Protective Associations of HDL with Blood Brain Barrier Injury in Multiple Sclerosis Patients

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Running Head: Lipids and Blood Brain Barrier Breakdown in MS


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ABSTRACT

Purpose: To investigate the associations of serum cholesterol and apolipoproteins with measures of blood brain barrier (BBB) permeability and central nervous system (CNS) inflammation following the first clinical demyelinating event.

Methods: This study included 154 patients (67% female, age: 29.5 ± SD 8.2 years) enrolled in a multi-center study of interferon beta-1a (IFN) treatment following the first demyelinating event. Blood and cerebrospinal fluid (CSF) were obtained at screening prior to treatment. A comprehensive serum lipid profile and multiple surrogate markers of BBB breakdown and CNS immune activity were obtained.

Results: Higher levels of serum high-density lipoprotein cholesterol (HDL-C) and apolipoprotein A-I (ApoA-I) were associated with lower CSF total protein level, CSF albumin level, albumin quotient and CSF IgG level (all $p \leq 0.001$ for HDL-C and all $p < 0.01$ for ApoA-I). HDL-C was also associated with CSF CD80+ ($p < 0.001$) and with CSF CD80+CD19+ ($p = 0.007$) cell frequencies.

Conclusions: Higher serum HDL is associated with lower levels of BBB injury and decreased CD80+ and CD80+CD19+ cell extravasation into the CSF. HDL may potentially inhibit the initiation and/or maintenance of pathogenic BBB injury following the first demyelinating event.
INTRODUCTION

Blood brain barrier (BBB) injury creates a permissive environment for inflammation and the extravasation of immune cells (1) in multiple sclerosis (MS), a chronic inflammatory and neurodegenerative disease of the CNS (2). Compromised BBB structural integrity is necessary for the formation of contrast-enhancing lesions (CEL), which are frequently found on brain MRI from MS patients even in the absence of clinical relapses. CEL represent focal areas with compromised BBB structural integrity and are associated with parenchymal and meningeal inflammation (3). Increased BBB water permeability precedes the appearance of CEL and is pervasive in normal-appearing white matter (4).

In CNS regions with loss of BBB structural integrity, there is increased permeability to macromolecules such as proteins, albumin and immunoglobulins that are normally excluded by the BBB. These macromolecules enter more readily into the CNS and can be detected in CSF. CSF levels of proteins produced exclusively outside the CNS, such as albumin, provide useful surrogate measures of the pathological injury to BBB structural integrity. The presence of T and B cells and immunoglobulins in CSF resulting from extravasation through the BBB provides surrogate measures of immune activity in the CNS. The presence of immunoglobulin oligoclonal bands (OCB) in CSF is used as a paraclinical diagnostic criterion in MS (5).

Chronic hypercholesterolemia can promote exaggerated immune responses, stronger leukocyte-vascular endothelial cell adhesion and immune cell extravasation in the microvasculature (6, 7). In this research, we leverage a range of CSF measures to understand the contributions of lipid profile variables to pathophysiological increases in BBB permeability and to immune activity in CSF. We focused on serum cholesterol profiles because there is an emerging body of data suggesting associations between serum cholesterol profiles and MRI measures of lesional and neurodegenerative injury in MS patients (8-13). In our previous work we found an adverse association between greater serum cholesterol and MS disease progression. Higher low-density lipoprotein cholesterol (LDL-C) and total cholesterol (TC) levels were
positively associated with worsening disability measured on the Expanded Disability Status Scale (EDSS) and MS Severity Scale (MSSS) (10, 13). Higher HDL levels were associated with fewer CEL (9, 13). We also investigated the role of cholesterol profiles in patients following the first demyelinating event, prior to disease-modifying treatment, and found that greater LDL-C, TC and ApoB levels were associated with greater number of T2 lesions after 2-years (12, 14). The main goals of this study are to investigate the associations of cholesterol and apolipoprotein levels with CSF-derived measures of increased BBB permeability and cellular and humoral immune activity.
METHODS

Study Population

Study Setting: Multi-center, prospective, longitudinal observational study.

Informed Consent: The Medical Ethics Committees of the General University Hospital and 1st Faculty of Medicine of Charles University, Prague, Czech Republic, approved the study protocol and the informed consent procedure. Additionally, approvals were obtained from local medical ethics committees of all other participating centers. Written informed consent was obtained from all patients at enrollment.

Clinical Study Design: The Observational Study of Early Interferon beta 1-a Treatment in High Risk Subjects after Clinically Isolated Syndrome (CIS) (SET study, clin.gov # NCT01592474) and its design have been previously described (15, 16). The study was coordinated by Charles University in Prague, Czech Republic. The study screened 259 patients and enrolled 220 patients from 8 Czech Republic MS centers.

Inclusion Criteria: Patients with the following characteristics: 18-55 years of age, enrolled within 4 months from the clinical event, presence of ≥ 2 T2-hyperintense lesions on diagnostic MRI, and presence of ≥ 2 OCB in CSF obtained prior to corticosteroid treatment and Expanded Disability Status Scale (EDSS) ≤ 3.5.

This sub-study included 154 patients for whom lipid profiles and CSF-derived measures of increased BBB permeability were available.

Treatments: All patients were treated with 3-5 g of methylprednisolone for the first symptom and baseline MRI was performed ≥ 30 days after steroid administration.

All patients were started on 30 µg, once-weekly, intramuscular interferon beta 1-a (AVONEX®) treatment at baseline.
MRI Acquisition and Analysis

This sub-study was limited to MRI measures obtained at baseline. MRI methods are summarized in Supplementary Methods. We investigated the associations of the number of CEL and T2 lesion volume (LV) at baseline with CSF measures of BBB permeability in statistical analyses.

Serum Lipids and Apolipoproteins

Serum for lipid and apolipoprotein analyses was obtained in the non-fasted state at the screening visit prior to the start of corticosteroid or interferon. The methods for lipid profile and apolipoprotein analyses have been previously described (14) but are succinctly recapitulated here.

Immunoturbidimetric diagnostic kits (Kamiya Biomedical, Thousand Oaks, CA) were used for the apolipoprotein (AI, AII, B and E), Lpa and high sensitivity C-reactive protein (CRP) assays. Diagnostic reagent kits (Sekisui Diagnostics, Lexington, MA) were used to measure serum total cholesterol, HDL-C, phospholipids and triglycerides. These assays were conducted on an automated chemistry analyzer (ABX Pentra 400, Horiba Instruments, Irvine, CA). The coefficient of variation of these assays is < 5%. LDL-C was obtained from the Friedewalde equation (17).

Specific probes for both single nucleotide polymorphisms rs7412 and rs429358 (OpenArray, Applied Biosystems, Life Technologies, Foster City, California, USA) were used to genotype APOE gene variants ε2, ε3 and ε4.

The arylesterase and paraoxonase activities of the human serum paraoxonase-1 (PON1) enzyme were measured using phenyl acetate (arylesterase activity) and paraoxon (paraoxonase activity) as substrates, respectively. The assay CV is 0.6 to 1.4%. The PON1 Q192R polymorphism was obtained from the paraoxonase and arylesterase activities as previously described (18).

Clinical data collected included height and weight for body mass index (BMI) calculations, and history of statin use.
Cerebrospinal Fluid Assays

Lumbar Punctures: All lumbar punctures were performed prior to treatment with corticosteroids at the study-coordinating center during the morning hours. CSF was drawn from L5-S1, L4-5 or L3-4 interspace with the patient sitting upright using a standard sterile preparation and 20G Sprotte atraumatic needle. A total of 20-25 ml of CSF and 5-ml volume of blood were obtained.

Biochemical, Immunological and Cellular Assays: Total protein in CSF was determined photometrically using the pyrogallol red-molybdate reaction method (Synchron LX 20, Beckman Coulter analyzer). Albumin, immunoglobulin G (IgG) and immunoglobulin M (IgM) concentrations were quantified in serum and CSF by immunonephelometry (IMMAGE Immunohistochemistry system, Beckman Coulter).

The albumin quotient \( Q_{Alb} \) was defined as the ratio of the CSF albumin concentration to the serum albumin concentration (19, 20):

\[
Q_{Alb} = \frac{\text{CSF Albumin (mg/L)}}{\text{Serum Albumin (g/L)}}
\]

The IgG Quotient \( Q_{IgG} \) and IgM Quotient \( Q_{IgM} \) were analogously defined as the ratio of CSF IgG or CSF IgM concentration to their corresponding serum IgG or IgM concentrations:

\[
Q_{IgG} = \frac{\text{CSF IgG (mg/L)}}{\text{Serum IgG (g/L)}}
\]

\[
Q_{IgM} = \frac{\text{CSF IgM (mg/L)}}{\text{Serum IgM (g/L)}}
\]

The IgG index and IgM index, which can be used to assess CSF IgG and IgM synthesis (21), were obtained using the following:

\[
\text{IgG Index} = \frac{\text{CSF IgG (mg/L)/Serum IgG (g/L)}}{\text{CSF Albumin (mg/L)/Serum Albumin (g/L)}} = \frac{Q_{IgG}}{Q_{Alb}}
\]
IgM Index = \frac{CSF \ IgM (mg/L)/Serum \ IgM (g/L)}{CSF \ Albumin (mg/L)/Serum \ Albumin (g/L)} = \frac{Q_{IgM}}{Q_{Alb}}

Isoelectric focusing (IEF) with ultra-sensitive immunofixation (Sebia, Hydrasys Focusing) was used to identify CSF-restricted OCB.

**CSF Cell Phenotyping:** Cell surface markers were measured using flow cytometry within 3 hours following CSF collection. Cells were concentrated by centrifugation (5 min at 1000 rpm), resuspended in BD Cell-Wash (BD Biosciences) and stained without lysing with fluorochrome-labeled antibodies for 20 minutes in the dark at room temperature. After washing (twice in BD Cell-Wash), the CSF lymphocytes were immediately analyzed without fixation.

Fluorochrome-labeled antibodies against CD80, CD80CD19, CD4, CCR5 (all from Becton-Dickinson Biosciences, San Jose, CA, USA) and CXCR3 (R&D Systems, Minneapolis, MN, USA) antigens were used. Six-color flow cytometric analysis was performed with FACSCanto flow cytometer and BD FACS Diva 5.03 software (BD Biosciences). For FACS analyses, 3,000-10,000 events were acquired at a fluid flow rate of 60 µl/min. The frequencies of CD80+, CD80+CD19+, CD4+, CCR5+ and CXCR3+ cell subsets in CSF were computed for analyses.

CSF cell subset immunophenotyping data were available for 81 patients.

**Data Analysis**

SPSS (IBM Inc., Armonk, NY, version 19.0) statistical program was used. In view of the multiple testing, the Benjamini-Hochberg method was used to assess significance with a target false discovery rate of $q \leq 0.05$ (22). The Tables and Results summarize the raw, unadjusted $p$-values. Adjusted $p$-values ($q$-values) are shown only for variables with unadjusted $p$-values $\leq 0.05$.

All CSF variables were logarithm (base 10) transformed to reduce skew. T2-LV was cube root transformed.
The associations of CSF variables with lipid profile variables (HDL-C, LDL-C, TC, ApoA-I, ApoA-II, ApoB, ApoE, CRP or PON1 arylesterase activity) were assessed in linear regression analyses. The CSF variable of interest was the dependent variable whereas the individual lipid profile variable of interest, age, gender and BMI were treated as predictor variables in these analyses.

Negative binomial regression was used to assess associations of lipid profile variables with CSF cell frequency variables (CD80+, CD80+CD19+, CD4+, CCR5+ and CXCR3+). Individual CSF cell frequency variables were treated as the dependent variable with the individual lipid profile variable of interest, age, gender, and BMI as predictors.

The associations of CEL number and T2-LV were individually assessed as dependent variables in negative binomial regression and linear regression, respectively. The CSF variable of interest, age and gender were treated as predictor variables.
RESULTS

Demographic and Clinical Characteristics: The clinical, demographic and MRI characteristics of the study sample at baseline and the CSF measures and lipid profile variables at screening are summarized in Table 1.

The mean time ± SD between disease onset and lumbar puncture was 28.2 ± 23.2 days (median = 20.0, inter-quartile range = 34 days). None of the subjects were on statins.

The clinical and demographic characteristics of subjects with lipid profile and CSF measures who were included in the study were similar to the SET study sample that was not included (data not shown).

Associations of CSF Variables with Lipid Profile Variables: Table 2 summarizes the associations of CSF variables with the serum cholesterol variables (HDL-C, LDL-C, TC), serum apolipoprotein variables (ApoA-I, ApoA-II, ApoB, ApoE) and CRP.

Greater HDL-C and TC levels were associated with lower CSF total protein level, CSF albumin level, albumin quotient and CSF IgG level (Table 2). Additionally, TC was negatively associated with alkaline OCB (23) \( p = 0.003, q = 0.007 \). The CSF variables that were negatively associated with increased TC were also negatively associated with LDL-C with the exception of albumin quotient \( q = 0.053 \).

ApoA-I was associated with the same CSF variables as HDL-C. This provides corroborative support for the HDL-C findings. ApoA-II was associated with CSF IgG levels \( p = 0.003, q = 0.036 \) but no other CSF measures. ApoB and CRP were not associated with any of the CSF variables.

The associations of albumin quotient, IgG Index, IgM Index and CSF leukocytes with HDL-C and ApoA-I are summarized in Figure 1 and Figure 2, respectively.

Human serum Paraoxonase-1 (PON-1), a hydrolytic enzyme exclusively associated with HDL-C, exhibits a genotype-independent arylesterase activity and a paraoxonase activity that depends on its Q192R genotype. To further confirm that HDL was associated with protective associations on CSF measures of
BBB permeability, we examined the corresponding associations of PON1 arylesterase activity. Higher PON1 arylesterase activity was associated with lower albumin quotient \((p = 0.015)\), CSF total protein level \((p = 0.014)\), CSF albumin level \((p = 0.015)\) and CSF IgG level \((p = 0.001)\). The results from this analysis provide additional evidence for the associations between HDL-C and CSF variables.

To assess the relative importance of HDL-C and LDL-C to the associations with CSF variables, we conducted additional regression analyses that included both HDL-C and LDL-C as predictors. In these analyses, the associations of HDL-C with BBB variables remained significant but LDL-C was no longer significant.

Based on the corroborating evidence from the additional regression analyses and the ApoA-I, PONI arylesterase activity and ApoB results, we surmise that the negative associations of TC with albumin quotient, CSF total protein and CSF albumin levels are the result of a salient contribution from HDL-C and that the contributions from LDL-C are potentially secondary in comparison. The negative associations of alkaline OCB with TC are mediated primarily by LDL-C. Both HDL-C and LDL-C appear to contribute to the negative associations of TC with CSF IgG (Table 2).

ApoE levels (Table 2) were negatively associated with CSF total protein level \((p = 0.003, q = 0.012)\), CSF albumin \((p = 0.002, q = 0.012)\) level, albumin quotient \((p = 0.001, q = 0.012)\) and CSF IgG level \((p = 0.027, q = 0.065)\). The APOE4 allele was present in 21.8% of the patient group. However, there were no significant associations of APOE4 genotype status with any of the CSF variables.

**Associations of Lipid Profile Variables with Cell Variables:** The associations of cell variables (CD80+, CD80+CD19+, CD4+, CCR5+ and CXCR3+) with the serum cholesterol variables (HDL-C, LDL-C, TC), serum apolipoprotein variables (ApoA-I, ApoA-II, ApoB, ApoE) and CRP were assessed. The Wald \(\chi^2\) and \(p\)-values from the regressions are shown in Table 2.

HDL-C was associated with CSF CD80+ cell frequencies \((p <0.001, q = 0.002)\) and with CSF CD80+CD19+ cells \((p = 0.007, q = 0.028)\). HDL-C was not associated with CSF CD4+, CCR5+ or
CXCR3+ cells frequencies. Similarly, ApoA-I was associated with CSF CD80+ ($p = 0.001$, $q = 0.004$) and CD80+CD19+ ($p = 0.015$, $q = 0.04$) frequencies. ApoA-I levels were not associated with CSF CD4+, CCR5+ and CXCR3+ cell frequencies. ApoA-II levels were not associated with any of the immune cell subset frequencies.

Interestingly, only CD80+CD19+ cell frequencies were associated with ApoB ($p < 0.001$, $q < 0.001$). None of the immune cell subset frequency variables were associated with LDL-C, TC, ApoE or CRP. The associations of CD80+ and CD80+CD19+ cells frequencies and lipid variables HDL-C, ApoA-I and ApoB are summarized in Figure 3.

The regression results for the cell frequency and lipid profile variables suggest that higher HDL-C levels results in less extravasation of CD80+ and CD80+CD19+ cells into the CSF. There was no evidence for associations of HDL-C levels with CD4+, CCR5+ or CXCR3+ frequencies in CSF.

**Associations of CSF Variables with MRI:** We investigated whether CSF measures were associated with baseline CEL number and T2-LV to assess the potential clinical relevance of altered BBB permeability and immune activity. The regression analyses are summarized in supplementary Table 1.

The mean time ± SD between disease onset and baseline MRI was 82.5 ± 23.6 days (median = 79.0, interquartile range = 37.5 days).

CEL number was associated with all but one of the CSF variables: total protein level, albumin level, albumin quotient, IgG level, IgM level, IgM quotient and IgM index (all $p < 0.001$, $q < 0.001$), as well as IgG quotient ($p = 0.007$, $q = 0.008$), total OCB ($p = 0.004$, $q = 0.005$), alkaline OCB ($p = 0.003$, $q = 0.005$) and leukocytes ($p = 0.017$, $q = 0.019$). CEL number was not associated with IgG index. The associations of albumin quotient and CSF leukocytes with CEL number and T2-LV are summarized in Figures 4A and 4C, respectively.

Only a subset of the CSF variables associated with CEL number were associated with T2-LV. CSF total protein level ($p = 0.016$, $q = 0.048$), albumin level ($p = 0.014$, $q = 0.056$), albumin quotient ($p = 0.006$, $q$
= 0.072), CSF IgG level ($p = 0.017, q = 0.041$) and IgG quotient ($p = 0.012, q = 0.072$) were associated with T2-LV. The associations of albumin quotient and CSF leukocytes with T2-LV are summarized in Figures 4B and 4D, respectively. We did not find evidence for associations for CSF IgM level, IgM quotient, IgM index, total OCB, alkaline OCB and leukocytes with T2-LV.

These results are consistent with a role for increased BBB permeability (as assessed by albumin quotient) and CSF humoral immunity (as assessed by OCB and IgG) in CEL formation following the first demyelinating event.
DISCUSSION

We investigated the role of cholesterol and apolipoproteins in BBB breakdown following the first demyelinating event suggestive of MS. Greater HDL-C was associated with less BBB permeability as assessed by several CSF measures including CSF albumin quotient. High levels of HDL-C and ApoA-I were associated with lower CSF frequencies of CD80+ and CD80+CD19+ cells. To our knowledge, the associations of cholesterol and cholesterol biomarkers with BBB breakdown have not been extensively investigated.

Although our results demonstrate protective associations between greater HDL-C levels and lower BBB permeability, the strengths and limitations of our study design merit discussion. The SET study was a well-controlled longitudinal, treatment study that accrued a wealth of clinical MRI and blood biomarkers over a four-year period. However, a potential criticism of the study design is the lack of a placebo-controlled group. A four-year placebo-controlled trial would clearly be ethically infeasible given that proven disease-modifying therapies are now available for MS. However, it would have been useful to compare interferon treatment in the SET trial to a different treatment such as glatiramer acetate to establish whether our results were generalizable to other MS disease-modifying treatments. Our study was also limited because the lipid profile and CSF measures were obtained only at screening making this analysis cross-sectional in nature. Although our statistical results are consistent with a protective role for high HDL levels against BBB injury after the first demyelinating event these associations are not proof of causation.

Our study would be further strengthened if we had also obtained cholesterol and lipoprotein profiles in the CSF. The CSF from SET study patients at the screening visit was primarily used to assess oligoclonal band status of patients for meeting inclusion criteria. Additionally, measures of BBB breakdown and immune cell extravasation were also obtained. Although the CNS and peripheral cholesterol compartments are relatively segregated by the BBB, there is evidence for regulatory interactions and
homeostatic mechanisms. Glial cells play a critical role in cholesterol production and homeostasis in the brain where an HDL-like particle containing ApoE mediates cholesterol transport. The ApoE that comprises this HDL-like particle is secreted by astrocytes and microglia. Although ApoA-I is not produced in the brain, recent evidence suggests that circulating ApoA-I can enter the CSF through the choroid plexus (24). The ApoA-I entering the brain is likely derived from SR-BI-mediated uptake of circulating discoidal HDL particles into CSF at the choroid plexus (24, 25). ApoA-I and ApoE measurements in CSF would have yielded information regarding these interactions between the CNS and blood compartments. It would also be interesting to know if HDL particle size, particularly small, discoidal, pre-β-HDL HDL, are more predictive of BBB integrity than total HDL-C alone.

MS is associated with significant cerebral vascular endothelial cell dysfunction (26-29). In atherosclerosis, HDL-C plays an important protective role in the immune cell-vascular endothelial interactions that mediate lesion formation. HDL can modulate immune cell phenotype by altering cellular cholesterol because it stimulates cholesterol removal from macrophages and down-regulates foam cell production. Plasma HDL-C has been found to be negatively associated with baseline monocyte counts (30). HDL-C mediated cholesterol transport also preserves active endothelial nitric oxide synthase (eNOS) dimer levels that maintain endothelial cell function (31).

The mechanisms by which these protective processes occur in MS remain unknown. Meyers et al, reported that ApoA-I levels were lower in the MS patients compared to healthy controls and the primary progressive MS group had lower levels than relapsing-remitting and secondary-progressive MS groups (32). Interestingly, in a small study of 36 Alzheimer’s disease patients, low HDL was associated with increased BBB breakdown as assessed by albumin quotient (33). This provides independent evidence, albeit from a different neurological disease, that HDL and lipid profiles can modulate BBB breakdown. However, Alzheimer’s disease is prevalent in the elderly, and 47% of the study group in (33) had metabolic dyslipidemia and 22% were on statins. In contrast, our patient group was younger and none were on statins. Results in the induced experimental allergic encephalomyelitis (EAE) animal model also
provide supporting evidence. In EAE-induced ApoA-I-deficient mice, there was increased T cell penetration into the CSF that led to an increase in demyelination (32).

We used a diverse range of CSF-derived measures of altered BBB permeability and immune activity. For example, we used the albumin quotient ($Q_{\text{Alb}}$), a calculated measure that normalizes CSF albumin levels to serum albumin. Because serum albumin is synthesized in the liver, any albumin present in CSF enters via CNS regions of increased BBB permeability. In contrast, immunoglobulins can be synthesized in the CSF by extravasating B cells in addition to entering the CSF from blood at regions where BBB integrity is compromised. CD80+ is a co-stimulatory molecule for T cell activation that is expressed on activated B cells and monocytes, whereas CD19+ is found on B cells; CCR5 and CXCR3 are chemokine receptors that are expressed on T cells, particularly pro-inflammatory Th1 cells. Studies have demonstrated that CD80+ cells and CD19+ cells are increased in peripheral blood mononuclear cells (PBMC) in MS (34, 35). CXCR3 and CCR5 expressing T cells are found in MS lesions and are higher in PBMC of progressive MS patients (36).

Interestingly, increased HDL-C and ApoA-I were associated with lower IgG quotient; no associations were found for IgG index, IgM quotient or IgM index. We attribute the differences between IgG and IgM to the approximately 5-fold higher molecular weight of IgM (approximately 970 kD vs. 150 kD for IgG) and lower prevalence of intrathecal synthesis of IgM in MS patients.

Weinstock-Guttman et al. (13) found that higher HDL-C levels were associated with decreased CEL activity in a large group of MS patients whereas Giubilei et al. (8) found a similar association in clinically isolated syndrome patients. The protective associations of the serum HDL compartment with CSF measures of BBB integrity were confirmed via three different HDL biomarkers, viz., HDL-C, ApoA-I and PON1 arylesterase activity. ApoA-I is the characteristic protein of HDL-C that mediates its important interactions with other lipoproteins and cells. ApoA-I was associated with all of the same BBB permeability measures and CSF immune cell subset frequencies as HDL-C. In contrast to ApoA-I, ApoA-II has only secondary supporting roles in HDL functionality (37). ApoA-II is not as anti-atherogenic as
ApoA-I and its associations with the risk of cardiovascular disease are considered weaker and more controversial than ApoA-I (38). These physiological and clinical findings related to ApoA-II provide the context for understanding the lack of ApoA-II associations with CSF measures. Taken together, the concordance of our HDL-C and ApoA-I results provides support for potentially protective roles for the HDL compartment on the CSF measures of altered BBB permeability (e.g. albumin quotient) and immune activity (e.g., CD80+ and CD80+CD19+ cell frequencies in the CSF). The TC associations with CSF measures are the result of key contributions from HDL.

In additional analyses (data not shown), we found that $Q_{Alb}$ was associated with clinical disability progression and with brain MRI measures of lesional injury and neurodegeneration. Higher baseline $Q_{Alb}$ was associated with increased EDSS over the 4 years following the initial demyelinating event. These findings suggest that CSF measures may have prognostic importance in MS.

Multiple groups have independently reported that high LDL-C and TC levels are associated with increased disability and T2 lesion burden (8, 10-13). ApoB, the characteristic protein of LDL-C, was associated with greater CSF CD80+CD19+ cell frequency suggesting a possible role for the LDL compartment in promoting extravasation, proliferation or survival of CD80+CD19+ cells in the CSF milieu. We surmise that while the HDL compartment is important for protecting against increased BBB permeability at the earliest stages of MS, the LDL-C and TC compartments are more important to the subsequent processes that promote T2 lesion burden.

Our results indicate a protective role for HDL-C in the pathophysiological BBB injury that precedes the formation of MS lesions. The findings are consistent with the intriguing possibility that loss of BBB structural integrity is nucleated in membrane sub-domains actively involved in cholesterol homeostasis or at pathophysiologically dyslipidemic tissue regions.
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AUTHOR CONTRIBUTIONS

Kelly Fellows – Data acquisition and analysis, manuscript drafting.

Tomas Uher – Data acquisition and analysis, manuscript drafting.

Richard W. Browne – Data acquisition.

Bianca Weinstock-Guttman, – Data interpretation, manuscript preparation

Robert Zivadinov – MRI data analysis, manuscript preparation

Dana Horakova – Clinical study design, data acquisition and interpretation, manuscript preparation.

Helena Posova – Flow cytometry and immunological data acquisition

Manuela Vaneckova – Data acquisition

Zdenek Seidl – Data acquisition

Jan Krasensky – Data acquisition

Michaela Tyblova – Data acquisition

Eva Havrdova – Clinical study design, manuscript preparation.

Murali Ramanathan – Study concept and design, data analysis, manuscript preparation.
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FIGURE LEGENDS

Figure 1. Associations of HDL cholesterol quartiles with albumin quotient (Figure 1A), IgG Index (Figure 1B), IgM Index (Figure 1C) and CSF leukocytes (Figure 1D). The quartile boundaries were: the lowest quartile corresponds to HDL-<sub>C ≤ 56.27 mg/dl</sub>, 56.27 mg/dl < Quartile 2 ≤ 66.25 mg/dl, 66.25 mg/dl < Quartile 3 ≤ 80.69 mg/dl, Highest quartile > 80.69 mg/dl. The bars compare mean values of the CSF measures shown on the y-axis for the quartiles of HDL cholesterol shown on the x-axis. The error bars indicate the standard error of the mean.

Figure 2. Associations of apolipoprotein A-I quartiles with albumin quotient (Figure 2A), IgG Index (Figure 2B), IgM Index (Figure 2C) and CSF leukocytes (Figure 2D). The ApoA-I quartile boundaries were: the lowest quartile corresponds to ApoA-I ≤ 128.3 mg/dl, 128.3 mg/dl < Quartile 2 ≤ 151.1 mg/dl, 151.1 mg/dl < Quartile 3 ≤ 175.8 mg/dl, and the highest quartile > 175.8 mg/dl. The bars compare mean values of the CSF measures shown on the y-axis to the quartiles of ApoA-I shown on the x-axis. The error bars indicate the standard error of the mean.

Figure 3. Associations of mean CSF CD80<sup>+</sup> (top row) and CSF CD80<sup>+</sup> CD19<sup>+</sup> (bottom row) cell frequencies in percent with HDL-C quartiles, ApoA-I quartiles and ApoB quartiles. The HDL-C quartile boundaries were: the lowest quartile corresponds to HDL-C ≤ 56.27 mg/dl, 56.27 mg/dl < Quartile 2 ≤ 66.25 mg/dl, 66.25 mg/dl < Quartile 3 ≤ 80.69 mg/dl, Highest quartile > 80.69 mg/dl. The ApoA-I quartile boundaries were: the lowest quartile corresponds to ApoA-I ≤ 128.3 mg/dl, 128.3 mg/dl < Quartile 2 ≤ 151.1 mg/dl, 151.1 mg/dl < Quartile 3 ≤ 175.8 mg/dl, and the highest quartile > 175.8 mg/dl. The ApoB quartile boundaries were: the lowest quartile corresponds to ApoB ≤ 54.0 mg/dl, 54.0 mg/dl < Quartile 2 ≤ 70.75 mg/dl, 70.75 mg/dl < Quartile 3 ≤ 87.0 mg/dl, and the highest quartile > 87.0 mg/dl. The bars compare mean values of the CSF cell frequencies in percent shown on the y-axis to the quartiles of HDL-C, ApoA-I or ApoB on the x-axis. The error bars indicate the standard error of the mean.

Figure 4. Associations of albumin quotient and CSF leukocytes with CEL number and T2-LV MRI measures. The bars compare mean values of the MRI measures shown on the y-axis for the quartiles of
the CSF variable shown on the x-axis. The albumin quotient quartile boundaries were: the lowest quartile corresponds to albumin quotient \( \leq 3.36 \, \text{mg/g} \), \( 3.36 \, \text{mg/g} < \text{Quartile 2} \leq 4.25 \, \text{mg/g} \), \( 4.25 \, \text{mg/g} < \text{Quartile 3} \leq 5.58 \, \text{mg/g} \), Highest quartile > 5.58 mg/g. The CSF leukocytes quartile boundaries were: the lowest quartile corresponds to CSF leukocytes \( \leq 9.0/3 \, \mu l \), \( 10.0/3 \, \mu l < \text{Quartile 2} \leq 17.0/3 \, \mu l \), \( 18.0/3 \, \mu l < \text{Quartile 3} \leq 33.5/3 \, \mu l \), Highest quartile > 33.5/3 \, \mu l. The error bars indicate the standard error of the mean.
Table 1. Demographic and clinical characteristics at baseline. Lipid profile and CSF variable values at disease onset.

| Demographic Characteristics | Mean ± SD  
|:-----------------------------|--------:|----------:|
| Sample Size n                |        | 154      |
| % Female                     |        | 67%      |
| Age, years                   | 29.5 ± 8.2 |        |
| EDSS                         | 1.71 (0.67) |        |

<table>
<thead>
<tr>
<th>MRI Characteristics</th>
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<tr>
<td>CEL number</td>
<td>1.07 ± 3.4</td>
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<tr>
<td>T2-LV, cm³</td>
<td>5.15 ± 6.44</td>
<td></td>
</tr>
<tr>
<td>Brain volume, cm³</td>
<td>1505 ± 72</td>
<td></td>
</tr>
<tr>
<td>Gray matter volume, cm³</td>
<td>791 ± 47</td>
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</table>

<table>
<thead>
<tr>
<th>Lipid Profile Characteristics</th>
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<tbody>
<tr>
<td>HDL cholesterol (mg/dl)</td>
<td>70.3 ± 19.0</td>
<td>40-83</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dl)</td>
<td>134 ± 40.1</td>
<td>57-189</td>
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<tr>
<td>Total cholesterol (mg/dl)</td>
<td>204 ± 52.9</td>
<td>133-234</td>
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<tr>
<td>Triglycerides (mg/dl)</td>
<td>110 ± 50.9</td>
<td>37-321</td>
</tr>
<tr>
<td>Apolipoprotein A-I (mg/dl)</td>
<td>158 ± 40.6</td>
<td>115-224</td>
</tr>
<tr>
<td>Apolipoprotein A-II (mg/dl)</td>
<td>37.3 ± 8.65</td>
<td>25-35</td>
</tr>
<tr>
<td>Apolipoprotein B (mg/dl)</td>
<td>73.9 ± 23.5</td>
<td>60-130</td>
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<tr>
<td>Apolipoprotein E (mg/dl)</td>
<td>3.28 ± 1.05</td>
<td>3.3-6.1</td>
</tr>
<tr>
<td>C-reactive protein (mg/l)</td>
<td>2.77 ± 4.40</td>
<td>&lt; 2.5</td>
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<table>
<thead>
<tr>
<th>CSF Variable Characteristics</th>
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</tr>
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<tbody>
<tr>
<td>CSF-leukocytes (in 3 mm³)</td>
<td>26.5 ± 34.1</td>
<td>&lt; 12</td>
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<tr>
<td>CSF-total protein (mg/l)</td>
<td>345 ± 119</td>
<td>150-450</td>
</tr>
<tr>
<td>CSF-albumin (mg/l)</td>
<td>217 ± 86</td>
<td>120-300</td>
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<tr>
<td>Albumin quotient</td>
<td>4.75 ± 1.84</td>
<td>≤ 6.5 if age &lt;40 years</td>
</tr>
<tr>
<td></td>
<td></td>
<td>≤ 8.0 if age ≥40 years</td>
</tr>
<tr>
<td>CSF-restricted total OCB</td>
<td>12.1 ± 5.1</td>
<td>Not available</td>
</tr>
<tr>
<td>CSF-restricted total alkaline OCB</td>
<td>8.33 ± 3.7</td>
<td>Not present</td>
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<tr>
<td>IgG index</td>
<td>0.892 ± 0.39</td>
<td>&lt; 0.65</td>
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<tr>
<td>IgG quotient</td>
<td>4.21 ± 2.3</td>
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<tr>
<td>IgM index</td>
<td>0.251 ± 0.32</td>
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</tr>
<tr>
<td>IgM quotient</td>
<td>1.11 ± 1.1</td>
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</table>
a All continuous variables (age, disease duration, T2-LV, T1-LV) are mean ± standard deviation. For the ordinal EDSS, the median (inter-quartile range) is given.

b Reference range provided by diagnostic reagent kit manufacturers or clinical laboratory.
Table 2. Associations of lipid profile variables with CSF variables and cell frequencies.

<table>
<thead>
<tr>
<th>CSF Variablea</th>
<th>HDL-C</th>
<th>LDL-C</th>
<th>TC</th>
<th>ApoA-I</th>
<th>ApoA-II</th>
<th>ApoB</th>
<th>ApoE</th>
<th>CRP</th>
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<td>$r_p$</td>
<td>$p$</td>
<td>$r_p$</td>
<td>$p$</td>
<td>$r_p$</td>
<td>$p$</td>
<td>$r_p$</td>
<td>$p$</td>
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<tr>
<td>CSF total protein</td>
<td>-0.29&lt; 0.001</td>
<td>-0.23 0.005</td>
<td>-0.28 0.001</td>
<td>-0.27 0.001</td>
<td>-0.19 0.018</td>
<td>-0.13 0.13</td>
<td>-0.24 0.003</td>
<td>-0.092 0.27</td>
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<tr>
<td>CSF albumin</td>
<td>-0.26 0.001</td>
<td>-0.20 0.014</td>
<td>-0.25 0.002</td>
<td>-0.25 0.002</td>
<td>-0.19 0.018</td>
<td>-0.12 0.13</td>
<td>-0.25 0.002</td>
<td>-0.099 0.23</td>
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<tr>
<td>Albumin quotient</td>
<td>-0.27 0.001</td>
<td>-0.17 0.04</td>
<td>-0.23 0.006</td>
<td>-0.22 0.008</td>
<td>-0.18 0.028</td>
<td>-0.08 0.36</td>
<td>-0.27 0.001</td>
<td>-0.042 0.61</td>
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<tr>
<td>CSF IgG</td>
<td>-0.31 &lt; 0.001</td>
<td>-0.28 0.001</td>
<td>-0.33 &lt; 0.001</td>
<td>-0.29 &lt; 0.001</td>
<td>-0.24 0.003</td>
<td>-0.17 0.035</td>
<td>-0.18 0.027</td>
<td>-0.062 0.46</td>
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<tr>
<td>IgG quotient</td>
<td>-0.28 0.001</td>
<td>-0.28 0.001</td>
<td>-0.31 &lt; 0.001</td>
<td>-0.25 0.002</td>
<td>-0.21 0.01</td>
<td>-0.18 0.03</td>
<td>-0.21 0.01</td>
<td>-0.015 0.86</td>
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<tr>
<td>IgG index</td>
<td>-0.12 0.15</td>
<td>-0.22 0.008</td>
<td>-0.21 0.011</td>
<td>-0.14 0.088</td>
<td>-0.11 0.19</td>
<td>-0.17 0.03</td>
<td>-0.13 0.068</td>
<td>0.031 0.71</td>
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<tr>
<td>CSF IgM</td>
<td>-0.11 0.18</td>
<td>-0.14 0.087</td>
<td>-0.15 0.072</td>
<td>-0.089 0.29</td>
<td>-0.19 0.02</td>
<td>-0.095 0.26</td>
<td>-0.05 0.53</td>
<td>-0.025 0.77</td>
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<tr>
<td>IgM quotient</td>
<td>-0.05 0.53</td>
<td>-0.13 0.14</td>
<td>-0.12 0.17</td>
<td>-0.12 0.15</td>
<td>-0.16 0.053</td>
<td>-0.02 0.81</td>
<td>-0.12 0.17</td>
<td>0.006 0.95</td>
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<tr>
<td>IgM index</td>
<td>0.08 0.35</td>
<td>-0.065 0.45</td>
<td>-0.021 0.81</td>
<td>-0.02 0.82</td>
<td>-0.086 0.31</td>
<td>-0.003 0.98</td>
<td>0.004 0.96</td>
<td>0.046 0.59</td>
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<tr>
<td>Total OCB</td>
<td>-0.09 0.30</td>
<td>-0.18 0.027</td>
<td>-0.17 0.039</td>
<td>-0.092 0.27</td>
<td>-0.082 0.33</td>
<td>-0.06 0.47</td>
<td>0.03 0.69</td>
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<tr>
<td>Alkaline OCB</td>
<td>-0.14 0.11</td>
<td>-0.26 0.002</td>
<td>-0.25 0.003</td>
<td>-0.18 0.036</td>
<td>-0.12 0.16</td>
<td>-0.063 0.46</td>
<td>0.044 0.60</td>
<td>-0.046 0.59</td>
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<tr>
<th>Cell Variableb</th>
<th>$\chi^2$</th>
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<tbody>
<tr>
<td>CSF leukocytes</td>
<td>5.75 0.017</td>
<td>6.05 0.014</td>
<td>7.56 0.006</td>
<td>7.35 0.007</td>
<td>4.19 0.041</td>
<td>2.3 0.13</td>
<td>1.15 0.28</td>
<td>0.695 0.404</td>
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<td>CD80</td>
<td>13.4 &lt; 0.001</td>
<td>0.004 0.95</td>
<td>1.39 0.23</td>
<td>11.6 0.001</td>
<td>3.61 0.05</td>
<td>3.14 0.077</td>
<td>0.81 0.37</td>
<td>0.65 0.42</td>
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<td>CD80 CD19</td>
<td>7.21 0.007</td>
<td>0.779 0.38</td>
<td>0.13 0.72</td>
<td>5.97 0.015</td>
<td>2.71 0.1</td>
<td>15.5 &lt; 0.001</td>
<td>0.776 0.38</td>
<td>0.071 0.79</td>
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<td>CD4</td>
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<td>0 0.99</td>
<td>0.012 0.91</td>
<td>0.002 0.97</td>
<td>0.38 0.54</td>
<td>0.206 0.65</td>
<td>0.536 0.46</td>
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<td>CCR5</td>
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<td>0.001 0.97</td>
<td>0.032 0.86</td>
<td>0.002 0.97</td>
<td>0.427 0.51</td>
<td>0.627 0.43</td>
<td>0.806 0.37</td>
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<td>CXCR3</td>
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<td>0.015 0.9</td>
<td>0.018 0.9</td>
<td>0.002 0.97</td>
<td>0.613 0.43</td>
<td>0.141 0.71</td>
<td>0.367 0.54</td>
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</table>

a Partial correlation ($r_p$) and $p$-value from linear regression are shown.

b Negative binomial regression was used and Wald Chi-Square ($\chi^2$) values are provided instead of partial correlation.
FIGURE 3

A

HDL-C Quartiles

B

ApoAI Quartiles

C

ApoB Quartiles

D

HDL-C Quartiles

E

ApoAI Quartiles

F

ApoB Quartiles
FIGURE 4

A

Number of CEL

Low Q2 Q3 High

B

T2-LV, ml

Low Q2 Q3 High

Albumin Quotient Quartiles

C

Number of CEL

Low Q2 Q3 High

D

T2-LV, ml

Low Q2 Q3 High

CSF Leukocytes Quartiles