminogen activator urokinase (uPA). Plasmin is responsible for intravascular fibrinolysis and contributes to numerous physiological and pathological processes, including tissue remodeling, cell migration, thrombolysis, wound healing, and cancer progression (120, 121). Invasive forms of *B. burgdorferi* are known to expresses multiple plasminogen-binding surface proteins that likely assist pathogen dissemination through host tissues (120, 122). Enolase is an integral enzyme of the glycolysis and gluconeogenesis pathways, and a multifunctional protein found in both prokaryote and eukaryotes (123). In eukaryotic cells, surface enolase acts as a plasminogen receptor in certain tumor cells (123, 124). Similarly, this enzyme is also localized on the cell surface of different microorganisms including *B. burgdorferi* (118, 125–127). The surface-localized enolase acts as a plasminogen receptor contributing to spirochetal survival in feeding ticks (118). Although dispensable for infection, plasminogen is required for dissemination in ticks, and its absence is associated with a decreased spirochetemia in plasminogen-deficient mice (128). Surface-associated plasmin on *B. burgdorferi* degrade fibronectin, which is an important component of the ECM, laminin and vitronectin (129, 130). *B. burgdorferi* also induces the release of host matrix metalloproteases 9 (MMP-9) and MMP-1, and plasmin-coated *B. burgdorferi* activates pro-MMP-9, leading to degradation of basement membranes (131).

B. burgdorferi exhibits a specific affinity for the CNS as demonstrated by the presence of spirochetes in the human CSF within 14–18 days after the tick bite (18, 132, 133). From the initial site of entry in the skin the spirochetes can reach the CNS either through the bloodstream or, alternatively, by the peripheral nerves (114).

Hematogenous dissemination from the tick bite on the skin to the CNS is a key pathogenetic event in LNB (114). However, it has been proposed that, at least for *B. garinii* which is mostly

responsible for LNB in Europe, spirochetes can pass along the peripheral nerves (114). To penetrate the brain, spirochetes must first cross the blood-brain barrier reaching the brain microvascular endothelium and astrocytes (134). This barrier is composed by the brain microvascular endothelial cells (BMEC), astrocytes, basement membrane, pericytes, and neurons. The BMEC are firmly held together by tight junctions, presenting with a reduced transcytotic vesicles and an absence of fenestrae. All of these elements contribute to reduce the transport of solutes defending the brain from most pathogens or toxic agents (135, 136). Invasion of the blood-brain barrier by *B. burgdorferi* is still a matter of debate. Some studies suggest that *Borrelia* uses a paracellular route of translocation (134, 137), although other evidences suggest a possible transcellular passage of the spirochetes (138). Neurotropic *B. burgdorferi* strains showed the activation of the host plasminogen system, MMPs, and calcium signaling pathway to facilitate an efficient translocation through the blood-brain barrier (120, 134, 139). Compelling evidence suggest that *spirochetes* can adhere to murine neural and glial cell lines, primary neural cells, and primary rat brain cultures (140). In addition, *in vitro* studies show that *B. burgdorferi* can promote an intracellular invasion of human fibroblast, umbilical vein endothelial, synovial, neuronal, and glial cells without affecting the cell viability. This suggests that spirochetal cellular invasion may provide a mechanism for immune evasion and disease pathogenesis (140–142).

Lyme Neuroborreliosis

A common clinical and pathological manifestations of LNB in Europe is painful lymphocytic meningoradiculitis also known as Bannwarth syndrome, frequently accompanied by CSF signs of inflammation (13, 14, 143). The early manifestation of LNB generally appears within 2–18 weeks after infection (13, 143). The clinical description of painful meningoradiculitis was first reported in 1922, but the etiology remained unknown till the isolation of spirochetes by Burgdorfer in 1982 and the isolation in 1984 of spirochetes from the CSF of a patient with Bannwarth syndrome (144–146).

In addition to Bannwarth syndrome, other important neurological symptoms of the early stages of LNB include meningitis, meningeal perivascular, and vasculitic lymphoplasmocytic infiltrates, neuritis, and in rare acute LNB cases encephalitis and myelitis (143, 147). CNS vasculitis are rare in LNB, affecting mainly the large/medium-sized vessels and are associated with ischemia and stroke (19, 148). However, in European patients with LNB the mortality rate is comparable to that of the general population. Nevertheless, LNB is associated with increased risk of hematological and non-melanoma skin cancers (149).

Treatment with conventional intravenous antibiotic therapy, leads, in most cases, to a gradual improvement of the symptoms after several weeks or months, accompanied frequently by a normalization of CSF findings (150, 151). However, <2% of patients treated for LNB experience late neurological manifestations that persist months or years after *B. burgdorferi* infection (14, 143). The clinical symptoms of late LNB include several neurological and psychiatric symptoms such as meningoradiculitis, encephalomyelitis, chronic meningitis, and cerebral vasculitis (152–154). The presence of depressive states was described in 26–66% of patients with late LNB together with psychosis, schizophrenia, hallucinations, paranoia, anorexia nervosa, obsessive-compulsive disorder, and dementia (155–163). A frequent manifestation appearing in the late stage of LNB is the chronic vascular damage, clinically characterized by recurrent stroke or transient ischemic attacks (153, 154). Other distinctive findings in patients with late LNB are inflammatory CSF changes (CSF pleocytosis and elevated total protein content) and the presence of specific *B. burgdorferi* intrathecal antibody (150, 151).

The clinical outcome of antibiotic treatment of either early or late manifestations of LNB may include progression to a chronic form characterized by nonspecific and persistent fatigue, arthralgia, myalgia, musculoskeletal, and cognitive symptoms. This condition, frequently defined as posttreatment Lyme disease syndrome (PTLDS), can be intermittent or persistent, lasting for at least six or more months after completion of antibiotic treatment (143, 164).

Specific diagnostic criteria for PTLDS proposed by the Infectious Disease Society of America relies on the objective proof of previous LB, the presence of subjective symptoms that compromise function in daily life, and the absence of clinical evidences for another underlying illness (7). However, those criteria have rarely used in clinical studies, contributing to confusion and controversy about the clinical significance of PTLDS syndrome (7). The frequency of PTLDS among patients with LB varies largely, ranging from 0 to 50%, depending upon differences in study design and enrollment criteria (165, 166). A long-term follow-up study of patients with early presentation of erythema migrans and treated with antibiotics at the time of diagnosis showed an excellent rate of remission, with only 4% of patients remaining symptomatic during follow-up evaluation (167). Conversely, other trials reported rates of PTLDS ranging from approximatively 10–20% (168). Nevertheless, in the community medical practice, where prompt LB diagnosis and treatment are not common, PTLDS rates may reach 50% (169, 170). Notably, xenodiagnoses demonstrated the presence of *B. burgdorferi* DNA in a patient with PTLDS, despite repeated cycles of antibiotic treatments (171). *B. burgdorferi* DNA was detected in mice after prolonged (up to 12 months) treatment with antibiotics despite the persistence of non-cultivable *bacteria.* Moreover, the study revealed *B. burgdorferi* DNA and the presence of RNA transcripts of multiple spirochetal genes in host tissues (172). These findings suggest that *B. burgdorferi* persist within the host indicating that the immune system and antimicrobial treatment may not be effective at eradicating *B. burgdorferi*. This may contribute to antibiotic-refractory arthritis, as observed in a murine model in which spirochetal antigens, but not infectious spirochetes, were recovered near cartilage for extended periods after LB therapy (173).

According to the guidelines of the European Federation of Neurological Sciences and the Infectious Diseases Society of America, treatment with beta-lactams antibiotics, like ceftriaxone, penicillin, or cefotaxime, or oral doxycycline for 14–21 days is recommended for the treatment of LNB

(7, 25, 174). Intravenous administration of ceftriaxone is often recommended for the treatment of Lyme meningitis. Oral treatment with doxycycline demonstrated to be as effective as ceftriaxone for Lyme meningitis in adults in Europe, although not recommended as first-line therapy in the United States (175). Nevertheless, four NIH-sponsored trials aimed at assessing the administration of antibiotic treatment in patients with persistent unexplained symptoms despite previous antimicrobial treatment of LB indicated that the new treatment cycle provides little if any clinical benefit (176). A randomized, double-blinded, placebo-controlled trial conducted in Europe, in patients with persistent symptoms attributed to Lyme disease showed that longer-term antibiotic treatment did not have a better outcome as compared with shorter-term treatment (177).

Biofilm Production and Antimicrobial Tolerance in *Borrelia burgdorferi*

B. burgdorferi can switch from motile cellular forms into several defensive morphological forms such as round bodies, stationary phase, persister cells, and biofilm (23, 24, 178–182). Transition between different morphologies represents an adaptation strategy to survive in unfavorable environmental conditions, including pH variations, nutrient starvation, host immune system attacks, or the presence of antimicrobial agents (21, 23, 24, 172, 179, 181).

Notably, within the biofilm, bacteria are physically joined together producing a matrix, characterized by the presence of an extracellular polymeric substance (EPS) composed by polysaccharides, proteins, and extracellular DNA (183). Bacterial biofilms are intrinsically more resistant to environmental agents and antimicrobials than the corresponding planktonic counterpart and this can lead to chronic and recurrent infections (184–186). *In vitro* and *in vivo* studies revealed that both *B. burgdorferi* sensu stricto and sensu lato (*B. afzelii* and *B. garinii*) aggregates, but not free-floating spirochetes, present typical markers found in the EPS of other pathogenic bacteria such as sulfated mucins, non-sulfated mucins (mainly alginate), extracellular DNA and calcium (22, 23, 187). *B. burgdorferi* biofilm is also characterized by the presence of a distinctive architecture with channel-like elements that in mature biofilm are required for oxygen and nutrient diffusion and waste removal (22, 23, 187). Biofilm formation by *B. burgdorferi* follows the same evolution described for other bacteria. Initially, individual spirochetes adhere to biotic or abiotic surfaces forming microcolonies, coated by the EPS. From this point, *Borrelia* aggregates expand undergoing changes in the growth rate, gene expression and structural rearrangements in the EPS components (22, 23). The rapid rearrangements occurring within the biofilm matrix, culminate in a complex three-dimensional structure with common traits observed among *Borrelia* genera (**Figure 1**) (23, 189). The existence of biofilm-like structures was further found in human skin biopsies obtained from patients with borrelial lymphocytoma, a common manifestation of LB in Europe, revealing the presence of *Borrelia*-positive aggregates characterized by mucopolysaccharides, especially alginate (22). Other reports demonstrated that *Borrelia* DNA is deposited in the Alzheimer brain showing structural similarities between spirochetal aggregates and the profiles of amyloid plaques in patients with Alzheimer disease (188, 190). Nevertheless, the specific contribution of biofilm to borrelial persistent infections remain unclear. Besides, additional *in vivo* studies showed the presence of *Borrelia* aggregates in the midguts of naturally-infected nymphs during their blood meal (191). These results strongly suggest that biofilm may contribute to the spirochetal successful transmission to the mammalian host and to the ensuing disease manifestations (191).

Biofilm production in *Borrelia* requires the modulation of a complex array of signaling processes which allows spirochetes to communicate with the surrounding environment. The RpoN–RpoS alternative sigma factor and the LuxS quorum-sensing pathways, which are involved in several cellular functions in response to environmental stresses (pH and temperature variations, high osmolarity, oxidative stress, high cell density, nutrient starvation, host infection), participate in biofilm production in *B. burgdorferi* (22, 192). The RpoN–RpoS pathway, also known as the σ^{54}-σ^{S} cascade, regulates adaptive changes in *B. burgdorferi* during the transition between the tick vector and mammalian host (91, 95). The RpoN–RpoS pathway relies on the activity of RpoN (σ^{54}), which controls the transcription of RpoS (σ^{S}) through the binding to a canonical −24/−12 RpoN-type promoter sequence (95, 193). The activation of the σ^{54}-σ^{S} cascade, in turn, is modulated by a bacterial enhancer-binding protein (bEBP)/σ54-dependent activator (Rrp2) in concert with BosR (91, 95, 193–198). After the activation, RpoS acts as a global gene regulator controlling the expression of over 100 different genes involved in stress responses, host infection and survival, including biofilm formation (87, 95, 182).

Mutant strains of *B. burgdorferi* lacking RpoN, RpoS, presented a less compact biofilm with loose and dispersed small aggregates compared to wild-type strains (182). Notably, all mutants expressed *Borrelia* biofilm markers such as alginate, extracellular DNA, and calcium, although they showed significantly higher sensitivity to low MIC dose of doxycyline (0.1 μg/ml) than the wild-type strain (182). In addition, the quorum sensing (QS) molecules LuxS also contributes to *B. burgdorferi* biofilm. The QS signaling system is a cell-to-cell communication mechanism, shared by different bacteria, which is based on the release of small molecules called autoinducers (AI) in environment (199). LuxS pathways regulate biofilm formation in various ways according to bacterial species and environmental conditions (183). Specifically, *luxS* mutant strains of *Streptococcus gordonii* and *Porphyromonas gingivalis*, which are two important components of dental plaque, are unable to produce a mixed-species biofilm (200). Besides, in *Helicobacter pylori* the presence of *luxS* mutation leads to a more efficient biofilm formation than the wild type whereas a *luxS* mutant of *Streptococcus mutans* shows an altered biofilm structure (183, 201–203). *B. burgdorferi* significantly increases transcription of *luxS* during transition from ticks to mammalian hosts where it is involved in the regulation of several genes such as *vlsE*, *erpA*, and *ipLA7* (204–207). *luxS* mutant strains in stationary cultures of *B. burgdorferi* showed a higher tendency

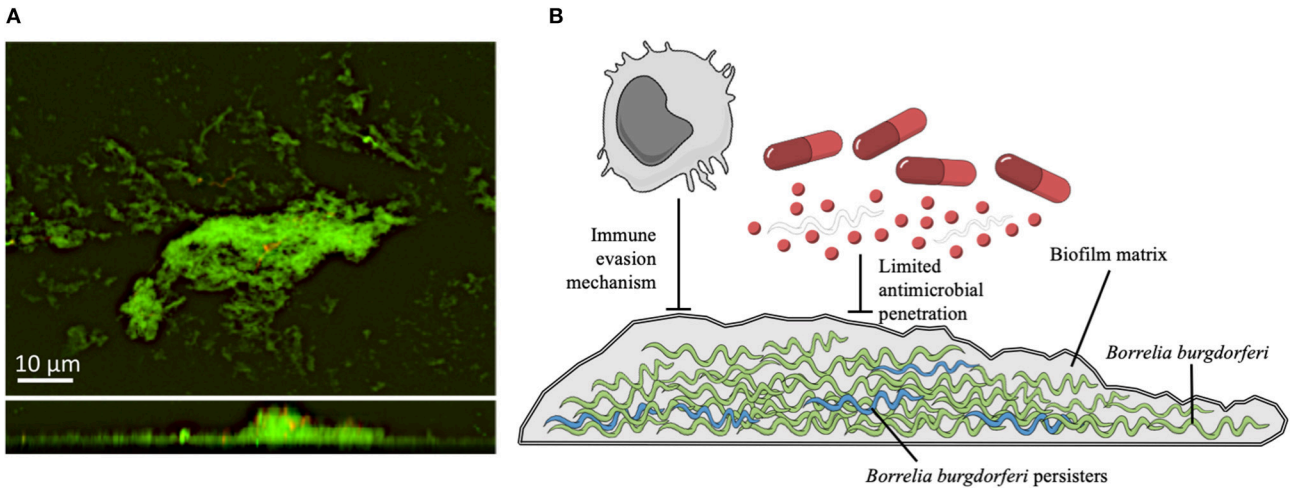

FIGURE 1 | (A) Confocal microscopy images of *B. burgdorferi* B31 strain (American Type Tissue Collection 35210) biofilms. Upper panel show the X-Y planes (top view), while the lower panel show the Z section (side view). The sample was stained with *BacLight* Live/Dead (Invitrogen Life Technologies, Carlsbad, CA, USA) (188). Representative images of biofilms developed on polystyrene pegs following 72 h incubation at 37°C. Spirochetes were grown in a µ-Slide 8-well system (ibidi, Germany) and cultured in BSK-H medium containing 6% rabbit serum (Sigma-Aldrich). **(B)** Schematic representation of a *B. burgdorferi* biofilm. The biofilm matrix produced by spirochetes (green) provides shelter from host defenses, and reduces the diffusion of antibiotics. Persister forms of *B. burgdorferi* (in blue), exhibit multidrug tolerance and are likely responsible for the recalcitrance of chronic LB. The illustration is adapted from Mind the Graph (https://mindthegraph.com) under the Creative Commons License.

to form smaller and looser aggregates and a greater sensitivity to antibiotics than the wild-type counterpart (182).

Although antibiotic treatment resolves most of clinical manifestations of LB, persistent forms occur in ∼10% of patients after treatment for erythema migrans disease (208, 209). The long-term persistence of symptoms and failure of the antibiotic therapy are reminiscent of chronic biofilm-associated infections. Biofilm aggregates display an enhanced tolerance to various antibiotics, which, conversely, are effective against the planktonic spirochetes and round body forms of *B. burgdorferi* (210). In particular, doxycycline and amoxicillin were found to effectively kill the motile spirochete forms *in vitro*, but failed to completely remove *B. burgdorferi* in biofilms (20, 21, 24, 26, 210–214). High throughput screens of *B. burgdorferi* identified several promising Food and Drug Administration (FDA)-approved drugs that have excellent anti-persister activity (24, 181, 213, 215). Among them, daptomycin, which is a lipopeptide targeting bacterial cell membranes, clofazimine, carbomycin, sulfa drugs such as sulfamethoxazole, and certain cephalosporins such as cefoperazone, showed higher activity against *B. burgdorferi* persister cells resulting more effective than doxycycline or amoxicillin (213). Although the combination of these drugs was found to be active against *B. burgdorferi* persisters, they showed poor activity when used individually (24). Daptomycin was found to be the most active antibiotic when combined with doxycycline plus either beta-lactams like cefoperazone or carbenicillin or alternatively with clofazimine (212). Daptomycin in combination with doxycycline and cefoperazone was found to be able to completely eradicate *B. burgdorferi* persisters, revealing a durable killing activity that was not achieved by any other drug combinations (212). These results where further supported by prospective randomized clinical studies which failed to demonstrate significant beneficial effect of additional prolonged therapy with doxycycline, amoxicillin or ceftriaxone in monotherapy, in patients with Lyme encephalopathy and post-treatment symptoms of Lyme disease (176, 215).

In addition to biofilm formation, the ability of *Borrelia* to localize intracellularly in the host has been proposed as a mechanism which might favor chronic or persistent infection and may contribute in reducing the efficacy of antibiotics. However, *Borrelia* predominantly occupies the extracellular matrix, and the antibiotics recommended for the treatment of LB are first-line drugs in several intracellular infections (216, 217). Doxycycline and azithromycin are commonly used for the treatment of *Mycoplasma*, *Chlamydia*, and *Legionella*, while ceftriaxone is effective against *Salmonella* and *Neisseria*, and amoxicillin is used to treat *Listeria* infections (217, 218). Nevertheless, biofilm production by extracellular bacteria and intracellular localization of *Borrelia* are not mutually exclusive and may both participate in supporting chronic bacterial persistence in the host.

On the other hand, a polymicrobial infection is a frequent occurrence in ticks (219, 220). Chronic and persistent forms of Lyme have been also associated to infections caused by *Babesia* spp. and *Anaplasma phagocytophilum*, *Bartonella henselae*, or other minor pathogens (217, 219, 221). This condition may add a further level of complexity to the clinical and therapeutic management of LB since it may lead to inappropriate diagnoses and apparent failure of the antibiotic treatment targeted exclusively against *Borrelia*. However, the real clinical relevance of these coinfections is unclear and requires further, more in depth evaluation.

CONCLUDING REMARKS

LNB is the most dangerous manifestation of Lyme disease. Although the early antimicrobial treatment is effective in the majority of patients, persistent forms are relatively common. The mechanisms underlying chronic LNB and other persistent forms of Lyme are unknown. Patients who have late manifestations of LB generally show a slower response to therapy with incomplete resolution. Persistent *Borrelia* infection requires prolonged antimicrobial treatment, with limited and controversial clinical efficacy. Recent evidences suggest that the antibiotic resistance and the reoccurrence of LB are associated with biofilm-like aggregates, which allow *Borrelia* spp. to resist to adverse environmental conditions. Several promising FDA-approved drugs have been shown to have excellent anti-persister activity when used in combination while their use in monotherapy regimens showed a poor effectiveness. This notion should be taken into careful consideration for the clinical management of Lyme Disease in order to prevent long-term complications.

In preliminary studies by the clinical Biofilm Ring Test® (cBRT), we found that *Borrelia* is able to readily produce biofilm within 24–48 h. Diagnostic procedures such as the cBRT, which allow for a rapid biofilm measurement may represent very useful tools for clinical applications (222, 223), since the rapid identification of biofilm-producing *Borrelia* strains, may help identify forms of LB which are at risk of chronicity (224). Further, characterization of *Borrelia* biofilm as well as the ensuing inflammatory process will likely provide novel insight to better understand the mechanism(s) concurring to LNB pathogenesis and may offer new therapeutic targets for intervention.

AUTHOR CONTRIBUTIONS

Conceived and designed the study: ED, IC, LT, FP, and FE. Performed the confocal microscopy analysis: ED, IC, MP, GP, and MG. All authors analyzed data. Wrote the paper: ED, IC, LT, VB, GD, ET, and FE. All the authors read and approved the final version of the manuscript.

ACKNOWLEDGMENTS

This work was partially supported by L'Associazione Nazionale Contro le Infezioni Ospedaliere (L'ANCIO).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neurology

REVIEW
published: 26 November 2018
doi: 10.3389/fneur.2018.01001

Antiphospholipid Syndrome and the Neurologist: From Pathogenesis to Therapy

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Antiphospholipid syndrome (APS) is an autoimmune antibody-mediated condition characterized by thrombotic events and/or pregnancy morbidity in association with persistent positivity to antiphospholipid antibodies (aPL). The nervous system is frequently affected, as intracranial vessels are the most frequent site of arterial pathology. Over the course of years, many other neurological conditions not included in the diagnostic criteria, have been associated with APS. The pathogenic mechanisms behind the syndrome are complex and not fully elucidated. aPL enhance thrombosis, interfering with different pathways. Nevertheless, ischemic injury is not always sufficient to explain clinical features of the syndrome and immune-mediated damage has been advocated. This may be particularly relevant in the context of neurological complications. The reason why only a subgroup of patients develop non-criteria nervous system disorders and what determines the clinical phenotype are questions that remain open. The double nature, thrombotic and immunologic, of APS is also reflected by therapeutic strategies. In this review we summarize known neurological manifestations of APS, revisiting pathogenesis and current treatment options.

Keywords: APS, antiphospholipid syndrome, aPL, antiphospholipid antibodies, neurological manifestations, pathogenic mechanisms, therapy

OPEN ACCESS

Edited by:
Tatiana Koudriavtseva, Istituto Nazionale del Cancro Regina Elena, Italy

Reviewed by:
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Specialty section:
This article was submitted to Multiple Sclerosis and Neuroimmunology, a section of the journal Frontiers in Neurology

Received: *31 July 2018*
Accepted: *06 November 2018*
Published: *26 November 2018*

Citation:
Fleetwood T, Cantello R and Comi C (2018) Antiphospholipid Syndrome and the Neurologist: From Pathogenesis to Therapy. Front. Neurol. 9:1001. doi: 10.3389/fneur.2018.01001

INTRODUCTION

Antiphospholipid syndrome (APS) is an autoimmune antibody-mediated disorder defined by the occurrence of thrombosis and/or pregnancy morbidity in presence of persistent antiphospholipid antibodies (aPL) (1). The estimated incidence of APS is approximately 5 cases every 100,000 subjects/year with a prevalence of 40–50 every 100,000 subjects (2). The diseases can occur alone (primary APS) or in the context of other autoimmune conditions, in particular systemic lupus erythematosus (SLE), Sjögren's syndrome and rheumatoid arthritis (secondary APS). Veins, arteries and small vessels can all be affected by thrombosis, with deep veins of the legs and intracranial arteries being the most common sites of venous and arterial thrombosis, respectively. Pregnancy morbidity includes embryonic losses, fetal death, and premature birth. The diagnosis is made according to the updated international Sydney consensus criteria (**Table 1**) (1). However, patients with persistent aPL may present with clinical manifestations not included in the criteria (the so called "non-criteria" symptoms), among which thrombocytopenia, hemolytic anemia, cardiac valve disease, renal microangiopathy, livedo reticularis, and neurologic disturbances other than ischemic cerebrovascular accidents (CVA) (3).

In this paper we summarize current concepts regarding the pathogenesis of APS, reviewing neurological clinical features and related therapeutic implications.

PATHOGENESIS OF APS: THROMBOSIS AND OTHER MECHANISMS

Clinical manifestations in APS are associated with aPL presence. aPL, namely lupus anticoagulant (LA), anti-cardiolipin (aCL), anti-β2-glycoprotein-I (anti-β2-GPI), are an heterogeneous group of auto-antibodies directed against phospholipid binding proteins (4). The current hypothesis is that susceptible individuals develop aPL upon exposure to an external trigger, such as an infective agent, through a mechanism of molecular mimicry (2). β2-GPI, one of the main antigens recognized by aPL, is a plasma protein composed of five domains (I–V) (5). Circulating β2-GPI presents a circular form, in which epitopes present in domain I and recognized by B-cells are hidden. Upon binding to an anionic phospholipid surface, β2-GPI undergoes a conformational change exposing the cryptic epitopes (6). Oxidative stress seems to enhance the immune reaction, leading to the formation of disulfide bonds in domain I that further increase its immunogenic potential (7). Prevalence of aPL in the general population is around 1–5% (8) but only a minority of subjects develops APS, suggesting that the presence of autoantibodies alone is not sufficient to cause pathology. Following aPL formation, a "second hit" thus is necessary. It has been demonstrated that β2-GPI only binds its ApoE receptor 2 (ApoER2) when vessel endothelial cells become activated (9), inducing dimerization of the receptor and activation of intracellular signaling. A number of conditions that increase oxidative stress, including infection, malignancy, pregnancy and smoking, may act as triggers for endothelium priming (10, 11). The mechanisms through which aPL enhance thrombosis is complex and in part still to be understood. Different mediators are likely to be involved, among which endothelium, platelets, complement, and innate immune systems (9) (**Figure 1**). First, aPL stimulate the expression of proadhesive, procoagulant, and proinflammatory molecules. Tissue factor (TF) is a key component of the extrinsic coagulation pathway, implicated in the activation of thrombin, that becomes exposed upon vessel injury. aPL enhance surface expression of TF on endothelial cells by binding to annexin A2 and toll-like receptor 4 (TLR4) and activating the nuclear factor κB (NF-κB) signaling pathway (12–14). aPL also seem to interact directly with monocytes and neutrophils, inducing mitochondrial dysfunction and subsequent expression of TF and proinflammatory tumor necrosis factor α (TNF-α) (15). Annexin A5 is a protein involved in many biological processes. On endothelial cells it binds to phosphatidylserine molecules, forming a shield that inhibits the activation of procoagulant complexes. *In vitro* studies have shown that aPL binding to annexin A5 disrupts the shield leading to thrombosis (16). Endothelial cells also contribute to modulate the activity of vessel wall muscular cells through the production of nitric oxide by endothelial nitric oxide synthase (eNOS). In murine models, aPL inhibit the activity of eNOS (17). This can lead to impaired regulation of vascular tone, increase in superoxide and peroxynitrite, and cell adhesion (18, 19). Confirming this hypothesis, APS patients have reduced plasma levels of nitric oxide compared to controls (20). Platelets play a pivotal role in thrombus formation. β2-GPI binds to the von Willebrand factor (vWF) receptor glycoprotein Ibα and to the ApoER2 on the platelet surface inducing the release of thromboxane A2 and enhancing aggregation and adhesiveness (21). Interestingly, binding of aPL to platelet membrane phospholipids leads to activation and possible dysregulation of serotonin metabolism, which could be involved in the pathogenesis of aPL-mediated migraine (22). Some authors have also identified anti-platelet antibodies in the setting of APS (23). aPL can activate the classical complement pathway, inducing production of C5a, which in turn can bind to neutrophils and stimulate the expression of TF (24). Previous studies have also suggested that aPL impair thrombolysis by interfering with tissue plasminogen activator (tPA) and plasmin (25). Despite the amount of evidence gathered over the course of years, thrombosis alone is perhaps not sufficient to explain all of the clinical effects of aPL. Direct binding of aPL to nervous system antigens has been proposed to explain some neurologic manifestations of APS, which may be mediated by inflammation and neurodegeneration (26, 27). An experimental model of APS (eAPS) can be obtained by immunizing normal mice with β2GPI, therefore inducing aPL and typical clinical features of APS (28). Interestingly, in eAPS mice, long-term exposition to aPL also leads to behavioral hyperactivity and decline in cognitive performances (29, 30), which resolve after elimination of aPL through ultraviolet irradiation (26). A proposed mechanism of neuronal injury focuses on the disruption of the blood-brain barrier (BBB), secondary to diffuse endothelial dysfunction caused by aPL binding (31). Katzav et al. have demonstrated impaired integrity of the BBB and accumulation of aPL in cortical and inhibitory hippocampal neurons of immunized mice (32), which may be linked to behavioral changes and cognitive impairment. Furthermore, in a previous study eAPS mice displayed higher brain levels of proinflammatory TNF-α and prostaglandin E (PGE) and lower levels of thrombin inhibitors compared to controls (33). Treatment with aspirin and enoxaparin ameliorated concentrations of TNF-α, PGE and thrombin inhibitors, as well as behavioral patterns. An interesting correlation between coagulation and autoimmunity has been investigated in eAPS mice carrying the factor V Leiden mutation (FVL), either in homozygosis ($FVL^{Q/Q}$) or in heterozygosis ($FVL^{Q/-}$). Induced aPL levels were higher in $FVL^{Q/Q}$ mutated mice compared to $FVL^{Q/-}$ and controls, as well as the burden of behavioral and cognitive impairment and of neurodegenerative changes on histological examination (34). It is known from previous studies that aPL have the potential to bind to myelin, brain ependyma and choroid epithelium epitopes in the animal model (35). A direct interaction between aPL and specific, yet still unidentified, basal ganglia epitopes, may lead to the development of movement disorders in APS patients. Supporting this hypothesis Dale et al. in 2011 demonstrated binding of IgG from the serum of pediatric APS patients with chorea to neuronal cell-surface antigens of cultured neuronal cells with

TABLE 1 | Revised classification criteria for APS [adapted from Miyakis et al. (1)].

Clinical criteria (one or more of the following):
VASCULAR THROMBOSIS
• One or more clinical episodes of arterial, venous, or small vessel thrombosis, in any tissue or organ.
PREGNANCY MORBIDITY
• One or more unexplained deaths of a morphologically normal fetus at or beyond the 10th week of gestation • One or more premature births of a morphologically normal neonate before the 34th week of gestation because of eclampsia or severe preeclampsia (placental insufficiency). • Three or more unexplained consecutive spontaneous abortions before the 10th week of gestation.
LABORATORY CRITERIA (ONE OR MORE OF THE FOLLOWING):
1. Lupus anticoagulant (LA) in plasma, on two or more occasions at least 12 weeks apart, detected according to the guidelines of the International Society on Thrombosis and Haemostasis. 2. Anticardiolipin antibody (aCL) of IgG and/or IgM isotype in serum or plasma, present in medium or high titer (i.e., >40 GPL or MPL, or >the 99th percentile), on two or more occasions, at least 12 weeks apart, measured by a standardized ELISA. 3. Anti-beta-2-glycoprotein-I antibody (anti-β2-GPI) of IgG and/or IgM isotype in serum or plasma (in titer >the 99th percentile), present on two or more occasions, at least 12 weeks apart, measured by a standardized ELISA.

dopaminergic characteristics (36). Furthermore it has been shown that aPL can impair GABA receptor activity and induce depolarization of synaptoneurosomes, disrupting neuronal function by acting on nerve terminals (37, 38), with possible implications in APS-associated epileptogenesis. Energetic dysfunction in neuronal cells and altered neurotransmission may also play a role in APS pathology, since it has been demonstrated that aPL from patients with neurological involvement also bind to adenosine triphosphate (ATP) (39).

NEUROLOGICAL MANIFESTATIONS OF APS

The nervous system is a major target of APS. This appeared clear since the first description of the syndrome by Hughes in 1983 in which he described CVA and transverse myelitis (40). Although thrombotic damage has been advocated to explain many neurologic manifestations, direct immune-mediated processes may also be involved (41). Neurologic symptoms have been therefore classified in thrombotic and non-thrombotic according to the supposed primary pathogenic mechanism (**Table 2**) (42). The reason why some patients develop neurological symptoms is unknown as well as what determines the site of lesion (e.g., central vs. peripheral nervous system) and the clinical phenotype. Possible explanations may be related to antibody subtypes, individual susceptibility or even genetic predisposition.

Cerebrovascular Disease

Acute ischemic stroke and transient ischemic attack (TIA) are the most common manifestations of arterial pathology in APS (2). According to the Euro-Phospholipid Project Study Group the cumulative prevalence of stroke and TIA in APS patients are 19.8 and 11.1%, respectively, (43). On the other hand, aPL may be detected in up to 13.5% of stroke patients (44), with higher rates in young subjects. Moreover, age at onset in aPL-positive patients is anticipated. A previous study on 128 patients with CVA and aPL showed a mean age was 46 years (45). It has been suggested that APS accounts for over 20% of cases in the young population (46). A 2015 systematic review of 43 studies showed that the presence of aPL in subjects under the age of 50 increased the risk for thrombotic cerebrovascular events by 5.48-fold (47). The association between aPL and stroke incidence in older patients is less clear, due to the higher prevalence of other vascular risk factors (48). Clinical manifestations depend on the site and entity of the lesion. In a sequence of 110 SLE and non-SLE aPL-positive patients undergoing neuroradiological evaluation, the most common finding was large infarcts (22%), followed by white matter changes (17%), small cortical infarcts (10%), and lacunar infarcts (9%) (49). In a cohort of 55 APS patients, 25 of which suffered an ischemic stroke, the most common site of occlusion was the middle cerebral artery (31%) (50). *In situ* thrombosis is thought to be the most frequent pathogenic mechanism, whereas in other cases, cardiac embolisms can arise from involvement of the cardiac valvular apparatus, with thickening of valve leaflets by deposition of immune complexes (Liebman-Sacks endocarditis) (51). Besides stem or branch occlusion of intracranial arteries, a vasculitis-like pattern, with multiple sites of narrowing and dilation has also been described through arteriography (52). Alterations of extracranial arteries appear to be less frequent (52), though early atherosclerosis seems to affect APS patients (53), and some authors have proposed a direct role of the autoantibodies through oxidative damage (54). Although rare, cerebral venous thrombosis (CVT) can complicate APS or, in some cases, be the presenting symptom (55). Presence of aPL in non-SLE patients with CVT has been reported in previous studies (56). aCL positivity may be found in 7–22% of patients (51). APS accounts for ∼6–17% of all CVT cases, being one of the most frequent prothrombotic conditions associated (57). Other vascular conditions have also been reported in association with APS, including Sneddon's syndrome and reversible cerebral vasoconstriction syndrome (RCVS). Sneddon's syndrome is a rare non-inflammatory thrombotic vasculopathy, characterized by livedo reticularis and recurrent cerebral infarctions (53). Other clinical manifestations include headache, seizures and cognitive decline (58). Details of the pathogenetic mechanisms are still to be clarified but non-inflammatory thrombotic vasculopathy is seen in medium- and small-sized arteries in the brain and skin (59). Indeed, neuroradiological findings show that leukoaraiosis and small lacunar infarcts are more common than infarcts in the territory of large cerebral arteries (60). Reversible cerebral vasoconstriction syndrome (RCVS) is a neurological disorder marked by severe headaches variably associated with seizures, ischemic stroke, and subarachnoid hemorrhage (61). The cause has been related to a possible disturbance in the regulation of cerebrovascular tone (62).

Headache

Headache, specifically in the form of migraine, is the most common neurologic symptom of APS patients, with an estimated

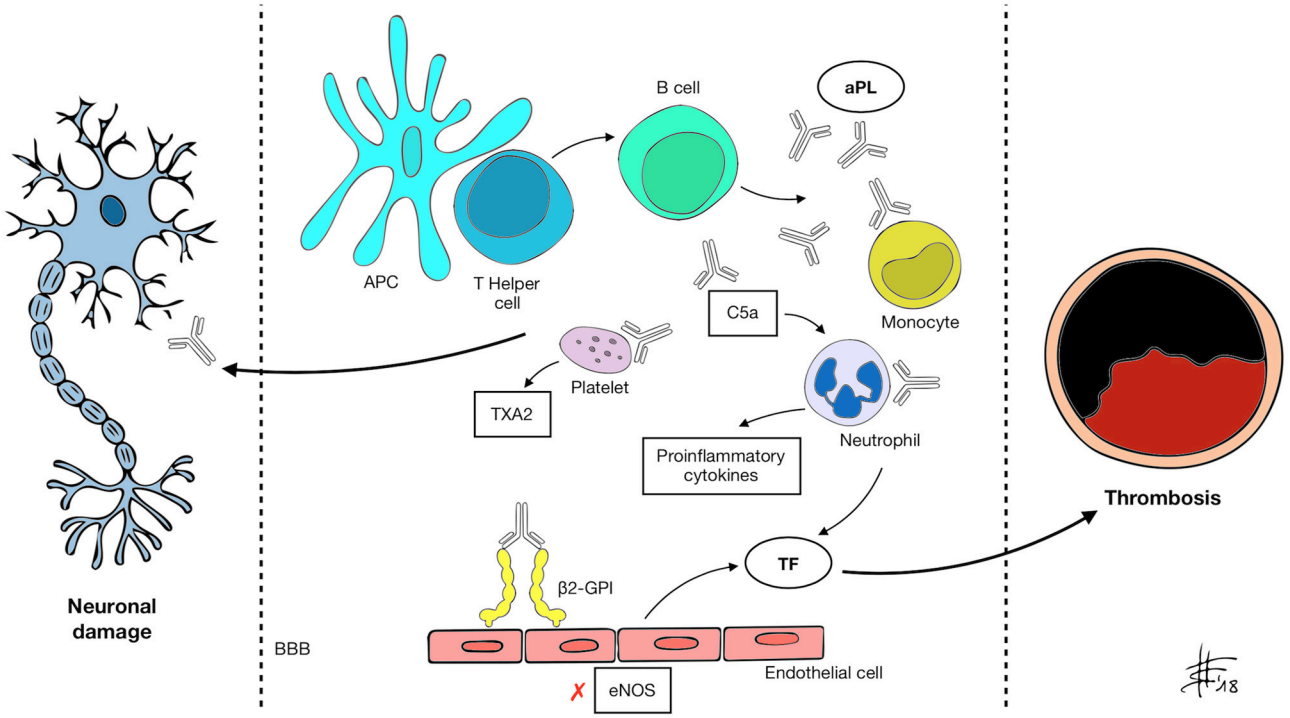

FIGURE 1 | APC, antigen presenting cell; aPL, antiphospholipid antibody; β2-GPI, β2 glycoprotein-I; BBB, blood-brain barrier; C5a, activated complement 5 fraction; eNOS, endothelial nitric oxide synthase; TXA2, thromboxane A2; TF, tissue factor. A mechanism of molecular mimicry may underlie B cell activation by the APC-T helper system and subsequent aPL production in susceptible individuals. Through recognized antigens (mainly β2-GPI) aPL interact with different cell types and activate the classical complement pathway, inducing expression of pro-inflammatory cytokines, enhancing platelet adhesiveness and aggregation, impairing vascular tone by inhibiting eNOS and increasing the expression of TF. These changes determine a prothrombotic state and increase the risk of vascular events. aPL also appear to be able to recognize and target nervous system antigens, possibly through the disruption of the BBB. Neurological manifestations of APS may be determined by both mechanisms.

prevalence of 20.2% (43). Indeed, aPL antibodies are more frequently found in migraineurs than in age-matched controls (63). However, given its high prevalence in the general population, it is difficult to establish whether APS is a risk factor for developing migraine or if this represents a comorbid condition. Previous CVA represent a strong risk factor for developing migraine. However, not all patients have a positive history for stroke or TIA, which suggests non-ischemic mechanisms (64). On the contrary some authors have found long-standing migraine to be a risk factor for stroke in APS patients (65). Further studies are necessary to elucidate such interplay. Notably, APS-associated migraine may be difficult to control with classic analgesic regimens (66).

Seizures and Epilepsy

APS seems to confer a higher risk of developing seizures, with an estimated prevalence of ∼8% (67), which may further increase in APS secondary to SLE (68). All forms of epilepsy may be seen, including subclinical forms, determined by the presence of abnormal electroencephalography findings alone (46). It has been suggested that 20% of idiopathic juvenile epilepsy cases may be associated with aPL (69). According to existing studies, previous CVA have been identified has the most solid risk factor for developing seizures (68). It is therefore easy to speculate that the most plausible pathogenic mechanism is ischemic damage to brain tissue, leading to the formation of cortical epileptogenic foci (64). However, seizures may also develop in structurally normal brains, suggesting an antibody-mediated mechanism (70).

Movement Disorders

Movement disorders are a possible, though rare, neurological manifestations in the setting of APS. Chorea in particular occurs in 1.3–4.5% of patients (71), and may be the first symptom of the syndrome (72, 73). Other less frequently reported conditions include parkinsonism (corticobasal-like syndrome and progressive supranuclear palsy phenotype), dystonia, ballismus, paroxysmal dyskinesias, tremor, tic, myoclonus, cerebellar ataxia (41). Mixed clinical presentations have also been described (74). The pathogenesis of movement disorders in the setting of APS is matter of debate. On the one hand, cerebral infarctions and white matter changes on MRI suggest a thrombo-occlusive mechanism, which could account for the majority of cases (51, 75). On the other, immune-mediated attack against basal ganglia epitopes has been suggested and reported in some cases (41). No specific correlations between the type of antibody and clinical features have been reported

TABLE 2 | Neurological manifestations of APS.

Thrombotic	Non-thrombotic
• Acute ischemic stroke • Transient ischemic attack (TIA) • Cerebral venous thrombosis (CVT) • Sneddon's syndrome • Reversible cerebral vasoconstriction syndrome (RCVS)	• Headache • Seizures and epilepsy • Movement disorders • Multiple sclerosis-like disease • Transverse myelitis • Cognitive impairment and dementia • Neuropsychiatric symptoms • Peripheral neuropathy • Autonomic dysfunction

(41). Nevertheless, it is likely that genetic predisposition might partially explain phenotypic variability, in analogy to what was reported in Parkinson's disease (76).

Multiple Sclerosis-Like Disease

Overlap of clinical and laboratory findings of multiple sclerosis (MS) and SLE have been described long ago, leading to the proposal of a hybrid condition named "lupoid sclerosis" (77, 78). Diagnostic criteria, as well as the role of anti-nuclear antibodies (ANA) and aPL are still matter of debate (79). Several studies have provided different estimates of aPL prevalence in definite MS patients, ranging from 2 to 88% (80), with higher titers observed during exacerbations of the disease (81). aCL and anti-β2GPI appear to be more prevalent compared to LA, although the latter have been studied to a lesser extent and the real prevalence remains to be clarified (80). Some authors have suggested that aPL may alter the integrity of the BBB and facilitate the access of immune cells to the central nervous system (CNS) compartment (81, 82). APS can also present with symptoms resembling MS, including visual, sensitive or motor deficits with a relapsing remitting course, with similar MRI T2 lesions (83–87). This condition is referred to as MS-like disease (64). Indeed, the differential diagnosis between these two conditions may be challenging. Acute onset of atypical MS symptoms, coexistence of other typically APS-related neurological manifestations (for example headache or epilepsy), connective tissue-like features or a history of thrombosis, pregnancy morbidity should orientate toward APS (88, 89). Neuroimaging studies may also help in the differential diagnosis. APS lesions on MRI are smaller, frequently localized in the subcortical area, are stable over time and may also improve with anticoagulation therapy (80, 90). Normal cell count and absence of oligoclonal bands on CSF analysis also suggests APS (91). Some authors have suggested that aPL may alter the integrity of the BBB and facilitate the access of immune cells to the central nervous system (CNS) compartment (81, 82).

Transverse Myelitis

Transverse myelitis (TM) is an inflammatory condition affecting the gray and white matter of the spinal cord (92). Symptoms include motor and sensory level deficits and sphincter abnormalities. Estimated prevalence in APS is around 0.4–4% (71, 93). Although the exact pathogenesis is unsure, vasculitis and arterial thrombosis resulting in ischemic cord necrosis have been suggested (89). Furthermore, APS has been described in overlap with neuromyelitis optica spectrum disorder (NMOSD) (94, 95), another autoimmune condition characterized by recurrent episodes of optic neuritis and longitudinally extensive transverse myelitis (that is, extended over 3 or more spinal cord segments) and positivity for anti-aquaporin-4 (anti-AQP4) or anti-myelin oligodendrocyte glycoprotein (anti-MOG) (96). Therefore, some authors suggest screening APS patients presenting with optic neuritis or myelitis for NMO-associated autoantibodies (95).

Cognitive Impairment and Dementia

A high percentage of patients with primary APS (which may reach 42–80%) develop some degree of cognitive impairment, usually with a subcortical pattern (64, 97). In some cases, deficits may even precede the diagnosis of APS, as demonstrated in a study by Jacobson et al. on aPL-positive non-elderly subjects who displayed differences in executive functioning, verbal learning and memory, and visuospatial ability compared to age- and education-matched controls (98). Dementia frequency has been estimated around 2.5% in APS patients (71). MRI studies have shown a high burden of white matter lesions in APS patients with cognitive impairment (99) resembling multi-infarct dementia. However, vascular damage may not be the only mechanism. Findings of degenerative rather than multi-infarct dementia have also been described in aPL-positive elderly subjects (100). Other studies and a meta-analysis have underlined a strong association with aCL antibodies (101). Furthermore, some animal models showed that cognitive dysfunction can be induced by intraventricular injection of neuronal-binding antibodies from APS patients (26), while other failed to demonstrate an association with ischemic lesions (102). Such findings support the idea of a direct effect of aPL on congnition. aPL-mediated dysregulation of the dopaminergic system has also been proposed (103). Given the clinical overlap, MS-like disease should also be considered in the differential diagnosis.

Neuropsychiatric Symptoms

Psychiatric symptoms, including psychosis, mania, depression, bipolar disorders, obsessive–compulsive disorders, and schizophrenia have been described in APS patients (64). Older age, cerebral lesions, and triple aPL positivity (i.e., anti-β2-GPI, aCL, and LA) are considered risk factors (104). A high prevalence of aPL has been described in patients with psychosis, though such finding must be interpreted carefully as antipsychotic medications are though to induce aPL and aCL in particular (66).

Peripheral Neuropathy

A small study by Santos et al. in 2010 has investigated the involvement of the peripheral nervous system in APS (105). The most frequent findings were sensori-motor neuropathy and isolated carpal tunnel syndrome. Most patients were asymptomatic and showed no sign of pathology upon physical examination. The underlying pathogenic mechanism is not clear, and may be linked to thrombosis of the vasa nervorum, vasculitis or even targeting of lipidic components of myelin by aPL. Reports of Guillain-Barré syndrome in APS patients support this hypothesis (106).

Autonomic Dysfunction

Several reports exist of autonomic dysfunction in the context of APS. A 2017 work by Schofield describes the clinical findings in 22 patients with autonomic dysfunction as the initial symptom of APS (107). Manifestations included postural tachycardia syndrome, neurocardiogenic syncope, inappropriate sinus tachycardia, labile hypertension, complex regional pain syndrome, severe gastrointestinal dysmotility, and neurogenic bladder, with 45% of subjects presenting more than one disorder. Reduction in autonomic and sensory small fibers, assessed by skin punch biopsy was widespread among subjects. Ten patients (45%) subsequently developed arterial thrombosis, of which 8 (36%) presented with stroke, TIA, or amaurosis fugax. Pathogenesis of small fiber dysfunction may derive either from microthrombosis or from direct antibody binding to neuronal epitopes leading to nerve dysfunction. The latter hypothesis is supported by reported improvement of symptoms with immune modulatory therapy (107).

TREATMENT: CURRENT CONCEPTS

To date, antithrombotic therapy represents the cornerstone of APS management. This is primarily based on vitamin K antagonists (VKA), generally warfarin (108). The high risk of thrombosis recurrence, which can be seen in 5–16% of subjects (109), warrants long-term anticoagulation. In the acute phase, ischemic cerebrovascular events are managed according to clinical standards as for patients without APS. Reports of intravenous thrombolysis in APS patients who develop acute ischemic stroke date back to 1997 (110). aPL-induced thrombocytopenia or prolongation of prothrombin time are issues to be considered before starting treatment (111). Primary endovascular thrombectomy may be considered as an alternative in these patients (112). Anticoagulation with warfarin and INR 2–3 is the most common strategy for secondary thromboprophylaxis (113). Risk stratification combining antibody profile and comorbidities could help to identify patients needing a more aggressive treatment but to date no model has been validated (3). Although no strong evidence exists in this sense, long-term antiplatelet therapy with low-doses aspirin may be beneficial in addition to warfarin (114). Recurrence of thrombosis frequently depends from inadequate anticoagulation (115), sometimes linked to aPL artifactual prolongation of prothrombin time. Nevertheless, in a series of 66 APS patients treated to a target INR of 3.5 a recurrence rate of 9.1/100 patient-years was detected, with recurrences often affecting the same vascular bed as the original thrombosis (116). Due to the lack of solid evidence, management of recurrences is not standardized and may include higher-intensity warfarin therapy (INR 3–4), the addition of low-dose aspirin or the use of low-molecular-weight heparin (3). Prophylactic treatment in asymptomatic aPL carriers is matter of debate. The risk of a first thrombotic event is likely liked to the presence of other concomitant factors (117–120). The use of low-dose antiplatelet therapy for primary prophylaxis is controversial, due to the lack of solid evidence of efficacy and some authors suggest management following guidelines for prevention of cardiovascular disease in the general population (3). Under specific conditions, immunosuppressive treatment may be an option in addition to antithrombotic therapy. The paradigm of this is represented by catastrophic APS (CAPS), a rare condition characterized by widespread small vessel thrombosis and subsequent multiorgan failure in the context of APS (2) with a mortality rate up to 37% (121). In the case of CAPS, aggressive treatment is recommended and consists of a "triple therapy" composed by anticoagulants plus glucocorticoids plus plasma exchange (PE) and/or intravenous immunoglobulins (IVIG) (122). Rituximab (RTX) is an anti-CD20 monoclonal antibody that depletes B-cells, currently used to treat several autoimmune diseases and hematologic malignancies. B-cells seem to play a pivotal role in the pathogenesis of APS (123). Several case reports describe the use of RTX in APS patients, suggesting a beneficial role in the management of hemolytic anemia (124), thrombocytopenia (125) (126), thrombotic microangiopathy (127). Despite no strong evidence, RTX may be used in patients with thrombotic relapses while on AVK and adequate INR, especially in the setting of APS secondary to SLE (128–130). No standard treatment exists for non-thrombotic neurological manifestations of APS and available evidence mostly derives from retrospective non-randomized trials or case reports (64). Besides cerebrovascular events, anticoagulation has proven effective in the treatment of conditions that are not primarily thrombotic, including migraine, transverse myelitis, and neuropsychiatric disturbances (131–133). Improvement of cognitive performance with anticoagulation therapy has also been described (46). Notably, response to anticoagulation may help distinguish APS from atypical presentations of CNS inflammatory disorders (80). The combined use of neuroleptics and antiplatelet or anticoagulant therapy, with or without steroids, can help control aPL-associated chorea (74, 134, 135). On the other hand the potential role of aPL-mediated damage provides the rational for the use of immunosuppressive therapy (136). Reports describe clinical remission of pediatric aPL-associated chorea achieved with mycophenolate mofetil, an immunosuppressant agent primarily used to prevent rejection in organ transplant induced (137), and IVIG (138). Steroids have been successfully used to treat psychotic symptoms in the context of APS (139). Furthermore, a 2013 non-randomized pilot study (RITAPS) demonstrated partial or complete remission of cognitive dysfunction in a small sample of patients treated with RTX (140).

Future Directions in the Treatment of APS

Antithrombotic Agents

Direct oral anticoagulants (DOACs) represent in interesting possible alternative to warfarin, especially considering the young age of many patients and the need to monitor INR with VKA. Furthermore, it has been suggested that the therapeutic effect of DOACs may go beyond anticoagulation and exert an anti-inflammatory and anti-angiogenic effect (141). Many case reports and case series have been published on the use of DOACs (mainly rivaroxaban) in APS, with heterogeneous

results (142). However, only a few solid randomized controlled trials are available. In 2016 the RAPS trial by Cohen et al. failed to demonstrate the non-inferiority of rivaroxaban over warfarin in APS patients (143). Indeed, questions have been raised regarding thrombotic risk and safety issues related to DOACs, especially in triple-positive patients (144). Given the lack of solid evidence, the 15th International Congress on Antiphospholipid Antibodies Task Force on Antiphospholipid Syndrome Treatment Trends in 2017 did not support the use of DOACs instead of warfarin (142). Another trial by Pengo et al. on high-risk APS patients treated with rivaroxaban or warfarin was prematurely terminated because of the higher rate of thromboembolic events and major bleeding in the rivaroxaban group (145). Although other studies are currently ongoing investigating the effect of rivaroxaban and other DOACs, warfarin represents at the moment the only approved anticoagulant agent. Platelet activation and aggregation represents a further target for therapeutic agents. Glycoprotein IIb/IIIa (GPIIb/IIIa) receptor inhibitors are a relatively new class of antiplatelet drugs compared to aspirin, mainly used in the field of coronary disease. In murine models aPL-enhanced thrombosis can be inhibited by infusion of a GPIIb/IIIa antagonist monoclonal antibody and GPIIb/IIIa-deficient mice are resistant to aPL-mediated thrombosis (146, 147). Management of acute myocardial infarction in APS can represent a challenge due to the high risk of restenosis after angioplasty (148). Abciximab, a monoclonal antibody targeting GPIIb/IIIa on platelets, has been proposed as a promising alternative to stenting (149, 150). However, Abciximab is not approved for the management of cerebrovascular disease due to the negative results of previous studies (151) limiting its possible use in aPL-ischemic stroke.

Agents Targeting Inflammation

Several agents with anti-inflammatory and anti-oxidative properties may also prove beneficial in APS. Among them, one of the most studied is hydroxichloroquine (HCQ), which has been used for the treatment of SLE for many years. Available data show a potential role in the inhibition of toll-like receptors (TLRs) (152), and reduction of proinflammatory IL-1, TNF-α, and IL-2 (153). HCQ may also target the coagulation pathway by inhibiting platelet aggregation (154) and preventing the disruption of the annexin A5 shield by anti-β2-GPI antibodies (155). Interestingly, a 2013 non-randomized study by Schmidt-Tanguy et al. demonstrated a lower rate of recurrence of thrombosis in APS patients treated with oral anticoagulation plus HCQ vs. oral anticoagulation alone (156). Other potential agents include N-Acetylcysteine (NAC) and mitochondrial cofactor coenzyme Q10 (9). Statins, a class of HMG-CoA reductase inhibitors commonly used to treat dyslipidemia and atherosclerosis, have shown a potential to inhibit aPL-mediated thrombogenesis and modulate the proinflammatory milieu in APS, and both *in vitro* and *in vivo*, through the down-regulation of the expression of intracellular adhesion molecule 1 (ICAM1), vascular endothelial growth factor (VEGF) and of pro-inflammatory cytokines, including IL-1β, TNF-α, and interferon (IFN)-α (157, 158).

Monoclonal Antibodies

Belimumab (BEL) is a monoclonal antibody directed against the B-cell activating factor (BAFF or BLyS) which promotes B-cell survival and differentiation, approved for unresponsive SLE. A report by Yazici et al. described its use in two primary APS patients with pulmonary and skin manifestations, respectively. One of the patients showed partial benefit while the other complete remission of symptoms, while aPL profile remained unchanged (159). On the contrary, a report by Sciascia et al. demonstrated disappearance of aPL in three patients with APS secondary to SLE treated with belimumab (160). Eculizumab is a monoclonal antibody directed against the C5 fraction of complement approved for the treatment of paroxysmal nocturnal hemoglobinuria and atypical hemolytic uremic syndrome. To date a series of case reports have been published regarding its use in APS, specifically in the setting of CAPS and post-transplantation renal thrombotic microangiopathy, with encouraging results (161–170).

Other Agents

Sirolimus is a macrolide with inhibiting properties on the mammalian target of rapamycin (mTOR), a kinase involved in many signaling pathways related to cellular growth, proliferation, and survival (112). Previous studies have highlighted the possibility of the involvement of mTOR in the genesis of vascular stenosis in the context of endothelial injury (171, 172). Vascular cellular infiltrates and changes in the vessels intima and media layers have been observed in APS patients, especially in the context of CAPS (110, 111), suggesting a vasculopathic pathogenic mechanism. In a 2014 publication Canaud et al. described a series of patients with APS nephropathy undergoing transplantation. Interestingly, those receiving sirolimus had no recurrence of vascular lesions and showed decreased vascular proliferation on biopsy (173). Ongoing researches aim at identifying other therapeutic targets, including TLR 4 inhibition, TF inhibition, protease-activated receptor (PAR) antagonists, intracellular signaling blockers, and tolerogenic dendritic cells (142).

CONCLUSIONS

APS is a rare and heterogeneous condition the neurologist may have to deal with. The diagnosis is not easy and a high level of suspicion must be held. APS should always be considered in young patients with CVA, especially in the absence of other vascular risk factors. Moreover, publications from the past decades have shown that many neurologic disorders not comprised in the original description by Hughes may harbor the disease. Elements such as autoimmune and connective tissue comorbidities, a positive history for obstetrical complications and refractoriness to standard therapy may guide the neurologist toward the correct diagnosis. A great amount of research aims at clarifying the complex etiopathogenesis of APS but many aspects remain obscure. The mechanism behind aPL-mediated pathology appears to be strongly, but not exclusively, thrombotic. Immune-mediated damage may be the key to some aPL-related neurologic manifestations.

What favors the attack against nervous structures and what lies beneath the great phenotypic variability even among neurological presentations are open questions for researchers in the near future. On the one hand, mechanisms of immunological surveillance might be involved, as it was demonstrated in other more common forms of nervous system autoimmunity (174). On the other, genetic variability in neurobiological pathways may be worth careful investigation (76, 175). The double nature, thrombotic and immune-mediated, of APS is exemplified by current therapeutic strategies. Anticoagulation with warfarin represents at the moment the most effective therapy in the setting of thrombotic events. Anticoagulation has also been reported to reverse non-criteria neurologic manifestations that are not primarily thrombotic in their origin, further supporting the role of thrombosis in the pathogenesis of APS. On the other hand targeting the immune response through steroids, rituximab, IVIG or PE, can improve many non-criteria symptoms. A great number of other treatments are under investigation. DOACs represent an appealing alternative to warfarin, especially considering the young age of APS patients and the necessity of INR monitoring. Nevertheless, available evidence does not support their use. Other interesting options include antioxidant agents, monoclonal antibodies, and several agents targeting specific cells or molecules in the complex pathogenetic pathway of APS. One last interesting issues concerns the relationship between APS and SLE. A 2010 report by Veres et al. on a group of 165 patients with primary APS showed that 23% of cases converted to definite SLE within 10 years (176). The correlation between these two entities is not fully understood. The term neurolupus indicates the involvement of the nervous system in the setting of SLE. Pathogenic mechanisms, similarly to APS, include thrombosis, vascular proliferative changes and, possibly, anti-neuronal and anti-glial antibody activity (177). Further studies are needed to understand the relation between these two entities, shared mechanisms of nervous system injury and possible therapeutic options.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

SYSTEMATIC REVIEW
published: 26 July 2018
doi: 10.3389/fimmu.2018.01731

The Coagulation Factors Fibrinogen, Thrombin, and Factor XII in Inflammatory Disorders—A Systematic Review

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Background: The interaction of coagulation factors has been shown to go beyond their traditional roles in hemostasis and to affect the development of inflammatory diseases. Key molecular players, such as fibrinogen, thrombin, or factor XII have been mechanistically and epidemiologically linked to inflammatory disorders like multiple sclerosis (MS), rheumatoid arthritis (RA), and colitis.

Objectives: To systematically review the evidence for a role of coagulation factors, especially factor XII, fibrinogen, and thrombin in inflammatory disorders like MS, RA, and bowel disorders.

Methods: A systematic literature search was done in the PubMed database to identify studies about coagulation factors in inflammatory diseases. Original articles and reviews investigating the role of the kallikrein–kinin and the coagulation system in mouse and humans were included.

Results: We identified 43 animal studies dealing with inflammatory disorders and factors of the kallikrein–kinin or the coagulation system. Different immunological influences are described and novel molecular mechanisms linking coagulation and inflammation are reported.

Conclusion: A number of studies have highlighted coagulation factors to tip the balance between hemostasis and thrombosis and between protection from infection and extensive inflammation. To optimize the treatment of chronic inflammatory disorders by these factors, further studies are necessary.

Keywords: coagulation factors, neuroinflammation, contact system, fibrinogen, factor XII, thrombin

OPEN ACCESS

Edited by:
Tatiana Koudriavtseva, Istituto Nazionale del Cancro Regina Elena, Italy

Reviewed by:
Tomas Per Olsson, Karolinska Institutet (KI), Sweden
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Specialty section:
This article was submitted to Multiple Sclerosis and Neuroimmunology, a section of the journal Frontiers in Immunology

Received: *28 February 2018*
Accepted: *12 July 2018*
Published: *26 July 2018*

Citation:
Göbel K, Eichler S, Wiendl H, Chavakis T, Kleinschnitz C and Meuth SG (2018) The Coagulation Factors Fibrinogen, Thrombin, and Factor XII in Inflammatory Disorders—A Systematic Review. Front. Immunol. 9:1731. doi: 10.3389/fimmu.2018.01731

INTRODUCTION

The coagulation system is a highly regulated cascade that ultimately leads to blood clot formation. The primary purpose of coagulation is hemostasis, i.e., to stop bleeding from a damaged blood vessel.

The concept of a stepwise process or cascade of the coagulation system was first described in 1964 (1). While this traditional model described two separate pathways, the intrinsic and the extrinsic pathway, which culminate in a common pathway, current views support an interconnected

relationship between the two (2, 3). The contact pathway (also called the kallikrein–kinin system) is composed of three zymogens [factor XII (FXII), plasma kallikrein (PK), and high-molecular-weight kininogen (HMWK)]. *In vitro*, the initial triggering event leads to clot formation through the activation of FXII on an artificial surface. However, *in vivo*, the activation of this factor is still under discussion, so that current studies consider tissue factor (TF), a transmembrane glycoprotein expressed in perivascular tissue, to be the main initiator of *in vivo* blood clotting (4). TF forms a complex with factor VII (FVII) to activate factor X (FX) either directly or by activating factor IX (FIX). Both pathways interlace with the activation of FX, which leads to the cleavage of prothrombin (factor II) to thrombin (factor IIa). In the last step, thrombin mediates the cleavage of fibrinogen to fibrin monomers that, upon polymerization, form a fibrin clot and stop bleeding. The formation of these clots is dependent on the availability of thrombin, calcium, and negatively charged phospholipid membranes. The whole coagulation cascade is very tightly regulated with several checkpoints that function in a positive or negative feedback loop (5).

However, in recent years, significant evidence has emerged implicating coagulation factors also in tissue repair and inflammatory responses. In line with this, several of the major coagulation factors, like TF, thrombin, or fibrinogen, are described as potential drivers of inflammation in disease models, such as sepsis, endotoxemia, encephalomyelitis, or multiple sclerosis (MS) (2, 6–8). Thereby, these factors not only enhance inflammation in the bloodstream, but also within tissues. Furthermore, it is known that FXII triggers the release of bradykinin (BK) from HMWK through cleavage by PK, leaving two chain HMWK behind, which has numerous adhesion-regulatory properties (9, 10) including inhibitory activity on the interaction between fibrinogen and the leukocyte integrin CD11b/CD18 (11). Binding of BK to the BK receptors can activate proinflammatory pathways that induce chemotaxis of leukocytes and increase vascular permeability (12).

A proteomic analysis has been performed on human brain material from individuals with MS identifying a dysregulation of several proteins of the coagulation cascade, such as TF or protein C inhibitor (13). Furthermore, in an animal model of MS, i.e., experimental autoimmune encephalomyelitis (EAE), it could be shown that other factors, such as FXII or thrombin, are upregulated in the central nervous system (CNS) (14, 15). It has been demonstrated that this dysregulation of the coagulation system is not restricted to the CNS but can also be found in the peripheral blood (15). Both FXII and thrombin are highly upregulated in the plasma of patients with MS (16). Moreover, dysregulation of BK receptors was found to be relevant in MS (17, 18).

Although further studies using animal models of MS are required, the available data indicate that the interplay between coagulation factors and immune cells and/or brain endothelial cells may modulate initiation and/or the course of neuroinflammatory disorders.

In this review, we summarize key links between inflammation and coagulation, with a specific focus on the molecular roles of the clotting factors FXII, fibrinogen, and thrombin in neuroinflammation as well as in neuroinflammatory disorders. The role of coagulation factors in non-neurological inflammatory disorders is also discussed. The evidence presented here suggests that manipulation of components of the coagulation system could be potentially therapeutically exploitable not only in inflammatory disorders of the CNS but also in autoimmune diseases in general.

METHODS

A literature review was done in December 2017 searching the PubMed database using the search items: BK, coagulation factors, colitis, complement, Crohn's disease, EAE, FXII, fibrinogen, inflammatory bowel disease, kallikrein–kinin system, thrombin, MS, and rheumatoid arthritis (RA). The search terms were used in different combinations and plural forms, and the search was limited to articles in English. References were screened for additional articles. Studies in mouse and human were included.

RESULTS AND DISCUSSION

Factor XII and Neuroinflammation

Factor XII is a soluble zymogen with a molecular weight of approximately 80 kDa that is produced in the liver (3). FXII consists of a heavy chain (353 residues) and a light chain (243 residues) held together by a disulfide bond (**Figure 1**) (19). It has several domains, namely, a leader peptide, a fibronectin type II domain, an epidermal growth factor (EGF)-like domain, a fibronectin type I domain, a second EGF-like domain, a kringle domain, a proline-rich region, and the catalytic domain (**Figure 1**). Proteolytic cleavage of its R353–V354 site converts the zymogen FXII to activated FXII (FXIIa). This cleaved protein circulates as a two-chain protein, a heavy and a light chain, held together by a disulfide bond (19). *In vitro*, FXII can be activated by PK, plasmin, or on negatively charged surfaces, while *in vivo* activation is still under debate (20, 21). FXIIa is suggested to initiate the intrinsic coagulation, the contact, and complement systems (**Figure 1**). Thus, FXIIa leads to the cleavage of PK to generate active PK (contact system, **Figure 2**), triggers fibrin formation through the activation of factor XI (FXI; **Figure 1**), and activates the complement pathway. The serine protease C1 inhibitor (C1-INH) is the major inhibitor of FXII, and thereby controls its proteolytic activity. Besides C1-INH, antithrombin III and plasminogen activator inhibitor I also have FXII-inhibitory capacity. Despite its contribution to fibrin formation *in vitro*, FXII seems not to be essential for hemostasis *in vivo* (21, 22). However, under pathological conditions, FXII participates in thrombus formation and thromboembolic disorders, such as stroke (23).

In terms of neuroinflammation, we have been able to show that FXII deficiency leads to an attenuated disease severity in EAE, accompanied by reduced numbers of interleukin (IL)-17A-producing effector T helper cells (T_H17). The role of FXII in EAE was mediated by its ability to shift the cytokine profile

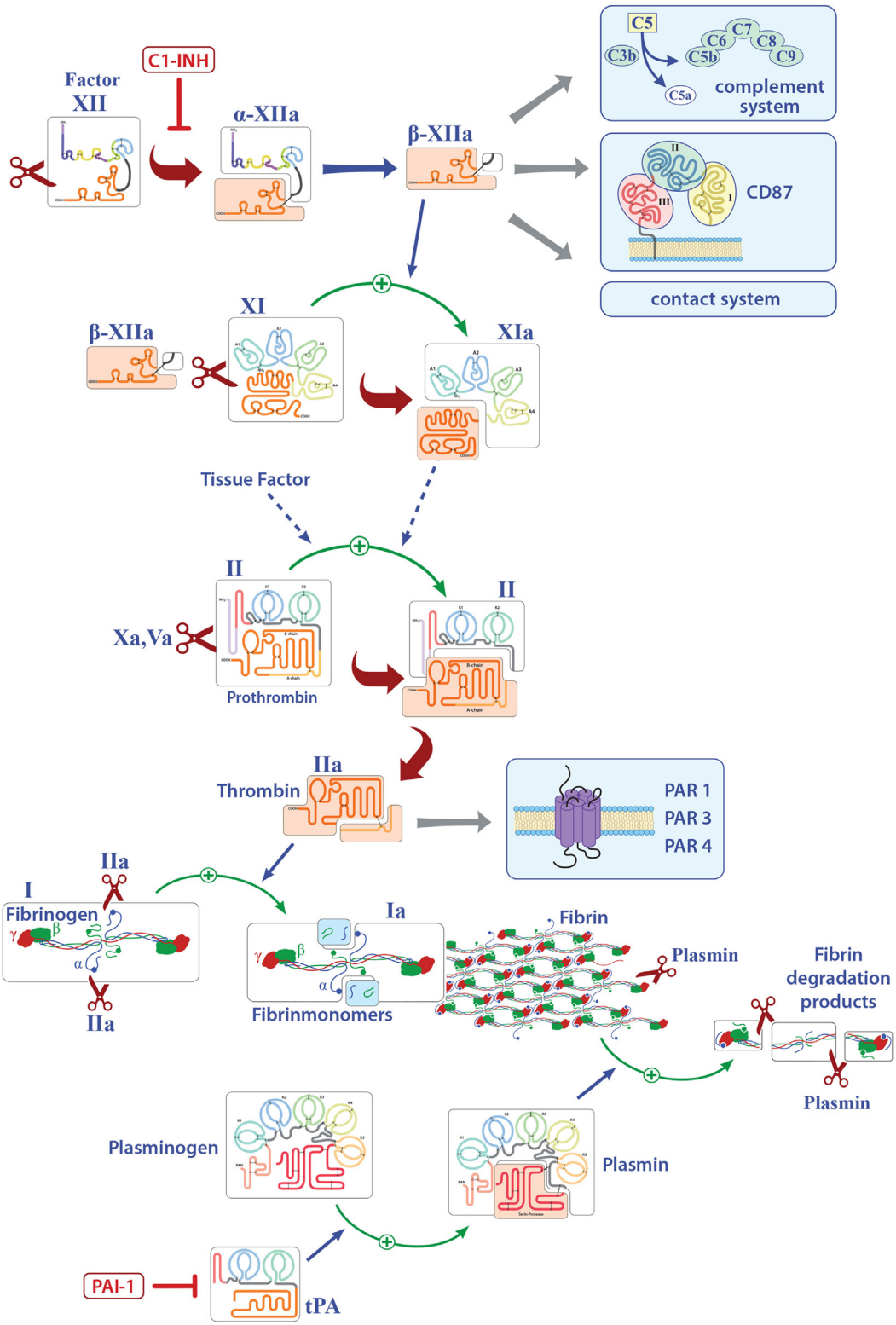

FIGURE 1 | Overview of the known pathways activated by factor XII (FXII) and the extrinsic coagulation system. Only factors that are involved in inflammation are shown. Activated FXII leads to the cleavage of factor XI, activates the intrinsic, the contact, and complement systems and can bind to CD87. Tissue factor finally leads to the release of thrombin (FIIa) that can directly bind several receptors and activates fibrinogen to fibrin. Deposition of fibrin is regulated by plasmin. Abbreviations: C1-INH, serine protease C1 inhibitor; CD87, urokinase-type plasminogen-activator receptor; tPA, tissue plasminogen activator; PAI-1, plasminogen-activator inhibitor 1; PAR, protease-activated receptor.

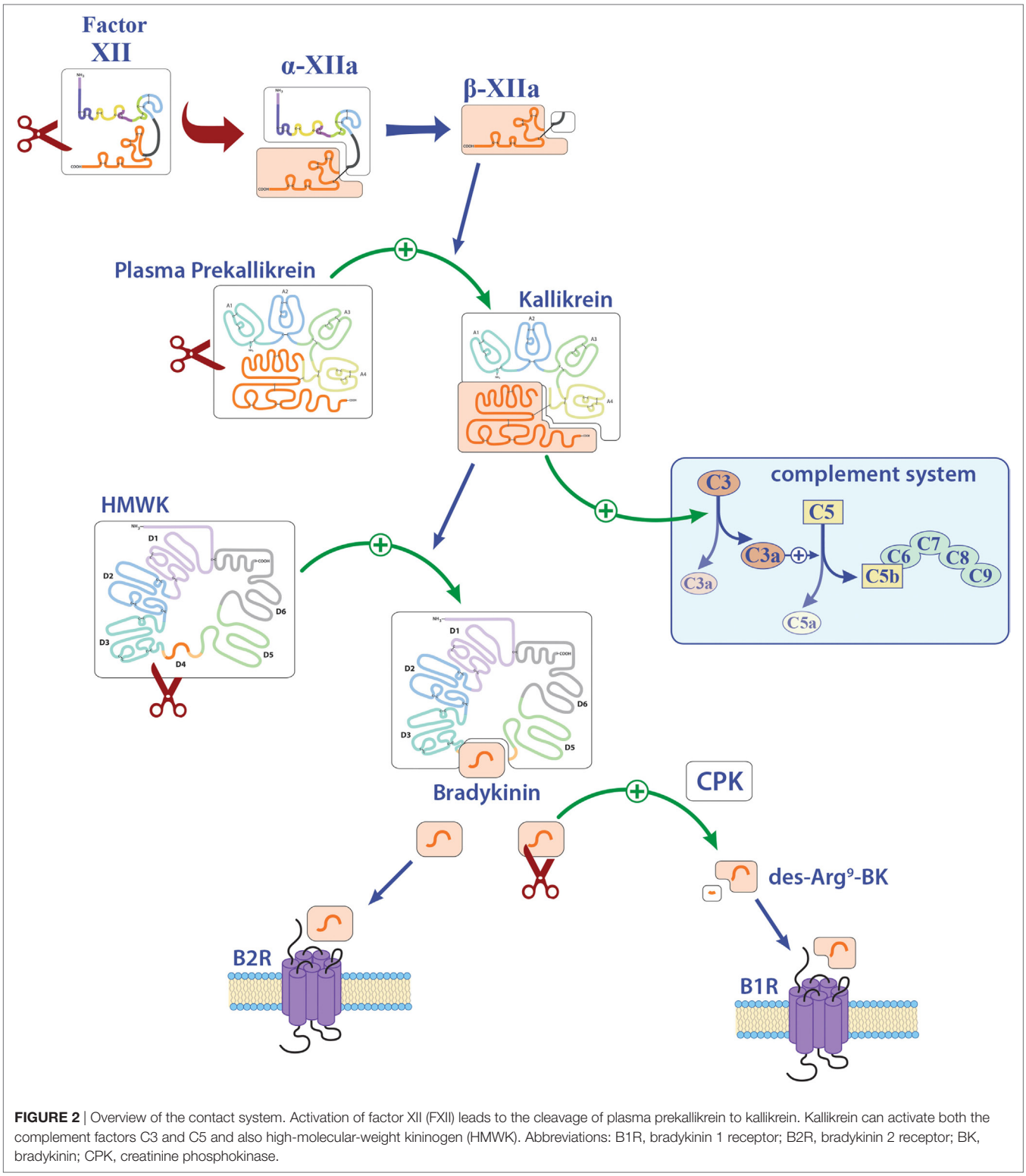

FIGURE 2 | Overview of the contact system. Activation of factor XII (FXII) leads to the cleavage of plasma prekallikrein to kallikrein. Kallikrein can activate both the complement factors C3 and C5 and also high-molecular-weight kininogen (HMWK). Abbreviations: B1R, bradykinin 1 receptor; B2R, bradykinin 2 receptor; BK, bradykinin; CPK, creatinine phosphokinase.

of dendritic cells (DC) necessary to induce differentiation of effector T cells (see also **Table 1**) (15). Pharmacologic inhibition of FXII by recombinant human albumin-tagged infestin-4 (24) resulted in decreased EAE severity as well (see **Table 1**). These findings suggest a potential novel link between FXII and the immune system in neuroinflammation. Strikingly, we also found significantly increased FXII plasma activity in individuals with relapsing–remitting MS and secondary progressive MS, as compared to healthy donors, thus, indicating a role for this factor in human MS pathogenesis (15).

TABLE 1 | Studies of intrinsic and contact system factors: effects on neuroinflammatory processes in transgenic mice or using pharmacological substances.

Mouse line or treatment	Model (peptide)	Genetic background	Effects in models of autoimmune neurodegeneration	Reference
B1R$^{-/-}$	Active EAE (MOG)	C57BL/6	Delayed EAE onset, reduced clinical deficits, reduced inflammation and demyelination, decreased expression of endothelial adhesion molecules, reduced migration of lymphocytes	(25)
			Earlier EAE onset, severe clinical disease course with enhanced inflammation, demyelination, glial activation, increased migration of CD4$^+$ T cells, especially T_H17 cells	(26)
			Delayed EAE onset, reduced clinical deficits, reduced inflammation and demyelination, decreased cytokine production of CD4$^+$ T cells	(27)
			Delayed EAE onset, reduced clinical deficits, reduced glial activation, decreased release of proinflammatory mediators by astrocytes	(28)
Des-Arg^9-BK (B1R activator)	Active EAE (MOG)	C57BL/6	No clinical effect	(27)
R838 (B1R agonist)	Active EAE (MOG)	C57BL/6	Aggravated disease course, enhanced inflammation, demyelination, axonal damage	(25)
			Milder clinical deficits	(26)
	Active RR-EAE (PLP)	SJL	Reduced clinical deficits	(26)
Des-Arg^9-[Leu^8]-BK (B1R inhibitor)	Active EAE (MOG)	C57BL/6	Delayed EAE onset, reduced clinical deficits, decreased release of proinflammatory mediators by astrocytes	(28)
			Delayed EAE onset, reduced clinical deficits, reduced inflammation and demyelination, decreased cytokine production of CD4$^+$ T cells	(27)
R715 (B1R inhibitor)	Active EAE (MOG)	C57BL/6	Reduced clinical deficits, reduced inflammation, demyelination, axonal damage	(25)
			Accelerated disease onset	(26)
B2R$^{-/-}$	Active EAE (MOG)	C57BL/6	No effect on clinical course, immune cells, cytokine production	(25, 26)
			Moderate reduced clinical deficits, reduced inflammation, reduced leukocyte adhesion, decreased chemokine (CCL2, CCL5) production	(29)
HOE-140 (B2R inhibitor)	Active EAE (MOG)	C57BL/6	Moderate or no effect on clinical disease course, immune cells, cytokine production	(25–28)
			Moderate reduced clinical deficits, reduced inflammation, reduced leukocyte adhesion, decreased chemokine (CCL2, CCL5) production	(29)
F12$^{-/-}$	Active EAE (MOG)	C57BL/6	Delayed EAE onset, reduced clinical deficits, reduced inflammation and demyelination, reduced T_H17 cells, decreased cytokine production (IL-6, IL-23) of DC	(15)
rHA-Infestin 4 (FXIIa inhibitor)	Active EAE (MOG)	C57BL/6	Delayed EAE onset, reduced clinical deficits, reduced inflammation and demyelination, reduced cytokine production (IL-6, IL-17A)	(15)
	Active EAE (PLP)	SJL	Ameliorated disease course	(15)
F11$^{-/-}$	Active EAE (MOG)	C57BL/6	No effect on clinical symptoms, inflammation and demyelination	(15)
C1q$^{-/-}$	Active EAE (MOG)	C57BL/6	No effect on clinical symptoms	(30)
C3$^{-/-}$	Active EAE (MOG)	129SVJ/ C57BL/6	Reduced clinical deficits, decreased inflammation and demyelination	(31)
			No clinical effect, but higher mortality, tendency to enhanced inflammation and demyelination	(32)
C3$^{-/-}$	Active + AT-EAE (MOG)	C57BL/6	Reduced clinical deficits, reduced infiltration of T cells	(33, 34)
C5$^{-/-}$	Active EAE (guinea pig myelin)	B10.D2/oSnJ	Moderate reduction of clinical deficits, narrow zones of inflammation and demyelination, gliosis, reduced remyelination, enhanced apoptosis of oligodendrocytes, axonal damage	(35, 36)
C5a/GFAP	Active EAE (MOG)	C57BL/6	No clinical effect	(37)
Cd87$^{-/-}$	Active EAE (MOG)	C57BL/6	Aggravated disease course, enhanced inflammation, axonal damage, reduced T-cell proliferation and cytokine production	(38)
			Delayed disease onset, reduced clinical deficits, reduced inflammation, enhanced demyelination and axonal damage	(39)
	Active EAE (MOG) in BM-chimera		Reduced clinical deficits with *Cd87*$^{-/-}$ BM, partial protection of *Cd87*$^{-/-}$ hosts	(15)

AT, adoptive transfer; B1R, bradykinin receptor 1; B2R, bradykinin receptor 2; BK, bradykinin; BM, bone marrow; CCL, chemokine (C–C) motif ligand; DC, dendritic cells; EAE; experimental autoimmune encephalomyelitis; FXIIa, activated factor XII; IL, interleukin; MOG, myelin oligodendrocyte glycoprotein; PLP, proteolipid protein; rHA, recombinant human albumin; RR, relapsing–remitting; T_H17, IL-17A-producing effector T helper cells.

As aforementioned, FXIIa leads to the cleavage of FXI (**Figure 1**). However, studies from our laboratory indicate that the latter factor has no significant role in EAE (see also **Table 1**), suggesting that not the entire intrinsic coagulation system is involved, but rather that the effect of FXII in neuroinflammation is dependent on other pathways triggered by FXII (15).

In particular, besides hemostasis, FXII leads to the activation of the contact system and hereby to the release of BK (**Figure 2**). Reports on the function of BK in MS and EAE remain contradictory. While three reports described a protective role of genetic or pharmacological inhibition of one distinct bradykinin receptor (bradykinin receptor 1, B1R), another study demonstrated enhanced inflammation by B1R blockade (see also **Table 1**) (25–28). For MS patients, B1R has been shown to have a detrimental effect, as it is upregulated on T-lymphocytes from patients with either secondary progressive MS or relapsing–remitting MS during active relapse (17). Levels of B1R expression on mononuclear cells correlate positively with the Expanded Disability Status Scale, with occurrence of clinical relapses and lesion volumes on T2-weighted images, but not with gadolinium-enhancing lesions (40). Furthermore, a potential role for B1R has been described in the regulation of blood–brain barrier permeability and chemokine production (18), indicating this factor's involvement in neuroinflammation.

FXII has the capacity to activate the classic complement pathway by direct cleavage of C1q (20). However, it has been shown that C1q has no influence on neuroinflammation, at least in terms of clinical symptoms (30). It is known that PK can activate the complement components C3 and C5 (20). However, reports on these members of the complement system in the context of neuroinflammation remain elusive. While three reports showed a significant role of C3, as C3-deficient animals had an attenuated EAE disease course and reduced T-cell infiltration (31, 33, 34), another study showed no clinical differences, but a tendency to enhanced inflammation and demyelination (32). For C5, a dual role in EAE has been suggested: One study revealed that C5 leads to reduced inflammation and tissue repair in acute lesions, while this factor seemed to be responsible for increased axonal damage and enhanced gliosis in chronic lesions (35). Furthermore, it has been shown that C5 can limit oligodendrocyte apoptosis in EAE, thus promoting remyelination (36). Use of transgenic mice that express C5 under the astrocytic-specific glial fibrillary acidic protein promoter revealed no significant contribution to disease development of this component in the CNS (37), so that the role of complement in EAE remains contradictory.

Although most investigations focus on FXII as a serine protease, FXII can interact with cells independently of its enzymatic activity. In line with this, FXII can bind to urokinase plasminogen activator receptor (uPAR, CD87; **Figures 1** and **3**) (20). Studies from our laboratory have demonstrated high levels of CD87 on DC. In this context, we could show that FXII exerts its immunoregulatory effects directly *via* CD87 and by regulating cyclic adenosine monophosphate (cAMP) and thereby cytokine levels (e.g., IL-6, IL-23) in DC. In contrast, we could rule out the involvement of alternative FXII-triggered pathways, such as the intrinsic coagulation, the contact and complement systems, for EAE pathogenesis.

However, reports on the relevance of CD87 inhibition, *per se*, in EAE remain contradictory: while two reports indicated a protective role in terms of clinical score and inflammation, when CD87 was missing (15, 39), and another showed enhanced inflammation (38).

In conclusion, the data so far indicate a significant role of FXII and downstream factors and pathways in neuroinflammation. However, further studies are needed to clarify remaining contradictions.

Fibrinogen and Neuroinflammation

Fibrinogen (Factor I) is a 340-kDa glycoprotein that is synthesized in the liver (41). It is activated to fibrin by thrombin, exposing several polymerization sites that are crosslinked to an insoluble fibrin clot under the involvement of activated factor XIII (41, 42). Although activation of the coagulation system and thereby fibrin formation is essential for stopping lethal hemorrhage, the deposition of fibrin is carefully regulated to avoid thrombotic incidents (43). This is achieved by the fibrinolytic system in which plasmin especially counterbalances the procoagulatory signals, leads to clot dissolution, and results in the generation of soluble fibrin fragments, such as fragments D and E, and D-dimers (44). Plasmin generation is regulated by two proteases, tissue plasminogen activator (tPA) and uPA (45), which are controlled by plasminogen activator inhibitor-1 (PAI-1; **Figures 1** and **4**) (46).

Under physiological conditions, the plasma concentration of fibrinogen is between 2 and 4 g/l; however, it is known that this concentration can rapidly increase under pathological conditions (acute phase reactions), such as injury, infection, or inflammation (47, 48). Similarly, elevated levels of fibrin degradation products, such as D-dimer, are used in clinical practice as indicators of inflammation and risk predictors of thrombotic events (49). In the majority of cases, the proinflammatory function of fibrin/fibrinogen is mediated by its ability to bind to different immune cells for instance to the CD11b/CD18 integrin receptor (also termed Mac-1) on macrophages, monocytes, or microglia that induces the release of reactive oxygen species and is required for axonal damage in EAE (8, 47, 50). In this context, it has been shown that binding of fibrin/fibrinogen to the CD11b/CD18 integrin receptor results in activation of proinflammatory cascades, such as nuclear factor κB, which leads to the release of inflammatory cytokines, like tumor necrosis factor (TNF)-α or IL-1β (51, 52) and can thereby influence diseases such as RA (53) or colitis-associated cancer (**Figure 4**) (54). In addition, fibrinogen-dependent effects of platelets may also contribute to EAE disease pathogenesis (55).

A detrimental role of fibrin/fibrinogen has also been suggested for neuroinflammation, as fibrin deposition in the CNS correlates with microglial activation in active MS lesions (56, 57). In line with this finding, it has been shown that fibrinogen can directly activate microglia, enhance their phagocytic ability, induce peripheral macrophage recruitment and local CNS activation of myelin antigen-specific T_H1 cells (58, 59). Moreover, genetic deletion of fibrinogen resulted in reduced inflammation

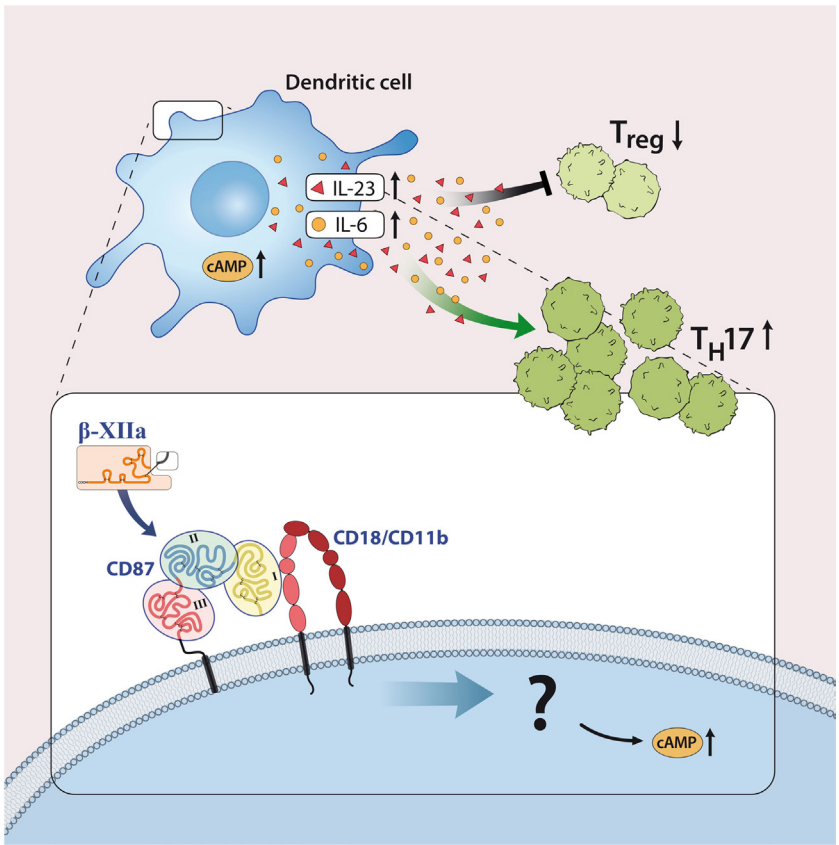

FIGURE 3 | Factor XII (FXII) as a mediator of inflammatory disease. FXII acts on dendritic cells through the urokinase-type plasminogen-activator receptor (CD87) to enhance the release of interleukin (IL)-6 and -23. This cytokine shift leads to increased amounts of IL-17A-producing $CD4^+$ effector T helper cells (T_H17). Abbreviations: AC, adenylate cyclase; CD11b/CD18, leukocyte integrin adhesion molecule.

and demyelination using a TNF transgenic model of MS (mice that lack the TNF receptor, develop spontaneous clinical symptoms of paralysis, and die by 5 weeks of age; *TgK21fib*$^{-/-}$; see also **Table 2**) (60). Furthermore, inhibition of fibrinogen binding to CD11b/CD18 by genetic mutation of the CD11b/CD18-binding motif (*Fib*$\gamma^{390-396A}$) (61) or a peptide ($\gamma^{377-395}$) results in reduced microglial activation and an attenuated disease course in EAE (see also **Table 2**) (58).

Interestingly, none of these inhibitory approaches interferes with the clotting function of fibrinogen (53, 58). Moreover, staphylococcal-derived extracellular adherence protein, which, among others, interferes with the interaction between CD11b/CD18 and fibrinogen, also suppressed murine EAE disease severity (67), while pharmacological treatment strategies with snake venom-derived defibrinogenating agents, such as ancrod or batroxobin, suppress clinical symptoms in different animal models of MS (see also **Table 2**) (60, 62–64, 68).

Enhanced fibrin deposition is usually counterbalanced by plasmin that is generated by tPA and uPA. Interestingly, uPA as well as PAI-1 are significantly increased in acute MS lesions, while tPA levels are unchanged (69, 70). Results concerning tPA activity remain contradictory; while one report indicates a reduction in tPA activity in normal-appearing white and gray matter and lesions of individuals with MS (70), others describe a significant increase of activity in lesions and the cerebrospinal fluid of MS patients during the acute, but not the chronic disease phase (71).

When EAE was induced in *uPA*$^{-/-}$ mice, these animals displayed an aggravated disease course. This finding was accompanied by enhanced microglial activation (see also **Table 2**) (38). In line with these results, treatment with a PA inhibitor-derived peptide (PAI-1-dp) that increases plasminogen activation ability of uPA, suppressed the development of EAE symptoms (see also **Table 2**) (38). In contrast, another publication using ε-aminocaproic acid, an inhibitor of plasminogen and trypsinogen activator, reported a suppression of EAE severity (see also **Table 2**) (65).

Results of EAE experiments in tPA-deficient animals remain contradictory: while two publications described increased severity and a delayed recovery with enhanced demyelination and axonal damage after genetic depletion of tPA, disease onset was reported to be either earlier or delayed in the literature (see also **Table 2**) (39, 66). Due to the significant upregulation of PAI-1 in MS patients, EAE induced in PAI-1-deficient animals was shown to have moderate clinical protection with reduced perivascular cuffs, but no difference in terms of demyelination or axonal damage was observed (39).

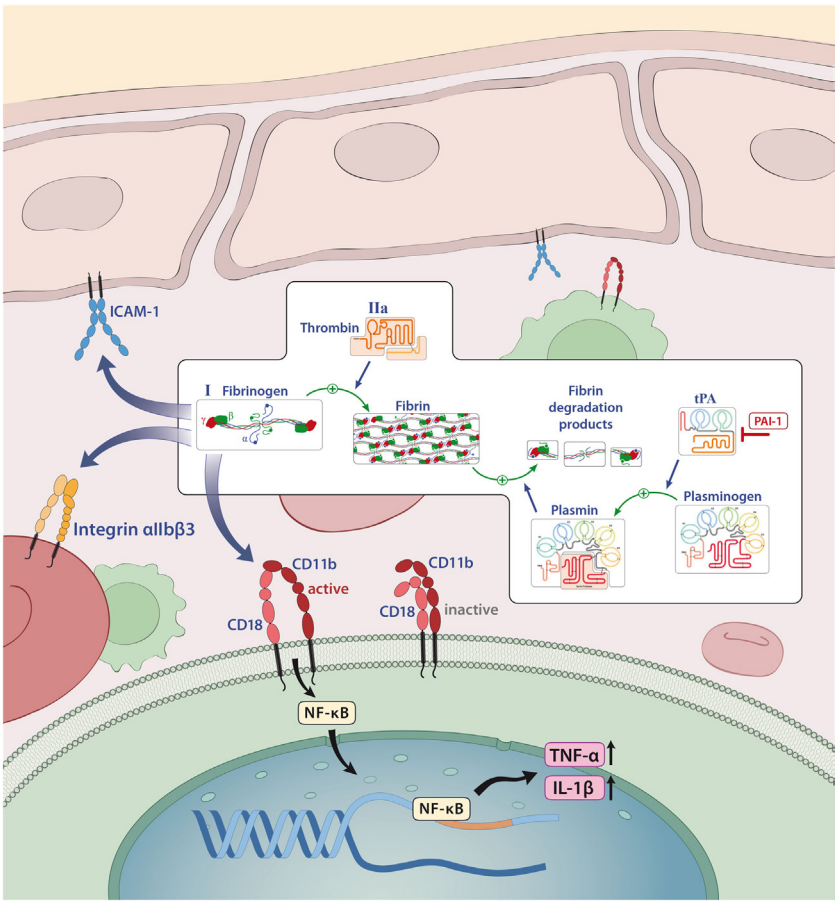

FIGURE 4 | Fibrinogen as a mediator of inflammation. Fibrinogen acts on different cells through integrin and non-integrin receptors to induce specific inflammatory effects. Abbreviations: CD11b/CD18, leukocyte integrin adhesion molecule; ICAM-1, intercellular adhesion molecule-1; IL-1β, interleukin-1β; NF-κB, nuclear factor κB; tPA, tissue plasminogen activator; PAI-1, plasminogen-activator inhibitor 1; TNF-α, tumor necrosis factor α.

Nonetheless, data so far indicate a significant role of local fibrin deposits in neuroinflammation and indicate a promising anti-inflammatory therapeutic potential of targeting this pathway.

Thrombin and Neuroinflammation

Prothrombin (factor II) is a soluble 72-kDa protein that is produced by the liver. It is activated to thrombin (factor IIa) *via* enzymatic cleavage of two sites by activated FX (FXa). Activated thrombin leads to cleavage of fibrinogen into fibrin monomers that, upon polymerization, form a fibrin clot. Therefore, activation of prothrombin is crucial in physiological and pathophysiological coagulation. For instance, various rare disorders, such as congenital hypoprothrombinemia (a blood disease in which deficiency of prothrombin results in impaired blood clotting) and acquired hypoprothrombinemia (e.g., in autoimmune diseases with lupus anticoagulant) have been described (72, 73).

Beyond its key role in coagulation, thrombin can mediate further effects, e.g., thrombin is a potent vasoconstrictor and is implicated in vasospasms following subarachnoid hemorrhage (74).

In terms of neuroinflammation, thrombin activity was found to be significantly increased in the spinal cord of mice with EAE (14). Thrombin activity precedes the onset of neurological signs and correlates with the amount of fibrin deposition, microglial activation, demyelination, axonal damage, and clinical severity. Interestingly, inhibition of thrombin activity by hirudin leads to a significant improvement of disease severity (13) (see also **Table 2**). This is accompanied by decreased immune cell proliferation and cytokine secretion, as well as a reduction in the number of inflammatory lesions (13). Furthermore, it has been shown that levels of thrombin inhibitors are significantly increased during EAE. For instance, antithrombin III (as well as protease nexin 1) were detected at higher levels in CNS homogenates during EAE compared with controls (75). Additionally, it was recently shown that prothrombin levels are elevated in plasma of patients suffering from relapsing-remitting MS or secondary progressive MS indicating a prominent role of this coagulation factor in neuroinflammation (16).

Coagulation Factors in Non-Neurological Inflammatory Diseases

An increasing body of evidence also supports a decisive role of coagulation factors in regulating inflammatory responses

TABLE 2 | Studies of coagulation system factors: effects on neuroinflammatory processes using transgenic mice or pharmacological substances.

Mouse line (genetic background)	Model (peptide)	Genetic background or species	Neuroinflammatory effect	Reference
$\gamma^{377-395}$	Active RR-EAE (PLP)	SJL	Reduced clinical symptoms, decreased inflammation, reduced microglial activation	(58)
Ancrod	Active RR-EAE (PLP)	SJL	Reduced clinical symptoms, decreased microglial activation and demyelination, no impact on immune cell proliferation	(58)
	TNF-transgenic model (no peptide, spontaneous)	Tg6072 TNF transgenic mice	Reduced demyelination, downregulation of MHC-I	(60)
	Active EAE	Rat	Reduced clinical symptoms, decreased inflammation and fibrin deposition	(62)
Batroxobin	Active EAE (MOG)	C57BL/6	Reduced clinical symptoms, decreased inflammation, demyelination, no effect on axonal damage	(63)
	AT-EAE (GP-MBP)	Rat	Attenuated disease course, reduced fibrin depositions, no effect on inflammation and immune cell proliferation	(64)
ε-Aminocaproic acid	Active EAE	Rat	Attenuated disease course	(65)
Fib$\gamma^{390-396A}$	Active EAE (MOG)	C57BL/6	Reduced clinical symptoms, decreased inflammation, demyelination, reduced microglial activation	(58)
Hirudin	Active EAE (PLP)	SJL/J	Improvement of clinical deficits, reduced inflammation, decreased immune cell proliferation and cytokine production	(13)
PAI-1$^{-/-}$	Active CREAE (spinal cord homogenate)	ABH	Reduced incidence, moderately delayed onset, reduced inflammation, no effect on demyelination and axonal damage	(39)
PAI-1-dp (uPA activator)	Active EAE (MOG)	C57BL/6	Attenuated disease course, reduced Tcell proliferation and cytokine production	(38)
TgK21fib$^{-/-}$	TNF-transgenic model (no peptide, spontaneous)		Increased lifespan, delayed onset of clinical symptoms, reduced inflammation and demyelination	(60)
tPA$^{-/-}$	Active EAE (MOG)	C57BL/6	Aggravated disease course, enhanced demyelination, axonal damage, fibrin deposition	(39)
			Delayed disease onset, aggravated disease course, delayed, but prolonged demyelination and axonal damage, reduced microglial activation	(66)
uPA$^{-/-}$	Active EAE (MOG)	C57BL/6	Aggravated disease course, enhanced inflammation and microglial activation	(38)

AT, adoptive transfer; CREAE, chronic relapsing experimental allergic encephalomyelitis; EAE, experimental autoimmune encephalomyelitis; MHC-I, major histocompatibility complex class I; MOG, myelin oligodendrocyte glycoprotein; PAI-I, plasminogen activator inhibitor I; PAI-I-dp, plasminogen activator inhibitor I-deprived; PLP, proteolipid protein; TNF, tumor necrosis factor; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator; RR, relapsing–remitting.

in non-neurological inflammatory diseases. For instance, a substantial contribution of different coagulation factors has been suggested in RA or inflammatory joint disease as fibrin depositions can be found in the joints of patients with RA (76). Moreover, the degradation products of fibrin, such as D-dimer, are used as common biomarkers for disease activity (77, 78). *In vitro*, it was shown that fibrinogen can enhance IL-8 secretion and intercellular adhesion molecule 1 expression from human synovial fibroblasts, leading to enhanced lymphocyte adhesiveness (79). A further direct proinflammatory role of fibrin/fibrinogen was suggested in RA pathogenesis as its genetic depletion in mice leads to an improvement in the clinical symptoms in animal models of RA and results in decreased synovial inflammation (see **Table 3**) (53). Interestingly, it was shown that the interaction of fibrinogen with immune cells *via* CD11b/CD18 is the relevant partner for this effect. Furthermore, pharmacological inhibition of thrombin *via* hirudin resulted in a significant reduction in synovial inflammation and disease severity in two different animal models of RA (see **Table 3**) (80, 81). In this context, it could be shown that the plasmin activity is decreased, while PAI-1 levels are increased in both blood and inflamed joints of mice with collagen-induced arthritis (CIA) (82).

While treatment with uPA and tPA improves plasmin activity and removes fibrinogen depositions in joints, disease severity remains unchanged, challenging the pathophysiological role of fibrinogen in this context (82). Nonetheless, a significant contribution of uPA could be seen in other studies, but this remains contradictory for different arthritis models: in monoarticular models, uPA-deficient mice had an aggravated disease course (90, 93). In contrast, other studies using polyarticular animal models demonstrated resistance to or suppression of disease and reduced inflammation in animals lacking uPA, indicating a distinct role of uPA in different types of arthritis (91, 92, 94–96).

TABLE 3 | Studies of coagulation system factors: effects on inflammatory processes using transgenic mice or pharmacological substances.

Mouse line or treatment	Model (peptide)	Genetic background or species	Effect in arthritis and colitis models	Reference
B1R/B2R$^{-/-}$	CAIA (anti-CII antibodies)	C57BL/6	Decreased clinical symptoms, reduced inflammation and cytokine levels	(83)
C11C1 (HMWK antibody)	Reactive arthritis (PG-PS) Spontaneous arthritis	Lewis rats HLA-B27 transgenic rats	Reduced joint diameter, local inflammation Reduced joint destruction, inflammation	(84) (85)
Fib$^{-/-}$	CIA (type II collagen)	DBA/1J	Reduced incidence, severity, joint destruction, synovial inflammation	(53)
Fib$^{-/-}$	Colitis-associated cancer (DSS)	C57BL/6	Reduced inflammation-driven adenoma formation	(54)
Fib$^{\Delta 5}$	CIA (type II collagen)	DBA/1J	No effect on incidence and severity	(53)
Fib$\gamma^{390-396A}$	CIA (type II collagen)	DBA/1J	Reduced incidence, severity, joint destruction, synovial inflammation	(53)
Fib$\gamma^{390-396A}$	Colitis (DSS)	C57BL/6	Diminished inflammatory disease, reduced ulceration, cytokine levels and neutrophil infiltration	(54)
Fib$\gamma^{390-396A}$	Colitis-associated cancer (DSS)	C57BL/6	Reduced inflammation-driven adenoma formation	(54)
Heparin	CIA (type II collagen)	DBA/1	No effect on clinical score	(82)
Hirudin	AIA (mBSA)	C57BL/6	Reduced knee joint inflammation, fibrin deposition	(80)
	CIA (type II collagen)	DBA/1J	Decreased disease incidence, severity, reduced loss of articular cartilage, inflammation, fibrin deposition and PAR-1 expression	(81)
HMWK$^{-/-}$	Reactive arthritis (PG-PS)	Rats	No clinical signs of arthritis, absence of inflammation	(86)
MEN16132 (B2R antagonist)	Inflammatory arthritis (carrageenan)	Rats	Reduced knee diameter and myeloperoxidase activity	(87)
P8720 (PK inhibitor)	Reactive arthritis (PG-PS)	Lewis rats	Decreased joint swelling, reduced inflammation	(88)
PKSI-527 (PK inhibitor)	CIA (type II collagen)	DBA/1	Reduced severity of arthritis, reduced PK and HMWK plasma levels	(89)
Plg$^{-/-}$	AIA (mBSA)	C57BL/6	Enhanced inflammation, bone erosion, synovial thickness, fibrin deposition	(90)
Plg$^{-/-}$	CIA (type II collagen)	C57BL/6xDBA/1	No clinical symptoms of disease, no signs of inflammation	(91)
	CAIA (anti-CII antibodies)		No clinical symptoms of disease, no signs of inflammation	(91)
	AIA (mBSA)		Enhanced synovial thickness	(92)
	CIA (type II collagen)		Reduced synovial thickness	(92)
Tg197Fib$^{-/-}$	TNF-transgenic model, spontaneous polyarthritis		No effect on incidence, severity, inflammation, joint destruction	(53)
uPA	CIA (type II collagen)	DBA/1	No effect on clinical score, reduced fibrin deposits in joints, decreased plasma D-dimer level	(82)
uPA$^{-/-}$	AIA (mBSA)	Ola129xC57BL/6	Enhanced joint inflammation, bone erosion, synovial thickness, fibrin deposition	(90)
uPA$^{-/-}$	Monoarticular arthritis (mBSA/IL-1)	C57BL/6	Enhanced arthritis, fibrin deposition, increased numbers of macrophages	(93)
	CIA (type II collagen)		Reduced clinical symptoms, decreased inflammation, cartilage destruction, bone erosion, reduced cytokine production	(94)
	CAIA (M2139, CIIC1 antibodies)		No clinical signs of disease	(95)
	AIA (mBSA)		Enhanced proteoglycan loss, inflammation, bone erosion	(92, 96)
uPA$^{-/-}$	CIA (type II collagen)	C57BL/6xDBA/1	Reduced clinical symptoms, no histological changes	(91)
	K/BxN serum transfer arthritis (K/BxN serum)		No clinical signs of disease, reduced inflammation, bone erosion, fibrin deposition	(95, 96)
tPA	CIA (type II collagen)	DBA/1	No effect on clinical score, reduced fibrin deposits in joints	(82)

(*Continued*)

TABLE 3 | Continued

Mouse line or treatment	Model (peptide)	Genetic background or species	Effect in arthritis and colitis models	Reference
tPA$^{-/-}$	Monoarticular arthritis (mBSA/IL-1)	C57BL/6	Enhanced arthritis, fibrin deposition, increased numbers of macrophages	(93)
	CIA (type II collagen)		Aggravated clinical symptoms, enhanced inflammation, cartilage destruction, bone erosion, fibrin deposition	(94)

AIA, antigen-induced arthritis; B1R, bradykinin receptor I; B2R, bradykinin receptor 2; CAIA, type II collagen mAb-induced arthritis; CIA, collagen-induced arthritis; CII, collagen type II; DSS, dextran sulfate sodium; HLA, human leukocyte antigen; HMWK, high molecular weight kininogen; mBSA, methylated bovine serum albumin; PAR-1, protease-activated receptor; PG-PS, peptidoglycan-polysaccharide; PK, plasma kallikrein; TNF, tumor necrosis factor; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator.

The same result was observed in plasminogen-deficient animals (90–92). In contrast to uPA, studies using tPA-deficient animals have so far indicated an aggravated disease course with enhanced inflammation (93, 94).

A substantial role for the contact system in arthritis has been discussed. For instance, FXIIa levels were significantly increased in RA patients compared with healthy controls (97). Furthermore, pharmacological blockade of PK by different inhibitors revealed reduced disease severity and inflammation in different models of arthritis (88, 89). In line with these findings, genetic or pharmacological inhibition of HMWK leads to an attenuation of PK–kinin system activation, local and systemic inflammation, indicating a therapeutic potential in RA (84–86). Moreover, arthritis severity is significantly attenuated in mice lacking B1R and B2R (83) or by treatment with a B2R antagonist (87, 98).

In addition to RA, potential drugability of the coagulation system and its factors is under consideration for the treatment of inflammatory bowel disease. Interestingly, it was shown that patients with Crohn's disease have significantly higher levels of C1-inhibitor and intestinal tissue kallikrein, while plasma levels of prekallikrein, FXI, and HMWK are unaltered (99). Furthermore, inflammatory bowel disease in humans is associated with higher plasma levels of fibrinogen, prothrombin, factor V, factor VIII, plasminogen, and platelets (100). In line with these findings, animal models of colitis have demonstrated reduced inflammation in animals with a genetic disruption to the binding of fibrinogen to the CD11b/CD18 integrin receptor (see **Table 3**) (54). Since a link between chronic inflammation and tumor development, e.g., colitis and colorectal cancer, could be established (101), it is interesting that both fibrinogen-deficient mice and mice with a genetic disruption of the interaction between fibrinogen and the CD11b/CD18 integrin receptor develop significantly fewer adenomas (54).

Collectively, these results demonstrate a clear role of the coagulation system, not only in neuroinflammation, but also in other autoimmune and inflammatory disorders.

CONCLUSION

In this review, we have discussed the links between coagulation and inflammation, focusing on the role of different coagulation factors in neuroinflammatory disorders. Overall, it becomes increasingly clear that the deposition of different coagulation factors in the CNS tissue may trigger exacerbation of inflammation, thereby limiting regenerative mechanisms. A prominent role was especially described for fibrinogen, thrombin, and factor XII. As novel molecular and cellular binding partners are identified, the role of coagulation factors is evolving from hemostasis regulators to multi-faceted signal molecules, which control the balance between immune defense mechanisms and extensive inflammation.

Interestingly, the binding of coagulation factors to their cellular targets requires distinct non-overlapping epitopes and is usually independent of their protease function. Taking advantage of this knowledge, targeted inhibition of coagulation factors that facilitate disease pathogenesis without affecting their protease activity represents an ideal strategy for pharmacological intervention in different neuroinflammatory disorders without unwarranted side-effects like bleeding. Therefore, future studies are needed to elucidate the exact contribution of blood proteins to autoimmune neurodegeneration.

AUTHOR CONTRIBUTIONS

KG drafted the manuscript. SE, HW, TC, CK, and SM extensively revised the manuscript. KG and TC funded the study. All authors provided substantial input throughout the process.

ACKNOWLEDGMENTS

This work was supported by the Else-Kröner-Fresenius-Stiftung EKFS (2015_A113 to KG), the Deutsche Forschungsgemeinschaft DFG (GO2505/1-1 to KG), the Medical Faculty (Junior Research Group to KG), and the European Community's Seventh Framework Program (under grant agreement No. 602699—DIREKT to TC).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neurology

ORIGINAL RESEARCH
published: 20 April 2018
doi: 10.3389/fneur.2018.00245

Coagulation Factor XII Levels and Intrinsic Thrombin Generation in Multiple Sclerosis

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Background: Factor XII (FXII) activation initiates the intrinsic (contact) coagulation pathway. It has been recently suggested that FXII could act as an autoimmunity mediator in multiple sclerosis (MS). FXII depositions nearby dentritic cells were detected in the central nervous system of MS patients and increased FXII activity has been reported in plasma of relapsing remitting and secondary progressive MS patients. FXII inhibition has been proposed to treat MS.

Objective: To investigate in MS patients multiple FXII-related variables, including the circulating amount of protein, its pro-coagulant function, and their variation over time. To explore kinetic activation features of FXII in thrombin generation (TG).

Methods: In plasma from 74 MS patients and 49 healthy subjects (HS), FXII procoagulant activity (FXII:c) and FXII protein (FXII:Ag) levels were assessed. Their ratio (FXII:ratio) values were derived. Intrinsic TG was evaluated by different triggers.

Results: Higher FXII:Ag levels ($p = 0.003$) and lower FXII:ratio ($p < 0.001$) were detected in MS patients compared with HS. FXII variables were highly correlated over four time points, which supports investigation of FXII contribution to disease phenotype and progression. A significant difference over time was detected for FXII:c ($p = 0.031$). In patients selected for the lowest FXII:ratio, TG triggered by ellagic acid showed a trend in lower endogenous thrombin potential (ETP) in MS patients compared with HS ($p = 0.042$). Intrinsic triggering of TG by nucleic acid addition produced longer time parameters in patients than in HS and substantially increased ETP in MS patients ($p = 0.004$) and TG peak height in HS ($p = 0.008$). Coherently, lower FXII:ratio and longer lag time ($p = 0.02$) and time to peak ($p = 0.007$) point out a reduced response of FXII to activation in part of MS patients.

Conclusion: In MS patients, factor-specific and modified global assays suggest the presence of increased FXII protein level and reduced function within the intrinsic coagulation pathway. These novel findings support further investigation by multiple approaches of FXII contribution to disease phenotype and progression.

Keywords: multiple sclerosis, coagulation, factor XII, intrinsic pathway, thrombin generation

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Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Multiple Sclerosis and Neuroimmunology, a section of the journal Frontiers in Neurology

Received: *31 January 2018*
Accepted: *28 March 2018*
Published: *20 April 2018*

Citation:
Ziliotto N, Baroni M, Straudi S, Manfredini F, Mari R, Menegatti E, Voltan R, Secchiero P, Zamboni P, Basaglia N, Marchetti G and Bernardi F (2018) Coagulation Factor XII Levels and Intrinsic Thrombin Generation in Multiple Sclerosis. Front. Neurol. 9:245. doi: 10.3389/fneur.2018.00245

INTRODUCTION

Multiple sclerosis (MS) is a chronic autoimmune disorder, characterized by immune-mediated inflammation and multifocal demyelinated lesions within the central nervous system (CNS) (1, 2). Growing evidences suggest the crosstalk between hemostasis components, inflammation, and immune system, which appear to be involved in MS pathophysiology (3–8). The relationship of the coagulation pathway with disease processes could be further supported by recent findings, showing that anticoagulation ameliorated clinical course of experimental autoimmune encephalomyelitis (EAE), an animal model of MS (9, 10).

Among coagulation factors, a key role seems accomplished by factor XII (FXII), the initiator of the "old" contact (intrinsic) coagulation pathway (11), which cooperates redundantly with the extrinsic pathway, thus re-defining the cascade model [reviewed in Ref. (12)]. Upon contact with negative charged surfaces, including nucleic acids (NAs) released by damaged cells, zymogen FXII is converted to activated FXII (FXIIa), which starts the sequential proteolytic reactions within coagulation cascade with activation of factor XI, and subsequent thrombin generation (TG) and final fibrin formation. Moreover, FXII regulates fibrinolysis, complement activation, and the kallikrein–kinin pathway (11, 13).

Recently, histological analysis of CNS tissue from MS patients identified FXII light chain depositions nearby dendritic cells (DCs) (14). The basic FXII protein structure consists of an N-terminal heavy chain with six domains for substrates interaction, and a C-terminal light chain, which includes the catalytic domain (15).

In EAE, FXII depletion had protective effects, reducing susceptibility to CNS inflammation, delaying disease onset, decreasing disease severity and production of T helper 17 (Th17) cells (14). In mice, FXII stimulates expression of CD87 receptor on DCs, which is crucial for inducing Th17 cells differentiation. To note, both FXIIa and zymogen FXII forms were found to modulate conventional DCs function inducing excessive production of cytokines during neuroinflammation in CNS. In support of these findings, deficiency of factor XI, directly activated by FXIIa, did not alter the disease course in EAE model.

These results, indicating that FXII could not contribute to the MS animal model through the intrinsic coagulation pathway, suggest that the FXII procoagulant activity "*per se*" is not involved in MS. Nevertheless, the FXII levels in plasma are usually assessed by a procoagulant assay (FXII:c). In fact, FXII:c has been found significantly increased in patients with relapsing-remitting MS (RR-MS) and secondary progressive MS (SP-MS) compared with healthy donors. Additionally, enhanced FXII:c was associated with relapses and shorter relapse-free period, independently from immune-modulatory therapy (14).

It has been proposed that FXII inhibition could represent a new approach in MS therapy, as indicated by the reduced number and severity of relapses in the EAE mouse model by injection of a recognized FXII inhibitor (14, 16). This would probably not cause bleeding tendency in patients, because it is well known that FXII deficiency does not compromise effective hemostasis (17, 18).

Interestingly, clinical and genetic reports point at a FXII role in thrombosis (19–21). Local and systemic thrombotic events has been described in MS potentially in relation to the overstimulation of innate immunity for both its inflammatory and coagulant components (22). Recently, the hypercoagulability and potentially prothrombotic state in MS patients has been investigated by TG, triggered by extrinsic activation (23).

The poorly defined role of FXII forms and features and paucity of studies in patients, strongly support investigation of FXII in MS. We investigated multiple FXII-related variables, as well as FXII activation in the intrinsic TG, to explore their association with MS.

MATERIALS AND METHODS

Study Population

The study population included MS patients, the majority of which participated in the RAGTIME study (https://ClinicalTrials.gov ID:NCT02421731) (24). This clinical trial compares robot-assisted gait training vs. conventional therapy on mobility in severely disabled progressive MS patients.

All MS patients underwent to neurological visits, MRI examinations, and assessment of the Expanded Disability Status Scale (EDSS).

Inclusion and exclusion criteria for RAGTIME study were previously reported (24). The selection criteria for the present study included: age between 18 and 79 years, MS diagnosis according to the revised McDonald criteria (25), lack of MS worsening in the previous 3 months.

The healthy subjects (HS) group was represented by healthy volunteers, who were never diagnosed with MS, neurological disorder, other chronic inflammatory disease, and cardiovascular disease.

All subjects were of Caucasian origin. Patients were not under treatment with anticoagulant drugs. Written informed consent was obtained from all subjects, and the study was approved by the Ethical Committee of the S. Anna University-Hospital, Ferrara, Italy. The demographic and clinical characteristics of the study populations, which included 74 MS patients (12 relapsing remitting, RR-MS; 28 primary progressive PP-MS; 34 secondary progressive SP-MS) and 49 HS, are summarized in **Table 1**. Age was significantly different between MS and HS ($p < 0.001$, Student's *t*-test, **Table 1**), while gender difference was not significant ($p = 0.138$, Fisher's exact test). The total number of patients under disease-modifying treatments (DMTs) at blood sampling was 12 (5 patients under DMTs and 7 patients under both DMTs and symptomatic treatments), as detailed in **Table 1**. Five patients (three RR-MS and two SP-MS) with discontinuation of DMTs before their enrollment in the present study were included in the group "None treatment," since at sampling they were not under treatment.

Plasma Samples

Venous peripheral blood samples from both MS patients and HS were collected into sodium citrate tubes. Patients enrolled in the RAGTIME study provided blood sampling at four time point: (T0) baseline point, prior to the first rehabilitative session; (T1) intermediate point, after six training sessions; (T2) end of

TABLE 1 | Demographic and clinical characteristics.

	All MS	RR-MS	SP-MS	PP-MS	HS
Sample size, *n*	74	12	34	28	49
Female, *n* (%)	48 (64.9)	8 (66.7)	19 (55.9)	21 (75)	25 (51)
Age, mean ± SD	53.5 ± 10.7	43.5 ± 9	52.2 ± 8.9	59.3 ± 9.9	40.6 ± 13.3
EDSS, median (IQR)	6 (0.5)	3 (2)	6.5 (0.5)	6 (0.5)	–
Disease duration, mean ± SD	14.4 ± 10.0	7.4 ± 5.2	18.0 ± 8.1	13.5 ± 11.7	–
Treatment, *n* (%)					
Disease-modifying	5 (6.8)	–	1 (2.9)	4 (14.3)	–
Symptomatic	17 (22.9)	–	7 (20.6)	10 (35.7)	
Both	7 (9.5)	1 (8.3)	5 (14.7)	1 (3.6)	
None	45 (60.8)	11 (91.7)	21 (61.8)	13 (46.4)	

MS, multiple sclerosis; RR-MS, relapsing-remitting multiple sclerosis; SP-MS, secondary progressive multiple sclerosis; PP-MS, primary progressive multiple sclerosis; HS, healthy subjects; n, number; EDSS, Expanded Disability Status Scale; IQR, interquartile range.

Age and disease duration in years are reported as mean ± SD. For the ordinal EDSS, the median (interquartile range) is given.

Disease-modifying treatments were as follow: 1 (RR) Rituximab; 1 (SP) interferon-beta; 1 (SP) glatiramer acetate; 1 (SP) methotrexate; 1 (SP) teriflunomide; 2 (1 SP and 1 PP) fingolimod; 2 (1 SP and 1 PP) azathioprine; 2 (PP) natalizumab; 1 (PP) cyclophosphamide.

Symptomatic treatments were as follow: 16 (1 RR, 6 SP, and 9 PP) oral baclofen; 2 (SP) Pregabalin; 1 (SP) oral baclofen plus gabapentin; 1 (SP) tetrahydrocannabinol plus cannabidiol; 1 (SP) combination of clonazepam, tetrahydrocannabinol and cannabidiol; 2 (1 SP and 1 PP) oral baclofen plus amitriptyline; 1 (PP) amantadine.

Descriptive analysis between MS and HI were performed using Fisher's exact test and Student's t-test.

treatment, 12 completed rehabilitative sessions, 1 month after T0; (T3) follow-up, after 3 months from the end of training program. Plasma samples were obtained after two consecutive centrifugations of blood samples, at room temperature (2,500 *g* for 15 min and 11,000 *g* for 5 min). Aliquots were stored at −80°C until use.

FXII Activity

Coagulant activity of FXII (FXII:c) in plasma samples was assessed by an activated partial thromboplastin time (aPTT)-based assay (HemosIL aPTT SynthASil kit, Instrumentation Laboratory, Lexington, MA, USA). Activity and coagulation times were recorded by the ACLTOP 700 instrument (HemosIL, Instrumentation Laboratory). The inter-assay coefficients of variation assessed over multiple runs was 2.1%.

FXII Antigen

Plasma FXII antigen (FXII:Ag) concentrations were determined using a sandwich enzyme-linked immunosorbent assay kit (LS-F10418, LifeSpan Biosciences, Seattle, WA, USA), following the manufacturer's instructions. The assay uses a polyclonal capture antibody for FXII and a mouse primary monoclonal antibody raised against the heavy chain of FXII as detection antibody. The plasma samples were tested with a dilution of 1:3,000. The results were expressed as relative units in percentage generated from concentration values normalized to a pool of normal plasma loaded in all plates. The inter-assay coefficient of variation for plasma measurements was 2.6%.

Intrinsic TG

Thrombin generation in plasma samples was evaluated by the addition of a specific thrombin fluorogenic substrate (Calbiochem-Novobiochem, La Jolla, CA, USA) (26, 27). Plasma samples were diluted (1/5) in a HBS buffer (Hepes 20 mM, NaCl 150 mM, PEG-8000 0.1%, pH 7.4) and incubated for 5 min at 37°C. TG through intrinsic activation was conducted by addition of a volume mixture of ellagic acid (Dade Actin FS, Siemens) and phospholipid vesicles (4 μM, MP-reagent, Stago), as previously reported (28, 29). TG was also evaluated by further addition of NA as trigger (1 μM) for the activation. Final concentrations of $CaCl_2$ and thrombin fluorogenic substrate were 2.5 mM and 250 μM, respectively. The fluorescence was measured overtime in a fluorometer (Fluoroskan Ascent BioMed) and the amount of the generated thrombin was calculated using a normal pooled human plasma (Hyphen BioMed) as a standard. As negative control of contact activation, the FXII inhibitor "corn trypsin inhibitor" was added to the normal pooled plasma in each assay condition (single/double trigger).

Specific parameters of TG-lag time, time to peak (TTP), peak height, and endogenous thrombin potential (ETP) (area under the curve) were obtained by a nonlinear regression analysis of the first derivative of relative fluorescence units using the software version 6.01 (GraphPad Software, Inc., La Jolla, CA, USA).

Statistical Analysis

All statistical analyses were performed using IBM® SPSS® Statistics version 24 software (IBM Corp., Armonk, NY, USA) and figures were produced by GraphPad Prism version 6.01 (GraphPad Software, Inc., La Jolla, CA, USA).

The Shapiro–Wilk test was used to test for normality of continuous variables. The Fisher's exact test was used to compare differences in categorical variables and Student's *t*-test was used to compare age between total MS and HI groups.

Comparisons of MS vs. HS and of males vs. females of FXII:c, FXII:Ag, and FXII:c/FXII:Ag ratio (FXII:ratio) were conducted with the ANCOVA test using age as covariate. Comparisons for FXII levels among clinical subgroups were performed with ANCOVA test using age as covariate and, in case of a significant *p*-value, pairwise comparisons were Bonferroni corrected for multiple testing (*q*-values).

To assess whether FXII levels were significantly different among patients receiving DMTs, symptomatic treatments, or none current treatment, one-way ANOVA was used and, in case of a significant *p*-value, pairwise comparisons were Bonferroni corrected for multiple testing (*q*-values).

Pearson's test was used to assess correlation over time for FXII:c and FXII:Ag. ANOVA for repeated measures was used to test FXII:c, FXII:Ag, and FXII:ratio across the four time points and, in case of a significant *p*-value, pairwise comparisons were Bonferroni corrected (*q*-values). Student's *t*-test was used to compare TG parameters of MS patients with those of HS, while paired Student's *t*-test was used to assess differences in TG after NA addition in MS and HS.

RESULTS

FXII Activity and Antigen Levels

Factor XII coagulant activity (FXII:c), FXII protein concentration (FXII:Ag), and their ratio, providing quantitative information about the FXII activity in relation to the amount of circulating protein, were evaluated in plasma of MS patients and of HS and summarized in **Table 2**. Comparison between MS and HS groups revealed significant differences in FXII:Ag ($p = 0.003$) and FXII:ratio ($p < 0.001$) but not in FXII:c ($p = 0.421$). No differences within clinical subgroups (RR-MS, SP-MS, and PP-MS) were detected for FXII:c ($p = 0.296$), FXII:Ag ($p = 0.248$), and FXII:ratio ($p = 0.765$). Comparison between male and female within MS and HS groups, after age adjustment, did not reveal difference in FXII:c (MS $p = 0.74$, HS $p = 0.374$), FXII:Ag (MS $p = 0.256$, HS $p = 0.622$), and showed a trend in difference in FXII:ratio in HS ($p = 0.045$), but not in MS ($p = 0.11$).

The potential modulation of FXII levels by treatments was investigated. Patients under both DMTs and symptomatic treatments (**Table 1**) were categorized in DMTs group for the purpose of the analyses. No difference according to DMTs, symptomatic treatments, or none current treatments were detected for FXII:c ($p = 0.98$), FXII:Ag ($p = 0.81$), and FXII:ratio ($p = 0.97$).

A large portion of patients under study were characterized by a small range of EDSS (6–6.5), which does not favor the investigation of the relation between EDSS and FXII levels.

Factor XII:c, FXII:Ag, and FXII:ratio levels were also investigated over four time points (**Table 3**) in 49 MS (23 PP-MS and 26 SP-MS). A significant difference over time was detected for FXII:c ($p = 0.031$, **Table 3**). In particular, pairwise analysis revealed differences between T0 and T1 ($p = 0.004$; $q = 0.023$) and T0–T3 ($p = 0.005$; $q = 0.027$). The potential influence on FXII:c over time variations of MS phenotype or drug treatments was investigated. Differences were not detected within each clinical MS group (SP-MS, $p = 0.079$; PP-MS, $p = 0.093$), as well as within drug treatment groups (DMTs, $p = 0.188$; symptomatic treatment, $p = 0.345$; none, $p = 0.142$).

No differences over time were detected for FXII:Ag ($p = 0.596$) and FXII:ratio ($p = 0.151$) (**Table 3**).

Noteworthy, analysis of correlations among time points for each FXII parameter (**Table 4**) showed that FXII:c levels were highly correlated (T0–T1, $r^2 = 0.90$; T1–T2, $r^2 = 0.82$; T2–T3, $r^2 = 0.75$; $p < 0.001$) as well as FXII:Ag levels (T0–T1, $r^2 = 0.81$; T1–T2, $r^2 = 0.79$; T2–T3, $r^2 = 0.84$; $p < 0.001$).

Intrinsic TG

The decreased FXII:ratio values in MS patients prompted us to investigate potential variation of FXII specific activity through a global plasma assay (TG), which describes all phases of coagulation process and the integrated amount of generated thrombin (30). In particular, to provide kinetic information about the coagulation pathway triggered by intrinsic activation, a single classic activation (ellagic acid) was conducted in parallel with a double activation (**Figure 1**), by adding as trigger molecules of NA. This natural substance, released after cell death, is able to activate the contact pathway (31, 32).

To magnify FXII-related differences in TG, 10 patients' plasma, obtained at T0 (4 PP-MS, 6 SP-MS), were selected for the lowest FXII:ratio (≤0.93), virtually undetectable in HS, and compared with 10 HS plasma with the highest FXII:ratio (≥1.4), which on the other hand was rare in MS patients.

TABLE 2 | FXII activity, antigen, and ratio in MS patients and HS.

	MS						HS			MS vs. HS
		Female	Male	RR	PP	SP		Female	Male	*p*-value
N	**74**	**48**	**26**	**12**	**28**	**34**	**49**	**25**	**24**	
FXII:c %										0.421
Mean	115.0	113.9	116.9	111.3	115.0	116.2	123.7	127.5	119.8	
Lower 95% CI	110.3	108.7	107.1	97.3	108.5	108.3	116.5	115.3	111.7	
Upper 95% CI	119.7	119.1	126.7	125.2	121.5	124.1	130.9	139.8	127.8	
FXII:Ag %										0.003
Mean	106.7	102.7	114.2	106.2	108.1	105.8	99.3	99.0	99.6	
Lower 95% CI	99.3	93.6	101.0	85.3	95.2	94.8	91.4	85.5	90.5	
Upper 95% CI	114.1	111.7	127.4	127.1	121.0	116.8	107.2	112.5	108.8	
FXII:ratio										<0.001
Mean	1.14	1.18	1.06	1.09	1.15	1.15	1.30	1.36	1.24	
Lower 95% CI	1.07	1.09	0.98	0.96	1.02	1.06	1.22	1.23	1.14	
Upper 95% CI	1.21	1.28	1.13	1.21	1.27	1.25	1.38	1.48	1.33	

MS, multiple sclerosis; HS, healthy subjects; RR-MS, relapsing-remitting multiple sclerosis; SP-MS, secondary progressive multiple sclerosis; PP-MS, primary progressive multiple sclerosis; FXII:c, factor XII activity; FXII:Ag, factor XII antigen; FXII:ratio, FXII:c/FXII:Ag; CI, confidence interval; N, number.
Analysis were conducted with the ANCOVA test, using age as covariate.

TABLE 3 | FXII activity, antigen, and ratio in multiple sclerosis patients over four time points.

	Time points				
N 49	**T0**	**T1**	**T2**	**T3**	***p*-value**
FXII:c %					0.031
Mean	114.5	110.4	114.1	110.0	
Lower 95% CI	108.6	104.5	107.3	103.8	
Upper 95% CI	120.4	116.3	120.8	116.2	
FXII:Ag %					0.596
Mean	105.1	107.8	108.8	105.8	
Lower 95% CI	95.87	98.06	99.54	96.87	
Upper 95% CI	114.4	117.5	118.0	114.8	
FXII:ratio					0.151
Mean	1.16	1.10	1.10	1.09	
Lower 95% CI	1.07	1.01	1.03	1.02	
Upper 95% CI	1.25	1.18	1.17	1.16	

FXII:c, factor XII activity; FXII:Ag, factor XII antigen; FXII:ratio, FXII:c/FXII:Ag; CI, confidence interval; N, number.
ANOVA for repeated measures was used to test FXII:c, FXII:Ag, and FXII:ratio across the four time points.

TABLE 4 | Correlations of factor XII activity and antigen over four time points in multiple sclerosis patients.

	T0	**T1**	**T2**
FXII:c			
T1	0.90		
T2	0.80	0.82	
T3	0.88	0.84	0.75
FXII:Ag			
T1	0.81		
T2	0.81	0.79	
T3	0.63	0.70	0.84

Pearson's test was used to assess correlation over time for factor FXII activity (FXII:c) and antigen (FXII:Ag). (T0) baseline point, prior to the first rehabilitative session; (T1) intermediate point, after six training sessions; (T2) end of training, 12 completed rehabilitative sessions, 1 month after T0; (T3) follow-up, after 3 months from the end of training program.

Thrombin generation curves and parameters are reported in **Figure 1** and in **Table 5**, respectively. TG activated by ellagic acid showed only a trend in lower thrombin potential (ETP) in MS patients compared with HS (2,631 ± 166 vs. 2,780 ± 136, $p = 0.042$).

The TG triggered, in the same experiment, with the addition of NA, produced a clear decrease in main time parameters both in MS patients and in HS. As compared with the single ellagic acid trigger, both lag time and TTP were shorter in MS patients (612 ± 97 vs. 561 ± 81, $p = 0.006$; 750 ± 109 vs. 706 ± 91, $p = 0.014$, respectively) and in HS (564 ± 44 vs. 487 ± 44, $p < 0.0001$; 690 ± 51 vs. 605 ± 51, $p < 0.0001$). After the double induction, the increase in thrombin peak height and ETP differed between patients and HS. Particularly, the ETP value increased only in MS patients (2,631 ± 166 vs. 2,748 ± 133, $p = 0.004$), whereas the peak height was significantly increased only in HS (13.4 ± 1.4 vs. 14.3 ± 1.5, $p = 0.008$).

The comparison between MS patients and HS of TG, after the double trigger, showed longer time parameters in MS patients. Lag time was longer as a trend (561 ± 81 vs. 487 ± 44 in HS, $p = 0.02$) and TTP was around 100 s longer (706 ± 91 vs. 605 ± 51 in HS, $p = 0.007$). To note, three out of four PP-MS patients showed the most prolonged time parameters (**Figures 1A,C**). Worth noting that the significant differences in TG parameters between MS and HS (**Table 5**) were observed in the presence of high correlations between time parameters, both in MS patients and in HS (**Figure 2**).

DISCUSSION

Prompted by the potential contribution of FXII in MS, in this study we provide the investigation of multiple FXII-related variables, to better define the relation between FXII and disease. This approach was coupled with global evaluation of the intrinsic pathway, with FXII activation obtained by artificial and natural molecules. Our main aims were to reveal differences between MS patients and HS, among MS clinical phenotypes, and in addition to evaluate in MS patients the variation over time of FXII-related variables.

The investigation on FXII:Ag revealed significantly increased levels in MS patients. FXII:Ag provides information about the concentration of circulating FXII protein independently from its activation and activity, presence of inhibitors, and other factors participating in the coagulation pathway. We did not observe, even as a trend, higher levels of FXII:c in RR-MS and SP-MS patients compared to HS as reported by a previous study (14). However, our cohorts were smaller (with exception of the PP-MS group) than those of the German study and, in accordance with our study design, we did not investigate FXII:c during relapse. Further comparison between data in German and Italian MS patients is hampered by absence of information about FXII protein levels (FXII antigen) in German patients. Nevertheless, the increased FXII:Ag levels detected in Italian MS patients and the increased FXII:c detected in German MS patients are both candidate to increase FXII-related immunomodulatory function. Of note, both FXII protein forms, the zymogen and the active ones, would express the immunomodulatory role independently from FXII activation in the coagulation pathway.

Repeated evaluation over 4 months of FXII:c, FXII:Ag levels, and FXII:ratio were instrumental to investigate their variation overtime in patients. We observed high correlation among time points for each FXII parameter. This feature could support a meaningful investigation of FXII contribution to disease phenotype and progression in future prospective studies.

Interestingly, FXII:c displayed a trend for variation across the time points. This could highlight changes dependent on the rehabilitative treatment, as inferred by comparison of FXII:c at T0 and T1 time points, as well as independent from treatment, as inferred by measurements prior and after 3 months of the rehabilitative training program (T0 vs. T3). Aimed at improving knowledge about the FXII role in the disease, we provided quantitative information about the FXII procoagulant activity in relation to the amount of circulating protein, by evaluating their ratio. This analysis indicated a significantly lower FXII:ratio in MS patients. This novel finding prompted us to investigate in selected groups of MS patients and HS the intrinsic pathway by TG, which provides kinetic information and potentially

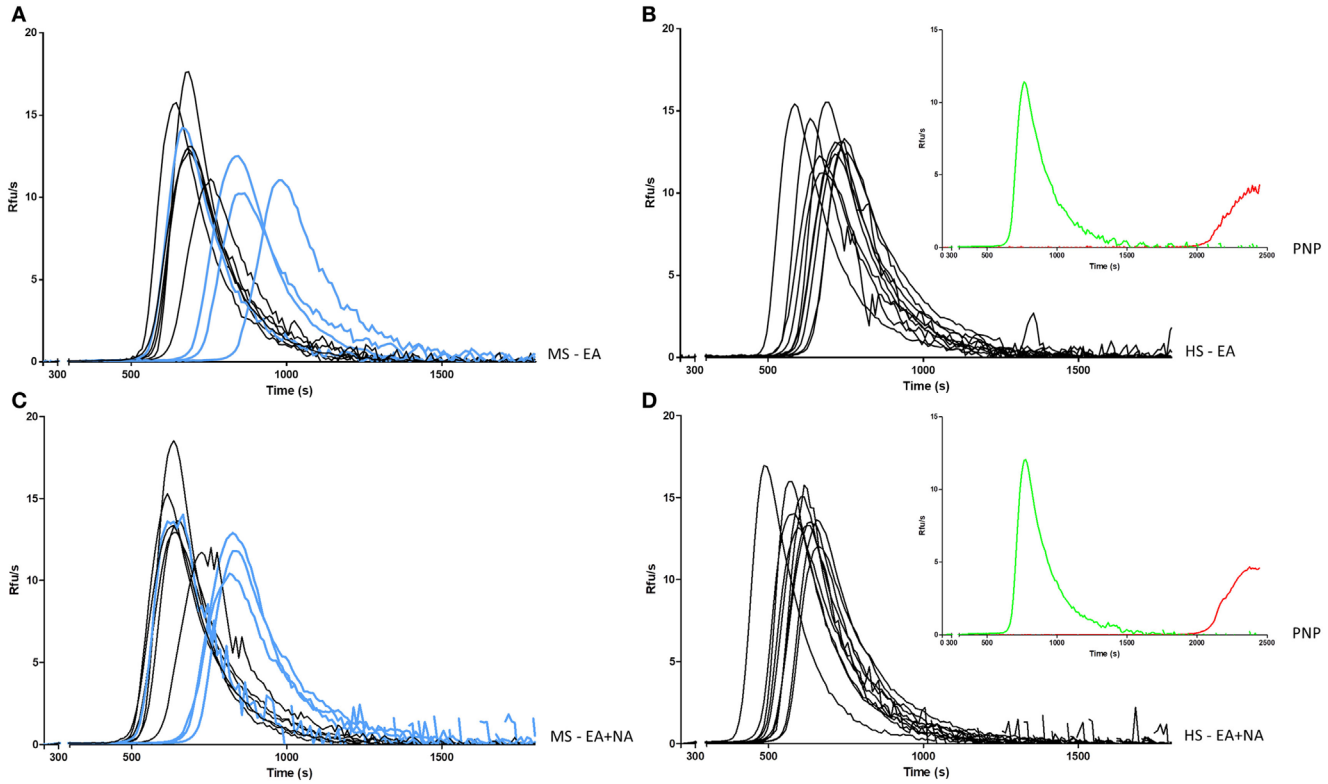

FIGURE 1 | Intrinsic generation of thrombin in plasma of MS patients and HS. Thrombin generation activity in plasma samples was triggered by EA in MS patients **(A)** and in HS **(B)**, or EA plus NA in MS patients **(C)** and in HS **(D)**. Curves from PP-MS are shown in light blue. Curves of PNP (insets) in the same conditions of MS patients and HS (green), and after addition of FXII inhibitor (red) are reported as control. MS, multiple sclerosis; PP-MS, primary progressive multiple sclerosis; HS, healthy subjects; EA, ellagic acid; NA, nucleic acid; PNP, pooled normal plasma; RFUs, relative fluorescence units; s, seconds.

TABLE 5 | Generation of thrombin in plasma of multiple sclerosis patients and HS.

	EA			EA + NA			EA vs. EA + NA *p*-Value	
	MS	**HS**	***p*-Value**	**MS**	**HS**	***p*-Value**	**MS**	**HS**
Lag time (s)	612 ± 97	564 ± 44	0.175	561 ± 81	487 ± 44	0.02	0.006	<0.0001
TTP (s)	750 ± 109	690 ± 51	0.132	706 ± 91	605 ± 51	0.007	0.014	<0.0001
Peak (RFU/s)	13.1 ± 2.3	13.4 ± 1.4	0.804	13.5 ± 2.2	14.3 ± 1.5	0.335	0.153	0.008
ETP (RFU)	2,631 ± 166	2,780 ± 136	0.042	2,748 ± 133	2,746 ± 38	0.964	0.004	0.506

MS, multiple sclerosis; HS, healthy subjects; ETP, endogenous thrombin potential; EA, ellagic acid; NA, nucleic acid; TTP, time to peak; RFUs, relative fluorescence units; s, seconds. Student's t-test was used to compare TG parameters of MS patients with those of HS, while paired Student's t-test was used to assess differences in TG after NA addition in MS and HS (p-value EA vs. EA + NA).

mechanistic interpretation of differences. Coherently with the decreased FXII:ratio, we report in MS patients a trend of lower amounts of thrombin potential, ETP, a stable and highly affordable parameter.

We introduced in this study a modified TG assay with double activation, obtained by the addition of NA, which has been recognized among true physiological activators of the contact pathway (31, 32).

Interestingly, NAs released from dead and dying cells may induce an autoimmune response by activating specific sensing receptors (33), thus representing candidate molecules of the complex crosstalk between coagulation pathway, inflammation, and immune system.

Additional trigger by NA shortened time parameters less in MS patients as compared with HS. Overall, the lower FXII:ratio and longer TG time parameters suggested that in part of MS patients (i) FXII could be less active per antigen unit and (ii) FXII response to contact activation and its support to the intrinsic coagulation pathway could be reduced. Interestingly, it has been recently reported that in TG, triggered by extrinsic activation,

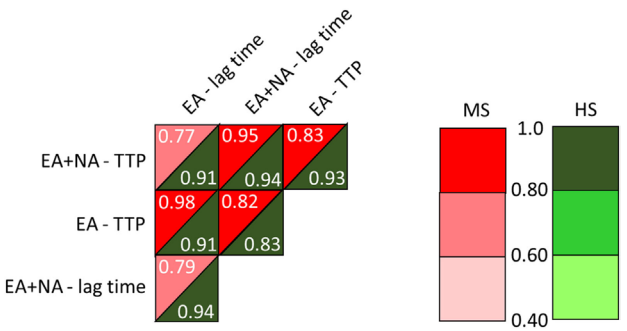

FIGURE 2 | Correlations between time parameters from the intrinsic thrombin generation (TG) triggered by EA or by ellagic plus NA. MS, multiple sclerosis; HS, healthy subjects; EA, ellagic acid; NA, nucleic acid; TTP, time to peak. Linear models were generated to determine the relationship between TG parameters.

time parameters were shorter in MS patients (23), which does not conflict with our data because extrinsic TG does not explore FXII contribution. Noteworthy, both the intrinsic TG, first reported in our study, and the extrinsic TG (23) tightly depend on activation and activity of coagulation factors in the common pathway, essential to generate thrombin. Although indirectly, our study does not support the presence of a prothrombotic state in the MS patients under study.

In light of the increased FXII protein levels and decreased activation that we report, pharmacological inhibition of FXII, proposed as a potentially new approach to MS treatment, needs deep investigation.

The low number of patients under DMTs and the extremely heterogeneous DMTs did not permit us a productive analysis of FXII levels in relation to DMTs. Nevertheless, these study features enabled us to obtain FXII-related values reasonably independent from drugs, like interferon that is known to heavily influence gene expression in several tissues. These values could better reflect the "biological" relation between FXII and (untreated) disease. On the other hand, the investigation of DMTs effects on FXII-related variables in a properly designed study would provide a comprehensive picture of this poorly defined field.

In conclusion, our study points toward FXII-related differences between MS patients and HS, with the limitation of the small sample size. Multiple specific and global coagulation assays could help stratification of patients to better define FXII contribution to disease phenotype and progression.

ETHICS STATEMENT

This study was approved by the Ethics Committee of Ferrara province with approval number 101-2012. Written informed consent was obtained from all participants.

AUTHOR CONTRIBUTIONS

NZ, MB, GM, and FB conceived the study design and wrote the manuscript; NZ, RV, and PS collected plasma samples and evaluated pre analytical variables; NZ and MB set up ELISA and aPTT; NZ performed ELISA, aPTT, and analyzed data; RM set up and performed aPTT; MB performed thrombin generation and analyzed data; SS, FM, PZ, and NB designed and supervised the rehabilitation study, recruited patients, and performed their clinical evaluation; SS and EM collected and analyzed instrumental and clinical data for patients classification. All authors critically evaluated the manuscript.

FUNDING

This study was supported by the grant 1786/2012 from the strategic 2010–2012 Research Program of Emilia Romagna Region, Italy.

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