Protective associations of HDL with blood-brain barrier injury in multiple sclerosis patients[S]

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Abstract The purpose of this work was to investigate the associations of serum cholesterol and apolipoproteins with measures of blood-brain barrier (BBB) permeability and CNS inflammation following the first clinical demyelinating event. This study included 154 patients [67% female; age, 29.5 ± 8.2 years (mean ± SD)] enrolled in a multi-center study of interferon β1-a 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. Higher levels of serum HDL cholesterol (HDL-C) and 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. 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.—Fellows, K., T. Uher, R. W. Browne, B. Weinstock-Guttman, D. Horakova, H. Posova, M. Vaneckova, Z. Seidl, J. Krasensky, M. Tyblova, E. Havrdova, R. Zivadinov, and M. Ramanathan. **Protective associations of HDL with blood-brain barrier injury in multiple sclerosis patients. *J. Lipid Res*. 2015.** 56: **2010–2018.**

Supplementary key words high density lipoprotein • cholesterol • apolipoproteins • clinically isolated syndrome

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 (CELs), which are frequently found on brain MRI from MS patients, even in the absence of clinical relapses. CELs 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 CELs 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

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Abbreviations: BBB, blood-brain barrier; CEL, contrast-enhancing lesion; CRP, C-reactive protein; CSF, cerebrospinal fluid; EDSS, Expanded Disability Status Scale; HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; LV, lesion volume; MS, multiple sclerosis; OCB, oligoclonal band; PON1, paraoxonase-1; TC, total cholesterol; SET, Study of Early Interferon β 1-a Treatment.

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excluded by the BBB. These macromolecules enter more readily into the CNS and can be detected in cerebrospinal fluid (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 (OCBs) 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 LDL 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 (10, 13). Higher HDL levels were associated with fewer CELs (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 a 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. This was a multi-center, prospective, longitudinal, and observational study.

Informed consent. The Medical Ethics Committees of the General University Hospital and First 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 β 1-a Treatment (SET) in High Risk Subjects after Clinically Isolated Syndrome (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 eight Czech Republic MS centers.

Inclusion criteria. Patients included in the study had the following characteristics: 18–55 years of age, enrolled within 4 months from the clinical event, presence of two or more T2-hyperintense lesions on diagnostic MRI and presence of two or more OCBs in CSF obtained prior to corticosteroid treatment, and EDSS $\leqslant$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 $\geqslant$30 days after steroid administration.

All patients were started on a 30 μg once weekly intramuscular interferon β 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 the supplementary Methods. We investigated the associations of the number of CELs 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 nonfasted 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.

Immunoturbidometric diagnostic kits (Kamiya Biomedical, Thousand Oaks, CA) were used for the apolipoprotein (ApoA-I, ApoA-II, ApoB, and ApoE), lipoprotein(a), and high sensitivity C-reactive protein (CRP) assays. Diagnostic reagent kits (Sekisui Diagnostics, Lexington, MA) were used to measure serum TC, HDL 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 Friedewald equation (17).

Specific probes for both single nucleotide polymorphisms, rs7412 and rs429358 (OpenArray; Applied Biosystems, Life Technologies, Foster City, CA), 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 coefficient of variation was 0.6–1.4%. The *PON1* Q192R polymorphism was obtained from the paraoxonase and arylesterase activities, as previously described (18).

Clinical data collected included height and weight for BMI calculations, and history of statin use.

CSF 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 inter-space with the patient sitting upright using a standard sterile preparation and 20 gauge Sprotte atraumatic needle. A total of 20–25 ml of CSF and a 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, IgG, and 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} = CSF albumin (mg/l)/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} = CSF IgG (mg/l)/serum IgG (g/l) and Q_{IgM} = CSF IgM (mg/l)/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: IgG index = [CSF IgG (mg/l)/serum IgG (g/l)]/[CSF albumin (mg/l)/serum albumin (g/l)] = Q_{IgG}/Q_{Alb} and IgM index = [CSF IgM (mg/l)/serum IgM (g/l)]/[CSF albumin (mg/l)/serum albumin (g/l)] = Q_{IgM}/Q_{Alb}.

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

CSF cell phenotyping. Cell surface markers were measured using flow cytometry within 3 h following CSF collection. Cells were concentrated by centrifugation (5 min at 1,000 rpm), resuspended in BD Cell-Wash (BD Biosciences), and stained without lysing with fluorochrome-labeled antibodies for 20 min 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), and CXCR3 (R&D Systems, Minneapolis, MN) antigens were used. Six-color flow cytometric analysis was performed with a 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 ≤ 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 variables 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 predictor variables.

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 days, interquartile range = 34 days). None of the subjects were on statins.

The clinical and demographic characteristics of subjects with lipid profile and CSF measures that 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, and TC), serum apolipoprotein variables (ApoA-I, ApoA-II, ApoB, and 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 OCBs (23) ($P = 0.003$, $q = 0.007$). The CSF variables that were negatively associated

TABLE 1. Demographic and clinical characteristics at baseline, lipid profile and CSF variable values at disease onset

	Mean ± SD[a]	Reference Range[b]
Demographic characteristics		
Sample size (n)	154	—
Female (%)	67	—
Age (years)	29.5 ± 8.2	—
EDSS	1.71 (0.67)	—
MRI Characteristics		
CEL number	1.07 ± 3.4	—
T2-LV (cm^3)	5.15 ± 6.44	—
Brain volume (cm^3)	1,505 ± 72	—
Gray matter volume (cm^3)	791 ± 47	—
Lipid profile characteristics		
HDL-C (mg/dl)	70.3 ± 19.0	40–83
LDL-C (mg/dl)	134 ± 40.1	57–189
TC (mg/dl)	204 ± 52.9	133–234
Triglycerides (mg/dl)	110 ± 50.9	37–321
ApoA-I (mg/dl)	158 ± 40.6	115–224
ApoA-II (mg/dl)	37.3 ± 8.65	25–35
ApoB (mg/dl)	73.9 ± 23.5	60–130
ApoE (mg/dl)	3.28 ± 1.05	3.3–6.1
CRP (mg/l)	2.77 ± 4.40	<2.5
CSF variable characteristics		
CSF-leukocytes (in 3 mm^3)	26.5 ± 34.1	<12
CSF-total protein (mg/l)	345 ± 119	150–450
CSF-albumin (mg/l)	217 ± 86	120–300
Albumin quotient	4.75 ± 1.84	≤6.5 if age <40 years, ≤8.0 if age ≥40 years
CSF-restricted total OCBs	12.1 ± 5.1	Not available
CSF-restricted total alkaline OCBs	8.33 ± 3.7	Not present
IgG index	0.892 ± 0.39	<0.65
IgG quotient	4.21 ± 2.3	—
IgM index	0.251 ± 0.32	—
IgM quotient	1.11 ± 1.1	—

[a] All continuous variables (age, disease duration, T2-LV, and T1-LV) are mean ± SD. For the ordinal EDSS, the median (interquartile 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

CSF Variable[a]	HDL-C		LDL-C		TC		ApoA-I		ApoA-II		ApoB		ApoE		CRP	
	r_p	P	r_p	P	r_p	P	r_p	P	r_p	P	r_p	P	r_p	P	r_p	P
CSF total protein	−0.29	<0.001	−0.23	0.005	−0.28	0.001	−0.27	0.001	−0.19	0.018	−0.13	0.13	−0.24	0.003	−0.092	0.27
CSF albumin	−0.26	0.001	−0.20	0.014	−0.25	0.002	−0.25	0.002	−0.19	0.018	−0.12	0.13	−0.25	0.002	−0.099	0.23
Albumin quotient	−0.27	0.001	−0.17	0.04	−0.23	0.006	−0.22	0.008	−0.18	0.028	−0.08	0.36	−0.27	0.001	−0.042	0.61
CSF IgG	−0.31	< 0.001	−0.28	0.001	−0.33	< 0.001	−0.29	< 0.001	−0.24	0.003	−0.17	0.035	−0.18	0.027	−0.062	0.46
IgG quotient	−0.28	0.001	−0.28	0.001	−0.31	< 0.001	−0.25	0.002	−0.21	0.01	−0.18	0.03	−0.21	0.01	−0.015	0.86
IgG index	−0.12	0.15	−0.22	0.008	−0.21	0.011	−0.14	0.088	−0.11	0.19	−0.17	0.03	−0.034	0.68	0.031	0.71
CSF IgM	−0.11	0.18	−0.14	0.087	−0.15	0.072	−0.089	0.29	−0.19	0.02	−0.095	0.26	−0.05	0.53	−0.025	0.77
IgM quotient	−0.05	0.53	−0.13	0.14	−0.12	0.17	−0.12	0.15	−0.16	0.053	−0.02	0.81	−0.12	0.17	0.006	0.95
IgM index	0.08	0.35	−0.065	0.45	−0.021	0.81	−0.02	0.82	−0.086	0.31	−0.003	0.98	0.004	0.96	0.046	0.59
Total OCBs	−0.09	0.30	−0.18	0.027	−0.17	0.039	−0.092	0.27	−0.082	0.33	−0.06	0.47	0.03	0.69	−0.041	0.62
Alkaline OCBs	−0.14	0.11	−0.26	0.002	−0.25	0.003	−0.18	0.036	−0.12	0.16	−0.063	0.46	0.044	0.60	−0.046	0.59
Cell Variable[b]	χ^2	P	χ^2	P	χ^2	P	χ^2	P	χ^2	P	χ^2	P	χ^2	P	χ^2	P
CSF leukocytes	5.75	0.017	6.05	0.014	7.56	0.006	7.35	0.007	4.19	0.041	2.3	0.13	1.15	0.28	0.695	0.404
CD80	13.4	<0.001	0.004	0.95	1.39	0.23	11.6	0.001	3.61	0.05	3.14	0.077	0.81	0.37	0.65	0.42
CD80 CD19	7.21	0.007	0.779	0.38	0.13	0.72	5.97	0.015	2.71	0.1	15.5	<0.001	0.776	0.38	0.071	0.79
CD4	0	0.98	0	1	0	0.99	0.012	0.91	0.002	0.97	0.38	0.54	0.206	0.65	0.536	0.46
CCR5	0.045	0.83	0.003	0.96	0.001	0.97	0.032	0.86	0.002	0.97	0.427	0.51	0.627	0.43	0.806	0.37
CXCR3	0.001	0.97	0.036	0.85	0.015	0.9	0.018	0.9	0.002	0.97	0.613	0.43	0.141	0.71	0.367	0.54

[a] Partial correlation (r_p) and P value from linear regression are shown.
[b] Negative binomial regression was used and Wald chi-square (χ^2) values are provided instead of partial correlation.

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 **Fig. 1** and **Fig. 2**, respectively.

Human serum PON1, 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 OCBs 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 level ($P = 0.002$, $q = 0.012$), 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, and TC), serum apolipoprotein variables (ApoA-I, ApoA-II, ApoB, and ApoE), and CRP were assessed. The Wald χ^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+ cell 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.

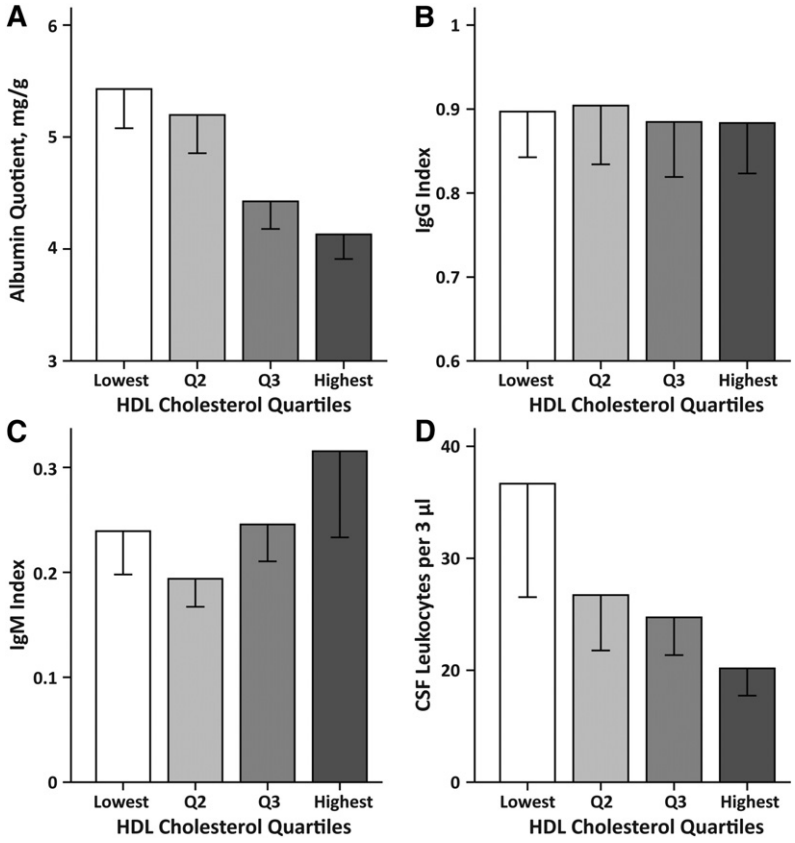

Fig. 1. Associations of HDL-C quartiles with albumin quotient (A), IgG index (B), IgM index (C), and CSF leukocytes (D). The quartile boundaries were: the lowest quartile corresponds to HDL-C ⩽ 56.27 mg/dl, 56.27 mg/dl < quartile 2 (Q2) ⩽ 66.25 mg/dl, 66.25 mg/dl < quartile 3 (Q3) ⩽ 80.69 mg/dl, and the 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-C shown on the x axis. The error bars indicate the standard error of the mean.

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+ cell frequencies and lipid variables, HDL-C, ApoA-I, and ApoB, are summarized in **Fig. 3**.

The regression results for the cell frequency and lipid profile variables suggest that higher HDL-C levels result 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 days, 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 OCBs ($P = 0.004$, $q = 0.005$), alkaline OCBs ($P = 0.003$, $q = 0.005$), 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 are summarized in **Fig. 4A** and Fig. 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 Fig. 4B and Fig. 4D, respectively. We did not find evidence for associations for CSF IgM level, IgM quotient, IgM index, total OCBs, alkaline OCBs, 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 OCBs 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.

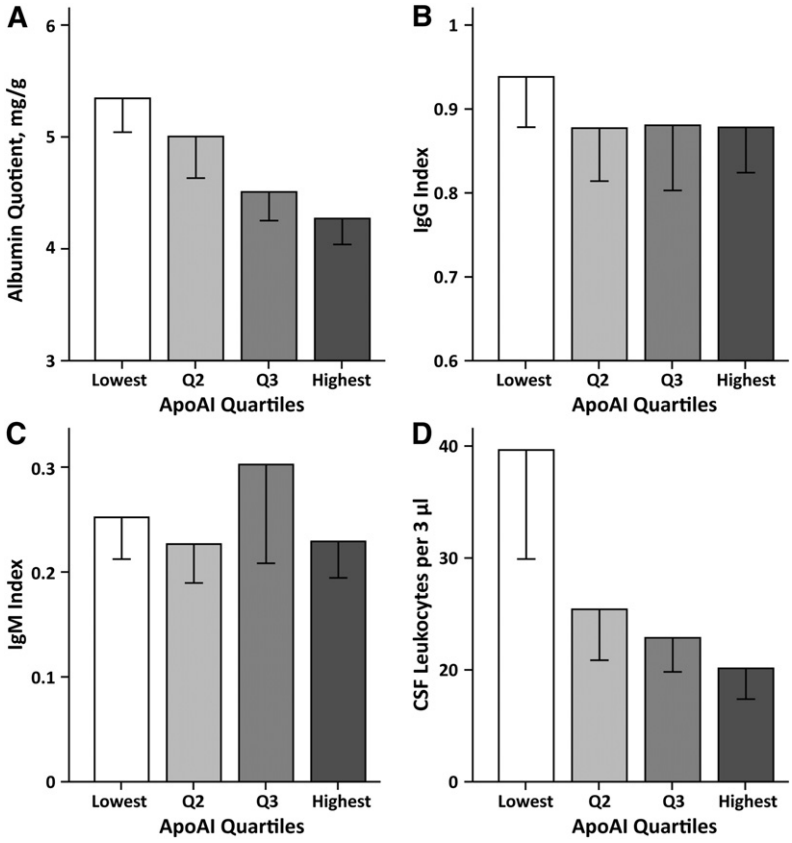

Fig. 2. Associations of ApoA-I quartiles with albumin quotient (A), IgG index (B), IgM index (C), and CSF leukocytes (D). The ApoA-I quartile boundaries were: the lowest quartile corresponds to ApoA-I ⩽ 128.3 mg/dl, 128.3 mg/dl < quartile 2 (Q2) ⩽ 151.1 mg/dl, 151.1 mg/dl < quartile 3 (Q3) ⩽ 175.8 mg/dl, 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.

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 4 year period. However, a potential criticism of the study design is the lack of a placebo-controlled group. A 4 year placebo-controlled trial would clearly be ethically infeasible given that proven disease-modifying therapies are now available for MS.

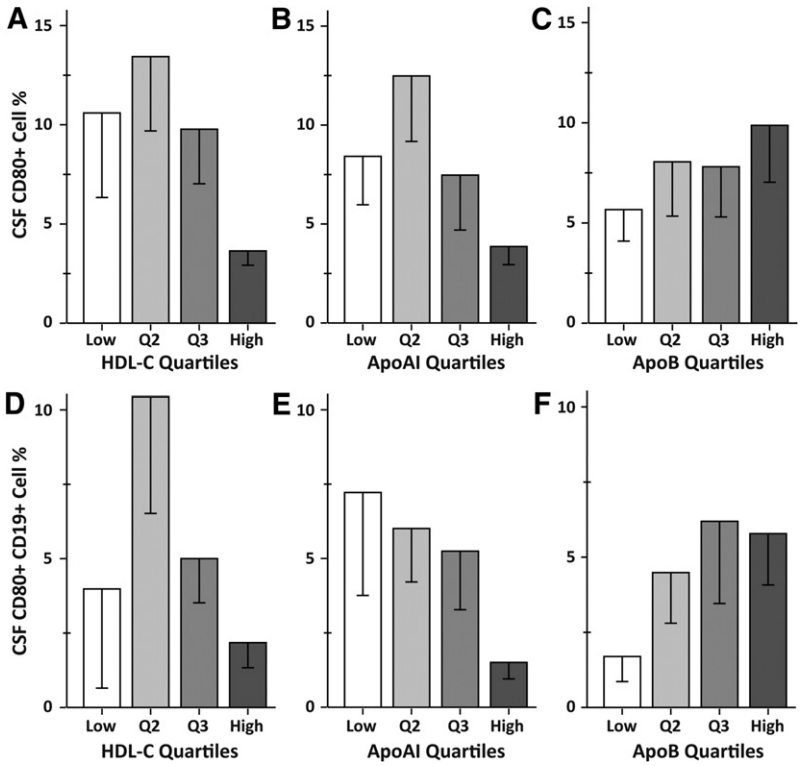

Fig. 3. Associations of mean CSF CD80+ (A–C) and CSF CD80+CD19+ (D–F) cell frequencies in percent with HDL-C quartiles, ApoA-I quartiles, and ApoB quartiles. A, D: The HDL-C quartile boundaries were: the lowest quartile corresponds to HDL-C ⩽ 56.27 mg/dl, 56.27 mg/dl < quartile 2 (Q2) ⩽ 66.25 mg/dl, 66.25 mg/dl < quartile 3 (Q3) ⩽ 80.69 mg/dl, and the highest quartile > 80.69 mg/dl. B, E: 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. C, F: 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.

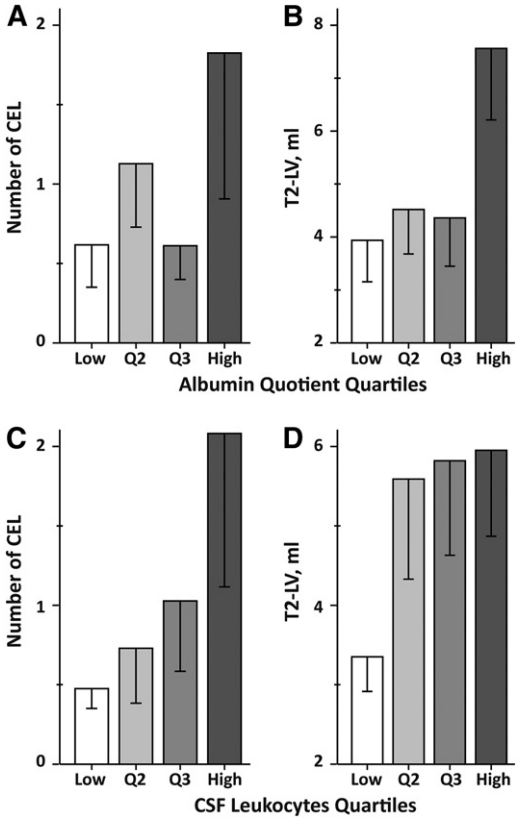

Fig. 4. Associations of albumin quotient (A, B) and CSF leukocytes (C, D) with CEL number and T2-LV MRI measures. The bars compare mean values of the MRI measures shown on the y axis for the quartiles of the CSF variable shown on the x axis. A, B: The albumin quotient quartile boundaries were: the lowest quartile corresponds to albumin quotient ⩽ 3.36 mg/g, 3.36 mg/g < quartile 2 (Q2) ⩽ 4.25 mg/g, 4.25 mg/g < quartile 3 (Q3) ⩽ 5.58 mg/g, and the highest quartile > 5.58 mg/g. C, D: The CSF leukocytes quartile boundaries were: the lowest quartile corresponds to CSF leukocytes ⩽ 9.0/3 μl, 10.0/3 μl ⩽ quartile 2 ⩽ 17.0/3 μl, 18.0/3 μl ⩽ quartile 3 ⩽ 33.5/3 μl, and the highest quartile > 33.5/3 μl. The error bars indicate the standard error of the mean.

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 OCB 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 scavenger receptor class B member 1-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 whether HDL particle size, particularly small discoidal preβ-HDL, is 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 downregulates 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 eNOS dimer levels that maintain endothelial cell function (31).

The mechanisms by which these protective processes occur in MS remain unknown. Meyers et al. (32) reported that ApoA-I levels were lower in the MS patients compared with healthy controls and the primary progressive MS group had lower levels than relapsing-remitting and secondary-progressive MS groups. 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 animal model also provide supporting evidence. In experimental allergic encephalomyelitis-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_{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 costimulatory 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 in MS (34, 35). CXCR3- and CCR5-expressing T cells are found in MS lesions and are higher in the peripheral blood mononuclear cells 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 mass of IgM (approximately 970 kDa vs. 150 kDa 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, namely, 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 greater EDSS over the 4 years following the initial demyelinating event. These findings suggest that CSF measures may have prognostic importance in MS (39).

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 subdomains actively involved in cholesterol homeostasis or at pathophysiologically dyslipidemic tissue regions.

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