Supplementary Data 1. Systemic Biomarker Summary

**Oligoclonal bands**

During inflammatory processes including MS, B cells are believed to migrate into the CNS to produce immunoglobulins. IgG is typically the most abundant immunoglobulin in CSF, and detection of a specific oligoclonal IgG profile in CSF that is not apparent in serum is the main biochemical biomarker for MS because it indicates intrathecal synthesis of IgG.\(^1\) Oligoclonal bands (OCBs) are detected frequently in MS patients and serve as a supportive diagnostic biomarker for disease. Oligoclonal bands are detected less frequently in humans with the fulminant forms of MS than classic MS. Oligoclonal bands have been identified in dogs with MUO at a significantly higher percentage than other neurological diseases, supporting an inflammatory B cell response, however oligoclonal bands have not been evaluated specifically in dogs with NME.\(^2\)

**Kappa free light chain (k-FLC) Neurofilament Light (NfL) Chain**

\(\kappa\)-Free Light Chain (\(\kappa\)-FLC) and Neurofilament light (NfL) chain have emerged as biomarkers in MS.\(^3\) Free light chain analysis within CSF reflects the intrathecal synthesis of immunoglobulins, and elevated levels of \(\kappa\)-FLC have been associated with increasing disability in humans with MS.\(^3\) Approximately 50\% of people without OCB in the CSF have elevated \(\kappa\)-FLC, and \(\kappa\)-FLC can be used to predict early MS disease activity.\(^1,3\) Neurofilament light chain concentrations reflect axonal damage and show a good correlation with early MS disease activity.\(^3\) Elevated NfL concentrations have been identified in dogs with MUO and may have promise as a diagnostic biomarker and indication of response to therapy; similar changes in dogs with NME have yet to be evaluated.\(^4\)

**Cytokine profiling**

In neuroinflammatory diseases including MS, leukocytes are believed to invade the CNS and trigger the release of inflammatory mediators.\(^5\) Immune-mediated reaction by CD4+ specific pro-
inflammatory T helper 1 (Th1) and Th17 cells are thought to induce inflammation via release of various cytokines including interleukins (IL), interferons (IFN) and tumor necrosis factors (TNF). A variety of inflammatory cytokines including IL-1β, IL-6, IFN-γ, IL-23 and IL-17 are elevated in the blood of humans with MS. 

Blood leukocyte and cytokine profiling in Pug dogs asymptomatic for NME demonstrated lower CD4+ T lymphocyte and higher IL-10 concentrations in Pugs at increased genetic risk to develop NME. The majority of these dogs were beyond the expected age to develop NME, suggesting this anti-inflammatory profile may have been protective in older genetically at-risk dogs that did not develop NME. Cytokine profiling in young Pugs across genetic risk for NME showed no statistical difference between low risk and at-risk Pugs and no statistical difference between normal Pugs and those with early NME, however one Pug with early NME demonstrated marked elevations in pro-inflammatory cytokines (IFN-γ, TNF-α, IL-6) that improved with MSC treatment and is the subject of ongoing research.

Evaluation of mRNA expression of T helper cells and related cytokines and chemokines in dogs with NME has shown increased expression of the Th1 proinflammatory cytokine IFN-γ in NME compared to NLE and GME and elevated chemokine receptor 3 (CXCR3), a receptor expressed primarily by activated T cells producing large amounts of IFN-γ, suggesting the important role of Th1-related immune responses in the pathogenesis of NME. IL-17 has also been identified as a pro-inflammatory cytokine of interest in MUO but has yet to be studied for NME specifically. CSF cytokine profiles are complex with a variety of reported patterns in humans with MS; they may show promise in future NME studies.

**T-cell receptor repertoire**

The major histocompatibility complex (MHC) is crucial for shaping adaptive immune responses, and the MHC haplotype constitutes a major risk factor for MS. In humans, HLA-class II molecules on antigen presenting cells present peptide antigen to CD4+ T cells, resulting in the selection and proliferation of distinct lymphocyte clones. Each T-cell clone expresses a unique T cell receptor (TCR)
that can recognize the target peptide presented by the MHC. In MS, CNS antigens are presented by HLA molecules, which drive the selection of auto-reactive lymphocyte clones. Given the central role of T cells in the pathology of MS, it is pertinent to understand the shape and dynamics of the TCR repertoire. TCR repertoire differences have been identified between MS patients and healthy controls and certain TCR clones have been associated with a protective role in individuals with a specific HLA-DRB1 haplotype. Changes in TCRβ repertoire has also been identified in response to immunomodulatory therapy in humans with MS, indicating they may be a useful monitoring tool. Confounding factors for identifying meaningful TCR signatures are the enormous diversity of TCR repertoires and the fact that a unifying autoantigen may not exist in MS. Given the fact that samples are more readily accessible in dogs than in humans, the dog might represent a worthwhile model species to contribute to our understanding of TCR repertoire dynamics and lymphocyte trafficking in neuroinflammatory diseases. Potential alterations in TCR repertoire are currently being explored by our group in Pugs with early and classic NME compared to healthy controls.

**Anti-astrocyte antibodies**

Serum Glial Fibrillary Acid Protein (s-GFAP), an intermediate astrocyte cytoskeletal protein, can correlate with degree of disability and be used to differentiate progressive MS from RRMS. Anti-astrocyte antibodies against GFAP are less reliable in Pugs with NME, as they have been found in Pugs with NME, healthy Pugs, and dogs with other neuroinflammatory diseases and neoplasia, suggesting GFAP elevations may result from damaged astrocytes during disease and have a lesser role in the pathogenesis of disease. Transglutaminase 2 (TG2), a protein within astrocytes, has also been identified as an auto-antigen in CSF, however there is poor specificity between NME, NLE and GME.

**Glutamate**

Glutamate plays a role in the pathology of MS by damaging neurons and oligodendrocytes through excitotoxic mechanisms, and CSF glutamate levels correlate with MS disease severity and
Elevated glutamate has identified in dogs with NME which may be attributed in part to reduced expression of the excitatory amino acid transporter EAAT2. The significance of this finding is unclear but it may contribute to brain pathology and intractable seizures in NME patients.

**Lactate**

Impaired mitochondrial functioning may increase anaerobic metabolism and drive neurodegeneration in MS and measurement of serum lactate has been proposed as an easy and inexpensive means to monitor disease progression and response to therapy. Elevations in blood and CSF lactate have been identified in dogs with CNS inflammatory disease and lactate has potential as a prognostic indicator for MUO; NME has not been evaluated specifically.

**Proteomics**

Proteomics is the study of the composition of proteins detected in a biospecimen. Because proteins/peptides are the effector molecules in biological processes, proteomics may provide a better understanding of pathological processes than genomics. Several potential protein biomarkers have been identified that may show promise in the diagnosis and monitoring of progression and response to therapy in humans with MS. Proteomics are emerging in veterinary medicine; blood and CSF proteomic analysis may show promise in the early detection and management of NME and other immune-mediated neuroinflammatory diseases.

**Fecal microbiome**

Several studies have explored the relationship between the gut microbiome and the development and progression of MS. General conclusions are that humans with MS have a decreased abundance of bacteria with immunomodulatory properties, and the relative number of some microbiota correlates positively or negatively with the degree of disability in some MS patients. Bacterial abundance in MS patients has also been associated with pro-inflammatory leukocyte and cytokine profiles.
transplant has shown promise to alter the gut microbiome by increasing anti-inflammatory butyrate-producing bacteria, reducing intestinal permeability, and improving clinical signs in humans with MS. Prevotellaceae was found to be significantly less abundant in dogs with MUO, supporting the suggestion that high abundance of Prevotellaceae is associated with a lower risk for immune-mediated neuroinflammatory disease. Comparison of fecal microbiome in healthy Pugs compared to Pugs with early and classic NME may provide useful information about the potential contribution of altered gut microbiome in the development and progression of NME.


