PLATELETS AS PLAYERS IN NEUROPATHOLOGIES: NOVEL DIAGNOSTIC AND THERAPEUTIC TARGETS

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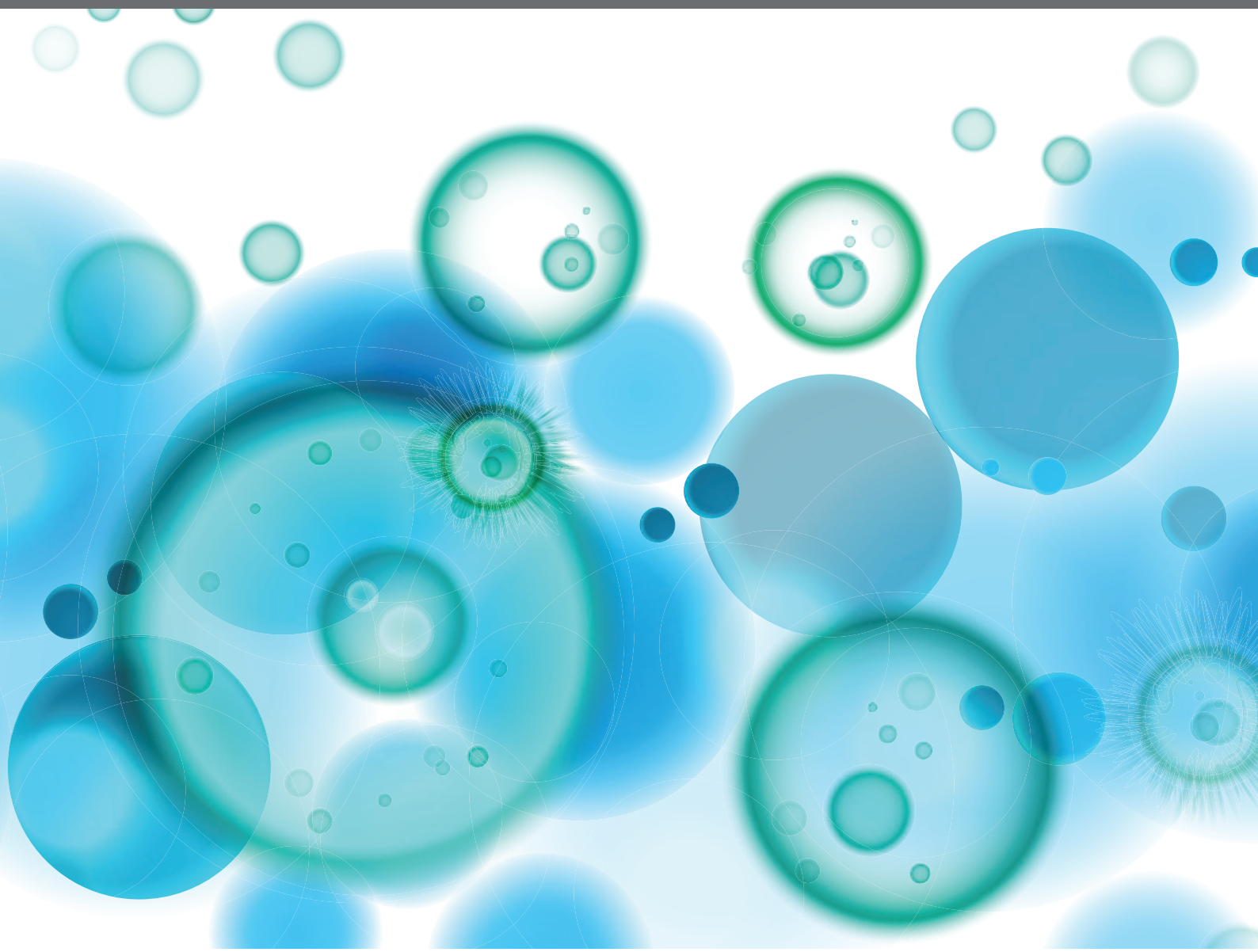

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PLATELETS AS PLAYERS IN NEUROPATHOLOGIES: NOVEL DIAGNOSTIC AND THERAPEUTIC TARGETS

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EDITORIAL
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Editorial: Platelets as Players in Neuropathologies: Novel Diagnostic and Therapeutic Targets

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Editorial on the Research Topic:

Platelets as Players in Neuropathologies: Novel Diagnostic and Therapeutic Targets

The revised view of platelets, or thrombocytes, from megakaryocytes-derived cell fragments dedicated to hemostasis, to pivotal elements in inflammation and autoimmunity is now well-documented (1, 2). Over recent decades, unexpected evidence has highlighted the multifaceted functions of these unique cells (3–5):

1. Platelets are anucleate cells of 1.5-3 μm in diameter in humans (0.5-1.0 μm in mice), of high abundance (150-450 x10^3/μl in humans; 1,000-1,500 x10^3/μl in mice) and short lifespan (8-9 days in humans; 4-5 days in mice). However, their estimated 3,700 different proteins, relate not only to hemostasis, but to defence, cell-cell communication and the inflammatory response (6, 7).
2. Platelets carry rough endoplasmic reticulum, polyribosomes and stable megakaryocyte-derived mRNA transcripts, selected during thrombopoiesis. Also identified are 284 miRNA species regulating protein expression *via* miRNA-mRNA pairings (8, 9).
3. Platelets are high extracellular vesicle (EV) producers. Platelet-derived microvesicles account for up to 70–90 % of total EV in peripheral blood (10).
4. Platelets exhibit rapid changes of phenotype by acquiring unique mRNA and protein profiles, depending on pathological status (11).

Consequently, platelets instantaneously sense danger signals and respond by recruitment of innate immune cells, triggering an adaptive immune response. In this context, we organized a Research Topic in Frontiers in Immunology with a focus on two themes: (1) similarities between platelets and neurons in expression profile and (2) their potential as biomarkers and therapeutic targets. We gathered five original papers and four reviews on the role of platelets in neuroinflammatory diseases, such as multiple sclerosis (MS) and neurodegenerative/neuropsychiatric disorders, particularly stroke, Alzheimer disease (AD) and Parkinson's disease (PD).

The review by Leiter and Walker provides an updated overview of current evidence on platelet function and details how platelets are pivotal to immune responses, tissue remodeling and healthy brain function. Significantly, platelets express multiple components regarded as bona fide neuronal proteins, including neurotransmitters for central nervous system intercellular communication,

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neurogenesis-enhancing molecules, components promoting neuronal plasticity and Alzheimer's precursor protein (APP) and its metabolite beta-amyloid (Aß). Additionally, there are shared mechanisms between platelets and neurons in neurotransmitter storage and release, secretory pathways and uptake and packaging (Leiter and Walker) (12). Therefore, platelet hyperactivation has major implications in neurodegenerative conditions.

Consequently, the identification of release markers of platelet activation is a major pursuit. Inyushin and colleagues explore the role of systemic platelet-derived APP and Aß peptides, particularly the $Aß_{1-40}$ peptide predominant in platelets (as opposed to $Aß_{1-42}$ predominant in brain), in various forms of amyloidosis (Inyushin et al.). Platelet-derived Aß has immune functions in infection, where APP processing is non-amyloidogenic. However, this changes in amyloidosis disorders, when systemic Aß contributes to vascular damage. The consensus is that the platelet shift in APP processing to Aß represents an excellent model to study blood-based AD biomarkers. In contrast, evidence also suggests a complex picture whereby changes in platelet components are incompletely replicated in plasma. This is the case for brain-derived neurotrophic factor (BDNF) and its precursor proBDNF, where the cerebrospinal fluid proBDNF/BDNF ratio is a candidate AD biomarker. Plasma and platelets also contain proBDNF and BDNF, but studies from the Lordkipanidze group (Le Blanc et al.; Fleury et al.) show that unlike BDNF, proBDNF is not released from platelets upon activation showing a different proBDNF/BDNF regulation between CNS and plasma.

With similar objectives in mind, Humpel's group (Foidl et al.) used a lipidomic approach to profile the lipid expression pattern in a murine model of sporadic cerebral amyloid angiopathy (CAA), a vascular pathology which occurs independently, or as a frequent AD co-morbidity. CAA diagnosis relies on vascular deposition of $Aß_{1-40}$. Alterations in lipid profiles in both platelets and plasma (6 platelet lipids and 15 plasma lipids) were identified in the CAA model, which does not exhibit AD pathology, but with differential signatures. Given the difficulty of diagnosing pure CAA, the identification of a unique lipid profile in this disorder may lead to earlier differentiation between CAA and AD.

The potential of classical platelet parameters such as mean platelet volume, platelet count and platelet distribution width as early disease markers is also being explored. Gialluisi's group (Tirozzi et al.) identified a significant genetic correlation between platelet distribution width and PD risk, but not between AD and platelet parameters. Given that platelet distribution width is an index of platelet procoagulant activity, this parameter may represent a risk indicator for certain neurodegenerative/neuropsychiatric disorders.

The Langer group describes the interplay between platelets and the complement system as well as plasmatic coagulation factors and the potential clinical benefit of targeting platelet-mediated neuroinflammation as an adjunct therapy to mitigate disease severity in MS and stroke-associated brain injury (Rawish et al.). Such developments prompted the design of an MS study by Koudriavtseva and colleagues seeking to establish a link between the pathogenetic role of coagulation and hemodynamic abnormalities in MS. This study aims to correlate magnetic resonance imaging-identified brain hemodynamic changes with altered coagulation/complement factor profiles and related damage markers, with the long-term goal of validating the coagulation system as a therapeutic target in MS (Koudriavtseva et al.).

Concurrently, as described by Orian and collaborators, avenues for platelet imaging and targeting are being explored. The platelet-specific GPIIb/IIIa receptor undergoes conformational changes during activation, thereby exposing a ligand binding pocket enabling differential targeting of the activated counterpart, but not resting platelets. Since activated platelets accumulate at the site of injury, platelet imaging when combined with other imaging approaches may provide improved sensitivity for longitudinal monitoring and candidate therapeutic evaluation. The concept of platelet targeting for therapeutic ends has been hampered by the risk of bleeding complications, but refined targeting of activation-specific epitopes warrants further investigation.

While the link between platelets and neuropathologies is strengthening, similar revelations are being made in other fields. Studies have highlighted the cross-talk between platelets and cancer cells and the role of platelets in tumor metastasis (13, 14). Work in cerebral malaria has shown that both platelets and platelet-derived EV contribute to pathology (15). Therefore, advances in determining the potential of platelets in diagnosis, patient monitoring and as therapeutic targets, would benefit from improved understanding of shared mechanisms across conditions where platelets drive pathological progression and of platelet interaction with their target organs over disease evolution.

AUTHOR CONTRIBUTIONS

JMO wrote the first complete draft of the article. SCW and CH contributed to the final draft and edited the article. All authors contributed to the article and approved the submitted version.

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REVIEW
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Platelets in Neurodegenerative Conditions—Friend or Foe?

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It is now apparent that platelet function is more diverse than originally thought, shifting the view of platelets from blood cells involved in hemostasis and wound healing to major contributors to numerous regulatory processes across different tissues. Given their intriguing ability to store, produce and release distinct subsets of bioactive molecules, including intercellular signaling molecules and neurotransmitters, platelets may play an important role in orchestrating healthy brain function. Conversely, a number of neurodegenerative conditions have recently been associated with platelet dysfunction, further highlighting the tissue-independent role of these cells. In this review we summarize the requirements for platelet-neural cell communication with a focus on neurodegenerative diseases, and discuss the therapeutic potential of healthy platelets and the proteins which they release to counteract these conditions.

Keywords: platelets, neurodegeneration, neuroinflammation, brain function, neuroimmune crosstalk

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INTRODUCTION

Platelets are small anucleate blood cells that have been gaining recognition as important mediators of several regulatory processes. Emerging research has identified novel functions that reach well beyond the traditional role of platelets in hemostasis and wound closure, revealing them to be crucial players during immune responses and tissue remodeling processes. We have recently summarized the evidence highlighting the capacity of platelets to contribute to brain homeostasis under physiological circumstances (1). Whereas, their versatile functions make platelets important regulators of cellular processes under normal conditions, platelet dysfunction is linked to a number of pathologies, including neurodegeneration. In the following review we briefly discuss the prerequisites of intercellular communication between platelets and cells from the central nervous system and summarize the research that demonstrates the involvement of impaired platelet function in several neurodegenerative conditions, including Alzheimer's disease (AD), Huntington's disease (HD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), and prion diseases (**Figure 1**). Finally, we highlight the emerging role of platelet preparations in the development of therapeutic interventions for the treatment of neuropathologies.

PLATELETS—THE DIVERSE PROPERTIES OF A SMALL BLOOD CELL

Until recently, platelets were primarily known for initiating coagulation following tissue injury and endothelial disruption. Although the platelet count in healthy humans ranges from 150,000 to 400,000 platelets per microliter of blood (2), only a small fraction of these (about 10,000 platelets per microliter) are necessary to act during hemostasis (3), supporting reports that platelets

also exert other functions. Platelets are produced in the bone marrow by megakaryocytes which equip them with cytoplasm, including messenger ribonucleic acid (mRNA), mitochondria and secretory vesicles such as lysosomes, dense granules and α-granules, before they are released into the blood. Mouse and human platelets are functionally similar (4) and have short lifespans of 4–5 days and 8–12 days, respectively (5). However, a recent study found that platelets can return to the circulation following activation by thrombotic and immunological stimuli, suggesting that their lifespan could be longer than traditionally thought and that their elimination is not a direct consequence of the activation process (6). Platelet activation is required to fulfill particular functions; however, the outcome is specific to the trigger which initiates the activation. The most common platelet responses to activating stimuli include changes in shape, the upregulation of cell surface molecules, protein synthesis from mRNA, endo- and exocytosis, and the release of molecules from granule contents. In particular, the context-dependent secretion from α-granules, which provide a storage compartment for abundant bioactive molecules including growth and coagulation factors, chemokines, immune molecules and adhesion molecules, is highly regulated. Consequently, the stimulation of platelet preparations with three common agonists, adenosine diphosphate, collagen and thrombin receptor-activating peptide, results in distinct protein secretion profiles (7). In another study, it was shown that subpopulations of α-granules exist, in which proteins are stored in distinct clusters such as pro- or anti-angiogenic protein clusters (8). The selective release of these granule subtypes was triggered by the stimulation of different receptors with specific agonists, indicating that α-granule cargo is secreted in a context-dependent manner to either inhibit or promote angiogenesis as required (8). The finely tuned mechanisms whereby bioactive molecules are released from platelets represent a crucial asset in orchestrating regulatory processes across different tissues. However, disturbances in the regulation of platelet responses or hyperactivation of platelets have implications in numerous diseases, including during neurodegenerative conditions, as described in more detail below.

PLATELETS ARE EXPERTS IN CELL-CELL COMMUNICATION

Platelets can communicate with other cell types in multiple ways, with their flexibility and mechanistic diversity suggesting that they likely act as inter-tissue messengers, including between blood and brain cells. Although the secretion of bioactive molecules from α- and dense granules represents a likely route of intercellular communication, additional mechanisms via which platelets may support crosstalk between the brain and the systemic environment are possible. Platelets release extracellular vesicles containing active cytoplasm components such as exosomes and microparticles (9). Both represent common ways of intercellular communication between organs and tissues in health and disease. Platelet exosomes and microparticles can also contain microRNAs, which when dysregulated are involved in various neurodegenerative disorders, including AD, PD, MS, HD, and ALS (10). Moreover, platelet-released particles, as well as platelets themselves which measure ∼0.5 μm in diameter in mice (5) and from 1 to 5 μm in humans (11), are small enough to travel deep within the microcapillaries that span the brain. Thus, platelets and their released factors could interact with specific receptors in the cerebral vasculature to exert local, receptor-mediated effects. In conditions where the vascular integrity is altered or disturbed direct interactions with neural cells are possible. Platelet activity has been observed within the brain parenchyma following lesion (12) and stroke (13), as well as in the brain of experimental autoimmune encephalomyelitis (EAE)-induced mice (14). Furthermore, a direct interaction between platelets and neuronal cells has been reported, as they can bind central nervous system-specific glycolipid structures that are present in the lipid rafts of neuronal processes (15). This interaction was recently shown to stimulate the growth of new dendritic spines (16). The proposed mechanisms via which platelets communicate with neural cells have been discussed in more detail elsewhere (1); however, these mechanisms could also influence neural cell properties under neurodegenerative conditions. Moreover, as reviewed below, platelets exhibit neuron-like properties that further facilitate crosstalk between these cells and the central nervous system.

THE NEURON-LIKE PROPERTIES OF PLATELETS—BRIDGING THE GAP BETWEEN THE SYSTEMIC ENVIRONMENT AND BRAIN PATHOLOGIES?

Despite their distinct location and function, platelets and neural cells are remarkably similar, suggesting a potential path of cross-communication between the systemic environment and the brain. In particular the intercellular storage compartments in neurons, which contain neuropeptides, neurohormones and neurotransmitters, are comparable to platelet granules, including the use of similar vesicle trafficking mechanisms. Platelet dense granules resemble the small dense-core synaptic vesicles of neurons in terms of their serotonin and adenosine triphosphate contents, among other features, whereas the large dense-core vesicles of neurons are comparable to platelet α-granules. Both storage compartments carry a large variety of bioactive peptides, and stimulus-specific secretion processes are observed in both neurons (17) and platelets (8). This indicates that the strict regulation of selective exocytosis is a conserved mechanism in both cell types (18). Platelet and neuronal exocytosis are both triggered by an increase in the internal calcium concentration (19), leading to the rapid activation of the secretory machinery. Moreover, the mechanism whereby the internal vesicles fuse with the plasma membrane is highly conserved, occurring via specific

Abbreviations: $A_{2A}R$, adenosine A receptor; AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; APP, amyloid precursor protein; EAE, experimental autoimmune encephalomyelitis; GABA, γ-aminobutyric acid; HD, Huntington's disease; mHtt, mutant huntingtin protein; MAO, monoamine oxidase; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MS, multiple sclerosis; PD, Parkinson's disease; PrP, prion protein; TDP-43, TAR DNA-binding protein of 43kDa.

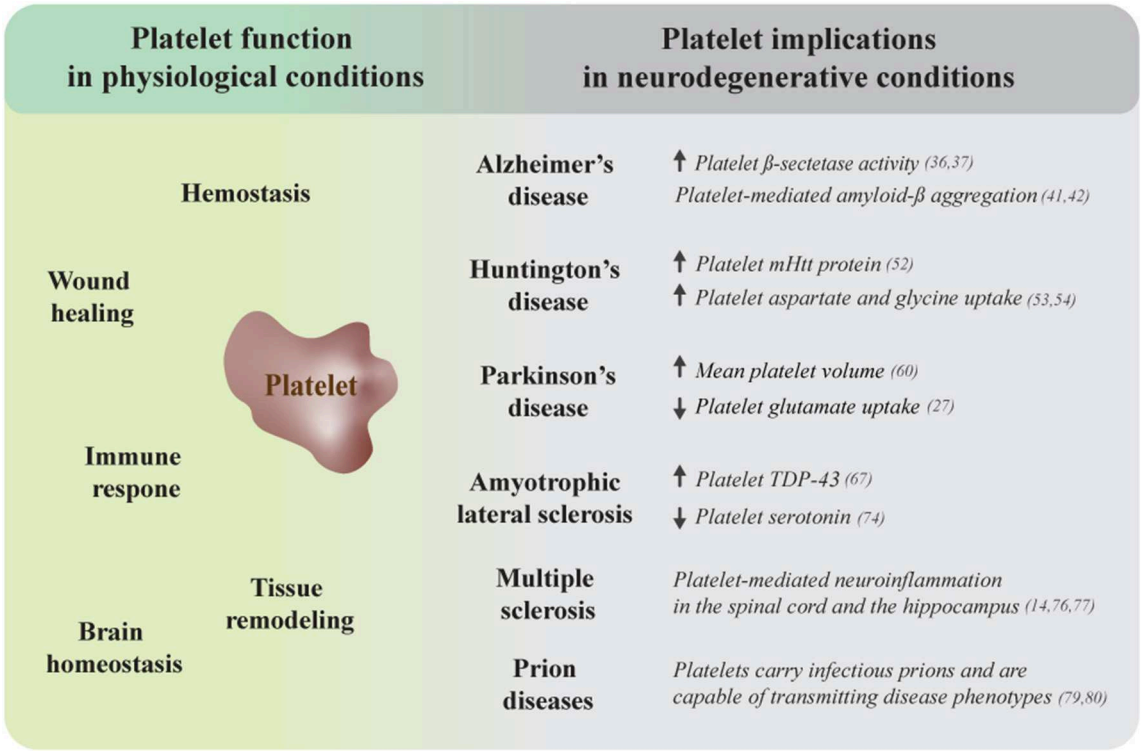

FIGURE 1 | Platelet dysfunction is associated with several neurodegenerative disorders. Platelets are complex cells that exert numerous regulatory functions under physiological conditions, ranging from their traditional roles in hemostasis and wound healing to fundamental contributions to immune and tissue remodeling processes and brain homeostasis (left side). Platelet dysfunction, including mitochondrial abnormalities, is a common observation during neurodegeneration. The right side of this figure summarizes additional platelet-related impairments that link these cells to several neurodegenerative conditions. mHtt, mutant huntingtin protein; TDP-43, TAR DNA-binding protein of 43 kDa.

docking molecules such as SNAREs, VAMPs and syntaxins (19). Other review papers have discussed the molecular similarities between platelets and neuronal cells in more detail (18–20), and have proposed that platelets could even be considered "neuronal cells" themselves, with the interaction between platelets and T cells representing a novel "neuroimmunological" synapse in the periphery (20). Likewise, platelets could act as messengers, transferring signals between the peripheral environment and brain cells. We have shown that platelet-rich plasma has direct stimulating effects on a pure population of flow cytometry-isolated hippocampal dentate gyrus-derived neural precursor cells *in vitro*, and that mice which have been depleted of platelets fail to show the expected exercise-induced increase in neural precursor cell proliferation *in vivo* (21). This work suggests that platelet-neural stem cell communication is an important regulatory mechanism in these brain cells, although the precise molecular mechanisms underlying this communication are still unclear.

Platelets carry several neurotransmitters that are essential for the intercellular communication between brain cells, including γ-aminobutyric acid (GABA), glutamate, serotonin, epinephrine, dopamine, and histamine. This suggests that platelets can send and receive signals to and from the nervous system and may act as an important relay between the brain and peripheral organs. The monoamine neurotransmitter serotonin is stored in dense granules, and peripheral serotonin release-associated regulatory functions of platelets have been described (6, 22). Although the peripheral and central nervous system serotonergic systems are thought to be separated, platelets release serotonin in response to brain-specific glycolipid structures, which are integrated into the lipid rafts of neurons and astrocytes (15). Such interactions could occur in conditions in which cerebral microvessels become leaky, including during neurodegenerative diseases (23), suggesting that platelets could act as communicators between blood and brain. This hypothesis becomes more cogent when considering the two major neurotransmitters GABA and glutamate, both of which are taken up by platelets (24). Glutamate is the most abundant excitatory neurotransmitter in the brain, and substrate-induced glutamate uptake has been demonstrated in human platelets, likely via specific glutamate receptors (25), similar to what is observed in neuronal cells (26). Platelets express various glutamate receptor subtypes and exhibit high affinity glutamate uptake activity, a process which is impaired in disorders such as PD (27), AD (28) and ALS (29). GABA, an inhibitory neurotransmitter, is crucial for healthy brain function, with perturbances in GABA receptor signaling being associated with neurodegenerative conditions [reviewed in Kim et al. (30)]. Platelets carry considerable amounts of GABA, although the

concentration is 30% lower than that found in neurons (31). In both neurons and platelets GABA is metabolized by GABA transaminase (31). Moreover, similar to neurons, platelets appear to take up GABA in a substrate-induced manner, with an *in vitro* study reporting that the GABA concentration in platelets is negligible when the peripheral benzodiazepine receptor blocker PK11195 is present in the cell culture medium (31).

Given these similar mechanisms of neurotransmitter uptake and metabolism, platelets have been suggested as a model system of glutamate and GABA transport in patients suffering from neurodegenerative conditions (25, 31). A more recent review article has extended these concepts to other conserved mechanisms between platelets and neurons that are associated with neurodegenerative diseases, with platelet dysfunction mirroring the abnormalities observed in neurons (32). However, to date it is unclear whether platelet dysfunction occurs first or whether functional impairments in platelets arise as a consequence of other defects that occur during neurodegenerative processes.

PLATELETS IN NEURODEGENERATIVE CONDITIONS

It is becoming clear that neurodegenerative diseases do not solely involve cells and tissue of the central nervous system, but rather that systemic influences also play a fundamental role in the development and exacerbation of brain pathologies. As discussed above, platelets are of particular interest as important mediators of this two-way relationship. Several review papers have concluded that these blood cells can serve as potent systemic biomarkers of neurodegenerative diseases, mirroring the pathological phenotypes of neural cells (32–34). In this section we describe the studies that link platelets to neurodegenerative conditions, with a particular focus on platelet dysfunction in these disorders (summarized in **Table 1**).

Alzheimer's Disease

AD is a slowly developing progressive form of dementia that is accompanied by unpredictable behavior, lack of enthusiasm and memory loss. The neuropathological hallmarks of AD include neuronal and synaptic loss, neuroinflammation, the formation of intracellular neurofibrillary tangles and the deposition of amyloid-ß in brain tissue and cerebral vessels. Increasing evidence has linked platelet dysfunction to this disease, in particular in the context of amyloid-ß secretion from platelets.

Although neural cells, including astrocytes and neurons, produce and secrete amyloid-ß (81), the peptide can also be released by activated platelets (82). Platelets have been suggested to be the primary source of amyloid-ß peptide in the blood (83). The cells produce this peptide through the cleavage of its precursor protein, amyloid precursor protein (APP), which is abundantly present in platelets and is secreted following platelet activation, similar to its metabolite amyloid-ß (82, 84, 85). Both APP and amyloid-ß peptide are associated with platelet functions. Whereas, APP is involved in the regulation of thrombosis and coagulation (46–48), amyloid-ß peptide has the ability to promote platelet activation (41, 49–51), adhesion (43, 48, 50), aggregation (47, 48) and to induce reactive oxygen species generation (45, 51).

Rather than alterations in platelet count or size, changes in platelet activation appear to play a prominent role in AD, with increases in activation detected in the blood of AD patients, likely as a result of increased lipid peroxidation (35). Similarly, platelets have been shown to be hyperactive in aged APP23 transgenic mice, a model of AD (38). A subsequent study confirmed abnormalities in platelet function in a more complex mouse model of AD, 3xTg-AD mice, with increased platelet adhesion to components of the subendothelial matrix and accelerated thrombus formation, although the platelet count remained unchanged (39). In patients with mild cognitive impairment and AD, the activity of ß-secretase, one of the major enzymes required for the cleavage of APP, is significantly increased in the membranes of platelets (36, 37), suggesting further platelet-related systemic changes during the disease.

A recent parabiosis study, in which the blood circulation of APPswe/PS1dE9 transgenic AD model mice was connected with that of their wildtype counterparts demonstrated that human amyloid-ß originating from the transgenic mice accumulated in the brains of their healthy littermates, forming amyloid-ß plaques and amyloid angiopathy following 12 months of parabiosis (86). Moreover, the parabiotic wildtype mice exhibited impaired long-term potentiation in the hippocampal cornu ammonis 1 area, suggesting a reduction in synaptic plasticity, which is thought to underlie deficits in learning and memory (86). Although this study did not investigate the origin of the blood-derived amyloid-ß, the authors suggested platelets as a likely source.

Prior to amyloid-ß plaque formation, platelet inclusions in cerebral blood vessels are among the first symptoms to appear in the brains of APP_SweDI AD model mice (40). Another study demonstrated that platelets enhance the formation of amyloid-ß aggregates in the brain vasculature and that amyloid-ß itself can activate platelets (41). In the same study, the plaque burden of cerebral vessels in APP23 mice was significantly reduced following a 3-month treatment with clopidogrel, a known inhibitor of platelet activation (41). Interestingly, a trend toward reduced plaque formation was also observed within the hippocampus, a brain region which is crucial for learning and memory and is profoundly affected by AD (41). More recent work has shown that platelets isolated from APP_SweDI mice promote vessel damage and neuroinflammation in the healthy mouse brain, leading to amyloid-ß-like immunoreactivity at the damaged vessel sites (42). Together these data suggest that hyperactive AD platelets release and interact with amyloid-ß specifically at sites of vessel damage, thereby accelerating the progression of the disease (38, 39, 41, 42). This is in line with work suggesting that AD may, at least in part, be a slowly developing thrombohemorrhagic disorder (87, 88), highlighting the need to expand research beyond the brain and consider treatment of the systemic environment in AD patients. In this regard, platelets represent a potential target, with a reduction in platelet count

TABLE 1 | Platelet abnormalities linked to neurodegenerative conditions.

Condition	Implication of platelets	Species/model	Reference
Alzheimer's disease	Increased platelet activation	Human	(35)
	Increased platelet β-secretase activity	Human	(36, 37)
	Platelet hyperactivity	APP23 mice	(38)
	Increased adhesion to subendothelial matrix components	3xTg-AD mice	(39)
	Platelet inclusions in cerebral blood vessels	APP_SweDI mice	(40)
	Platelets enhance formation of amyloid-β aggregates in cerebral vessels	APP23 mice	(41)
	Platelets promote neuroinflammation and vessel damage	APP_SweDI mice	(42)
	APP and amyloid-β influence platelet function	Human/APP-KO, C57BL/6 and APP23 mice	(41, 43–51)
Huntington's disease	Increased platelet mHtt protein levels	Human	(52)
	Increased platelet aspartate and glycine levels	Human	(53, 54)
	Platelets promote blood brain barrier permeability	Human	(52)
	Impaired platelet adenosine A receptor signaling	Human	(55, 56)
	Impaired platelet nitric oxide metabolism	Human	(57)
	Elevated platelet mitochondrial monoamine oxidase activity	Human	(58, 59)
Parkinson's disease	Increased mean platelet volume	Human	(60)
	Decreased platelet glutamate uptake	Human	(27)
	Reduction in vesicular monoamine transporter 2 mRNA	Human	(61)
	Platelet mitochondrial dysfunction	Human/ Cybrid model	(62–66)
Amyotrophic lateral sclerosis	Increased platelet TDP-43 levels	Human	(67)
	Reduced complex IV activity in platelet mitochondria	Human	(68)
	Altered platelet mitochondrial membrane potential	Human	(69)
	Altered platelet mitochondrial morphology	Human	(70)
	Altered platelet activation and morphology	Human	(70)
	Enlarged mitochondria, degenerating mitochondrial vacuoles and neurofilament aggregations	Cybrid model	(71–73)
	Decreased platelet serotonin levels	Human	(74)
Multiple sclerosis	Increased platelet activation	Human	(75)
	Platelets drive neuroinflammation in the spinal cord	EAE mice	(76, 77)
	Platelet-neuron associations are associated with neuroinflammation in the hippocampus	EAE mice	(14)
	Altered serotonin release from dense granules	Human/EAE mice	(78)
Prion diseases	Platelets carry infectious prions	Deer	(79)
	Platelets are capable of transmitting disease phenotypes	Deer and sheep	(79, 80)

AD, Alzheimer's disease; APP, amyloid precursor protein; EAE, experimental autoimmune encephalomyelitis, mHtt, mutant huntingtin protein; TDP-43, TAR DNA-binding protein of 43 kDa.

being suggested as a means to counteract the overproduction of amyloid-β (87).

An interesting alternative theory is that amyloid-ß release represents a defense mechanism against septic agents (89, 90). Recent research indicates that amyloid-ß may be a normal component of the innate immune system, protecting individuals against microbial and viral infection (91–94). Given the emerging evidence that platelets act as fundamental immune cells, including in the brain [summarized in Leiter and Walker (1)], they could accumulate at damaged cerebral vessel sites and release amyloid-ß as a defense peptide. This is in line with a study which suggests that the release of amyloid-ß from platelets is triggered by pre-existing tissue damage and inflammation and represents a natural protective mechanism against infection during thrombosis (92). However, the platelet hyperactivity that is associated with AD may lead to the overproduction of amyloid-ß, thereby exacerbating inflammation and eventually promoting the development of plaque formation.

Although the studies described above focused on amyloid-ß, this peptide does not represent the only known link between platelets and AD, with other investigators examining the involvement of neurofibrillary tangles and impaired neurotransmitter homeostasis. These studies have been reviewed elsewhere (95).

Huntington's Disease

HD is a hereditary autosomal dominant neurodegenerative disorder caused by a CAG repeat expansion in exon 1 of the huntingtin gene, resulting in the production of a mutant huntingtin protein (mHtt). This protein accumulates in neurons, thereby leading to their eventual death and a progressive loss of motor and cognitive functions. Extensive research has shown that a number of cell subpopulations in the blood are altered in HD patients, with platelets having the highest levels of mHtt (52).

The platelets of HD patients exhibit a number of abnormalities, including aberrant amplification of adenosine A receptor ($A_{2A}R$) signaling (55, 56). Given that the $A_{2A}R$ is expressed in GABA/enkephalin spiny neurons, it has been proposed that it may play a role in HD pathogenesis. Other studies have also reported a correlation between the density of $A_{2A}R$ in platelets and the rate of disease, age at onset and CAG repeat expansion (55, 96). However, whether or not $A_{2A}R$ activity provides a useful biomarker remains to be determined.

Dysfunction of the nitric oxide /nitric oxide synthase pathway and monoamine oxidase (MAO) have also been suggested to be critical contributors to HD pathology. Nitric oxide metabolism has been found to be dysregulated in platelets during the late stages of HD progression (57), and MAO activity has been associated with neuronal damage in a number of degenerative conditions. MAO is a mitochondrial enzyme that catalyzes the oxidative deamination of monoamines such as dopamine. MAO exists in the MAO-A and MAO-B isoforms. Whereas, some cell types express both isoforms, only MAO-B is found in platelets. Significantly elevated platelet MAO activity has been observed in HD patients during disease progression (58, 59), with the levels negatively correlating with the clinical response to drug treatment (97).

A proposed model of HD pathogenesis is the "excitatory hypothesis," based on the observation that excitatory amino acids and N-methyl-D-aspartate receptor agonists, including aspartate and glutamate, recapitulate the striatal neuron degeneration observed in HD (98). Although early studies found no differences in glutamate and aspartate activity between normal and HD platelets (99, 100), later studies have reported significantly increased aspartate and glycine in HD platelets (53, 54).

Mitochondrial dysfunction has also been implicated in the pathogenesis of HD. A significant decrease in mitochondrial complex I activity per platelet was observed when patients were grouped according to disease severity; however, when normalized to mitochondrial DNA content, no differences were detected (101). In contrast, an earlier study found no difference in platelet mitochondrial complex activity in HD patients (102). Given the relatively small group sizes, further data are required to determine whether mitochondrial function in platelets provides a useful biomarker of HD. However, increased mitochondrial-dependent apoptosis has also been reported in HD cybrids (103).

Platelets are also important in maintaining normal vascular integrity (104). Recently, an initial study investigating the potential impact of mHtt on platelet function showed that platelets can promote blood brain barrier permeability in HD, pointing toward their potential contribution to disease pathogenesis (52).

Parkinson's Disease

PD is a degenerative disorder caused by the loss of dopaminergic neurons in the substantia nigra, thereby resulting in an impairment in motor and cognitive functions. Although the cause of sporadic PD, the most common form of the disease, is unknown, one major causal factor is mitochondrial dysfunction. This was first suggested by the finding that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxin that selectively kills dopaminergic neurons, acts by inhibiting complex I of the electron transport chain (105). A plethora of studies have reported reduced complex I activity in the platelets of patients with PD (62–64), although it should be noted that other studies did not find such alterations (65, 66). Supporting the former observation, a PD cybrid model in which mitochondrial DNA from PD platelets was expressed in rho 0 human teratocarcinoma cells showed a reduction in complex I activity (106, 107). In addition, 1-methyl-4-phenyl-pyridinium ion MPP(+), the metabolite of MPTP, was shown to induce adenosine triphosphate depletion in platelets and attenuate platelet aggregation and activity, providing a potential mechanism underlying the anti-aggregation effect observed in PD patients (108).

Several studies have suggested that MAO also plays an important role in MPTP toxicity and the etiology of PD. Increased MAO-B activity has been observed in PD patients (109–111), potentially due to a G/A single nucleotide polymorphism in intron 13 which results in a splicing enhancer that stimulates intron 13 removal efficiency (110). However, the data concerning platelet MAO-B activity in PD patients are not consistent, with other studies reporting that platelet MAO-B activity is unchanged in PD patients (112, 113).

A number of other alterations in the platelets of PD patients have also been suggested as potentially useful biomarkers. These include a reduction in vesicular monoamine transporter 2 mRNA (61), an increase in mean platelet volume (60), and decreased glutamate uptake (27).

Amyotrophic Lateral Sclerosis

ALS is a fatal neurodegenerative disorder that is characterized by progressive and selective loss of motor neurons in the brain and spinal cord. Patients suffer from progressive muscle weakness and paralysis of their voluntary muscles, ultimately leading to respiratory failure and death. There is accumulating evidence that in addition to affecting motor neurons, ALS also affects platelets.

Almost all ALS cases (∼97%) are characterized by pathology due to the TAR DNA-binding protein of 43 kDa (TDP-43) (114, 115). In diseased neurons, TDP-43 is relocated from its normal nuclear location to the cytoplasm, where it is phosphorylated and ubiquitinated, subsequently aggregating to form insoluble intracellular inclusions (115). A recent study found that the TDP-43 levels in platelets from patients with sporadic ALS are significantly higher than those of non-ALS age-matched controls (67). Interestingly, the TDP-43 levels in platelets tended to increase with disease progression, although a larger cohort of patients is required to confirm this observation (67).

Mitochondrial abnormalities, particularly impairments of complex IV (cytochrome c-oxidase) activity, have been

implicated in ALS, although the exact role of mitochondrial dysfunction remains unclear. In addition to mitochondrial dysfunction in motor neurons of ALS patients, mitochondrial changes have also been reported in muscle, liver and blood cells, suggesting systemic involvement (116–118). Complex IV activity was found to be decreased in platelets from ALS patients in a small case-control study (68). Interestingly, the cellular mitochondrial content increased, indicating a potential compensatory mechanism (68). Further supporting the notion of mitochondrial dysfunction, a change in the mitochondrial membrane potential has been reported in platelets from ALS patients (69), as well as changes in the ultrastructure and morphology of platelets and their mitochondria (70). This is in line with an earlier study which observed platelet activation and morphological changes in ALS platelets (119). ALS cybrids (platelets fused to the rho neuronal cell lineage) also show similar cytoskeletal deformities to those found in ALS patients and transgenic superoxide dismutase 1 mice, including enlarged mitochondria, degenerating mitochondrial vacuoles and neurofilament aggregations (71–73). Despite these links between platelet mitochondrial dysfunction and ALS, larger cohort studies are required to conclusively determine whether mitochondrial function can be used as a biomarker for ALS.

Thrombospondin is a glycoprotein that is released from platelet α-granules following thrombin-induced platelet activation. Changes in blood thrombospondin levels have been detected in a number of pathological conditions, including a marked increase in thrombospondin deposition in the muscles of ALS patients (120, 121). The neurotransmitter serotonin is also decreased in the brain and spinal cord of ALS patients (122, 123). Platelets are a major source of serotonin and platelet serotonin levels have been shown to be significantly lower in ALS patients and to positively correlate with patient survival (74). However, the cause of this decrease in serotonin remains elusive. Glutamate excitotoxicity has also been implicated in the pathogenesis of the disease. Platelets contain a glutamate uptake system and express components of the glutamate-glutamine cycle, including the excitatory amino acid transporter 2 and glutamine synthetase. Increased glutamine synthetase, but normal excitatory amino acid transporter 2 expression, has been reported in the platelets of ALS patients (124). However, given that this finding is in contrast to an earlier study which reported a reduction in glutamate uptake in ALS patients (29), these data need to be confirmed.

Multiple Sclerosis

MS is an inflammatory disease, where the immune system attacks the myelin sheaths that cover nerve axons in the spinal cord and brain. The resulting nerve damage leads to communication deficits between the brain and other tissues, and depending on the affected nerves provokes a range of symptoms, including impairments in vision, deficits in motor control of the arms and legs and neuropsychological symptoms such as depression and memory loss. To date, there is no known cure for MS, as the underlying cause is still unknown.

A few studies targeting platelets and their involvement in MS and its mouse model, EAE, have shown that these conditions are associated with abnormalities in platelet function. One of these investigations found increased platelet activation in the blood of clinically stable relapsing-remitting MS patients who had not yet received treatment (116). This was evidenced by significantly larger numbers of CD62P-positive platelets and CD41-positive platelet microparticles (75). Subsequent evidence in EAE mice revealed that platelets exacerbate the development of the disease via the recruitment of leukocytes to the neural tissue (76). A more recent study cemented the involvement of platelets in EAE, demonstrating that platelets not only aggravate (76) but also drive neuroinflammation in the spinal cord (77). Possible mechanisms via which platelets could exacerbate the pathophysiology of MS are discussed in a review by Wachowicz et al. with one interesting concept being an impaired antioxidant mechanism in combination with inflammation-induced platelet activation as an additional source of reactive oxygen species to further accelerate tissue damage (125). Moreover, the secretion of serotonin from dense granules has been shown to modulate immune cell responses in a stage-depended manner. During the early stages of EAE and MS, high levels of platelet-released serotonin stimulate the proliferation and differentiation of pathogenic T cell subsets, thereby promoting proinflammatory responses (78). During later phases of the disease, however, platelets exhibit reduced serotonin levels and appear to suppress T cell activation and central nervous system inflammation (78).

Recent work investigating the brains of EAE-induced mice demonstrated that platelets were also present in the parenchyma of the hippocampus, including in the fimbria and in close proximity to neuronal cell bodies in the dentate gyrus and CA1 region (14). This phenotype was associated with the formation of a neuroinflammatory environment, supposedly due to platelet-neuron associations (14). However, this occurred in the absence of inflammatory cell infiltration, further highlighting the role of platelets in the initiation of EAE (14). In the same study, the pro-inflammatory environment in the hippocampus of EAE-induced mice, as well as their increased anxiety-like behavior, were improved following platelet depletion with polyclonal anti-platelet glycoprotein Ib α chain antibodies, suggesting that platelets could serve as a potential target for the amelioration of the symptoms of MS (14).

Prion Diseases

Prion proteins (PrPs) comprise a class of amyloid-forming proteins, with some isoforms being associated with a group of fatal neurodegenerative diseases termed transmissible spongiform encephalopathies. Once diagnosed, these conditions progress rapidly and are characterized by the chronic deterioration of physical and mental abilities, including profound memory impairments. The scrapie isoform of PrP is an abnormal, misfolded, protease-resistant isoform (126, 127) which is believed to be responsible for transmissible spongiform encephalopathies. Although considered transmissible, the paths through which prion diseases spread are unknown, with the transfusion of blood from infected donors presenting a concern.

Cellular PrP (PrP^c) is carried by blood cells, including platelets, in which PrP^c is present on the membranes of α-granules (128, 129). Following activation, PrP^c can be released

from activated platelets, mainly in the form of microparticles and exosomes (128). The function of PrP^c under these circumstances is unknown, although it has been reported that the protein is unlikely to play a role in the aggregation or adhesive actions of activated platelets (128). The release of microparticles and exosomes represents a major route of intercellular communication, including crosstalk between platelets and neural cells (1). This suggests that in the course of transmissible spongiform encephalopathies, the less soluble scrapie prion isoform could be carried and released from activated platelets thereby contributing to the infection of the brain and the transmission of the disease through blood transfusion (128). Other work has confirmed that platelets and B cells in the blood of deer, infected with chronic wasting disease carry infectious prions, and are substantially involved in transmitting the disease phenotype (79). In a sheep model of variant Creutzfeldt Jakob disease, the disease could be transmitted through several blood components, such as whole blood, plasma, red blood cells, buffy coat and platelets (80). These data from animal studies suggest a high probability that spongiform encephalopathies are transmissible through blood (79, 80), even in pre-clinical stages of the disease (80). However, only a few cases suggest this possibility in humans, where the lack of a causal link between blood transfusions and the development of prion diseases makes it difficult to draw a conclusion (130–132).

PLATELETS—A NOVEL THERAPEUTIC AVENUE FOR THE TREATMENT OF NEURODEGENERATIVE CONDITIONS?

Impairments in platelet function are a common observation in neurodegenerative disorders; however, healthy platelets and their secreted factors also represent a possible approach for the development of therapeutic interventions for the treatment of neurodegenerative conditions. Among the primary applications are the use of platelet lysate and platelet-rich plasma, both of which are easy to obtain from immune-compatible healthy donors. The beneficial effects of platelet-rich plasma treatment are likely to be attributable to the abundant variety of growth factors that platelets carry in their granules. Neural and glial cells express surface receptors for a range of these growth factors, including vascular endothelial growth factor, epidermal growth factor, fibroblast growth factor-2, platelet-derived growth factor, brain-derived neurotrophic factor, platelet factor 4, transforming growth factor-ß, insulin-like growth factor-1, connective tissue growth factor and bone morphogenetic protein-2,−4, and−6, suggesting a fundamental role of platelets in tissue growth and regeneration, including in the brain (133–135). Moreover, human platelet lysate comprises a plethora of growth factors, including those with neuroprotective properties. Although emerging research has shown promising results, diverse protocols for the isolation of platelet-rich plasma and platelet lysates exist, resulting in products which contain variable ranges of growth factors (136). Moreover, novel protocols are continuously being published, describing optimized preparations for specific use in different applications (137–139). These factors therefore represent an important consideration when evaluating study outcomes and planning future clinical trials across different fields.

Platelet-Rich Plasma

Platelet-rich plasma can easily be prepared from whole blood using a slow centrifugation speed and physiological washing buffers that support platelet purification. This method achieves a nearly pure population of platelets [>99.99% purity (140)], and the platelet preparation can be used immediately or stored. However, upon freezer storage and subsequent thawing of the samples, a substantial number of cells will be lyzed, leading to the release of growth factors from platelet granules. These are also present in frozen/thawed platelet-rich plasma preparations, making them a physiological cocktail of intact cells and released bioactive molecules.

Beneficial therapeutic effects of platelet-rich plasma treatment have been reported in numerous tissues, including during burn healing (141, 142), cartilage repair (143) and healing following dermal injuries (144). Other studies have demonstrated that platelet-rich plasma treatment enhances the recovery of peripheral nerves following injury, including cavernous nerve injuries (145) and damage of the facial (146) and sciatic (147) nerves. Moreover, platelet-rich plasma injections into the injured spinal cord of rats have been shown to promote locomotor recovery, local angiogenesis and neuronal regeneration (148). Another study in mice suggested the therapeutic use of platelet-rich plasma in neuroinflammatory central nervous system diseases, as platelet-rich plasma treatment considerably improved the clinical symptoms in the EAE mouse model of MS (149). This effect was accompanied by significantly lower gene expression and a decrease in the protein levels of inflammatory markers in the lumbar parts of the spinal cord, including the microglial marker Iba1 and the pro-inflammatory cytokine interleukin 1-β, as well as the reduced infiltration of inflammatory cells (149). The platelet-rich plasma injection also protected the cells from demyelination in the affected area (149). Other studies which used the plasma rich in growth factors Endoret® technology to isolate platelet-rich plasma from human blood have demonstrated that treatment with these preparations significantly reduces amyloid-β plaque density in the hippocampus and improves cognitive function in APP/PS1 AD model mice (150). Another study complemented this finding showing that the same preparations enhanced adult neurogenesis in the hippocampus of APP/PS1 mice, a process known to be affected during AD, and that this enhancement was likely due to a reduction in amyloid-β-mediated neurotoxicity (151). The same method also promoted neuronal survival and diminished the inflammatory responses in a mouse model of PD, as well as reducing the associated motor impairments (152). These data suggest that platelet-rich plasma treatment represents a promising approach which could be applied to several neurodegenerative disorders.

Platelet Lysate

Similar to platelet-rich plasma, platelet lysate can be easily obtained from whole blood samples. Platelets are first enriched by centrifugation steps, followed by freezing and thawing of the

samples. An additional centrifugation step then separates the freeze/thaw-triggered secreted platelet factors, which constitutes the platelet lysate, from the remaining cell debris.

Given their essential role in wound healing and tissue repair, platelet lysates are being investigated as a therapy for a number of neurodegenerative diseases. Human platelet lysates have been investigated as a novel biotherapy for ALS and PD patients. In an NSC-34 cell-based model of ALS, human platelet lysates conferred a neuroprotective effect against staurosporine-induced apoptosis and menadione-induced oxidative stress, indicating that neuronal loss can be diminished by platelet factors in those conditions (153). In a Lund human mesencephalic cell-based model of PD, pre-treatment of the cells with human platelet lysates also protected again erastin-induced ferroptotic cell death (153). The authors further optimized the isolation protocol to produce platelet lysate preparations which are more enriched for neurotrophins and at the same time depleted of plasma proteins, thereby preventing potential adverse thrombotic effects during *in vivo* applications (137). Following intranasal administration of the optimized platelet lysate, obvious protective effects were observed on dopaminergic neurons in the substantia nigra and the striatum of PD model mice (137). The intranasally administered platelet factors were also found in several other regions of the brain, including the striatum, olfactory bulb, and cortex (137), making this treatment method a promising tool for application in various neurodegenerative conditions.

Although we have not addressed stroke and other brain injuries in this review, human platelet lysate treatment has also been shown to produce positive outcomes in these conditions. Following stroke, human platelet lysate injections into the lateral ventricles of rats had neuroprotective effects (154). The platelet lysate-treated rats exhibited a larger number of proliferating neural precursor cells in the subventricular zone, accompanied by increased angiogenesis (151). They also displayed lower motor function deficits (154). Another study demonstrated that administrating human platelet lysate decreased apoptosis and stimulated the survival of proliferating neural precursor cells in the same brain region after a lysolecithin-induced demyelination lesion in the corpus callosum (12), further suggesting a neuroprotective role of platelets after cerebral damage.

Platelets and Platelet Microparticles—Potential Vehicles for the Delivery of Therapeutic Drugs?

In addition to platelet-rich plasma and platelet lysate preparations, an interesting approach is emerging, whereby platelets are used as a physiological vehicle to deliver molecules to target regions that might otherwise be difficult to access. With their context-dependent and specific cell-cell communication capacity, platelets could serve as a selective and non-toxic drug delivery system in order to target specific cells and tissues. This approach has been extensively discussed previously, with a particular focus on the use of platelets to deliver chemotherapeutic agents to tumors (155). However, this novel strategy still requires additional studies to confirm its efficacy. Microparticles, which are released by platelets upon activation, have also been proposed as a natural delivery system for drugs (155, 156). The majority of all microvesicles in the blood are platelet-derived (157), indicating a vital contribution of platelets to intercellular communication. Platelet microparticles, which are 0.1–1 μm in diameter, are shed from the plasma membrane (158) and contain cytoplasm, microRNA, mRNA, lipids and proteins. These can be transferred to other cells, thereby affecting their function (159–162). Given their capacity to influence and communicate with neural cells, platelets and their secreted microparticles could also be engineered as drug carriers for the treatment of neurodegenerative disorders. However, until the exact mechanisms of the specific cell-cell communication between platelets and brain cells are fully understood, the value of this approach remains speculative. Furthermore, in order to develop human therapies with drug-loaded blood cells, extensive studies are needed to establish clinical grade protocols which standardize the varying methods of isolation and storage of platelets and platelet microparticles prior to their regulated reintroduction into individuals. Drug loading protocols for these natural vehicles, in terms of their capacity and compatibility with the drugs required to target neurodegenerative phenotypes, also need to be established. Nonetheless, in the field of regenerative medicine, considerable headway has already been made toward engineering extracellular vesicles and blood cell-inspired nanoparticles for therapeutic use (163–166).

CONCLUSION

As summarized in this review, data connecting platelets and the factors they secrete to neurodegeneration have accumulated over recent years. However, it remains unclear whether platelet malfunction initiates the pathophysiological events that occur in neurodegenerative conditions, or whether platelet dysfunction arises as a consequence of other unfavorable changes that occur at early stages of these disorders. More data regarding the origin of platelet dysfunction are therefore required. During the onset of neurodegenerative conditions, factors released from healthy platelets could also have a protective role, as suggested by recent studies of AD (92) and cancer, where platelets initially suppress tumor angiogenesis (167). Moreover, platelets exhibit a sophisticated endocytic machinery (168) via which they could collect products that are released into the blood from other malfunctioning cells in an attempt to clear the systemic environment of cytotoxic components in the early stages of disease.

Although platelets and their released factors are gaining recognition for their potential therapeutic value in regenerative medicine, research is still in its infancy. Furthermore, the origin of platelets, the bone marrow, should not be overlooked, as a functional predisposition may also be inherited from their parent cells, the megakaryocytes. In conclusion, it remains highly interesting, but at the same time extremely challenging, to understand how platelets exert manifold actions across different tissues in physiological as well as pathological conditions.

Their functional complexity clearly demands interdisciplinary approaches in order to develop novel therapeutic interventions which benefit from the multifaceted nature of platelets, including their capacity to facilitate crosstalk between the systemic environment and the brain.

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Platelet and Plasma Phosphatidylcholines as Biomarkers to Diagnose Cerebral Amyloid Angiopathy

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Alzheimer's disease is a severe neurodegenerative brain disorder and characterized by deposition of extracellular toxic β-amyloid (42) plaques and the formation of intracellular tau neurofibrillary tangles. In addition, β-amyloid peptide deposits are found in the walls of small to medium blood vessels termed cerebral amyloid angiopathy (CAA). However, the pathogenesis of CAA appears to differ from that of senile plaques in several aspects. The aim of the present study was to analyze different lipids [phosphatidylcholines (PCs) and lysoPCs] in platelets and plasma of a novel mouse model of sporadic CAA (1). Our data show that lipids are significantly altered in plasma of the CAA mice. Levels of eight diacyl PCs, two acyl-alkyl PCs, and five lysoPCs were significantly increased. In extracts of mouse blood platelets, four diacyl and two acyl-alkyl PCs (but not lysoPCs) were significantly altered. Our data show that lipids are changed in CAA with a specific pattern, and we provide for the first time evidence that selected platelet and plasma PCs may help to characterize CAA.

Keywords: Alzheimer's disease, cerebral amyloid angiopathy, platelets, biomarker, metabolomics, lipids, mouse model

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INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder in the brain. The major hallmarks are extracellular β-amyloid (Aβ) plaques, intracellular tau neurofibrillary tangles, cholinergic neurodegeneration, and cerebrovascular damage (2). In many cases, a comorbid cerebral amyloid angiopathy (CAA) is found, which is characterized by the accumulation of $A\beta_{40}$ in the vessels. However, CAA is also present as an independent pathology without AD and can be characterized as a subform of vascular dementia (vaD) (3). The origin of AD, vaD, and CAA is still not fully understood. Approximately 95% of all CAA cases are sporadic, and only 5% are explained by a genetic background (2). More and more research suggests that vascular risk factors may play a role in the development of both sporadic CAA and AD (3–6).

At present, the diagnosis of AD and/or CAA combines psychological tests, brain imaging, and the analysis of four biomarkers ($A\beta_{40}$, $A\beta_{42}$, total tau, and phospho-tau-181) in the cerebrospinal fluid (CSF). For CAA especially, $A\beta_{40}$ levels in CSF are investigated, as the values are lower in CAA compared to controls and AD patients (7). Furthermore, for CAA, the Boston criteria are used, which classify "definite CAA" in autopsy postmortem, "possible CAA" with brain imaging

combined with clinical exclusions, and "probable and possible CAA" by tissue biopsy or the detection of multiple intracerebral hemorrhages (8, 9). However, the problem arises that the complete procedure is time consuming, and the collection of CSF is an invasive procedure. Moreover, in CSF samples, it is difficult to distinguish between AD and CAA or mixed forms of dementia (10, 11). Thus, the search for blood/plasma biomarkers is of great interest, as those biomarkers may offer a fast and non-invasive diagnostic method. In addition, platelets are of special interest, as they contain a high amount of the amyloid precursor protein (APP) and release approximately 90% of peripheral Aβ (mainly $A\beta_{40}$) (12, 13). Recently, we and others have shown that lipidomics may offer a promising tool to diagnose AD not only in CSF (14), in plasma (15, 16), or in platelets (17) but also in saliva (18).

Animal models are of great interest in studying mechanisms and potential treatments for CAA. In the last years, transgenic mouse models expressing the human APP have been developed. Many of these mouse models develop CAA in addition to senile plaques (19). Recently, we have generated a mouse model to study specifically CAA pathogenesis (1). Mice exposed to five vascular risk factors (hypercholesterolemia, copper in the drinking water, diabetes, inflammation, and social stress) showed drastic vessel pathology, cognitive decline, vascular bleedings, and the deposition of Aβ in the vessels (**Figure 1**) (1). A comparison in the expression pattern of lipids between the mouse model and AD patients can provide important information about the pathogenesis of CAA. We will use a well-established metabolomic lipid platform to identify changes in phosphatidylcholines (PCs), lysoPCs and sphingomyelins in plasma and platelet extracts. Specific changes in the lipid expression pattern can be helpful for diagnosis, course of disease, and treatment. Thus, in the present study, we aimed to measure plasma and platelet lipids in this CAA mouse model to differentiate and identify CAA-specific biomarkers.

METHODS

Collection of Plasma and Platelets From Sporadic CAA Mouse Model

Sporadic CAA mice ($n = 8$) were generated as described in detail by us (1). All animal experiments were approved by the Austrian Ministry of Science and Research (BMWF-66.011/011_WF/V/3b/2015) and conformed to the Austrian guidelines on animal welfare and experimentation. Briefly, 5-month-old wild-type mice (C57BL/6N) were either not treated (controls) or treated with vascular risk factors for 35 or 56 weeks (**Figure 1**). Vascular risk factors contained 2% cholesterol food, copper in the drinking water (1 mg/L), streptozotocin (to induce diabetes; maximum dose 50 mg/kg), lipopolysaccharides (to induce inflammation; 1.25 mg/kg), and social stress (induced by changing the cage partners). Mice were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine. Blood was taken directly from the heart and collected in EDTA tubes. Subsequently, the blood was centrifuged (10 min, 100 × *g*) at room temperature (RT) to obtain platelet-rich plasma (PRP). The supernatant was taken, and 500 nM prostaglandin (PGI2; Sigma, Vienna, Austria) was added. Plasma was centrifuged again (10 min, 400 × *g*, RT) to isolate platelets from PRP, and then the plasma supernatant and platelet pellets were frozen at −80°C until use.

FACS Analysis

Fluorescence Activated Cell Sorting (FACS) analysis was performed as reported by us previously in detail (20). Immediately after isolation, two microliter mouse platelets were incubated with antibodies against immunoglobulin G1 (IgG1)–fluorescein isothiocyanate (FITC) (BD 555748) or CD41/CD61-FITC (LeoF2, Emfret M025-1) or CD42a-FITC (XiaB4, Emfret M051-1) or CD42b-FITC (XiaB2, Emfret M043-1) in 50 μL FACS buffer [2 mM EDTA, 0.5% FCS ad 100 mL phosphate-buffered saline (PBS), pH 7.1] for 30 min at 4°C in dark. All samples were centrifuged at 300 × *g* for 10 min, and the pellets were resuspended in 100 μL of FACSFlow (BD FACSFlow, Erembodegem, Aalst, Belgium). FACS analysis was instantly performed with a BD FACScan.

Targeted Metabolomic Analysis of Plasma and Platelets

The endogenous metabolites were analyzed with a targeted quantitatively and qualitatively controlled metabolomics assay by using the AbsoluteIDQ p150 Kit (Biocrates Life Science AG, Innsbruck, Austria). This validated assay allows the quantification and comprehensive identification of 163 endogenous metabolites including among others 77 PCs (PC aa = diacyl x:y; PC ae = acylalkyl x:y) and 40 acylcarnitines (Cx:y). The AbsoluteIDQ p150 Kit was performed according to the manufacturer's instructions as reported by us (16, 17). In short, 10 μL of sample mixture was pipetted onto filter spots suspended in the wells of a 96-well filter plate. The filter plate was fixed on top of a deep-well plate serving as a receiving plate for the extract later on, that is, a combi-plate structure. After drying under a nitrogen stream for 30 min, 50 μL of a 5% phenylisothiocyanate solution was added to enable derivatization of amino acids. After 20 min of shaking and nitrogen drying, 300 μL of 5 mM ammonium acetate in methanol was added to the wells. After 30 min of incubation, the combi-plate was centrifuged to move the extracts into the lower receiving deep-well plate, which was then detached from the upper filter plate. After adding another 300 μL of 5 mM ammonium acetate in methanol to the extracts and briefly shaking, the plate was placed in the autosampler of the flow injection analysis (FIA)–tandem mass spectrometry (MS/MS) system for analysis. The FIA-MS/MS system consisted of a Knauer K-1001 LC pump (Knauer, Berlin, Germany), a CTC-PAL HTS9 autosampler (CTC Analytics AG, Zwingen, Switzerland), and a QTrap 3200 mass spectrometer (Sciex, Toronto, Ontario, Canada). The injection volume was 30 μL. The flow rate was set to 30 μL/min. Metabolite concentrations (μM) were automatically calculated by the MetIDQ software package part of the AbsoluteIDQ p150Kit.

Western Blot Analysis

Western blot analysis was performed as previously described by us (21). Platelet samples (−80°C) were thawed and tubes dissolved in 100 μL ice-cold PBS containing a protease inhibitor

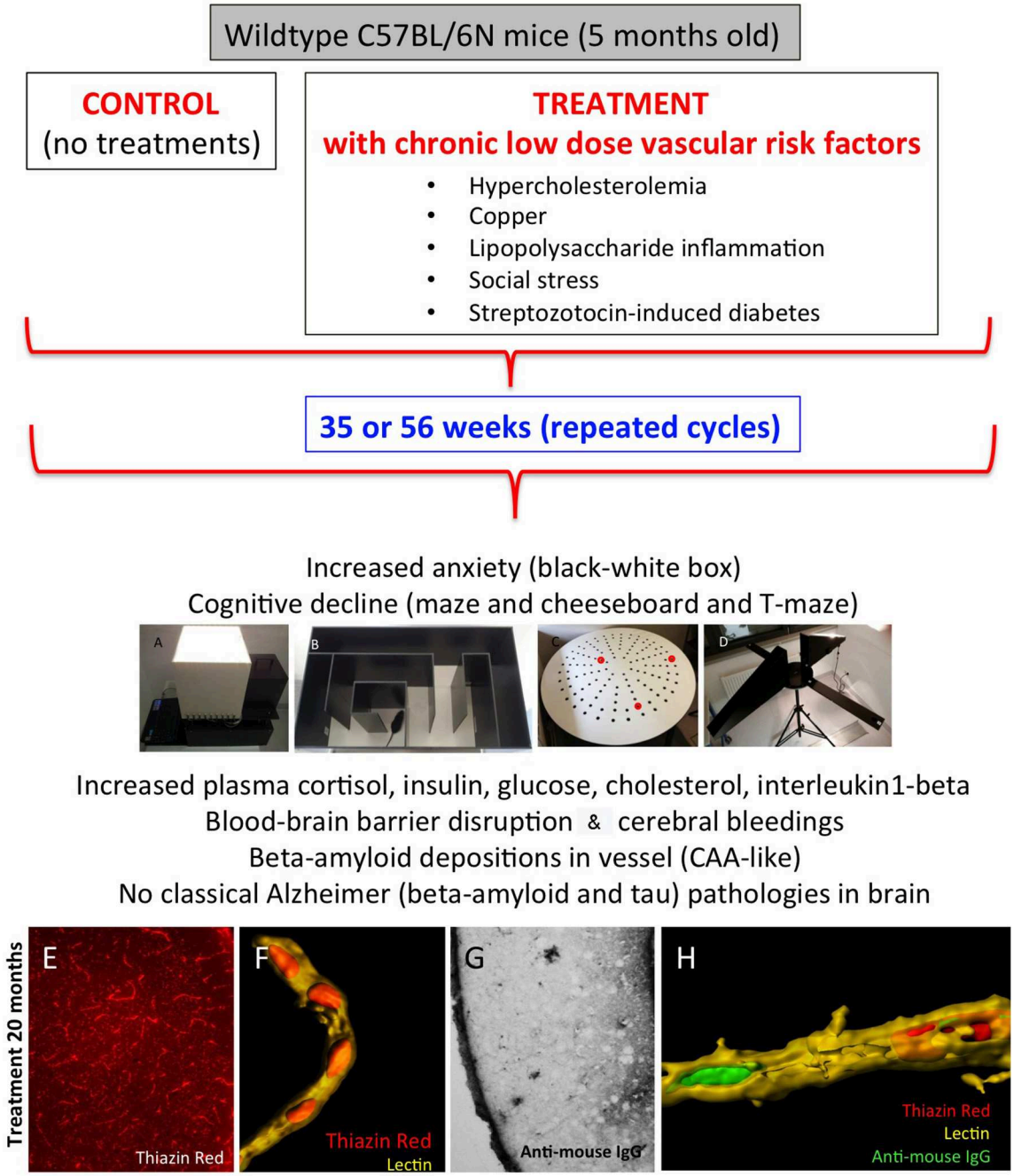

FIGURE 1 | Characterization of the novel sporadic mouse model of cerebral amyloid angiopathy (CAA). Healthy 5-month-old C57BL/6N mice were treated for 35 or 56 weeks with different low-dose vascular risk factors (treatments) or without (controls). The behavior was tested all 6 weeks using a black–white box **(A)**, labyrinth maze **(B)**, cheeseboard maze **(C)**, and at the end of the experiment with a T-maze **(D)**. Mice were analyzed for plasma levels and brain vessels and AD-like pathologies. Note that these mice exhibit a severe pathology for CAA, as seen by thiazine red staining **(E)** in lectin + vessels **(F)** and blood–brain barrier disruptions as seen by anti–mouse IgG staining **(G)**. **(H)** a representative confocal microscopy staining of a lectin + vessel (yellow) with thiazine red inclusions (red) and anti–mouse IgG (green). For methodological details and details on the model, refer to our original publication (1).

cocktail (P-8340; Sigma). Samples were then sonicated using an ultrasonic device, centrifuged at 14,000 × *g* for 10 min at 4°C; the extracts were denatured (10 min, 70°C), and 18 µg was loaded onto 10% *bis–tris* SDS-polyacrylamide gels (Thermo Fisher Scientific, Vienna, Austria), separated for 35 min at 200 V and finally electrotransferred to nylon-PVDF Immobilon-PSQ membranes for 20 min at 30 V in 20% methanol blotting buffer. Next, blots were blocked for 30 min in blocking buffer; incubated

TABLE 1 | Plasma levels of selected lipids altered in CAA mice.

		Controls (56 weeks)	Sporadic CAA (56 weeks)	*p*-value
n		8	8	
lysoPC	C16:0	138 ± 5	193 ± 11	0.0005***
	C16:1	4 ± 0	6 ± 0	0.001***
	C18:0	67 ± 3	120 ± 13	0.001***
	C18:1	34 ± 2	58 ± 5	0.0004***
	C20:4	40 ± 3	69 ± 8	0.006**
PCaa	C30:2	2 ± 0	4 ± 0	0.002**
	C34:1	54 ± 5	74 ± 4	0.01**
	C36:1	11 ± 1	19 ± 1	0.0003***
	C38:4	67 ± 4	118 ± 13	0.003**
	C38:5	21 ± 2	31 ± 2	0.002**
	C38:6	58 ± 6	86 ± 4	0.002**
	C40:4	0.9 ± 0.08	1.4 ± 0.1	0.002**
	C40:6	20 ± 1	38 ± 5	0.002**
PCae	C38:0	1 ± 0	2 ± 0	0.01**
	C40:4	1.03 ± 0.08	1.5 ± 0.1	0.005**

*Significantly altered lipids [phosphatidylcholines (PCs)] in plasma of mice with sporadic CAA (treated for 56 weeks) compared to same age-matched controls. Values are given as mean ± SEM (in μM); n represents the number of animals or patients per group. Statistical analysis was performed with ANOVA with a Fisher LSD post-hoc test (**p < 0.01; ***p < 0.001). PCaa, diacyl x:y; PCae, acylalkyl x:y.*

TABLE 2 | Platelet levels of selected lipids altered in CAA mice.

		Controls (56 weeks)	Sporadic CAA (56 weeks)	*p*-value
n		8	8	
PCaa	C36:5	0.02 ± 0.005	0.06 ± 0.01	0.01**
	C38:0	0.17 ± 0.01	0.08 ± 0.009	0.0002***
	C38:5	0.09 ± 0.03	0.3 ± 0.06	0.003**
	C38:6	0.14 ± 0.04	0.48 ± 0.11	0.01**
PCae	C36:1	0.14 ± 0.008	0.07 ± 0.01	0.0002***
	C40:2	0.05 ± 0.004	0.02 ± 0.0003	0.0001***

*Significantly altered lipids [phosphatidylcholines (PCs)] in isolated platelets of mice with sporadic CAA (treated for 56 weeks) compared to the same age-matched controls. Values are given as mean ± SEM (in μM per 1 mg platelets); the n represents the number of animals or patients per group. Statistical analysis was performed with ANOVA with a Fisher LSD post-hoc test (**p < 0.01; ***p < 0.001). PCaa, diacyl x:y; PCae, acylalkyl x:y.*

with primary antibody against APP (Abcam ab32136, 1:2,000, Cambridge, UK), or CD41 (Abcam ab63323, 1:2,000), or actin (1:1,000, A2066; Sigma, Vienna, Austria) at 4°C overnight; washed; and then incubated in alkaline phosphatase–conjugated anti–rabbit IgG for 30 min. After washing, bound antibodies were detected using an enhanced chemiluminescence system and visualized by using a cooled CCD camera (SearchLight; Thermo Fisher Scientific).

Statistical Analysis

Statistical analysis was performed with analysis of variance (ANOVA) and a subsequent Fisher least significant difference (LSD) *post-hoc* test and comparing controls vs. treatments. Statistical results were considered significant at $p < 0.05$.

RESULTS

Lipids in Plasma of CAA Mice

Approximately 100 lipids were determined in the plasma of well-characterized CAA mice and compared to control mice (**Table 1**). Levels of eight aaPCs (PCaaC30:2, PCaaC34:1, PCaaC36:1, PCaaC38:4, PCaaC38:5, PCaaC38:6, PCaaC40:4, PCaaC40:6) and two aePCs (PCaeC38:0, PCaeC40:4) were significantly elevated. Five lysoPCs were significantly enhanced compared to the controls (lysoPC C16:0, lysoPC C16:1, lysoPC C18:0, lysoPC C18:1, lysoPC C20:4).

Lipids in Platelets of CAA Mice

Approximately 100 lipids were determined in the platelets of well-characterized CAA mice and compared to control mice (**Table 2**). Platelets were evaluated by FACS analysis for CD41/61, CD42a, and CD42b (**Figure 2A**). Levels of six PCs (PCaaC36:5, PCaaC38:0, PCaaC38:5, PCaaC38:6, PCaeC36:1, PCaeC40:2) were significantly changed in platelet extracts taken from 56 week-old sporadic CAA mice, compared to age-matched controls (**Table 2**). As a positive control, APP was analyzed in isolated platelets and compared to CD41 and actin (**Figure 2B**). Western blot analysis showed a 130-kDa APP protein, and its expression was significantly higher in the CAA mice compared to the controls (**Figure 2C**), both in the 35 and 56 week treatment groups.

DISCUSSION

In the present study, we examined lipid levels in the plasma and platelets taken from a well-characterized novel mouse model of sporadic CAA. Our data show that 15 plasma lipids and 6 platelet lipids may help to characterize CAA.

Pathology of Sporadic CAA

We recently characterized a novel mouse model of sporadic pure CAA pathology without AD pathology (**Figure 1**) (1). In the human form, CAA mostly comes along with a concurrent AD pathology, and the general problem arises that the two pathologies cannot be discriminated easily. Because CAA is a disease of the elderly, it overlaps with a variety of cardiovascular risk factors (e.g., hypertension, diabetes, hypercholesterolemia) that could contribute to CAA pathology. However, there is a strong suggestion that platelets contribute to the onset of CAA and AD (22). Specifically, in CAA dysfunctional platelets may play a crucial role. We hypothesize that due to lesions in brain vessels occurring over years, it also comes to a blood–brain barrier disruption and furthermore to vascular bleedings. These bleedings can cause dysfunction and overactivation of platelets. Subsequently, a production and release of Aβ and deposition of $A\beta_{40}$ and possibly $A\beta_{42}$ may cause CAA (22). Therefore, it is supposed that platelets and a preceding CAA pathology may be the first stage before progression to the AD pathology, and platelets may serve as putative biomarkers.

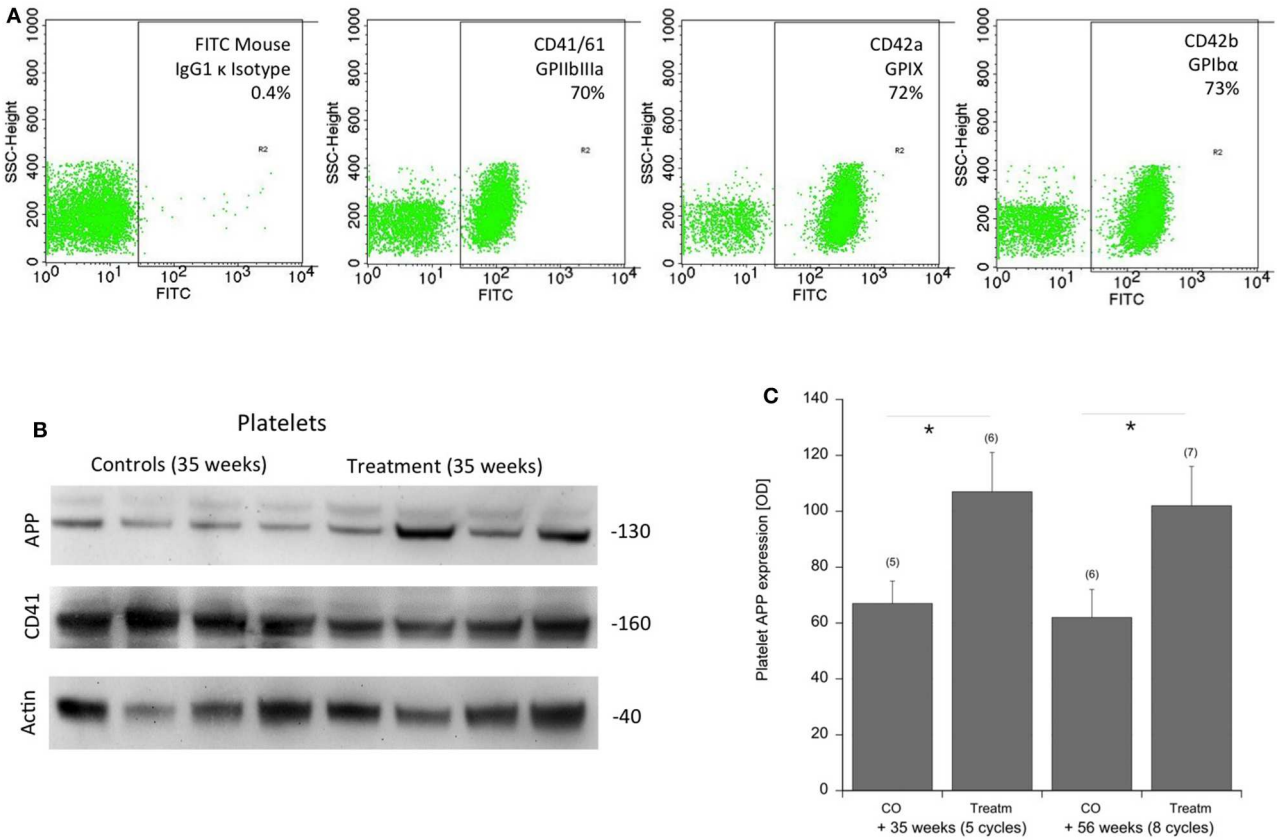

FIGURE 2 | Characterization of platelets by FACS and APP expression in the mouse CAA model. **(A)** Platelets were characterized by FACS analysis for CD41/61 GPIIbIIIa, CD42a GPIX, CD42b GPIbα compared to an IgG control. **(B)** Amyloid precursor protein (APP) was measured by Western blots and showed a 130-kDa protein. Actin and CD41 served as controls. Size markers are given as kDa. **(C)** Statistical analysis was performed by ANOVA with a subsequent Fisher LSD *post-hoc* test (*$p < 0.05$). Note an increased expression of APP in platelets of sporadic CAA mice in both treatment groups at 35 and 56 weeks of age (Treatm) compared to age-matched controls (CO). Values are given as mean ± SEM optical density. Numbers in parenthesis show numbers of animals.

Plasma Lipids

Recent data suggest lipid measures in plasma may produce detectable signatures in AD patients. A groundbreaking work has been published some years ago, where it has been postulated that a set of 10 lipids (C3, lysoPCaC18:2, PCaaC36:6, C16:1-OH, PCaaC38:0, PCaaC38:6, PCaaC40:1, PCaaC40:2, PCaaC40:6, and PCaeC40:6) from peripheral blood predicted the conversion to mild cognitive impairment (MCI) or AD within a 2 to 3 year timeframe with >90% accuracy (15). However, this study was very enthusiastic, and so far, the pattern of these 10 lipids could not be reproduced from other laboratories, and also this set did not go into routine analysis. We ourselves could not find the same pattern of lipids but found that the ratio of PCs to lysoPCs (PCaaC34:4 and lysoPCaC18:2) in plasma differentiated healthy controls from patients with AD and MCI (16). Very recently, a study showed that three serum lipids [SM(OH)C24:1, SMC24:0 and PCaeC44:3] differentiated MCI and early-stage AD patients (23). So far, no data have been published for lipids in CAA. Our data show for the first time that in a novel mouse model of sporadic CAA 15 plasma lipid metabolites are altered, which may distinguish CAA from the human AD pathology.

Platelet Lipids and APP

It has been well-established that platelets play a major role in the progression of AD and CAA (22). Indeed, we and others have shown that platelets from an AD mouse brain are able to damage healthy brain vessels (20). It is well-known that platelets release Aβ into the blood, where it may play a role in blood clotting (24). Interestingly, platelets contain a high amount of APP, and it has been shown that the APP expression is altered in AD patients (25, 26). However, so far, these findings have never entered routine analysis, as the APP expression and AD pathology are very heterogeneous with a high variance. In the present study, we confirm the expression of APP in platelets but show that the APP expression is also altered in a sporadic CAA mouse model. While this is an important control experiment to show the physiological role of platelets, it also shows for the first time that platelet APP is altered in CAA without any AD pathology. This also further strengthens the hypothesis that platelet pathology contributes to vessel damage and subsequent deposition of plaques and AD progression (22).

In a previous study, we have already demonstrated that the lipid metabolism is altered in platelets of MCI and AD patients

(17). We showed that soluble platelet PCaeC40:4 can be used as a marker for AD in platelets (17). However, it was very difficult to diagnose pure forms of CAA in humans. Thus, by generation of this mouse model of pure CAA, we are in a position to characterize the pattern of the platelet lipidom and to directly point to putative biomarkers in human CAA. Our data suggest that the lipidomic examination of platelets may allow diagnosing early changes of CAA. As it is very difficult to diagnose pure CAA in humans, our data may provide a differential diagnostic pattern, which could be useful to differentiate CAA in humans. The pathophysiology of CAA in humans is a complex process that is triggered by various risk factors, including aging, hyperlipidemia, hypertension, and diabetes. Thus, our CAA mouse model mimics this aspect of AD pathophysiology. Nevertheless, mice also display major genetic and physiological differences compared to humans.

Limitations of the Study

Definitely, this study had some limitations. (a) A limitation of our mouse model is that despite developing specific changes, they do not progress to advanced stages that are observed in humans. Consequently, no spontaneous plaque rupture is observed in our mouse model. Furthermore, one evident difference between mice and humans resides in the lipoprotein metabolism. Mice are considered as a high-density lipoprotein (HDL) model because most of the cholesterol is transported in HDL particles, and not in low-density lipoprotein (LDL) as in humans. However, a recent study showed that the mouse exhibits protein diversity across the LDL and HDL size ranges that are generally similar to those in humans (27). (b) The question arises how mouse models correlate with humans. In neurobiology research, we all rely on animal models to study human diseases. Transgenic mouse models are well-known AD models, but do not exhibit a sporadic origin (1). No mouse models for sporadic AD have yet been reported. We (1) recently developed a mouse model of sporadic CAA without any AD pathology, which mimics some aspects of CAA. (c) As mentioned, all these models only partly reflect a full pathology of a disease. In our model, we exposed wild-type mice chronically to five vascular risk factors, which caused a vessel pathology. But definitely, we do not know which risk factors cause CAA in humans, and again our model only partly reflects a human-related CAA.

CONCLUSION

Taken together, our results show that 15 plasma lipids and 6 platelet lipids may help to characterize CAA. Our model provides the basis for further studies in humans, where the results can be compared and pathophysiological changes in the plasma and platelets could be a useful tool for the early diagnosis of CAA.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Austrian Ministry of Science.

AUTHOR CONTRIBUTIONS

BF developed the sporadic CAA mouse model, isolated and analyzed platelets, evaluated all data, and wrote the manuscript. HO performed the lipidomic analysis. JM designed the study and wrote the MS. CH financed the study, designed the project, and wrote the manuscript.

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REVIEW
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On the Role of Platelet-Generated Amyloid Beta Peptides in Certain Amyloidosis Health Complications

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As do many other immunity-related blood cells, platelets release antimicrobial peptides that kill bacteria, fungi, and even certain viruses. Here we review the literature suggesting that there is a similarity between the antimicrobials released by other blood cells and the amyloid-related Aβ peptide released by platelets. Analyzing the literature, we also propose that platelet-generated Aβ amyloidosis may be more common than currently recognized. This systemic Aβ from a platelet source may participate in various forms of amyloidosis in pathologies ranging from brain cancer, glaucoma, skin Aβ accumulation, and preeclampsia to Alzheimer's disease and late-stage Parkinson's disease. We also discuss the advantages and disadvantages of specific animal models for studying platelet-related Aβ. This field is undergoing rapid change, as it evaluates competing ideas in the light of new experimental observations. We summarized both in order to clarify the role of platelet-generated Aβ peptides in amyloidosis-related health disorders, which may be helpful to researchers interested in this growing area of investigation.

Keywords: amyloid-beta, platelets, Alzheimer's disease, natural antibiotics, animal models

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INTRODUCTION

Amyloidosis represents a diverse group of diseases characterized by the common factor of deposition of twisted β-pleated sheet fibrils (amyloid) and their aggregates. The β-pleated sheet itself is not abnormal; it is a common motif, usually conserved across species, and a standard secondary structure in proteins, allowing different protein strands (subunits) of a functioning protein to be joined together with hydrogen bonds. The β-pleated sheet forms the basis for uniting subunits in many enzymes and immunoglobulins, as well as channel-forming subunits of specific ion channels and pores. Formation of β-pleated sheet hydrogen bonds between two or more parallel protein strands requires standard spacing between amino acids in these parallel polypeptides. It also requires correct subunit assembly and the right organization of the process (1). Unfortunately, this bond formation between parallel chains may occur pathologically because of mutations augmenting the binding propensity of particular polypeptides or the elevated concentration or overproduction of specific peptide chains, allowing the formation of polymeric ß-pleated sheets consisting mainly of multiple copies of the same type of chain. This interaction causes the proteins to form misfolded pathologic polymers, usually fibrils and aggregates, in a process called amyloidosis. The different forms of amyloidosis are classified by the composition of the amyloid fibrils and the manner of their

deposition, which may be local or systemic. In amyloid light-chain (AL) amyloidosis (also known as primary amyloidosis, as it is the most common form), the free light chain of the immunoglobulin molecule (termed in clinical practice the Bence Jones protein) is hyper-secreted by lymphocyte cells in blood plasma. In many cases, it is linked to cancer) (2, 3). While in the immunoglobulin fold, on which the β-sheet formation is healthy, the high concentration of only the light chain makes this process abnormal (2, 4). The accumulation of AL amyloid, which can be local or systemic, disrupts the tissue architecture and, in conjunction with a toxic effect from the oligomeric light chains (5), leads to severe organ damage that may involve the kidneys, heart, liver, peripheral nerves, and even bones. Systemic amyloidosis (which can be of the senile type or an early-onset familial type) is the result of the deposition of transthyretin (TTR) protein. TTR is a serum and cerebrospinal fluid carrier known for its transport of retinol, the thyroid pre-hormone thyroxine (T4), and also some peptides. It usually circulates as a homo-tetramer, but, due to genetic mutation, tetramers can dissociate into monomers that then misassemble into amyloid fibrils (6). In their senile form, TTR monomers become fragmented and mix with full-size monomers, leading to misfolded aggregates (7). Reactive systemic amyloidosis is the result of an overproduction of a non-immunoglobulin protein, AA, which is associated with blood serum. There can also be amyloidosis related to the overproduction of β2 microglobulin (B2M amyloidosis), a free protein with an antibacterial activity that is a light chain of the major histocompatibility complex protein (8). The production of amyloidogenic proteins in all the abovementioned forms of amyloidosis directly originates in blood cells or is related to blood plasma. Generally speaking, the depositions, in many cases, spread from the blood to inside the organs, with the highest concentration around blood vessels. In previously described types of amyloidosis, blood vessel damage is also common (9, 10).

Alzheimer's disease (AD) is the only well-known form of severe amyloidosis in which the amyloidogenic peptide is believed to be produced in organ tissue and not systemically in blood plasma. The main component of amyloid fibrils and other amyloid aggregates in AD are the amyloid beta (Aβ) peptides. Another common component of these aggregates is the amyloid P component (AP), a normal blood plasma constituent (11) produced by the liver, and its concentration in blood plasma has been shown to be about five-fold elevated in AD (12).

This exclusive association of brain tissue with the production of materials that form plaques in AD may be explained historically. Amyloid cerebrovascular senile plaques were described by Dr. Alois Alzheimer in the brain of dementia patients a century ago, and it was found later that these plaques contain Aβ peptides (13), while both neurons and astrocytes can produce these peptides (14). In addition, animal models that used neuron-associated promoters to generate the aggregation-prone mutated Aβ had shown many similarities in morphology and pathophysiology with the brains of AD patients [for review see: (15)]. This mechanism was therefore extrapolated to late-onset AD. Recently, multiple findings have emerged suggesting that there may be a flow of Aβ from blood to the brain in AD in which platelets are vital players [reviewed in (16)]. Platelets were also suggested as the most important source of Aβ in glaucoma [reviewed in (17)]. In this review, we used the Web of Science, PubMed, and Google patent databases to search for studies examining the role of systemic release of Aβ in a variety of health complications that exhibit Aβ accumulation of oligomers or plaque deposition. We ask related questions that have not been discussed in previous reviews and discuss the advantages and disadvantages of existing animal models for studying platelet-related Aβ in AD and other diseases.

Aβ PEPTIDE ACCUMULATION IS ASSOCIATED WITH A VARIETY OF DISEASES

Aβ peptides may be of varying length (<46 amino acids) and have a specific sequence, which differs only slightly across mammalian species (18). Due to hydrogen bonding between the parallel monomers, Aβ peptides are prone to form dimeric, tetrameric, or higher-order oligomers, even at very low concentrations (μm range), while at higher concentrations they associate into filaments that tend to join in misfolded aggregations known as amyloid plaques (19–21). The presence of Aβ extracellular plaques suggest that the concentrations of Aβ are elevated in the affected tissues. However, Aβ aggregation can start at lower concentrations due to specific mutations within Aβ and its precursor. For example, such mutations are the basis of hereditary early-onset familial AD (22). Aβ peptides of different lengths also have different propensities to aggregate (15), and the amyloidogenic properties of Aβ peptides from humans and other mammals may be different. For example, the propensity of murine Aβ to produce insoluble amyloid aggregations is limited (23), (also see below), and the majority of murine transgenic AD models involve the expression of mutated human Aβ.

However, besides AD, a variety of health problems have, as a common component, the accumulation of Aβ in tissues at elevated concentrations, sometimes leading to its aggregation. It was discovered that plasma levels of Aβ peptides in pancreatic, as well as in esophageal, colorectal, hepatic, and lung cancer patients were significantly higher than in healthy controls (24, 25). In glioblastoma, Aβ was found in both oligomeric and aggregated forms to be associated with glioma cells as well as localized in the tumor extracellular space, and it was proposed that blood could be the source of this peptide (26, 27). It was shown that platelets are activated near cancer tumors, playing the role of "first responders" during cancer development and metastasis (28).

Aβ is elevated near blood vessels and forms transient amyloid plaques in the zone of traumatic brain injury or stroke (29–35). It was proposed that Aβ accumulation in astrocytes and on blood vessel walls is related to ischemia in these processes, while both brain cells (30) and platelets (35–38) can be the source of Aβ. Using immunocytochemistry, we detected a massive release of

Aβ peptides in and around blood vessels in the brain and skin after experimental thrombosis, and we determined the source of these peptides to be platelets (39, 40). Interestingly, according to evidence in the literature, murine Aβ deposits are transient after traumatic brain injury, while in humans, they are relatively stable.

Aβ peptides also accumulate in the myocardium with ischemic heart failure, while circulating levels of Aβ are predictive of cardiovascular mortality in patients with coronary heart disease (41, 42). The sources of Aβ involved in this process are still not known, but we propose that Aβ generated from a platelet precursor could be at least one of these sources.

Aβ (and other amyloidogenic proteins) also accumulate in the placenta during preeclampsia, a leading contributor to maternal and perinatal morbidity and mortality worldwide. There are malformations to placental blood vessels in this condition. The attempt of the body to compensate these malformations probably leads to extremely high blood pressure. This induces vessel damage and inflammation in the placenta, leading to local amyloid accumulation, including Aβ (43). This condition usually produces hemolysis and affects blood composition (44).

During glaucoma, Aβ accumulates in the retina, mainly within the layer of apoptotic retinal ganglion cells (RGC) near the region of microvascular changes in the eye. During this disease, the rearrangement of damaged blood vessels occurs in the zone of the entrance of blood vessels and the optic nerve into the retina, producing anatomic changes, termed cupping. Aβ released in this area thus may be the cause of retinal cell death, previously associated only with the effects of high intraocular pressure (17, 44–46). It was found that application of synthetic Aβ induces significant RGC apoptosis *in vivo*, while anti-Aβ treatment was effective in the prevention of RGC apoptosis in glaucoma patients (17, 47–51). Additionally, some anti-glaucoma medicines have apparent anti-platelet effects, suggesting that platelets participate in glaucoma development (52).

Also, accumulation of Aβ is evident in the advanced stages of Parkinson's disease (PD) (53–55). While PD motor impairment, which develops due to α-synucleinopathy and dopamine deficiency, is devastating, later progressive cognitive impairment and dementia (PDD) eventually become the major debilitating symptoms for 80% of PD patients, and these have no cure (54, 56). From the early stages, after α-synucleinopathy advances in PD patients, Aβ becomes visible in the brain as well (57), and after 20 years approximately 50% of PDD patients develop extensive neuropathologies similar to AD. These include misfolded Aβ plaques and tau neurofibrillary tangles, mainly in the frontal cortex and striatum (58, 59), while the scale of Aβ-produced damage and its effects on PDD development are still being debated (54, 55, 57, 60–62). It was also found that there is an accumulation of insoluble Aβ around blood vessels (cerebral amyloid angiopathy, CAA) in 53% of PD patients (63). In sporadic AD, striatal depositions are rare (but common in early-onset AD, (64), while they are predominant in PD and PDD. Although the striatum and frontal cortex are the zones of massive degeneration of the neuronal processes of dopamine neurons as well as inflammation in PD (65), it is still difficult to differentiate the role of Aβ in "pure" AD from PD with Aβ depositions and to determine the source of these depositions in PD.

Here it should be remarked that, while it is known that Aβ peptides in humans can be of different lengths, with different properties, reported measurements of the Aβ40/Aβ42 ratio in many pathologies (except AD) are unfortunately rare, and we will not discuss this issue here. Moreover, the buildup of extracellular plaques due to Aβ aggregation occurs in brain tissue, in the vicinity of skin blood vessels, or in peripheral blood vessels in internal organs (40, 66). Most likely, it is related to the difference in blood vessel wall structure in these areas and in other parts of the body. It is known that brain blood vessels and peripheral blood vessels have a size barrier formed by the inter-endothelial junctions (IEJs) between endothelial cells (67, 68). This junction barrier defines paracellular permeability, not allowing phagocytes to enter the nearby tissue and producing a "no-cleanup" zone in brain and around peripheral blood vessels, shifting the balance between accumulation and removal of extracellular plaques.

There are other health conditions in which the occurrence of Aβ oligomers, fibrils, and plaques are common (16). Nevertheless, the best-studied disease related to Aβ is AD.

Aβ IN ALZHEIMER'S DISEASE

Aβ was found to be the major component of amyloid depositions described in the brain of AD patients (13), while Aβ oligomers at high concentrations probably ignite the disease itself (15, 21). Aβ oligomers damage neurons, inducing tangle formation. Neuronal tangles start to appear (those that correlate with brain impairment) when amyloid concentration is high, and greater concentrations of Aβ oligomers and amyloid plaques correlate with tangle spread (69).

While Aβ deposition in AD was discovered first in the brain, deposits or high concentrations of oligomers of Aβ were later described in peripheral tissues during the course of this disease. It can be found in the skin, certain muscles, heart tissue, the eye (in the retina and the lens), and even the intestines of patients (66, 70–73).

The presence of Aβ aggregates locally or systemically during many health problems, together with the known antibiotic activity of Aβ (see below), led many researchers to suggest that hyperproduction of Aβ is a typical defensive reaction of innate immunity (16, 17). The generation and release of Aβ in large quantities (hyperproduction) in pathological cases results in its aggregation and accumulation as a side effect of this response. The ultimate cause of the disease can be various infections or mechanical damage that activates this systemic release of Aβ. Released for protection against multiple invasions, Aβ later becomes the damaging factor for the tissue, creating a positive feedback in the vicious cycle of the disease. The question arises: where is the systemic production of Aβ concentrated, and how does it work?

Aβ IS AN INNATE IMMUNITY WEAPON RELEASED BY PLATELETS

Aβ Is an Antibiotic Agent

Aβ peptides have strong antibiotic activity against both Gram-negative and Gram-positive bacteria, as well as fungi and viruses (74–77). Aβ also combats mouse microbial infections *in vivo* (78). Extracellular entrapment of the invading agent may be one of the mechanisms of this antibiotic effect. As an example, it was shown that certain defensins, peptides produced by neutrophils and certain other blood cells, have a propensity to arrange themselves in amyloids. For instance, human α-defensin 6 forms ß-pleated sheet fibrils with antimicrobial properties entangling the bacteria in net-like structures (79, 80). Similarly, it was shown that Aβ peptide oligomers aggregated into fibrils entrap microbes (78) or can bind herpes virus surface glycoproteins, accelerating Aβ deposition and leading to protective viral entrapment (81). Other defensins can form large, weakly anion-selective ion channels, and this channel-forming ability contributes to their antimicrobial properties (82). Equally, we have shown that a synthetic Aβ peptide perforates the external membrane of yeast (40), and it is known that natural peptide antibiotics with channel-forming activity kill target cells, including fungi, by this same mechanism (83, 84). It was shown earlier that soluble Aβ peptide oligomers at low concentrations perforate cell membranes by forming tetrameric/octameric channels penetrable by K^+ ions, while at higher concentrations they form large, non-selective pores (85–89). An excess of Ca^{++} permeability through these pores induces calcium dyshomeostasis and is extremely toxic (90, 91). Large pores also allow large molecules entry into the cell. Based on these findings, it has been suggested that, like defensins, Aβ is a previously unrecognized antimicrobial agent that usually functions in the innate immune system (16, 38, 75, 78, 92). Other researchers and our group believe that Aβ may be released as a response to infection (16, 81), and this release is likely triggered by tissue damage and inflammation (17, 40).

Platelets Are the Primary Source of Systemic APP and Aβ

Amyloid beta (Aβ) peptides may be of various lengths (<46 amino acids) but have a specifically conserved sequence, with 90% similarity between vertebrate species but still with significant differences [see (18)]. These peptides are produced by a two-step (ß+γ) cleavage from a longer amyloid precursor protein (APP), a process occurring in many cell types, for example in neurons and astrocytes in the brain (15). This APP processing is known as the amyloidogenic pathway, because it produces Aβ and is enhanced during pathology; for example, it was found to occur in AD (93), while the same APP is processed differently (the non-amyloidogenic pathway) under normal physiological conditions. Due to hydrogen bonding between parallel monomers, Aβ may form dimeric, tetrameric, or higher-order oligomers, even at very low concentrations. At higher concentrations, it associates into larger β-pleated sheets, forming filaments tending to join in misfolded aggregations known as amyloid plaques (19, 20). The buildup of extracellular plaques in AD and other conditions (e.g., brain trauma and cancer) suggests that the concentration of Aβ is elevated in an affected individual's tissue. Aβ aggregation can start at a lower concentration, due to specific mutations within Aβ and its precursor that augment the propensity of Aβ peptides to aggregate, forming the basis for hereditary early-onset familial AD (22). Our group and others have already reviewed the literature on the possible sources of Aβ in AD and certain other diseases (16, 46, 94), and it has been suggested that there is significant local production of Aβ by neurons and probably astrocytes and that APP processing can be found in the brain and enteric nervous system (15, 95, 96). There is strong evidence that cultured neurons may produce Aβ and even form "plaques in the dish" (16). Multiple AD murine transgene models with human mutant Aβ generated in neurons under the control of specific neuronal promoters have shown important characteristics of AD, such as extracellular amyloid plaques, cerebral amyloid angiopathy (CAA), and sequential development of tauopathy (97–99). Although none of the animal models fully replicates the human disease, they have contributed essential insights into the pathophysiology of Aβ biology and toxicity.

However, there is another systemic source of APP and Aβ: platelets, which are small nuclear cells formed from the pro-platelet processes of the megakaryocyte (MK) precursor cell (100, 101). While MK cells originate in the bone marrow, and many researchers believe that platelets also originate there (102), it has been shown that at least 50% of platelets are generated from megakaryocyte-type extravascular progenitors in the pulmonary capillary bed of the lungs at the site of high oxygen tension (103–106). Platelet production from MK cells is tightly regulated by diverse humoral factors (100, 101). Platelets contain various types of granules, including α-granules, dense granules, and lysosomes (107). Besides coagulation factors, platelet α-granules contain APP, which is expressed predominantly as two isoforms of increasing length (751 and 770 amino acids), both containing a Kunitz proteinase inhibitor (KPI) domain (108, 109). APP can be liberated upon platelet degranulation (110–115) and represents about half of all protein secreted from agonist-treated platelets (111). APP with a Kunitz-type protease inhibitor can effectively inhibit chymotrypsin, trypsin, and other proteolytic enzymes (111, 116) and promotes activation of coagulation factor XII, affecting the hemostasis and temporal stability of the thrombus (117, 118). Platelets may also generate Aβ peptides and are the primary source (~90%) of this peptide in human blood (119). While APP processing in platelets under normal physiological conditions is mostly non-amyloidogenic, it changes during the response to pathology. Investigators studying AD biomarkers used platelets to examine the components of both the non-amyloidogenic and amyloidogenic cascades, finding that platelets are an excellent model with which to study blood-based AD-related biomarkers, reflecting a shift in Aβ production during AD (120). It was previously suggested that whether platelets generate soluble APP or either of the Aβ peptides is determined by a specialized regulated secretory vesicle pathway (121, 122) different from any found in neurons. In either setting, APP or its cleavage products are released

mainly within extracellular vesicles, although with a different type of γ-secretase and localization of APP during the two-step (ß+γ) cleavage:

(1) In its neuronal secretory pathway, APP is always a type 1 transmembrane protein and is located in the membrane. First, cleavage of APP by β-secretase occurs in a soluble environment, while secondary cleavage by γ-secretase occurs within the transmembrane domain of the APP when inserted into the membrane, thus liberating Aβ outside the cell or inside certain cellular vesicles (123, 124). In neurons, γ-secretase is a proteolytic complex consisting of four proteins. Presenilin (PS) is the active core, while the other three proteins provide support functions (125). In neurons, Aβ is released at nerve terminals in the CNS after the precursor APP is transported there by axonal transport (126, 127). Cleavage processing most probably occurs in a type of endosome known as a multi-vesicular body (MVB) in the terminals, the intracellular structures that contain smaller vesicles released from the cell in the form of exosomes when the MVB fuses with the plasma membrane (128, 129). These exosomes contain mainly APP cleavage products and have a variety of receptors reacting with nearby neurons and astrocytes (130).

(2) In the secretory pathway, vesicles may release both full-length, soluble APP, and/or Aβ. This event is known to occur in platelets (110, 111) and in chromaffin cells (131), and both cell types have specialized secretory vesicles. Full-length APP within vesicles exists mainly in its soluble form. It has β- and γ-secretase sites accessible for proteolytic cleavage inside the vesicle's soluble environment, thereby also releasing Aβ inside the vesicle lumen (131). The content of vesicles is released by the cell in a regulated process, and it may be APP that is released, or APP may be processed further inside the vesicle. In platelets, α-granules represent the final evolution of MVBs and contain exosomes, similar to the MVBs in neurons (132). The α-granule content can be extruded or fused to the external membrane (133), liberating exosomes, as also occurs in neurons. The cathepsin B and D enzymes, which can cleave soluble APP, are described as a β-secretases in this pathway. It was suggested that this regulated secretory pathway (121, 122, 134) produces the major portion of secreted, extracellular Aβ peptides.

It was also found that macrophages may engulf platelets and process APP to produce Aβ in atherosclerosis (135). In addition, brain vessel endothelial cell enzymes can cleave the platelet-released APP, forming Aβ, most efficiently if the activated platelets adhere directly to the endothelial cells (136). Leukocytes can also produce and release Aβ, but the amounts are small relative to that produced by platelets (137). Similarly, many other cells, such as fibroblasts and endothelial cells, may produce small amounts of Aβ (138). Summarizing, we can say that platelet-generated Aβ may be a significant component of systemic Aβ. Now the question arises: what is the role of systemic Aβ?

Aβ IS A VITAL DEFENSE PROTEIN WITH MULTIPLE ROLES

Evolutionarily, mammalian platelets became denucleated and reduced in size to small (1–2 μm) cells, thereby having a high surface-to-volume ratio that accelerated the speed of reception and granule secretion, with the further ability to easily transit from tissue to blood and back through gaps between endothelial cells everywhere except in the brain. These advantages made them useful as first responders, which are most important in hemostasis and innate immunity. In this review, we are primarily focused on the link between tissue damage and inflammation and the generation of platelet-associated Aβ peptides. Many comorbid bacteria and viruses were found in patient brains during AD or glaucoma (17). We suggest that Aβ peptides can be generated from APP released by platelets in response to inflammation of septic, mechanical, or chemical origin.

Aβ Is Generated by Platelets During Coagulation

We used immunostaining to visualize Aβ after photothrombosis in mouse brains and found that, upon coagulation, the increased concentration of platelets allows enhanced release of Aβ. Aβ immunostaining was intense inside and near blood vessels in the thrombotic zone, with the maximum intensity near the vessel walls (39). Similarly, Aβ generated from precursors released from platelets might be the source of its accumulation in mouse skin, as it was found to be concentrated around blood vessels after experimental thrombosis (40). A similar accumulation of Aβ around blood vessels in the skin of AD patients and generally in older patients was described many years ago (66, 139). Moreover, we recently reported that Aβ immunofluorescence accumulated on blood vessel walls in the damaged part of the brain and on nearby astrocytes after middle cerebral artery occlusion (35). Temporary accumulation of Aβ in GFAP-positive astrocytic bodies and processes that formed clusters with specific small vessel-like structures was reported previously (29, 140–143), see also review: (38). Aβ-containing plaques, as determined by immunofluorescence, but not plaques staining positive for Congo red or thioflavin (aggregation-specific amyloid stains) can persist for up to 9 months after arterial occlusion (144). Also, temporary Aβ plaques appeared in the brain of an AD mouse model after mild brain trauma. They then disappeared after 7 days, which was correlated with the post-traumatic concentration of soluble Aβ oligomers in the brain (145). Aβ plaques and oligomers may also be found in the brains of human patients within hours of traumatic brain injury (TBI) in non-AD patients (33, 146, 147). These findings, taken together, suggest that trauma followed by coagulation is an important cause of Aβ accumulation in tissues.

Platelets in the Immune Response

It is known that platelets act as important mediators of innate defenses: platelet adhesion, activation, and degranulation are the essential steps in this process, in which platelet-associated surface receptor molecules play a pivotal role in the development of inflammation (148).

Platelets express CD40L and toll-like receptors (TLR), which recognize microbe-associated threats and may modulate innate immunity or directly interact with microorganisms and viruses (17, 149–152). Platelets can engulf bacteria and viruses in endosome-like vacuoles that fuse with α-granules with

antimicrobial contents (153). When directly activated by viral and bacterial antigens, platelets release microbicidal peptides (16, 154–162). We have shown that Aβ peptides perforate yeast cell membranes while not affecting somatic cell membranes at the same concentration (40). Apart from Aβ peptide, there are other antibacterial peptides released by platelets. Like Aβ, one of these antibacterial peptides from rabbit platelets is cleaved from a longer precursor and has a variable length of 72–73 amino acids (159).

Moreover, platelets 1) interact with other immune cells using cell-specific adhesion molecules, 2) attach themselves to neutrophils and monocytes at the site of lesion and also activate these cells as well as themselves, 3) release multiple antibacterial factors, and 4) participate in both innate and acquired immune responses (163, 164). In addition, platelets have close interactions with the innate complement system, while being protected themselves from complement-mediated damage by soluble and membrane-expressed complement regulators. Still, they also bind several complement components on their surface and trigger complement activation in the fluid phase (165). The best-studied mechanism is the joint work of platelets and neutrophils in forming circulating platelet–neutrophil complexes: stimulation of the neutrophil surface receptor TLR type 2 (TLR2) amplifies the release of α-granules and membrane expression of P-selectin on the surface of platelets. P-selectin allows adhesive interactions with leukocytes and endothelial cells *via* P-selectin glycoprotein ligand 1, which activates leukocyte production of cytokine cascades and initiates or further promotes inflammation (166). At the same time, platelets promote the recruitment of neutrophils to sites of tissue damage. They bind with activated neutrophils and endothelial cells on vessel walls, forming platelet–neutrophil aggregations and stimulating the production of filamentous neutrophil extracellular traps (NETs), which trap and kill pathogens (132, 151, 167–170). It has been shown that aggregated platelets at high density secrete mainly Aβ peptides ending at residue 40 (Aβ40) as a final product, while the Aβ42 level is not affected by cell density (171).

Additionally, an unusual reverse influence of neutrophils on platelets, known as emperipolesis, was reported. In this process megakaryocytes engulf neutrophils, fusing with their membranes and subsequently producing "daughter" platelets containing neutrophil membrane and membrane receptors. The entire process of emperipolesis takes a few minutes, after which the neutrophil liberates itself and egresses intact from the megakaryocyte. This process enables neutrophils passing through the megakaryocyte cytoplasm to modulate the production and membrane content of platelets (172). All these interactions between neutrophils and platelets in normal blood and during infection, inflammation, and thrombosis are the pillars of the immune–hemostatic continuum [**Figure 1**, (166, 173–175)]. The connection between neutrophils and platelets led us to compare their antimicrobial arsenals, and they showed striking similarities.

The Similarities Between Aβ Peptides and Defensins

While there are a variety of mammalian defensins, all are synthesized as a larger precursor molecule and then cleaved a varying number of times to obtain the final product. They are active against bacteria, fungi, and many different viruses. For example, human neutrophil peptides (HNP)-1–3 are first synthesized as the 94-amino-acid (aa) preproHNP, which is

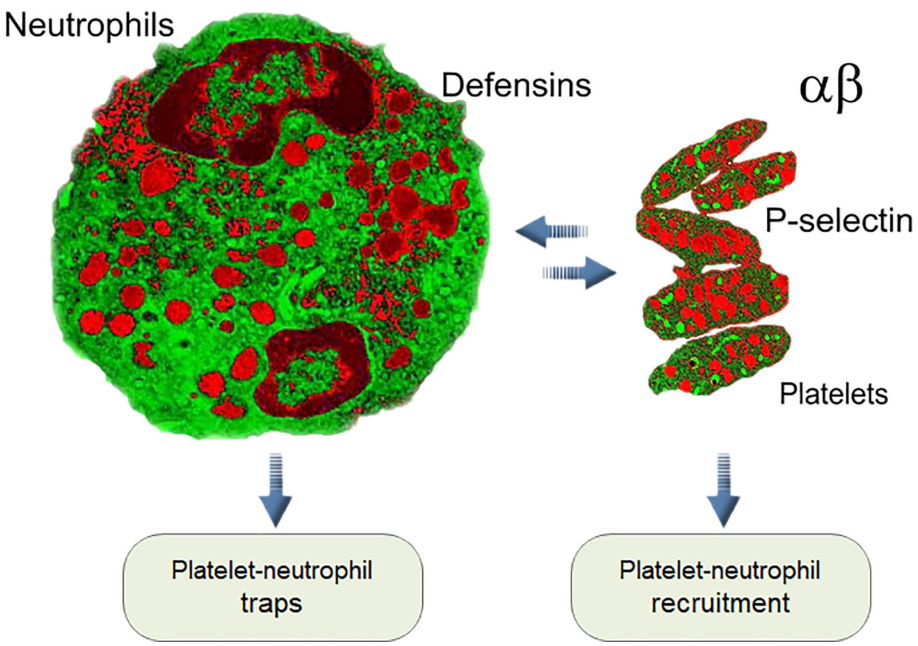

FIGURE 1 | Interaction between platelets and neutrophils is one of the key elements of innate immunity in mammals (see text).

converted to 75-aa proHNPs by cotranslational removal of a 19-aa endoplasmic reticulum signal peptide. At the promyelocytic stage of myelopoiesis, proHNPs are further cleaved and accumulate in azurophil granules in neutrophils as 29–30-aa HNPs. By contrast, the proHNPs produced by more mature myeloid cells undergo a high degree of constitutive exocytosis without cleavage. These prodefensins have no antimicrobial potential, and the significance of their secretion is unknown (176, 177). Antimicrobial action is mediated *via* several mechanisms, including pore formation or aggregation. For example, the antimicrobial peptide human defensin 6 (HD6) can aggregate, forming amyloid filaments with a strong affinity for bacterial surfaces and thereby trapping bacteria (69). By contrast, (HNP)-1–3 at low concentrations form a lipophilic β-sheet-rich dimer with additional disulfide bonding, but at higher concentrations they can oligomerize into tetramers, hexamers, and larger oligomers, creating a variety of pores or less-well-defined apertures, termed "giant aggregate channels," in plasma membranes, thereby killing cells (178).

Aβ peptides, while relatively short, are synthesized as longer (680–780 aa) APPs. Then, like defensins, the APPs are cleaved twice (with β- and γ-secretases) to obtain a final length of 36–43 aa for the mature Aβ peptide. They are also active against bacteria, fungi, and many different viruses, and their antimicrobial action is mediated *via* several mechanisms, including pore formation and aggregation. Soluble Aβ peptide oligomers at low concentrations (50–200 nM) perforate cell membranes by forming tetrameric channels penetrable by K^+ ions and do so at higher concentrations by creating Ca^{++}-permeable hexameric pores, while they may also form large pores (86–88). The main toxic effect that has been suggested is related to the excess Ca^{++} permeability through these pores, which induces calcium dyshomeostasis (90, 91). Other toxic agents may also enter the membrane aperture to kill the cell (179). In our experiments, the external membrane of the yeast was perforated by synthetic Aβ at a 5-mM concentration (40). A similar range of concentrations (10–40 μM) was shown for synthetic defensin-forming channels in fungal membranes (180). We also suggest that the effective concentration of peptides (lipophilic defensins and Aβ) for pore formation can be much lower if they are solubilized with selective carriers, such as transthyretin or apolipoproteins. Recently, it was shown that certain external compounds that react with Aβ might modulate its effects by working as carriers (181).

It is known that small and double-bridging peptides are resistant to many proteases, tolerating digestion, even following oral administration (182). A structure with four sulfide bridges and multiple β-strands linked to an α-helix is typical of defensins, making them resistant to proteases. Additionally, certain defensins have antipeptidase activity themselves or may regulate secretory leukocyte protease inhibitor α2 macroglobulin, which allows them to block microbial proteases with synergistic combinations of defensin and protease inhibitor (183) but also allows them to resist host proteases.

Aβ oligomers usually lack disulfide bridges, except for certain mutant peptides (184), but they have multiple β-strands reinforced with salt bridges (185). Besides, in many cases Aβ peptides are released jointly with a full-size APP or its fragments with Kunitz-type domains, which block protease activity and protect the released Aβ peptide. It was shown that the amount of released Kunitz-APP is vital for AD development and is correlated with the number of neurotic plaques (186).

It is common knowledge that Aβ concentration is augmented in AD and certain other conditions, but the same is true for defensins. Rapid accumulation of defensins proximal to the site of brain inflammation occurs with neurodegeneration (187), including in AD (188), bacterial and viral infection, and brain trauma (188–190). Antimicrobial peptide β-defensin-1 expression is also upregulated in AD brain, especially in the choroid plexus but also in astrocytes and blood vessel walls (191, 192). Under physiological conditions, dendritic cells are restricted to the meninges and choroid plexus of the brain and are generally not present within the brain parenchyma (193). In addition, there are several antimicrobial peptides with a clear structural resemblance to defensins, with similar pore-forming and mesh-forming activities [for a review see: (194)].

A POSSIBLE RODENT MODEL OF PLATELET-GENERATED Aβ

Studies of platelet-generated Aβ must reproduce the following effects: 1) induced APP is expressed in platelets; 2) platelet-generated Aβ is prone to aggregation; 3) platelet-generated Aβ can be transported from the blood to the brain or some other tissue of interest, as some Aβ mutants are not transportable.

Expressing an APP of Interest in Platelets Using Different Promoters

The expression of Aβ in a transgenic model depends on the type of promoter used to control its expression. Different promoters have a stably recurring expression in specific cells, while some have remarkable variation in expression patterns (195). Of the most common promotors used in mouse transgenes, the prion promoter element (PrP) is most promising. It is mainly active in brain neurons but also in extraneuronal regions, especially in cells with secretory granules (196). It was found that exosomes release cellular prion protein from activated platelets (197, 198). Similarly, APP was found to be concentrated in exosomes of a specific size in platelets (199). This gives hope that a transgene with an inserted variant of APP and under control of the PrP promoter can generate both APP and Aß in association with platelets as well as with neurons.

Another promising promoter is the rat platelet factor 4 promoter element (rPF4). A transgenic mouse that generated modest overexpression of induced human wild type APP (770 isoforms) in platelets was constructed (200). However, in this animal model, mouse and not human Aß was found in the brain (201), raising the possibility that human wild type Aß has a transport impediment at the blood–brain barrier (BBB) in mice.

The popular mouse Thy 1.1 promoter is used in many murine transgenes that develop Aβ accumulation in brains of mouse and

rat and CAA-type aggregation in blood vessels (202, 203). However, this promoter does not transcribe well in platelets and is usually manipulated (by intron 3 deletion) to remove its transcription in cells other than neurons (204, 205). Therefore, platelets have no expression of transgenic APP, but express only endogenous wild type APP. There are reports that truncated Thy 1 can also be activated in endothelial cells by inflammation (206). Interestingly, blood vessel damage in organotypic wild type brain slices was ascribed to platelets because of their platelet-generated Aβ (207). Platelets were harvested from Tg-SwDI mice with APP expressed under a Thy 1 promoter, and therefore we suggest that Aß in platelets from these animals was mainly wild-type and not transgenic. Kniewallner et al. showed that these AD-derived platelets more aggressively damage healthy vessels in any case and that matrix metalloproteinase hyperactivation was involved. Thus, even wild-type platelet-generated Aß can produce damage if platelets are hyperactivated.

Summarizing, the majority of murine transgenic models of AD use the insertion of mutated human APP variants, and many of these transgenes do not express human Aβ in platelets. This must be taken into account when evaluating platelet-related studies of Aβ accumulation.

Aggregation of Generated Aβ and Transit Barriers

It is known that Aβ wild type and variants have different tendencies to aggregate. Human Aβ(1–40) and Aβ(1–42) differ in their ability to form amyloid fibrils (208), while it was also shown that both variants can co-aggregate, creating mixed β-sheets (209). In addition, there is a species-related difference: the propensity of murine Aβ to produce amyloid deposits is limited, even in aged mice. This is because human and murine APPs differ at three amino acid residues within the Aβ peptide sequence and are cleaved differently by β-site APP cleaving enzyme 1 (BASE1), thereby producing mainly shortened Aβ fragments not prone to aggregation or easily soluble aggregates in wild type rodents (23, 210). Therefore, practically all transgenic mouse models of AD amyloid deposition use somewhat humanized APP. It can be a mutated human APP or a murine APP that is chimerized to include human-type early-onset mutations to generate Aβ deposits. Human presenilin (a component of the cleaving mechanism) must be added to produce longer Aβ peptides. For example, when expressed in mouse APP695, a transgene with mutations resembling Swedish human mutations leading to early-onset AD (APPswe) and reinforced by a human presenilin exon-9-deletion variant (PS1dE9) can produce amyloid deposits consisting entirely of mouse Aβ peptides that are morphologically similar to deposits found in humans during early-onset AD (211). Recently, using a parabiosis procedure on this APPswe/PS1dE9 transgenic AD mouse with their wild-type littermates, it was directly established that human Aβ originating from the transgenic AD mouse model entered the circulation, accumulated in the brains of the wild-type mice, and formed cerebral amyloid angiopathy and Aβ plaques after 12 months of parabiosis (212). The authors did not determine the source of blood-derived Aβ but suggested that the source may be platelets. This chimerical mouse/human amyloid precursor protein (Mo/HuAPP695swe), together with mutant human presenilin 1 (PS1-dE9), was directed to CNS neurons and platelets with a PrP promoter. It is possible that the Aβ in this model first penetrated the BBB from the brain of the transgenic mouse and then once again the BBB of the littermate, passing through this barrier twice. Alternatively, Aβ may simply be transported from platelets in the circulation to the littermate brain. In any case, at least one BBB transit mechanism was involved. The same mouse model (APPswe/PS1dE9) was used to show that thrombotic cerebrovascular lesions induce a rapid transient increase in amyloid plaque burden and amyloid angiopathy in the area immediately surrounding the infarcted area, (213). These and other results suggest that this model (APPswe/PS1dE9) is the best for studying the effects of platelet-generated Aβ.

Another interesting problem is hybrid aggregation. Wild type Aβ from one cell type and a mutant Aβ from neurons may aggregate, forming hybrid (hetero-)oligomers, thus affecting amyloid formation. For example, if a heterozygote animal has two different Aβ variants, one variant could reduce self-assembly of the fibrils of the other variant. Some Aβ mutants even have opposite parallel or antiparallel β-sheet arrangements in oligomers [as was shown for the Italian E22K and Iowa D23N mutations; (214)]. It is known that shorter Aβ fragments can aggregate with full-length Aβ, and the resulting oligomers will block self-assembly of the fibrils and amyloid (215). Thus, wild type Aβ fragments from platelets being transported to the brain may interfere with fibril formation by mutant Aβ from a neuronal source in transgenic animals. Are Aβ peptides transported to the brain and back? Fragments of mutant and hybrid Aβ oligomers may have transit barriers at the BBB, and this possibility has been largely unstudied.

Aβ may be transported in and out of the brain parenchyma by several physiological mechanisms. The vascular luminal receptor for advanced glycation end products (RAGE) is thought to be a primary transporter of Aβ across the BBB into the brain from the systemic circulation. The low-density lipoprotein receptor-related protein (LRP)-1 (expressed mainly at the abluminal side of the BBB) mediates transport of Aβ out of the brain (216–219).

The Italian E22K and Iowa D23N mutations can result in the formation of Aβ oligomers and fibrils, with an antiparallel β-sheet structure predisposing them to be deposited in cerebral blood vessels rather than accumulating mainly in plaques through distinct interactions with the receptors responsible for Aβ clearance across the BBB (214). As already mentioned, human Aβ probably encounters a transit barrier in murine models. For example, poor clearance of human Dutch/Iowa mutant Aβ40 peptides from mouse and rat brain was shown (203, 220). This factor may also be important for studying platelet-generated amyloid peptides in murine models.

CONCLUSIONS

- There are a number of health complications in which high levels of Aβ peptides and Aβ amyloid aggregates occur.

- While many cells may produce Aβ, including neurons and astrocytes, platelets are the primary source of systemic APP and Aβ.
- Platelets are a vital part of intrinsic immunity, and Aβ is an essential defense protein released during trauma and coagulation and as a response to inflammation. Aβ has evident antimicrobial and antiviral properties, suggesting that inflammation-related tissue accumulation of Aβ may be an overreaction against microbial or other aseptic causes.
- Platelets are essential players in tissue Aβ accumulation in AD, glioma, and glaucoma and may be involved in other neurodegenerative diseases, such as PD.
- While the direct release of APP and its non-amyloidogenic products is prevalent in platelets under normal physiological conditions, our literature review suggests that, in many pathologies, platelet activity shifts to Aβ production and that inflammation is one of the triggers.
- The propensities of Aβ from different animal species and humans to aggregate are different, and murine Aβ does not form stable aggregates. Thus, the majority of murine transgenic models of AD use the insertion of mutated human APP variants, and many of these transgenes do not express human Aβ in platelets. This must be considered when interpreting the results of platelet-related studies of Aβ accumulation. Some human Aβ may also encounter a transport filter at the mouse blood–brain barrier.

AUTHOR CONTRIBUTIONS

MI, AZ-S, LR, and LK reviewed the literature and wrote this review; MI and LR prepared the figure.

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REVIEW
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Platelets as Mediators of Neuroinflammation and Thrombosis

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Beyond platelets function in hemostasis, there is emerging evidence to suggest that platelets contribute crucially to inflammation and immune responses. Therefore, considering the detrimental role of inflammatory conditions in severe neurological disorders such as multiple sclerosis or stroke, this review outlines platelets involvement in neuroinflammation. For this, distinct mechanisms of platelet-mediated thrombosis and inflammation are portrayed, focusing on the interaction of platelet receptors with other immune cells as well as brain endothelial cells. Furthermore, we draw attention to the intimate interplay between platelets and the complement system as well as between platelets and plasmatic coagulation factors in the course of neuroinflammation. Following the thorough exposition of preclinical approaches which aim at ameliorating disease severity after inducing experimental autoimmune encephalomyelitis (a counterpart of multiple sclerosis in mice) or brain ischemia-reperfusion injury, the clinical relevance of platelet-mediated neuroinflammation is addressed. Thus, current as well as future propitious translational and clinical strategies for the treatment of neuro-inflammatory diseases by affecting platelet function are illustrated, emphasizing that targeting platelet-mediated neuroinflammation could become an efficient adjunct therapy to mitigate disease severity of multiple sclerosis or stroke associated brain injury.

Keywords: platelets, neuroinflammation, thrombosis, stroke, therapy, cytokines, encephalomyelitis, Alzheimer's

INTRODUCTION

Platelets, also called thrombocytes, are produced by megakaryocytes as tiny anucleate cells that, however, contain mRNA and a translational machinery; hence, they are capable of synthesizing proteins (1). After leaving the bone marrow, platelets circulate for about 7 to 10 days (2), subsequently they are eliminated by macrophages mainly in the spleen and liver (3). Platelets are

classically regarded as the major actor of primary hemostasis. Thus, their main function is stopping hemorrhage following vascular injury by rapidly binding to damaged blood vessels and forming thrombi (4). However, activated platelets also aggregate during atherosclerotic plaque erosion or rupture, stimulating thrombus formation and promoting severe atherothrombotic diseases such as acute limb ischemia or myocardial infarction (5, 6).

Beyond their importance in hemostasis and thrombosis, an increasing body of evidence points to a crucial role of platelets for inflammatory and immune responses (7, 8). For instance, platelets have been demonstrated to mediate inflammatory response in arthritis (9) or sepsis (10). Furthermore, thrombosis itself is pathophysiologically linked with inflammation in most diseases associated with ischemia-driven organ damage (11, 12). Accordingly, platelets have been shown to be of decisive importance for thrombo-inflammatory diseases such as stroke (13). Emerging evidence indicates a detrimental role of platelets not only in the context of neurovascular thrombosis but also in other neuro-inflammatory conditions, e.g., multiple sclerosis (MS) (14). Considering the severity of these diseases and the diminished patients' quality of life, there is an urgent need for novel therapeutic options.

Therefore, this review summarizes recent insights into the pathophysiological role of platelet receptors and related downstream signaling as well as platelet-mediated cell-cell interactions in neurovascular inflammation. Furthermore, translational and clinical applications are portrayed in order to delineate future therapeutic strategies for neuro-inflammatory diseases such as stroke or MS by targeting platelet function.

Abbreviations: 5-HT, serotonin; ADAMTS13, a disintegrin and metalloprotease with thrombospondin type 1 repeats 13; ADP, adenosine diphosphate; ALS, amyotrophic lateral sclerosis; AP, activated platelet; ApoE, apolipoprotein E; BBB, blood-brain barrier; BK, bradykinin; C3aR, complement receptor for C3a; C5aR, complement receptor for C5aR; cAMP, cyclic adenosine monophosphate; CCL, CC-chemokine ligand; CD40L, CD40 ligand; CLEC-2, C-type lectin-like receptor-2; CNS, central nervous system; CSF, cerebrospinal fluid; CX3CL1, chemokine (C-X3-C motif) ligand 1, fractalkine; CXCL, chemokine (C-X-C motif) ligand; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; F, coagulation factor; FasL, Fas ligand; FasR, Fas receptor; FOXP3, Forkhead box P3; GP, glycoprotein; HIV, human immunodeficiency virus; HUVEC, human umbilical vein endothelial cell; ICAM, intercellular adhesion molecule; IL, interleukin; JAM-C, junctional adhesion molecules-C; KKS, kallikrein–kinin system; LFA-1, lymphocyte function-associated antigen 1; LPS, lipopolysaccharide; Mac-1, macrophage-1 antigen; MBL, mannan-binding lectin; MCP1, monocyte chemotactic protein 1; MRI, magnetic resonance imaging; MS, multiple sclerosis; MΦ, macrophage; Ne, neutrophil; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PAF, platelet activating factor; PAMP, pathogen-associated molecular pattern; PAR, protease-activated receptor; PCI, percutaneous coronary intervention; PDE, phosphodiesterase; PECAM-1, platelet endothelial adhesion molecule-1; PF4, platelet factor 4; PLA, forming platelet-leukocyte-aggregates; PMP, platelet-derived microparticle; polyP, polyphosphates; PPX, recombinant *Escherichia coli* exopolyphosphatase; PSGL-1, P-selectin glycoprotein ligand-1; RANTES, regulated and normal T cell expressed and secreted; ROS, reactive oxygen species; sCD40L, soluble CD40L; TF, tissue factor; TIA, transient ischemic attack; TJs, tight junctions; TLR, toll-like receptor; tMCAO, transient middle cerebral artery occlusion; TNF, tumor-necrosis factor; Treg, regulatory T cell; TTP, thrombotic thrombocytopenic purpura; VCAM, vascular cell adhesion protein; vWF, von Willebrand factor; WT, wild-type.

MECHANISMS OF PLATELET-MEDIATED THROMBOSIS AND INFLAMMATION

As injuries require both an efficient hemostasis and an inflammatory immune response against entering pathogens, the close linkage between inflammatory and thrombotic processes is assumed to have an evolutionary origin (15, 16). Following vasoconstriction, platelets are the first immunomodulatory cells at the side of injury sealing damaged blood vessels by aggregation and forming a thrombus. Thereby, platelets promote inflammatory activity by an intimate crosstalk with leukocytes (17): In case of vascular injury, neutrophils or monocytes are suggested to interact either with endothelium-adherent platelets or, prior to endothelial contact, directly with platelets forming platelet-leukocyte-aggregates (PLA) which are recruited to the inflamed vessel wall (18). Thus, platelets orchestrate the inflammatory response by regulating the further adhesion of innate immune cells to the inflamed endothelium, which is regarded to be critical for the atherosclerotic disease process (19). For instance, macrophage pro-inflammatory cytokine secretion is enhanced following interaction with activated platelets *in vitro*, suggesting that the presence of activated platelets at sites of inflammation exacerbates pro-inflammatory macrophage activation (20). Further molecular mechanisms and receptors participating in the crosstalk between innate immune cells and platelets are outlined below.

Interaction of Platelets With Cells of Acquired Immunity

In addition to the interaction with the innate immune system, a crosstalk between platelets and B cells as well as T cells has been reported (21). Platelets have been demonstrated to induce B cell isotype switching (22). When platelets are co-incubated with B-cells *in vitro*, B-cells increase their production of IgG1, IgG2, and IgG3, indicating that platelet content can contribute to B-cell function and alter adaptive immunity (23). T-cell activation increases platelet aggregation *via* both T cytolytic and T helper cells mediated by platelet GPIIb/IIIa, CD40L, and lymphocyte integrin alpha M (24). Experimental approaches indicate that platelets may facilitate the recruitment of lymphocytes to an injured vessel at a site of vascular inflammation, constituting a central step in T-cell trafficking (25). Furthermore, activated platelets can modulate T-cell functions by releasing platelet factor 4 (PF4, chemokine [C-X-C motif] ligand 4, CXCL4), RANTES (CC-chemokine ligand 5, CCL5), or serotonin (26–28). For instance, PF4 is necessary for the limitation of Th17 expansion and differentiation (29). Serotonin, which is largely stored in platelet δ-granules, can activate naïve T-cells to stimulate their proliferation (26, 27). Hence, the interaction between platelets and lymphocytes should be considered as a relevant intersection in thrombo-inflammatory processes; therefore, receptors in platelet-immune cell interaction are further delineated in the following chapters.

Platelets and the Humoral Immunity

Platelets have been identified as a major source of chemokines and cytokines at the site of inflammation (30). For instance, activated platelets mediate inflammatory signaling and cell

recruitment by secreting RANTES, PF4, and IL-1β (31, 32). Emphasizing the role of platelets at the intersection between thrombosis and inflammation, their IL-1 activity yielded an exacerbation of atherosclerotic lesions as well as an upregulation of adhesion molecules and chemokine expression by human umbilical vein endothelial cells (HUVECs) (33, 34). Remarkably, platelet activation of brain endothelium *via* IL-1 has been recognized to promote the release of CXCL1, which plays an essential role in the subsequent leukocyte recruitment during neuroinflammation (35, 36). Furthermore, platelets contain nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) family members (37) that are critically involved in both inflammatory and thrombotic responses, which has recently been reviewed elsewhere (38). Moreover, a crosstalk between platelets and the complement system conduces to platelet-mediated inflammation (39, 40). Thus, the interaction of platelets with the complement system will be discussed here in the context of neurovascular inflammation, whereas further aspects have been comprehensively portrayed elsewhere (11).

Overall, a broad range of mechanisms contribute to platelet-mediated inflammation, revealing several fields for future research on diseases associated with thromboinflammation.

PLATELET RECEPTORS AND INTERACTIONS IN THE CONTEXT OF THROMBOINFLAMMATION

Both mechanisms of hemostasis respectively thrombosis and mechanisms of platelet-mediated inflammation require a close interaction of platelets with endothelial and immune cells but also with the extracellular matrix. Platelet adhesion receptors constitute the major determinants of these interactions. Commonly, four types of platelet receptors are considered as being crucial for hemostasis, thrombosis and inflammation: integrins, leucine-rich glycoproteins (GPs), selectins as well as receptors of the immunoglobulin type.

Under flow conditions, especially at high shear stress (>500 s^{-1}) as in small arteries and arterioles, the initial adhesion of platelets to the injured blood vessel wall requires the interaction between immobilized von Willebrand factor (vWF) on the surface of the endothelium or in the subendothelial matrix with its platelet receptor GPIbα, which is part of the GPIb-IX-V complex (41, 42). In addition, exposed subendothelial collagen binds reversibly to platelet GPIa/IIa recptor (also known as integrin $\alpha_2\beta_1$) and GPVI receptor, a member of the immunoglobulin superfamily (43). The firm binding of collagen to platelet GPVI receptor allows resistance towards high shear rates, and furthermore, induces platelet activation by a rise in the cytosolic Ca^{2+} concentration. Thus, platelet shape changes and P-selectin, platelet endothelial adhesion molecule-1 (PECAM-1), vWF, and fibrinogen from α-granules as well as ADP, calcium and serotonin from dense granules are released, which in turn fuels further platelet activation *via* autocrine and paracrine signaling by G-protein coupled receptors (44, 45). The final common pathway of platelet activation is the conformational change in platelet GPIIb/IIIa (also named integrin $\alpha_{IIb}\beta_3$) receptor which results in the cross-link of fibrinogen or vWF between GPIIb/IIIa receptors, leading to platelet aggregation (46). Thereby, platelet integrin receptors $\alpha_2\beta_1$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$ stabilize thrombus formation by binding to components of the extracellular matrix (47–49).

Importantly, platelet-mediated leukocyte recruitment is initiated by binding of platelet P-selectin to leukocyte P-selectin glycoprotein ligand-1 (PSGL-1) (50), inducing activation of β_1 and β_2 integrins and increasing adhesion of leukocytes to activated endothelium (51). Contrariwise, PSGL-1 on platelets can interact with P-selectin on endothelial cells as well (52). Interestingly, fractalkine (CX3CL1) expressed by inflamed endothelial cells can bind to the fractalkine receptor CX3CR1 on platelets triggering an increased P-selectin expression on platelets, thereby initiating local accumulation of leukocytes (53). Besides, another member of platelet selectin family, C-type lectin-like receptor-2 (CLEC-2), is thought to be a major player in thrombo-inflammatory disorders (54): Using a murine model of systemic *Salmonella Typhimurium* infection, it has been demonstrated that inflammation in several tissues triggers thrombosis within vessels *via* activation of CLEC-2 on platelets by its ligand podoplanin exposed to the vasculature following breaching of the vessel wall (55). Thus, targeting CLEC-2 could be a potential therapeutic approach in order to control infection-driven thrombosis. Interestingly, mice with general inducible deletion of CLEC-2 or platelet-specific deficiency in CLEC-2 were protected against deep vein thrombosis (56). With respect to neuroinflammation, it has recently been demonstrated that inhibition of spleen tyrosine kinase (Syk), which is part of the CLEC-2 downstream pathway, reduces neuroinflammation and infarct volume after ischemic stroke in mice (57). On the other hand, platelet CLEC-2 has been shown to diminish trauma-induced neuroinflammation and restore blood–brain barrier integrity following controlled cortical impact injury (58). Thus, the potential of CLEC-2 as a target in the context of neuroinflammation remains uncertain.

GPIb interacts with the leukocyte integrin macrophage-1 antigen (Mac-1, also known as $\alpha_M\beta_2$ or CD11b/CD18); thereby promoting a firm leukocyte/platelet adhesion (59). Accordingly, GPIb inactivation leads to reduced leukocyte adhesion to the vessel wall as well as to diminished development of atherosclerotic lesions in atherosclerosis-prone apolipoprotein E-deficient ($ApoE^{-/-}$) mice (60). Underlining the importance of GPIb for cerebral inflammation, it has recently been reported that platelet-mediated neutrophil infiltration to the brain can be reduced by 44% when platelet receptor GPIb is blocked in an *in vivo* model of lipopolysaccharide (LPS)-induced neuroinflammation (61).

In addition to GPIb, fibrinogen bound to platelet GPIIb/IIIa receptor can also interact with leukocyte Mac-1 in a platelet activating factor (PAF) regulated manner (62). Mac-1 on monocytes and neutrophils were identified as critical molecular links between inflammation and thrombosis, e.g., in myocardial infarction (62) or else in thrombotic glomerular injury (63). Strikingly, recent experimental approaches have demonstrated

that both Mac-1 deficiency and mutation of the Mac-1-binding site for GPIb delay thrombosis after carotid artery injury without affecting parameters of hemostasis (64). Thus, targeting Mac-1-mediated leukocyte/platelet interaction is suggested to have an anti-thrombotic therapeutic potential with reduced bleeding risk (64).

Fascinatingly, platelet-derived microparticles (PMPs), that are generated from the plasma membrane upon platelet activation, harbor functional GPIIb/IIIa receptors which can be acquired by neutrophils and cooperate in neutrophil-induced inflammation *via* NF-κB activation (65). Accordingly, GPIIb/IIIa receptor antagonists reduced thrombo-inflammatory processes, as the formation of PLA, in patients with acute coronary syndromes undergoing percutaneous coronary intervention (PCI) (66). In the course of neurovascular inflammation, magnetic resonance imaging (MRI) studies demonstrated the presence of activated platelet GPIIb/IIIa receptor in the inflamed brain of malaria-infected mice using a specific antibody conjugated to iron oxide microparticles (67). Elevated PMP levels have also been detected in stroke patients (68, 69). However, a prognostic value of plasma PMP on recurrence of stroke, neurological outcome or survival is not established (70). Therefore, the pathophysiological significance of PMPs in stroke remains elusive.

In addition, a contribution of platelet GPVI receptor to thrombo-inflammatory disorders has been repeatedly shown (54). For instance, inhibition of GPVI causes a reduction in inflammatory cell recruitment and infarct size following myocardial ischemia-reperfusion injury by improving microperfusion (71). Further receptors of the immunoglobulin superfamily are also of importance for platelet interactions: Under low shear stress platelets interact with leukocytes by binding of intercellular adhesion molecule 2 (ICAM-2, also known as CD54) on platelets to lymphocyte function-associated antigen 1 (LFA-1) on leukocytes (72). Moreover, junctional adhesion molecules-C (JAM-C) expressed on platelets are critical for the recruitment of DCs to the vascular wall *via* an interaction with Mac-1 on DCs (73).

Intriguingly, platelets express functional toll-like receptors (TLRs) (74), which are a major family of receptors that recognize pathogen-associated molecular patterns (PAMPs). In the context of thrombosis and inflammation, it has lately been revealed that platelet TLR2 can accelerate thrombosis in hyperlipidemic $ApoE^{-/-}$ mice (75). Further interactions of platelet TLRs in thrombo-inflammatory responses have been extensively reviewed elsewhere (76). In addition, complement receptors for C3a (C3aR) and C5a (C5aR) have been detected on platelets (77, 78); whereby platelet C5aR has been correlated to markers of platelet activation (79). Interestingly, a strong positive correlation of platelet C3aR expression with activated GPIIb/IIIa has been reported in thrombi obtained from patients with myocardial infarction (77). Besides, C3 on platelets has been shown to be elevated in ischemic stroke (80), further indicating an intimate relation between the complement system and platelets in cardiovascular diseases.

CD40 and CD40L (a member of the tumor-necrosis factor [TNF] superfamily, also named as CD154) are a receptor and its corresponding ligand which are decisive mediators of interactions between lymphocytes and antigen-presenting cells (81). Remarkably, CD40L has been implicated in numerous inflammatory conditions, such as atherothrombotic diseases (82) or else neuro-inflammatory disorders including cerebral malaria (83), Alzheimer's disease (AD) (84, 85) as well as HIV-associated CNS-inflammation (86). CD40L is present in the granules of resting platelets (87) and is rapidly translocated to the platelet surface upon activation (88). Platelet-expressed CD40L has been indicated to affect DCs, B cells as well as T cells, providing a crosslink between innate and adaptive immunity (89). Moreover, platelet CD40L interacts with CD40 on endothelial cells, promoting secretion of chemokines, such as IL-8 and monocyte chemotactic protein 1 (MCP1) as well as expression of adhesion molecules such as E-selectin, vascular cell adhesion molecule 1 (VCAM-1), and ICAM-1 (88). Platelet CD40L also contributes to neuroinflammation by inducing activation of astrocytes and microglia (90). Furthermore, activated platelets express soluble CD40L (sCD40L) which in turn induces endothelial secretion of IL-6 and surface expression of P-selectin. Thus, CD40L-mediated interactions promote migration of leukocytes to the site of vascular injury and subsequent adhesion (46).

The potential role of platelets in (neuro-) inflammation can be underlined by findings from neurologic complications of malaria. In Patients with Malaria, platelets were observed binding directly with and killing intraerythrocytic parasites of each of the *Plasmodium* species studied, a process which seems to be dependent on PF4 (91). In fact, thrombocytopenia is a hallmark of blood-stage plasmodium infection, and malaria is characterized by a procoagulant state that is most pronounced in Plasmodium falciparum (Pf) infections, the most virulent of the 5 species of Plasmodium infecting humans (92, 93). Other studies do not favor the hypothesis of direct killing of bacteria by platelets, but rather suggest an indirect inflammation-activating effect. Recently, it was demonstrated that platelets elicit the pathogenesis of malaria and that platelet CD40 is a key molecule in this process using an adoptive transfer model of WT platelets into CD40-KO mice, which are resistant to experimental cerebral malaria, whereby experimental cerebral malaria mortality and symptoms in CD40-KO recipients was partially restored (94). Platelet depletion experiments demonstrated that platelets contribute to the inflammatory response of experimental cerebral malaria, particularly in the early phase (95, 96).

Summarized, the diversity of platelet receptors participating in platelet interactions reveals various interesting targets within the context of platelet-mediated inflammation. Thus, the most promising targets during neurovascular inflammation are illuminated below.

Platelets, the Coagulation Cascade and Thrombosis

The classical plasmatic coagulation cascade of secondary hemostasis consists of the contact activation (intrinsic) pathway, the tissue factor (TF; extrinsic) pathway as wells as

the final common pathway (97). This traditional theory of blood coagulation is suitable for describing coagulation *in vitro* but it has flaws as a model of the hemostatic process *in vivo* (98). For instance, the model cannot explain why hemophilia A patients bleed although they have an intact "extrinsic" pathway (99). Thus, a current cell biological model of coagulation divides coagulation into three overlapping phases: Firstly, the initiation phase, in which low amounts of active coagulant factors are generated. At this stage, TF in damaged vessel binds "extrinsic" factor (F)VIIa to activate "intrinsic" FIX as well as FX. In the second stage, the amplification phase, levels of active coagulation factors, such as thrombin are boosted, leading to platelet-activation by cleaving protease-activated receptor 1 (PAR1). Finally, in the propagation phase, coagulation factors bind to procoagulant membranes of activated platelets driving formation of fibrin clots (100). Hence, according to the cellular model of coagulation, the "intrinsic" pathway mainly serves as an amplification loop initiated by the TF pathway (100).

Nevertheless, one should not undervalue the role of the "intrinsic" pathway. For instance, platelets are able to activate FXII as they contain negatively charged polyphosphates (polyP) which can be externalized onto the cell membrane upon platelet activation (101). Thereby, platelets promote subsequent activation of plasma kallikrein, FIX and further downstream coagulation factors of the 'intrinsic' pathway (102). Interestingly, polyP-dependent FXII activation does not yield a faster clot formation, but rather an increased fibrin clot stability (100). Accordingly, high levels of FXII were associated with thrombosis, whereas FXII inhibition reduces thrombus formation in mice (103) as wells as in primate thrombosis model (104). However, FXII deficiency is not associated with bleeding (105). Thus, targeting FXII might be a pharmacological option in order to reduce arterial thrombosis risk without influencing hemostasis (106). In line with this, deficiency or inhibition of FXII protected mice from ischemic brain injury (107, 108): Using a transient middle cerebral artery occlusion (tMCAO) modell (109), Kleinschnitz et al. found that the volume of infarcted brain in FXII-deficient ($FXII^{-/-}$) and FXII inhibitor–treated mice are substantially less than in wild-type (WT) controls, without an increase in infarct-associated hemorrhage (107). Furthermore, treating $FXII^{-/-}$ mice with human FXII "rescued" the WT phenotype regarding infarct volume as well as intravascular fibrin and platelet deposits leading to vessel occlusion (107). The importance of FXII in neurovascular thrombo-inflammatory diseases is underlined by the notion that a lack of its downstream coagulation factor XI has protective effects against stroke in humans (110) as well as in tMCAO mice model (107). Besides, activation of the kallikrein–kinin system (KKS) by FXII stimulates the production of the potent proinflammatory peptide bradykinin (111). Strikingly, bradykinin receptor B1 knockout mice have been shown to develop reduced brain infarct volumes after tMCAO compared with WT controls (112); thereby, crosslinking FXII-mediated thrombotic activity to inflammation.

Further strengthening the hypothesis that an interaction of platelets with the intrinsic pathway of coagulation could contribute to neurovascular inflammation and stroke, Choi et al. have demonstrated that polyP secreted by activated human platelets also accelerates factor XI activation mediated by thrombin (113). However, a potential direct crosslink between synthesis of polyP in platelets and the involvement of the coagulation cascade in stroke has not yet been investigated, as the protein(s) responsible for the polyP synthesis in higher eukaryotic species have not been identified so far (114). Nevertheless, neutralizing polyP using recombinant *Escherichia coli* exopolyphosphatase (PPX) (115) in tMCAO mice model could be an absorbing alternative experimental approach.

Beside interacting with the "intrinsic" pathway of the coagulation system, activated platelets may release TF after *de novo* synthesis (116). However, this assumption is the subject of a controversial discussion, as other, flow cytometric based, investigations indicated that no TF would be expressed on activated platelets (117). Only recently has the debate whether platelets can release TF by themselves been portrayed elsewhere in detail (118, 119). Regardless of this debate, platelet CD40L expression has been reported to induce monocyte expression of tissue factor, which in turn activates the extrinsic coagulation cascade (120); thus, emphasizing the intimate interaction between platelets, immune cells and the plasmatic coagulation system.

CONTRIBUTION OF PLATELETS TO NEUROVASCULAR THROMBOSIS AND THROMBOINFLAMMATION

Stroke is the second leading cause of death and third most common cause of disability worldwide. Approximately 80% of all strokes are caused by cerebral ischemia, whereas hemorrhagic events account for the remainder (121). Most nonlacunar ischemic strokes are of thromboembolic origin, with common sources of embolism being cardiac diseases, particularly atrial fibrillation, as well as symptomatic extracranial large artery atherosclerosis (122). Immediately after intracranial vessel occlusion by an embolus the lack of oxygen and glucose in the affected brain tissue leads to focal neurological deficits such as hemiparesis or aphasia. The mainstay of treatment for ischemic stroke is prompt recanalization by thrombolysis or mechanical thrombectomy (123). Unfortunately, many patients display secondary infarct growth despite successful vessel recanalization. As indicated above, reperfusion injury has been attributed to the thrombo-inflammatory activity of platelets and immune system cells (124). In particular, evidence suggests that T cells crucially contribute to reperfusion injury in stroke as immunodeficient $Rag1^{-/-}$ mice, which are lacking of T cells and B cells, developed smaller infarcts after tMCAO compared with WT mice (125, 126). Additionally, the critical contribution of T cells to brain injury in stroke had been further highlighted, as adoptive transfer of T cells, to $Rag1^{-/-}$ mice restored susceptibility to reperfusion injury after tMCAO (125, 126). Later on, particularly Forkhead box P3 (FOXP3)positive regulatory T (T_{reg}) cells have been identified as the detrimental type of T cells in ischemia–reperfusion injury (127). Strikingly, the removal of platelets from the circulation of $Rag1^{-/-}$ mice that received adoptive transfer of T_{reg} cells has led to infarcts that were as

small as in naive Rag1$^{-/-}$ mice after tMCAO (127). Thus, this investigation of Kleinschnitz et al. first discovered that the harmful effects of T cells in ischemia–reperfusion depend on platelets; thereby, underlining the determining role of platelets in stroke-associated thromboinflammation in a compelling fashion.

However, blockade of platelet GPIIb/IIIa receptor has led to intracranial hemorrhage and has not reduced cerebral infarct sizes following tMCAO in mice (128). In line with this, anti-GPIIb/IIIa treatment of patients with acute ischemic stroke is associated with a significant risk of intracranial hemorrhage with no evidence of any reduction in death or disability in survivors (129). Thus, final platelet aggregation *via* GPIIb/IIIa is not the critical mechanism underlying thromboinflammation and reperfusion injury in stroke.

In view of the delineated GPIb-mediated interaction between platelets and leukocytes, the vWF/GPIb axis could, however, be a potential pathomechanism of thromboinflammation in stroke. Indeed, blockade of vWF binding site on GPIb using p0p/B has reduced infarct size and improved reperfusion as well as neurological status after tMCAO (128). These effects were detected both in prophylactic (1 h before tMCAO) and therapeutic (1 h after tMCAO) setting. Furthermore, it has recently been revealed that inhibition of GPIb not only reduces infarct size but also limits the local inflammatory response in the ischemic brain, since levels of inflammatory cytokines and infiltration of T cells as well as macrophages were reduced after GPIb inhibition (130). Notably, GPIb blockade has not been accompanied by an increase in intracerebral bleeding complications (128). In line with these findings, both GPIb-deficient (131) and vWF-deficient mice (130) displayed smaller infarcts and a better neurological outcome than WT mice after tMCAO. Accordingly, apoptosis in the brain tissue was reduced in GPIb-deficient mice (132). Thereby, Schleicher et al. revealed that platelets induce neuronal apoptosis *via* expression of membrane bound Fas ligand (FasL) (132).

Exemplifying the suggested importance of the interaction between leukocyte Mac-1 and platelet GPIb in neurovascular thromboinflammation, mice deficient in Mac-1 have been found to be less susceptible to cerebral ischemia (133). Further supporting the role of the vWF-GPIb axis, mice lacking *A disintegrin and metalloprotease with thrombospondin type 1 repeats 13* (ADAMTS13), an enzyme that cleaves highly thrombogenic large vWF to smaller and less active vWF, are more vulnerable to brain damage following tMCAO (134). The reperfusion injury in ADAMTS13-deficient mice has further been accompanied by an increased accumulation of immune cells in the ischemic brain (134), underscoring the role of inflammation in neurovascular thrombosis. In accordance with experimental findings, high serum levels of vWF in patients as well as autoantibodies against ADAMTS13 have been identified as risk factors for stroke (135, 136).

As outlined above, further platelet activation following vWF-GPIb interaction is mainly driven by GPVI. Displaying GPVI as another key player in the neuronal damage during stroke, its inactivation by GPVI antibody (JAQ1) caused reduced brain infarct volumes after tMCAO without increasing the risk for cerebral hemorrhage (128). In addition, Kraft et al. have demonstrated that both GPVI and GPIb blockade protect from stroke in aged mice, mice with diabetes mellitus as well as hypertensive mice, suggesting that targeting GPVI or GPIb may be a future therapeutic option for patients with accompanying common metabolic diseases (137). Accordingly, inhibition of phospholipases D1 and D2, which are downstream signals of the vWF-GPIb axis in platelets (138), has yielded reduced susceptibility to stroke progression following tMCAO again without increasing bleeding risk (139). Likewise, the blockade of GPVI dependent downstream pathways has been reported to protected from stroke progression after tMCAO by reducing Ca^{2+} responsiveness in platelets (140). Platelet granule secretion depends on intracellular Ca^{2+} mobilization (141) and has been demonstrated to be crucial in ischemic-reperfusion injury (142). For instance, mice showing deficiency in both platelet dense granule secretion (143) and α-granule secretion (144) were protected from cerebral ischemia after tMCAO without observation of intracranial hemorrhage.

A role in cerebral ischemia-reperfusion injury has also been described for CD40L. According to Ishikawa et al., both CD40 and CD40L-deficient mice showed reduced infarct volume after tMCAO compared with WT mice (145). This notion was accompanied by diminished platelet/leukocyte adhesion, blood cell recruitment and neurovascular permeability in CD40(L)-deficient mice. Supporting the role of CD40/CD40L in thromboinflammation, plasma levels of sCD40L were significantly higher in patients with acute cerebral ischemia compared with controls. Furthermore, CD40 expression on monocytes was higher in stroke group, accompanied by significantly increased amount of prothrombotic platelet-monocyte aggregates (146).

The insinuated contribution of the complement system to platelet-mediated thromboinflammation has recently been depicted in a gripping fashion: Using C3aR$^{-/-}$ mice, Sauter et al. demonstrated not only that complement activation fragment C3a regulates bleeding time but also that C3aR$^{-/-}$ mice are less prone to experimental stroke and myocardial infarction (77). Notably, reconstitution of C3aR$^{-/-}$ mice with C3aR$^{+/+}$ platelets has restored bleeding time and susceptibility to reperfusion injury after tMCAO (77). In this context, it is worthwhile to mention the association of high serum levels of complement lectin pathway activator mannan-binding lectin (MBL) with cardiovascular diseases such as stroke (147). In accordance, infarct volumes and neurological deficits after tMCAO were smaller in MBL$^{-/-}$ mice than in WT controls. Remarkably, Orsini et al. have recently demonstrated that protection of MBL$^{-/-}$ mice against cerebral ischemia-reperfusion injury is accompanied by a less inflammatory phenotype of platelets as indicated by reduced IL-1α content in platelets (148). Furthermore, cultured human brain endothelial cells subjected to a lack in oxygen/glucose and exposed to platelets from MBL$^{-/-}$ mice displayed less cell death and lower CXCL1 release than those exposed to WT platelets (148). These observations distinctly underscore the importance of the pathophysiological crosstalk between platelets, brain endothelial cells, and mediators of the immune system in reperfusion injury of the brain.

Taken together, particularly GPIb, GPVI, C3aR, and MBL are crucial for platelets orchestration of thromboinflammation in

stroke (**Figure 1**). Therefore, corresponding translational approaches that may provide novel therapeutic strategies in stroke treatment and prevention are depicted further below.

CONTRIBUTION OF PLATELETS TO NEUROVASCULAR INFLAMMATION IN NEURODEGENERATIVE DISEASES

Neuroinflammation has been associated with a variety of further diseases including amyotrophic lateral sclerosis (ALS), epilepsy, traumatic brain injury, Parkinson's disease, and Huntington's chorea (149) but also with non-neurological chronic conditions such as rheumatoid arthritis, obesity and diabetes (150, 151). While the contribution of platelets to central nervous system (CNS)-inflammation in some of these diseases has recently been reviewed elsewhere (152), this review focuses on MS and Alzheimer's disease (AD).

Platelets in Experimental Autoimmune Encephalomyelitis and Multiple Sclerosis

MS is a chronic demyelinating and neurodegenerative disease. Although, the pathogenesis of MS is still not completely understood, it is commonly accepted as a heterogeneous, immune-mediated condition which is caused by gene–environment interactions (153). Focal areas of demyelination (plaques) constitute a pathological hallmark of MS. These areas are typically characterized by breakdown of the blood-brain barrier (BBB), whereby antigenpresenting cells (APCs) such as B cells and myeloid cells (macrophages, dendritic cells and microglia) pass through the BBB and initiate the differentiation of memory T cells into pro-inflammatory T helper (Th) lymphocytes (Th1 and Th17). Subsequent recruitment of

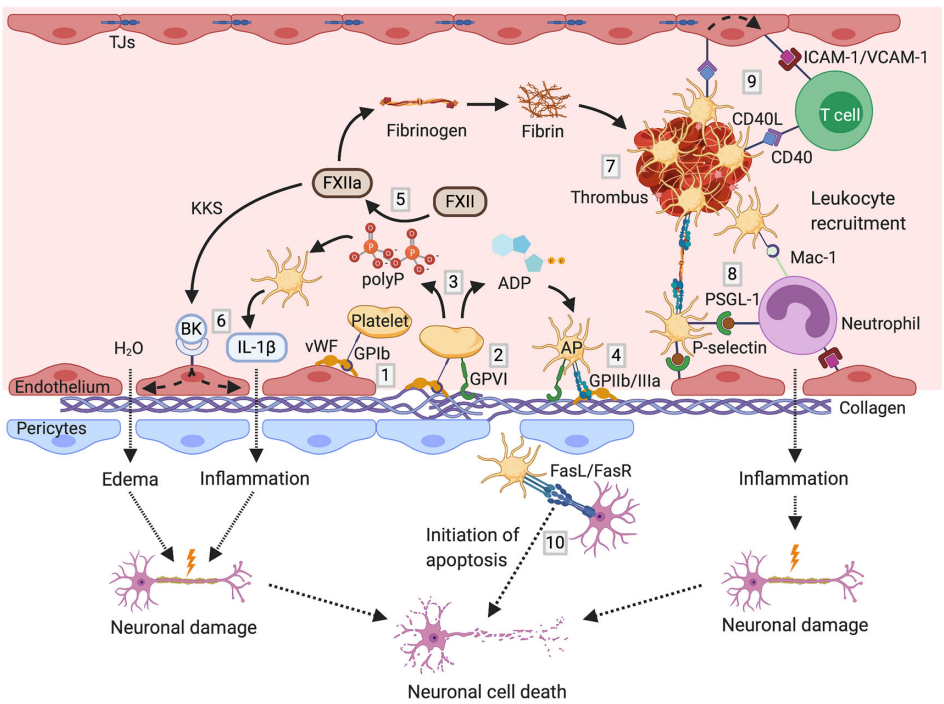

FIGURE 1 | Mechanisms of thromboinflammation in stroke; partially adopted and modified from (206, 207): Initial tethering of platelets to the extracellular matrix or endothelium at the site of ischemic vascular injury is mediated by GPIb binding to exposed vWF (1). The interaction between platelet GPVI receptor and subendothelial collagen triggers platelet activation (2). Activated platelets release paracrine factors including ADP and polyP (3), promoting functional upregulation of GPIIb/IIIa (4). Negatively charged polyP activate coagulation FXII (5). FXIIa stimulates the activation of the KKS, thereby promoting the release of the proinflammatory peptide bradykinin. In company with further cytokines such as IL-1ß, bradykinin causes endothelial cell damage leading to vascular edema and neuronal damage (6). On the other hand, FXIIa initiates the intrinsic coagulation pathway, triggering thrombus formation by fibrin engenderment (7). Activated platelets mediate thromboinflammation also by recruitment of leukocytes *via* binding of platelet P-selectin to leukocyte PSGL-1 as well as *via* GPIb/Mac-1 interaction (8). Stable tethering of leukocytes to the vessel wall is achieved by the interaction between platelet CD40L with CD40 on endothelial cells, promoting the expression of adhesion molecules such as ICAM-1 and VCAM-1 on endothelial cells (9). Thereby, platelets orchestrate the infiltration of immune cells into the brain parenchyma leading to further neuronal damage. Besides, platelets can initiate apoptosis *via* the expression of death receptor FasL on their surface (10). ADP, adenosine diphosphate; AP, activated platelet; BK, bradykinin; FasL, Fas ligand; FasR, Fas receptor; FXII, factor XII; GP, glycoprotein; ICAM-1, intercellular adhesion molecule 1; IL, interleukin; KKS, kallikrein–kinin system; Mac-1, macrophage-1 antigen; polyP, polyphosphates; PSGL-1, P-selectin glycoprotein ligand-1; TJs, tight junctions; VCAM-1, vascular cell adhesion protein 1; vWF, von Willebrand factor. Figures created with BioRender.com.

inflammatory effector cells into the CNS parenchyma is mediated by leukocyte or endothelial adhesion molecules and accompanied by pro-inflammatory stimulation of microglial cells which promotes destruction of axonal myelin sheath (153).

Platelet abnormalities in MS patients were already reported decades ago (154, 155). These observations are supported by more recent reports that have detected platelet specific GPIIb (CD41) in MS plaque of patients as well as in brain tissue of mice with experimental induced autoimmune encephalomyelitis (EAE, a counterpart of MS in mice) (14, 156). Accordingly, cerebrospinal fluid (CSF) levels of PAF have been correlated with both EAE (157) and MS disease activity (158). Interestingly, PAF receptor knockout have yielded a diminished severity of inflammation and demyelination in EAE mice (157). Recently, it was demonstrated that brain-abundant gangliosides GT1b and GQ1b were specifically recognized by platelets and platelets recognize brain-specific glycolipids in area of perivascular space thereby, triggering immune response cascades (159).

Unequivocally demonstrating a crucial contribution of platelets to EAE disease pathogenesis, platelet depletion has attenuated EAE in mice, particularly in the effector phase of the disease; thereby, reducing CNS mRNA levels of CCL-2, CCL-5, CCL-19, CXCR-4, and IL-1β as well as the expression of adhesion molecule ICAM-1 (14) (**Figure 2**). Consistently, recruitment of leukocytes to the inflamed CNS has been diminished by platelet depletion (14, 160). Furthermore, administration of blocking antibodies against GPIIb/IIIa as well as platelet GPIb and its interaction with leukocyte counterreceptor Mac-1 has ameliorated EAE; thus, the involvement of platelets in EAE is regarded to be multi-faceted (14). By contrast, P-selectin is not required for the development of EAE (161).

Besides, serotonin from platelets dense granules may also induce neuroinflammation in EAE, since platelet serotonin has been reported to promote neutrophil recruitment to sites of acute CNS inflammation in mice (162). Remarkably in this context, serotonin transporter depleted mice were less susceptible to EAE (163), and in addition, treatment with selective serotonin-reuptake inhibitor fluoxetine reduced disease activity of relapsing MS patients (164). Interestingly, the secretion of serotonin by platelets has been demonstrated to stimulate differentiation of T cells toward pathogenic Th1, Th17, and interferon-γ/interleukin-17–producing CD4 T cells in a stage-depended manner: Early in MS and EAE, high levels of platelet-derived serotonin stimulate differentiation of pathogenic T cell subsets, promoting proinflammatory responses (165). At the later stages of MS and experimental autoimmune encephalitis, platelets became

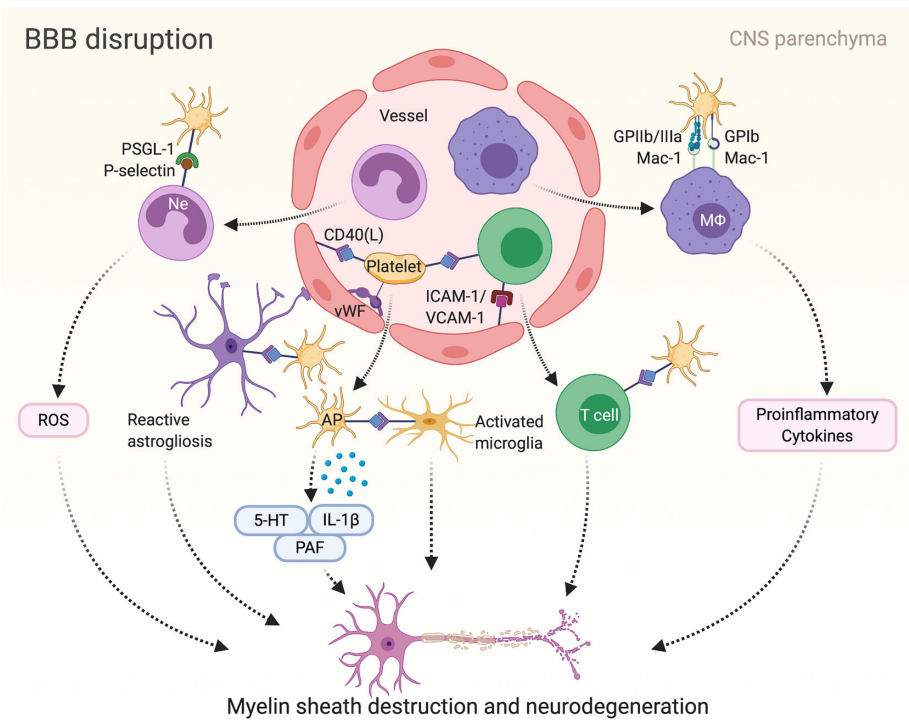

FIGURE 2 | Platelet mediated inflammation in multiple sclerosis (MS) and corresponding mice model of experimental autoimmune encephalomyelitis (EAE): Autoimmune T cells induce the breakdown of the blood-brain barrier (BBB) in multiple sclerosis. Consequently, inflammatory cells such as lymphocytes, macrophages (MΦ) and neutrophils (Ne) penetrate the BBB, promoting reactive activation of astrocytes and microglial cells and finally leading to myelin sheath destruction and axonal damage. Platelets can mediate neuroinflammation in MS/EAE by adhering to the endothelium and interacting with inflammatory and endothelial cells in various ways as depicted here. Furthermore, platelets release serotonin (5-HT), interleukin (IL)-1β and platelet activating factor (PAF), which in turn have been associated with disease progress in MS. AP, activated platelet; GP, glycoprotein; ICAM-1, intercellular adhesion molecule 1; Mac-1, macrophage-1 antigen; PSGL-1, P-selectin glycoprotein ligand-1; ROS, reactive oxygen species; VCAM-1, vascular cell adhesion protein 1; vWF, von Willebrand factor.

exhausted in their ability to produce proinflammatory factors and stimulate CD4 T cells but increase their ability to form aggregates with CD4 T cells, thereby decreasing T-cell activation and downmodulating EAE (165).

Furthermore, Sotnikov et al. demonstrated a new role of platelets in in the pathogenesis of EAE as P-selectin on platelets can interact with sialated glycosphingolipids (gangliosides) that are integrated in astroglial and neuronal lipid rafts which may constitute a new type of the neuronal damage danger signal (159). During neuroinflammation, platelets recognize these specific cerebral glycolipid structures and accumulate in the central nervous system parenchyma triggering further immune response cascades. Fascinatingly, preventing the interaction between platelets and brain-derived lipid rafts in the CNS substantially ameliorated EAE development (159).

Addressing neuropsychiatric symptoms of MS, such as anxiety and depression, it has recently been shown that GPIb antibody-mediated platelet depletion prevented the EAE-induced increase in anxiety-like behavior which was associated with reduction of the pro-inflammatory environment to control levels in the hippocampus of mice (166).

However, it is suggested that platelets are only one player in the interaction of coagulatory and thrombo-inflammatory systems with neuroinflammation in MS. For instance, tissue factor as well as thrombin were highly expressed in chronic active lesions of MS patients (167). Interestingly, thrombin inhibition by hirudin has ameliorated EAE (167). Furthermore, Göbel et al. have reported that deposition of FXII is detectable in CNS tissue of MS patients (168). Grippingly, deficiency, or pharmacologic blockade of FXII have rendered mice less susceptible to EAE (168). Considering the above depicted interaction of platelets and FXII, a FXII-mediated contribution of platelets to EAE might be feasible.

To recapitulate, both platelet GPIIb/IIIa and GPIb receptor embody promising targets for future MS therapy. Furthermore, the $P2Y_{12}$ receptor antagonists clopidogrel and ticagrelor have recently been shown to alleviate disease severity of EAE in mice (169). However, neither glycoprotein inhibitors nor ADP receptor antagonists have yet been investigated in clinical trials for treatment of MS patients. But interestingly, glatiramer acetate (Copaxone), an FDA and EMA approved drug for the treatment of MS, has been demonstrated to inhibit thrombin-induced calcium influx in human and mouse platelets. Furthermore, glatiramer acetate also decreased thrombin-induced PECAM-1, P-selectin, and active form of GPIIb/IIIa surface expression and formation of platelet aggregates for both mouse and human platelets, suggesting that glatiramer acetate inhibit neuroinflammation by affecting not only immune cells but also platelets (170).

Implications of Platelet Activation for Alzheimer's Disease

AD is a neurodegenerative brain disorder that slowly leads to severe cognitive impairment. The neuropathological hallmarks of AD constitute the formation of intracellular neurofibrillary tangles and the deposition of amyloid-ß (Aβ) in brain tissue and cerebral vessels (so-called cerebral amyloid angiopathy, CAA), accompanied by neuroinflammation as well as neuronal and synaptic loss. Interestingly, platelets constitute the primary source of amyloid-ß peptide (Aß) and its precursor protein, amyloid precursor protein (APP), in the blood (171, 172), as they are secreted following platelet activation (173, 174). Evidence suggests that both and APP play a role in regulating thrombosis and hemostasis (175, 176).

Two decades ago enhanced platelet activation was demonstrated in AD patients (177). Later, this was referred to an increased lipid peroxidation (178). In accordance, platelets have shown enhanced activity and increased adhesion to subendothelial matrix components in transgenic mice model of AD (179, 180). Further pointing to a pathophysiological relevance of platelets in AD progression, activity of ß-secretase, an enzyme which is required for the cleavage of APP, has been shown to be elevated in peripheral blood platelets of patients suffering AD compared to controls (181).

Interestingly, prior to Aß plaque formation, aggregated platelets were shown as a first pathological sign in AD mouse model, suggesting platelets as therapeutic target in early AD (182). Indeed, Donner et al. found that synthetic monomeric $A\beta_{40}$ can bind through its RHDS (Arg-His-Asp-Ser) sequence to GPIIb/IIIa, stimulating the secretion of ADP and the chaperone protein clusterin from platelets (183). This was accompanied by the formation of fibrillar Aβ aggregates and further $A\beta_{40}$ binding to platelets in a feed-forward loop (183). Strikingly, clopidogrel inhibited Aβ aggregation in platelet cultures; and further, platelet inhibition diminished the amount of clusterin in the circulation as well as the incidence of CAA in transgenic AD model mice (183). Underscoring anti-platelet drugs potential as useful therapeutic targets in counteracting CAA and AD, it has been demonstrated that platelets isolated from AD mice promote severe vessel damage, matrix metalloproteinases activation and neuroinflammation in wildtype mice brain, in an organotypic *ex vivo* brain slice model, thereby inducing Aß-like immunoreactivity at the damaged vessel sites (184).

Beyond the illustrated potential therapeutic relevance of platelets, recent metabolomic analysis revealed that platelet phosphatidylcholines constitute promising biomarkers to diagnose AD (185) and CAA (186).

PLATELETS IN THE MODULATION OF NEURONAL ELECTRIC ACTIVITY, SYNAPTIC FUNCTIONS, AND PLASTICITY

As already discussed, brain-enriched glycosphingolipids within neuronal lipid rafts were shown to induce platelet degranulation and secretion pro-inflammatory factors (159). In traumatic brain injury (TBI) - induced inflammation model the interaction of platelets with neuronal lipid rafts has been displayed to stimulate neurite growth, increase the number of postsynaptic Sontikov Idensity protein 95-positive dendritic spines, and intensify neuronal activity (187). Using adoptive transfer and blocking experiments the authors demonstrated that platelet-derived serotonin and platelet activating factor play a key role in regulation of neuroinflammation and neuronal plasticity after TBI (187).

With respect to the modulation of neuronal electric activity, a more recent study demonstrated that platelets substantially enhance epileptic seizures in a mouse model of pentylenetetrazole (PTZ) -induced seizures (188). Thereby, platelets ecreted serotonin, contributed to increased BBB permeability. In addition, platelets directly stimulated neuronal electric activity and induced the expression of genes related to early neuronal response and neuroinflammation. Grippingly, intracranial injection of platelets was sufficient to induce severe seizures, demonstrating to a novel role of platelets in the development of epileptic seizures, and pointing to potential new therapeutic approaches by targeting platelets to prevent and treat epilepsy (188).

POTENTIAL TRANSLATIONAL AND CLINICAL APPLICATIONS

To date, patients with non-cardioembolic ischemic stroke or transient ischemic attack (TIA) receive antiplatelet therapy with acetylsalicylic acid (aspirin) or clopidogrel for secondary prevention (189). However, experimental *in vivo* studies in mice have revealed that treatment with ticagrelor reduces infarct size and recovers neurological function after tMCAO to a greater extent than aspirin (190). Nevertheless, the SOCRATES clinical trial demonstrated that ticagrelor is not superior to aspirin in reducing the rate of stroke, myocardial infarction, or death at 90 days after acute ischemic stroke or TIA (191). However, current results of the THALES trial have demonstrated that the risk of the composite of stroke or death within 30 days in patients with a mild-to-moderate acute noncardioembolic ischemic stroke or TIA was lower with ticagrelor and aspirin than with aspirin alone, while severe bleeding was more frequent with ticagrelor (192).

Emphasizing the portrayed role of GPVI, the novel GPVI-Fc fusion protein Revacept, which blocks the collagen target for GPVI binding, has been shown to improve cerebral infarct volume and functional outcome in murine stroke model (193). Furthermore, Revacept has enhanced the efficacy of thrombolysis treatment after tMCAO in mice (194). Therefore, a clinical phase II trial aims to examine whether patients suffering from symptomatic carotid artery stenosis, TIA or stroke take advantage of Revacept plus antiplatelet therapy compared to antiplatelet therapy alone (NCT01645306). A further phase II trial will assess the efficacy and safety of Revacept in patients undergoing elective PCI (195). In addition, a complete blockade of platelet GPVI using a monoclonal anti-GPVI antibody (ACT017) constitutes an alternative therapeutic approach, although bleeding risk might be higher than in therapy with Revacept (196). Therefore, a clinical phase II trial assessing the safety of ACT017 application in patients with an acute ischemic stroke has recently begun (NCT03803007).

With respect to GPIb, Caplacizumab is an anti-vWF humanized single-variable-domain immunoglobulin (so called nanobody) that inhibits the interaction between ultra large vWF multimers and GPIb on platelets (197). Considering the portrayed significance of the vWF-GPIb axis in preclinical ischemic-reperfusion injury models, caplacizumabs platelet-protective effect in thrombotic thrombocytopenic purpura (TTP) (197) raises hope that this novel vWF-inhibitor might be protective in patients with ischemic stroke as well.

Furthermore, vorapaxar, a PAR-1 inhibitor, has been beneficial in the secondary prevention of atherothrombotic events in a phase III clinical trial (198). However, the PAR-1 inhibitor increased the risk of moderate or severe bleeding, including intracranial hemorrhage; thus, vorapaxar should not be used in persons with history of stroke, transient ischemic attack or intracranial hemorrhage (199). In addition, the PAR-4 inhibitor BMS-986141 is currently being investigated in a phase II trial, examining whether it is effective in reducing the recurrence of stroke in patients that have recently suffered an acute stroke or TIA and receive aspirin (NCT02671461).

Intriguingly, the phosphodiesterase (PDE)-3 inhibitor Cilostazol, which diminishes platelet aggregation by decreasing levels of cyclic adenosine monophosphate (cAMP), has been suggested to reduce stroke recurrence in patients with a prior ischemic stroke (200). In accordance, Bieber et al. have only recently concluded that another novel PDE-3 inhibitor (substance V) protects mice from infarct injury after tMCAO (201). Surprisingly, substance V did not affect platelet function (201).

In respect of MS, the treatment with PDE-4 inhibitor ibudilast (MN-166), that has been reported to inhibit platelet aggregation as well (202), was associated with slower progression of brain atrophy in patients with progressive MS (203). Furthermore, aspirin has latterly been demonstrated to ameliorate EAE in mice (204). As the effect of aspirin on general disease activity is inconclusive (205), further studies are needed to determine the benefits and risks of aspirin but also GPIIb/IIIa, GPIb and $P2Y_{12}$ receptor antagonists in patients with MS.

CONCLUSION

In conclusion, growing evidence suggests a crucial involvement of platelets in orchestration of neuroinflammation. Therefore, platelets could be considered as immune cells. A broad range of recent experimental approaches indicate that platelets participate in pathogenesis of AD, MS, and stroke associated neuroinflammation. Expanding our knowledge about these novel concepts will help to further understand mechanisms of neuro-inflammatory diseases and could reveal feasible therapeutic strategies with the aim of improving patient's quality of life.

AUTHOR CONTRIBUTIONS

ER wrote the manuscript in consultation with HL. HL conceptualized and submitted the manuscript. All authors contributed to the article and approved the submitted version.

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Assessing Genetic Overlap Between Platelet Parameters and Neurodegenerative Disorders

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Neurodegenerative disorders such as Parkinson's disease (PD) and Alzheimer's disease (AD) suffer from the lack of risk-predictive circulating biomarkers, and clinical diagnosis occurs only when symptoms are evident. Among potential biomarkers, platelet parameters have been associated with both disorders. However, these associations have been scarcely investigated at the genetic level. Here, we tested genome-wide coheritability based on common genetic variants between platelet parameters and PD/AD risk, through Linkage Disequilibrium Score Regression. This revealed a significant genetic correlation between platelet distribution width (PDW), an index of platelet size variability, and PD risk (r_g [SE] = 0.080 [0.034]; p = 0.019), which was confirmed by a summary-summary polygenic score analysis, where PDW explained a small but significant proportion PD risk (<1%). AD risk showed no significant correlations, although a negative trend was observed with PDW (rg [SE] =-0.088 [0.053]; p=0.096), in line with previous epidemiological reports. These findings suggest the existence of limited shared genetic bases between PDW and PD and warrant further investigations to clarify the genes involved in this relation. Additionally, they suggest that the association between platelet parameters and AD risk is more environmental in nature, prompting an investigation into which factors may influence these traits.

Keywords: neurodegenerative disorders, Parkinson disease, Alzheimer disease, platelets, genetics, platelet distribution width, genetic correlation

INTRODUCTION

Common neurodegenerative disorders due to accumulation of neurotoxic protein aggregates, such as Alzheimer's disease (AD) and Parkinson's disease (PD), suffer from the lack of risk-predictive circulating biomarkers, and clinical diagnosis occurs only when symptoms are evident, at an advanced stage of neurodegeneration (1, 2). Therefore, it is important to identify potential biomarkers that are easy to measure and that could predict the incident risk of such diseases,

e.g., circulating biomarkers (3). Among these, platelets have received increasing attention (4–6), and their link with neurodegenerative disorders has long been hypothesized (7). Indeed, platelets are considered "circulating mirrors of neurons" and share many similarities with neural cells (8). These include the molecular machinery that controls the secretory system (5), patterns of reciprocal interactions, and the metabolism of different neurotransmitters like serotonin and dopamine, but also of neurologically important proteins like the Amyloid Precursor Protein (4).

In spite of these interesting cues, the relation between neurodegenerative disorders and classical blood platelet parameters like mean platelet volume (MPV), platelet count (Plt), and platelet distribution width (PDW) has been scarcely investigated at the epidemiological level, also with relatively common disorders like AD and PD. Observational studies consistently revealed an inverse association of PDW with AD and other forms of mild/severe dementia (9–11), and a positive association with cognitive performance (9, 11). An association of MPV with higher PD risk (12) was also reported but was not replicated in a later study, where MPV showed an increase with PD severity (13). At the genetic level, only two studies have previously investigated the relationship of blood platelet parameters with AD (14) and PD risk (15). In a large Genome Wide Association Scan (GWAS) testing associations of common genetic variants like Single Nucleotide Polymorphisms (SNPs) and small insertions/deletions (indels) with different blood cell measures ($N_{max} \sim 170{,}000$) (14), the authors observed no evidence of a causal effect of Plt, MPV, or PDW on AD risk based on a multivariable Mendelian Randomization analysis. However, this technique may suffer from low power since it is usually based on a low number of variants (16). More recently, Nalls et al. (15) investigated genetic links of platelet parameters with PD risk through Linkage Disequilibrium (LD) Score Regression, which is a more robust approach based on hundreds of thousands of variants genome-wide (16) (see below). They reported non-significant genetic correlations with both Plt and MPV in the largest PD case-control GWAS meta-analysis carried out so far (involving ~ 56,300 PD cases and ~ 1.4 million controls) (15). Of note, in spite of the previous implication of PDW in neurodegenerative disorders (4, 9–11) and, more recently, in comorbid disorders like major depression (16, 17), this parameter has never been investigated with reference to PD risk at the genetic level.

Here, we tested the genetic relationship between the above mentioned platelet parameters, PD and AD risk, making use of summary statistics of large GWAS previously carried out on these traits (14, 15, 18). We first applied LD-score regression analysis to detect significant genome-wide co-heritability based on common genetic variants, and then we further investigated the significant correlations through polygenic risk association analysis (19). The aim of our investigation was twofold. First, we provided a comprehensive re-visitation of the genetic relationship between the most common neurodegenerative disorders—AD and PD—and platelet parameters commonly tested like Plt, MPV, and PDW, in a systematic and homogeneous way. Second, we provided hints into new potential biomarkers of such disorders, to drive future epidemiological, functional, and clinical studies.

METHODS

We applied LD-score regression (20, 21) to summary statistics of large independent GWAS previously conducted on AD (71,880 cases and 383,378 controls) (18), PD (54,376 cases and 1,474,097 controls), and platelet parameters, namely Plt, MPV, and PDW (N_{max} = 166,066) (14) (see **Table S1**). LD score regression models genetic correlation between two traits as a function of LD score among SNPs in 1 cM bins genome-wide, through the formula

$$r_g = \rho_g / \sqrt{h_1^2 * h_2^2},$$

where ρ_g is the genetic covariance between trait 1 and trait 2, and h_1^2 and h_2^2 represent the SNP-based heritability of the two traits (20, 21). SNP-based heritability is in turn computed as the slope of the linear function between χ^2 association statistics and LD score (i.e., the sum of r^2 of a given SNP with all the other SNPs in a 1 cM window), for every SNP tested genome-wide (i.e., for which the association statistics are available in a given GWAS study). For this analysis, we filtered out variants that were not SNPs (e.g., indels), strand-ambiguous SNPs, and SNPs with duplicated rs numbers or Minor Allele Frequency (MAF) ≤ 1%. Moreover, SNPs with low values of sample size were also removed, when detailed information by SNP was available in the summary statistics file (N < 321,820 and < 301,340 for the PD and the AD GWAS, respectively). Finally, we retained only common SNPs (MAF > 5%) in the HapMap 3 EUR reference panel (22)—excluding the HLA region—since these variants have good imputation quality stats (r^2>0.9) in most studies (21). LD scores of these variants were derived using the 1000G phase 1 v3 EUR panel (available at https://data.broadinstitute.org/alkesgroup/LDSCORE/w_hm3.snplist.bz2). Details on the number of variants available before and after quality control (QC) for each study are reported in **Table S1**.

Pairwise comparisons showing significant correlations were further investigated at a more fine-grained resolution, through a summary-summary polygenic risk score (Sum-Sum PRS) analysis using PRSice v1.25 (19). This method tests genetic overlap between two traits by making use of GWAS summary statistics: a training GWAS is used to build the PRS, which is then tested as a linear predictor of another trait in an independent study (target GWAS) (23). We performed Sum-Sum PRS analysis using only SNPs with association p-values (P_T) ≤ 0.05 in the training GWAS (on PDW) (14), in linkage equilibrium ($r^2 < 0.05$) with the local top hit within a 300 kb window, and shared between the training (14) and the target GWAS (on PD risk) (15). To verify the robustness of our results, we repeated the analysis at increasing association significance thresholds in the training GWAS (with P_T = 0.001, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0), as in (23). The number of SNPs meeting these criteria ranged from 2,813 (for $P_T \leq 0.001$) to 213,317 (for $P_T \leq 1$), respectively.

RESULTS AND DISCUSSION

LD score regression analysis revealed a significant genetic correlation between PDW and PD risk (r_g [Standard Error] = 0.080 [0.034]; p = 0.019; see **Tables 1A, B**) suggesting the existence of a genomic overlap based on common genetic variants. When we further analyzed this genetic relationship at a more fine-grained resolution, through Sum-Sum polygenic risk analysis, we observed that a modest but significant proportion of PD susceptibility (<1%) was explained by genetic variants nominally associated with PDW (at P_T = 0.05: p = 7.0×10^{-4}). This association was quite robust across varying p-value thresholds (P_T ranging from 0.001 to 1.0; **Figure 1**). Overall, the evidence reported here suggests PDW as a new potential biomarker for Parkinson's disease and is consistent with previous studies reporting positive associations between PDW and depression risk and/or symptoms, both at the epidemiological level (17, 24) and at the genetic level (16). Indeed, depression represents one of the main non-motor symptoms of PD, often presenting in its prodromal phase (25), and shows progressive patterns of microglial activation like other neurodegenerative disorders (26). Of note, previous epidemiological studies reported negative associations between PDW and the risk of cognitive impairment (9–11), which is co-morbid and partly

TABLE 1 | Genetic correlations of platelet parameters with **(A)** Parkinson's disease and **(B)** Alzheimer's disease risk, based on LD score regression analyses.

A Platelet parameter	#SNPs[a]	r_g	SE	Z-score	p
Plt	916,946	-0.033	0.031	-1.07	0.28
MPV	916,936	0.046	0.031	1.47	0.14
PDW	**916,712**	**0.080**	**0.034**	**2.35**	**0.019**
B Platelet parameter	#SNPs[a]	r_g	SE	Z-score	p
Plt	1,123,504	0.016	0.052	0.30	0.77
MPV	1,123,487	0.010	0.051	0.19	0.85
PDW	1,123,214	-0.088	0.053	-1.66	0.096

Significant genetic correlations (p < 0.05) are highlighted in bold.

[a]*Exact numbers of SNPs used to compute each pairwise genetic correlation (i.e., in common between the studies analyzed).*

Plt, platelet count; MPV, mean platelet volume; PDW, platelet distribution width; SNPs, Single Nucleotide Polymorphisms; rg (SE), genetic correlation and relevant Standard Error.

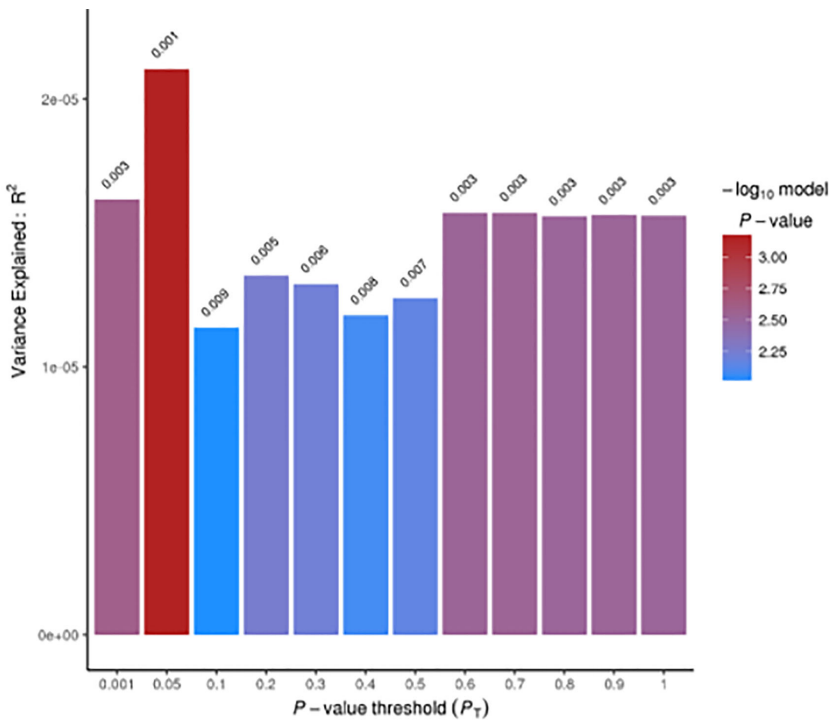

FIGURE 1 | Summary-Summary polygenic risk score (Sum-Sum) analysis between PDW and Parkinson's disease (PD) risk. No direction of effect could be inferred from Sum-Sum analysis, as per PRSice output (19).

shares biological bases with PD (27). While these two relations may appear in contrast, our knowledge of AD and PD is very limited from this point of view, and further epidemiological and clinical studies are needed to clarify the relation of PDW with the different neurodegenerative disorders, possibly through machine learning approaches including other platelet parameters and potential circulating biomarkers, to evaluate their prognostic value simultaneously. Similarly, genetic studies are warranted to identify specific genes influencing both PDW and PD risk.

By contrast, here we did not detect any significant genetic correlations between platelet parameters and AD risk (**Table 1B**), although PDW variability showed a trend of significance (rg [SE] = -0.088 [0.053]; p=0.096). This evidence suggests that the significant associations observed in previous epidemiological studies, which anyway showed a concordant sign (9–11), may be mainly due to shared environmental influences between platelet parameters and AD risk, and that common genetic influences are likely very limited, at least those of common variants. Indeed, a genetic link may have not been detected due to other (possibly rare or structural) genetic variants being at the basis of this. This hypothesis has been supported by recent findings for other complex traits like general cognition, educational attainment (28), and dyslexia (29), where only half of the heritability has been explained by common SNPs. Even so, this study rules out any large genetic overlap between PDW and AD risk.

In spite of the interesting findings reported here, the functional meaning of PDW and its potential usefulness as a biomarker remains to be clarified, beyond the neurodegenerative and neuropsychiatric landscape. As an index of heterogeneity of platelet size, reported associations of this marker with indices of platelet activation (30) suggest PDW might be a useful index of platelet function and procoagulant activity. This open issue, along with the modest co-heritability observed here, suggests caution in the interpretation of these findings and warrants further epidemiological, genetic, and functional studies to substantiate the potential usefulness of PDW as a new biomarker of neurodegeneration.

DATA AVAILABILITY STATEMENT

GWAS summary statistics analyzed in the present study are publicly available at the links reported in **Table S1**.

AUTHOR CONTRIBUTIONS

AG formulated the hypothesis, designed and performed statistical analyses. AT provided theoretical background and reviewed available literature. AG and AT wrote the manuscript, with contributions from all the co-authors. All the authors participated in discussion and interpretation of the results. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Coagulation/Complement Activation and Cerebral Hypoperfusion in Relapsing-Remitting Multiple Sclerosis

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Introduction: Multiple sclerosis (MS) is a demyelinating disease of the central nervous system with an underlying immune-mediated and inflammatory pathogenesis. Innate immunity, in addition to the adaptive immune system, plays a relevant role in MS pathogenesis. It represents the immediate non-specific defense against infections through the intrinsic effector mechanism "immunothrombosis" linking inflammation and coagulation. Moreover, decreased cerebral blood volume (CBV), cerebral blood flow (CBF), and prolonged mean transit time (MTT) have been widely demonstrated by MRI in MS patients. We hypothesized that coagulation/complement and platelet activation during MS relapse, likely during viral infections, could be related to CBF decrease. Our specific aims are to evaluate whether there are differences in serum/plasma levels of coagulation/complement factors between relapsing-remitting (RR) MS patients (RRMS) in relapse and those in remission and healthy controls as well as to assess whether brain hemodynamic changes detected by MRI occur in relapse compared with remission. This will allow us to correlate coagulation status with perfusion and demographic/clinical features in MS patients.

Materials and Methods: This is a multi-center, prospective, controlled study. RRMS patients (1° group: 30 patients in relapse; 2° group: 30 patients in remission) and age/sex-matched controls (3° group: 30 subjects) will be enrolled in the study. Patients and controls will be tested for either coagulation/complement (C3, C4, C4a, C9, PT, aPTT, fibrinogen, factor II, VIII, and X, D-dimer, antithrombin, protein C, protein S, von-Willebrand factor), soluble markers of endothelial damage (thrombomodulin, Endothelial Protein C Receptor), antiphospholipid antibodies, lupus anticoagulant, complete blood count, viral

serological assays, or microRNA microarray. Patients will undergo dynamic susceptibility contrast-enhanced MRI using a 3.0-T scanner to evaluate CBF, CBV, MTT, lesion number, and volume.

Statistical Analysis: ANOVA and unpaired t-tests will be used. The level of significance was set at $p \leq 0.05$.

Discussion: Identifying a link between activation of coagulation/complement system and cerebral hypoperfusion could improve the identification of novel molecular and/or imaging biomarkers and targets, leading to the development of new effective therapeutic strategies in MS.

Clinical Trial Registration: Clinicaltrials.gov, identifier NCT04380220.

Keywords: multiple sclerosis, coagulation, complement, platelets, relapse, infection, cerebral hypoperfusion

INTRODUCTION

Multiple sclerosis (MS) is a chronic demyelinating and degenerative disease of the central nervous system (CNS) with an underlying immune-mediated and inflammatory pathogenesis (1). For many years, self-reactive T cells have been considered to have an exclusive role in the pathogenesis of MS, mainly due to findings from animal disease models such as that on experimental autoimmune encephalomyelitis (EAE) (2). Recent evidence has pointed out the crucial role of both B cells and innate immunity (3–6). In particular, it is hypothesized that innate immunity, which can be considered the immediate non-specific defense against any *noxa patogena* including infections and dangerous agents, not only stimulates and modulates adaptive immunity at MS onset but also mediates its neurodegenerative progressive phase (5, 6). Both inflammation and coagulation are the main effector processes of innate immunity acting synergistically through mutual regulation (7–10). Unlike what is commonly thought, direct and functional vascular injury, such as that caused by hypoxia, sepsis, malignancy, and inflammation, can activate coagulation (11, 12). Thus, thrombosis represents a physiological process in some conditions, "immunothrombosis," an intrinsic effector mechanism of innate immunity (13). Immunothrombosis is aimed at recognizing the pathogens and counteracting their tissue invasion, dissemination, or survival, and it should be limited to only a restricted microvascular area to ensure sufficient overall organ perfusion. It is activated by blood-borne pathogens as well as by circulating self-components that are altered, and through its action on a platform consisting of fibrin, monocytes, neutrophils, and dendritic cells lead to fibrin formation and platelet activation.

In fact, many decades ago a relevant role of both coagulation and vascular thrombosis was hypothesized in MS (14). Subsequently, a number of studies focused on the role of either thrombin, fibrin(ogen), or other coagulation factors in MS due to the findings of both a close association between perivascular fibrin(ogen) deposition and clinical manifestations in EAE and its prompt improvement after the inhibition of thrombin generation by heparin and several anticoagulant agents (15). The discovery of some clotting factors in chronic active MS lesions by a proteomic approach has further strengthened this line of investigation (16).

Fibrinogen, produced by hepatocytes and cleaved off by thrombin, is an acute-phase reactant that increases during the inflammatory response and leads to the formation of insoluble and stable fibrin facilitating the formation of a platelet plug (17–19). In a recent study, high plasma fibrinogen levels resulted in a high specificity but a low sensitivity for detection of active lesions on MRI during relapses, suggesting a role of fibrinogen in the development of MS lesions (20). Moreover, fibrinogen transcripts were found to be present in chronic lesions of MS patients (21). Fibrinogen directly activated microglia *in vitro* and increased its phagocytic ability (22). Fibrinogen also induced the release of reactive oxygen species (ROS) in microglia, necessary for the formation of perivascular microglial clusters and axonal damage in EAE (23), stimulating the production of both tissue factor (TF) (24) and tumor necrosis factor (25) by monocytes. The conversion of fibrinogen to insoluble fibrin is fundamental for the binding of fibrin to the integrin receptor CD11b/CD18 expressed by microglia (18), leading to an increase of several cytokines that modulate cell adhesion and migration (26). Fibrin deposition in MS may precede and coincide with the formation of demyelinating lesions (27, 28) and with the area of axonal damage (29). In fact, a little deposition of extravascular fibrin has been observed in chronic, non-active MS lesions as a consequence of persistent blood-brain barrier (BBB) damage (28). Finally, fibrin-targeting monoclonal antibody immunotherapy could inhibit autoimmunity without suppressing innate immunity or interfering with coagulation (30).

Furthermore, significantly higher plasma levels of prothrombin and factor X have been found in relapsing-remitting (RR) MS (31). Of note, relapse-free time negatively correlated with levels of prothrombin, factor XII, or factor X, indicating that disease exacerbation is characterized by increased coagulation activity (31). Interestingly, the speed of thrombin generation was higher in relapsing-remitting than in primary progressive MS or healthy controls and correlated with time from clinical diagnosis, likely suggesting a differential active proinflammatory state in each MS subtype (32). By the

proteomics approach, some serum proteins such as anti-thrombin, ceruloplasmin, clusterin, apolipoprotein E, and complement C3 were differently expressed in RRMS patients compared to controls (33). Besides, anti-thrombin was oxidatively modified in relapse compared with remission.

Protein C (PC) is a vitamin K–dependent zymogen of a serine protease activated by thrombin when both bind to endothelial cell thrombomodulin (TM) (34). PC also binds to the endothelial protein C receptor (EPCR). Activated PC (APC) is a natural anticoagulant and with its cell membrane localizing cofactor, protein S (PS), binds to both endothelium and activated platelet membranes and interferes with the degradation of procoagulant factor Va and VIIIa, thus limiting further thrombin formation. Recombinant TM ameliorated EAE clinically and pathologically by suppressing plasma levels of inflammatory cytokines (35).

Moreover, APC contributes to endothelial cell integrity (36), inhibits leukocyte adhesion and BBB crossing (37), reduces the production of pro-inflammatory cytokines (36, 38–41), and has anti-oxidant properties (42). A potential role of APC in MS pathogenesis has been hypothesized (43) since it was found reduced in MS patients regardless of their lupus-anticoagulant (LA) activity or factor Va resistance (44).

There are conflicting results in MS regarding the role of antiphospholipid antibodies (APLs), markers of increased coagulation activity, mostly due to methodological issues and to the type of antibodies used in the assays (45). Recently, a consensus has been reached among experts that APL reactivity is higher in MS than in healthy controls. However, this finding could be variable depending on the different disease forms and phases. In particular, a higher APL reactivity appeared to be associated with a more severe clinical and MRI disease progression (46), and with clinical exacerbations, sometimes followed by its decrease in the next months after the relapse (47–50). These thrombogenic mechanisms seem to correlate with neurodegenerative processes (51) enough to consider APLs as a new attractive therapeutic target in MS for use, for example, of hydroxychloroquine, an anti-infective, anti-inflammatory, and anti-thrombotic drug with specific protective property for annexin-V anticoagulant shield (52).

There are a number of studies confirming the involvement of complement in the pathogenesis of MS, highlighting its important role due to interrelation with coagulation as well as with both innate and adaptive immunity (53–57). Its components have been proposed as biomarkers of both MS disease activity and patient therapeutic response.

Ingram and collaborators have demonstrated augmented plasma levels of either C3, C4, C4a components, C1 inhibitor, or factor H as well as reduced levels of C9 in MS patients compared with controls (55). Based on the correlations between their plasma and cerebrospinal concentrations, synthesis of these components was suggested to be localized both systemically and intrathecally. A derived statistical model combined this complement profiling with patient demographic data reaching a predictive value of 97% for MS diagnosis and 73% for clinical exacerbation.

Moreover, an immunohistochemical analysis identified the reactivity for complement proteins (C3, factor B, C1q), activation products (C3b, iC3b, C4d, terminal complement complex), and regulators (factor H, C1-inhibitor, clusterin) within and around MS lesions even in the absence of evident ongoing inflammation (56). Complement staining was also present in normal-appearing white matter (NAWM) and cortex of MS patients, albeit to a lesser extent than in MS plaques, indicating its persistent local synthesis, activation, and regulation. Reactive astrocytes, frequently adjoining to both microglia clusters and damaged myelin/axons, were largely positive for cellular complement staining. This suggests a role of complement in the pathogenesis of cell, axon, and myelin damage.

As a part of innate immunity, platelets play a relevant role in MS pathogenesis (58–62). They increase both BBB permeability and CNS inflammation by either releasing proinflammatory mediators (matrix metalloproteinases, chemokines, and adhesion molecules), displaying inflammatory molecules on their surface, or interacting with endothelial cells and leukocytes, thus, triggering the latter to infiltrate the CNS (63). Alpha-granules are the most abundant platelet secretory granules. They contain numerous soluble factors involved in coagulation such as prothrombin, TF, high molecular weight kininogen, chemokines, proangiogenic and antiangiogenic proteins, growth factors, vWF, fibrinogen, and inhibitory proteases including antithrombin III, protein S, plasminogen, and TF pathway inhibitor.

Platelets participate in the acute phase of the inflammatory response in MS by producing significant amounts of IL-1alpha and other bioactive mediators that activate brain endothelium and promote the recruitment of leukocytes triggering and amplifying cerebrovascular inflammation and brain injury (64). Moreover, platelet-activating factor receptors are up-regulated in MS lesions, and abundant platelets have been shown within the CNS inflamed area of MS patients (62).

Through the production of ROS, activated platelets represent an additional source of oxidative stress for the CNS that has antioxidant mechanisms (65). Oxidative stress is dramatically increased during neuroinflammation, leading to damage of several cellular structures, particularly myelin.

Degenerative disorders, including MS, are associated with platelet dysregulation and excessive release of extracellular vesicles containing RNA and miRNA (short single-strand sequences of non-coding RNA) constituting approximately 70–90% of all vesicles circulating in the blood (66). Among nine exosomal miRNA profiles identified as promising candidate biomarkers to distinguish relapsing-remitting from progressive MS, two platelet-enriched miRNAs, miR-30b-5p and miR-223, are drivers of platelet production (67). Moreover, various platelet-related miRNAs have been found to be associated with both MS activity and duration, and the platelet-enriched geromiR miR-155 seems to be up-regulated in MS patients contributing to MS-associated inflammation and neurodegeneration (68).

Finally, in the experimental settings, platelets have been demonstrated to be a potential therapeutic target since platelet depletion ameliorates the EAE course (69).

Neuroimaging studies have been fundamental for providing a better insight into the pathophysiology of MS. In particular, studies using quantitative contrast-enhanced MRI showed a BBB

leakage of small extent in non-enhancing MS lesions, which was not influenced by ongoing therapies and was different from an evident BBB damage of enhancing lesions, likely as a result of persisting reparative thickening of vessels within chronic MS lesions (70). These BBB abnormalities prevalently reflecting alterations of "tight" junctions (TJ) were demonstrated in NAWM (71) and even in the overall vascular CNS system, the latter probably due to the systemic effect of soluble pro-inflammatory mediators (72). It is not surprising that the chronic subtle BBB breakage can determine a persistent although soft discharge of inflammatory mediators and cells from blood to CNS with a slight but lasting fibrinogen leakage (73). Fibrinogen was found to be associated with both astrocyte processes and TJ abnormalities and correlated with diffuse microglial activation and weakened axonal and myelin integrity.

Moreover, dynamic-susceptibility contrast-enhanced (DSC) perfusion MRI showed delayed cerebral blood mean transit time (MTT) and reduced cerebral blood flow (CBF) in both NAWM and NA gray matter (NAGM) either in clinically isolated syndrome or in all forms of MS. These observations support a continuum of matter perfusion deceleration initiated in WM and spreading to GM (74–76). It is conceivable that the global hypoperfusion in GM and WM of MS patients may be determined by overall blood flow deceleration due to the inflammatory-thrombotic processes, which occur physiologically in the venous vessel bed, particularly during relapses frequently associated with the recurrent infections (77, 78).

Systemic infections can cause CNS damage so much so that peripheral inflammation resulted in being associated with disease exacerbations in experimental models of both MS and other neurodegenerative diseases (79, 80). Systemic immune activation influences local innate immunity, which, in turn, conditions adaptive immune response. During acute/subacute, thus delimited, CNS damage, neuroinflammation could be resolved and concluded with a regeneration area surrounding neurodegeneration (81). Conversely, chronic neuroinflammation inevitably leads to widespread neurodegeneration that, in the same way, spreads neuroinflammation and reduces CNS regenerative capacity. Natural regeneration in injured CNS tissue is insufficient in MS due to excessive extension of neuroinflammation and neurodegeneration.

Among the likely causes of acute and chronic neuroinflammation, there are recurrent and chronic infections accompanied by physiological immunothrombosis, as reported above (13). A continuous and close inter-correlated crosstalk between immune cells and coagulation is fundamental for an effective immune response aiming to restrain the dissemination of pathogens and to potentiate their elimination and tissue repair (12). Coagulation is activated during viral infections and plays multiple functions in the host immune system (82). The recent coronavirus (COVID-19) pandemic has confirmed a relevant role of coagulation activation during viral infection, especially in severe cases, with markedly elevated D-dimer and fibrin degradation product (83).

Temporal virus-BBB interlinkage during viral infections likely determines BBB breakdown triggering neuroinflammation and demyelination (84). A large Danish nationwide case-control study found that children who developed MS have had more infections than their peers 3 years prior, likely depending on their immune reaction to infections (85). Actually, some viruses have been identified to be responsible for the disease development by causing immune activation such as Epstein Barr virus, human herpesvirus 6, Torque teno virus, varicella zoster virus, poliovirus, Picornaviridae family including rhinovirus and enterovirus, coronavirus, adenovirus, influenza virus, and respiratory syncytial virus (86). For example, high anti-HHV-6 IgG titers indicative of HHV-6 infection as well as the immune response to HHV-6 antigens influenced the risk of MS relapses and likely MS progression (87).

Indeed, infections contribute not only to MS pathogenesis but also to disease exacerbation. It seems that about 30–40% of relapses occur after an upper-respiratory infection (88); these data are also confirmed by disease activity at MRI (89). Relapse rates are positively associated with upper-respiratory infections, and approximately two upper respiratory infections per year doubles the risk for MS relapse. After a peak in diagnosed influenza A cases in the general population, it was observed that the occurrence of MS relapse was 6.5 times more likely to occur (90).

In addition, a Cochrane review concluded that some pathogens such as human herpesvirus 6, Chlamydia pneumoniae, and Torque teno virus could contribute to MS progression (91). Therefore, not only viruses but also bacteria (e.g., *Chlamydia pneumonia*, *Staphylococcus aureus*, enterotoxin A) and fungal infections seem to have a role in both MS pathogenesis and course (92).

Altogether, these studies have pointed out the role of the coagulation pathway in close correlation with infections in the MS pathophysiology and its association with brain perfusion deceleration. This suggests possible therapeutic targets that may complement existing treatments. The aim of our study is to validate the pathogenetic role of coagulation together with brain hemodynamic abnormalities in MS.

METHODS AND ANALYSIS

Design

This is a multi-center, prospective, controlled study (**Figure 1**). The study has been approved by the Ethics Committee of the IRCCS Regina Elena National Cancer Institute and by the Ethics Committee of the Sapienza University of Rome.

Specific aims of our study are

Primary outcomes:

- To evaluate serum/plasma concentrations of complement (C3, C4, C4a, and C9), Fibrinogen, Factor VIII (FVIII), Factor X (FX), D-dimer (DD), PC, PS in relapsing-remitting MS (RRMS) patients and healthy people.

Key secondary outcomes:

- To evaluate serum/plasma concentrations of Prothrombin Time (PT), Activated Partial Thromboplastin Time (aPTT),

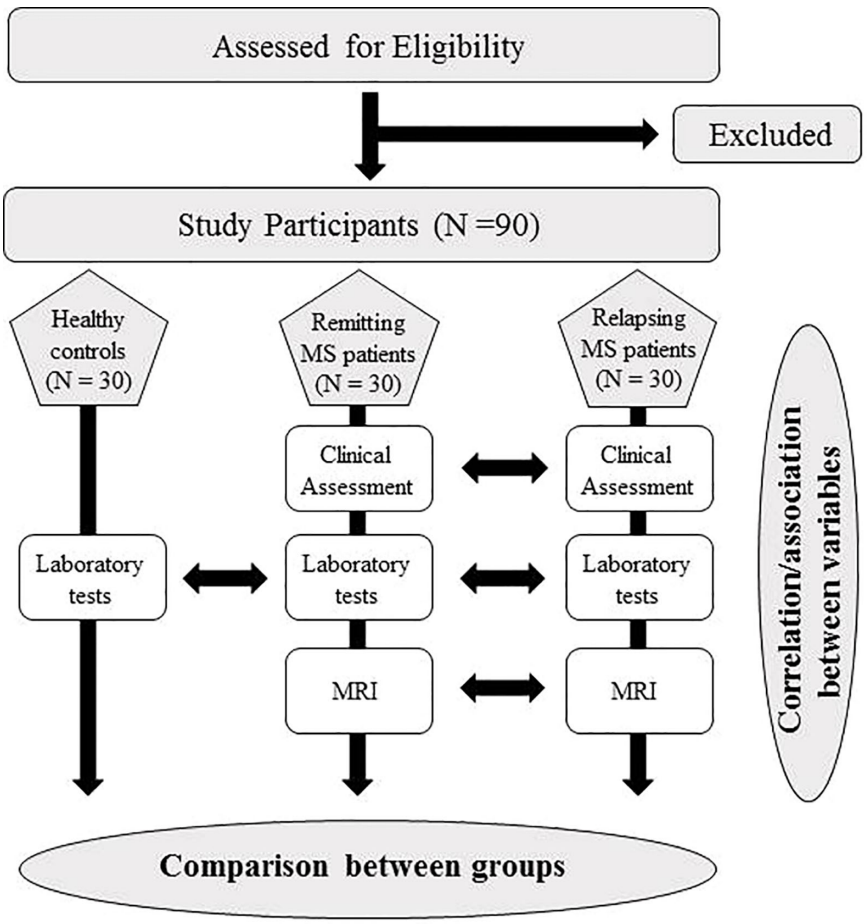

FIGURE 1 | Flow chart of the study protocol.

Factor II (FII), antithrombin III (ATIII), von Willebrand factor (vWF), soluble (s)TM and soluble (s)EPCR, Angiopoietin-1, Angiopoietin-2, FIII or TF, TM, Tie-2, Vascular Endothelial Growth Factor (VEGF), APLs, lupus anticoagulant (LA), complete blood count (CBC), viral serological assays, and microRNA microarray in relapsing-remitting MS (RRMS) patients and healthy people.

Additional secondary outcomes:

- To assess relative CBF, CBV, and MTT by DSC 3.0-T MRI in relapsing MS patients compared to remitting MS patients.
- To evaluate number and volume of enhancing lesions in relapsing MS patients compared to remitting MS patients.
- To evaluate the relationships between laboratory data, demographic/clinical (age, gender, disability and disease duration) features, and MRI perfusion findings in the patients' groups.

Selection of Subjects

Subjects of both genders will be recruited in two centers: the Multiple Sclerosis Center of the Sapienza University of Rome and the Multiple Sclerosis Center of the IRCCS Regina Elena National Cancer Institute. The planned recruitment period will be of three years.

Prior to enrollment, all participants will be screened to check their inclusion and exclusion criteria.

Patient inclusion criteria will be:

- Patients diagnosed with relapsing-remitting MS (93)
- Patients untreated or treated with only immunomodulatory therapy
- 18–60 years old

Patient exclusion criteria will be:

- pregnancy
- co-existing neoplastic, hematologic, thyroid, metabolic, thrombotic, or autoimmune diseases
- drug or alcohol addicted
- therapy with immunosuppressive drugs, steroids, or any medication interfering with coagulation

We will evaluate 3 groups of subjects: 30 RRMS patients in relapse (Group I); 30 RRMS patients in remission, i.e., without

relapse in the previous 2 months (Group II), and 30 sex- and age-matched healthy controls (Group III).

A relapse or exacerbation or "attack" was defined by the multiple sclerosis guidelines as a manifestation of new or worsened neurological symptoms lasting for more than 24 hours (94). Symptoms should be supported by subjective description and by objective clinical assessment with no other explanation for them. The relapse is separated from the previous "attack" by at least 30 days and usually persists for days or weeks, then slowly improves over weeks or a few months ending with partial or complete recovery, i.e., remission. MRI showed the new and/or active lesions in the majority of patients, and steroid therapy often accelerates the recovery of symptoms.

Relapse needs to be distinguished from a pseudo-exacerbation, which is defined as a temporary worsening of pre-existing symptoms without new MS-related neuroinflammation, thus an MRI does not detect active or new lesions (95). The causes of pseudo-relapses could be different and include infections with or without fever, increased body temperature due to over-exercising, sauna, hot shower/bath or weather, dehydration, hormonal changes during menstruation or severe psychological stress, surgery, trauma, medications, alcohol overuse, and several medical conditions as thyroid or other metabolic disorders. Pseudo-relapses usually last less than 24 hours and resolve after removing their triggering causes with no need for steroid therapy.

We will only include MS patients in relapse who have new symptoms for more than a day but no more than a month and possibly as soon as they communicate the neurological symptoms to their doctor to quickly perform all protocol procedures before starting treatment with steroids. Patients in relapse already on steroid therapy cannot be enrolled in the study. This includes both naïve and treated patients as they could raise some concerns regarding the possibility that treatment could affect levels of some of the biomarkers studied. Indeed, it would be ideal to recruit only untreated patients, although it should be taken into account that other concomitant factors (lifestyle, nutrition, current infections, physical activity, smoking, etc.) could influence these biomarkers, albeit to a lesser extent. The choice to include patients on immunomodulating (but not immunosuppressive) therapy was dictated by the actual difficulty in finding untreated patients out of those just diagnosed. However, since the two compared groups of patients, i.e., in relapse and in remission, are recruited from the same outpatient population, the treatment influence could be sufficiently balanced between these groups and, in any case, it will be weighed in the final analysis.

An informed written consent will be obtained by all participants. The study information will be provided to the patients by the investigator to explain the study aims and protocol procedures, risks, and benefits (96). We will encourage the patients to take time before signing the consent form to discuss their participation in the study with their trusted advisors. We want to highlight that the timing of our study procedures largely coincides with the routine clinical MS management. In fact, relapsing patients usually perform blood sampling to evaluate the general clinical status and Gd-enhanced MRIs to define the extent of radiological disease activity. Regarding remitting MS patients, study participation will be proposed only to patients who need to perform their routine periodic blood tests or neuroimaging. A greater amount of blood required for additional laboratory tests pre-specified in the study and perfusion sequences, both of them with their relative low risk, merely represent the only difference between study procedures and equivalent routine exams. The benefits of participating in the study could consist of more rapid MRI execution not always easily accessible in routine practice.

On the other hand, healthy people, potentially research subjects, will have only blood sample collection for the pre-specified laboratory assessment; they will not undergo Gd-enhanced MRIs since it represents, in any case, a reasonable low risk related to the use of contrast. Patients will be seen and assessed for physical disabilities using the Expanded Disability Status Scale (EDSS) (97) and Multiple Sclerosis Functional Composite (MSFC) (98).

Each subject included in the study will remain anonymous for privacy reasons and identified by a progressive numeric code (ID number) (associated with their own name, surname, and date of birth in a separate database) so that all serum/plasma samples and MRI data will be treated and processed blindly. An ad-hoc secure database has been established to collect standardized data from different centers, data storage, and sensitive data protection.

Interventional Methods

Laboratory Procedures

At enrollment, blood samples will be obtained from each participant. For most laboratory markers that will be investigated in this study (e.g., blood count test, coagulation factors, complement components, anti-cardiolipin antibody, anti-β2 glycoprotein I antibody, anti-prothrombin IgG/IgM and anti-AnnexinV IgG/IgM, RNA extraction, and Real Time polymerase chain reaction) technical procedures are already well established and are a part of the clinical laboratory practice. In regards to the technical procedures, we will use other specific molecular markers for measuring (i.e., angiopoietin-1, angiopoietin-2, FIII/TF, Tie-2, VEGF). We will refer to the available literature data and evidence.

Blood Processing

Plasma samples will be collected using sodium citrate as an anticoagulant at a concentration of 3.2%. Plasma and serum aliquots will be obtained within 3 hours of sample collection by centrifugation at 3,000 g for 10 minutes and 2,000 g for 10 minutes, respectively, at 25°C, and then they will be stored at -80° C up to the time of use. For specialized coagulation tests, a 20-minute double centrifugation will be performed.

Blood Count Test

The CBC is a routine examination of venous blood collected in tubes with EDTA anticoagulant and performed in our laboratory by automatic DxH 800 analyzer provided by the Beckman Coulter company (Brea, California).

The tool provides fast, reliable, and high-quality results thanks to Automated Intelligent Morphology (AIM) and

advanced algorithms, and it avoids cellular interference with Coulter's principle impedance and multi-angle scatter laser.

It analyzes the number of red blood cells (erythrocytes or RBC), white blood cells (leukocytes or WBC), platelets (thrombocytes), hematocrit (HCT), and hemoglobin (Hb) levels of the leukocyte formula (percentage of different white blood cells: neutrophils, lymphocytes, monocytes, eosinophils, and basophils). It determines the physical characteristics of the red blood cells by means of the following indices: MCV (average corpuscular volume), MCH (average corpuscular hemoglobin content), MCHC (Medium Corpuscular Hemoglobin Concentration), RDW (red blood cell distribution amplitude), and MPV platelets (mean platelet volume).

Measurement of Coagulation Factors

All routine coagulation factors such as DD, Fibrinogen, ATIII, aPTT, PT, and specialist test of coagulation as PC, PS, FII, FVIII, vWF, and LA will be performed by ACL TOP 750 analyzer. This instrument is fully automated, fast and reliable, and able to detect all coagulation, chromogenic, and immunological reactions using a photo optical detection system by HemosIL commercial kits (Instrumentation Laboratory Co.). This guarantees maximum precision and accuracy of the results with the transmission of data to the laboratory computer system.

Measurement of Complement Components: C3, C4, C4a, C9

Serum concentrations of complement C3 and C4 components will be evaluated with Cobas 8000 fully automated platform by Roche Diagnostics. This instrument is based on an immunoturbidimetric principle. The reference ranges include 90–180 mg/dL and 10–40 mg/dL for human C3 and C4, respectively. The sensitivity of the test is 4 mg/dL for C3 and lower than 0.02 g/L for C4.

The fragment C4-a and component C9 were separately quantified by sandwich enzyme-linked immune-sorbent assay technology (ELISA) using two different kits purchased by Nordic BioSite AB.

Sample Preparation. The serum samples have to be left for 2 hours at room temperature or overnight at 4°C before centrifugation for 20 minutes at about 1000 g, and the supernatant is collected and the test must be performed either immediately or stored at -80°C until use.

Assay Procedure. Before running the test, the reagents will be prepared and the samples will be diluted as required in the kit. In detail, the test is performed on a 96-well plate, pre-coated with anti-C4a or anti-C9 antibodies. Standards and diluted samples are added to each well and incubated at 37°C for 90 minutes followed by three incubations at 37°C by suitable washes: the first with 100 µl of biotin conjugated anti-C4a or anti-C9 antibody for 60 minutes, the second with 100 µl of HRP-Streptavidin Conjugate (SABC) working solution for 30 minutes, and the third with 90 µl of substrate (TMB) in dark for 15–30 min to visualize HRP enzymatic reaction. TMB is catalyzed by HRP and produces a blue colored product that changes to yellow after adding 50 µL acidic stop solution. The plate is read at 450 nm in a microplate reader. The density of yellow is directly proportional to C4a or C9 concentration of sample. The reference ranges are: 1.563–100 ng/mL and 0.234–15 ng/mL for human C4-a and C9, respectively. This assay has high sensitivity (< 0.938ng/ml) and excellent specificity.

Measurement of sEPCR

Plasma levels of sEPCR will be performed by quantitative sandwich enzyme immunoassay (ELISA) Kit (Cusabio Biotech CO., LTD.). The detection range established by the manufacturer ranges from 7.8 ng/mL to 500 ng/mL. The dosage has a sensitivity lower than 1.95 ng/mL and high specificity.

Measurement of Angiopoietin-1, Angiopoietin-2, FIII/TF, TM, Tie-2, VEGF

Angiopoietin-1, Angiopoietin-2, TF, TM, Tie-2, and VEGF biomarkers will be performed by Magnetic Luminex Assay multiplex kits supplied by R&D Systems, Inc. (USA) (**Figure 2**).

It is a premixed multi-analyte human kit, which can be used to evaluate up to 50 human biomarkers in a single supernatant, serum, plasma, and cell culture sample. The test is based on quantification immunoassay and the innovation of this test is represented by the use of magnetic microparticles. Determined antibodies for the analytes of interest are pre-coated to each specific region of the microparticle labeled with different fluorophores.

Sample Preparation. Fresh and previously frozen serum or plasma samples require centrifugation at 16000 g for 4 minutes immediately before use. Samples should be diluted correctly as required by the kit.

Procedure Assay. The diluted microparticle cocktail will be suspended by inversion or on a shaker, then 50 µl of microparticle cocktail, 50 µl standard, and 50 µl of samples will be added to each well of the microplate and incubated for 2 hours at room temperature on a horizontal orbital shaker for microplates (0.12 Orbit) set at 800 ± 50 rpm. Using a magnetic device, three washes with 100 µl of wash buffer will be performed to eliminate unbound substances, and 50 µl of the biotinylated antibody cocktail specific will be added to the analytes of interest to each well for 1 hour at room temperature on the shaker set at 800 ± 50 rpm. The washes will be repeated and 50 µl of the streptavidin-phycoerythrin conjugate will be added (Streptavidin-PE) to each well. Then it will be incubated for 30 minutes at room temperature on the shaker set at 800 ± 50 rpm. Final washes will be performed to remove unbound Streptavidin-PE. Finally, the microparticles will be resuspended in 100 µl of Wash Buffer for 2 minutes on the shaker set at 800 ± 50 rpm and within 90 minutes the plate will be read using a Luminex® MAGPIX® or Bio-Rad analyzer. A magnet in the analyzer captures and holds the superparamagnetic microparticles in a monolayer. Two spectrally distinct light emitting diodes (LEDs) illuminate the microparticles. One LED excites the dyes inside each microparticle to identify the region, and the second LED excites the PE to measure the amount of analyte bound to the microparticle. A sample from each well is imaged with a CCD camera with a set of filters to differentiate excitation levels. Analysis with the Luminex® 100/200™, Luminex® FLEXMAP 3D®, or Bio-Rad

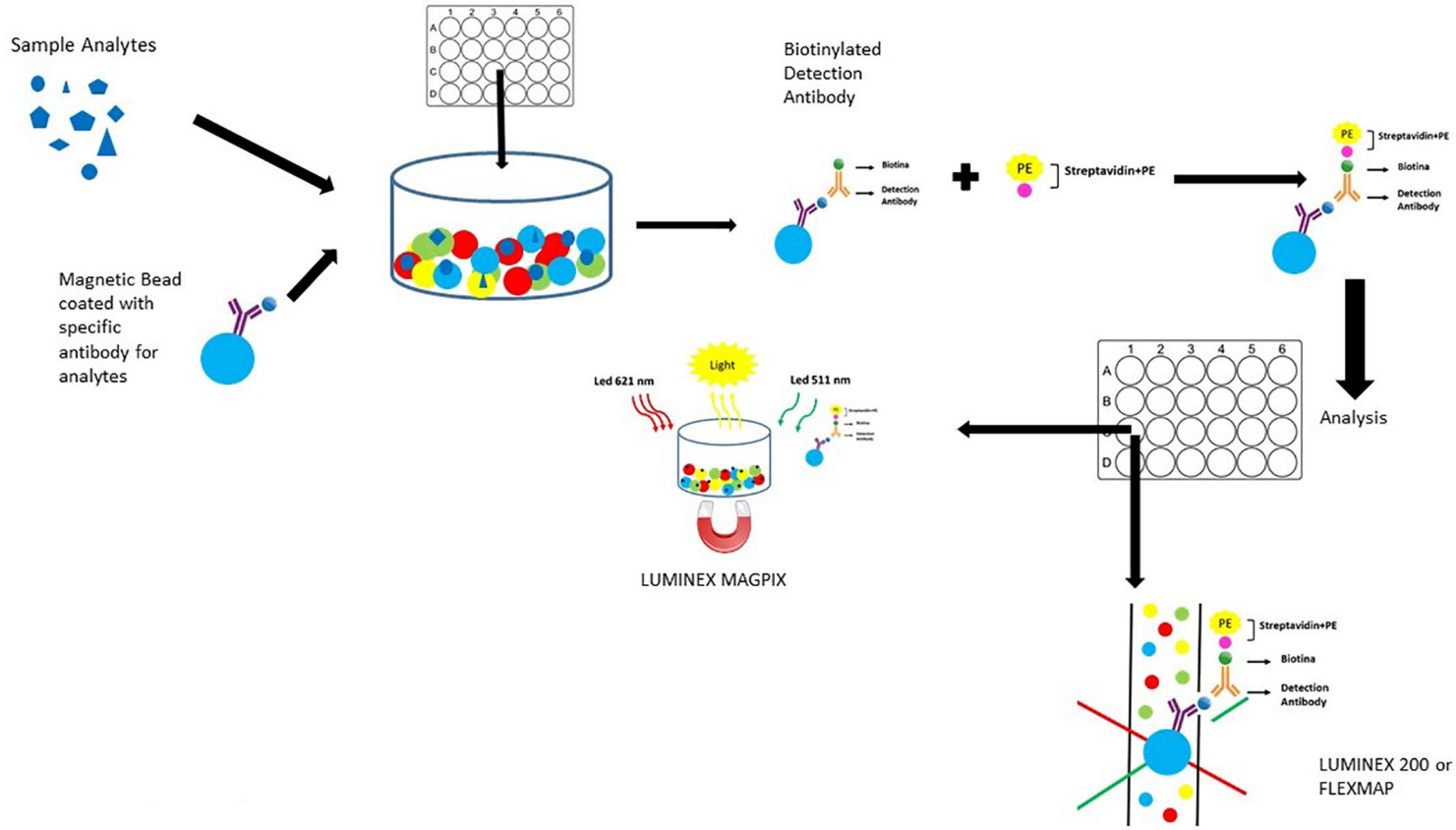

FIGURE 2 | Luminex Assay Principle. The sample is added to the mixture of colored beads coated with specific antibodies that bind the analyte of interest, the biotinylated detection antibodies in turn bind the analyte, and an analyte-antibody sandwich is formed. Streptoavidin conjugated with phycoerythrin (PE) binds biotinylated detection antibodies. The analysis of the beads is carried out either in a double laser flow with the Luminex 200 instrument or inside a magnet with the Luminex MAGPIX analyzer. The signal strength of phycoerythrin is directly proportional to the concentration of the specific analyte.

Bio-Plex uses one laser to excite the dyes inside each microparticle to identify the microparticle region and the second laser to excite the PE to measure the amount of analytes bound to the microparticle. All fluorescence emissions from each microparticle as it passes through the flow cell is then analyzed to differentiate emission levels using a Photomultiplier Tube (PMT) and an Avalanche Photodiode. The assay has a higher sensitivity than the traditional ELISA and requires a smaller sample volume, which is fast and efficient.

Measurement of APLs

Measurement of Anti-Cardilipin (aCL) and Anti-β2 Glycoprotein I (aβ2GPI). The semi-quantitative detection of aCL and aβ2GPI of both IgG and IgM classes will be evaluated in serum by Kit BioPlex 2200 System Antiphospholipid Syndrome (APS) supplied by BIO-RAD Laboratories, Marnes-la-Coquette, France. The test is based on the multiplex flow immunoassay that uses microspheres coated with aCL and aβ2GPI antigens. It is a fully automated test.

Procedure assay: The test requires two incubations steps at 37°C into a unique reaction vessel, separated by washing: in the first step the dyed microspheres are added to the diluted sample, and in the second step the IgG and IgM antibodies conjugated to phycoerythrin (PE) and added in the reaction vessel. The microspheres mixture passes in laminar flow and is analyzed by the detector with two lasers.

Signals from a laser classify the microspheres, while those from another laser measure the fluorescence of the conjugate. The system software converts the conjugate signal into a fluorescence value (RFI). The values of specific antibodies are established by the manufacturer and expressed in GPL/MPL (aCL) and U/mL (anti-Beta2GPI). Samples with values of aCL or aβ2GPI (either IgG or IgM) inferior to 20 are evaluated as negative, values ≥ 20 to 40 weakly positive, values ≥ 40 to 80 average positive, and values ≥ 80 to 160 strongly positive. The sensitivity and the specificity of aCL and aβ2GPI (IgG) test is 67.5% and 100%, respectively. The sensitivity and the specificity of aCL and aβ2GPI (IgM) test is 25.8% and 98.7%, respectively.

Measurement of Anti-Prothrombin IgG/IgM and Anti-AnnexinV IgG/IgM. The quantitative measurements of IgG/IgM class autoantibodies against AnnexinV and Prothrombin in the serum will be determined by an indirect enzyme linked immune reaction (ELISA) kit supplied by DRG International, Inc., USA. The measurement range of this ELISA test was established by the manufacturer for Anti-AnnexinV and Anti-Prothrombin IgG/IgM ranges from 0 to 100 U/mL. The cut-off for Anti-AnnexinV and Anti-Prothrombin IgG/IgM is 8 U/mL and 10 U/mL, respectively.

Samples with values of Anti-AnnexinV IgG/IgM <5 U/mL are considered as negative, with values between 5 U/mL and 8 U/mL as borderline, with values ≥ 8 U/mL as positive. Samples with values of Anti-Prothrombin IgG/IgM <10 U/mL are considered as negative and with values ≥ 10 U/mL as positive. Limit of detection of Anti-AnnexinV and Anti-Prothrombin is of 1U/mL either IgG or IgM. The specificity of Anti-AnnexinV is 95.8% and 96.7% for IgG and IgM test, respectively. The specificity of Anti-Prothrombin is 98% and 99.3% for IgG and IgM test, respectively.

RNA Extraction and Hybridization on Agilent Microarray

Total RNA extraction from sera will be performed by a column-based method that includes small RNAs and minimizes the carry-over of enzyme inhibitors typically contained in biofluids (miRNEeasy serum/plasma kit, QIAGEN) (**Figure 3**).

Total RNA will be labeled and hybridized to Human miRNA Microarray Release 21 (Agilent) containing probes for 2549 human microRNAs from the Sanger database. Each slide is an 8 x 15K format (~15,000 features printed in an 8-plex format, eight individual microarrays on a 1" x 3" glass slide) printed using Agilent's 60-mer Inkjet Technology, which, unlike competitive platforms, synthesizes 40–60-mer oligonucleotide probes directly on the array, resulting in high-purity, high fidelity probes. This miRNA platform requires small input amounts of total RNA—in the 100 nanogram range—because it uses a high-yield labeling method and does not require size fractionation or amplification steps that may introduce undesired bias during miRNA profiling.

Scanning and image analysis will be performed using the Agilent DNA Microarray Scanner (P/N G2565BA) equipped with extended dynamic range (XDR) software according to the Agilent miRNA Microarray System with miRNA Complete Labeling and Hyb Kit Protocol manual. Feature Extraction Software (Version 10.5) will be used for data extraction from raw microarray image files using the miRNA_105_Dec08 FE protocol. microRNA Microarray data will be subjected to stringent quality controls and then analyzed by the Bioinformatic Unit.

Real Time Polymerase Chain Reaction

We enable automated purification of DNA and RNA (Qiasymphony–Qiagen) from a broad range of sample types, amplifying the DNA sequences and then analyzing the products. For overcoming the challenges of limited samples and a costly analysis we will choose, when possible, Multiplex PCR that enables the amplification of more than one target in a single reaction using different reporters with distinct fluorescent spectra (Seegene Korea). Multiplex qPCR requires the use of probe-based assays, in which each probe is labeled with a unique fluorescent dye, resulting in different observed colors for each assay. The signal from each dye is used to quantitate the amount of each target separately in the same tube or well. The availability to multiplex therefore allows the measurement of the expression levels of several targets or genes of interest quickly. In particular, we will analyze the following markers by qPCR: Epstein Barr virus, human herpesvirus 6, Torque teno virus, varicella zoster virus, poliovirus, Picornaviridae family including rhinovirus and enterovirus, coronavirus, adenovirus, influenza virus, and respiratory syncytial virus, Chlamydia pneumoniae, Staphylococcus aureus, and enterotoxin A.

MRI Procedures

All RRMS patients will undergo the 3.0-T MRI within two weeks of enrollment. We will use DSC perfusion technique acquired

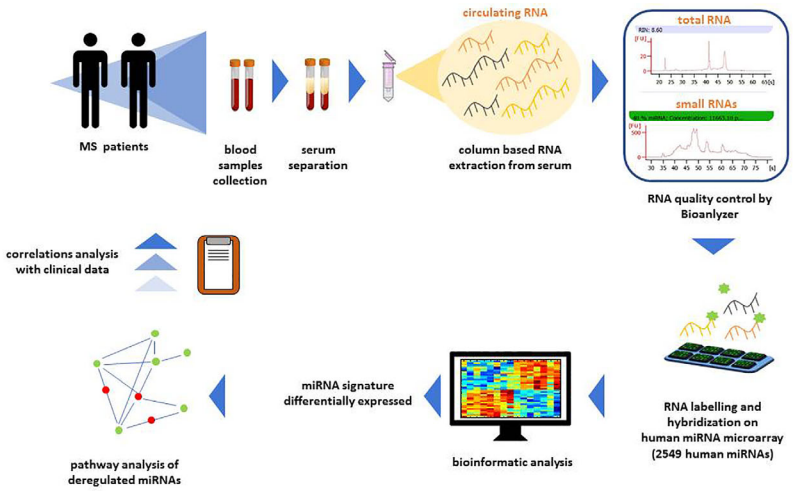

FIGURE 3 | Workflow of circulating miRNA profiling. For circulating miRNA profiling blood samples from patients will be processed. In particular, circulating RNA will be extracted from serum samples with a column-based extraction method. Total and small RNA quality will be assessed by Bioanalyzer. Then, total RNA will be labelled for the hybridization to Human miRNA Microarray Release 21 (Agilent) containing probes for 2549 human miRNAs. Microarray data will be subjected to bioinformatic analysis to identify a signature of miRNAs differentially expressed. Deregulated miRNAs pathway analysis and correlations analysis with clinical variables will be performed.

during the first pass of gadolinium to estimate perfusion features inside the damaged tissue of relapsing and remitting MS patients.

DSC MR images will be acquired on the axial plane during the first pass of a standard-dose bolus (0.1 mmol/kg) of gadopentetate dimeglumine (Magnevist; Berlex Laboratories, Wayne, NJ, USA) with a gradient-echo T2-weighted echo-planar imaging sequence. A contrast will be injected at a rate of 3,5 mL/sec, followed by a 20-mL bolus of saline also at a rate of 3,5 mL/sec. A total of 60 images will be acquired at 1-sec intervals, with the injection occurring at the fifth image, for a total acquisition time of 2 min 16 s. The imaging parameters will be as follows: TR/TE = 2140/30 ms, flip angle = 30°, slice thickness = 4 mm, FOV = 280 mm, matrix = 128x128.

The following hemodynamic parameters will be obtained from the concentration–time curves: the relative CBV (rCBV, i.e., the fraction of the tissue volume occupied by blood), the relative CBF (rCBF, i.e., the volume of blood in a given amount of tissue per unit of time), and MTT (the average time it takes for the contrast agent to travel through the tissue vasculature, for the ideal case of an instantaneous bolus injection) by using NordicIce software package.

Thus, we will use the leakage correction function provided by NordicIce software to minimize this effect both on Gadolinium and on non-Gadolinium enhancing lesions, in which a subtle leakage of contrast agent cannot be excluded.

Finally, rCBF, rCBV, and MTT values will be extracted from different sites of the brain damaged by MS (hyperintense T2-w lesions, Gadolinium enhancing T1-w lesions, periventricular and frontal NAWM, thalamus, and putamen nuclei and head of the caudate bilaterally). To exclude interobserver and to minimize intra-observer variability, each data set will be reviewed by two expert radiologists at the same time (**Figure 4**).

Data Analysis

Sample Size Calculation

Overall 90 subjects (30 for each group) will be enrolled to compare the level of complement C4a (55). By using the ANOVA test, this sample size will allow detection of effect size values [delta=(miA–miB)/sigma] equal to at least 0.71, with a statistical power of 80%, to a level of significance of 5%.

Statistical Methods

Descriptive statistics will be used to summarize pertinent study information. Correlations between quantitative variables will be assessed with the Pearson r correlation. The associations will be analyzed by the Fisher exact test or Chi Square test for trends. Comparisons between disease subgroups and control group will be carried out for different variables, using either Student's *t*-test or analysis of variance (ANOVA). If the ANOVA shows a statistical difference between subgroups, a post-hoc analysis with Bonferroni correction for multiple comparisons will be performed. For non-normally distributed data, non-parametric (Mann Whitney-U or Kruskal-Wallis-H) tests will be used. The level of significance is set at $p \leq 0.05$ (SPSS version 20.0, SPSS Inc., Chicago, Illinois, USA).

DISCUSSION

While we await our results, we discuss here the methods chosen to conduct our study.

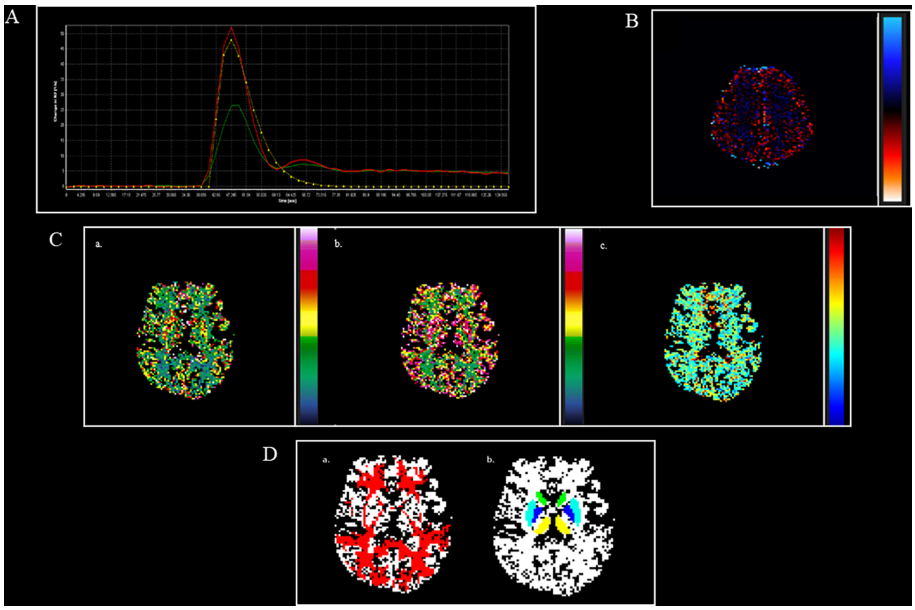

FIGURE 4 | DSC perfusion maps and their overlap with structural masks in an MS patient. **(A)**. AIF curve created by using global, automatic, and outside-artery technique **(B)**. Leakage map, obtained through the leakage correction function, to minimize leakage effect both on Gadolinium and no Gadolinium enhancing lesions **(C)**. a. CBV map; b. CBF map; c. MTT map. **(D)**. a. NAWM (red), obtained by subtracting from white matter (WM binary) masks the different types of lesions, linearly registered to CBV map; b. thalamus (yellow), caudate (green), putamen (light blue), globus pallidus (blue), obtained by using FIRST software, linearly registered to CBV map. AIF, arterial input function; CBV, cerebral blood volume; CBF, cerebral blood flow; MTT, mean transit time; NAWM, normal appearing white matter.

Blood Count Test

Gens et al. examined 2.145 whole blood samples to evaluate the analytical performance between two hematology analyzers: Sysmex XN 3000 and UniCel DxH 800. For both analyzers, the variation coefficients (CV%) for hemoglobin, RBC, MCV, WBC were <5%, for WBC less than 10%, while the variation coefficients for platelets and monocytes were <5%, 15%, 6%, and 9.5%, 45%, respectively for Sysmex XN 3000 and DxH 800. The analyzers are equally precise (R>.86), with the exception of monocytes and basophils (99).

Barnes and his collaborators furthermore evaluated a better sensitivity and specificity of the DxH 800 analyzer compared to the previous Beckman Coulter LH 750 series. The DxH 800 instrument is more skilled in capturing explosions; in fact, out of 95 samples containing a burst percentage > or = 1%, the LH 750 detected 6.4% of false negatives, while the DxH 800 0.0%. The advantage of having a low false positive number has reduced peripheral blood smears for microscopic blood analysis, a time-consuming technique, and the use of an expert operator (100).

In another study, the Beckman Coulter UniCel® DxH 800 analyzer was compared with the Coulter® LH 780 and flow cytometry (FCM), and it was found that the DxH 800 instrument has greater sensitivity and specificity for counting WBC, PLT, and NRBC with fewer false negatives for NRBC compared to LH 780 and greater accuracy for PLT and NRBC counting than FCM (101).

Angiopoietin-1, Angiopoietin-2, FIII/TF, TM, Tie-2, VEGF

The ELISA is the most commonly used method in both diagnostics and clinical research. However, it has some limitations. This technique requires large volumes of sample to capture an antigen of interest. In some cases, the larger surface of the wells can favor non-specific bonds, and the resulting fluorescence signal is not always linear and may invalidate the test. The assay performed on multiplex platforms also allows greater flexibility, reduced sample volume, and lower cost, with a similar workflow. Luminex xMAP technology is an array platform that allows both monoplex and multiplex assays that can be applied to either protein or nucleic acid applications. Microspheres have a smaller surface and the non-specific bond is significantly reduced (102).

Using multi-array and electrochemiluminescence technologies, the MSD platform offers multiplex capability with a consistency similar to that observed in ELISA with reduced costs and labor (103).

Coagulation Parameters

Laboratory automation began many years ago and has since spread across other fields such as hematology, immunology, molecular biology, and coagulation tests. The advantages of automation are either standardization, error reduction, cost reduction, or productivity increase, whereas the only disadvantage is its high maintenance costs (104).

The hemostatic measurements are influenced by the technique of the instrument and the reagents used for individual analyzes (105).

Geens and his collaborators compared the performance of System CS5100 and Stago STA-R analyzers for determining routine coagulation parameters such as aPTT, PT, FBG, DD, and AT (106).

All parameters including imprecision, accuracy, and total error were deemed acceptable for the two methods; however, the difference between them consists of both high sensitivity of the CS5100 for APTT towards the deficiencies of the factors and presence of unfractionated heparin.

Unlike Stago STA-R analyzers, the CS5100 can automatically control pre-analytical variable such as sample volume and interfering substances such as hemolysis, hyperbilirubin-hememia, and lipemia but fails to analyze highly lipemic and icteric samples that represent a disadvantage in routine practice.

The APTT reagent of CS5100 showed sensitivity between 46% and 72% to FVIII, IX, XI, and XII factors while the PT reagent showed sensitivity between 34% and 52% to FII, FV, FXII, and FX factors. This explains the reference interval for APTT between the two instruments (23–31s on CS5100 vs. 30–42s on STA-R Evolution).

Furthermore, a small increase in the percentage of PT and a slight decrease in PT (INR) and FBG on the CS5100 was observed compared to the STA-R Evolution instrument (106).

Hemolysis (4%), hyperbilirubinemia (11%), and lipemia (13%) are the main preanalytical variables that cause errors in blood sample coagulation tests (107).

Woo-Jae Kwoun and coworkers assessed the performance of the pre-analytic module of the ACL TOP 750 analyzer where the reference values obtained were compared with those of the XPT instrument for chemistry, which uses an enzymatic method, and with those of ADVIA2120i, which uses a spectrophotometric method (108). The researchers concluded that an efficient control of the pre-analytical variables is exercised by the ACL TOP family series 50 spectrophotometric apparatus module ensuring sample quality monitoring, while accurate test results from the interference of the HIL sample.

The ACL-TOP analysis system produces three types of curves. The first curve shows changes in absorbance during aPTT measurement. The second one, derived from absorbance, is related to the speed of coagulation. The third one measures the acceleration of coagulation. Tokunaga and his collaborators noted the second derivative curve's utility for detecting factor deficiencies (109). Shortcomings were found not only in FVIII but also in FIX, FXI, FXII, and FV. It has been reported that the ACL TOP system that uses the APTT-SP reagent comprising silica to be the most suitable for detecting intrinsic deficiencies of coagulation (110). Monitoring vWF on plasma from patients with acquired von Willebrand syndrome was evaluated and showed less than 10% of the activity of the vWF cofactor of both ristocetin and vWF antigen (111). After 15 minutes of desmopressin infusion (vasopressin 1-deamin-8-D-arginine; DDAVP), and based on the variations of the waveforms in an aPTT assay on the second derivative curve, levels of vWF and FVIII remarkably increased to 54% and 84%, respectively (111). These results explain that the waveform analysis of the second derivative curves of an APTT assay provide useful information relating to both reduction of coagulation factors and therapeutic treatment.

The ACL TOP instrument efficiently performs specific and routine coagulation tests for up to 120 samples simultaneously with high quality starting from smaller samples and reagent volumes rather than manual methods. Its use in many laboratories is determined by either its precision, reliability, high productivity, or daily maintenance of 4 minutes.

It is also equipped with a software capable of rerunning automatically multiple tests simultaneously using different dilutions.

The intra-assay and inter-assay precision (coefficients of variation) were less than 5% for most coagulation parameters in both the normal and pathological range (112). The results of coagulation tests obtained by the ACL TOP are well correlated with those obtained on the STAR analyzer characterized by a correlation coefficient (r) ranging from 0.876 to 0.990 (113).

Complement

It is necessary to standardize tests evaluating the function of the classical, alternative, or lectin pathway since the analysis of the complement system, with the exception of some proteins such as C3 and C4, varies widely between laboratories. Autoantibodies such as anti-C1q, convertases C3 and C4, or regulatory proteins like inhibitor of anti-C1, anti-factor H, are relevant in defining autoimmune processes and diseases based on complement dysregulation. The standardization committee of the International Complement Society (ICS) and the International Union of Immunological Societies (IUIS) have provided guidelines that ensure the quality of tests for the complement analysis (114).

Laboratory analyses for triggering the activation of the classical complement pathway are performed with methods based on the principles of nephelometry, turbidimetry, and ELISA (115). Complement deficiency is commonly detected in the laboratory by quantifying the main soluble fragments C3 and C4 formed during activation.

Li H. and his colleagues measured complement levels C3 and C4 in Chinese patients with systemic lupus erythematosus (SLE). The complement assay was performed based on the dispersion turbidimetry immunization rate using the Beckman Coulter instrument (Inc. Brea, CA, USA). The reference values for C3 were between 0.79 and 1.52 g/L and those for C4 were between 0.16 and 0.38 g/L, in line with those used in our laboratory measured by immunoturbidimetry and the COBAS 8000 instrument (116).

A recent study compared the levels of C3 and C4 fragments of patients diagnosed with SLE with those of healthy subjects by nephelometry. C3 and C4 values were significantly higher ($p < 0.001$) in healthy subjects than in patients.

Sensitivity and specificity for complement C3 are 87.11% and 82.74%, respectively, and for complement C4 are 88.66% and 77.43%, respectively (117).

Myriam and his collaborators studied the activation product of plasma complement C4d of patients with SLE as a marker of lupus nephritis by ELISA. The test found significantly higher C4d values in SLE patients compared with healthy subjects, whose C4d levels were negligible.

Levels of C4d discriminated the highest and lowest clinical disease activity with a positive predictive value of 68% and a sensitivity of 79% for identifying patients with nephritis. The test could detect the lowest concentration of 5.6 μg/L. The precision of the test was demonstrated by the intra-assay and inter-assay coefficients of variation of 13.2% and 16.7%, respectively (118).

According to a previous study regarding good monitoring of C4d marker disease, the availability and ease of the test, long execution times, probable sources of error by the operator, and detectable false positives are all disadvantages.

Antiphospholipid Antibodies

The laboratory criteria for APS were revised and published in 2006 due to the heterogeneity of aPL, plasma proteins, or protein complexes related to them, as well as to the harmonization of the assays diagnosing the APS in order to improve the detection of aPL antibodies and the interpretation of the results (119).

The new criteria include the LAC test, aCL IgG/IgM, and aβ2GPI IgG/IgM, measured by different types of solid phase immunoassays. Currently, these immunoassays have not been entirely standardized (120).

The aPL immunological test provides information that was not obtained from the LAC test such as specific antiphospholipid analytes, isotypic class (IgM or IgG), and their concentration levels. The solid phase of the immunoassay is important and should not be influenced by analytical variables, like anticoagulant or anticoagulant therapy, as they represent interference factors for the LAC test.

In addition to the ELISA test, either chemiluminescence immunoassays (CIA), enzyme fluorescence immunoassays, or new emerging technologies such as multiple dosing through microspheres are widely used in clinical diagnostic laboratories (119). CIAs have recently been developed for the detection of aPL antibodies and are currently used in a number of clinical laboratories. CIAs are advantageous due to their extreme sensitivity and capability to be automated (121).

A number of studies have demonstrated that the performance of CIAs is similar to both commercial and laboratory developed ELISAs for aPL criteria (122).

It has recently been suggested that CIAs improve reproducibility and inter-laboratory correlations for these analytes (123).

Despite these developments, there are still no generally accepted reference reagents for the development and calibration of these tests. It is necessary to compare the performance of the new methods used for detecting aPL antibodies with the more traditional ELISA adopted to identify the commutability in the diagnosis of the APS.

Testing for aPL antibodies has traditionally been performed by ELISA due to both easy use and widespread availability (124).

Thomas B. Martins and his collaborators evaluated methods for detecting aCL and aβ2GPI antibodies in patients with APS, and concluded that the two methods are comparable; however, CIA was found to be more sensitive in detecting aβ2GPI IgG while ELISA was more sensitive to aCL IgM. Lastly, the CIA compared to ELISA method was associated with a higher number of LAC-positive APS patients.

In agreement with this, a more recent study showed a good correlation (> 80%) between the ELISA and CIA methods (125). This is also evident in the study of the Iwaniev et al., which, however, reported a significantly lower detection of IgM aCL antibodies (126).

MRI

We decided to use DSC perfusion, which is the most popular perfusion imaging technique applied (127), particularly due to its very fast acquisition time (approximately 1 min acquisition time), as well as the use of conventional and widely available MRI sequences (e.g., gradient-echo echo-planar imaging, EPI), and its very good contrast-to-noise ratio compared with other perfusion imaging methods, such as ASL and Dynamic contrast-enhanced (DCE). The DSC technique relies on drop in the T2 signal after passing a gadolinium-based contrast agent (128).

Indeed, when the contrast agent reaches the vessels, it makes them more paramagnetic, and field inhomogeneities around the vessels are created. Thus, the concentration of the contrast agent may be derived from the loss in the signal intensity–time curve due to susceptibility effects of the contrast agent itself.

DSC perfusion may be obtained by using both gradient-echo and spin-echo sequences, which uses a spin-echo-planar scan. On the gradient-echo sequence, the effect of a contrast agent is stronger compared to the spin-echo signal due to the fact that the former has an additional static dephasing of spins in the same inhomogeneous environment. Different studies demonstrated that sensitivity of gradient-echo DSC is similar for a broad range of vessel sizes while spin-echo DSC is particularly sensitive to capillary-sized vessels (129).

A key role in quantifying CBF by using this technique is played by the so-called arterial input function (AIF), which describes the contrast agent input to the tissue of interest. Due to its fundamental role, many studies in recent years have focused on how and where to measure the AIF (global or regional, inside or outside the artery, manually or automatically), how DSC-MRI quantification may be influenced by AIF determination, what artefacts may be related to it, and the design of automatic processes to measure the AIF (127) In this study, we decided to perform a global, automatic, and outside-artery determination of AIF (127).

One of the main limitations of the DCS perfusion technique relies on the possible extravasation of the contrast agent due to the damage of BBB that may lead to T1 and T2* relaxation effects and, thus, to underestimating or overestimating rCBV, respectively (127). These leakage effects may partially be corrected by using a preload contrast bolus OR leakage correction algorithm.

CONCLUSION

In this study, it will be important to identify the exact links between activation of coagulation/complement system and brain hemodynamic changes with cerebral hypoperfusion.

We hypothesized that cerebral hypoperfusion in all forms of MS could be the result of the blood flow deceleration mostly in the venous vessel bed during brain inflammatory-thrombotic processes in the course of relapses. Systemic immune activation during the infections influences innate brain immunity and, consequently, adaptive immune response (79, 80). When recurrent and chronic infections, which manifest systemically with immunothrombosis (13), directly or indirectly involve the CNS, it could lead to acute and chronic neuroinflammation. Constant crosstalks between immune cells and coagulation are seminal for an effective immune response (12). While many efforts have been carried out to better define the function of innate immune cells in order to modulate their potential pathogenetic role in MS with specific therapeutic action (5, 6), the coagulant component of innate immunity, which is well studied in animals, has not been sufficiently evaluated in humans. Our working hypothesis is that relapsing patients could have a pro-coagulant condition that may be correlated with blood flow deceleration and the presence of serological indicators of ongoing infection. Whether or not we found correlations between laboratory and MRI parameters, we may see the difference between the relapsing and the remitting MS patient groups. One could argue that peripheral laboratory parameters measured in this study may not fully reflect nor be specific for pathophysiological events occurring in the CNS. However, we hypothesize and believe that events at the CNS level, particularly in MS, could partially represent or be a result of systemic diseases such as infections.

Even if the activation of the coagulation system linked to innate immunity is a mandatory process following different types of tissue damage, interfering with the coagulation system could represent a new therapeutic target in MS. This approach may lead to improved treatment options (e.g., polytherapy) and the development of new therapeutic perspectives for MS and demyelinating diseases in general, but also for other neurodegenerative conditions. It is already possible to interfere with the coagulation system at various levels of the cascade. Therefore, clinical trials trying to transfer the promising results on EAE to humans are needed.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Local ethical committee of IRCCS Regina Elena National Cancer Institute, Rome, Italy. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TK and MI devised the project, the main conceptual ideas, and proof outline. TK designed and directed the project. AS, MF, CL, CM, LC, GD, FP, SZ, SD, and GB worked out almost all of the technical details. DG performed the numerical calculations for the suggested experiment. TK and AS wrote the manuscript with input from all authors. CL, SL, FP, SD, MI, MF, MS, and ED contributed to the writing of the manuscript. AS, SD, and CL designed the figures. All authors contributed to the article and approved the submitted version.

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BRIEF RESEARCH REPORT
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Platelets Selectively Regulate the Release of BDNF, But Not That of Its Precursor Protein, proBDNF

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Background: Brain-derived neurotrophic factor (BDNF) plays a role in synaptic plasticity and neuroprotection. BDNF has well-established pro-survival effects, whereas its precursor protein, proBDNF, induces apoptosis. Thus, it has been suggested that the proBDNF/BDNF ratio could be an indicator of neuronal health. Access to neurons is, understandably, limited. Because of their similarities, platelets have been put forward as a non-invasive biomarker of neuronal health; indeed, they store large quantities of BDNF and can release it into circulation upon activation, similarly to neurons. However, whether platelets also express the precursor proBDNF protein remains unknown. We therefore sought to characterize proBDNF levels in human platelets and plasma.

Methods: The presence of proBDNF was assessed by immunoblotting, cell fractionation, flow cytometry, and confocal microscopy in washed platelets from 10 healthy volunteers. Platelets from 20 independent healthy volunteers were activated with several classical agonists and the release of BDNF and proBDNF into plasma was quantified by ELISA.

Results: Platelets expressed detectable levels of proBDNF (21 ± 13 fmol/250 x 10^6 platelets). ProBDNF expression was mainly localized in the intracellular compartment. The proBDNF to BDNF molar ratio was ~1:5 in platelets and 10:1 in plasma. In stark contrast to the release of BDNF during platelet activation, intraplatelet and plasma concentrations of proBDNF remained stable following stimulation with classical platelet agonists, consistent with non-granular expression.

Conclusions: Platelets express both the mature and the precursor form of BDNF. Whether the intraplatelet proBDNF to BDNF ratio could be used as a non-invasive biomarker of cognitive health warrants further investigation.

Keywords: platelets, brain-derived neurotrophic factor, secretion, plasma, pro-BDNF

INTRODUCTION

The brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family initially identified in the central nervous system where it is known to play a role in synaptic plasticity, long-term memory, cognition and neuroprotection (1–3). It is produced as a precursor protein, proBDNF, which is then cleaved by intracellular or extracellular proteases to release the pro-domain and the mature BDNF protein (2, 4). Like BDNF, proBDNF can be released by neurons following an action potential (5, 6), and has an active biological function, which is in opposition with the pro-survival functions of BDNF. Indeed, proBDNF induces neuronal apoptosis (5), reduces dendritic arborization (6), and negatively regulates synaptic plasticity (6, 7) and transmission (6).

The contrast between the role of BDNF and that of its precursor has led to the hypothesis that the regulation of the proBDNF/BDNF ratio is important for maintenance of a healthy nervous system (4, 5, 7, 8). In line with this hypothesis, it has been shown that the regulation of proBDNF cleavage is a key process in long-term hippocampal synaptic plasticity (9) and in memory (10, 11). Furthermore, some authors suggest that impairment in proBDNF cleavage could be important in the pathophysiology of cognitive disorders (12, 13). Several studies report a change of the proBDNF/BDNF ratio in neurons or cerebrospinal fluid in diverse neurocognitive disorders such as Alzheimer's disease (13, 14), major depressive disorder (8), autism (15), and affective disorders (4).

Although it was at first discovered in the brain, we now know that BDNF is also present in blood, where it is essentially stored in platelets (16, 17). BDNF levels in platelets can reach 100- to 1,000-fold those of neurons, making platelets the most important peripheral reservoir of BDNF (17–20). Similarly to neurons, platelets store BDNF mainly in α-granules (21) and release it into the bloodstream during platelet activation (16). While proBDNF has also been reported in circulation (22, 23), the origin of peripheral proBDNF is unknown. It is particularly puzzling, as the presence of proBDNF in platelets has never been confirmed.

We therefore sought to investigate the presence of proBDNF in platelets; to compare the plasma *vs*. platelet levels of proBDNF in healthy volunteers; and to investigate the release profiles of proBDNF *vs*. BDNF in response to platelet activation induced by different agonists. As several studies have suggested that platelets could be a potential biomarker of neuropsychiatric disorders, this study could open up new avenues of research on the intraplatelet proBDNF/BDNF ratio as a biomarker of neurocognitive health (24–28).

Abbreviations: AA, arachidonic acid; ACD, acid citrate dextrose; ADP, adenosine diphosphate; BDNF, brain-derived neurotrophic factor; BSA, bovine serum albumin; Ctrl, control; DABCO, 1,4-diazabicyclo(2,2,2)octane; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; IB, immunoblotting; IQR interquartile range; kDa, kilodaltons; PBMC, peripheral blood mononuclear cell; PBS, phosphate buffered saline; PFA, paraformaldehyde; PPP, platelet-poor plasma; proBDNF, pro-brain-derived neurotrophic factor; PRP, platelet-rich plasma; RIPA buffer, radioimmunoprecipitation assay buffer; rProBDNF, recombinant pro-brain-derived neurotrophic factor; RT, room temperature; SD, standard deviation; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBS-T, tris buffered saline-tween; TRAP, thrombin-related activating peptide; Tris, tris (hydroxymethyl)aminomethane.

MATERIALS AND METHODS

Participant Selection

This study was approved by the Montreal Heart Institute Scientific and Research Ethics Committee (REC reference: #2016-1996) and all participants gave written informed consent. A total of 30 participants were included in this study; in 10 subjects, platelets were isolated and washed to better characterize the presence of proBDNF in platelets, and in 20 subjects, proBDNF and BDNF levels were quantified in platelets and plasma following platelet activation. Participants were exempt of chronic diseases, did not require chronic medical treatment, had refrained from drugs influencing platelet function in the 2 weeks preceding blood sample collection, and had normal platelet count and hemoglobin levels. Participants with a history of bleeding were excluded.

Blood Collection and Platelet Isolation

Using a 21G needle, blood was drawn by venipuncture into 30 ml syringes containing either acid citrate dextrose (ACD-A) in a 1:5 volume ratio for experiments carried out in washed platelets, or sodium citrate 3.2% in 1:9 volume ratio for experiments in platelet-rich plasma (PRP). Blood samples were gently mixed by inversion, transferred to 50 ml tubes and centrifuged at 200 g for 15 min without a brake to prepare PRP and at 1,000 g for 10 min to prepare platelet-poor plasma (PPP).

Native citrated PRP was used without adjustment of platelet count for platelet aggregation experiments, with autologous PPP used to set baselines.

To obtain washed platelets, prostaglandin E_1 (1 μM) was added to ACD-anticoagulated PRP prior to centrifugation at 1,000 g for 10 min. Platelets were resuspended in Tyrode's buffer (137 mM NaCl, 11.9 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 2.7 mM KCl, 1.1 mM $MgCl_2$, 5.6 mM glucose, pH 7.4). This washing procedure was repeated three times. Platelets were counted using a Beckman Coulter hematology analyzer (Ac-T 5diff AL) and adjusted to a final concentration of 2.5 x 10^8/ml for flow cytometry experiments or 2 x 10^9/ml for cell fractionation and deglycosylation experiments. Platelets were allowed to rest at room temperature (RT) for 60 min prior to functional experiments.

Cell Fractionation

Washed platelets (2 x 10^9/ml) resuspended in phosphate buffered saline (PBS) were lysed by three freeze-thaw cycles (temperatures of −80 and 37°C). The samples were then centrifuged at 200,000 g for 90 min at 4°C. The supernatant representing the cytosolic fraction was transferred to a new tube, while the pellet representing the cytoskeleton and membranes was solubilized in radioimmunoprecipitation assay (RIPA) buffer [150 mM NaCl, 5 mM EDTA pH 8.0, 50 mM Tris-HCl pH 8.0, 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)]

and incubated on ice for 15 min to dissolve the membranes. The solubilized sample was then centrifuged at 100,000 g for 30 min at 4°C. The supernatant in which the membranes were dissolved was transferred to a new tube, while the pellet representing the cytoskeleton was resuspended in 1X Laemmli buffer and heated at 95°C for 5 min with vigorous vortexing. Samples were then assessed by immunoblotting using antibodies against proBDNF (as described below), BDNF (Biosensis, M-1744, monoclonal mouse antibody, 1 µg/ml), p-selectin (Santa Cruz, sc-6941 polyclonal goat antibody, 0.26 µg/ml), α-tubulin (Sigma-Aldrich, T5168 monoclonal mouse antibody, clone B-5-1-2, 1.225 µg/ml), and p65 NF-κB (Santa Cruz, sc-372 polyclonal rabbit antibody, 0.4 µg/ml). The equivalent in the protein content of 3 x 10^7 platelets was loaded for each fraction on the gel.

Deglycosylation

Washed platelets and U87-MG cells were lysed with ice-cold RIPA buffer for 20 min. Platelet or U87-MG cell lysates (100 µg) were denatured at 100°C for 10 min in a glycoprotein denaturating buffer (0.5% SDS, 40 mM DTT, B1704S, New England Biolabs, MA, USA) and allowed to cool to RT. GlycoBuffer 2 with 1% NP-40 (New England Biolabs, MA, USA) was added to denatured proteins, and PNGase F (P0704S, New England Biolabs, MA, USA) was then added to a final concentration of 50 units per µg of total protein. The mixture was incubated at 37°C for 1 h, frozen and kept at −80°C. Immunoblotting was performed using antibodies against proBDNF (Biosensis, R-176, polyclonal rabbit antibody, 0.25 µg/ml), CD42b (Santa Cruz, sc-59051, monoclonal mouse antibody, clone PM6/40, 1 µg/ml), or sortilin/NT3 (Abcam, ab16640, polyclonal rabbit antibody, 2 µg/ml). The equivalent of the lysate of 3 x 10^7 platelets was loaded in each well.

ProBDNF and α_2-Macroglobulin Immunoblotting

Human recombinant proBDNF (Alomone Labs, Israel) and Human Brain Cerebral Cortex Whole Tissue Lysate (Novus Biologicals, Bio-Techne, Oakville, ON, Canada) were used as positive controls. Native platelet whole cell lysates were incubated in Laemmli loading buffer (250 mM Tris pH 6.8, 8% SDS, 40% glycerol, 20% β-mercaptoethanol, and 0.02% bromophenol blue) before heating at 95°C for 5 min. Protein samples were separated on a 12% SDS-polyacrylamide gel electrophoresis (PAGE), transferred onto a PVDF 0.2 µm membrane and fixed with glutaraldehyde 0.5% for 30 min. Membranes were washed three times with TBS-T (Tris-buffered saline, 0.1% Tween 20) for 10 min and blocked for 1 h at RT in blocking solution [3% bovine serum albumin (BSA) in TBS-T or 5% milk in TBS-T] before incubation with anti proBDNF or anti α_2-macroglobulin antibodies (Biosensis, anti-proBDNF R-176 polyclonal rabbit antibody, 0.25 µg/ml; R&D systems, anti-proBDNF mab31751 monoclonal mouse antibody, clone 584412, 0.5 µg/ml; Abbexa, anti-α_2-macroglobulin abx132389, monoclonal mouse antibody, 1 µg/ml) overnight at 4°C. The blots were then washed and incubated with HRP-conjugated goat anti-rabbit or goat anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, diluted 1:10,000 in 5% milk) for 1 h at RT. Luminata Classico Western HRP Substrate (Millipore) was used for chemiluminescent detection.

Flow Cytometry

Washed platelets (2.5 x 10^8/ml), U87-MG or U251-MG cells were fixed with 1% paraformaldehyde (PFA) for 20 min at RT, a fraction of which was also permeabilized using 0.1% Triton-X for 15 min at RT. Platelets and neuroblastic cells were then incubated at RT with mouse anti-human proBDNF antibody (R&D System, mab31751, monoclonal mouse antibody, clone 584412, diluted 1:25 or Biosensis, R-176 polyclonal rabbit antibody diluted 1:25) or mouse IgG_{2b}/rabbit isotype control (R&D System, MAB004 and AB-105-C diluted 1:25) for 30 min. Alexa Fluor 488 or 647 conjugated donkey anti-mouse or rabbit secondary antibody (Invitrogen, diluted 1:100) was added for 30 min at RT in the dark. Samples were analyzed using the MACSQuant Analyzer 10 (Miltenyi Biotec, Germany).

Confocal Microscopy

Glass coverslips were pre-coated with 0.1% poly-L-lysine for 15 min at RT. Platelets fixed with 1% PFA for 20 min at RT were transferred onto pre-coated coverslips and allowed to adhere overnight at 4°C. Fixed U251-MG cells were plated on the pre-coated coverslips and incubated overnight at 37°C/5% CO_2 incubator. Platelets and U251-MG cells were permeabilized using 0.1% Triton X-100 in PBS for 10 min at RT. Coverslips were then washed twice with PBS and blocked with 3% donkey serum in PBS for 30 min at RT. Coverslips were then washed two times with PBS and proBDNF labelling was performed using an anti-proBDNF primary antibody (R&D, mab31751, monoclonal mouse antibody, clone 584412, 5 µg) for 2 h at RT followed by two PBS washes and an incubation with an Alexa Fluor 488-conjugated donkey anti-mouse secondary antibody (1:200) for 90 min at RT. Mouse IgG_{2b} was used as isotype control antibody (R&D System, MAB004, clone 20116, 5 µg). After two more washes, U251-MG coverslips were incubated with diluted DAPI (10 mg/ml, 1:1,000) for 5 min and washed again twice with PBS. Coverslips were then treated with 1,4-diazabicyclo(2,2,2)octane (DABCO) mounting medium (25 mg/ml DABCO in 90% glycerol/10% PBS solution) overnight in the dark. Fluorescence was visualized using a Zeiss LSM510 confocal microscope.

Light Transmission Aggregometry

Platelet aggregation was measured using a Chronolog Aggregometer (Model 700, Havertown, PA, USA) at 37°C with continuous stirring at 1,200 rpm. Platelet aggregation traces were recorded for 6 min using the AGGRO/LINK®8 Software package. The following agonists were used: adenosine diphosphate (ADP, Sigma Aldrich) 10 µM, arachidonic acid (AA, Cayman Chemical) 1 mM, collagen (Chronolog) 5 µg/ml, or thrombin-related activating peptide (TRAP-amide, Bachem) 3 µM. A vehicle-treated PRP sample was used under stirring conditions as a control. Ethylenediaminetetraacetic acid (EDTA, 5 mM) was added at the end of the incubation to stop platelet activation and agitation was continued for 1 min. Platelets and plasma were separated by centrifugation at 1,000 g for 5 min and

placed into separate tubes. Platelets were lysed for 30 min on ice with lysis buffer (1% NP40, 20 mM Tris pH 8.0, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate) containing Pierce Protease and Phosphatase Inhibitor Mini Tablets (Thermo Scientific). Lysed platelets and supernatants containing the platelet releasate were kept frozen at −80°C until analysis.

Assessment of BDNF and proBDNF Levels

Plasma and intraplatelet concentrations of BDNF and proBDNF were determined by ELISA (R&D System, DY248 and DY3175). Plasma and platelet lysate samples were centrifuged at 3,000 rpm for 5 min to remove cellular debris. Samples were diluted 1:15 in reagent diluent (**Supplementary Figures 1** and **2**). Assays were performed in accordance with manufacturer's instructions. Cross-reactivity for mature BDNF was negligible (1.4%) in the proBDNF assay, and low (13%) for proBDNF in the BDNF assay. Each condition was tested in duplicate. Colorimetric reading was performed with the Infinite F50 plate reader (Tecan, Männedorf, Switzerland) at 450 nm, with a reference at 620 nm.

Statistical Analyses

Continuous variables are presented as mean and standard deviation (SD) or median and interquartile range (IQR) when distribution deviated from normal. N refers to the number of independent experiments with each experiment representing a different biological sample. Repeated-measures analysis of variance (ANOVA) with Geisser-Greenhouse correction for sphericity and Dunnett's correction for multiple comparisons was performed to assess differences between agonist-stimulated conditions. Pearson correlation was used to explore the association between proBDNF and BDNF levels. All analyses were carried out with GraphPad Prism Software version 8 for MacOS (GraphPad Software, San Diego, CA, USA). A multiplicity-adjusted p value < 0.05 was considered significant.

RESULTS

Human Platelets Contain proBDNF

To assess the presence of proBDNF in platelets, we performed immunoblotting experiments on washed platelets using two different proBDNF antibodies (**Supplementary Figure 3**). To verify that detection of proBDNF in platelets was not due to plasma protein contamination, we cross-checked for immunoreactivity for α_2-macroglobulin in platelet lysates (**Figure 1F**), showing absence of this plasma protein in the washed platelet preparation. Human cortex used as positive control, expressed proBDNF at ~32–35 kDa (**Figure 1A**). Recombinant proBDNF produced in *Escherichia coli* was detected at a lower molecular weight (~25–27 kDa). In platelets, we detected a band at ~32–35 kDa (**Figure 1A**). Following incubation with PNGase F, a clear shift was seen for CD42b, a platelet membrane glycoprotein used as a positive control for N-deglycosylation (**Figure 1C**). Although a lower band did appear below the 32–35 kDa band for proBDNF, the same band was also visible in the absence of platelet protein, likely due to non-specific binding to PNGase F (expected molecular weight 36 kDa). Treatment of U87-MG human glioblastoma cell lysates with PNGase F also failed to show N-deglycosylation of proBDNF. This suggests that the differences in molecular weight between platelet and neuronal BDNF *vs.* recombinant proBDNF were not attributable to N-glycosylation.

Platelet proBDNF Is Localized in the Cytoplasm

The cytosolic, membrane (including granular membranes), and cytoskeletal fractions of platelets were obtained by differential ultracentrifugation and analyzed by immunoblotting. To confirm the cellular compartmentation of the fractions, we used NF-κB as a marker for the cytosolic fraction, P-selectin for the membrane fraction, and α-tubulin for the cytoskeletal fraction (**Figure 1B**). Mature BDNF was mainly found in the membrane fraction (presumably in α-granules), but also at lower levels in the cytosolic and cytoskeletal fractions (**Figure 1B**). In contrast, proBDNF was distributed similarly in each of the three fractions (**Figure 1B**). Interestingly, a lower band around the 15-kDa marker which could correspond to the cleaved pro-domain of proBDNF (29, 30), exclusively segregated into the cytoplasmic fraction (**Figure 1B**).

Immunoblotting for α-tubulin showed immunoreactivity in the membrane and cytosol fractions, suggesting suboptimal cytoskeletal fraction separation. Therefore, to further investigate proBDNF localization, we performed flow cytometry and confocal microscopy on fixed and permeabilized platelets. A mean of 13 ± 8% of platelets expressed proBDNF at their surface, and 40 ± 20% were positive for proBDNF once cells were permeabilized (**Figure 1D**). To validate that detection of proBDNF was not due to plasma protein adsorption on platelet plasma membranes, acid-washed platelets were compared with platelets washed in Tyrode's buffer at physiological pH, with no significant drop in proBDNF signal in acid-washed platelets (**Supplementary Figure 4**). Confocal microscopy imaging also suggested that proBDNF was present at the membrane and in the cytosol of platelets (**Figure 1E**). Human glioblastoma U87-MG and U251-MG cells used as positive controls showed mainly intracellular expression by flow cytometry, and microscopy further confirmed that expression was essentially cytosolic and nuclear (**Figures 1D, E**).

Human Platelets Contain Less proBDNF Than BDNF

To assess the relative abundance of proBDNF and BDNF in platelets, we quantified both proteins by ELISA in 20 healthy volunteers (**Table 1**). The levels of proBDNF were variable from one individual to the other (**Figure 2A**), but under basal conditions, proBDNF levels were significantly lower than BDNF levels (proBDNF 1,085 ± 672 pg/250 x 10^6 platelets equivalent to 21 ± 13 fmol/250 x 10^6 platelets compared to 4,516 ± 2,915 pg/250 x 10^6 platelets equivalent to 167 ± 108 fmol/250 x 10^6 platelets for BDNF). The mean intraplatelet proBDNF/BDNF molar ratio was 0.18 ± 0.14 (**Figure 2B**), meaning that there was ~1 molecule of proBDNF for ~5 molecules of BDNF in platelets (**Table 2**).

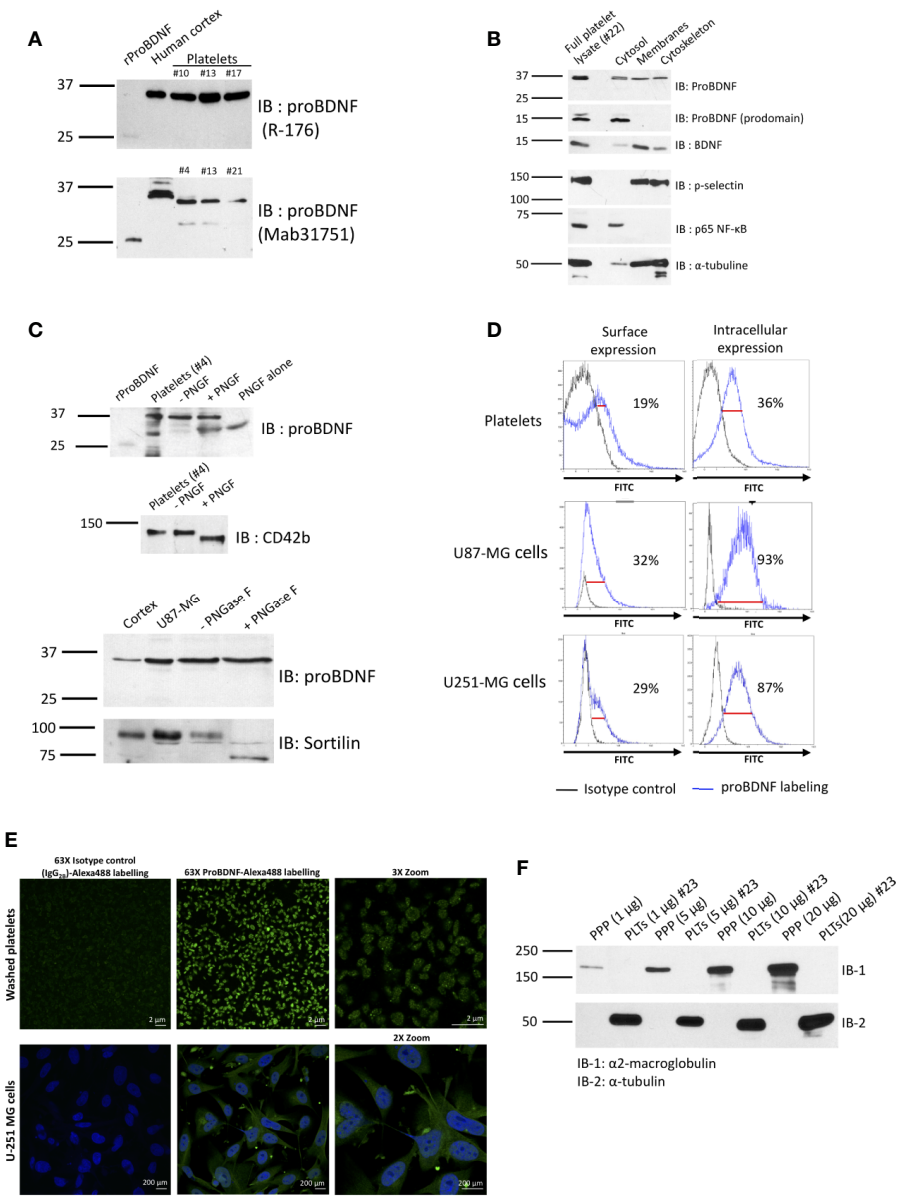

FIGURE 1 | Human platelets contain proBDNF. **(A)** ProBDNF immunoblotting of human washed platelet lysates (15 µg) from six different healthy volunteers. Recombinant proBDNF (3 ng) and human cortex lysate (3 µg) were used as positive controls. Molecular weight is indicated on the left (kDa) and primary antibody on the right. Experiments representative of n=9 for R-176 and n=10 for mab31751 antibody. IB, immunoblotting. **(B)** Immunoblotting of proBDNF and BDNF in different fractions of washed human platelets. P-Selectin was used as control protein in the membrane fraction, p65 NF-κB was used as control protein in the cytosol, and α-tubulin was used as control protein in the cytoskeleton. The equivalent of the protein content of 3 x 10^7 platelets was loaded for each fraction on the gel. Representative experiment of n=4 different volunteers. **(C)** ProBDNF treatment with PNGase F in washed human platelet lysates. U87-MG glioblastoma cells were used as a control. rProBDNF, recombinant proBDNF (3 ng); cortex, human cortex lysate (3 µg); platelets, whole human platelet lysate (representative experiment of n=4 different volunteers, 7.5 x 10^8 platelets per well); –PNGF, platelets treated with GlycoBuffer, and incubated at 37°C for 60 min without PNGase F; +PNGF, platelets treated with GlycoBuffer and incubated at 37°C for 60 min with PNGase F; PNGF alone, PNGase F incubated at 37°C for 60 min without platelet lysate. CD42b and sortilin were used as controls of protein deglycosylation in platelets and in U87-MG cells, respectively. n=3 different volunteers for PNGase treatments in human platelets and n=4 independent experiments for U87-MG cells. **(D)** Representative flow cytometry experiment showing surface and intracellular proBDNF in human washed platelets and in U87-MG and U251-MG glioblastoma cell lines. Mouse IgG_{2b} was used as isotype control. Percentage of expression are indicated on the figure. n=10 different healthy volunteers for human platelets; n=3 independent experiments for each glioblastoma cell line. **(E)** Confocal microscopy imaging of proBDNF in human permeabilized washed platelets (top) and in permeabilized U-251 MG cells (bottom). Mouse IgG_{2b} was used as isotype control. ProBDNF was labelled using Alexa488 fluorochrome (in green). Nuclei were stained with DAPI (in blue). Scale bar = 2 µm and 200 µm for washed platelets and U-251 MG cells images, respectively. **(F)** Immunoblotting of α_2-macroglobulin at increasing quantities of loaded proteins (1–20 µg) obtained from a washed platelet lysate or platelet-poor plasma (PPP) from the same individual (#23). α-tubulin was used as loading control. Molecular weight is indicated on the left (kDa) and primary antibody on the right. PPP, platelet poor plasma; PLTs, platelets; IB, immunoblotting.

TABLE 1 | Characteristics of the healthy volunteers included in the ELISA quantification study (n = 20).

	Number of participants, n (%)
Sex	
Female	13 (65)
Male	7 (35)
Age (years)	
20–29	9 (45)
30–39	4 (20)
40–49	5 (25)
50–59	2 (10)
Ethnicity	
French Canadian	18 (90)
North African	1 (5)
Caribbean	1 (5)
Body mass index (kg/m^2)	
<18.5 (underweight)	1 (5)
18.5–24.9 (normal weight)	10 (50)
25.0–29.9 (overweight)	8 (40)
30.0–34.9 (class I obesity)	1 (5)
35.0–39.9 (class II obesity)	0 (0)
>40.0 (class III obesity)	0 (0)
Smoking status	
Smoker	0 (0)
Ex-smoker	3 (15)
Non-smoker	17 (85)
Daily physical activity level	
Sedentary	4 (20)
Light	1 (5)
Moderate	15 (75)
Vigorous	0 (0)

Plasma Concentrations of proBDNF Are Higher Than Those of BDNF

We then investigated whether a similar proBDNF/BDNF ratio was found in circulation. Under basal conditions, we found the opposite pattern in plasma to that observed in platelets (**Figure 2C**), i.e., concentrations of proBDNF in plasma were much higher than those of BDNF (proBDNF 28,019 ± 19,695 pg/ml or 541 ± 380 fmol/ml compared with BDNF 2,064 ± 1,825 pg/ml or 76 ± 68 fmol/ml). We calculated the mean proBDNF/BDNF ratio to be ~10 molecules of proBDNF for ~1 molecule of BDNF in plasma (**Figure 2D**, **Table 2**).

BDNF and proBDNF Concentrations Are Correlated in Platelets But Not in Plasma

Figures 2E, F show the association between intraplatelet and plasma levels of proBDNF and BDNF. While a linear correlation was seen between BDNF and proBDNF in platelets (r=0.71; p=0.0005, **Figure 2E**), no such association was seen in plasma (r=0.14; p=0.58, **Figure 2F**), suggesting the regulation of proBDNF/BDNF ratio is different in the cellular and plasma compartments.

Unlike BDNF, proBDNF Is Not Released During Platelet Activation

We next studied whether platelets have the ability to release proBDNF in the same manner they release BDNF during their activation. Platelet responses to four different platelet agonists (ADP, TRAP, AA, and collagen) were assessed in platelet-rich plasma from 20 healthy volunteers. As expected, intraplatelet concentrations of BDNF decreased with the addition of platelet agonists and plasma concentrations increased, confirming that BDNF was released from platelets during their activation (**Figures 3A, B**). As shown in **Figure 3C**, resting platelets contained ~70% of total BDNF present in PRP, and this proportion decreased to ~20% after platelet activation.

In contrast, intracellular and plasma levels of proBDNF remained stable in response to platelet activation (**Figures 3D, E**). Only ~10% of proBDNF in PRP appeared to be stored in platelets, regardless of platelet activation status (**Figure 3F**). As a consequence, platelet activation significantly changed the proBDNF to BDNF ratio in plasma from 10:1 to close to 2:1 (**Table 2**).

DISCUSSION

In the present study, we have shown that: 1) platelets contain proBDNF in a 1:5 ratio to BDNF; 2) the levels of intraplatelet proBDNF correlate strongly with those of BDNF, whereas no such association was seen in plasma; 3) the pool of intraplatelet proBDNF represents approx. 10% of total circulating proBDNF, whereas approx. 70% of circulating BDNF is stored in platelets; and 4) platelet activation does not lead to proBDNF secretion, in contrast to BDNF that is largely released following platelet activation.

Presence of proBDNF in Platelets

Since the early 1990's, numerous reports have conclusively shown platelets to contain large quantities of BDNF as to represent the major peripheral reservoir of this neurotrophin (16, 17, 31, 32). Circulating proBDNF, on the other hand, has received considerably less attention. While it is known that proBDNF is produced by many cell types including neurons (5, 6), megakaryocytes (18), lymphocytes (33), skeletal muscle (34), and endothelial cells (35), the contribution of these cells to circulating proBDNF levels is not elucidated.

To our knowledge, there is only one group that has investigated the presence of proBDNF in platelets (18). Chacón-Fernández *et al.* have shown platelet precursor cells, megakaryocytes, to express proBDNF, but failed to detect proBDNF in mouse, rat, and human platelets with an antibody targeting the mature BDNF protein (18). With the use of antibodies targeting the prodomain of proBDNF, we were able to show that platelets do contain proBDNF, albeit in a much lesser proportion to its mature counterpart. The origin of BDNF in platelets remains debated. Chacón-Fernández *et al.* have shown BDNF to be present in megakaryocytes, their proplatelet extensions, and in platelets, suggesting BDNF is inherited from megakaryocytes (18). Fujimura *et al.* have shown platelets to internalize exogenous BDNF, suggesting they may acquire it from the bloodstream (16). The relative contribution of inherited *vs.* internalized BDNF in platelets remains unknown.

Our results show a high variability in circulating BDNF and proBDNF levels among healthy individuals. While the

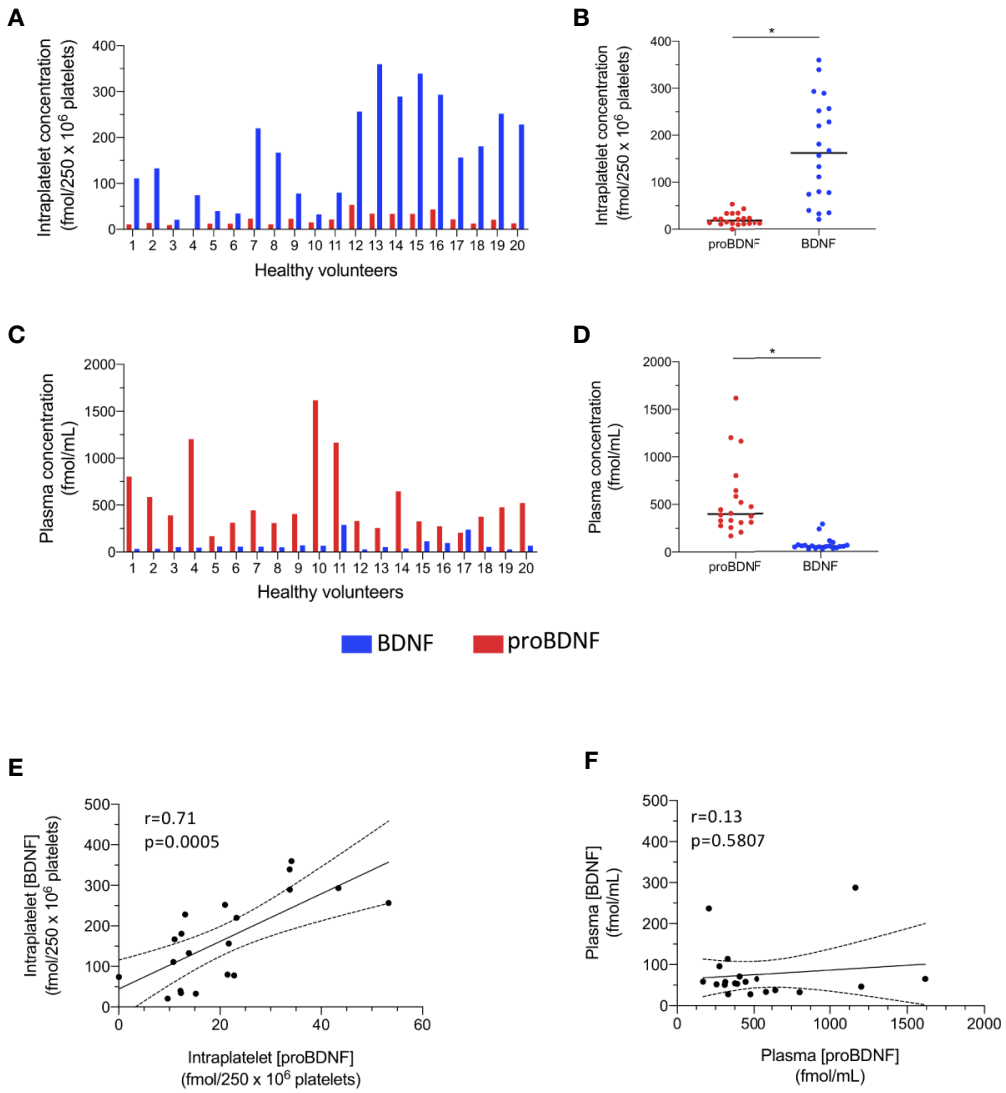

FIGURE 2 | Molar concentrations of proBDNF are lower in platelets and higher in plasma than those of BDNF. ELISA quantification of proBDNF and BDNF levels in the intraplatelet **(A, B)** and plasma **(C, D)** compartments. Concentrations are normalized for 250 x 10^6 platelets. Horizontal bar represents median, *p<0.05. **(E, F)** Correlation between BDNF and proBDNF molar concentrations in human platelets **(E)** and in plasma **(F)**. Dotted lines represent 95% confidence intervals, n=20 participants (#1 to #20).

TABLE 2 | proBDNF/BDNF ratio as a function of platelet activation status.

	Platelet state	Mean proBDNF/BDNF ratio (n=20)	SD	95% CI	p-value
Intraplatelet	**Basal**	0.18	0.14	0.12–0.24	
	Activated				
	ADP (10 µM)	0.46	0.33	0.31–0.60	0.0003
	TRAP (3 µM)	0.56	0.43	0.37–0.75	0.0003
	AA (1 mM)	0.60	0.37	0.43–0.76	0.00001
	Collagen (5 µg/ml)	0.59	0.43	0.40–0.78	0.0002
Plasma	**Basal**	10.2	7.9	6.7–13.7	
	Activated				
	ADP (10 µM)	2.5	4.3	0.60–4.4	0.0001
	TRAP (3 µM)	2.3	3.6	0.70–3.8	0.00005
	AA (1 mM)	3.6	7.4	0.38–6.8	0.003
	Collagen (5 µg/ml)	2.4	3.1	1.02–3.8	0.0002

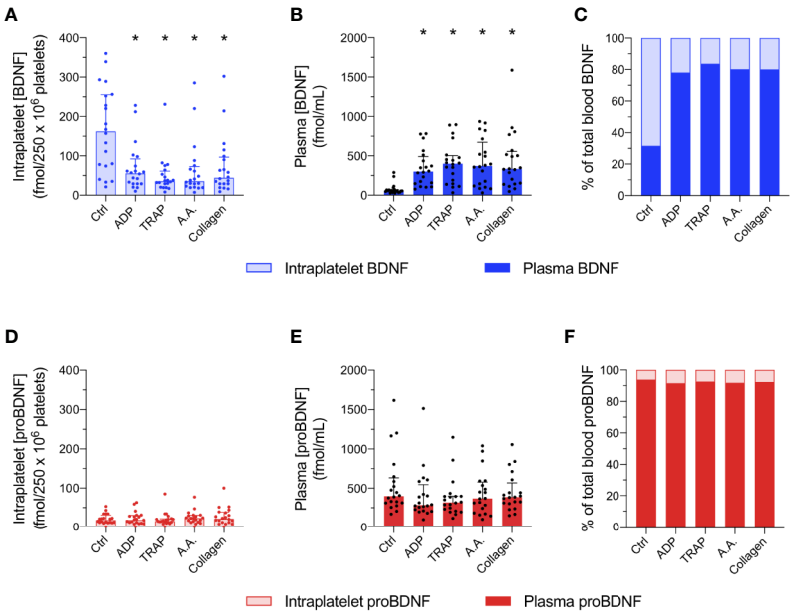

FIGURE 3 | Unlike BDNF, intraplatelet proBDNF is not released during platelet activation. Intraplatelet **(A, D)** and plasma **(B, E)** concentrations of BDNF and proBDNF following platelet activation by different agonists. Intraplatelet concentrations are normalized for 250 x 10^6 platelets. Proportion of BDNF **(C)** and proBDNF **(F)** in plasma *vs*. in platelets are expressed in percentage. Error bar represents IQR, *p < 0.05 *vs*. ctrl, n=20 participants (#1 to #20). Ctrl, control; ADP, adenosine diphosphate; TRAP, thrombin receptor-activating peptide; AA, arachidonic acid.

parameters influencing proBDNF circulating levels have not been the object of investigation, several factors are known to affect blood levels of mature BDNF, including age (36–39), sex (37–39), smoking status (37), and body mass index or weight (38, 39). Our small sample size precludes multivariate analyses to explore the contribution of these characteristics to circulating levels of proBDNF/BDNF in this study, but this should be explored in larger cohorts. Attention should also be given to ethnicity, as it appears to be an important determinant of circulating proBDNF levels (40).

Notwithstanding, for the 20 individuals tested, we found significantly less proBDNF than BDNF in platelets, resulting in a mean 1:5 ratio of proBDNF to BDNF. This ratio is in line with levels observed in the central nervous system, where the proBDNF/BDNF ratio was 1:10 in hippocampal cells (29, 41). However, while proBDNF was shown to be N-glycosylated in human neurons (42) and saliva (43), the platelet proBDNF did not appear to be sensitive to PNGase F treatment, thus arguing against N-glycosylation in these cells. It should be noted however that PNGase treatment of U87-MG glioblastoma cells also failed to induce changes in molecular weight, suggesting that other post-translational modifications may explain the differences of molecular weight with recombinant proBDNF produced in bacteria (25–27 kDa). Five different isoforms of proBDNF have been repertoried in the literature, each with a different molecular weight (44–46). Whether differences in molecular weight seen in this study are due to different isoforms expressed or different post-translational modifications of proBDNF in megakaryocytes and platelets requires further investigation.

In stark contrast to intraplatelet levels, plasma levels of proBDNF were 10 times those of BDNF. This is surprising, in view of the short half-life of BDNF in circulation (47, 48) suggesting that proBDNF is protected from degradation in plasma. It has been shown that mature neurotrophins including BDNF bind reversibly to α_2-macroglobulin (49), a plasma protease inhibitor and transporter, protecting them against proteolytic degradation and clearance pathways (50). Whether proBDNF can similarly bind to α_2-macroglobulin and thus be protected from proteolytic cleavage, or uses a different mechanism to avoid degradation, is worthy of investigation.

Not All Platelets Express proBDNF

Our flow cytometry experiments showed that a mean of 13 ± 8% of platelets had proBDNF on their surface and 40 ± 20% were proBDNF-positive upon permeabilization. Although platelets underwent gentle washing procedures to avoid platelet activation, we can not eliminate the possibility that some of the proBDNF found on the platelet surface is in fact residual from plasma. However, no plasma protein contamination was detected in our platelet lysates (**Figure 1F**), and acid washing to remove plasma proteins adsorbed on cell membranes did not reduce the proBDNF signal in platelets (**Supplementary Figure 2**), thus limiting this possibility. We observed a high level of correlation between intracellular BDNF and proBDNF levels, and found a proBDNF fragment of approx. 15-kDa consistent with the proBDNF pro-domain in the platelet cytosol, both suggesting that an intracellular regulation mechanism of the proBDNF/BDNF ratio is present in

platelets, or is vestigial from their precursor megakaryocytic cells and inherited by platelets during thrombopoiesis. In neurons, proBDNF cleavage is processed by furin, metalloproteinases (MMP-9) (51, 52), and protein convertases (PC1, PC5, PACE4, and PC7) (4). Platelets express several proteases that could possibly participate in BDNF maturation, such as furin-like proprotein convertases (53) and other proteases stored in platelets granules such as MMP-2 and MMP-9 (54). Thus, it is conceivable that proBDNF cleavage occurs in platelets in a regulated manner. While the body of evidence on the role of the pro-domain in the nervous system is growing, it was undetected for many years (55), and its characterization required a complex optimization of techniques (29). In the peripheral nervous system, the cleaved pro-domain is 10-fold more abundant than proBDNF, and both the pro-domain and BDNF are secreted from the same synaptic intracellular vesicles (29). The pro-domain appears to contribute to regulation of neuronal growth, and to essential mechanisms of depression and psychological disorders (30, 56, 57). Therefore, a thorough investigation of the presence and the potential role of the pro-domain in platelets is warranted.

Unlike BDNF, proBNDF Is Not Released Upon Platelet Activation

Our results confirm that platelets release approximately 50% of their BDNF content during activation. Tamura *et al.* have made the same observation and found that there are two distinct pools of BDNF in human platelets: a releasable pool of BDNF stored in α-granules and a non-releasable pool of BDNF localized in the platelet cytoplasm (21). However, Tamura *et al.* used an antibody raised against the mature portion of BDNF, and therefore could not distinguish between the precursor and mature proteins. In our experiments using antibodies raised against the proBDNF pro-domain, we have found a significant proportion of proBDNF to be present in the cytoplasm, as well as in the membrane and cytoskeletal fractions, which might explain the absence of release upon platelet activation. Considering that intraplatelet proBDNF represents only approximately 10% of total circulating proBDNF, and that platelets do not release significant levels of proBDNF upon activation, it is unlikely that plasma proBDNF comes from platelets.

Several groups have explored the possibility that plasma proBDNF is neuronal in origin. However, while it has been suggested by some authors that mature BDNF might cross the blood-brain barrier in mice and rats (58, 59), this finding was not supported by others (47, 48), and none have specifically investigated the permeability of the blood-brain barrier to proBDNF. Thus, the origin of the high levels of proBDNF seen in plasma remains to be elucidated. Notwithstanding, platelet activation induces a dramatic change in the plasma proBDNF to BDNF ratio, by releasing large quantities of mature BDNF (**Table 2**). Any future use of plasma proBDNF to BDNF ratio as a potential biomarker for neurocognitive health will thus need to take into account platelet activation status.

Limitations

Although we were able to show proBDNF in human platelets by three different techniques, there are limitations that require pause. First, the platelet release experiments were performed in plasma, which naturally contains BDNF and proBDNF. Thus, it is possible that the experimental model does not allow the detection of a weak release of proBDNF. Second, we have added EDTA at the end of the platelet activation experiments to inhibit calcium-dependent proteases. We cannot exclude the possibility that proBDNF was rapidly cleaved into BDNF following platelet activation and thus might not be measurable in the supernatant. However, intraplatelet proBDNF levels remained unchanged during platelet activation, which lends credence to the fact that there is no detectable proBDNF release upon platelet activation and argues against the two previous limitations. Our cell fractionation experiments suggest that proBDNF is equally present in cytoplasmic, membrane and cytoskeletal fractions. We used a crude technique for separation of cell fractions, with good resolution of the cytoplasmic from the membrane fraction, but with residual contamination from the cytoskeletal fraction. These results should therefore be interpreted alongside flow cytometry and microscopy assessments. Finally, it is possible that proBDNF and BDNF bind to plasma proteins, which might mask epitopes from detection by ELISA. However, both stimulated and control samples were handled in the same way, and the rise of BDNF levels was readily detectable in plasma. Thus, it is unlikely that release of proBDNF was missed in agonist-stimulated *vs*. resting platelets.

CONCLUSIONS

To our knowledge, this study is the first to report the presence of proBDNF in human platelets. Granted we do not provide the certainty of sequencing through mass spectrometry, but within the limitations of our assays, we are confident that what we are seeing is indeed proBDNF in platelets. It seems however unlikely that platelets contribute to a significant extent to circulating proBDNF levels, since only ~10% of the total circulating levels of proBDNF were found within platelets. Furthermore, in contrast to BDNF, platelets did not release proBDNF during activation, reinforcing the likelihood that the high levels of proBDNF observed in plasma originate from another cell type. The correlation between BDNF and proBDNF concentrations within platelets leads us to hypothesize that there is an intracellular mechanism regulating the proBDNF/BDNF ratio, albeit it could be vestigial from megakaryocytes. Further studies are required to elucidate the role of proBDNF in platelet function and the contribution of the intraplatelet proBDNF/BDNF ratio as a determinant of platelet biology. How the platelet proBDNF/BDNF ratio relates to neuronal levels, and whether platelets could be used as non-invasive biomarkers of neuronal health, remain open questions.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Montreal Heart Institute Scientific and Research Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JL has performed assays and collected data, analyzed and interpreted data, and wrote the manuscript. SF, IB, J-CB, and MW have performed assays and collected data, analyzed and interpreted data, and critically revised the manuscript. ML has overseen the research group, designed the research, obtained funding, analyzed and interpreted data, and critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.575607/full#supplementary-material

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BRIEF RESEARCH REPORT
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Tissue-Specificity of Antibodies Raised Against TrkB and p75^{NTR} Receptors; Implications for Platelets as Models of Neurodegenerative Diseases

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Platelets and neurons share many similarities including comparable secretory granule types with homologous calcium-dependent secretory mechanisms as well as internalization, sequestration and secretion of many neurotransmitters. Thus, platelets present a high potential to be used as peripheral biomarkers to reflect neuronal pathologies. The brain-derived neurotrophic factor (BDNF) acts as a neuronal growth factor involved in learning and memory through the binding of two receptors, the tropomyosin receptor kinase B (TrkB) and the 75 kDa pan-neurotrophic receptor (p75^{NTR}). In addition to its expression in the central nervous system, BDNF is found in much greater quantities in blood circulation, where it is largely stored within platelets. Levels 100- to 1,000-fold those of neurons make platelets the most important peripheral reservoir of BDNF. This led us to hypothesize that platelets would express canonical BDNF receptors, i.e., TrkB and p75^{NTR}, and that the receptors on platelets would bear significant resemblance to the ones found in the brain. However, herein we report discrepancies regarding detection of these receptors using antibody-based assays, with antibodies displaying important tissue-specificity. The currently available antibodies raised against TrkB and p75^{NTR} should therefore be used with caution to study platelets as models for neurological disorders. Rigorous characterization of antibodies and bioassays appears critical to understand the interplay between platelet and neuronal biology of BDNF.

Keywords: platelet, neurotrophin receptors, tropomyosin receptor kinase B, brain-derived neurotrophic factor, pan-neurotrophic receptor p75^{NTR}

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INTRODUCTION

Platelets are circulating anucleate cells originating from megakaryocytes. In addition to their crucial role in hemostasis, platelets have been proffered as a peripheral model for the study of neuronal processes as they share many similarities with neurons (1–3). These include similar secretory granule types with homologous calcium-dependent secretory mechanisms (4–7) as well as internalization, sequestration and secretion of many neurotransmitters (8–10). Moreover, platelet abnormalities are reported in multiple neurological pathologies (11), suggesting common pathophysiological mechanisms. Indeed, platelets express many proteins found in neurons, including serotonin transporter SERT (12), amyloid precursor protein (APP), and amyloid β (2). The brain-derived neurotrophic factor (BDNF) is one such protein present in the central nervous system that is also found within platelets (13), with concentrations reaching up to 1,000-fold those of neurons (13–15).

In the brain, BDNF is involved in axonal growth through the binding of the tropomyosin receptor kinase B (TrkB). This receptor has a highly glycosylated extracellular domain (ECD) and an intracellular domain (ICD) consisting of a SRC homology 2 domain-containing-transforming protein C (Shc)-binding domain and a tyrosine kinase region (16, 17). Two truncated isoforms are also found in the central nervous system: the 95 kDa TrkB-T1 isoform, lacking both the Shc-binding and tyrosine kinase domains (18, 19) and the 100 kDa TrkB-T-Shc isoform that also lacks the tyrosine kinase domain but expresses the Shc-binding domain (19, 20). Additionally, BDNF is involved in the myelination of peripheral axons through the binding of the 75 kDa pan-neurotrophic receptor ($p75^{NTR}$). This receptor is composed of an ECD consisting of four cysteine-rich domains (CRD) containing sites for both *N*- and *O*-linked glycosylation (21, 22). The intracellular domain consists of a palmitoylated chopper domain followed by a death domain (23, 24). The full-length isoform of the $p75^{NTR}$ receptor has a monomeric molecular weight varying between 72 and 85 kDa (21, 25–30). A 62–65 kDa splice variant lacking CRD 2 through 4 has also been reported (27, 31).

Platelets internalize BDNF and secrete it upon activation (32, 33). While the contribution of the brain-borne BDNF to the platelet pool is still unclear, circulating levels of BDNF are associated with multiple neurological diseases, suggesting that peripheral BDNF could be used as a model of neuronal BDNF levels (34, 35). TrkB-T1 is found in megakaryocytes (36) and was recently reported in a platelet proteomic dataset (37); the presence of the $p75^{NTR}$ mRNA has been reported in platelet transcriptomic studies (38–41). Nevertheless, BDNF receptors have not been reported using antibody-based approaches at the protein level (32, 42). To assess these divergences, we tested multiple antibodies against TrkB and $p75^{NTR}$ receptors on platelets by immunoblotting and flow cytometry. Herein, we report important tissue-specificity among the multiple antibodies raised against TrkB and $p75^{NTR}$ receptors, highlighting the importance of thorough antibody characterisation when investigating these receptors.

METHODS

Antibodies and Reagents

Acid citrate dextrose solution A (ACD-A) was purchased from the Montreal Heart Institute pharmacy (DIN: 00788139). Prostaglandin E_1 (PGE_1, catalog no. 1620) was obtained from Tocris Bioscience. Antibodies against TrkB and $p75^{NTR}$ are presented in **Table 1**. Allophycocyanin (APC)-Vio770 isotype control (catalog no. 130-104-618) was from Miltenyi Biotec. Mouse IgG_1 (catalog no. MAB002) and IgG_{2B} (catalog no. MAB004) isotypes were from R&D Systems. Alexa Fluor 488-conjugated donkey anti-mouse IgG and donkey anti-rabbit IgG were from Thermo Fisher Scientific (catalog no. A21202 and A21206). The healthy human brain cerebral cortex full tissue lysate was obtained from Novus Biologicals (cat. NB820-59182). Proteins were extracted from the cortex of a healthy 66 years old male using the total protein extraction kit (cat. NBP2-37853). Proteins were aliquoted and conserved at −80°C until western blot analyses. U87-MG and U251-MG cells were a gift from Dr. Gaëlle V. Roullin. Eagle's minimum essential medium (EMEM) and fetal bovine serum (FBS) were obtained from Wisent and Thermo Fisher Scientific. Deglycosylation kits were obtained from New England Biolabs (catalog no. P0704S and P6044). Platelet integrin Ibα (CD42b) and integrin αIIb (CD41) antibodies were from Santa Cruz Biotechnology (catalog no. sc-59051 and sc-365938). Platelet integrin β3 (CD61) antibody coupled to phycoerythrin (PE) and corresponding control isotype were from Miltenyi Biotec (catalog no. 130-110-749 and 130-104-613). Sortilin antibody was from Abcam (catalog no. ab16640) and lysosomal-associated membrane protein 1 (LAMP-1) was from the Developmental Studies Hybridoma Bank (catalog no. H4A3).

Participant Selection

The study protocol was approved by the Montreal Heart Institute Scientific and Research Ethics Committee (#2018-2368) and written informed consent was obtained from each participant. Participants (four females and three males) were healthy adults aged between 22 and 43 years old, refrained from taking drugs known to affect platelet function in the 14 days before sampling, had not undergone major surgery in the last 6 months, did not have a history of bleeding symptoms and had platelet counts and hemoglobin levels within normal ranges.

Blood Collection and Platelet Isolation

Whole blood was collected in syringes containing ACD-A anticoagulant (1:5 ratio) with 21G needles. Blood was centrifuged at 200g for 15 min and platelet-rich plasma (PRP) was collected. PGE_1 (1 μM) was added to PRP to prevent platelet activation during isolation. PRP was centrifuged for 10 min at 1,000g to pellet platelets. The supernatant was discarded, and platelets were resuspended gently in Tyrode's buffer (137 mM NaCl, 11.9 mM $NaHCO_3$, 0.4 mM $NaH_2PO_4 \bullet 2H_2O$, 2.7 mM KCl, 5.6 mM glucose, 1.1 mM $MgCl_2$, pH 7.4). The purity of the platelet preparation was verified by flow cytometry. The mean percentage of events contained within the platelet gate and verified by platelet

TABLE 1 | List of antibodies tested.

Target	Manufacturer	Catalog number	Clone	Immunoblotting concentration	Flow cytometry concentration
TrkB	Abcam	ab134155	EPR1294	0.5 µg/ml in 5% skim milk	–
	Abnova Corporation	H00004915-M02	3D12	1 µg/ml in 3% BSA	–
	Alomone Labs	ANT-019	Polyclonal	1 µg/ml in 5% skim milk	–
	Biosensis	R-1834	Polyclonal	1 µg/ml in 3% BSA	–
	Millipore Sigma	HPA007637	Polyclonal	(1 µg/ml in 5% skim milk)	–
	Novus Biologicals	NBP2-52524	10B6C4	0.5 µg/ml in 3% BSA	–
	R&D Systems	AF1494	Polyclonal	1 µg/ml in 3% BSA	–
	R&D Systems	FAB397G * (FITC)	75133	–	80 to 330 µg/ml
	R&D Systems	FAB3971G (FITC)	72509	–	40 µg/ml
	R&D Systems	MAB397	75133	0.5 µg/ml in 3% BSA	(20 µg/ml)
	R&D Systems	MAB3971	72509	(0.5 µg/ml in 3% BSA)	20 µg/ml
	Sino Biologicals	10047-MM12	7H6E7B3	(1 µg/ml in 5% skim milk)	40 µg/ml
p75NTR	Alomone Labs	ANT-007	Polyclonal	0.8 µg/ml in 3% BSA	20 µg/ml
	Alomone Labs	ANT-011	Polyclonal	0.4 µg/ml in 3% BSA	–
	Biosensis	M-011-100	ME20.4	–	[20 to 100 µg/ml]
	EMD Millipore	05-446	ME20.4	–	80 to 200 µg/ml
	Millipore Sigma	HPA004765	Polyclonal	0.3 µg/ml in 5% skim milk	–
	Miltenyi Biotec	REA844 (APC-Vio770)	REA844	–	1:25
	Miltenyi Biotec	130-113-983 (PE)	ME20.4-1.H4	–	1:5
	Santa Cruz Biotechnology	sc-271708	B1	0.8 µg/ml in 5% skim milk	–

*All other antibodies were indirectly labeled with either donkey anti-mouse or donkey anti-rabbit secondary antibodies conjugated to Alexa Fluor 488. Antibody concentrations for immunoblotting and flow cytometry are listed either in µg/ml, or in dilution factor of the stock concentration. *custom-made antibody, PE, phycoerythrin; APC, allophycocyanin;— antibody not used for this application; ( ), Antibody not validated for this application by the manufacturer, [ ], antibody supported but not validated for this application by the manufacturer. For pre-conjugated antibodies, the associated fluorochrome appears in parenthesis next to the catalog number.*

integrin β3 (CD61) labeling was 99.53 ± 0.27%. A representative flow cytometry readout is shown as **Figure S1**.

Cell Culture

U87-MG and U251-MG human glioblastoma cells were grown in EMEM supplemented with 10% FBS and 1% penicillin/streptomycin mix at 37°C and a fixed CO_2 level of 5%. Cells were washed with phosphate-buffered saline (PBS) prior to trypsinization. Cells were then pooled down by 800g centrifugation and washed again in PBS prior to lysis for immunoblotting or fixation for flow cytometry experiments.

Deglycosylation

Platelets or U87-MG cells were lysed in RIPA buffer (150 mM NaCl, 5 mM EDTA pH 8.0, 50 mM Tris-HCl pH 8.0, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS). Proteins from whole cell lysates were denatured in denaturing buffer (0.5% SDS, 40mM DTT, B1704S, New England Biolabs, MA, USA) and heated to 100°C for 10 min. Lysates were put on ice and glycobuffer 2, 1% NP40 and protein *N*-glycanase F (PNGase F) (P0704S, New England Biolabs, MA, USA) were added to denatured proteins for *N*-deglycosylation. Protein deglycosylation mix II (P6044, New England Biolabs, MA, USA) containing PNGase F, *O*-glycosidase, neuraminidase A, β1-4 galactosidase, and β-*N*-acetylhexosaminidase f was used for *N* and *O*-deglycosylation assays. Samples were incubated overnight at 37°C and then conserved at −80°C until analysis. Glycosylation profiles were assessed by mass shift on western blots, using the ANT-019 antibody for the TrkB receptor and the HPA004765 antibody for the $p75^{NTR}$ receptor. Enzymatic activity was verified by reblotting membranes for *N*-glycosylated proteins sortilin and CD42b (*N*-glycosylation) or CD41 and LAMP-1 (*O* and *N*-glycosylation).

Gel Electrophoresis and Immunoblotting

Platelets were centrifuged in the presence of PGE_1 at 1,000g for 10 min, at room temperature (RT). The supernatant was discarded and platelets were lysed in ice-cold RIPA buffer containing protease and phosphatase inhibitors. 4X Laemmli's buffer (250 mM Tris pH 6.8, 8% SDS, 40% glycerol, 20% β-mercaptoethanol, 0.02% bromophenol blue) was added 1:4 to samples and heated at 95°C for 5 min. Proteins were resolved on 8% polyacrylamide gels and transferred onto 0.45 µm PVDF membranes. Membranes were blocked in 3% BSA or 5% non-fat dry milk depending of the primary antibody diluent and incubated at 4°C overnight with the primary antibody. Membranes were then washed thrice for 10 min in Tris-buffered saline containing 0.1% Tween (TBS-T) and incubated in secondary antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at a dilution of 1:10,000 in 5% milk for 60 min. Membranes were washed thrice in TBS-T and exposed to HRP substrate (Immobilon Classico Western HRP substrate, Luminata Classico, EMD Millipore, Etobicoke, ON, Canada). Chemiluminescence was captured on half-blue films (Mandel Scientific, Guelph, ON, Canada).

Flow Cytometry

Platelets or U87-MG cells were fixed in 1% paraformaldehyde (PFA) at RT for 15 min. Following fixation, cells for intracellular labeling were permeabilized by adding 0.1% Triton X-100 for 10 min. Permeabilization was stopped by adding 500 µl of PBS. Cells were than pooled down and resuspended in PBS. Samples were labeled with primary antibody or with the corresponding isotypes for 30 min in the dark at RT. For unconjugated antibodies, secondary antibodies conjugated to Alexa Fluor 488 were added at a dilution 1:200 and incubated in the dark for 30 min. Cells were gated based on size and granularity. A total of

10,000 events were acquired with the MACSQuant Analyzer 10 flow cytometer; data was analyzed using the MACSQuantify software (version 8.2.1).

Data Analysis

Immunoblotting data is representative of a minimum of three independent experiments and five healthy volunteers for platelets. For flow cytometry, data are presented as median (25^{th} percentile; 75^{th} percentile), corrected by isotype control and are representative of three or more independent experiments. Sample size varies between three for negative results (i.e., absent or very weak expression) and 10 for results suggestive of expression for increased precision.

RESULTS

Antibodies Targeting TrkB

TrkB in the Brain and Platelets

Except for mab3971, all antibodies tested identified the 95 kDa TrkB-T1 isoform in human cortex, while only half displayed the full-length isoform. Out of the 10 antibodies tested, seven showed a band directly under the 100 kDa weight marker in platelet samples (**Figure 1A** and **Figure S2**). This finding is in line with the observation of TrkB-T1 in primary human megakaryocytes by Labouyrie et al. (36). Furthermore, a band corresponding to the full-length TrkB receptor expected at a molecular weight of 145 kDa was displayed in platelets only by the NBP2-52524 antibody and was not present in platelets from all volunteers. As the full-length TrkB receptor has not yet been reported in platelets or megakaryocytes, further investigations to confirm the presence of this isoform in platelets is warranted. Interestingly, mab3971 displayed a band at the molecular weight of 95 kDa in platelet lysates but failed to detect the TrkB receptor in the human cortex. On the other hand, three antibodies identified at least the truncated isoform of the TrkB receptor in the cortex but failed to in platelet lysates. In addition, all six antibodies that identified the TrkB-T1 isoform in both cortex and platelets systematically displayed a lower band in the cortex, suggesting slight differences between TrkB-T1 in these samples. Interestingly, TrkB-T1 originating from brain tissues has also been observed to run at a slightly lower molecular mass than that of other cell types in mice, potentially due to differential glycosylation (18).

Deglycosylation of the TrkB Receptor

We then tested whether differences in TrkB mass could be explained by *N*-glycosylation. In both cell types, the protein identified by the TrkB antibodies was unaffected by PNGase F treatment (**Figure 1B**). Deglycosylation of glycoproteins sortilin in U87-MG and CD42b in platelets confirmed that PNGase F was active in the experimental settings. The absence of a mass shift following PNGase F treatment is in opposition to previous results reporting that TrkB is *N*-glycosylated (43), and argues against glycosylation as the primary cause of different molecular masses reported here and elsewhere (18).

Cellular Localization of the TrkB Receptor

Flow cytometry was used to assess the localization of the TrkB protein in human platelets. U87-MG cells were used as a positive control. A total of five antibodies were tested (**Figure 1C**, **Table 2**), including the mab397 and mab3971 in both direct (preconjugated to fluorochrome) and indirect labeling (conjugated with AlexaFluor488 secondary antibody).

All antibodies tested indicate either an absence or very weak TrkB expression at the membrane of U87-MG cells (**Table 2**). For permeabilized cell labeling, the 10047-MM12 antibody detected TrkB in approximately half of the cells, a result that was reproduced with mab397 through indirect labeling. However, the same clone failed to recognize TrkB when preconjugated to the fluorochrome. Similarly, mab3971 antibody showed a weak proportion of U87-MG cells expressing TrkB through indirect labeling and resulted in complete absence of this protein when preconjugated to FITC, thus failing to bind to the positive control.

In platelets, 10047-MM12 showed TrkB to be expressed at the surface of approx. half of the platelet population and this proportion increased to nearly 75% when platelets were permeabilized (**Table 2**). A similar pattern was seen with the indirectly conjugated mab397, albeit to a lesser extent; preconjugation with the fluorochrome abolished labeling, as for U87-MG cells. The other antibodies tested showed absence or very low expression of TrkB at the platelet membrane, and minimal increases in permeabilized platelets (**Table 2**).

In summary, only the unconjugated mab397 and 10047-MM12 showed convincing TrkB signals in U87-MG cells and both antibodies showed membrane and intracellular labeling of TrkB in platelets, with a stronger signal in the intracellular compartment, suggesting that TrkB is present both at the cell membrane and in the intracellular compartment.

Antibodies Targeting $p75^{NTR}$

$p75^{NTR}$ in the Brain and Platelets

We then sought to investigate whether platelets expressed the low affinity BDNF receptor $p75^{NTR}$ by western blotting (**Figure 2A** and **Figure S3**). The masses for the monomer of $p75^{NTR}$ reported in the literature vary between 72 and 85 kDa, with 75 kDa being the most reported (21, 25–30). HPA004765 raised against the ICD and ANT-007 raised against the ECD both displayed bands at the expected size of 75 kDa corresponding to the monomeric form of the full-length receptor in platelet samples. ANT-007 also resulted in bands at around 80 kDa, and the ANT-011 antibody, which targets the ICD of $p75^{NTR}$, resulted in a band at 70-72 kDa in platelets. sc-271708, also directed against the ICD, did not find any band in this range in platelets nor cortex lysates. Instead, it identified a protein running just above the 100 kDa marker that was only seen in platelet lysates. Interestingly, bands having the same mobility were also found with the HPA004765 and ANT-007 antibodies when used on platelets, but not on human cortex lysates. Furthermore, the intensity of this band in platelets is highly variable from an individual to another despite equal quantities of platelet lysates loaded into each well, as further supported by the β-actin used as a loading control. As with the TrkB receptor, the band found in the cortex lysates for $p75^{NTR}$

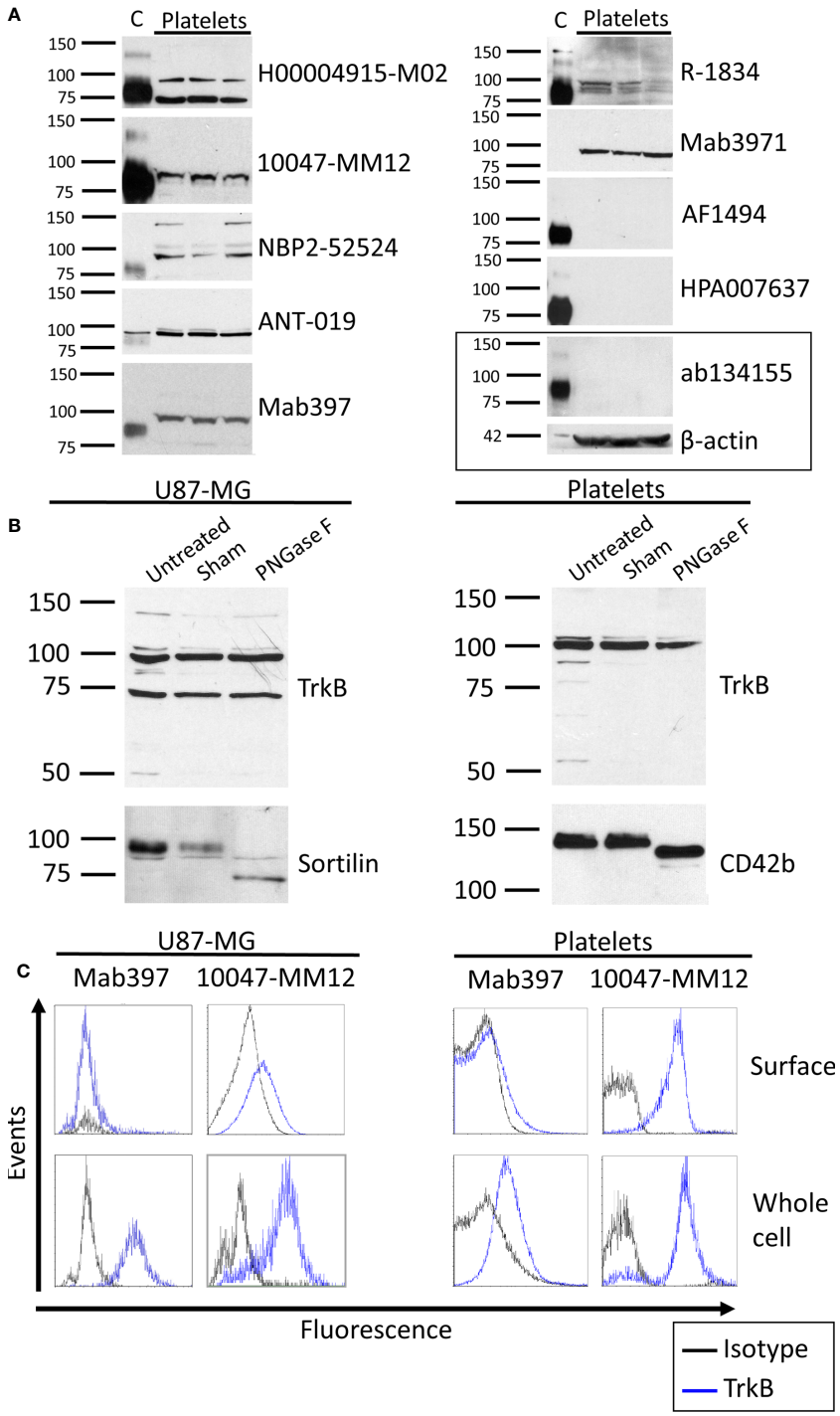

FIGURE 1 | TrkB expression in human brain and platelets. **(A)** Healthy human cortex (C; 3 µg) and platelets lysates (platelets; 30 to 75 µg) were analyzed in denaturing and reducing conditions and blotted with antibodies raised against TrkB extracellular domain. Left: molecular weight marker in kDa. Right: antibody catalog number. β-actin was used as a loading control. **(B)** Human glioblastoma cells U87-MG and healthy platelets lysates were either left untreated (Untreated) or submitted to 37°C overnight in absence (Sham) or presence of *N*-deglycosylation enzyme PNGase F (PNGase F). Membranes were blotted with ANT-019 antibody against TrkB ECD. PNGase F activity was confirmed by reblotting membranes with antibodies against *N*-glycosylated proteins sortilin for U87-MG and CD42b for platelets. **(C)** Human glioblastoma U87-MG cells and healthy human platelets isolated from whole blood were fixed or fixed and permeabilized. Cells were labeled with antibodies directed toward the extracellular domain of the TrkB receptor and analyzed by flow cytometry. Results shown are representative of **(A)** ≥ 3 independent experiments and ≥ 5 different platelet samples from different donors and **(B, C)** 3 independent experiments and 3 different donors for platelets samples.

TABLE 2 | TrkB and $p75^{NTR}$ expression assessed by flow cytometry.

Target	Catalog no./clone	Fluorochrome	Host	U87-MG cells		Platelets	
				Surface (%)	Whole cell (%)	Surface (%)	Whole cell (%)
TrkB	MAB397/75133	Alexa Fluor 488	Mouse IgG_{2B}	2.6 (0.0; 14.3)	43.3 (26.8; 76.3)	7.7 (0.7; 60.2)	58.0 (34.4; 86.5)
	FAB397G*/75133	Pre-conjugated to FITC	Mouse IgG_{2B}	0.1 (0.0; 1.2)	0.0 (0.0; 1.2)	0.0 (0.0; 0.0)	7.2 (0.0; 14.4)
	MAB3971/72509	Alexa Fluor 488	Mouse IgG_1	0.0 (0.0; 0.0)	0.0 (0.0; 15.5)	3.3 (0.0; 13.8)	1.1 (0.4; 6.6)
	FAB3971G/72509	Pre-conjugated to FITC	Mouse IgG_1	0.0 (0.0; 0.0)	0.0 (0.0; 0.0)	0.0 (0.0; 2.1)	0.0 (0.0; 18.9)
	10047-MM12/7H6E7B3	Alexa Fluor 488	Mouse IgG_1	2.2 (0.0; 23.4)	39.1 (26.8; 69.9)	66.4 (10.6; 90.9)	79.7 (41.3; 97.2)
$P75^{NTR}$	REA844/REA844	Pre-conjugated to APC-Vio770	Humanized IgG_1	68.9 (43.2; 72.3)	77.3 (67.1; 87.8)	19.6 (7.6; 62.5)	56.4 (23.4; 71.6)
	130-113-983/ME20.4-1.H4	Pre-conjugated to PE	Mouse $IgG_{1\kappa}$	16.9 (11.8; 23.7)	36.6 (21.4; 44.0)	16.2 (9.4; 27.8)	6.8 (5.1; 11.3)
	M-011-100/ME20.4	Alexa Fluor 488	Mouse IgG_1	0.0 (0.0; 9.6)	0.0 (0.0; 3.0)	0.0 (0.0; 0.2)	0.0 (0.0; 0.0)
	ANT-007/polyclonal	Alexa Fluor 488	Rabbit polyclonal	0.0 (0.0; 12.7)	0.0 (0.0; 3.0)	0.4 (0.0; 21.8)	0.4 (0.0; 36.4)
	05-446	Alexa Fluor 488	Mouse IgG_1	0.0 (0.0; 1.8)	0.0 (0.0; 15.7)	0.0 (0.0; 12.0)	33.2 (0.1; 82.7)

U87-MG cells and platelets were fixed in 1% paraformaldehyde for surface labeling and further permeabilized in 0.1% Triton X-100 for whole cell labeling. Cells were then labeled with antibodies raised against the extracellular domain of the TrkB or $p75^{NTR}$ receptor. Data represent percentage of positive cells, corrected for isotype control, and are presented as median (25^{th}; 75^{th} percentile). Data are representative of at least 3 independent experiments. FITC, fluorescein isothiocyanate; PE, phycoerythrin; APC, allophycocyanin.

runs slightly below that found in the platelet lysate samples, except for ANT-011 which identifies a single 72 kDa band both in both the human cortex and platelet lysates.

Deglycosylation of the $p75^{NTR}$ Receptor

We investigated whether the differences in apparent molecular weights in the brain and platelet lysates originated from a differential glycosylation pattern. The $p75^{NTR}$ receptor has a single *N*-glycosylation site on the first CRD of its ECD and multiple *O*-glycosylation sites on its stalk domain. We therefore used a deglycosylation mix that removes *N*-glycans as well as the majority of *O*-glycans. As shown in **Figure 2B**, the 75 kDa band identified in platelets did not shift following deglycosylation, while deglycosylation decreased the apparent molecular weight of this band in U251-MG cells. However, the higher band observed at approx. 100 kDa in platelets either disappeared or lost intensity in all 3 replicates. The fact that no new band appeared concomitantly to the loss of the 100 kDa band suggests that the mass shift engendered by deglycosylation caused the 100 kDa band to merge to the already present 75 kDa band, suggesting that this higher band could be a highly glycosylated form of the 75 kDa band observed in platelets.

Cellular Localization of the $p75^{NTR}$ Receptor

We then used flow cytometry to assess $p75^{NTR}$ localization in human platelets and U87-MG human glioblastoma cells used as a positive control. A total of five antibodies raised against the $p75^{NTR}$ receptor ECD were tested, including ANT-007 also tested by western blotting, and the well-characterized clone ME20.4 (**Table 2**). The percentage of $p75^{NTR}$-positive cells varied greatly depending on the antibody used, not only in platelet samples, but also in U87-MG cells (**Figure 2C** and **Table 2**). Only the humanized REA844 antibody and the clone ME20.4-1.H4 gave a positive expression signal in U87-MG cells. All the other antibodies tested showed close to no signal for both surface and whole cell labeling in U87-MG cells, including other ME20.4 clones. On platelets, antibodies REA844 and the clone ME20.4-1.H4 resulted in a signal at the cell surface. While the REA844 offered an increased signal in permeabilized platelets, clone ME20.4-1.H4 resulted in a weaker signal in permeabilized cells. In summary, only the REA844 antibody showed convincing signals in both U87-MG cells and platelets, with large disparities between antibodies in their ability to bind the receptor in U87-MG cells contributing to the uncertainty of the results seen in platelets.

DISCUSSION

We set out to identify TrkB and $p75^{NTR}$ on human platelets using antibody-based techniques and tested various commercial antibodies from different host species and targeting different epitopes. While both receptors could be detected on human platelets, we found major discrepancies among antibodies in their ability to detect BDNF receptors on platelets, but also on human cortex and U87-MG cells. These results highlight important challenges in using antibody-based assays to determine the expression pattern of these receptors, with a notable lack of reproducibility among the tested antibodies.

TrkB on Platelets

Our immunoblotting experiments suggest the presence of a truncated form of the TrkB receptor in human platelets (**Table 3**). However, major discrepancies were found among antibodies, as well as between brain and platelet lysates.

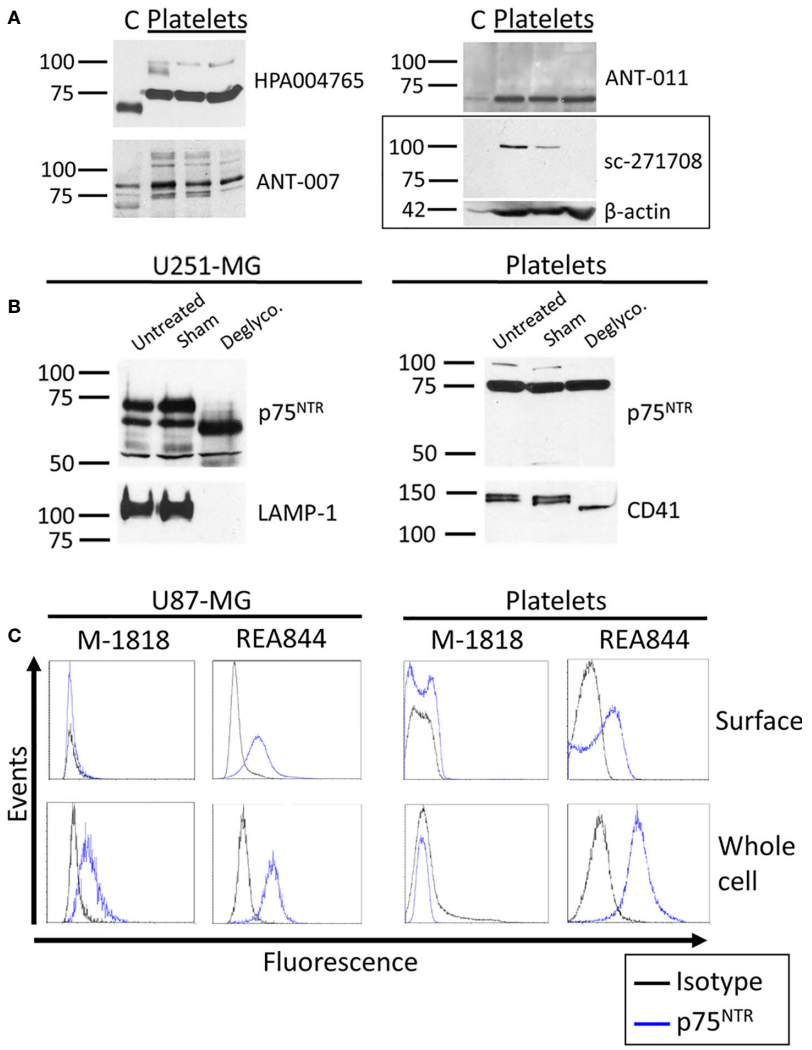

FIGURE 2 | p75^{NTR} expression in human brain and platelets. **(A)** Healthy human cortex (C; 10 µg) and platelets lysates (platelets; 30 µg) were analyzed in denaturing and reducing conditions and blotted with antibodies raised against p75^{NTR}. Left: molecular weight marker in kDa. Right: antibody catalog number. β-actin was used as a loading control. **(B)** U251-MG cells and healthy human platelet lysates were either left untreated (untreated) or submitted to 37°C overnight in absence (Sham) or presence of the protein deglycosylation mix II (Deglyco). Membranes were blotted with the HPA004765 antibody against p75^{NTR} ICD. Membranes were then stripped and reblotted for lysosomal-associated membrane protein 1 (LAMP-1) and CD41 as internal controls of enzymatic activity. **(C)** Human glioblastoma U87-MG cells and healthy human platelets isolated from whole blood were fixed or fixed and permeabilized. Cells were labeled with antibodies directed toward the p75^{NTR} receptor and analyzed by flow cytometry. Results displayed are representative of **(A)** ≥ 3 independent experiments and ≥ 5 different platelet samples from different donors and **(B, C)** 3 independent experiments and 3 different donors for platelets samples.

All antibodies that worked on both samples consistently identified TrkB in the cortex at a slightly lower mass to that seen in platelets. A similar band shift was also reported for TrkB-T1 in mice, with brain lysates running slightly below NIH/3T3 cells (18). The authors suggested the mass shift was likely due to differential glycosylation but did not confirm their hypothesis. A similar hypothesis was also raised by another group facing challenges to identify TrkB in glial cells (44). Glycosylation on TrkB appears to be important for antibody recognition, as Eager et al. showed that the glycans on the ECD of TrkB were necessary for antibody binding, either by being included in the epitope or by allowing the correct epitope conformation for antibody binding (45). However, PNGase F treatment did not alter the apparent molecular weight of the bands detected by TrkB antibodies in platelets nor in the U87-MG cell line in our study. Because the intensity of the observed 95 kDa band did not decrease following PNGase F treatment, it seems unlikely that this is the result of a lack of affinity of the tested antibodies for the deglycosylated TrkB receptor. A limitation regarding these experiments is that while PNGase F has a large spectrum, it does not cleave all *N*-linked glycans. In addition, we focused on the glycosylation profile but cannot exclude that the observed mass difference seen by western blotting could be due to other post-translational modifications.

TABLE 3 | Summary of antibody performance for TrkB.

TrkB Manufacturer	Catalog no.	Host	Detection in cortex		Detection in platelets	
			TrkB-FL	TrkB-T1	TrkB-FL	TrkB-T1
Abnova Corporation	H00004915-M02	Mouse	+	+	–	+
Sino Biologicals	10047-MM12		+	+	–	+
Novus Biologicals	NBP2-52524		–	+	+	+
R&D Systems	Mab397		–	+	–	+
R&D Systems	Mab3971		–	–	–	+
Alomone Labs	ANT-019	Rabbit	–	+	–	+
Biosensis	R-1834		+	+	–	+
Millipore Sigma	HPA007637		+	+	–	–
Abcam	ab134155		+	+	–	
R&D Systems	AF1494	Goat	–	+	–	–

This table summarizes the results obtained regarding the detection of the full-length (FL) and truncated (T1) isoforms of TrkB. +, detected; -, not detected.

It has been suggested that different TrkB glycosylation patterns could result in an alternative folding of the protein and alter the layout of certain epitopes (44, 45). This could explain the many differences between antibodies observed in flow cytometry, as this technique labels proteins in their native conformation, rather than under reducing conditions that can be used in immunoblotting. Nonetheless, it cannot explain the differences observed between the same clones through direct and indirect labeling. As we adjusted for non-specific binding by subtracting the isotype control fluorescence in our experiments, the difference is unlikely to arise from non-specific binding. Whether steric hindrance of fluorochrome-conjugated antibodies could explain the lack of binding would merit further attention with alternative fluorochrome conjugates. Taken together, these results highlight the importance of confirming findings with independent antibodies and characterizing them against known controls.

p75^{NTR} on Platelets

Our immunoblotting experiments suggest the presence of the p75^{NTR} receptor in human platelets (**Table 4**). The 72, 75, and 80 kDa bands identified in platelets by the HPA004765, ANT-007 and ANT-011 antibodies all correspond to molecular weights reported for the full-length p75^{NTR} receptor (21, 25, 26, 28–30). Previous studies found that the apparent mass of the receptor varied depending on reducing conditions (25, 30). While the masses reported herein are in the same range, all samples were subjected to identical reducing conditions. Thus, the differences in weights cannot be attributed to variable reducing conditions. Furthermore, HPA004765 and ANT-007 antibodies identified the isoform in the cortex to run slightly below the isoform found in platelets. Despite the many glycosylation sites, *O* and *N*-deglycosylation did not lower the apparent molecular weight of the protein in platelets, in contrast with U251-MG cells as previously reported (25, 29). Whether other post-translational modifications, such as palmitoylation, may be the cause of the mass differences observed (46), and could explain the important discrepancies observed among the tested antibodies in flow cytometry, have not been the center of investigation so far.

While bands between 72 and 85 kDa have all been associated to the monomeric p75^{NTR} receptor (21, 25–30), the band at 100 kDa is rather associated to a single p75^{NTR} receptor bound by dimeric nerve growth factor (NGF) (47, 48). However, the study of such a complex requires cross-linking, which we have not carried out, and the denaturing and reducing conditions used in our experiments render the possibility of a non-covalent complex unlikely. The intensity of the 100 kDa band was decreased or completely abolished by deglycosylation, suggesting either a highly post-translationally modified form of the receptor or a strong complex stabilized by glycans. The fact that this band is displayed by three antibodies targeting different epitopes suggest that this band is specific, and the nature of the protein or protein complex it identifies in platelets warrants further investigation.

Platelets as Neuronal Biomarkers for BDNF Receptors

Several studies have highlighted alterations in brain TrkB and p75^{NTR} receptors in neurological disorders. For instance, the TrkB receptor levels have been shown to be decreased in the brain of schizophrenic patients (49), while alterations in p75^{NTR} cleavage is believed to lead to neuronal death in Alzheimer's

TABLE 4 | Summary of antibody performance for p75^{NTR}.

p75^{NTR}					
Manufacturer	**Catalog no.**	**Host**	**Epitope**	**Detection in cortex**	**Detection in platelets**
Santa Cruz Biotechnology	sc-271708	Mouse	ICD	–	?
Millipore Sigma	HPA004765	Rabbit	ICD	+	+
Alomone Labs	ANT-007		ECD	+	+
Alomone Labs	ANT-011		ICD	+	+

This table summarizes the results obtained regarding the detection of the full-length p75^{NTR} receptor. +, detected; –, not detected; ?, uncertain. ECD, extracellular domain; ICD, intracellular domain.

disease through the production and binding of amyloid β (50). The identification of the TrkB and $p75^{NTR}$ receptors in platelets opens new avenues of research, using platelets as peripheral biomarkers of neurological expression patterns of these receptors.

Whereas circulating BDNF levels have been extensively studied (34), little is known about the interplay between BDNF, proBDNF, TrkB, and $p75^{NTR}$ in these easily available peripheral cells (51, 52). The results presented herein highlighting structural differences between TrkB and $p75^{NTR}$ receptors in human cortex and platelets, raise the possibility that these receptors in platelets might not be a true reflection of expression in the cerebral cortex. A better characterization of the activity of TrkB and $p75^{NTR}$ in platelets is warranted, to assess whether BDNF receptors in platelets have an inherent biological role, and could potentially be used to mirror receptor function in neuronal tissues.

LIMITATIONS

While the many antibodies characterized represent a strength of the present study, there are also noteworthy limitations. The fact that all antibodies were tested on the exact same cortex sample allowed a better comparison between antibodies themselves because the differences observed for the cortex sample were not due to unequal levels of receptors in the sample. However, we recognize that the cerebral cortex might not be representative of other brain regions, and the ratio between the multiple isoforms of BDNF receptors are known to vary from a region to another (53). Moreover, using a single donor does not allow representation of inter-individual variation in levels of these receptors in cortical tissues. The presence of TrkB and $p75^{NTR}$ has been shown solely with antibody-based techniques. Since platelets are anucleate cells, we could not verify antibody specificity in this cell type using classic molecular biology approaches, such as knockout models, and have had to rely on cross-verification with independent antibodies, in the presence of cells/tissues with confirmed TrkB and $p75^{NTR}$ expression. It is important to reconcile the results presented herein with reports of absence of TrkB or $p75^{NTR}$ receptors on human platelets (32, 42). The fact that only the truncated isoform of TrkB lacking its tyrosine kinase domain has been found in platelets in our study may explain why prior reports using antibodies targeting the intracellular domain of TrkB receptors would have failed, highlighting the importance of confirming results with independent antibodies targeting different epitopes of the receptor. Arguably, protein sequencing by techniques such as mass spectrometry would further increase confidence in the results.

CONCLUSION

Our results suggest that human platelets express a truncated form of the TrkB receptor and the full-length $p75^{NTR}$ receptor; structural or post-translational differences from the isoforms expressed in the central nervous system are apparent on receptor mass. An important aspect of this work is the tissue-specificity of some antibodies targeting BDNF receptors, and a lack of reproducibility between antibodies, even within the same clonal selection. This highlights the importance of careful characterization of antibodies when using immuno-based assays to study BDNF receptors, both within and beyond the central nervous system. A thorough characterization of the TrkB and $p75^{NTR}$ isoforms in platelets and other circulating cells is therefore critical before they can be recommended as models of neurocognitive health.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Montreal Heart Institute Scientific and Research Ethics Committee. The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SF has performed assays, collected data, analyzed and interpreted data, and wrote the manuscript. IB, JLB, and MW have performed assays, collected data, analyzed and interpreted data, and critically revised the manuscript. ML has overseen the research group, assured funding, designed the research, analyzed and interpreted data, and critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.606861/full#supplementary-material

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REVIEW
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Platelets in Multiple Sclerosis: Early and Central Mediators of Inflammation and Neurodegeneration and Attractive Targets for Molecular Imaging and Site-Directed Therapy

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Platelets are clearly central to thrombosis and hemostasis. In addition, more recently, evidence has emerged for non-hemostatic roles of platelets including inflammatory and immune reactions/responses. Platelets express immunologically relevant ligands and receptors, demonstrate adhesive interactions with endothelial cells, monocytes and neutrophils, and toll-like receptor (TLR) mediated responses. These properties make platelets central to innate and adaptive immunity and potential candidate key mediators of autoimmune disorders. Multiple sclerosis (MS) is the most common chronic autoimmune central nervous system (CNS) disease. An association between platelets and MS was first indicated by the increased adhesion of platelets to endothelial cells. This was followed by reports identifying structural and functional changes of platelets, their chronic activation in the peripheral blood of MS patients, platelet presence in MS lesions and the more recent revelation that these structural and functional abnormalities are associated with all MS forms and stages. Investigations based on the murine experimental autoimmune encephalomyelitis (EAE) MS model first revealed a contribution to EAE pathogenesis by exacerbation of CNS inflammation and an early role for platelets in EAE development via platelet-neuron and platelet-astrocyte associations, through sialated gangliosides in lipid rafts. Our own studies refined and extended these findings by identifying the critical timing of platelet accumulation in pre-clinical EAE and establishing an initiating and central rather than merely exacerbating role for platelets in disease development. Furthermore, we demonstrated platelet-neuron associations in EAE, coincident with behavioral changes, but preceding the earliest detectable autoreactive T cell accumulation. In combination, these findings establish a new paradigm by asserting that platelets play a neurodegenerative as well as a neuroinflammatory role in MS and therefore, that these

two pathological processes are causally linked. This review will discuss the implications of these findings for our understanding of MS, for future applications for imaging toward early detection of MS, and for novel strategies for platelet-targeted treatment of MS.

Keywords: platelets, multiple sclerosis, experimental autoimmune encephalomyelitis, neuroprotection, neuroinflammation, imaging, targeted therapy

INTRODUCTION

Multiple sclerosis (MS) is a chronic autoimmune CNS disorder and one of the commonest causes of neurological disability in the young adult population worldwide (1). The disease is typified by the presence of lesions disseminated in time and space, characterized by inflammation, microglial reactivity, demyelination, axonal injury and neuronal loss (2). This widespread distribution of lesions accounts for the broad range of symptoms exhibited by affected individuals, including for example, sensory loss, fatigue, or difficulties with balance, mobility, bladder and bowel control, vision, speech and cognition. Women are more commonly affected than men and this difference is increasing in some areas of the world. North America and Europe have the highest prevalence and Asia and sub-Saharan Africa the lowest, but regardless of prevalence, the incidence of MS is rising worldwide (3, 4). MS is therefore a major health care burden for the individual affected, as well as for the respective health care system.

A number of treatment options are available for this condition, however, progress in this direction has been hindered by the limited understanding of the disease pathophysiology which is very complex. Historically, MS was viewed as a disease of white matter targeting principally myelin, due to the evidence of lesions and demyelination, considerably more prominent in white relative to gray matter (5). More recently, however, as a result of ground-breaking confocal microscopic investigations (6) and advanced magnetic resonance imaging (MRI) the neurodegenerative component of MS was brought to the forefront (7). This resulted in the revised view of MS as a global CNS disorder, with different, but partially overlapping pathophysiological mechanisms evolving over the disease trajectory. One type of mechanism is driven by classical inflammatory processes which are increasingly understood and addressed with current therapeutics (8). A second type begins in early disease development, is associated with diffuse neuro-axonal degeneration and becomes gradually more significant with age and disease duration. However, neurodegenerative mechanisms are poorly understood and remain untreatable so far (9–11). The literature suggests the existence of a soluble factor, produced by inflammatory cells, which induces neurodegeneration via stimulation of microglial reactivity, but the identity of this factor has remained elusive (12). On the other hand, the recent identification of platelets as new players in the development of EAE, a rodent neuroinflammatory disease which serves as an MS model (13–15), suggests that these anuclear components circulating in blood represent a credible candidate for this proposed pathogenic factor.

THE CHANGING VIEW OF MS

MS nearly always first manifests in young adults aged between 18 and 35 (3). In the majority of cases, the first indication of the disease consists of an acute episode of neurological dysfunction affecting one or several CNS regions, commonly the optic nerve, brainstem or spinal cord, associated with white matter lesion(s) identified by magnetic resonance imaging (MRI) (16). This presentation is defined as a clinically isolated syndrome (CIS). Confirmation of a clinical diagnosis of MS (clinically definite MS) is made with the use of gadolinium-based contrast agents (GBCA), which allows, in a single study, the identification of lesions disseminated in time (GBCA-enhancing lesions) and lesions disseminated in space, which constitutes the hallmarks of MS diagnosis (17).

The disease exists as a number of subtypes (18). The most common one is the relapsing-remitting form (RR-MS), involving over 80% of total MS cases. It is associated with earlier onset and a strong sex bias, affecting over three females to one male. RR-MS exhibits a disease course characterized by the occurrence of episodes of neurological dysfunction (or relapses) with or without recovery. This pattern is observed for 15–25 years, then followed in about 50% of RR-MS patients by transition to a progressive form characterized by worsening neurological decline without remissions. This is known as secondary progressive (SP)-MS. A less common form termed primary progressive (PP)-MS, is observed in about 10–15% of total MS cases where patients enter the neurodegenerative stage from the onset (19). This form more commonly first manifests in early middle age and affects females and males equally. Other rare forms exist, for example benign MS where patients are not symptom-free, but do not reach an irreversible stage, or MS with childhood onset beginning by 16 years of age (20). The relationship between these different MS forms is ambiguous, particularly in the absence of defined biomarkers for this disease (21, 22). It is unclear whether they represent distinct diseases, or clinical variants of a single disorder (23). Major questions that remain unexplained are the relationship between the two forms of progressive disease and mechanisms underlying the transition from RR to SP-MS.

The pathological hallmark unique to MS is the focal lesion resulting from primary demyelination and astrocytic scarring in a context of chronic inflammation (24). Focal demyelinated lesions are found in both white and gray matter and are associated with large-scale T and B lymphocytic infiltration resulting from major loss of blood brain barrier (BBB) function, together with oligodendrocyte death, axonal and neuronal injury and loss, leading to brain and spinal cord atrophy. Astrogliosis and microglial reactivity are also

typical features of these lesions. These classic active lesions are particularly significant in white matter and in the early disease stage (in both acute and relapsing MS). Immunological and pathological studies, however, have revealed that inflammatory demyelination can result from a broad spectrum of mechanisms. Accordingly, profound heterogeneity has been demonstrated in lesions, whereby although all inflammatory infiltrates contain T cells and macrophages, the target can be either myelin or oligodendrocytes. Analysis of active lesions from biopsies (71 lesions from 51 cases) or autopsies (325 lesions from 32 cases) from early disease revealed multiple patterns (25), including Pattern I: lesions dominated by T cells and macrophages, Pattern II: lesions similar to Pattern I, but with accumulation of immunoglobulins and complement, suggestive of involvement of pathogenic antibodies, Pattern III: hallmarks of injury reminiscent of acute white matter stroke, characterized by reactive oxygen and nitric oxide radicals, or Pattern IV: severe oligodendrocyte degeneration in peri-plaque white matter, indicative of immune-mediated injury. In progressive stages, these active focal lesions become less prominent whilst increasing numbers of chronic active (slowly expanding plaques) and inactive lesions with reduced BBB damage are observed (2).

Gray matter lesions occur in multiple regions including deep gray matter nuclei, thalamus, hypothalamus, basal ganglia and spinal cord gray matter (26–30). They are already present from early disease stage, but with numbers and size increasing only moderately over disease progression. These lesions are also associated with perivascular inflammation, demyelination and microglial activation (2). However, the most significant aspect of gray matter pathology is in the form of cortical lesions, now identified as a major substrate of MS pathology (31, 32). This type of lesion occurs most commonly in the forebrain, cerebellum and hippocampus and is seen in biopsies or autopsies with disease duration of weeks to months, suggesting that they arise in early stages of disease development (33). These lesions result from a different type of inflammatory process, which is not coincident with large scale loss of BBB function, but with considerable meningeal accumulation of T and B cells. In their most severe form these accumulations can take the form of follicle-like structures containing distinct T and B cell sub-regions. The resulting meningeal infiltrates are associated with prominent microglial reactivity, the creation of a highly inflammatory parenchymal environment resulting in demyelination, together with major axonal and neuronal degeneration and extensive neuronal loss. However, the identity of the soluble inflammatory mediators which trigger microglial reactivity, remains unknown. These lesions are poorly detected by MRI and their extent and severity can only be fully gauged by post-mortem examination. They increase in number and size over time, thereby being more extensive in progressive disease, without apparent difference between PP and SP-MS.

In addition to the above, roles for $CD8^+$ T-lymphocytes and B cells relevant to progressive MS have emerged, but remains ambiguous. $CD8^+$ T-lymphocytes are the most abundant inflammatory immune cell sub-type in lesions, but are also associated with diffuse infiltration, active demyelination and slow accumulation of axonal damage in normal appearing gray and white matter, which also contribute to brain atrophy. B cells functions include antigen presentation, T cell activation and antibody-production (34–37). Furthermore as mentioned above, they become a core component of follicle-like structures, which eventually, may lead to compartmentalization of a B cell population independent of the peripheral B cell pool (38). It has been suggested that follicle-like structures develop during the RR phase as a result of recurring inflammatory activity and have been identified in SP-MS, in close association with gray matter lesions (39), thereby potentially acting as a source of antibodies and other pro-inflammatory components which can contribute to demyelination and neurodegeneration in the progressive stage.

The predominance of lesions, especially in white matter drove the early view of MS as a white matter disease. This plaque-centered approach to pathophysiological investigations, dramatically challenged by the identification of concurrent but apparent differential pathophysiological processes in different CNS regions and different disease stages, has been replaced by the view that there are two types of inflammation in MS, which are related but partly independent (2). The first type, associated with BBB breakdown and lymphocytic infiltration, is typical of the classic active demyelinated plaque. The second type is associated with meningeal accumulation of lymphocytes, absence of BBB breakdown, but severe demyelination and diffuse neuro-axonal degeneration. It is hypothesized that in the latter case, microglial responses to meningeal soluble factor(s) play a major role in neurodegeneration. The two types of inflammation occur in both RR and progressive disease forms. Future studies, therefore will need to focus on two key questions, namely: do the two types of inflammation reflect immune responses to different target antigens and what is the identity of the soluble factor which drives cortical demyelination and neurodegeneration?

A ROLE FOR PLATELETS IN MS AND EAE

The Non-hemostatic Functions of Platelets

Platelets are anuclear cells circulating in blood, historically associated with a role in hemostasis and more recently, with vascular inflammatory disorders and also cancer (40). They are derived by budding of megakaryocytes in the bone marrow and are unique in terms of their abundance (with a normal range of $150–400 \times 10^9$/L in humans), small size (2–3 μm in diameter) and rapid turnover (with a lifespan of 8–9 days). Under laminar flow conditions, they travel along the endothelial cell layer lining the blood vessel wall, thereby enabling immediate recruitment and local activation at the site of injury, in response to physical damage and/or invading pathogens. They are also characterized by three types of secretory compartments, namely the α granules, dense granules and lysosomes, as well as a complex membranous system, known as the open canalicular system. Together, these structures allow the storage of polypeptides in precursor or processed form and rapid release upon platelet activation (41). Platelet products have autocrine or paracrine functions, essential to activate platelet aggregation and trigger coagulation cascades fundamental to platelet hemostatic functions in pathogen surveillance and wound healing (42, 43).

A growing literature has highlighted previously unsuspected, non-hemostatic roles for these cells in inflammation and immunity. It was commonly believed that because platelets are devoid of nuclei, they would not synthesize new proteins and that their protein content was endowed from megakaryocytes upon platelet budding. It has since been demonstrated that they possess abundant mRNA, as well as microRNA and non-coding RNA and all the elements of the transcriptional machinery required for protein synthesis (42). Studies integrating proteomic and genomic studies of platelets from healthy human donors revealed ∼3,000 distinct mRNA species (44–47). This number illustrates the diverse repertoire of mediators, as well as mechanisms via which platelets modulate inflammatory functions. A number of the most abundant transcripts represent proteins already known to be produced in platelets, including adhesive proteins, coagulation factors, proteoglycans, immunoglobulins, proteases and protease inhibitors (48). However, such investigations also revealed expression of molecules with immunological functions. It is the discovery of these components that led to a paradigm change and the appreciation of the role of platelets in the continuum of immunity, namely in bridging innate to adaptive immunity. These components include for example, Toll-like receptors (TLR), a family of pattern recognition receptors, that identify and respond to conserved microbial pathogen-associated molecular patterns. To date TLR 1, 2, 3, 4, 6, 7, and 9 have been implicated in platelet responses (49). Platelets also express a wide range of cytokines, chemokines and their receptors. These molecules are central to inflammation by signaling leukocyte differentiation, migration and infiltration. Among the most potent molecules of that class are the cytokine IL-1ß, a key regulator of inflammatory responses, and others, originally known as platelet "growth factors," such as transforming growth factor-ß (TGF-ß) and platelet-derived growth factor (PDGF) with mitogenic properties. Platelet chemokines include CCL5 (RANTES), CCL3 (macrophage inflammatory protein-1α), and CXCL4 (PF4) (50). There was agreement among profiling studies of a high level of concordance between detectable proteins and their mRNA transcripts, although differences were observed between levels of individual message and protein. Messages for about 30% of proteins could not be detected, which could be accounted for by differences in half-lives of mRNA and their corresponding products, paracrine delivery of mRNAs (as well as microRNA) to target cells, proteins carried over from megakaryocytes, or proteins scavenged from plasma, such as albumin and fibrinogen (42, 46).

In addition, platelet mediators include lipid components and indeed, platelets are primary lipid carriers in the circulation (51). Examples include the thromboxane A2 (TXA2; a metabolite of arachidonic acid) and platelet activation factor (PAF). TXA2, notable for its half-life of 30 s, is produced by activated platelets and acts in an autocrine and paracrine manner by stimulating activation of new platelets and promoting platelet aggregation, resulting in platelet shape change and degranulation. PAF is a small phospholipid signaling molecule (acetyl-glyceryl-ether-phosphorylcholine) on the platelet surface with pro-inflammatory and vasoactive properties, also produced by neutrophils, monocytes, endothelial cells, and neurons. PAF causes both platelet and neutrophil adhesion and activation and platelet synthesis of IL-1ß and elevated PAF levels have been identified in RR-MS (52, 53). Thus, platelets can both generate and respond to signals at early time points in the inflammatory process.

A further mechanism of platelet action is via the use of signaling molecules anchored on the plasma membrane and mediating cell-cell interactions. This provides the advantage of signaling localized to the site of injury, while co-incidentally maintaining precision of interaction between platelets and their cellular partners. Upon vascular injury, exposure of sub-endothelial matrix proteins is immediately followed by platelet tethering to the subendothelial extracellular matrix through multiple receptors, such as glycoprotein GPVI and the GP1b/IX/V complex, together with integrins αIIbß3 (GPIIb/IIIa), α2ß1, α5ß1, and α6ß1, thrombin receptors, P2Y12 receptors, and thromboxane receptors (50). In addition to integrins which promote platelet aggregate formation, platelets express molecules facilitating their interactions with endothelial cells and leukocytes including platelet endothelial cell adhesion molecule 1 (PECAM-1), intercellular cell adhesion molecule 2 (ICAM-2) and junctional adhesion molecules (JAM) which belong to the Ig superfamily. These receptors mediate morphological changes, activation, adhesion and aggregation. Platelet activation causes release of signaling molecules from granules causing recruitment and activation of additional platelets resulting in clot formation (54). Adherent platelets release large amounts of P-selectin from α granules to the surface of activated platelets, which recognizes P selectin glycoprotein 1 (PGSL1) on target cells, for example neutrophils. Platelets are also a major source of the bioactive modulator CD40 ligand (CD40L) a transmembrane protein belonging to the TNF-α family. CD40L binds its cognate receptor CD40, which is widely expressed, resulting in the activation of monocytes (dendritic cells and macrophages), B and T cells and endothelial cells (54).

An Association Between Platelets and MS/EAE

Early Evidence of Platelet Involvement in MS

Historically, there has long been a postulate of a role for platelets in MS (55). Such evidence first emerged with reports by Putman (56, 57), suggesting a role for venule thrombosis in CNS demyelination. Subsequently, multiple studies demonstrated increased platelet adhesiveness in MS, relative to other neurological disorders, both in the percentage of patients exhibiting abnormalities in adhesiveness and in the extent of the differences. For example, a study of 60 MS patients and 12 healthy subjects was reported by Wright et al. (58), where MS patients were further classified as stationary, fluctuating or exhibiting acute exacerbation. Estimations of platelet adhesion indices revealed that levels of adhesiveness paralleled the clinical activity of the disease, whereby more than 80% of patients in the fluctuating group and over 90% of those in the group with acute exacerbation showed abnormal platelet adhesiveness, compared with 20% of patients identified as stable. Furthermore, the highest adhesive index was found in the group exhibiting exacerbation.

These findings have since been repeatedly confirmed by other investigators (59), but additionally, have been shown to coincide with biochemical abnormalities in the form of alterations in lipid composition of platelet membranes and serum, notably reduced cholesteryl linoleate (60, 61), a quantitatively minor cholesterol ester in platelets (62). A significant relationship between reduced cholesteryl linoleate levels, platelet adhesiveness and level of disease activity was established. In this context, imbalances in lipid composition are now associated with the initiation and progression of CNS disorders, such as MS, Alzheimer's disease, and Parkinson's disease (63). Consequently, lipidomic profiling to identify changes in plasma levels or plasma/CSF ratios are increasingly being explored as an approach for biomarker discovery of pathological pathways in these conditions (64). Given the consistent observation of platelet adhesiveness in MS, it would be of interest to extend these lipidomic and biomarker studies to platelets.

Ultrastructural Modifications and Alterations in Membrane Constituents

The above biochemical abnormalities are consistent with changes of platelet ultrastructure in MS patients, documented by scanning electron microscopy, in the form of pseudopodium formation, aggregation and lysis, which are all markers of platelet activation. The generation of pseudopods is associated with cytoskeletal collapse, fusion of granule membranes with surface-connected membranes of the open canalicular system or with the plasma membrane and transfer of granule contents to the platelet surface. Activated platelets are characterized by surface expression of P-selectin, which is critical to platelet-leukocyte interactions. Thus, further evidence of platelet activation in MS was provided by the demonstration of significant elevation of P-selectin in a study which compared 33 treatment-naïve, clinically stable RR-MS patients, with 92 control subjects using flow cytometry (65). The same study also identified significantly elevated platelet-associated IgM but not IgG. This IgM may represent autoimmunity, because anti-phospholipid antibodies in MS are predominantly of the IgM sub-class (65). Non-organ-specific anti-phospholipid (aPL) antibodies are recognized markers of increased coagulation activity and have been investigated particularly in the context of intravascular thrombosis. In MS, higher aPL antibody levels relative to healthy controls have been reported in both RR- and SP-MS (66) and associated with more severe clinical disease and MRI-identified disease progression. APL antibodies are predominantly directed against phosphatidylethanolamine, cardiolipin and ß2-glycoprotein 1 (a multifunctional apolipoprotein which binds cardiolipin) (67). Alternatively (or additionally), this IgM may originate from immunoglobulins normally stored within platelets, since chronic platelet activation has been reported to cause externalization of immunoglobulins normally stored within platelets. Surface expression of IgM may be part of an opsonisation mechanism which sensitizes platelets for destruction by complement (48). The complement system is part of the innate immune system and consists of a complex array of circulating proteins, which identify and kill target cells via cascades of proteolytic conversions of zymogens to their active forms. Platelets express multiple receptors for complement and are very sensitive to complement-mediated attack, which has the effect of increased procoagulant activity (67).

Given the evidence of alterations of platelet membranes, attention also turned to membrane-bound components that regulate mechanisms modulating platelet aggregation. A study of 20 RR-MS patients and 20 healthy subjects (68) examined changes in the enzymes ectonucleoside triphosphate diphosphohydrolase (NTPDase [CD39]), ectonucleotide pyrophosphatase/phospho-diesterase (E-NPP), 5′-nucleotidase and adenosine deaminase (ADA), known to modulate platelet aggregation via regulation of extracellular adenine nucleotide levels. Extracellular adenine nucleotides ATP, ADP and AMP (in particular ADP) are potent promoters of platelet aggregation, while their nucleoside derivative adenosine is a potent inhibitor of this process. Therefore, the platelet aggregation status is regulated by the equilibrium between adenine nucleotides and adenosine by the combined activity of these four enzymes. This investigation identified significant reduction in hydrolysis of ATP, ADP and AMP associated with reduced activity of NTPDase, 5′-nucleotidase, E-NPP and ADA in MS patients relative to normal controls. These data suggest the presence of a pro-inflammatory/pro-thrombotic milieu in MS by the absence of nucleotide hydrolysis, ADP accumulation and decreased adenosine production.

Evidence of Platelet Products in Plasma of MS Patients

It is therefore not surprising that as a result of platelet activation, significant changes in levels of plasma platelet-specific components have been reported. These include for example the α granule components soluble P-selectin (69), as well as ß-thromboglobulin (ß-TG) and PF4 (70), with PF4 levels being shown to correlate with disease severity. In addition, platelet microparticles (MP) have been investigated in MS (64). MP are a heterogeneous population of small vesicles between 100 nm and 1 μm in diameter, released by cells under physiological and pathological conditions. They are generated by budding of the plasma membrane and the cargo they carry includes proteins, lipids, miRNA, non-coding RNA and cell surface receptors and antigens. Many of these components function as bioactive molecules, hence the identification of microparticles as vectors for intercellular communication, mediating target cell activation, phenotypic modification, and reprogramming. A multitude of pathologies including inflammation and autoimmune diseases have been associated with significant increase in MP release and since these particles express markers pertaining to their cells of origin, there is considerable interest in their potential as disease biomarkers. Platelet MP (PMP) are the most abundant ones in the circulation under homeostatic conditions. There have been multiple reports of a relationship between elevated PMP in the circulation and MS. In a flow cytometry study of 95 patients (including 12 CIS, 51 RR, 23 SP, 9 PP cases) and 49 healthy subjects Marcos-Ramiro et al. (71) demonstrated that significantly increased PMP levels relative to healthy controls were associated with all clinically definite MS forms. PMP levels in CIS patients were elevated but this increase was

not significant. Therefore, this study concluded that platelet dysfunction manifests when patients are definitely progressing. Of interest was the additional finding that endothelial cell-derived MP were significantly increased in confirmed MS cases, as well as CIS cases, suggesting platelet activation to be secondary to endothelial cell damage. In a separate study, Sáenz-Cuesta et al. (72) identified elevated PMP in RR-MS, untreated and treated patients and furthermore demonstrated that numbers of circulating PMP, in particular, are indicative of treatment effect and clinical status in MS. Thus, the highest PMP levels were found during disease exacerbations while untreated MS patients also showed significantly higher PMP compared with controls. Treatments with IFN-ß, or the α-4-ß-1 integrin antibody blocker natalizumab was associated with higher MP levels, including PMP. On the other hand, this study showed that SP-MS patients exhibited no significant difference in PMP from healthy controls. These data were interpreted as being suggestive of a relationship between rising levels of platelet shedding and periods of active inflammation. The differences between the Marcos-Ramiro and Saenz-Cuenza may be related to methodological approaches, for example in the use of different markers for flow cytometric sorting of microparticle subsets. Additionally, they may be influenced by differential treatment effects between patient cohorts, since drugs, such as IFN-ß, or natalizumab are associated with increased PMP (73). The basis for the rise in PMP with drug treatment is still unclear, but may be a secondary effect of blockade of lymphocytic entry into the CNS and increasing blood lymphocytes and lymphocyte-derived MP. Nonetheless, despite the differences between studies in the PMP status in SP-MS, there is an overall consensus that platelets increase PMP generation during periods of exacerbations, suggesting a pathological function for this phenomenon (73).

In the study of Sheremata et al., it was found that P-selectin-positive PMP were capable of binding to PSGL-1 and PECAM-1 on lymphocytes by increasing levels of integrins, such as VLA-4, resulting in increased lymphocyte binding to the endothelium. Furthermore, PMP cargo was shown to contain PAF (48, 74). However, PAF is produced by a variety of cells involved in host defense and the coordinated secretion of PAF would enhance the opening of the BBB, since disruption of endothelial junctions is the most prominent effect of PAF. This evidence suggests that PMP serve as immunomodulatory agents engaged in the propagation of inflammation (74). To date however, there is still insufficient evidence to determine whether PMP are suitable as MS biomarkers.

Platelet-Specific Markers Found in MS Lesions and Normal Appearing White Matter (NAWM)

Platelets were shown to cross the BBB via the damaged vascular basal lamina, rather than the inter-endothelial cell junctions *in vivo* (75). Evidence of platelet-derived products has been demonstrated within MS lesions, or around lesions (that is in NAWM). In a study by Han et al. (76) proteomics analysis of laser-microdissected lesions including acute plaque, chronic active plaque and chronic plaque, proteins of the coagulation cascade, such as tissue factor and protein C were identified in chronic active plaques. The significance of this finding is that these platelet-related components are associated with an active stage of the disease. In a separate study the same laboratory identified the platelet-specific glycoprotein GPIIb (CD41) in chronic plaques (77).

Studies focused on the relationship between platelets and CNS demyelination demonstrated that fibrinogen is abundant in NAWM. Fibrinogen (plasma and platelet-derived) is emerging as a significant mediator of inflammation and potentially, a trigger of early lesion formation in MS (78). Fibrinogen is a 340-kDa multimeric glycoprotein that has critical functions in vascular hemostasis. Although fibrinogen normally circulates in plasma at concentrations approximating 3 mg/ml, its levels can exceed 7 mg/ml during inflammatory responses. At sites of inflammation, endothelial cell retraction permits extravasation of fibrinogen, leading to its extravascular deposition as mixed fibrin/fibrinogen polymers. Fibrinogen is known to promote innate immune activation, thereby driving local inflammation. Post-mortem studies have reported extensive fibrinogen deposits around blood vessels, not only in active and chronic MS lesions, but significantly in pre-active lesions, namely prior to inflammatory infiltration and demyelination.

Recent Novel Insights From EAE-Based Investigations

The EAE Model and Its Application to MS Research

EAE is a neuroinflammatory disease induced in susceptible species, which has been used as an MS model for several decades (13–15). It is generated by active immunization with CNS antigens, including spinal cord homogenate, purified myelin proteins, or their immunodominant epitopes, most commonly mice, rats, and non-human primates (13, 79). Depending on the mouse/rat strain and antigen combinations different clinical profiles can be generated (80, 81), but currently, the variant generated by the peptide containing amino acids 35–55 of the myelin oligodendrocyte glycoprotein (MOG) in the C57BL/6 mouse strain has taken the most prominent place in EAE-based investigations (82). Alternatively, the disease can be provoked by passive immunization with encephalitogenic $CD4^+$ T cells isolated from draining lymph nodes of actively immunized donor mice into syngeneic animals.

Common symptoms include ambulatory difficulties, impaired balance, bladder and bowel dysfunction, as well as behavioral deficits (83, 84). The pathology of EAE consists of meningeal and perivenous inflammation, dominated by T cells and macrophages, associated with severe and widespread microglial and astrocytic reactivity. Axonal injury and neuronal loss are additional features of disease pathology, beginning early in disease development (85). Of interest is the observation that depending on the genetic background of the host and immunization regimen, EAE lesions are most reminiscent of Types I and II MS lesions (25). These clinical, histological and immunopathological hallmarks reminiscent of MS, together with the potential to access a wide range of genetically modified mouse lines, have made EAE an attractive experimental model to gain insights into MS immunopathological mechanisms and validate candidate MS therapeutics.

However, although MS and EAE share multiple common pathological mechanisms, they are distinct diseases (86–89). The model has been criticized for discrepancies with MS from the genetic perspective and because of its partial recapitulation of MS (13–15), but also significantly, for its lack of reliability in prediction of the efficacy of candidate MS therapeutics (90). On the other hand, there is no doubt that EAE has provided valuable proof of concept for mechanisms of immune-mediated injury (89, 91). A classic example is the differentiation between the roles of $CD4^+$ and $CD8^+$ T cells in CNS autoimmunity. Originally thought to be the major drivers of the inflammatory process, $CD4^+$ T cells now appear to be involved in the initiation of the immune response rather than in the effector stage of brain inflammation. $CD4^+$ T cells are outnumbered by $CD8^+$ T cells in the ratio of 1 to 10 in MS lesions and data suggest that $CD8^+$ T cells proliferate in response to myelin antigens, subsequently trafficking to sites of inflammation. Using MOG_{35-55}-induced C57BL/6 mice, it was shown that MOG_{37-46} is a minimal peptide capable of inducing specific a $CD8^+$ T cell response, thereby supporting the notion of a major role for $CD8^+$ T cells in active tissue damage (92, 93). A second example is the identification of the Th17 subset, characterized by expression of the cytokines IL-17A, IL-17F, IL-21, and IL-22. Studies, mainly based on the use of the EAE model, have identified the multistep differentiation of Th17 cells including the pro-inflammatory cytokines driving this process, together with Th17-derived products which attract T cells and myeloid cell populations into lesions. Additionally, IL-17 acts directly on endothelial cells by induction of reactive oxygen species. Thus, whereas EAE was thought to be a prototypical Th1 driven autoimmune disease, it is now clear that Th17 cells play a more critical role in disease initiation than Th1 cells (93–95).

Therefore, whilst EAE recapitulates a limited number of facets of MS, no other currently available model demonstrates pathological features reminiscent of MS, or the capacity to address the autoimmune response. The consensus is that if used rationally, namely by recognizing the limitations of the model in the experimental design, EAE still represents a useful tool to elucidate specific mechanisms underlying MS pathophysiology and allow repair and neuroprotective strategies to be explored (96–98).

The Critical Role of Platelets in EAE Development

Overall, the sum of the evidence supports the concept that platelets are chronically active in MS and that their involvement begins early, since a number of these changes have even been identified at the CIS stage. Novel insight into the relationship between platelets and the development of neuroinflammation was provided by a series of EAE-based studies from several laboratories. Firstly, in a series of elegant experiments Langer et al. (99) demonstrated the presence of platelets in the mouse CNS via detection of the platelet-specific marker CD41. Subsequently, they demonstrated a beneficial effect of platelet depletion with anti-mouse platelet serum on EAE severity and progression, when platelets were depleted in the inflammatory phase of the disease. This was confirmed by evidence of reduced microgliosis, together with decreased spinal cord inflammatory components CCL2, CCL5, CXCR4, and IL-1ß and decreased axonal injury and demyelination. Furthermore, targeting of specific platelet components involved in platelet responses also ameliorated EAE. This was achieved by administration of blocking Fab to GP1bα, a component of the GP1b/IX/V complex, or Fab to the major platelet adhesion receptor GPIIb/IIIa. Importantly, this study also identified platelets in MS post-mortem tissue. These data, therefore, provided the first strong evidence that platelets contribute to the pathogenesis of EAE by promoting CNS inflammation.

In independent studies, Sotnikov et al. (100) identified $CD41^+$ platelets/platelet aggregates directly associated with neuronal cell bodies and astrocytes from as early as day 6 post-EAE initiation, co-incident with a 25-fold increase in PF4 expression. They demonstrated that this occurs via sialated gangliosides in lipid rafts, specifically the gangliosides GT1b and GQ1b, confirmed by the use of mice genetically deficient in sialyltransferase ST3Gal-V (a synthase of GM3 ganglioside). This resulted in significant reduction in EAE symptoms, together with $CD4^+$ T cells, lymphocytes and macrophages both in CNS tissues and in the circulation. The implications of these findings are that they demonstrated the presence of activated platelets in the CNS parenchyma and their direct association with CNS cells.

Our own studies began with mapping of platelet accumulation from day 0 of EAE to characterize the relationship between platelet changes and the development of inflammation. In D'Souza et al. (101) and Kocovski et al. (84) platelet accumulation in the circulation and its timing were identified from 3 days post-induction, peaking at about 7 days and remaining elevated over the rest of the disease course. This was distinctly earlier than the accumulation of autoreactive T cells in blood, lymphoid tissues and the CNS, which were only detectable beginning from 11 days after induction. Platelet entry into the CNS was also identified ahead of that of CD3 cells using a qPCR approach, but different outcomes were documented between white and gray matter. In white matter platelets were found to be disseminated throughout the tissue, while in gray matter they were closely associated with neurons. Both studies showed intimate platelet-neuronal associations in the spinal cord, retina (associated with retinal structural abnormalities) and hippocampus (a region associated with emotion, cognition and memory) immediately following platelet entry and at times preceding autoreactive T cell accumulation. Significantly, the efficacy of platelet depletion was directly related to the timing of platelet accumulation: thus, when depletion was performed only over the period preceding the peak of platelet accumulation, or initiated in the chronic stage of disease, the beneficial effect of treatment was reduced. On the other hand, when platelet depletion was initiated from the peak of accumulation, treatment eliminated parenchymal T cell accumulation, EAE development and the generation of a pro-inflammatory environment in the CNS. A functional relationship was confirmed when it was shown that platelet accumulation is associated with anxiety-like behavior and that this effect is reversed with platelet depletion.

An understanding of mechanisms underlying platelet effects on neuronal and glial cell functions is emerging, although some of these mechanisms have to be inferred from data

derived from experimental models more relevant to other neurodegenerative conditions. Firstly, the study by Sotnikov et al. (100) establish a new role of platelets by demonstrating that these elements directly recognize sialated gangliosides in lipid rafts on the surface of neuronal processes and end feet of astrocytes and that these interactions are essential for EAE development. Gangliosides are commonly found in many tissues but are most abundant in the brain; however only gangliosides GT1b and GQ1b were recognized by platelets, highlighting the specificity of these interactions (100). The recognition of these sialated gangliosides by platelets involved principally P-selectin, consistent with the established pro-inflammatory role of this component. These data therefore, identify lipid rafts of astrocytes and neurons as new ligands within the CNS that are recognized by platelets, thereby suggesting a mechanism for platelet-CNS cell communication. In separate studies, using a model of traumatic brain injury (102), the same group showed that interaction of platelets with neuronal lipid rafts occurs within minutes of injury, leading to immediate release of platelet-derived factors, via platelet degranulation and PMP shedding. Degranulation was associated with the release of neurotransmitters, such as serotonin (5-HT), from dense granules. On the other hand PMP production results in surface localization of PAF (74). In this context, however, platelet-neuron association stimulated neuronal activity, increased neuronal survival near the site of injury and promoted the formation of new dendritic spines on these cells, demonstrating an alternative role for platelets in synaptic plasticity. It is of interest that similar glycolipid structures have also been documented on neural precursor cells (103, 104), implying that platelets may directly communicate with neural precursor cells via a receptor-mediated interaction.

A study aimed at identifying signals responsible for early response to CNS injury (105) demonstrated platelet effects on oligodendrocyte precursor cells (OPC). First, damage to the BBB was caused by microinjections of vascular endothelial growth factor (VEGF) or lipopolysaccharide (LPS) in mouse or rat basal ganglia, followed by delivery of blood and blood components. Responses of glial cells were quantified by sub-type specific activation markers after 24 h in the injected side relative to vehicle-only contralateral side. Delivery of whole blood, platelets, or macrophages, as well as injury-related cytokines associated with macrophages and platelets, such as TNFα, TGFß, IL-1ß, and IFNγ, resulted in OPC activation while OPC mitogenic factors, including platelet-derived growth factor (PDGF) and fibroblast growth factor 2 (FGF-2), failed to produce a similar effect. As expected, these observations were associated with prominent microglial reactivity. The role of OPC is normally associated with repopulation of depleted oligodendrocytes following demyelination. The rapid response shown here suggests a platelet-mediated OPC injury response related to wound-healing in the brain.

In addition to PMPs, platelets release exosomes, which are 40–100 nm diameter, $CD63^+$ extracellular vesicles, originating from endosomal multivesicular bodies and α-granules. Platelet exosomes carry principally (but not exclusively) α-granule components (106), including several neurogenesis-promoting molecules. Therefore, exosome release represents a potential additional route for platelet targeting of CNS cells in response to BBB disturbances.

These recent findings, therefore, bring new insight into mechanisms underlying neuroinflammation. They demonstrate early platelet involvement in disease development which drives two distinct but related pathophysiological processes (**Figure 1**). First, platelets cross the BBB (and potentially other barriers, such as the blood-retinal barrier), become closely associated with and deliver pro-inflammatory products to neural (and potentially glial) cells via lipid rafts, PMP and exosomes, thereby using the full armamentarium of communicating molecules, namely proteins, lipids and nucleic acids and glycoconjugates. This results in early functional consequences which are independent of lymphocytic infiltration. Concurrently, platelets drive the generation of autoreactive T cells in the peripheral circulation. This presumably occurs by degranulation of platelets immediately upon BBB disturbance, resulting in the release of multiple soluble factors serotonin (5HT), PF4 and PAF, which specifically stimulate differentiation of T cells toward pathogenic Th1, Th17, and IFN-γ/IL-17-producing CD4 T cells (107). However, inflammatory infiltration into the CNS parenchyma occurs subsequently to that of platelets, but for unknown reasons remains less significant in gray relative to white matter. These are significant observations which highlight the multi-modal participation of platelets in the orchestration of neuroinflammation. The question that arises, therefore, is: what are the processes via which platelet responses are linked to the complex and as yet, unelucidated interactions between inflammation and neurodegeneration in MS, which potentially involves multiple immune cell types and their products?

POTENTIAL PATHWAYS FOR PLATELET INVOLVEMENT IN MS

Under inflammatory conditions, platelets bind to other platelets (aggregate) and multiple immune cell types. The interplay between platelets, endothelial cells and leukocytes is the direct cause of BBB damage (108). Platelet activation results within seconds in the expression of surface CD40L and cytokines, notably IL-1ß, leading to endothelial cell expression of adhesion molecules ICAM-1, vascular cell adhesion molecule-1 (VCAM-1), E-selectin and P-selectin (CD62P). IL-1ß release promotes endothelial cell permeability, together with recruitment and attachment, on the endothelium, of several classes of leukocytes, including neutrophils, monocytes, dendritic cells and B and T lymphocytes. Monocytes and neutrophils are the major leukocytes that form complexes with platelets, via P-selectin/PSGL-1 and CD40L/CD40 interactions, leading to monocyte differentiation into dendritic cells and concomitant polymorphonuclear cell activation. Platelet-neutrophil interactions also directly modulate dendritic cell maturation by CD40L/CD40, leading to dendritic cell antigen presentation to T cells. CD40L expression on platelets also enhance $CD8^+$ T cell responses, as well as isotype switching in B cells from IgM to IgG. Finally, interactions between platelets and their cellular partners are bi-directional, resulting in amplification and expansion

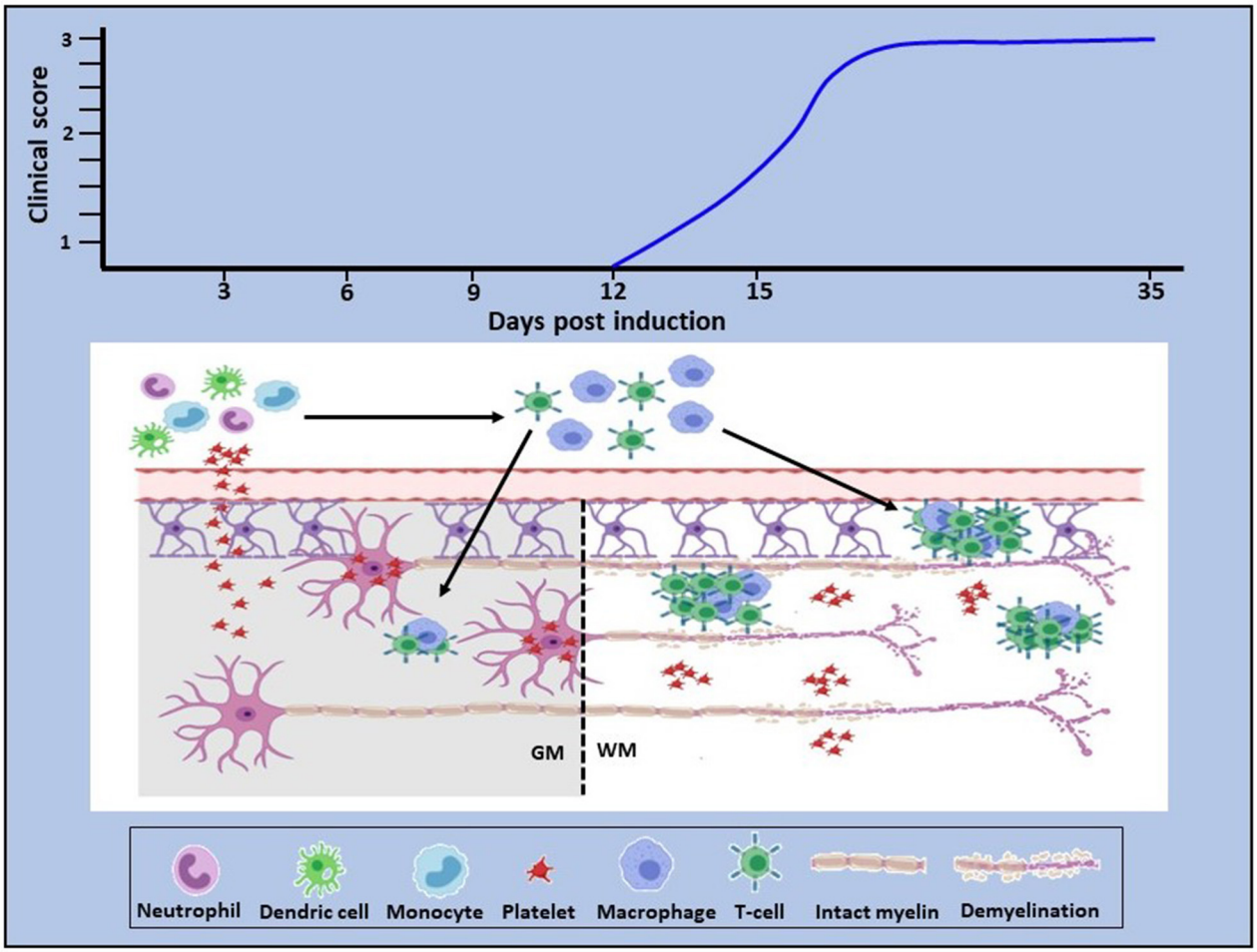

FIGURE 1 | Proposed role of platelets in neuroinflammation. In EAE, platelet activation is an early event which is quickly followed by platelet infiltration into the CNS white and gray matter. In white matter they disseminate throughout the parenchyma, whereas in gray matter they target neurons (bottom panel). Concurrently, platelets drive the generation of autoreactive immune cells which become detectable by day 11–12 post-disease induction, corresponding to clinical onset (top panel). This evidence provides proof of concept that platelets are neurodegenerative as well as pro-inflammatory and that these two pathological processes are causally linked. Additionally, these data suggest that platelet targeting would represent an effective anti-inflammatory and neuroprotective therapeutic approach for MS. GM, gray matter; WM, white matter.

of the inflammatory response (54, 108–112). Thus, platelets have evolved a range of mechanisms resulting in an extensive functional repertoire enabling signaling to multiple immune cell subsets. These mechanisms underlie the evolution of innate to adaptive immunity, thereby placing platelets in a central position for the development of inflammation and autoimmune disease.

Are there any data in the literature that would suggest early platelet involvement in MS? Evidence has been mounting for a vascular component in MS, whereby vascular abnormalities play a crucial role in lesion formation and progression (113). The evidence for this notion comes from reports of enhanced risk of cardiovascular events, such as ischemic stroke, myocardial infarction, and thrombosis in MS patients, directly associated with a dysfunctional coagulation cascade and aberrant platelet function and their increased pro-thrombotic activity (54, 114). Vice versa, platelet defects are known to be a risk factor in MS. For example, comparison of 165 Japanese patients diagnosed with clinically definite MS and 245 healthy controls demonstrated that the frequency of the missense mutation A224D, which impairs PAF-PAF receptor (PAFR) signaling, was significantly higher in MS patients (21.0%) than in healthy controls (13.5%). PAF has been intensely investigated for its proinflammatory response to various stimuli. However not all PAF effects are proinflammatory, since PAF is also involved in immunosuppressive mechanisms and consequently, mutations affecting PAF-PAFR signaling may enhance susceptibility to MS in some patients (115). Additional evidence is provided by the demonstration of an association between MS and mitochondrial mutations (most commonly in Complex 1 components), affecting platelet function. In one case study where a large mitochondrial DNA

mutation was identified, a patient diagnosed with myopathy and progressive external ophthalmoplegia (PEO, a condition characterized by eye muscle weakness) also exhibited MS-like features. The mutation caused respiratory chain deficiency in muscle and blood, together with reduced basal platelet mitochondrial membrane potential. However, cerebrospinal fluid analysis and MRI revealed inflammatory CNS demyelination indistinguishable from MS (116) while magnetic resonance spectroscopy showed absence of a significant lactate peak. This was a clear indication that the predominant pathology underlying the MRI data resulted from immune-mediated disease rather than hypoxic/ischemic mechanisms secondary to mitochondrial energy deficits. Thus, the mitochondrial mutation and platelet abnormalities were associated with MS, as well as myopathy and PEO. In this context it is important to remember that mitochondrial mutations and consequential compromised energy supply would also render the high energy-demanding neurons as well as glial cells more susceptible to apoptosis, thereby contributing to progressive disability. The coincidence of an MS-like phenotype with a primary mitochondrial defect (mitochondrial or genomic DNA encoded) suggests that some MS cases may be associated with multisystemic diffuse mitochondrial abnormality (117). Such cases have been reported in Harding's disease, where MS-like disease co-exists with Leber's hereditary optic neuropathy (LHON), and others where the primary mitochondrial disorder was identified as a mutation in the optic atrophy 1 (*OPA1*) gene, or the mitochondrial DNA polymerase gamma (*POLG1*) gene.

An alternative mechanism is suggested by recent findings on the role of the commensal microbiome in autoimmune disease and the gut-brain axis. There is now increasing evidence that dysbiosis (or reduction or loss of normally-residing gut microbiota) constitute an environmental factor, which can modulate immune processes relevant to MS by boosting the polarization of proinflammatory cells (118, 119). Reduced diversity of microbiota has been documented in MS patients with active disease. For example, *Parabactoroides distasonis*, which is frequently found to be decreased in MS patients enhances the differentiation of IL-10$^+$ Tregs. On the other hand, abundance of *Akkermensia municiphilia* boosts Th1 and Th17 differentiation, while suppressing that of Treg. Gut T cells traffic to lymphoid tissues and the CNS where they can have direct effects on inflammation. Studies by Linden et al. and Rumah et al. (120, 121) showed that toxic products from gut bacteria may initiate new lesion formation. They demonstrated that the gut bacterium *Clostridium perfringes* releases the epsilon protein which may play a pivotal role in triggering new lesions due to its tropism for myelin and the BBB. Epsilon toxin is a 33 KD precursor cleaved in the gut to a 28.6 KD product. In mammalian brain slices, cleaved epsilon toxin has been shown to bind myelin. It also has the capacity to enter the blood stream and bind to CNS microvessel endothelial cells forming a pore on the endothelial plasma membrane, thereby compromising BBB integrity. Such disruption of the BBB would have an immediate effect on platelet homeostasis, resulting in platelet activation, platelet adhesion/aggregation on the vascular surface and platelet interactions with multiple immune subsets, as described above, resulting in an inflammatory response and lesion development. A relationship has recently been demonstrated between platelet counts and alterations in the gut microbiome, whereby the reduction in "good" microorganisms is inversely related to platelet numbers (122). Overall, gut microbiome-directed therapeutic strategies based on microbiome profiling in patients with MS and in the EAE mouse model have attracted major interest, however, systematic clinical trials are yet outstanding (123).

It has also been documented that oligodendrocyte death appears to be an initial event in lesion development. Thus in the study of Lucchinetti et al. already described, Type III lesions were characterized by oligodendrocyte loss ahead of demyelination, whereas T cell infiltration was mild until large scale demyelination became evident. Similarly, in a study of new symptomatic lesions in patients who died shortly after a relapse, Barnett and Prineas (124) identified severe oligodendrocyte apoptosis, associated with reactive microglia, but few myelin-laden macrophages and essentially intact myelin. Strikingly, this occurred in the absence or minimal evidence of lymphocytic infiltration. Following oligodendrocyte, death large amounts of membrane become metabolically unsupported, with ensuing accumulation of myelin fragments and vesicles at the site of the early lesion. Therefore it can be envisaged that these events would impact the BBB, if the capacity of macrophages and microglia to remove myelin debris became exceeded (125).

Any of the above proposed scenarios, singly or in combination, would represent a candidate mechanism potentially triggering platelet involvement in disease development. For example:

(a) Steady release of toxic products from gut bacteria into the circulation would lead to failure to maintain vascular integrity and to compromised BBB function. This would be met with immediate platelet activation, platelet association with the endothelial layer and platelet inflammatory cascades.
(b) Vascular damage and extravascular accumulation of blood components, especially fibrin/fibrinogen is believed to drive local inflammation resulting in microglial activity, release of pro-inflammatory chemokines across a leaky BBB and recruitment of peripheral macrophages, again resulting in platelet activation.
(c) Myelin debris, for example by oligodendrocyte death or local inflammation resulting from fibrinogen accumulation first collect in the interstitial fluid. This is followed by migration of antigens into perivascular channels, eventually accumulating in lymph nodes via soluble and cellular routes. Alternatively, antigens are collected by dendritic cells within the CNS parenchyma and travel to lymph nodes. Antigens are then presented to CD4$^+$ and CD8$^+$ cells which enter the CNS, infiltrate the cerebrospinal fluid (CSF) entering the circulation via a damaged BBB, where they encounter platelets (125).

This diversity of mechanisms and myelin products which can be recognized as antigens are also in keeping with the heterogeneity of the disease.

THE POTENTIAL OF PLATELETS AS BIOMARKERS AND THERAPEUTIC TARGETS IN MS

MRI remains the gold standard for the diagnosis of MS and for patient monitoring. Nonetheless, MRI has a number of major limitations in the context of MS, such as discordance between lesion location and clinical presentation, as well as low sensitivity of conventional approaches to cortical lesions and diffuse white matter damage (126). Novel and often very advanced imaging approaches are continuously being explored to improve early diagnosis and better identification of MS phenotypes. This includes the combination of MRI with nuclear medicine imaging modalities, such as positron emission tomography (PET) and single photon emission computed tomography (SPECT), whereby MRI provides anatomical information about lesion topography and lesion load, while nuclear imaging can identify physiological changes at the single cell or single molecule level. For example, nuclear imaging represents an attractive candidate modality for improved detection and further characterization of cortical lesions. Accumulation of platelets at the lesion site is a very plausible hypothesis based on several potential mechanisms. (1) Meningeal accumulation of B and T cells via leakage of the BBB. It is known that platelets form aggregates with various immune cells and thus are transported via piggybacking (48, 50, 54). (2) Platelets have been shown to migrate and thus may associate with neurons (84, 100, 101). In the following paragraphs we will discuss improvements in nuclear medicine imaging and the potential for identification of adjunct biomarkers and molecular targets in MS.

Early platelet involvement in MS can potentially provide a unique opportunity to use platelets as biomarkers for diagnostic, therapeutic and combined theranostic approaches. Direct targeting of specific biomarkers for the diagnosis of MS has been demonstrated using the peripheral benzodiazepine receptor, also known as TSPO, a protein that is only minimally expressed in healthy brain (127). Currently more than 80 TSPO radiotracers are under development for molecular positron emission tomography imaging, with the aim to improve signal to background ratio, as well as to overcome low binding status in over 30% of the population with a single nucleotide polymorphism (127). Other molecular imaging approaches for MS include the targeting of adenosine receptors, which are key element in inflammation and thrombosis (128). These promising approaches are demonstrating that molecular imaging targeting specific markers has the potential to provide specific and early imaging of MS. We propose that molecular markers specific for activated platelets will be worth to be tested as novel diagnostic approach for the imaging and diagnosis of MS.

Amongst the various receptors expressed on platelets, P-selectin and GPIIb/IIIa receptors are the most often used biomarkers because they enable the differentiation between resting and activated platelets. Upon platelet activation, P-selectin expression is upregulated by translocation from the intracellular granules to the external membrane. P-selectin is, however, not platelet-specific because of its expression on other cells, including endothelial cells. The GPIIb/IIIa receptor, on the other hand, is only found on platelets and undergoes a conformational change during platelet activation (129). This exposes activation-specific epitopes, such as ligand-induced binding sites and the ligand (fibrinogen) binding pocket. In addition to this activation and platelet specificity, GPIIb/IIIa is highly abundant with about 60,000 receptor molecules/platelet. As such small numbers of platelets expose abundant molecular target epitopes, which is an important advantage toward a superior sensitivity of the respective molecular imaging. These features make GPllb/IIIa an ideal epitope for molecular targeting.

Across the cardiovascular and cancer fields, technologies used for the molecular imaging of platelets include ultrasound (130–134), positron emission tomography [PET (134, 135)], single photon emission computed tomography [SPECT (136, 137)] and optical imaging (136, 137). Each of these modalities have their strengths and weaknesses (138–142), which have to be assessed to determine their suitability for diagnosing MS. In particular, MRI has been commonly used for the detection of MS lesions (143), therefore the addition of molecular imaging to detect activated platelets may enable earlier and more sensitive diagnosis. Since the current imaging methods provide a readout only after anatomical/functional changes have occurred, molecular imaging of activated platelets might allow us to improve the specificity of MS diagnosis, even in areas of small lesions. Furthermore, activated platelet imaging might represent an approach for longitudinal monitoring of MS progression and provide the ability for the monitoring of treatment success or failure.

A wide selection of platelet targeting ligands have been conjugated onto contrast agents for the respective imaging technologies, and have provided direct *in vivo* visualization of platelets as major components of thrombi (128). These studies are helpful in directing us to the platelet contrast agent best suitable for the diagnosis of MS. Using fucodian (a sialyl Lewis X mimetic with a strong affinity to P-selectin), Letourneur et al. successfully imaged platelets *in vivo* in arterial thrombi via SPECT (144) and via MRI (138). More recently, Jing et al. conjugated microbubbles with commercially available antibodies that target P-selectin for *in vivo* molecular ultrasound imaging of platelets in left atrial thrombi in rats (138, 145). Arginine-glycine-aspartic acid (RGD) analogs have been used for visualization of platelets in vascular thrombosis via a range of ultrasound, MRI and nuclear imaging (129, 139, 146). However, RGD is not specific for the GPIIb/IIIa integrin and indeed has been used for the targeting of other integrins, such as the vitronectin receptor αvβ3 in cancer imaging (128). Furthermore, RGD analogs are not specific for activated platelets.

More interestingly, GPIIb/IIIa antagonists, such as elarofiban and abxicimab (147–149) have been employed as ligands for imaging. However, these antagonists similar to RGD analogs bind to all circulating platelets, thereby specificity of their diagnosis has been questioned. Peter and colleagues have generated single-chain antibodies (scFv) with specific targeting toward the activated GPIIb/IIIa integrin, and no binding to resting platelets in circulation (150). This scFv have been conjugated to microbubbles for ultrasound (151), iron oxides

particles or perfluorocarbon nanoemulsions for MRI (131), radiotracers for PET (137) and near-infrared dyes for optical imaging (137) of arteria and venous thrombi. Using this scFv, Yap et al. successfully visualized a board range of tumors via ultrasound, PET and optical imaging (133). Additionally, Yap et al. conjugated this scFv with a potent chemo-therapeutic microtubule inhibitor and demonstrated that these antibody-drug conjugates could prevent tumor growth and metastases (133). This concept of activated platelet targeting may be transferable and seems highly attractive as a diagnostic tool as well as targeted, site-specific therapy for MS. The latter promises to achieve high lesion-localized drug accumulation with low system drug concentrations.

As mentioned in the previous section, the depletion of platelets resulted in the elimination of EAE progression. Clinically, anti-platelet therapies including the cyclooxygenase 1 inhibitors aspirin, P_2Y_{12} receptor inhibitors, PAR1 antagonists, and GPIIb/IIIa inhibitors, are used for the prevention of platelet aggregation. As such, multiple anti-platelet therapies would be available for the potential treatment of MS patients. The activated-platelet targeting approach of CD39 has been successfully tested in cardiac ischemia/reperfusion injury in mice. In this setting the anti-inflammatory effect of the platelet-targeted construct contributed directly to the prevention of ischemia/reperfusion injury. Similarly, this construct would use platelets as targets to direct and accumulate the anti-inflammatory effect to lesion sites.

Preclinical research into targeted drug delivery systems and nano-/micro-carriers have been extensively conducted to overcome bleeding complications, and the employment of these side-effect free drugs may help to successfully treat MS. With genetic engineering of recombinant antibody-fusion drugs, scFvs targeting activated GPIIb/IIIa have been paired with/conjugated to several drugs, including urokinase-type plasminogen activator (134), tick anticoagulation peptide (152, 153), and CD39 (ectonucleoside triphosphate diphosphohydrolase-1) (154). These antibody-fusion drugs have been used successfully for side-directed low systemic dose treatment and prevention of thrombosis, myocardial ischemic/reperfusion injury and sepsis. Of the three different drugs, the scFv-CD39 seems to be the most ideal drug candidate to stop MS progression, because CD39 is both anti-thrombotic and anti-inflammatory (155, 156). It is known that inflammation plays an important role in MS and adenosine receptors have been proven to be upregulated in the area of MS (128–130). Therefore, the dual action of anti-platelet targeting of the scFv, together with the anti-inflammatory NTPase breakdown of adenosine by the CD39 component, will likely play a beneficial role in the treatment of MS. Respective preclinical studies are currently ongoing.

CONCLUSIONS

Historic and more recently emerging evidence indicate an early and important role of platelets in MS. This includes the consistent demonstration of platelet abnormalities and release of platelet inflammatory products in MS patients and of presence of platelet products in MS post-mortem lesions. Some of these biochemical and ultrastructural defects were identified from the CIS stage. Preclinical data generated proof of concept that platelet depletion prevents the development of EAE in mice and that early platelet infiltration into the CNS parenchyma is neurodegenerative. However, the direct translation of these findings into the use of anti-platelet drugs in patients with MS is clearly impeded by an increased bleeding risk, generally associated with the use of currently available anti-platelet drugs. On the other hand, activated platelets may provide a unique molecular epitope for early diagnosis of MS and for therapeutic drug-targeting, the latter potentially avoiding bleeding complications. As such we hypothesize that platelet targeting for diagnosis and therapy holds great promise and warrants further investigations.

DEDICATION

This work is dedicated to the memory of Christine Heim.

AUTHOR CONTRIBUTIONS

CD'S, PK, MH, and JO generated the data based on the EAE model discussed in section An Association Between Platelets and MS/EAE. XW contributed to the imaging data discussed in section The Potential of Platelets as Biomarkers and Therapeutic Targets in MS. GK provided the advice on the translational aspects of the research. JO wrote the sections Introduction to The Potential of Platelets as Biomarkers and Therapeutic Targets in MS. XW and KP wrote the section The Potential of Platelets as Biomarkers and Therapeutic Targets in MS. KP wrote the section Conclusions of the manuscript. JO and KP reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: JO, XW, and KP are inventors on a patent application on diagnosis and therapy of MS.

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.

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