Investigating lymphoid-like structures in the pathogenesis of Multiple Sclerosis

SHORT REPORT

Dr Rachael Kee, MBChB

Funded by: HSC R&D Division, Public Health Agency

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Evidence Brief

Why did we start?

Multiple Sclerosis (MS) is a disease of the central nervous system (CNS) characterised by areas of damage (lesions) that often contain inflammatory cells. Despite advances in treatments for relapsing-remitting MS (RRMS), few treatments have shown efficacy in the progressive disease stages. Furthermore, it is difficult to predict which patients will go on to have severe and progressive forms of MS. Further research is required to investigate pathological mechanisms of the progressive disease stage so that we can find better treatments targeted at progression and better ways of predicting which patients are more likely to develop progressive MS. The purpose of this research was to find out; what types of immune cells are contained in LLS, what genes are switched on in them, and how these structures may be influencing the disease course in MS.

What did we do?

Using post-mortem tissue from the Dame Ingrid Allen Tissue collection, we characterised the clinical and pathological features of a cohort of patients that had MS. We investigated the neuropathological features of grey matter pathology and how this was related to meningeal inflammation and LLS. Using cutting-edge technology, we applied Nanostring spatial transcriptomics to two post-mortem cases that harboured LLS to determine the gene expression profiles of LLS and other CNS compartments that harbour inflammatory cells. Twelve highly expressed genes in LLS were quantified using the RNAscope HiPlex assay to determine mRNA expression of these targets at the cellular level. These genes were then validated at the protein level and the possible immune cells expressing these targets was investigated by immunohistochemistry. Finally, the serum and cerebrospinal fluid (CSF) profiles of these protein targets were investigated in a cohort of patients with MS to determine if distinct profiles could predict patients more likely to develop progressive disease.

What answers did we get?

We identified that higher degrees of meningeal inflammation and the presence of LLS were associated with poorer clinical outcomes (earlier time to death and younger age at death) and a trend of greater extent of grey matter demyelination in the Dame Ingrid Allen post-mortem cohort. The highest expressed genes in LLS were revealed to be the immunoglobulin G (IgG) subclasses; IgG3 and IgG4. We found that the highest proportion of cells expressing IgG3 and IgG4 were located in LLS, although a gradient of expression was observed in perivascular spaces of the white matter and neighbouring grey matter. We identified that some of the cells expressing IgG3 and IgG4 were germinal centre B cells or plasma cells, indicating that LLS could be a potential source of these IgG subclasses in the CNS. Finally, we observed a difference in IgG3 CSF profiles in patients that had RRMS and progressive MS, indicating that distinctions in IgG3 profiles may differentiate patients at greater risk of developing progressive MS.

What should be done now?

The role that immunoglobulins play in MS remains unclear. Further studies are needed to determine if immunoglobulins are a reactive phenomenon (to the presence of antigen) or are mediating the disease process for example by initiating the complement cascade and release of cytotoxic factors. Further studies would also be required to determine if LLS are the sole source of IgG3 and IgG4 production in MS or could there be additional/alternative sources from other CNS and peripheral compartments. It would also be useful to determine in a larger cohort of patients with longitudinal sampling, whether serum and CSF IgG3 and IgG4 profiles change over time from the earliest to chronic stages of the MS disease process. This may reveal distinct profiles related to disease stage which could develop a better biomarker for progressive MS.

1. Background

MS is a chronic, inflammatory, demyelinating disease of the central nervous system (CNS) that strikes young adults in the prime of their lives, affecting over 100,000 people in the UK (1). Forty-three percent of patients leave employment by 12 years post-diagnosis with 55% of the remainder changing their roles or hours (2). Northern Ireland has one of the highest prevalence rates of MS globally (3). The cause of MS is unknown. Epidemiological data suggest combined genetic and environmental factors with a prominent immunological component (4). Whilst the development of disease-modifying therapies (DMTs) for the relapsing-remitting form of MS (RRMS) has continued at pace, all but two of these drugs have failed in progressive MS trials. Furthermore, it is difficult to predict which patients may get a higher disease burden with more relapses or when they may develop progressive disease. Progressive MS is characterised by continual accrual of permanent disability, thus, therapies to slow, halt or reverse disease progression are urgently needed. Understanding the immunological differences in different types of MS, and different phases of disease pathogenesis, will support efforts to develop such therapies and better biomarkers for disease prognostication.

Lymphoid-like structures in Multiple Sclerosis

A hallmark of progressive MS is accumulating neurodegeneration and cortical demyelination, which are related to the extent of overlying meningeal inflammation (5). Our collaborators and others have reported extensive immune infiltrates, termed lymphoid-like structures (LLS), in many progressive MS cases at post mortem (5-8). Cases harbouring LLS exhibit greater parenchymal inflammation, demyelination and a gradient of neurodegeneration, which is greatest in superficial layers of the cortex nearest the pial surface. Importantly, post-mortem studies have shown that the presence of LLS and the relative grade of meningeal lymphoid aggregate correlates with clinical disease severity. Post-mortem cases that have LLS experience earlier transition to progressive MS, become substantially disabled sooner and die following a shorter disease duration and at a younger age (5,9). Identification of immune signatures of LLS could be valuable predictors of disease outcome - a key unmet need in MS prognostics.

2. Aims and Objectives

- Establish the frequency of lymphoid-like structures in an archived CNS post-mortem MS tissue collection
- Undertake neuropathological characterisation of an archived CNS post-mortem MS tissue collection
- Determine the molecular profiles of lymphoid-like structures
- Determine the immunoglobulin profiles of lymphoid-like structures
- Determine if cellular and molecular profiles of lymphoid-like structures are distinct from other CNS inflammatory compartments
- Investigate serum and CSF profiles of IgG3 and IgG4

3. Methods

Tissue processing and histological staining

Post-mortem FFPE tissue from the Dame Ingrid Allen tissue collection and fresh-frozen tissue from the UK MS Society Brain Bank were sectioned at 4-6µm and 10µm thickness respectively. For immunohistochemical analysis, briefly FFPE tissue sections were dewaxed, rehydrated and underwent heat induced epitope retrieval. Tissue sections were blocked and incubated in primary antibody solutions overnight. Following a series of wash steps, species specific secondary antibodies were applied. Antibody binding was visualised with 3, 3'-diaminobenzidine (DAB), emerald green substrate or permanent red substrate. Fresh-frozen tissue sections were fixed in formalin, underwent blocking steps and incubated in primary antibody solutions overnight. Species-specific antibodies were applied and antibody binding was visualised with 3, 3'-diaminobenzidine (DAB) or permanent red substrate. For immunofluorescence studies following the application of primary antibodies, species specific fluorescently labelled secondary antibodies were applied, counterstained with DAPI and slides were mounted with ProLong Gold.

Whole transcriptome analysis

Two tissue sections from two FFPE LLS+ post-mortem cases were sent to Nanostring Technologies, Inc, Seattle, USA and underwent whole transcriptome analysis (WTA). Briefly slides were baked for 2hrs for paraffin removal, rehydrated, underwent antigen retrieval and enzymatic digestion. Tissue sections were then hybridised with UV-photocleavable barcode-conjugated oligonucleotide probes (human WTA, 18269 genes) overnight. Slides were blocked and then incubated with morphology marker antibodies (CD3 and CD20). Tissue sections were then loaded into the GeoMx platform and

regions of interest (ROIs) selected. Conjugated target-specific oligonucleotides for each ROI were cleaved and collected into 96-well plates. Library preparation was performed and sequenced on an Ilumina next generation sequencing (NGS) platform.

***In situ* hybridisation**

The RNAscope HiPlex V2 assay (ACD, 324445) was used to validate 12 selected mRNA targets from the Nanostring dataset. Tissue sections underwent deparaffinisation, rehydration and target retrieval. RNAscope probes were hybridised to tissue sections which then underwent a series of amplification steps. Fluorophores were hybridised and cleaved sequentially in four cycles, with images acquired prior to the next cycle. FFPE dampening reagent was applied to reduce autofluorescence.

ELISA

ELISAs were carried out according to manufacturer's instructions for serum and CSF IgG3 and IgG4 (Thermofisher, 991000). The human subclass specific antibody and standards and samples, diluted in reagent diluent if required, were added to each well for 30 mins at room temperaure (50 µl per well). The plate was washed 4 times with wash buffer and then 100 µl of diluted peroxidase anti-human IgG solution was added to each well for 30 mins at room temperature. The wash step was repeated and 100 µl of 3,3',5,5' tetramethylbenzidine (TMB) solution was added to each well for 10 mins at room temperature in the dark. Stop solution (100 µl) was added to each well.

Image acquisition and analysis

Brightfield images were acquired on Nikon Eclipse 80i microscope. Fluorescence images were acquired on a Stellaris 5 confocal microscope. Brightfield images of PLP, CD68 and H&E stained tissue sections were digitalised by whole slide scanning using an SCN400F scanner (Leica, Wetzlar, Germany), Imperial College London or a Hamamatsu Nanozoomer 2.0-HT slide scanner (Hamamatsu, Shizuoka, JPN), University of Glasgow. Images were processed and analysed in ImageJ or QuPath.

4. Personal and Public Involvement (PPI)

Prior to the submission of this research proposal, a focus group within the Northern Ireland MS Research Network (NIMSRN) was established. This is a group of service users, some of whom have academic backgrounds. Prior to undertaking the research, this focus group established priority research areas for focus. A common theme identified was progressive MS; why some patients develop this and the lack of effective treatments. Addressing knowledge gaps in the pathogenesis of progressive MS was therefore seen as a key research area. I also met with a local MS Society Support Group who also reiterated that a key research area should focus on progressive MS. In response to this, I designed this research proposal to specifically address this area, given the current gap in knowledge, and to complement the resources of Professor Fitzgerald's laboratory. The focus group also had a leading role in reviewing lay summaries and abstracts not only for the grant application to undertake this work but also for other grants that I applied to during the research project. Throughout the course of this research, key findings and the research being undertaken was disseminated to members of the public (participation in the NI Science Festival) and MS service users. The NIMSRN organise an annual public information evening which is free to attend by any person with MS, their families, carers and friends. It provides an opportunity to disseminate research findings, answer questions about our research and engage service users with the basic science research being undertaken in MS locally. This event is growing year on year and the participation from service users steers our research priorities and helps ensure that our research goals are focused on people with MS and how this can make a difference to their lives.

5. Findings

Substantial meningeal inflammation but not perivascular inflammation is associated with poorer clinical outcomes

In the Dame Ingrid Allen cohort screened for the presence of LLS; 7 cases had evidence of substantial meningeal inflammation and 4 cases had evidence of LLS. Cases that had substantial meningeal inflammation had significantly younger ages at death than cases with milder meningeal inflammation (p=0.0399) (figure 1). The median age at death was 60 years and 56 years in cases with mild and moderate meningeal inflammation respectively. In contrast, cases with substantial meningeal inflammation had a median age at death of 37 years. Cases with substantial meningeal inflammation also had significantly shorter disease duration than cases with mild and moderate meningeal inflammation (p=0.0428 and p=0.0153 respectively).

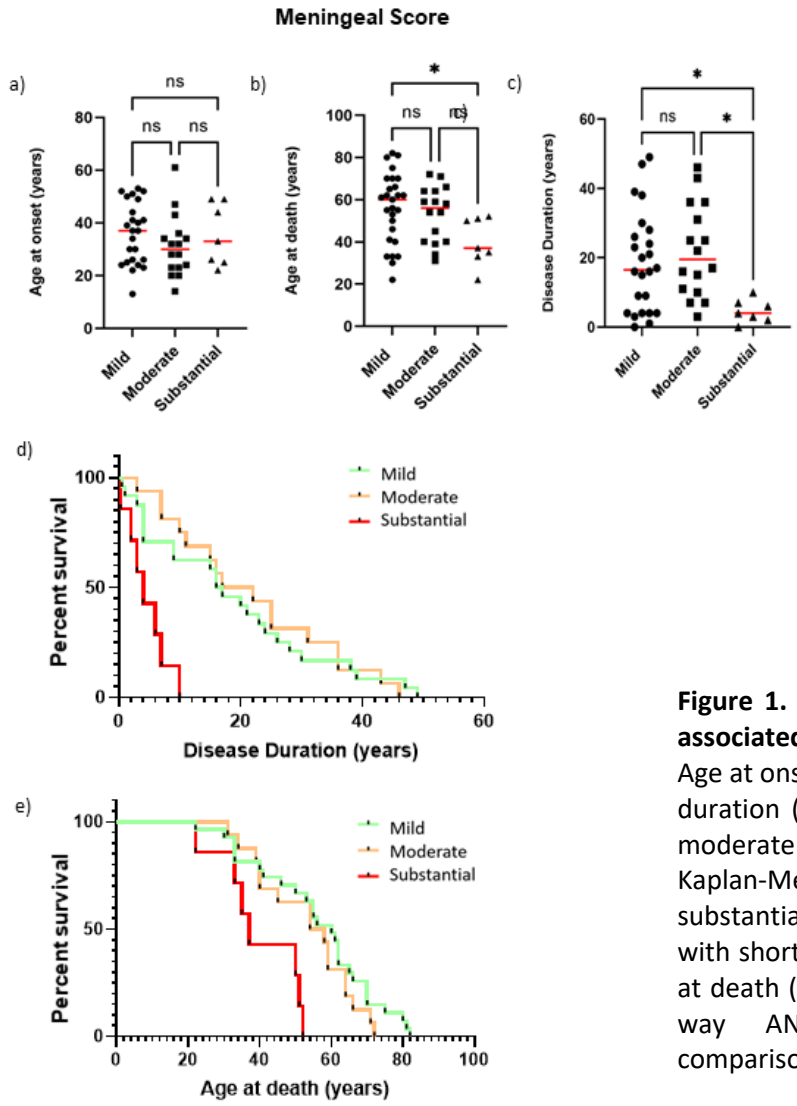

Figure 1. Substantial meningeal scores are associated with poorer clinical outcomes. Age at onset (a), age at death (b) and disease duration (c) were compared between mild, moderate and substantial meningeal scores. Kaplan-Meier survival curves showing substantial meningeal scores were associated with shorter disease duration and a younger at death (d-e). n=50, bars are median, one-way ANOVA with Tukey's multiple comparisons test (a-c).

There is a trend between extent of grey matter demyelination and grade of meningeal and perivascular inflammation

There was a trend observed between increasing meningeal inflammation and greater extents of grey matter demyelination, however, this was not statistically significant (figure 2). Cases with longer disease durations were also observed to have greater extents of grey matter demyelination, however, this was also not found to be statistically significant. Moderate but not substantial perivascular inflammation was associated with a greater extent of GM demyelination (p=0.0169). Due to the limitation of available tissue blocks per case, it is likely that this study is not sufficiently powered to determine associations between grey matter demyelination and meningeal and perivascular inflammation.

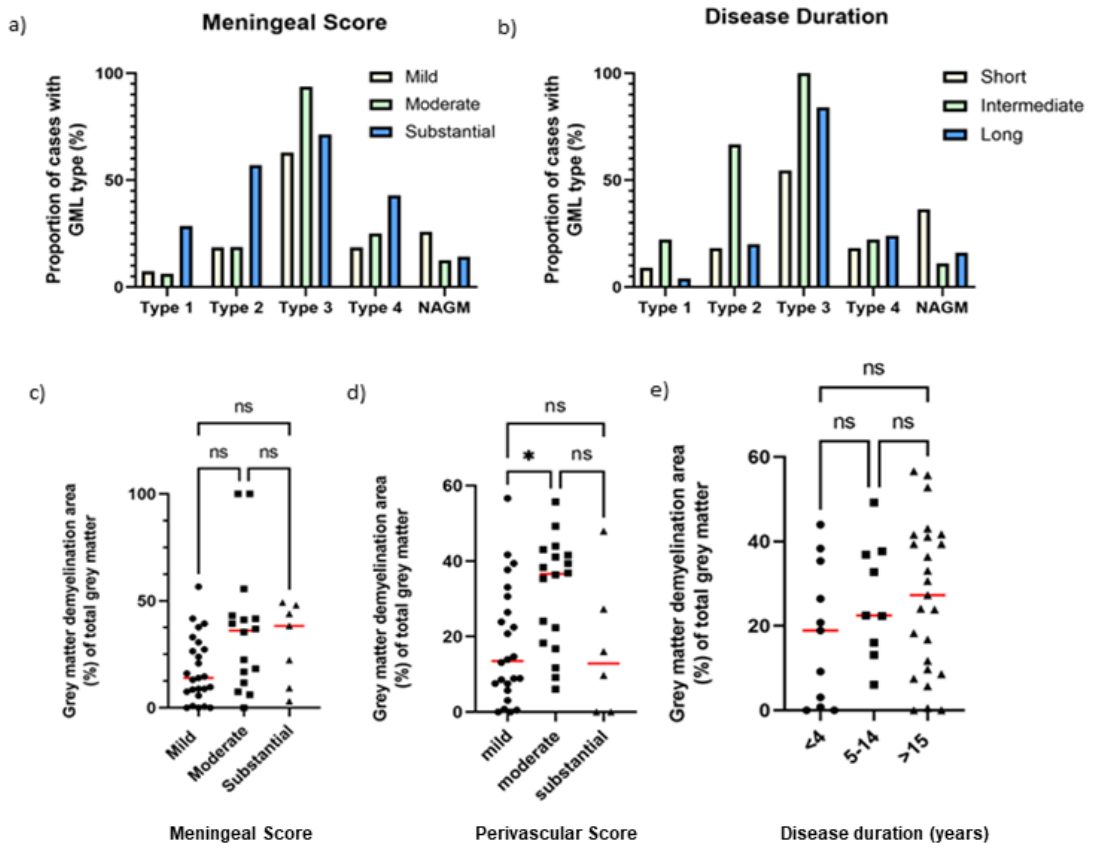

Figure 2. Grey matter lesion characterisation. Grey matter lesion types of cases based on available tissue sections, in mild, moderate and substantial meningeal inflammation (a) and disease duration cohorts (b). Percentage of grey matter lesion area in mild, moderate and substantial meningeal inflammation (c) and perivascular inflammation (d) and disease duration cohorts (e). n=50, Kruskal-Wallis with Dunn's multiple comparisons test (c), one-way ANOVA with Tukey's multiple comparisons tests (d-e) after arcsin transformation. ns, not significant

The immunoglobulin G subclasses are the highest expressed genes in lymphoid-like structures

Gene expression data of LLS and other inflammatory meningeal regions with evidence of immune cells (not in a lymphoid-like structure) were obtained. In LLS, the highest expressed genes were related to immunoglobulins (figure 3). *IGHG4* was the highest expressed gene in LLS followed by *IGHG3* and *IGHG2*.

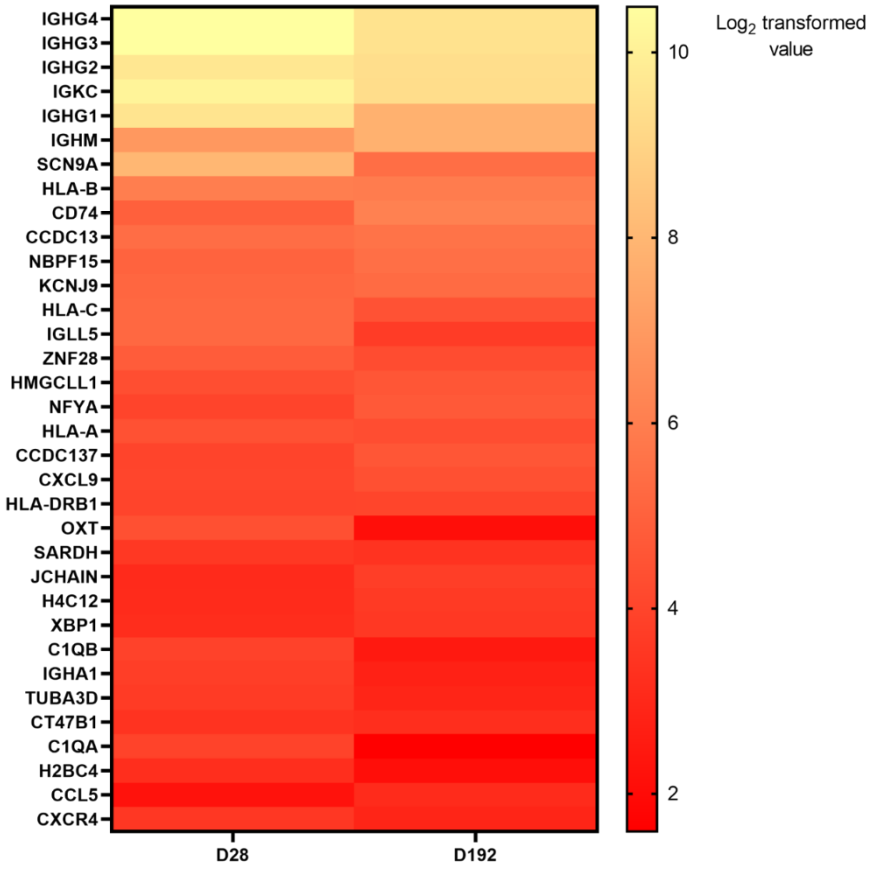

Figure 3. Highest expressed genes in lymphoid-like structures were related to immunoglobulin genes. Heatmap showing the highest expressed genes in lymphoid-like structures from two FFPE cases in the Dame Ingrid Allen cohort.

Immune cells expressing IgG3 and IgG4 in lymphoid-like structures

In LLS, a significantly higher IgG4$^+$ cell density was observed in comparison to IgG3$^+$ cell density (mean 9.75% vs mean 2.84%. There were no significant differences between IgG4$^+$ cell and IgG3$^+$ cell density in other meningeal regions, GM or WM perivascular spaces. Markers for germinal centre (GC) B cells (CD38) and plasma cells (CD138) were used to determine if GC B cells or plasma cells could be a source of IgG3 and IgG4 in the CNS. In LLS, approximately 40% of IgG3$^+$ cells co-localised with CD38$^+$ cells and approximately 20% of IgG3$^+$ cells co-localised with CD38$^+$CD138$^+$ cells (figure 4) suggesting GC B cells and plasma cells were the predominant source of IgG3. In LLS, the majority of IgG4$^+$ cells did not co-localise with CD38$^+$, CD138$^+$ or CD38$^+$CD138$^+$ cells suggesting plasma cells were not the predominant source of IgG4.

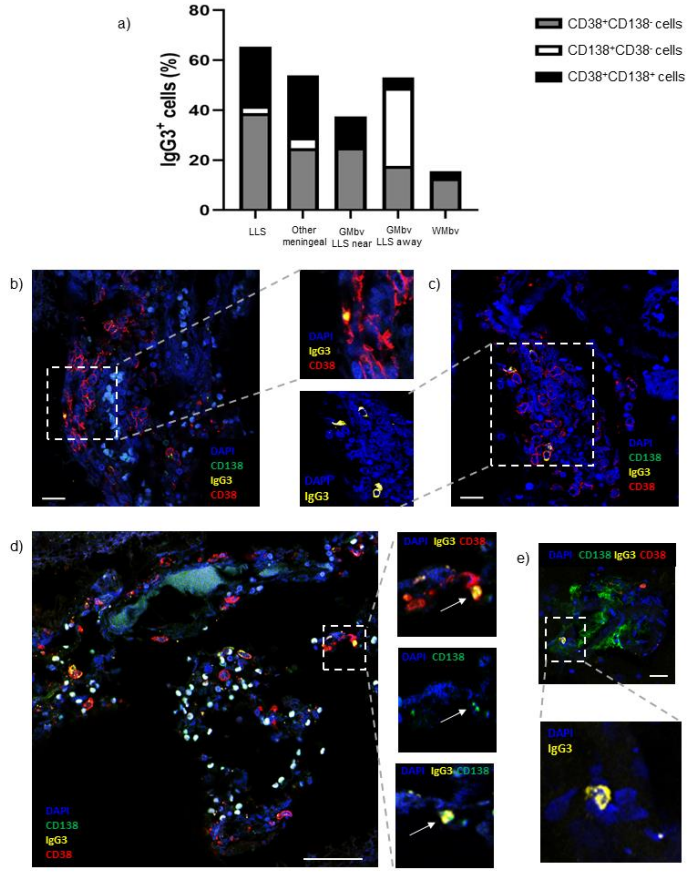

Figure 4. IgG3 co-localisation with CD38$^+$, CD138$^+$ and CD38$^+$CD138$^+$ cells. The majority of IgG3$^+$ cells co-localised with CD38$^+$, CD138$^+$ or CD38$^+$CD138$^+$ cells in LLS, other meningeal regions and GM perivascular spaces (a). Representative images from two LLS cases showing co-localisation of IgG3$^+$ cells with CD38$^+$ cells (b-c). CD38$^+$IgG3$^+$ cells and CD38$^+$CD138$^+$ IgG3$^+$ cells (arrows) were also observed in meningeal spaces (d). In WM perivascular spaces IgG3$^+$ cells were often not co-localised to CD38 or CD138 (e). Scale bar = 20 µm (b,c,e), 50 µm (d). In (a), number of cases analysed for each feature: LLS and GMbv near LLS n=5, other meningeal, GMbv distal to LLS and WMbv n=8.

Serum and CSF IgG3 and IgG4 levels

Serum IgG3 levels were similar across all cohorts and no statistically significant differences were observed (figure 5). A trend of higher serum IgG4 levels were observed in the RRMS and SPMS cohort in comparison to the control group, however, this was not statistically significant. CSF IgG3 levels were similar in the control and RRMS cohort. CSF IgG3 levels were significantly higher in the SPMS cohort in comparison to the control cohort (p=0.0153). CSF IgG3 levels had a trend to be higher in the SPMS cohort in comparison to the RRMS cohort but this was not statistically significant. There was no statistically significant difference in CSF IgG4 levels between the cohorts.

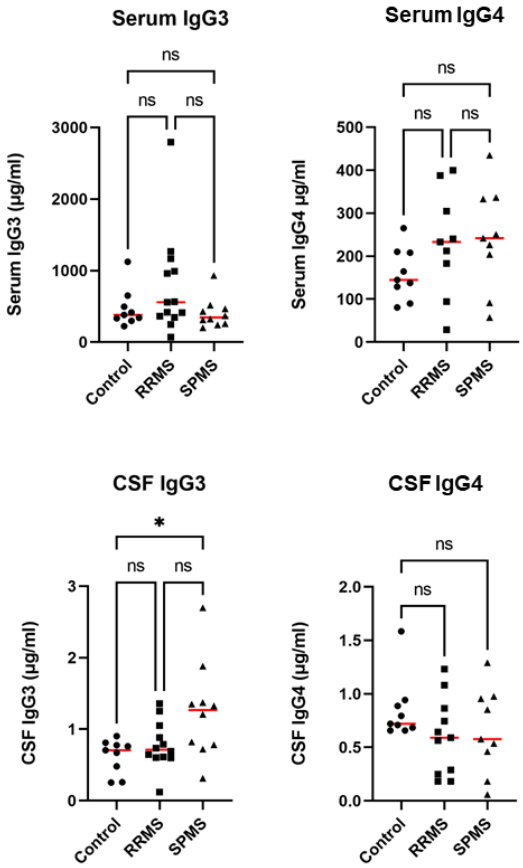

Figure 5. Serum and CSF IgG3 and IgG4 profiles. Serum and CSF IgG3 and IgG4 levels in cases with RRMS and RRMS that developed SPMS. Controls n=9, RRMS n=9-13, SPMS n=10-11. Serum IgG4 and CSF IgG3; one-way ANOVA with Tukey's multiple comparisons test. Serum IgG3 and CSF IgG4; Kruskal-Wallis with Dunn's multiple comparisons test. *p=0.0153.

6. Conclusion

Neuropathological characterisation of a Northern Ireland post-mortem MS cohort that predated DMTs, revealed an association between the presence of substantial meningeal inflammation and poorer clinical outcomes and a trend for greater grey matter pathology related to extent of meningeal and perivascular inflammation. Furthermore, screening this tissue collection for LLS identified four cases with LLS, three of which were at the earliest disease stages. The highest expressed genes in LLS were found to be *IGHG3* and *IGHG4,* a novel finding which could reveal important clues about sites of antigen presentation and antibody production in the CNS. Investigation of the serum and CSF IgG3 and IgG4 profiles identified a possible relationship between CSF IgG3 levels and the development of progressive MS. These studies suggest that LLS have a unique immune signature which may be detectable in the CSF of patients with MS and pave a way for future studies seeking to find biomarkers that can better stratify patients who are at greater risk of progressive disease.

7. Acknowledgments

Firstly, I would like to acknowledge the funders of this research, HSC R&D Division, Public Health Agency for giving me an opportunity to undertake this research. Fundamental to this research project was the work of the late Professor Dame Ingrid Allen and her team, who established a globally unique tissue collection which has facilitated seminal advances in the field of neuropathology. The high standard and attention to detail invoked by Professor Dame Ingrid Allen was fundamental for the successful application of transcriptomic technologies in this research and led to the discovery of LLS gene expression in the earliest disease stages of MS. I would also like to acknowledge my supervisors Professor Denise Fitzgerald, Dr Owain Howell and Dr Gavin McDonnell for their support, dedication and mentorship. Thank you to Dr Michelle Naughton (assistant supervisor) for providing me with expert guidance and training so that I could develop the skill set required to undertake this laboratory based research. Thank you also to the MS research group and immunology research cluster in the Wellcome-Wolfson Institute for Experimental Medicine, QUB for their technical expertise, help with experiments and providing learning opportunities from the breadth and wealth of their experience. I would also like to acknowledge the collaborators on this research grant Dr David Simpson and Dr Guillermo Lopez Campos who provided advice about transcriptomic studies. The advice and support from the imaging core technical unit, in particular Dr Ileana Micu and Dr Ryan Delaney was also instrumental in delivering this research project. I would also like to acknowledge the support of other awards that helped apply cutting-edge technologies within this research and elevate the capabilities of what we could achieve; The Lavinia Boyce Postgraduate Research Scholarship (awarded to RK), Nanostring digital spatial profiling award (awarded to MN), Biotechne Spatial Validation grant (awarded to RK), Emily Sarah Montgomery Travel Scholarship (awarded to RK) and the British Society for Immunology Career Enhancing grant (awarded to RK).

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