

Fibrin(ogen) and Neurodegeneration in the Progressive Multiple Sclerosis Cortex

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Objective: Neuronal loss, a key substrate of irreversible disability in multiple sclerosis (MS), is a recognized feature of MS cortical pathology of which the cause remains unknown. Fibrin(ogen) deposition is neurotoxic in animal models of MS, but has not been evaluated in human progressive MS cortex. The aim of this study was to investigate the extent and distribution of fibrin(ogen) in progressive MS cortex and elucidate its relationship with neurodegeneration.

Methods: A postmortem cohort of pathologically confirmed MS ($n = 47$) and control ($n = 10$) cases was used. The extent and distribution of fibrin(ogen) was assessed and related to measures of demyelination, inflammation, and neuronal density. In a subset of cases (MS, $n = 20$; control, $n = 10$), expression of plasminogen activator inhibitor 1 (PAI-1), a key enzyme in the fibrinolytic cascade, was assessed and related to the extent of fibrin(ogen).

Results: Motor cortical fibrin(ogen) deposition was significantly over-represented in MS compared to control cases in all compartments studied (ie, extracellular [$p = 0.001$], cell body [$p = 0.003$], and neuritic/glial-processes [$p = 0.004$]). MS cases with high levels of extracellular fibrin(ogen) had significantly upregulated PAI-1 expression in all cortical layers assessed ($p < 0.05$) and reduced neuronal density ($p = 0.017$), including in the functionally-relevant layer 5 ($p = 0.001$).

Interpretation: For the first time, we provide unequivocal evidence that fibrin(ogen) is extensively deposited in progressive MS motor cortex, where regulation of fibrinolysis appears perturbed. Progressive MS cases with severe fibrin(ogen) deposition have significantly reduced neuronal density. Future studies are needed to elucidate the prove-nance and putative neurotoxicity of fibrin(ogen), and its potential impact on clinical disability.

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Cortical tissue injury is a key pathological feature of progressive multiple sclerosis (MS) and correlates with irreversible physical and cognitive decline.¹ A widely held theory states that the progressive stage of MS is characterized by compartmentalization of central nervous system (CNS) inflammation behind an intact blood-brain barrier (BBB).² According to this view, the inaccessibility of the CNS in progressive disease might explain why immunomodulatory compounds fail to produce clinical benefit. However, there is limited experimental evidence to support this explanation. A greater understanding of the pathogenesis of cortical injury in MS is fundamental for developing novel disease-modifying

drugs that could slow or even halt disability accumulation in the progressive stage.

Meningeal inflammation appears to be a central driver of cortical pathology in MS.³ However, the role of BBB disruption in this process has been relatively overlooked. Given the anatomical relationship between the subarachnoid compartment and penetrating perivascular spaces, BBB disruption and meningeal inflammation could both be intricately linked to the initiation and/or exacerbation of cortical pathology. To date, the evidence of cortical BBB disruption in MS has been limited. Endothelial tight junction abnormalities have been detected in cortical normal-appearing gray matter

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TABLE 1. Details of MS and Control Cohort

	MS Cases (n = 47)	Controls (n = 10)
Age of death (y)	63 (range, 40–92)	74 (range, 57–91)
Duration of disease (y)	31 (range, 11–58)	n/a
Sex	M = 13, F = 34	M = 7, F = 3
Clinical course	PPMS: 4, SPMS: 38, RRMS: 1, unknown: 4	n/a
Brain weight (g)	1,157 (range, 894–1,380)	1,320 (range, 1,072–1,628)
PM interval (h)	18 (range, 7–38)	36.7 (range, 10–72)

MS = multiple sclerosis; PM = postmortem; M = male; F = female; PPMS = primary progressive MS; SPMS = secondary progressive MS; RRMS = relapsing-remitting MS; n/a = not applicable.

(NAGM), a finding suggestive of BBB disruption.⁴ However, attempts to detect surrogates of BBB disruption, such as extravasated plasma proteins, in postmortem cortical MS tissue have so far failed.⁵

The serum protein, fibrinogen, is a good surrogate marker of BBB disruption because of its abundance, large size (340 kDa), restriction to the intravascular compartment, and lack of expression in the healthy CNS. Thrombin mediates the polymerization of fibrinogen to insoluble fibrin, and thus helps maintain vascular hemostasis. Plasmin mediates the dissolution of fibrin, a process tightly regulated by plasminogen activator inhibitors (PAIs), such as PAI-1. It is not currently possible to distinguish between fibrin and fibrinogen in postmortem specimens, largely because of their molecular similarity and shared epitopes, and hence the commonly used term fibrin(ogen). Fibrin(ogen) can stimulate inflammatory sequelae in addition to their role in the coagulation cascade.^{6,7}

Animal models of MS have implicated fibrin(ogen) deposition and PAI-1 dysregulation in disease pathogenesis. In experimental autoimmune encephalomyelitis (EAE), fibrinogen extravasation induces activation of microglia and subsequent axonal damage before onset of clinical signs, as well as promoting recruitment of myelin specific T cells.^{8,9} Blocking the interaction of fibrinogen with microglia suppresses relapsing paralysis,¹⁰ and EAE can also be ameliorated by fibrin depletion¹¹ and PAI-1 inhibition.¹²

Studies investigating fibrin(ogen) and PAI-1 in animal models of MS are relevant to understanding MS in humans. Similar to EAE, BBB disruption and deposition of fibrin(ogen) are among the earliest pathological features of human MS and are linked with inflammation and demyelination.^{13,14} Proteomic assessment of actively demyelinating plaques further points to specific

dysregulation of coagulation proteins.¹⁵ PAI-1 is upregulated in acute white matter (WM) lesions, but also in cerebrospinal fluid (CSF) and serum, where it has been linked to disease activity.^{16–18} The potential clinical relevance of elevated PAI-1 is highlighted by the recent observation that PAI-1 polymorphisms are linked to MS susceptibility.¹⁹ These observations provide a compelling rationale to revisit cortical fibrin(ogen) deposition and PAI-1 expression and their relationships to neuronal damage in MS postmortem tissue. Assessment of fibrin(ogen) deposition at late stages of MS, wherein cortical parenchymal inflammation is sparse, has not been undertaken to date.

In the current study, we provide unequivocal evidence that fibrin(ogen) deposition is common and extensive in the progressive MS cortex and is significantly associated with the extent of neurodegeneration. We reinforce these findings by showing that cortical PAI-1 is upregulated in cases with abundant fibrin(ogen) deposition. Altogether, these findings suggest that fibrin(ogen) deposition in the MS cortex may play an important role in cortical neurodegeneration and therefore contribute to irreversible disability in the progressive phase of the disease.

Patients and Methods

Study Population

Human archival material of MS (n = 47) and control cases (n = 10) from the UK MS tissue bank, Imperial College, London (Research Ethics Code [REC] 08/MRE09/31 + 5), and Oxford Brain Bank (REC 07/0606/85) was used with ethical approval as per Human Tissue Act 2006 guidelines (Table 1).

Tissue Sampling

For each MS and control case, brain weight was recorded before sampling. Motor cortical tissue blocks from the mesial frontal cortex were dissected given the relevance of cortical

pathology and lower limb motor system dysfunction to progressive MS. Motor cortical location was confirmed by the presence of Betz cells in cortical layer 5 identified by hematoxylin and eosin staining. By matching the location of motor cortex between cases, intracortical pathological heterogeneity was minimized.

Immunohistochemistry and Immunofluorescence

Formalin-fixed, paraffin-embedded tissue blocks were cut into 6- μm -thick adjacent sections and labeled with primary antibodies for myelin (proteolipid protein [PLP]; AbD Serotec, Kidlington, UK), fibrinogen (monoclonal; Abcam, Cambridge, UK; polyclonal; Dako, Carpinteria, CA), microglia/macrophages (ionized calcium-binding adaptor molecule 1 [Iba1]; Wako, Osaka, Japan), activated microglia/macrophages (CD68; Dako, Carpinteria, CA), neurons (neuronal nuclei [NeuN]; Millipore, Billerica, MA), and plasminogen activator inhibitor-1 (PAI-1; Abcam), using 3,3'-diaminobenzidine (DAB) immunohistochemistry, as previously described.²⁰ Sections were counterstained with hematoxylin. Fluorescent double labeling for fibrinogen (monoclonal) and each of neurons (NeuN), astrocytes (glial fibrillary acidic protein; Dako), and microglia/macrophages (Iba1) was also performed on a subset of progressive MS cases ($n = 3$) that demonstrated the greatest extent of intracellular fibrin(ogen) staining on DAB-immunolabeled sections to probe cellular localization of fibrinogen using established protocols.

Assessment of Motor Cortical Demyelination

Foci of cortical demyelination were defined by complete loss of myelin in PLP-stained sections. A quantitative measure of cortical lesion burden area was related to total cortical length, as previously described.²⁰

Assessment of Motor Cortical Fibrin(ogen) Deposition, Microglial/Macrophage Density, and Neuronal Density

Fibrin(ogen) deposition (as assessed with the monoclonal antibody), microglial/macrophage density, and neuronal density were quantified in MS cases and controls within consistently spaced trajectories perpendicular to the subpial surface of the cortex. Each trajectory consisted of two fields of view (FOVs) per cortical layer, with the exception of layer 3 which was sampled using four FOVs because of its relatively large size. Supragranular cortex was defined as cortical layers 1 to 3 and infragranular cortex was defined as cortical layers 4 to 6 (Fig 1). Photomicrographs of each FOV were acquired using Axio-Vision software. Approximately 2,500 FOVs were assessed for each antigen under study across MS and control cohorts ($>12,500$ FOVs total), which were then analyzed as outlined below. The distribution and extent of fibrin(ogen), as immunolabeled with the monoclonal antibody, was validated using an additional fibrinogen antibody that was polyclonal. Relationships between (monoclonal) fibrin(ogen) and demyelination, microglial/macrophage density, and neurons were explored.

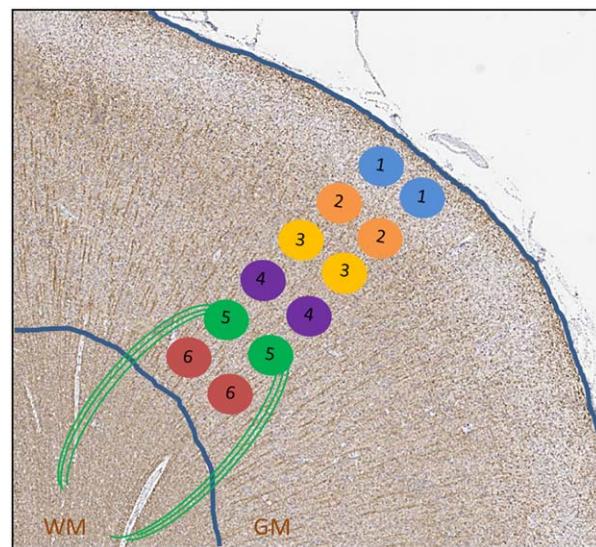


FIGURE 1: Assessment strategy of motor cortical pathology. Trajectories of analysis were spaced at systematic intervals perpendicular to the cortical surface. Two field of views (FOVs) (colored circles) in each cortical layer (numbered 1–6) were photographed at $400 \times$ magnification (four FOVs were imaged in layer 3 because of its relatively larger size). Approximately 2,500 images were acquired for each antibody under study. FOVs were analyzed as outlined. Supragranular and infragranular cortex were defined as layers 1–3 and layers 4–6, respectively. GM, gray matter; WM, white matter.

QUANTITATION OF FIBRIN(OGEN) DEPOSITION. Total fibrin(ogen) deposition was quantified in each FOV using a semiautomatic color-based extraction method and is reported as chromogen positive pixels/ mm^2 , as previously published.²⁰ On close inspection of the patterns of fibrinogen deposition (Fig 2), this method lacked the specificity needed to be able to quantify fibrin(ogen) deposition in separate cortical compartments, that is, extracellular spaces, cell bodies, and neuritic/glial processes. Therefore, a separate semiquantitative score of the severity of fibrin(ogen) deposition in each of the extracellular (Fig 3A), cell body (Fig 3B), and neuritic/glial process (Fig 3C) compartments in each FOV were used as follows: 0 (virtually absent), 1 (mild), 2 (moderate), or 3 (severe).

QUANTITATION OF MICROGLIAL/MACROPHAGE DENSITY. Microglial/macrophage density (Iba1^+ , CD68^+) was quantified in each FOV using semiautomatic color-based extraction and expressed as chromogen-positive pixels/ mm^2 .

QUANTITATION OF NEURONAL DENSITY. Neuronal density was quantified in each FOV by manually counting the number of NeuN $^+$ cells with a prominent nucleus and visible single nucleolus. Across the cohort, we quantified approximately 70,000 neurons, expressed as neurons/ mm^2 .

Assessment of Motor Cortical PAI-1

Motor cortical PAI-1 was assessed in all controls and in a subset of MS cases lying at the extremes of the extent of extracellular

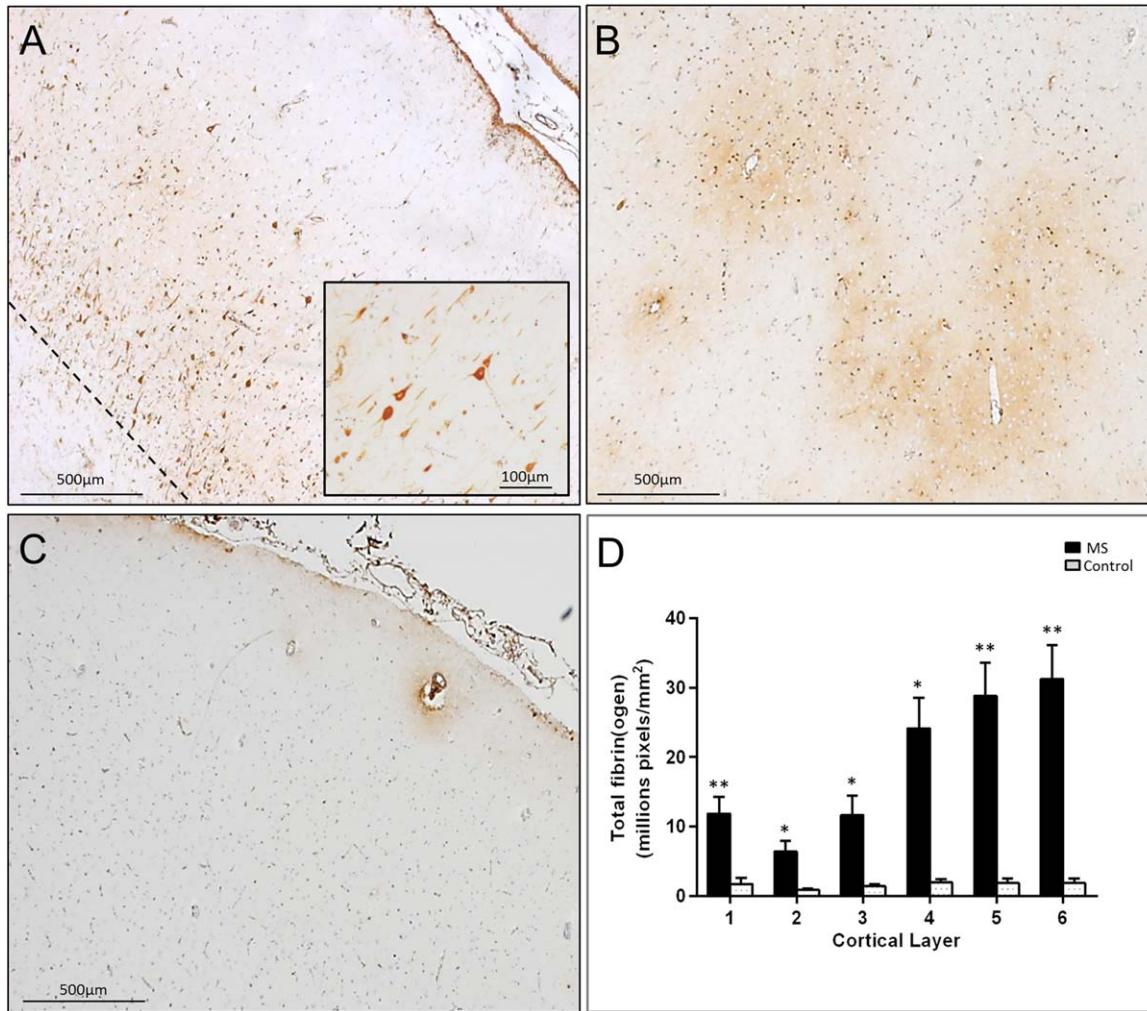


FIGURE 2: Fibrin(ogen) deposition in the MS motor cortex. Fibrin(ogen) was deposited diffusely in the MS motor cortex in both extracellular and intracellular spaces (A) and was commonly found to be in close proximity to blood vessels (B). Parenchymal fibrin(ogen) in control cases was typically confined to the perivasculation and subpial cortex (C). Quantitation of total fibrin(ogen) in MS cases and controls, using a semiautomatic color-based extraction algorithm, demonstrated more-severe deposition in MS throughout the cortical lamina (D). * $p < 0.05$; ** $p < 0.01$. MS = multiple sclerosis.

fibrin(ogen) deposition (ie, $n = 10$ MS cases with the lowest levels of extracellular fibrin(ogen) deposition; and $n = 10$ MS cases with the highest levels of extracellular fibrin(ogen) deposition) while controlling for age, duration of disease, sex, clinical course, brain weight, and postmortem (PM) interval (Table 2). FOVs were examined in trajectories that were consistently spaced and perpendicular to the subpial surface, as described for previous analyses. FOVs were assessed by a semiautomatic color-based extraction method, with data presented as chromogen-positive pixels/ mm^2 .

Statistical Analyses

Quantitative differences in continuous pathological variables between MS cases and controls was assessed using independent t tests where data were normally distributed and Mann-Whitney U tests where data were not normally distributed. Kruskal-Wallis tests were used to compare data within MS and control cases where independent variables had more than one value (ie,

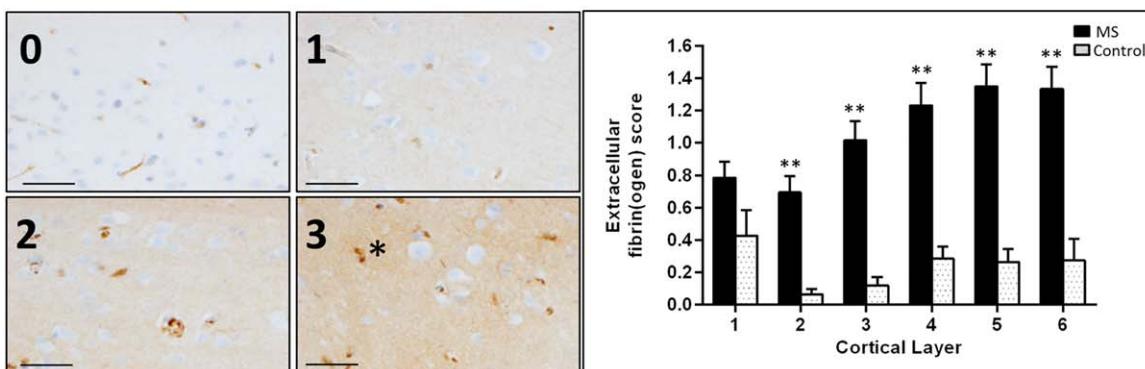
cortical layers 1–6) and data were not normally distributed. For comparisons of normal data within MS cases, regression models were fitted with age, sex, and postmortem interval as covariates. The relationships between continuous pathological variables were assessed using Spearman rank-correlation coefficients. Distribution of data was assessed analytically and graphically and transformed as required. Data are presented \pm standard error of the mean. Two-sided tests were used to test hypotheses and p values less than 0.05 were considered significant. Statistical analyses were done using SPSS (v22; SPSS, Inc., Chicago, IL) software.

Results

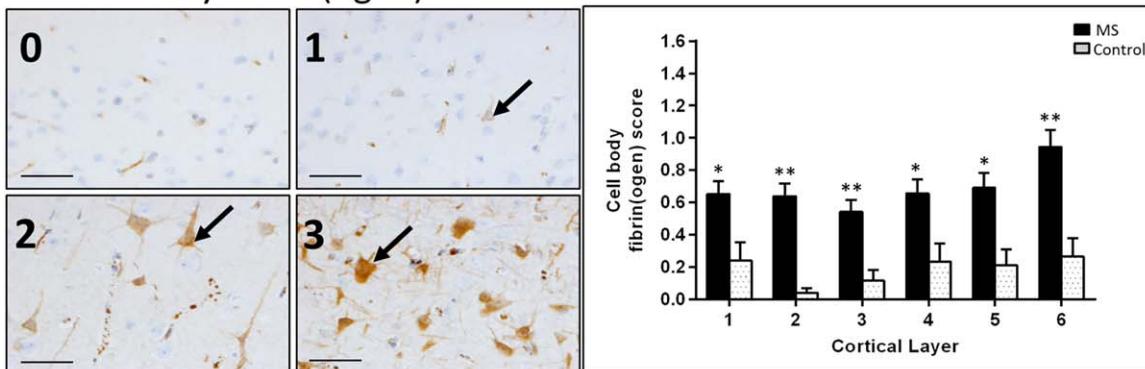
Demographic Features of MS Cohort

Clinical details of the cases used in this study are found in Table 1. In summary, the majority of MS cases were clinically classified as secondary progressive (38 of

A. Extracellular Fibrin(ogen)



B. Cell Body Fibrin(ogen)



C. Neuritic/Glial-process Fibrin(ogen)

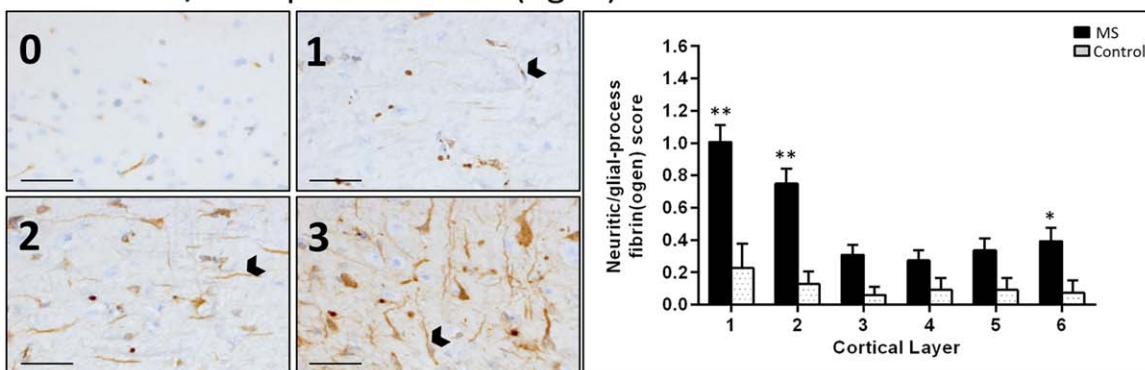


FIGURE 3: Semiquantitative scoring of fibrin(ogen) deposition. Automated quantification of total fibrin(ogen) lacked the specificity to enable quantitation of deposition in separate cortical compartments. To circumvent this issue, we devised a semiquantitative score for the severity of fibrin(ogen) in each compartment, that is, extracellular, cell body (black arrows), and neurites/glia processes (arrow heads). Star denotes vascular fibrin(ogen). For each FOV analyzed, fibrin(ogen) deposited in each compartment (extracellular, cell bodies, and neurites/glia processes) was scored on a scale of 0 (no deposition), 1 (mild deposition), 2 (moderate deposition), and 3 (severe deposition). Extracellular fibrin(ogen) was greater in MS throughout the cortical lamina, but was concentrated in the infragranular cortex (A). Cell body fibrin(ogen) was also greater in MS, but showed a consistent distribution throughout the cortical lamina (B). Neuritic/glia process fibrin(ogen) was greater in MS in cortical layers 1 and 2, as well as layer 6 (C). * p <0.05; ** p <0.01. FOV = field of view; MS = multiple sclerosis.

47 = 80.9%) with 4 of 47 (8.5%) being primary progressive and only 1 of 47 (2.1%) being relapsing-remitting. The clinical course was unknown in 4 (8.5%) cases. All

cortical plaques were chronic inactive save for a leuko-cortical lesion, which demonstrated microglial/macrophage activation at the lesion border.

TABLE 2. Details of MS and control cohort used in cortical PAI-1 analysis

	MS: Low EC Fib (n = 10)	MS: High EC Fib (n = 10)
Age of death (y)	65.7 (55–82)	65.9 (55–82)
Duration of disease (y)	33.5 (19–50)	31.1 (12–50)
Sex	8 F, 2 M	8 F, 2 M
Clinical course	SPMS = 10	SPMS = 10
Brain weight (g)	1,195 (1,000–1,380)	1,162 (1,045–1,370)
PM interval (h)	16.2 (7–25)	15.7 (7–21)

The extent of PAI-1 deposition was assessed in a subset of MS cases selected based on the extent of extracellular fibrin(ogen) deposition, while controlling for age, duration of disease, sex, clinical course, brain weight, and PM interval. In total, n = 10 cases were analyzed that had high levels of extracellular fibrin(ogen) deposition, and n = 10 cases were analyzed with low levels of extracellular fibrin(ogen). Available control cases (n = 10) were also analyzed.

PM = postmortem; MS = multiple sclerosis; EC Fib = extracellular fibrin(ogen); F = female; M = male; SPMS = secondary progressive MS.

Fibrin(ogen) Deposition: MS vs controls

DISTRIBUTION AND EXTENT OF FIBRIN(OGEN) DEPOSITION. Total fibrin(ogen) deposition was observed to a greater extent in MS motor cortex when compared to control cases (MS, 19.05 ± 3 million pixels/mm² vs control, 1.64 ± 0.41 million pixels/mm²; $p = 0.003$). The extent of total fibrin(ogen) deposition between cortical layers was significantly different within MS cases ($p = 0.001$), but not controls ($p = 0.701$; Fig 2). In MS, total fibrin(ogen) was greater in deep infragranular cortical layers compared to more superficial ones (layers 5 and 6 vs layer 2; Dunn's nonparametric comparison, $p < 0.05$).

Fibrin(ogen) deposition was frequently observed to respect the boundary between WM and gray matter (GM). Indeed, extensive infragranular cortical deposition was often contrasted sharply with negative WM immediately adjacent. Fibrin(ogen) was deposited diffusely in extracellular spaces as well as cell bodies and neurites/glial processes. The extent and distribution of fibrin(ogen) deposition in these specific motor cortical compartments is outlined below.

EXTRACELLULAR FIBRIN(OGEN). The extent of extracellular deposition in MS was significantly greater than in controls (MS, 1.07 ± 0.12 vs control, 0.24 ± 0.07 ; $p = 0.001$). The extent of extracellular fibrin(ogen) deposition differed significantly between cortical layers in MS cases ($p < 0.001$), but not controls ($p = 0.189$; Fig 3A). In MS, it was again deeper infragranular cortical layers that demonstrated more extracellular fibrin(ogen) deposition (layers 5 and 6 vs layer 2; Dunn's nonparametric comparison, $p < 0.01$).

CELL BODY FIBRIN(OGEN). The extent of cell body deposition was significantly greater in MS cases than in controls (MS, 0.69 ± 0.08 vs control, 0.19 ± 0.06 ; $p = 0.003$). Unlike extracellular fibrin(ogen), the extent of fibrin(ogen) within cell bodies did not differ between cortical layers within MS cases ($p = 0.157$) or controls ($p = 0.517$; Fig 3B).

NEURITIC/GLIAL-PROCESS FIBRIN(OGEN). The extent of neuritic/glial-process fibrin(ogen) was significantly greater in MS cases than controls (MS, 0.51 ± 0.07 vs control, 0.11 ± 0.07 ; $p = 0.004$). The extent of neuritic/glial-process fibrin(ogen) deposition was significantly different between cortical layers within MS cases ($p < 0.001$), but not controls ($p = 0.733$; Fig 3C). In MS, fibrin(ogen) in neuritic/glial processes was greater in superficial cortical layers compared to deeper ones (ie, layers 1 and 2 vs layers 4–6; Dunn's nonparametric comparison, $p < 0.05$; Fig 4A).

Total, extracellular, cell body, and neuritic/glial-process fibrin(ogen) deposition in each motor cortical layer (layers 1–6) in MS compared to control is summarized in Supplementary Table 1.

Double-Labeling Immunofluorescence With Fibrin(ogen) and Cell Markers

Fibrin(ogen) was found to affect different cell types using DAB immunohistochemistry (Fig 4A); therefore, double-labelling immunofluorescence with various cell markers was performed (Fig 4B–D). Figure 4 illustrates the key findings highlighted in the quantitative data derived from the >7,500 images analyzed and presented in Figure 3. Neuritic/glial-process fibrin(ogen) immunolabeling was most prominent in cortical layers 1 and 2. Astrocytic processes often contained dense fibrin(ogen) deposits in these layers of the subpial zone (Fig 4B).

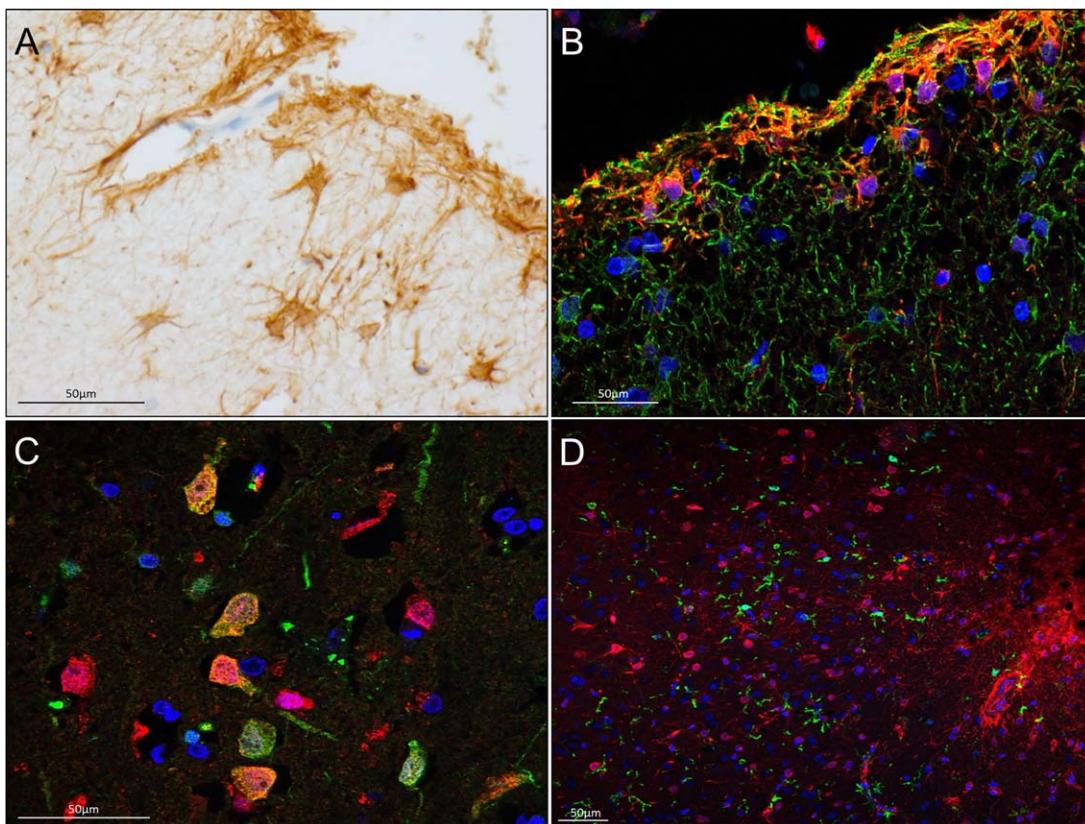


FIGURE 4: Fluorescent double labeling of fibrin(ogen) with astrocytes, microglia/macrophages, and neurons to demonstrate cellular localization from progressive MS cases ($n = 3$) with greatest intracellular staining on DAB-immunolabeled sections. Images shown are representative of pathology observed within the selected cases and are consistent with the observations made with DAB-labeled sections in the entire MS cohort. Fibrin(ogen) was found to affect different cell types using DAB immunohistochemistry (A). Because neuritic/glial-process fibrin(ogen) immunolabeling was more prominent in cortical layers 1 and 2, we herein provide evidence of representative neuritic/glial-process fibrin(ogen) (red) cellular uptake within astrocytes (green) in the subpial glia limitans (B). Given that cell body fibrin(ogen) immunolabeling was present in all cortical layers, we present a representative view of fibrin(ogen) (red) and neuronal (NeuN, green) cellular uptake in the infragranular cortex (C). In contrast, an assessment of fibrin(ogen) (red) and Iba1⁺ microglia/macrophages (green) did not provide evidence of cellular uptake (D). DAB = 3,3'-diaminobenzidine; Iba1 = ionized calcium-binding adaptor molecule 1; MS = multiple sclerosis; NeuN = neuronal nuclei.

Some neuritic/glial-process fibrin(ogen) was present outside of astrocytes, suggesting uptake within the neuritic compartment. Cell body fibrin(ogen) immunolabeling was present in all cortical layers (Fig 2A) and found within astrocytes and neurons (Fig 4B,C). In contrast, an assessment of fibrin(ogen) and Iba1⁺ microglia/macrophages did not provide evidence of cellular staining (Fig 4D). In control cases, cell body and neuritic-glial process fibrin(ogen) deposition was scant, but, when present, was detected within neurons and glia.

Validation of (Monoclonal) Fibrin(ogen) Findings With Additional (Polyclonal) Fibrin (Ogen) Antibody

To verify the specificity of the (monoclonal) fibrin(ogen) findings, we replicated our semiquantitative scores with a second, polyclonal, antibody targeting fibrin(ogen). There was a significant correlation of fibrin(ogen) scores between

antibodies in the extracellular (Spearman $r = 0.515$; $p < 0.001$), cell body ($r = 0.809$; $p < 0.001$), and neuritic/glial-process ($r = 0.76$; $p < 0.001$) compartments in MS cases and controls. The distribution of fibrin(ogen), as assessed by this second polyclonal fibrin(ogen) antibody, was consistent with the results of our monoclonal antibody (data not shown).

The polyclonal fibrin(ogen) antibody demonstrated a greater level of background staining relative to the monoclonal fibrin(ogen) antibody. As such, further analyses between fibrin(ogen) and other metrics of cortical pathology were restricted to the more specific monoclonal fibrin(ogen) antibody.

Relationships Between MS Motor Cortical Pathology and Fibrin(ogen) Deposition

Quantitative measures of brain weight, demyelination, microglial/macrophage density, and neuronal density

were obtained and related to the extent of fibrin(ogen) deposition as outlined below.

BRAIN WEIGHT AND FIBRIN(OGEN) DEPOSITION. No relationship was observed between brain weight and total fibrin(ogen) deposition ($r = -0.037$; $p = 0.806$). Similarly, no relationships were observed between brain weight and either extracellular ($r = 0.017$; $p = 0.909$), cell body ($r = 0.073$; $p = 0.628$), or neuritic/glial-process fibrin(ogen) deposition ($r = 0.154$; $p = 0.307$). No relationships between brain weight and fibrin(ogen) deposition were detected in controls.

DEMYELINATION AND FIBRIN(OGEN) DEPOSITION. No relationship was observed between overall cortical lesional burden and total fibrin(ogen) deposition ($r = 0.140$; $p = 0.349$). Furthermore, no relationships were observed between cortical lesional burden and either extracellular ($r = 0.142$; $p = 0.342$), cell body ($r = -0.001$; $p = 0.993$), or neuritic/glial-process fibrin(ogen) deposition ($r = -0.005$; $p = 0.973$) irrespective of cortical layer.

MICROGLIAL/MACROPHAGE DENSITY AND FIBRIN(OGEN) DEPOSITION. No relationship was observed between Iba1⁺ inflammation and total fibrin(ogen) deposition ($r = -0.145$; $p = 0.337$) nor CD68⁺ inflammation and total fibrin(ogen) deposition ($r = 0.018$; $p = 0.902$). In addition, no relationships were observed between microglial/macrophage density and either extracellular (Iba1⁺: $r = -0.062$; $p = 0.681$; CD68⁺: $r = 0.104$; $p = 0.485$), cell body (Iba1⁺: $r = -0.220$; $p = 0.142$; CD68⁺: $r = -0.039$; $p = 0.794$), or neuritic/glial process-associated fibrin(ogen) deposition (Iba1⁺: $r = -0.258$; $p = 0.084$; CD68⁺: $r = -0.097$; $p = 0.516$), irrespective of cortical layer or demyelination. Similar to MS cases, no relationships between microglial/macrophage density and fibrin(ogen) deposition were observed in controls.

NEURONAL DENSITY AND FIBRIN(OGEN) DEPOSITION. Although neuronal density was not statistically different between MS and controls, striking differences emerged within MS cases when considering the extent of fibrin(ogen). Neuronal density inversely correlated with total fibrin(ogen) deposition ($r = -0.401$; $p = 0.006$). This relationship was restricted to extracellular fibrin(ogen) deposition (extracellular, $r = -0.355$; $p = 0.015$; cell body, $r = -0.284$; $p = 0.056$; neuritic/glial process, $r = -0.234$; $p = 0.118$). No relationship between fibrin(ogen) deposition and neuronal density was observed in control cases (total, $r = -0.250$; $p = 0.589$; extracellular, $r = -0.679$; $p = 0.094$; cell body, $r = -0.222$; $p = 0.632$; neuritic/glial process, $r = 0.148$; $p = 0.751$).

To further assess the impact of extracellular fibrin(ogen) deposition on neuronal density in the motor cortex, MS cases were divided into high and low fibrin(ogen) groups based on the median value for extracellular fibrin(ogen) (EC fib) in the relevant anatomic area under study. MS cases with an (EC fib) score equal to the median were excluded from the analysis to ensure an equal number of cases in each group.

MS cases with a high burden of EC fib showed significantly reduced neuronal density when compared with MS cases with low fibrin(ogen), corrected for age, sex, and PM interval (high EC fib, 372 ± 19 neurons/ mm^2 vs low EC fib, 454 ± 27 neurons/ mm^2 ; $p = 0.017$). These findings were limited to the infragranular cortex where fibrin(ogen) deposition was more severe (high infragranular EC fib, 387 ± 24 infragranular neurons/ mm^2 vs low infragranular EC fib, 488 ± 33 infragranular neurons/

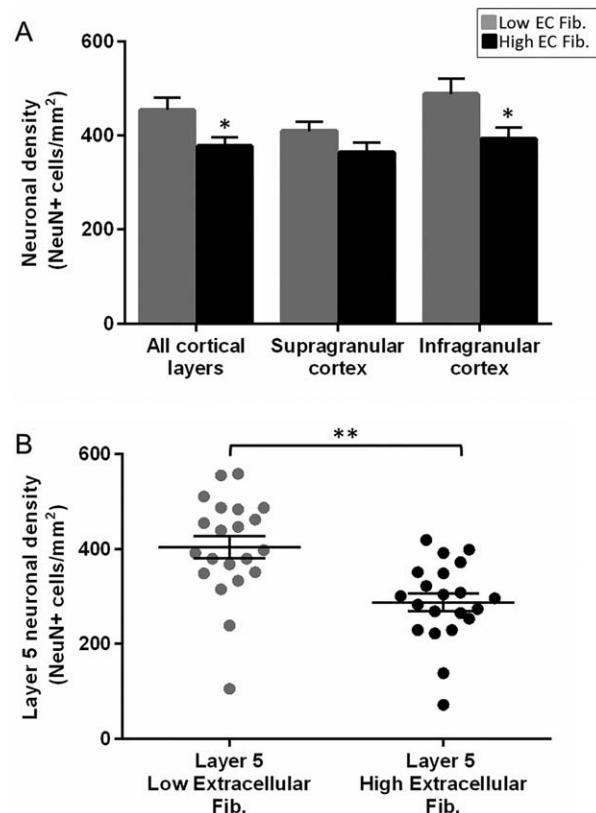


FIGURE 5: Association of fibrin(ogen) deposition with motor cortical neuronal density. MS cases were divided into high and low extracellular fibrin(ogen) groups based on the median extracellular score for the MS cohort. A significant difference in neuronal density was observed between high and low extracellular fibrin(ogen) MS groups when all cortical layers were considered, with the effect being driven by differences in infragranular cortex (A). Further assessment of layer 5 showed a significant reduction of neurons in the MS group with high levels of extracellular fibrin(ogen) (B). * $p < 0.05$; ** $p < 0.01$. MS = multiple sclerosis; NeuN = neuronal nuclei.

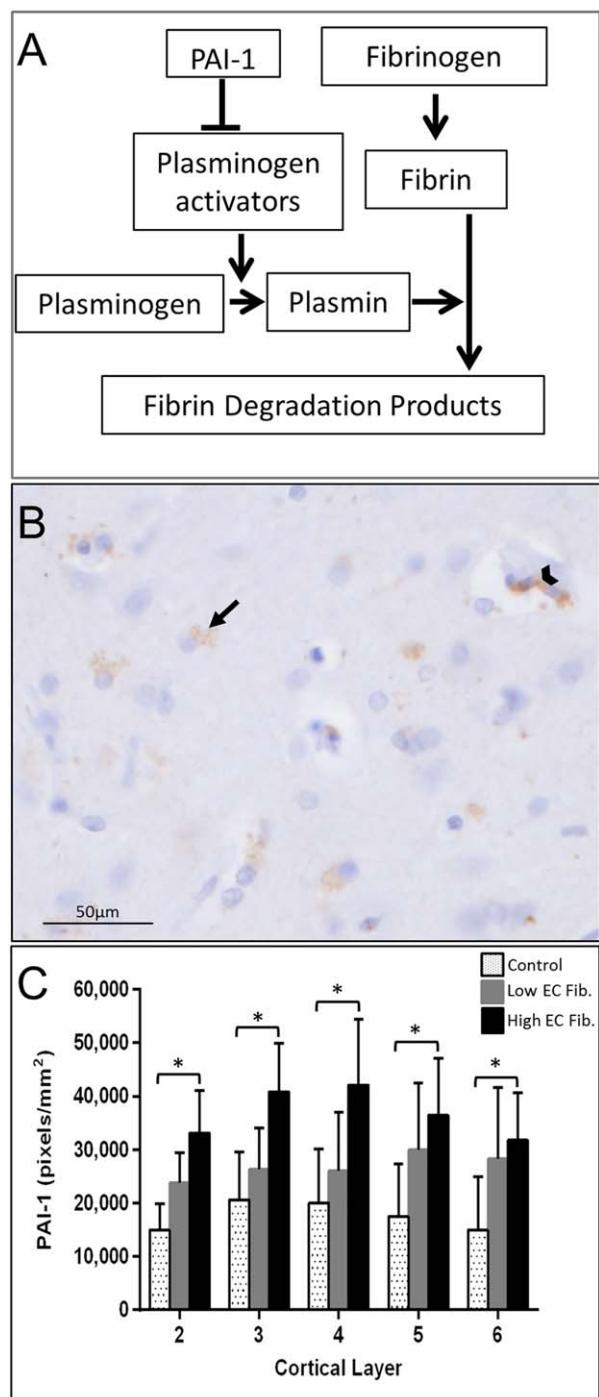


FIGURE 6: Motor cortical PAI-1. Fibrinolysis is under the close control of PAI-1, through its inhibition of plasminogen activators. A greater expression of PAI-1 would therefore be expected to coincide with greater deposition of fibrin(ogen) (A). PAI-1 was expressed diffusely throughout the cortex, with both intracellular (arrow head) and extracellular deposition (arrow) observed (B). In all cortical layers assessed, a significant difference was detected in the expression of PAI-1 in MS cases with the most extracellular fibrin(ogen) deposition compared to controls. Layer 1 was not assessed because of consistent artefactual immunolabeling at the section edge (C). * $p < 0.05$; ** $p < 0.01$. EC Fib = extracellular fibrin(ogen); PAI-1 = plasminogen activator inhibitor-1.

mm^2 ; $p = 0.039$). No significant difference in neuronal density was observed in supragranular cortical layers according to EC fib deposition (high supragranular EC fib, 362 ± 21 supragranular neurons/ mm^2 vs low supragranular EC fib, 409 ± 20 supragranular neurons/ mm^2 ; $p = 0.235$; Fig 5A). Given the importance of layer 5 neurons for motor function, it is intriguing that layer 5 neuronal density was also significantly reduced in the high fibrin(ogen) group (high layer 5 EC fib, 288 ± 18 layer 5 neurons/ mm^2 vs low layer 5 EC fib, 404 ± 23 layer 5 neurons/ mm^2 ; $p = 0.001$; Fig 5B).

Assessment of Motor Cortical PAI-1 in MS Cases and Controls

PAI-1 was similarly distributed throughout all cortical layers in MS cases and control. However, the extent of PAI-1 expression differed significantly between high fibrin(ogen) MS cases and controls in each cortical layer assessed (layer 2: MS, $33,147 \pm 7,881$ vs control, $14,990 \pm 4,862$ pixels/ mm^2 ; $p = 0.043$; layer 3: MS, $40,839 \pm 9,022$ vs control, $20,626 \pm 8,954$ pixels/ mm^2 ; $p = 0.035$; layer 4: MS, $42,148 \pm 12,299$ vs control, $20,038 \pm 10,103$ pixels/ mm^2 ; $p = 0.035$; layer 5: MS, $36,482 \pm 10,650$ vs control, $17,539 \pm 9,752$ pixels/ mm^2 ; $p = 0.035$; layer 6: MS, $31,766 \pm 8,874$ vs control, $14,986 \pm 9,00$ pixels/ mm^2 ; $p = 0.035$; Fig 6). Lesions had no influence on the extent of PAI-1 expression when compared to MS NAGM (lesional PAI-1, $31,957 \pm 8,277$ vs NAGM PAI-1, $26,828 \pm 14,339$ pixels/ mm^2 ; $p = 0.658$).

Discussion

We provide unequivocal evidence that fibrin(ogen) deposition in the MS cortex is frequent and extensive, and relates to the extent of neurodegeneration in progressive disease. Given the importance of cortical pathology to disease progression, the potential clinical relevance of these findings is substantial. Future investigations are now needed to elucidate the role of cortical fibrin(ogen) on clinical outcome and the impact of its elimination.

Our study identifies fibrin(ogen) deposition as a novel component of MS cortical pathology in the progressive phase of the disease. In early MS, BBB disruption and fibrin deposition are among the first pathological events and are linked to inflammation and demyelination in the WM.²¹ We provide an important extension to these findings by demonstrating that fibrin(ogen) deposition is a consistent feature of MS pathology in the cortex of human postmortem tissue at advanced disease stages (ie, end-stage progressive disease), wherein active demyelination is sparse.

We found markedly elevated levels of fibrin(ogen) in progressive MS cases compared to controls. Fibrin(ogen) was located in both the extracellular space and within cells and intracellular processes with varying distributions. Extracellular fibrin(ogen) deposition was predominantly located in the deeper infragranular cortical layers. A perivascular distribution was common, though more extensive staining beyond vessels was also frequently observed. In contrast, deposition of fibrin(ogen) within neuritic/glial processes was observed in superficial cortical layers where neuritic and astrocytic labeling was observed. Intracellular fibrin(ogen) was found within neurons and astrocytes throughout all cortical layers. Heterogeneity of the vasculature, glial and neuronal density, and variability of extracellular matrix protein composition in the different cortical layers could influence the deposition and/or clearance of fibrin(ogen), and may provide an explanation for the differential distribution of fibrin(ogen) observed in our series.^{22,23} Regardless, our findings challenge the idea that large-molecular-weight serum proteins are not deposited in the progressive MS cortex.⁵ The explanation for this inconsistency is not clear, but may relate to differences in tissue antigenicity that relate to fixation and/or processing protocols employed by different institutions.

The presence of fibrin(ogen) in the progressive MS cortex raises the question about its provenance. Fibrin(ogen) accumulation may be caused by increased deposition (ie, extravasation), impaired clearance, or both. The possibility that BBB disruption leads to increased fibrin(ogen) extravasation in the progressive MS cortex is supported by Leech et al, who demonstrated endothelial tight junction abnormalities in cortical GM in secondary progressive MS cases.⁴ Together with our own observations, these findings suggest that BBB disruption may be a feature of MS cortical pathology in the progressive phase. The deposition of other high-molecular-weight proteins in the cortex of progressive MS cases, such as immunoglobulin and albumin, remains controversial.⁵ This may be explained by the fact that most large serum proteins do not get converted to an insoluble matrix like fibrinogen does to fibrin, precluding accurate assessment in progressive MS. Conversion of soluble fibrinogen to insoluble fibrin in the brain requires activation of coagulation. Indeed, increased coagulation is reported in EAE and human MS lesions.^{15,24}

Impaired fibrin(ogen) clearance could also explain the excess fibrin(ogen) burden seen in the cortex of our MS cohort. Impaired clearance may be a late consequence of BBB disruption in the early phase of the disease. Several lines of evidence suggest that the coagulation pathway is perturbed in MS. Proteomic assessment of active plaques

has shown specific dysregulation of coagulation proteins,¹⁵ and PAI-1 is upregulated in white matter lesions, where it has been associated with a failure of fibrinolysis.¹⁸ Interestingly, PAI-1 is also upregulated in CSF and blood serum of MS patients, and polymorphisms of the PAI-1 gene have recently been linked to disease risk, providing further support to suboptimal clearance of coagulation proteins in MS.^{16,17,19} Our finding of significant upregulation of PAI-1 in progressive MS cases in which fibrin(ogen) deposition is most severe implies that dysregulation of PAI-1 might impair the clearance of fibrin(ogen) and allow its pathological accumulation in late-stage MS. Other possible sources of fibrin(ogen) include synthesis by resident neurons and glia and/or retrograde transport in damaged axons in WM areas exposed to accumulated serum proteins.²⁵ Experimental evidence for these alternative cellular sources of fibrin(ogen) is lacking.

Irrespective of its provenance, fibrin(ogen) deposition likely has deleterious downstream consequences. The extent of EC fib in our cohort significantly inversely related to neuronal density, suggesting a possible role of this protein in MS cortical pathology in progressive disease. Whereas previous studies have demonstrated neuronal loss in the MS cortex, the reduction is typically slight and predominantly in superficial cortical layers where it is topographically associated with tertiary lymphoid-like aggregates in the meninges.^{3,26} In our cohort, the inverse association of EC fib deposition with neuronal density was most exaggerated in the infragranular cortex, where fibrin(ogen) deposition was most severe. Specific loss of layer 5 projection neurons also appeared to segregate with the extent of EC fib deposition. These findings suggest that the cortical deposition of fibrin(ogen) could be an important driver of disease progression, a finding supported by a wealth of experimental data.⁶

A relationship between fibrin(ogen) and neuroaxonal demise has been shown in disease-relevant animal models. In EAE, fibrin(ogen) activates microglia, which, in turn, mediates axonal damage even before onset of clinical signs.⁸ The manipulation of the coagulation cascade to reduce CNS fibrin(ogen) deposition or inhibition of the interaction of fibrinogen with CD11b/CD18 in these animal models leads to clinical improvement.^{10,11,15,27} Human in vitro experiments have confirmed that fibrinogen mediates innate-immune responses through Toll-like receptor-4 expressed by interferon (IFN)- γ activated macrophages, with secretion of neurotoxic products.²⁸ IFN- γ can diffuse from the subarachnoid space into the subpial parenchyma.²⁹ Given that the perivascular space is continuous with the subarachnoid space, microglia/macrophages activated by IFN- γ may create a neurotoxic inflammatory milieu in the presence

of fibrin(ogen), even in cortical layers distant from the subpial surface. Although we could not demonstrate a relationship between fibrin(ogen) deposition and innate inflammation in our cohort, such relationships may be difficult to discern given that genetic factors, disease evolution, and aging likely impact the recognized phenotypic heterogeneity of microglial/macrophage inflammation.^{20,30,31} Furthermore, interactions of inflammatory mediators are likely short-lived and therefore impervious to detection in an autopsy cohort. Importantly, one must also consider ascertainment bias in that the significant majority of the MS cases included in the current study had late-stage progressive disease potentially confounding detection of a link between fibrin(ogen) deposition and microglial/macrophage inflammation, and the cohort size of our study may have been insufficient to detect such relationships, should they indeed exist in late-stage progressive MS. In contrast, the permanency of neuronal death allowed us to detect an inverse relationship between fibrin(ogen) deposition and neuronal density.

Fibrin(ogen), as a potent activator of microglial/macrophage inflammation, might be a novel therapeutic target that could limit ongoing maladaptive innate immune responses in advanced progressive MS.⁶ Activation of cortical microglia/macrophages by fibrin(ogen) might be an early interaction setting the stage for diffuse neuroaxonal loss characteristic of progressive disease through several potential mechanisms, including the generation of reactive oxygen species,³² glutamate excitotoxicity,³³ and deposition of complement as recently described.³⁴

In summary, we introduce cortical fibrin(ogen) deposition as a novel component of cortical pathology in progressive MS. We show that fibrin(ogen) is extensively deposited in the progressive MS motor cortex where regulation of fibrinolysis appears perturbed. The inverse relationship between neuronal density and fibrin(ogen) deposition in the MS motor cortex highlights the functional relevance of this protein in disease progression. Future studies aimed at elucidating the provenance and putative neuroaxonal toxicity of fibrin(ogen), and its potential impact on clinical disability in progressive MS, are urgently needed.

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Author Contributions

R.L.Y., M.M.E., and G.C.D. conceived and designed the study. R.L.Y. acquired the data. R.L.Y. and R.P. carried out statistical analyses and interpretation of the data. R.L.Y., J.P., M.M.E., B.J., and G.D. drafted the manuscript and/or figures.

Potential Conflicts of Interest

Nothing to report.

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