Age and maturation stage linked consequences of fibrinogen on human oligodendroglia
Running Title: Fibrinogen effect on human oligodendroglia

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Main Points

- Fibrinogen enhances myelination by mature oligodendrocytes, but prevents immature lineage cells from differentiating and myelinating.
- Fibrinogen deposition occurs on oligodendroglia in multiple sclerosis and amyotrophic lateral sclerosis.

Key Words: Oligodendrocytes, oligodendroglial precursors, fibrinogen, myelination, fibrinogen deposition in human central nervous system

Abstract

Our aim was to investigate the effect of a systemically circulating molecule fibrinogen, which is abnormally present in the central nervous system (CNS) as a result of blood-brain barrier breakdown, on human oligodendrocyte lineage cells.

Mature oligodendrocytes and late progenitors were derived from brain tissue from pediatric and adult donors. Early progenitors were generated from human induced pluripotent stem cells. Following fibrinogen addition to these cultures, cell viability and functional capacity was evaluated. Downstream signalling following fibrinogen exposure was confirmed by immunofluorescence microscopy and bulk RNA sequencing. In situ immunofluorescence was performed using anti-fibrinogen and anti-SOX10 antibodies to determine if fibrinogen is deposited on oligodendroglia in multiple sclerosis (MS) and amyotrophic lateral sclerosis (ALS), disorders known to have fibrinogen in the CNS.

In response to in vitro exposure to fibrinogen, mature oligodendrocytes from adults showed increased ensheathment capacity and upregulation of lipid synthesis, whereas pediatric-age late oligodendrocyte precursors showed a decrease. Functional effects were inhibited by the bone morphogenic protein (BMP)-pathway inhibitor, DHM1. Early precursors were unable to differentiate but expressed astrocytic markers and increased proliferation. Fibrinogen-exposed cells show BMP signalling, more prominently in mature oligodendrocytes. In situ studies showed fibrinogen on SOX10-positive oligodendroglia in MS, both in plaques and normal-appearing white matter, and white matter in ALS.

We demonstrate that fibrinogen has distinct functional consequences dependent on cell lineage stage. Our findings derived using human OL lineage cells suggest fibrinogen may benefit myelin maintenance by mature oligodendrocytes, while preventing earlier lineage cells from differentiating and remyelinating.

1. Introduction

Potential contributors to the injury and repair responses of oligodendroglia (OLs) are blood-derived factors that may access the CNS due to the breakdown of the blood-brain barrier (BBB) (1). Fibrinogen is a circulating blood clotting factor composed of polypeptide chains which have polymerization sites for clot formation (2), and various domains known to be involved in stimulation of multiple cell types (3). Fibrinogen has been described in multiple sclerosis (MS) and detected in the borders of chronic active white matter plaques (4) and leakage into diffusely abnormal white matter (5) and normal-appearing white matter (6). It has also been found in MS motor cortex, where its presence significantly correlated with reduced neuronal density (7), implicating its direct role in cellular damage. In MS, fibrinogen has been found at the cellular level on astrocytes (8, 9), axons (5), and macrophages (8), but has not been previously demonstrated on oligodendroglia. Moreover, plasma levels of fibrinogen are elevated in MS relapses (10); its role in MS pathogenesis has been linked to its function in the coagulation cascade and as a pro-inflammatory agent (11). The role of fibrinogen in MS and autoimmune disorders of the CNS has been extensively studied by Akassoglou and colleagues (12), and experimental studies in her laboratory have shown that fibrinogen can inhibit the differentiation of rodent OPCs into myelinating oligodendrocytes, primarily mediated via bone morphogenetic protein (BMP) signaling (13). While fibrinogen is thought to have an important role in neuroinflammation (12, 14) and the pathogenesis of MS (11, 15-18), it could also be relevant in the pathogenesis of other diseases with BBB, blood-spinal cord barrier, or blood-cerebrospinal fluid barrier breakdown (such as primary and secondary CNS tumors, infarcts, hemorrhages, trauma and infectious processes, to mention but a few) and even neurodegenerative conditions, such as Alzheimer's disease, Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis (ALS), where fibrinogen has been found to be deposited in the CNS and has been thought to be important in their pathogenesis (19).

The objectives of our study were to extend insights into the direct effects of fibrinogen on human OLs in relation to age and lineage stage. For our in vitro studies we employed primary human mature OLs (O4+A2B5-) and late lineage cells (O4+A2B5+) (20, 21) derived from surgically resected tissue samples together with early-stage progenitor cells derived from human induced pluripotential stem cells (iPSCs), as such primary cells cannot currently be derived from available human CNS sources. Primary cell studies were also considered in context of the donor age, as we previously reported that ensheathment capacity, as determined using cultures with synthetic nanofibers, and susceptibility to injury were donor age dependent (22). For iPSC studies, we also employed a previously characterized line expressing a reporter gene under the SOX10 promoter, where the degree of expression correlated with the differentiation stage of the cells (23). We documented that fibrinogen was deposited on OL lineage cells *in situ* in MS and ALS tissue samples.

2. Materials and Methods

This study was ethically approved by the Institutional Review Board of the McGill University Faculty of Medicine and Health Sciences (A09-M72-22B [22-08-053]).

Antibodies

Antibodies used in this study are listed in Table S3.

Human primary cell isolation and culture

Studies were conducted on OLs derived from pediatric (2.5 -18 years) and adult (26 - 65 years) tissue samples collected from patients undergoing surgery. The use of adult tissues was approved by the Montreal Neurological Institute Neurosciences Research Ethics Board and the use of paediatric tissues by the Montreal Children's Hospital Research Ethics Board. As previously described (21, 22), samples were dissociated by trypsin digestion and subjected to Percoll gradient (Sigma, Oakville, ON) separation. To enrich progenitor populations, MACS magnetic beads selection against the cell surface marker A2B5 (Miltenyi Biotec, Auburn, CA) was performed following cell isolation, and positive and negative fractions were cultured as described previously (21, 22) . Cells were treated with plasminogen-depleted fibrinogen (Millipore Sigma, Burlington MA, 341578, 80ug/ml to 4mg/ml) for 6 days. Cells were stained live for O4 (1:200, R&D systems, Oakville, ON) and the cell viability was determined by live staining with propidium iodide (PI, 1:200, Thermo Fisher Scientific, Mississauga, ON).

Our previous detailed flow cytometry based analysis of the adult and fetal cell populations (24) indicated that of the oligodendroglial (OL) population isolated from the adult brain 5-10% of the cells were recognized by the A2B5 antibody (referred to as A2B5+ cells), as also previously reported by others (25), and are considered late progenitor cells; 85-90% express detectible levels of O4 but none express myelin oligodendrocyte glycoprotein (MOG). Of the A2B5- cells, ~85% express both O4 and MOG. These cells do not express platelet derived growth factor receptor alpha (PDGFR□).

Nanofiber ensheathment cultures

Aliquots of A2B5+ and A2B5- cells were plated in multi-well aligned poly-L lactide nanofiber plates (The Electrospinning Company Ltd., Didcot, Oxfordshire, UK) (10,000 cells per microwell) and treated with plasminogen-depleted fibrinogen (2.5 mg/ml) in DMEM/F12+ N1 medium supplemented with B27 (Life Technologies, Grand Island, NY) and T3 (Sigma-Aldrich, Oakville, ON) for two weeks. Cell cultures were immunolabelled with O4 antibody diluted 1:200 (R&D Systems, Oakville, ON) for one hour, followed by corresponding secondary antibodies (1:500) conjugated with Alexa Fluor 647 (Thermo Fisher Scientific, Mississauga, ON) for two hours. To determine the percentage of O4+ cells ensheathing nanofibers in a culture, a blinded rater counted individual O4+ cells whose nanofibre-ensheathing processes could be identified as being connected to their cell bodies identified by Hoechst 33342 (Thermo Fisher Scientific, Mississauga, ON) staining of nuclei. Ensheathed processes were recognized by their straight alignment with underlying nanofibers and increased thickness of the segments (Figure 1F). Cells were assigned as ensheathing (one or more segments) or not and counted by the Plugin Cell Counter ImageJ program.

Human iPSC culture and OPC differentiation

iPSC-derived OL lineage cells were differentiated according to a recently published protocol (23). The use of iPSCs and stem cells in this research was approved by the McGill University Health Centre Research Ethics Board (DURCAN_IPSC/2019–5374). Briefly, from healthy control iPSC lines as well as the SOX10-mOrange reporter line (23) generated at the Montreal Neurological Institute's (MNI) Early Drug Discovery Unit (EDDU) (Table S2). According to a previously published protocol (23), early OL-lineage progenitors were generated, and plated on

poly-L-ornithine (Sigma-Aldrich, Oakville ON)/ laminin (Invitrogen, Waltham MA) coated culture vessels. Post day 75 *in vitro*, cells are used for up to a maximum of four passages, and differentiated to reach a majority of OL-lineage cells at a total of 21 days. As regards the composition of iPSC derived cells, at post day 75 in vitro (oligodendrocyte precursor cell [OPC] phase) ~50% of the cells express the early marker PDGFRα and ~36% the ganglioside A2B5. Between 20% and 40% of the total cells express O4 (23).
Cells were treated with plasminogen-depleted fibrinogen (Millipore Sigma, Burlington MA, 341578, 2.5 mg/ml) for 4 days prior to fixation (days 18-21 of differentiation). The cell viability (PI) and differentiation (O4) were determined as described above. Proliferation was determined by staining with Ki67 (Invitrogen, Waltham MA), post-fixation and permeabilization diluted 1:200, overnight. Secondary antibody (Alexa Fluor 488 or 647; Invitrogen, Waltham MA) was applied the following day, diluted to 1:400.

Immunofluorescence Analysis
Plates were imaged with a 10X objective using a Zeiss Axio Observer fluorescence microscope (Carl Zeiss Canada, Toronto) or the ImageXpress (Molecular Devices, San Jose, CA) high-content imaging platform following staining for O4, PI or Ki67. Cells were counted by a blinded individual using the ImageJ software or automatically using the ImageXpress software.

Signaling assessment

Staining: Primary human OLs and iPSC-derived OPCs following 21 days of differentiation were treated with molecules to induce signaling for 2 hours. The following concentrations were applied: plasminogen-depleted fibrinogen (Millipore Sigma, Burlington MA, 341578, 2.5 mg/ml), recombinant human BMP4 (Peprotech, Rocky Hill NJ, 50 ng/mL), BMP4-inhibitor DMH1 (Selleck Chemicals, Houston TX, 2 uM). Post-fixation, cells were permeabilized with 100% cold methanol and stained with anti-phosphorylated (p)SMAD1/5/9 (Cell Signaling Technology, Danvers MA) overnight at a 1:50 dilution, and secondary antibodies (Alexa Fluor 488, 555 or 647; Invitrogen, Waltham MA) diluted to 1:400 were added for two hours the following day.

To measure Mean Fluorescence Intensity (MFI): Images were acquired on a Zeiss Axio Observer fluorescence microscope (Carl Zeiss Canada, Toronto, ON) at 20X objective or the Zeiss LSM 900 confocal microscope at 20X objective. For pSMAD1/5/9 nuclear intensity, nuclei of O4+ cells were selected by a blinded individual on the ImageJ software and the mean grey value of the pSMAD1/5/9 channel was recorded.

To record pSMAD localization: Following image acquisition at the 20X objective, localization of pSMAD1/5/9 on O4+ cells was assessed by a blinded individual based on four criteria: no signal, cytoplasm only, nucleus and cytoplasm, nuclear only. 20-25 cells were counted for each replicate.

FACS Sorting and Flow Cytometry
For the iPSC-reporter line, cells were collected from the culture vessel using TrypLE (Thermo-Fisher, Mississauga, ON) added for 30 mins at 37□. The cells were passed through a 40µM mesh to ensure a single cell suspension. Following resuspension in PBS (Wisent, Saint-Jean-

Baptiste, QC), the cells were counted using the Attune flow cytometer (Thermo-Fisher Scientific, Mississauga, ON). Sorting was based on reporter intensity using the previously described protocol (23), with the Aria fusion cell sorter (BD Biosciences, Franklin Lakes NJ). For phenotyping, cells were stained as previously described (23), and acquired on the Attune flow cytometer (Thermo-Fisher Scientific, Mississauga, ON). Data was analyzed using the FlowJo software (Ashland, OR).

Bulk RNAseq preparation and analysis

Three subsequent passages of FACS-sorted SOX10-mOrange cells were collected as replicates, and primary cells from two different donors (53 yr, 58 yr) were collected as replicates following treatment with plasminogen-depleted fibrinogen (Millipore Sigma, Burlington MA, 2.5 mg/ml) for four and two days, respectively. Treated cells were collected and RNA was extracted using the Qiagen (Hilden, Germany) RNA-mini kit or Norgen (Thorold, ON) single cell RNA purification kit.

Library preparation, sequencing, quality check, alignment, quantification of raw read counts: Library preparation, sequencing, quality check, alignment, quantification of raw read counts, and normalization of read counts were performed using the same methods as described in Mohammadnia et al. (2024) (26).

Analysis of RNA-seq data from human primary oligodendrocytes: Samples were analyzed in a pairwise manner as described in Mohammadnia et al. (2024) (26).

Analysis of RNA-seq data from iPSC-differentiated cells: As these samples did not exhibit significant heterogeneity and the lack of pair information for samples, we used DESeq2 for differential gene expression analysis following the methodology outlined in Pernin et al. (2024) (27, 28). Significantly differentially expressed genes were identified using a threshold of log2 fold change > 1 and a p-value cutoff of < 0.05.

Heatmaps were generated following hierarchical clustering using the "Hierarchical Clustering Image" function in GenePattern (v3.9.11). Gene clustering was performed using Pearson correlation, and normalized values were transformed using a log transformation. Finally, row normalization was applied for the final visualization (29). Single-sample Gene Set Enrichment Analysis (ssGSEA) was performed using the same method described in Mohammadnia et al. (2024)(26). However, the signatures used in this analysis were BMP4 target genes extracted from the weighted matrix of ligand-target interactions in NichNet (30). We selected target genes based on their weighted values, specifically those with a weight > 0.1 (n = 107 genes).

Single-cell RNA-seq analysis: All single-cell RNA-seq analyses were performed using the same pipeline employed in our previous publication (28) on the same Jakel et al. (2019) data set (31).

Human MS and ALS brain tissue

Tissues from the Centre Hospitalier de l'Université de Montréal (CHUM) were obtained by a Material Transfer Agreement between CHUM and McGill University. Human brain tissue was obtained from patients diagnosed with MS according to the revised 2010 McDonald's criteria (32). Tissue samples were collected from one amyotrophic lateral sclerosis (ALS) patient and three MS patients (Table S1) with full ethical approval (BH07.001, Nagano 20.332-YP) and

informed consent as approved by the local ethics committee. Autopsy samples were preserved, and lesions classified using Luxol Fast Blue-Haematoxylin and Eosin (LFB-HE) staining as previously published (33, 34).

Immunohistochemistry

Frozen human brain tissue sections, previously briefly fixed in acetone, were further fixed in 100% acetone for 10 minutes, followed by 70% ethanol for 5 minutes. Subsequently, sections were washed with PBS and 0.05% PBST (phosphate-buffered saline + Tween20) for 3 minutes. Fc-receptors in sections were blocked with PBS containing 10% donkey serum. Primary antibodies, goat anti-SOX10 (R&D systems, Oakville ON, AF2864) and rabbit anti-fibrinogen (Agilent, Santa Clara CA, A0080) were diluted 1:500 (0.2 mg/ml) and 1:400 (1mg/ml) respectively in PBS containing 3% donkey serum and incubated overnight at 4□. Isotype controls consisted of the IgG of the same species and same concentration as each primary antibody. Following incubation, sections were washed with 0.05% PBST 3 times for 5 minutes each. Fluorophore-conjugated secondary antibodies (Invitrogen, Waltham MA) were prepared at a 1:200 dilution in PBS, donkey anti-goat IgG Alexa Fluor 488 to visualize SOX10 and donkey anti-rabbit IgG Alexa Fluor 647 to visualize fibrinogen and slides were incubated in the dark for 60 minutes at room temperature. Sections were washed with 0.05% PBST 3 times for 3 minutes and incubated with Hoechst (Invitrogen, Waltham MA; 1:5000) for 10 minutes at room temperature. Once washed with 0.05% PBST 3 times for 3 minutes to block lipofuscin autofluorescence, sections were incubated with 0.3% TrueBlack (Biotium, Fremont, CA) diluted in 70% ethanol for 3 minutes. Slides were washed with PBS and mounted with PermaFluor mounting medium (Thermo-Fisher, Missisauga, ON), coverslipped and stored in the dark at 4□.

Statistical analysis

Statistical analyses were performed using Excel or GraphPad Prism software. Cell culture studies were performed with at least three individual replicates per experiment. For iPSC-studies, three cell lines were used which contributed to values on graphs, and subsequent passages were used as biological replicates. Bars on graphs indicate means ±SEM. Student's t-test was used for comparisons between two groups. P-values <0.05 were considered statistically significant.

3. Results

Human primary OLs have an age and maturation-dependent functional response to fibrinogen

Dissociated OL cultures, comprised of 95 % mature OLs (22), were treated with fibrinogen. We observed no significant effect on cell viability (Fig. 1A) when fibrinogen was added over an 80ug/ml to 4mg/ml concentration range for 6 days. To determine a functional effect on OLs, we assessed their capacity to ensheath poly-L-lactide nanofibers. We separated pre-myelinating OLs (A2B5+) from mature OLs (A2B5-) to determine the relation of lineage maturity and donor age. In the presence of fibrinogen, we observed an increased capacity in adult-derived mature OLs (A2B5-) ($p < 0.05$) but no effect on adult A2B5+ cells (Fig. 1B). For pediatric donor cells, we observed a significant decrease in ensheathing A2B5+ cells ($p < 0.05$) but not mature OLs (A2B5-) in comparison to the age-matched untreated controls (Fig. 1C). Figures 1D-E show the overall age-related effect of fibrinogen on mature (A2B5-) cells (Fig. 1E) and immature (A2B5+) cells (Fig. 1D), with representative images in Fig. 1F, indicating that fibrinogen has its most

apparent positive effect on the older adult donors. Bulk RNAseq analysis of adult OLs shows upregulation of lipid metabolism pathways, including cholesterol synthesis, (Fig. 1G-H) consistent with this beneficial effect of fibrinogen on ensheathment by adult OLs.

Fibrinogen inhibits the differentiation of human OPCs

Since, as stated, we cannot isolate early OPCs from our surgical samples, we generated early OL-lineage cells from iPSCs, including a previously described human iPSC reporter line (SOX10mO) (23) where fluorescence intensity of the reporter correlates to the overall maturity of the cells. Fibrinogen treatment of early lineage cells, had no significant effect on OPC viability (Fig. 2A). Fibrinogen did significantly reduce the proportion of O4+ OPCs (Fig. 2B) as measured by immunofluorescence. To assess the effect of fibrinogen on overall differentiation into the OL lineage, we assessed level of expression of mOrange in our SOX10mO reporter line. As shown in Fig. 2C, placing these cells in differentiation media results in increased mOrange expression with a reduced proportion of undifferentiated (SOX10-neg) cells. Addition of fibrinogen resulted in an increase proportion of SOX10-med cells. We used the astrocyte-specific marker CD49f (35) to follow-up on the observation by Petersen et al. (13) that fibrinogen diverts the differentiation of primary post-natal rodent derived OPCs towards the astrocyte lineage. We observed that the overall decrease in O4+ cells was associated with a significant ($p < 0.05$, t-test) increase in proportion of O4-CD49f+ cells and O4+CD49f+ cells following fibrinogen exposure (Fig. 2D, E). The latter hybrid OPCs retained the expression of our reporter (Fig. S1). To explore the molecular response of our medium level SOX10-mOrange cells, they were FACS-selected as previously described (23). Bulk RNAseq analysis indicates an overall decrease in signalling and differentiation occurring in these early OL-lineage cells, alongside significant upregulation of cell-cycle pathways (Fig. 2G-I), confirmed by Ki67 staining (Fig. S2). The latter would account for the increased proportion of SOX10-medium cells evident in Fig 2C.

Fibrinogen signalling via the BMP pathway

To determine if the BMP pathway was activated throughout the OL lineage in response to fibrinogen, we measured the activation of a downstream signalling factor, SMAD1/5/9 (36). Nuclear localization of phosphorylated SMAD (pSMAD), as opposed to cytoplasmic, indicates activation. In mature OLs, we observed a significant ($p < 0.05$, t-test) increase in nuclear localization of pSMAD1/5/9 in response to fibrinogen (Fig. 3A-B) in comparison to untreated cells. To confirm pathway activation earlier in the OL lineage, human iPSC derived OPCs were exposed to fibrinogen in the same manner, and we detected increased signalling in the fibrinogen-treated cells in comparison to the untreated controls (Fig. 3C). Comparing the iPSC-derived cells and the primary cells (Fig. 3E), nuclear pSMAD localization was more frequent in the latter, indicating that the BMP pathway is more activated in these cells than iPSC-derived cells after exposure to fibrinogen. For both primary adult and iPSC-derived O4+ cells, BMP4 treatment induced similar changes and fibrinogen-induced responses were significantly reduced by the BMP-pathway inhibitor, DHM1 ($p < 0.05$, t-test) (Fig. 3).

Bulk RNA seq analyses of fibrinogen-treated mature OLs confirmed activation of the BMP pathway (Fig. 4A), as well as an array of BMP-regulated genes (Fig 4C-D). Analysis was based on paired samples given the diversity between donors. In regards to the early OL-lineage cells, we detected a non-significant but decreasing trend in BMP pathway enrichment (Fig 4B);

however, we did not observe significant changes in BMP regulated genes including those of the astrocyte lineage (data not shown). The early OL-lineage cells which were sequenced showed an overall gene suppression except for increase in cell proliferation related pathways (Fig. 2H-I).

OLs in the MS brain parallel the response seen *in vitro*

Mature oligodendrocytes identified in a publicly available RNAseq dataset from MS and control brains (31) were explored to determine if our in vitro responses could be paralleled (Fig 5A-C). Following re-clustering of the OLs, six sub-clusters were labelled Olig1-6, containing cells from both MS and non-neurologically diseased control samples. To explore BMP-pathway related signatures, each OL cluster was evaluated on the expression of top 107 target genes for the BMP4 ligand (Fig 5D). OL clusters 2-6 had more BMP genes expressed in cells derived from MS tissue rather than control. Distribution of known BMP-target genes *ID1* and *ID3* were evaluated and found to have an increasing trend in the cells derived from MS tissue (Fig 5E). To parallel this response to the signature found in our fibrinogen-treated adult OLs, the expression of lipid-related genes *ACAT2* and *MVD* was explored and found to have a similar increased trend in MS-derived samples (Fig 5F).

Fibrinogen is deposited on OL-lineage cells in the MS brain

To determine in vivo relevance of our findings, tissue sections of brain were co-immunostained to detect SOX10 as a marker of OL lineage cells (37) and fibrinogen. We noted that fibrinogen was found on SOX10-positive oligodendroglia in MS lesions, the adjacent white matter, and in ALS white matter (Fig. 6A). This was quantitated in all areas, and great variability in the numbers of SOX10+/Fibrinogen+ cells was observed (Fig. 6B).

4. Discussion

Fibrinogen, a blood coagulation protein, is deposited in many CNS diseases with BBB disruption and myelin abnormalities, including MS, stroke and traumatic brain injury (15). Its involvement throughout the MS disease course is highlighted by studies showing BBB disruption and fibrin deposition early in MS lesions and preceding demyelination (38), while in progressive MS with severe cortical fibrinogen deposition there is reduced neuronal density (7). Here we have demonstrated for the first time, to our knowledge, fibrin deposition on oligodendroglia.

Fibrinogen has been noted to have a functional effect on immune cells, and resident CNS glia such as astrocytes and microglia (15, 39, 40). This molecule has been tested on rodent OPCs and OLs (13), but, to our knowledge, no studies have documented a fibrinogen effect on OL-lineage cells in human samples. Therefore, we aimed to determine if fibrinogen localizes to OL-lineage cells within the MS brain and test its functional effects on human cells *in vitro*. Here we demonstrate that fibrinogen co-localizes with OL-lineage cells (SOX10+) in the MS brain. Fibrinogen was not only localized on SOX10+ cells in MS lesions, but also in MS NAWM as well as ALS tissue.

The mature OL plays important roles in myelin maintenance and can even contribute to re-myelination in the post-natal brain (41). To assess the functional effects of fibrinogen on OLs in relation to their stage in the cell lineage, we obtained mature human OLs from surgically resected materials as described previously (20, 21). These samples were characterized further

into late progenitors (A2B5+) and mature OLs (A2B5-) both from pediatric and adult donors. Our functional studies indicate a differential effect of fibrinogen in relation to OL lineage stage. Mature OLs (A2B5-), most apparent with adult donor cells, had increased ensheathment capacity following fibrinogen exposure, whereas fibrinogen had detrimental effects on ensheathment capacity of pediatric A2B5+ cells. Bulk RNAseq supported these positive observed effects in adult cells, showing enrichment for lipid metabolism pathways as would be upregulated in OL-mediated ensheathment and subsequent remyelination (42, 43). Pediatric and adult human OL-lineage cells differ on a transcriptomic level, with the indication that pediatric cells are more progenitor-like (22). These results indicate that both donor age and lineage stage underlie functional differences.

As our primary cell isolation technique is not conducive to isolation of early lineage cells (PDGFRα+ A2B5+ O4+), we derived OPCs from human iPSCs based on a recently published protocol and employed a previously characterized iPSC reporter line (SOX10mO) to aid in determining the overall culture maturation stage (23). We found reduced O4+ cells when fibrinogen was added during the last four days of differentiation, in line with previous rodent studies (13). This data is further compatible with the inhibition of ensheathment by pediatric A2B5+ cells, the most progenitor-like cells within our primary cell isolates. Using the SOX10mO line, we observed an increase in the proportion of SOX10-med cells. We relate this finding to increased proliferation in response to fibrinogen, as shown by Ki67 and bulk RNAseq, which matches the data observed in rodent OPCs (13). The decrease in proportion of O4+ cells was associated with the increase in cells co-expressing the astrocyte surface marker, CD49f. This deviation towards the astrocyte lineage parallels with the findings of Petersen et al. (13), although was not evident in our bulk RNAseq data. This may be due to this being a small sub-population of cells less prominent within the larger SOX10-medium sample, where overall cell cycling genes were more predominantly upregulated.

The work of the Akassoglou group determined that the effects of fibrinogen on OL-lineage cells are mediated by signaling via the BMP pathway. Using postnatal rat O4-selected cells (13), they confirmed this with a knockout model of the BMP type-1 receptor molecule, activin A receptor type I (ACVR1). Our analysis of this pathway indicates significant differences between the primary and the iPSC-derived OL-lineage cells. Using immunocytochemistry, we observed a strong nuclear translocation of the downstream BMP effector molecule, pSMAD 1/5/9 in mature OLs; this response was less marked in the iPSC-derived cells. For both groups, we showed fibrinogen signaling inhibition using the ACVR1 inhibitor DMH1, showing there is some functional signaling. Our RNAseq data provides insight into the basis of the observed functional responses. Extensive gene upregulation downstream of the BMP pathway was observed in the adult primary OLs, which was paralleled in a subset of OLs from an MS RNAseq dataset and coincided with upregulation of lipid synthesis pathways in both data sources. On the other hand, the RNAseq on early OL-lineage cells showed a downregulation of all signaling pathways in response to fibrinogen, and as a result there was no enrichment for BMP pathway associated genes.

As our results have shown, the effects of BMP pathway activation are highly context dependent. In the perspective of development, studies have determined that BMP signaling has different effects dependent on developmental stage such as promoting neural progenitor cells to proliferate

in early development while promoting astrocytic and neuronal differentiation in later stages (44). These previous findings in the literature may suggest a reason for our observed age-related effect of fibrinogen in human primary OLs. As seen in our iPSC-OPCs, BMP4 signaling also has the capacity to retain cells in a progenitor-like state (45), and promotes a subset of cells to gain an astrocytic phenotype. These observed differences can be attributed to ligand and receptor availability, as well as the presence of competing signaling molecules (46).

In the context of MS, the fibrinogen-driven increased ensheathment by mature OLs could positively contribute to the remyelination process that occurs during early inflammatory demyelinating activity with ongoing breakdown of the BBB, and at the borders of chronic active plaques. In these latter situations numbers of mature OLs are relatively preserved. Failure of subsequent remyelination would reflect a depletion of such cells, as evident in previously demyelinated regions of the plaque, that is not replenished by production of new OLs capable of remyelination due a block in oligodendroglial progenitor differentiation. This could reflect at least in part the effect of fibrinogen on OPCs. Whether other factors in the milieu of the MS lesion contribute to this differentiation block remains to be determined.

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Potential conflicts of interest: nothing to report.

Data Availability: data will be made available upon manuscript acceptance.

5. Figure Legends

Figure 1. Fibrinogen has an age and maturation-dependent effect on human OLs.
A. Cell viability of human A2B5+ pre-OLs (A+) and A2B5- mature OLs (A-) from both adult and pediatric samples following fibrinogen (Fib) treatment and quantified following immunofluorescence microscopy for propidium iodide (PI). 1:1= 4 mg/ml 1:2= 2 mg/ml 1:10= 0.4 mg/ml 1:50= 80 ug/ml. No increases in cell death were observed. Colours represent age and sex-matched samples. **B-C.** Capacity of (B) adult (26 - 65 years) and (C) pediatric (2.5 -18 years) pre-OLs (A2B5+ (A+)) and mature OLs (A2B5- (A-)) to ensheath nanofibers. Note the increase of ensheathment capacity in adult A- cells, and the decrease in pediatric A+ cells. * $p < 0.05$, ** $p<0.01$, *** $p<0.001$. Bars indicate SEM. Colours represent age and sex-matched samples. **D-E.** Correlation of (D) A2B5+ and (E) A2B5- cell ensheathment capacity to age, as normalized to age-matched controls following treatment with fibrinogen. Note the increasing trend in ensheathment capacity with age. **F.** Representative images of adult OLs on nanofibers. O4 stain in white, nuclei in blue. Top panels represent A2B5+ cells, bottom panels represent A2B5- cells. Arrows point to representative examples of ensheathing segments. **G**. GO term analysis following bulk RNA sequencing on adult OL samples. **H.** Significantly upregulated genes involved in lipid metabolism, following fibrinogen treatment on adult OL samples (matched donors).

Figure 2. Fibrinogen effect on human iPSC-derived OPCs.
A. Cell viability of OPCs (O4+) imaged and quantified following immunofluorescence microscopy for propidium iodide (PI). NG: no glucose. Bars indicate SEM. **B.** Proportion of OPCs (O4+) as quantified following immunofluorescence microscopy, showing a decrease in OPCs following fibrinogen treatment for 4 days. Bars indicate SEM. * $p < 0.05$ **C.** Proportions (%) of iPSC-derived cultures expressing the mOrange reporter as measured by flow cytometry, average of n=5 passages. **D.** Proportions of cells co-expressing OPC/astrocyte markers as measured by flow cytometry. Values normalized to the average of the untreated control, n=3 passages. Bars indicate SEM. **E.** Gating technique. **G.** Differentially expressed genes following bulk RNAseq analysis of sorted reporter-medium cells. **H-I.** GO term analysis of (H) downregulated and (I) upregulated genes.

Figure 3. Fibrinogen activates BMP signalling across the OL lineage.
A. Representative images of human primary OLs (O4+) following exposure to treatments for 2 hours, by Zeiss epifluoresence microscope. pSMAD 1/5/9 (green), O4 (white), nuclei (blue). **B**. Quantification of nuclear fluorescence intensity of pSMAD 1/5/9 in human primary OLs, where fibrinogen and BMP4 significantly increase pSMAD1/5/9 nuclear signal intensity. Colours represent sex and age-matched controls. **C.** Representative images of human iPSC derived OPCs (O4+) following exposure to treatments for 2 hours, by Zeiss confocal microscope. pSMAD 1/5/9 (green), O4 (white), nuclei (blue). **D.** Quantification of nuclear fluorescence intensity of pSMAD 1/5/9 in iPSC OPCs, where fibrinogen and BMP4 significantly increase pSMAD1/5/9 nuclear signal intensity. Colours represent matched replicates. **E.** Proportion of pSMAD signal localization following treatment with fibrinogen. n=3 donors for primary cells, n=4 passages for iPSC * $p < 0.05$, ** $p<0.01$, *** $p<0.001$. Bars indicate SEM.

Figure 4. Transcriptomic analysis of BMP signaling activation in the oligodendrocyte lineage.
A-B. Single-sample Gene Set Enrichment Analysis (ssGSEA) for BMP4 target genes in: (A) human primary oligodendrocytes treated with fibrinogen at 2.5 mg/mL, 2 days and (B) iPSC-differentiated SOX10-positive medium cells treated with fibrinogen at 2.5 mg/mL for 4 days. Normalized read counts were used for both analyses. Enrichment scores are visualized in the plots. One iPSC-OPC fibrinogen-treated sample was removed during quality control. **C.** Bar plot showing log2-transformed fold changes (ratios) of BMP4 target gene expression in human primary oligodendrocytes. Red bars indicate genes with higher expression levels in fibrinogen-treated cells, and blue bars indicate genes with higher expression levels in the control group. **D.** Example of BMP4 target genes in human primary oligodendrocytes showing upregulation upon treatment with fibrinogen (2.5 mg/mL, 2 days). Paired t-tests were applied to assess significance levels in all plots for human primary oligodendrocytes. In all plots, blue represents control samples, and red represents fibrinogen-treated samples.

Figure 5. Transcriptomic analysis of the BMP signaling pathway in mature oligodendrocytes using single-nucleus RNA-sequencing (snRNA-seq) data.
A. UMAP plots were generated based on the study by Jakel et al. (2019) to capture all relevant cell types in the context of multiple sclerosis (MS) and control tissue. **B.** Mature oligodendrocyte marker genes were used to identify mature cells within the snRNA-seq data. Myelin

oligodendrocyte glycoprotein (MOG) is visualized in this UMAP plot as an example of an oligodendrocyte-specific marker gene. **C.** All cell clusters contained a mixed combination of cells from MS and control patients, making them suitable for comparing BMP signaling within each oligodendrocyte population. **D.** Bar plot showing the distribution of BMP4 target genes across different oligodendrocyte populations in MS and control groups. We used the top 107 target genes for the BMP4 ligand (with interaction weight > 0.1) and extracted their average expression in each oligodendrocyte cluster from both MS and control groups. The Oligo1 population was excluded because most of its cells (85%) originated from control samples. In the bar plot, purple, red, and blue represent not expressed, higher expression in MS, and higher expression in control samples, respectively. **E.** ID1 and ID3 are examples of BMP signaling target genes that show an increasing trend in expression in most oligodendrocyte populations in MS compared to control groups. **F.** Example of genes related to lipid metabolism that exhibit a similar increasing trend in gene expression as BMP signaling target genes, consistent with the results from in vitro experiments using human primary oligodendrocytes. In all cases, normalized read count data were used to calculate average expression levels for each cluster for analysis and plot generation. Red represents MS, and blue represents control cells.

Figure 6. Deposition of fibrinogen on oligodendroglial lineage cells within the MS and ALS brain.

A. Representative images of MS plaque, MS NAWM, and ALS white matter. Sox10 (oligodendroglial nuclear marker) in green; fibrinogen in red. For each region, a higher magnification of the area within the box is shown in the panel immediately to the right. Plaque 1, an MS lesion that in sections stained for myelin showed active demyelination at its edge, consistent with a chronic active plaque; Plaque 2, an MS lesion that in sections stained for myelin showed no active ongoing demyelination, consistent with a chronic inactive plaque. Plaque 3, an MS lesion that in sections stained for myelin showed active demyelination throughout its extent, consistent with an active plaque. The representative NAWM illustrated is adjacent to Plaque 3, and the ALS white matter serves as a non-MS condition. Note the variable distribution of fibrinogen on SOX10+ cells in the MS plaques, MS NAWM and ALS white matter. Examples of SOX10-positive oligodendroglia that are positive for fibrinogen in their cytoplasm and/or nuclei are indicated with white arrows. SOX10+ Fibrinogen+ nuclei are yellow. The blue arrow indicates a prominent fibrinogen-positive cell, possibly an astrocyte or macrophage that has engulfed SOX10- and fibrinogen-positive debris: the debris double labelled with red and green, appearing yellow. Scale bars = 50µm. **B.** Quantification of the percentages of SOX10+ oligodendroglia that are positive for fibrinogen from the three MS plaques, in comparison to NAWM from the same tissue section, and from the ALS white matter illustrated in A, verifying the variability of the number of fibrinogen-positive oligodendroglia.

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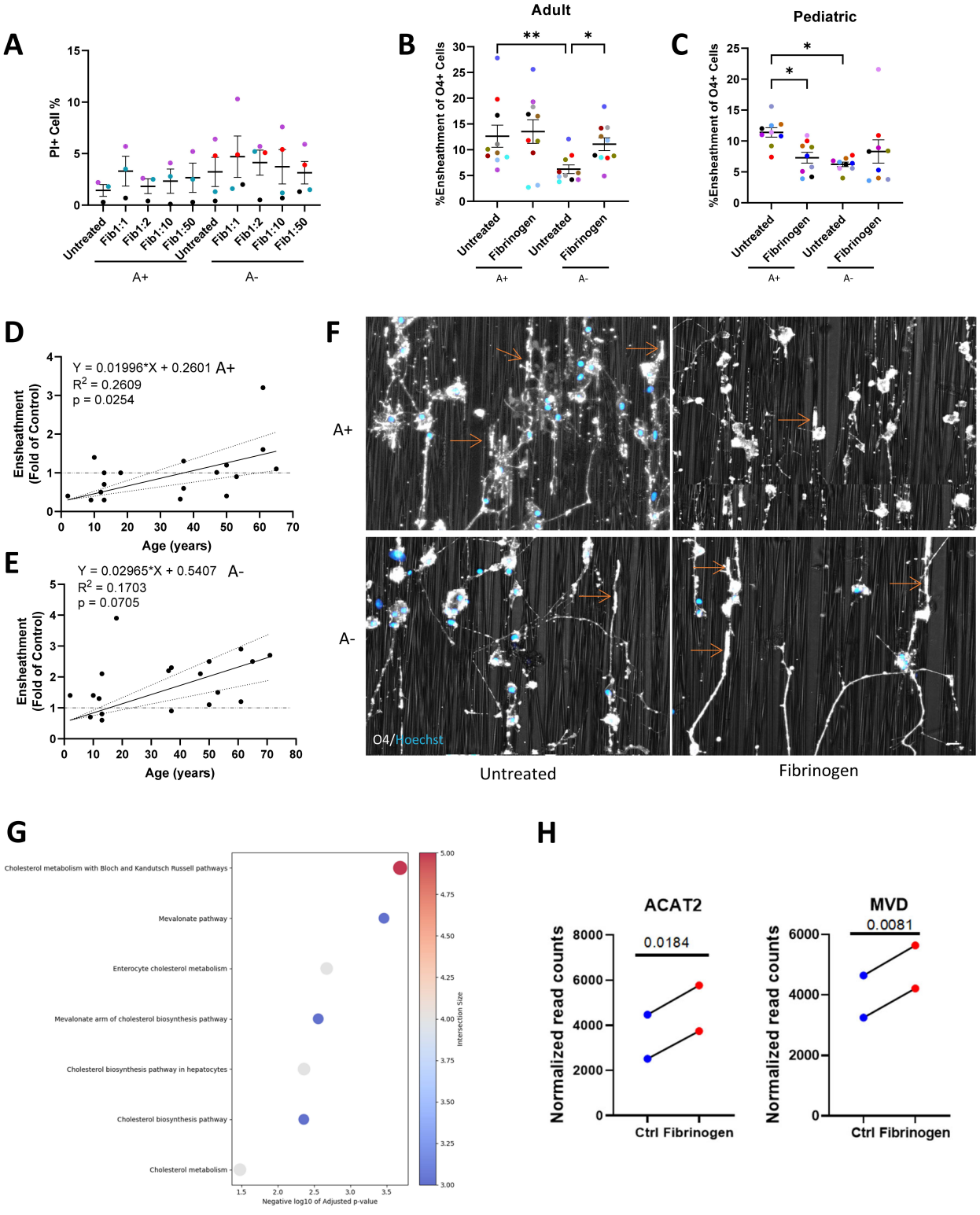
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PI+ Cell %
Untreated
Fib1:1
Fib1:2
Fib1:10
Fib1:50
A+
A-
B
Adult
%Ensheathment of O4+ Cells
Untreated
Fibrinogen
C
Pediatric
D
Y = 0.01996*X + 0.2601 A+
R2 = 0.2609
p = 0.0254
Ensheathment (Fold of Control)
Age (years)
E
Y = 0.02965*X + 0.5407 A-
R2 = 0.1703
p = 0.0705
F
A+
A-
O4/Hoechst
Untreated
Fibrinogen
G
Cholesterol metabolism with Bloch and Kandutsch Russell pathways
Mevalonate pathway
Enterocyte cholesterol metabolism
Mevalonate arm of cholesterol biosynthesis pathway
Cholesterol biosynthesis pathway in hepatocytes
Cholesterol biosynthesis pathway
Cholesterol metabolism
Negative log10 of Adjusted p-value
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MVD
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Normalized read counts
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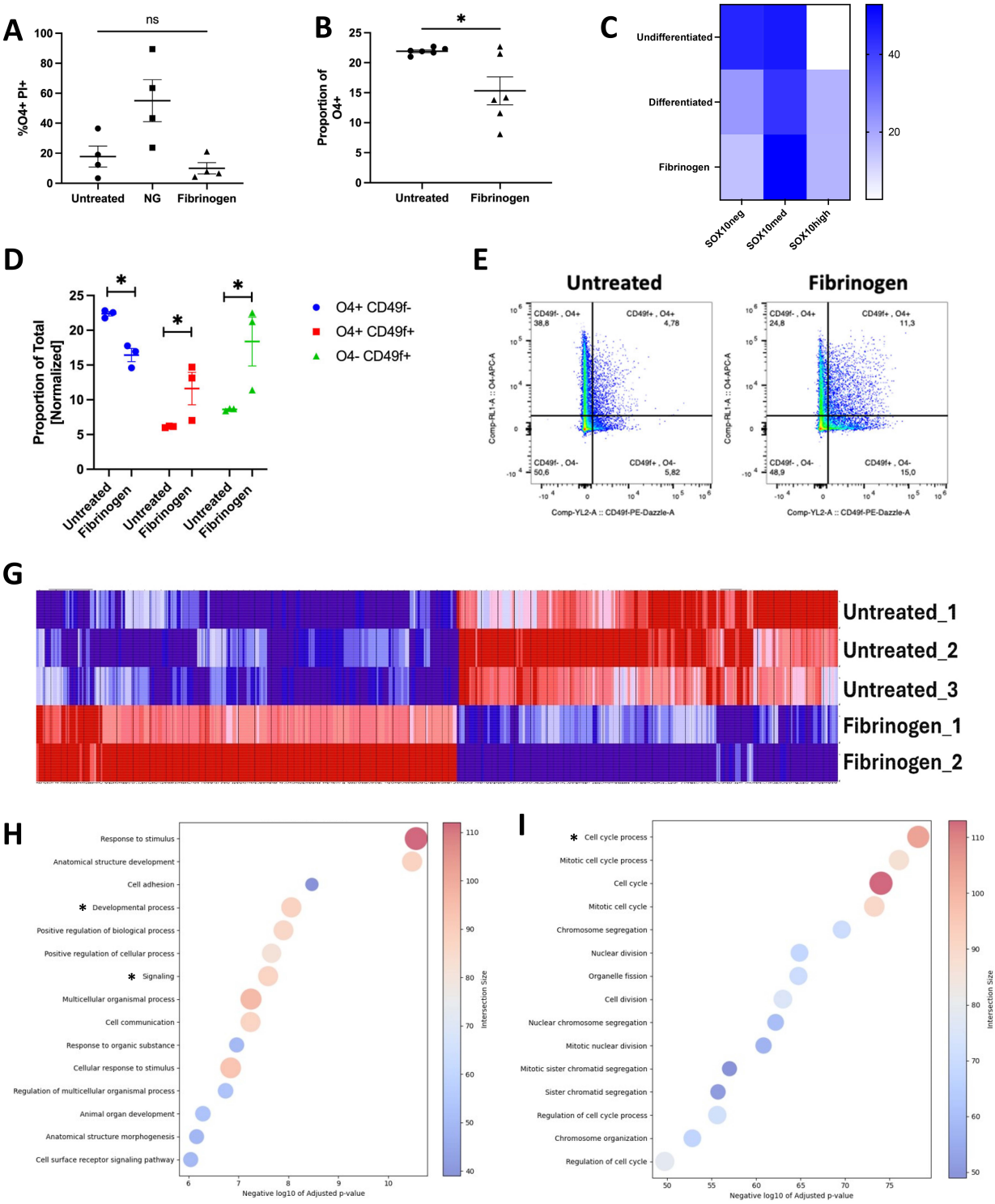
A
ns
%O4+ PI+
Untreated
NG
Fibrinogen
B
*
Proportion of O4+
Untreated
Fibrinogen
C
Undifferentiated
Differentiated
Fibrinogen
SOX10neg
SOX10med
SOX10high
D
Proportion of Total [Normalized]
O4+ CD49f-
O4+ CD49f+
O4- CD49f+
Untreated
Fibrinogen
E
Untreated
Fibrinogen
CD49f- , O4+ 38,8
CD49f+ , O4+ 4,78
CD49f- , O4- 50,6
CD49f+ , O4- 5,82
CD49f- , O4+ 24,8
CD49f+ , O4+ 11,3
CD49f- , O4- 48,9
CD49f+ , O4- 15,0
Comp-RL1-A :: O4-APC-A
Comp-YL2-A :: CD49f-PE-Dazzle-A
G
Untreated_1
Untreated_2
Untreated_3
Fibrinogen_1
Fibrinogen_2
H
Response to stimulus
Anatomical structure development
Cell adhesion
Developmental process
Positive regulation of biological process
Positive regulation of cellular process
Signaling
Multicellular organismal process
Cell communication
Response to organic substance
Cellular response to stimulus
Regulation of multicellular organismal process
Animal organ development
Anatomical structure morphogenesis
Cell surface receptor signaling pathway
Negative log10 of Adjusted p-value
Intersection Size
I
Cell cycle process
Mitotic cell cycle process
Cell cycle
Mitotic cell cycle
Chromosome segregation
Nuclear division
Organelle fission
Cell division
Nuclear chromosome segregation
Mitotic nuclear division
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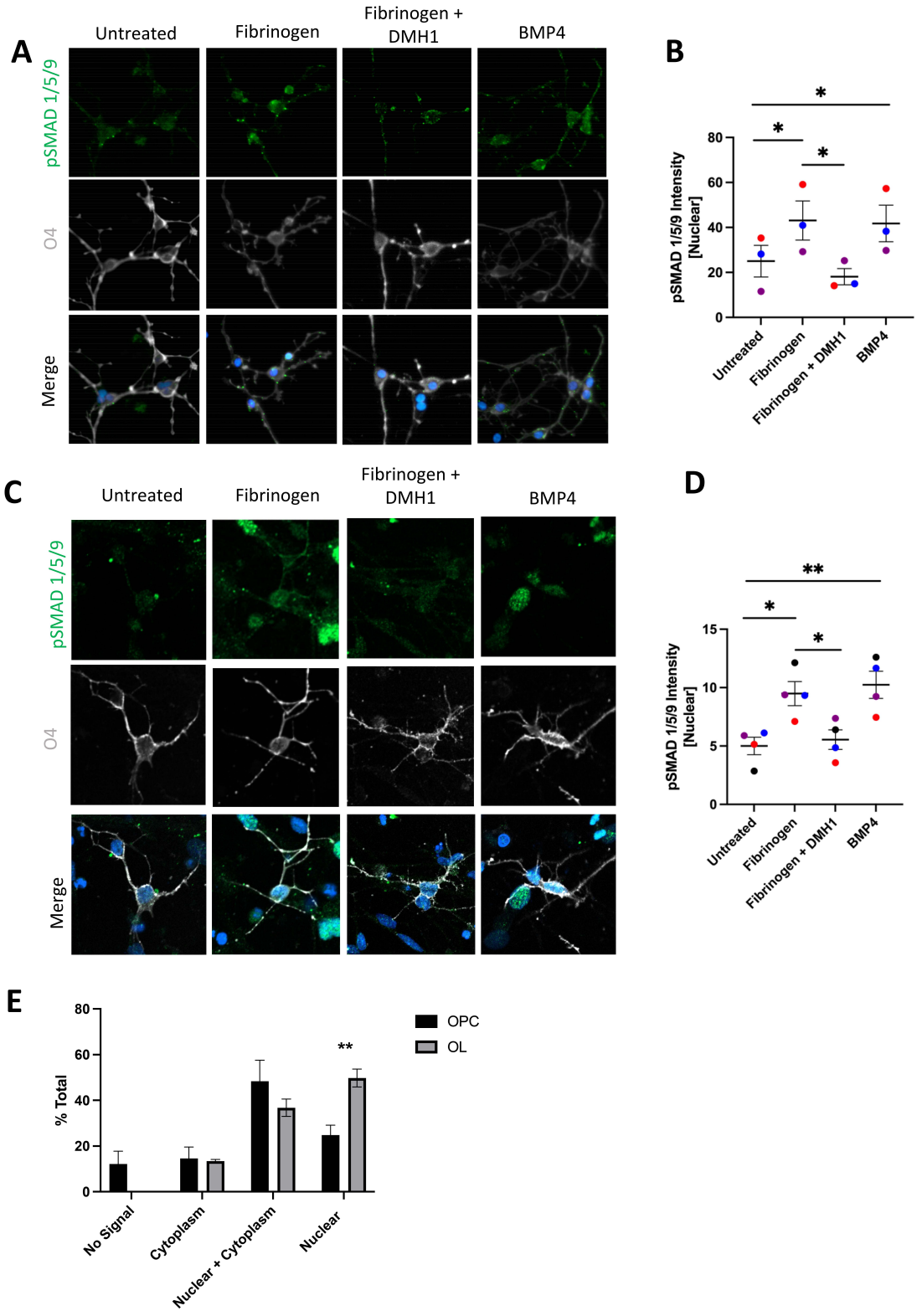
Sister chromatid segregation
Regulation of cell cycle process
Chromosome organization
Regulation of cell cycle
Negative log10 of Adjusted p-value
Intersection Size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Untreated
Fibrinogen
Fibrinogen + DMH1
BMP4
pSMAD 1/5/9
O4
Merge
B
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0
20
40
60
80
*
*
*
Untreated
Fibrinogen
Fibrinogen + DMH1
BMP4
C
Untreated
Fibrinogen
Fibrinogen + DMH1
BMP4
pSMAD 1/5/9
O4
Merge
D
pSMAD 1/5/9 Intensity [Nuclear]
0
5
10
15
*
*
**
Untreated
Fibrinogen
Fibrinogen + DMH1
BMP4
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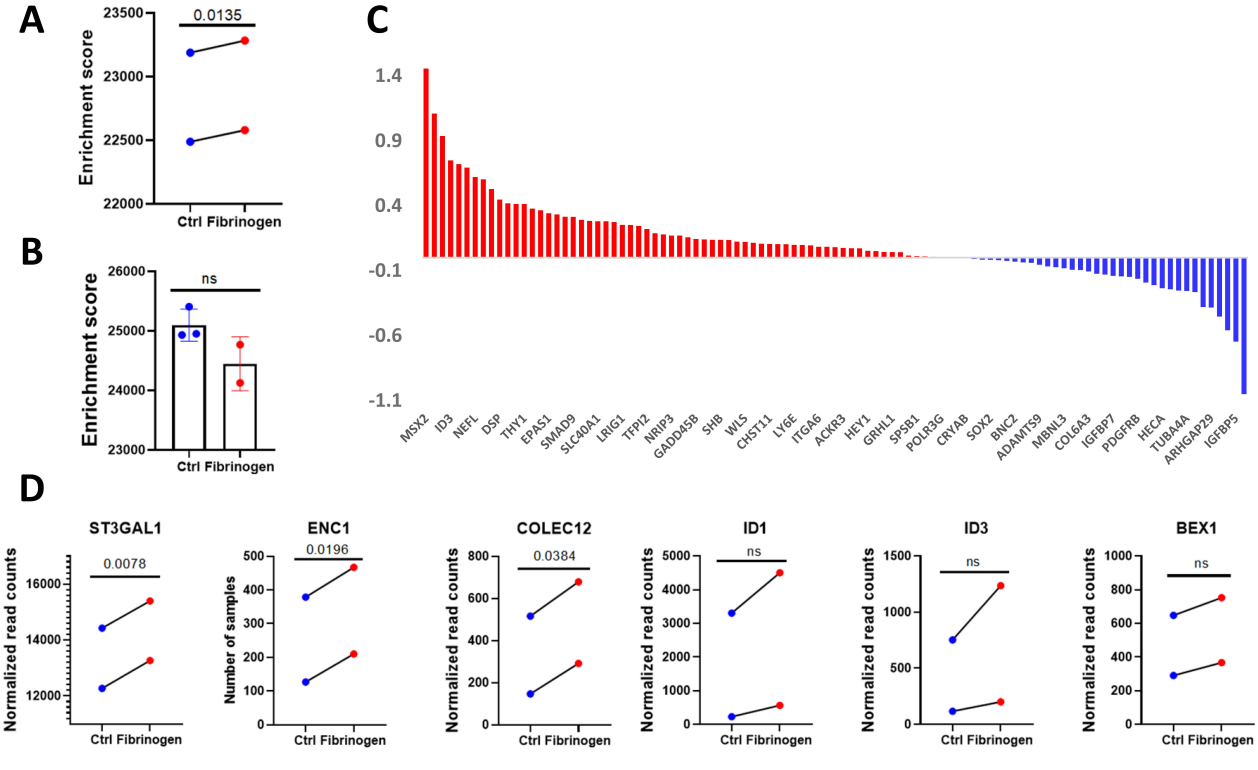
Nuclear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22000
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B
ns
Enrichment score
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25000
24000
23000
Ctrl Fibrinogen
C
1.4
0.9
0.4
-0.1
-0.6
-1.1
MSX2
ID3
NEFL
DSP
THY1
EPAS1
SMAD9
SLC40A1
LRIG1
TFPI2
NRIP3
GADD45B
SHB
WLS
CHST11
LY6E
ITGA6
ACKR3
HEY1
GRHL1
SPSB1
POLR3G
CRYAB
SOX2
BNC2
ADAMTS9
MBNL3
COL6A3
IGFBP7
PDGFRB
HECA
TUBA4A
ARHGAP29
IGFBP5
D
ST3GAL1
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Normalized read counts
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14000
12000
Ctrl Fibrinogen
ENC1
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Number of samples
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400
300
200
100
0
Ctrl Fibrinogen
COLEC12
0.0384
Normalized read counts
800
600
400
200
0
Ctrl Fibrinogen
ID1
ns
Normalized read counts
5000
4000
3000
2000
1000
0
Ctrl Fibrinogen
ID3
ns
Normalized read counts
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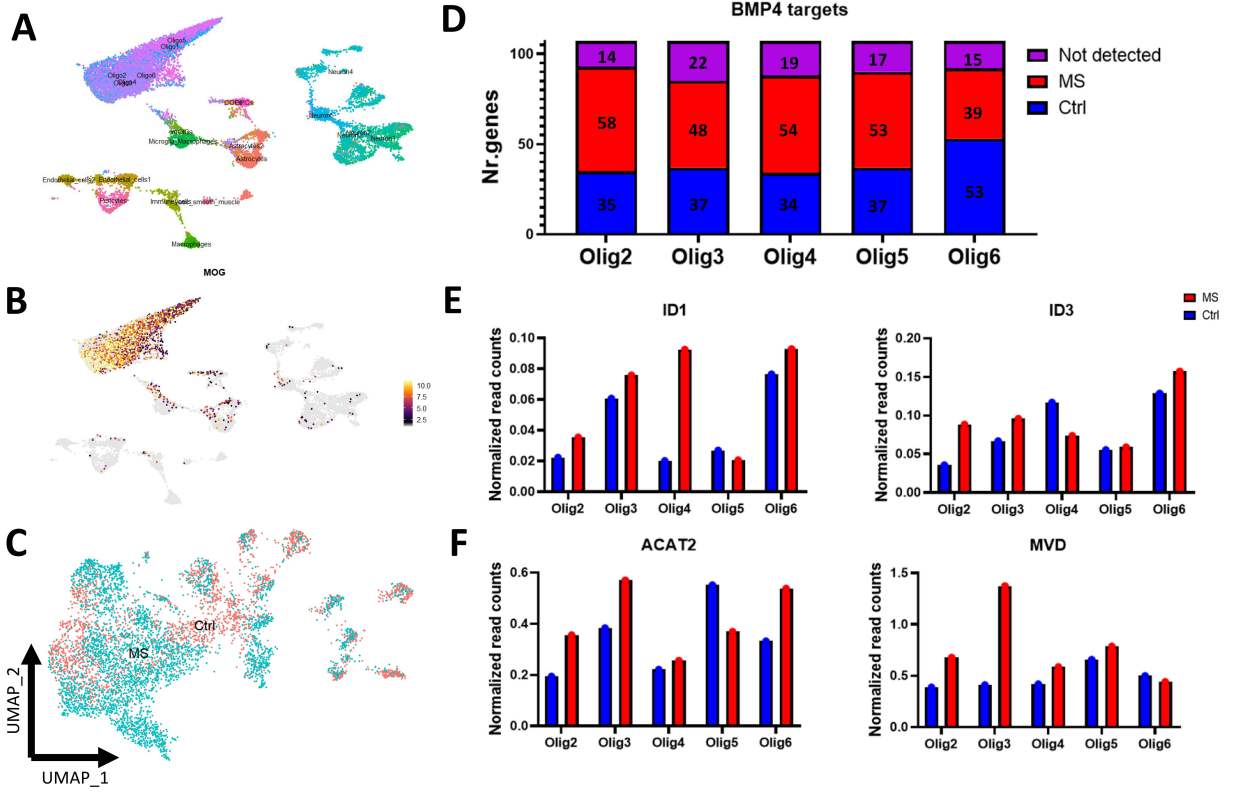
Normalized read counts
1000
800
600
400
200
0
Ctrl Fibrinogen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Oligo5
Oligo1
Oligo2
Oligo3
Oligo4
Neuron4
Neuron2
Neuron1
Neuron3
Microglia_Macrophages
Astrocytes2
Astrocytes
Endothelial_cells
Endothelial_cells1
Pericytes
Immune_cells_smooth_muscle
Macrophages
MOG
B
10.0
7.5
5.0
2.5
C
Ctrl
MS
UMAP_2
UMAP_1
D
BMP4 targets
Nr.genes
100
50
0
14
58
35
22
48
37
19
54
34
17
53
37
15
39
53
Olig2
Olig3
Olig4
Olig5
Olig6
Not detected
MS
Ctrl
E
ID1
ID3
MS
Ctrl
Normalized read counts
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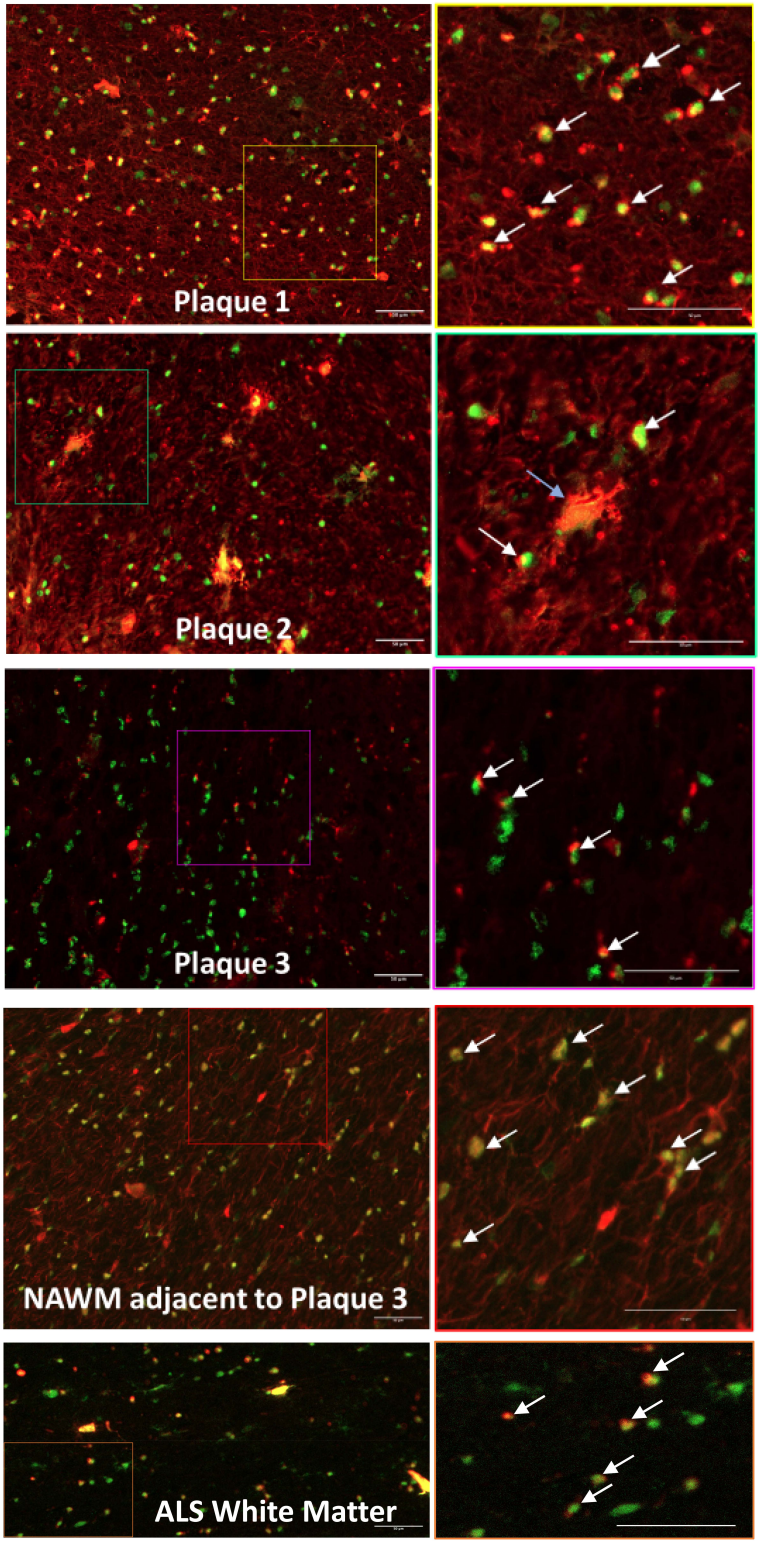
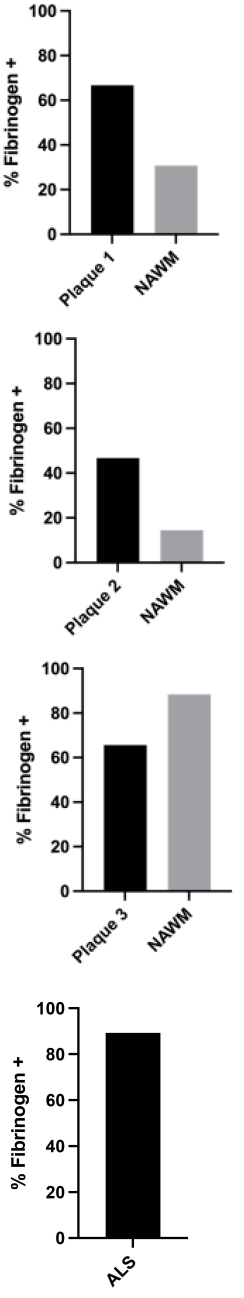
0.08
0.06
0.04
0.02
0.00
0.20
0.15
0.10
0.05
0.00
Olig2
Olig3
Olig4
Olig5
Olig6
F
ACAT2
MVD
Normalized read counts
0.6
0.4
0.2
0.0
1.5
1.0
0.5
0.0
Olig2
Olig3
Olig4
Olig5
Olig6

A


Plaque 1
Plaque 2
Plaque 3
NAWM adjacent to Plaque 3
ALS White Matter

B


% Fibrinogen +
100
80
60
40
20
0
Plaque 1
NAWM
Plaque 2
Plaque 3
ALS