Journal of Alzheimer's Disease 74 (2020) 563–578
DOI 10.3233/JAD-191246
IOS Press

Correlates and Predictors of Cerebrospinal Fluid Cholesterol Efflux Capacity from Neural Cells, a Family of Biomarkers for Cholesterol Epidemiology in Alzheimer's Disease

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Handling Associate Editor: Patricia Mecocci

Accepted 13 January 2020

Abstract.
Background: Basic research has implicated intracellular cholesterol in neurons, microglia, and astrocytes in the pathogenesis of Alzheimer's disease (AD), but there is presently no assay to access intracellular cholesterol in neural cells in living people in the context of AD.

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Objective: To devise and characterize an assay that can access intracellular cholesterol and cholesterol efflux in neural cells in living subjects.

Methods: We modified the protocol for high-density lipoprotein cholesterol efflux capacity (CEC) from macrophages, a biomarker that accesses cholesterol in macrophages in atherosclerosis. To measure cerebrospinal fluid (CSF) CECs from neurons, microglia, and astrocytes, CSF was exposed to, correspondingly, neuronal, microglial, and astrocytic cholesterol source cells. Human neuroblastoma SH-SY5Y, mouse microglial N9, and human astroglial A172 cells were used as the cholesterol source cells. CSF samples were screened for contamination with blood. CSF CECs were measured in a small cohort of 22 individuals.

Results: CSF CECs from neurons, microglia, and astrocytes were moderately to moderately strongly correlated with CSF concentrations of cholesterol, apolipoprotein A-I, apolipoprotein E, and clusterin (Pearson's $r = 0.53$–0.86), were in poor agreement with one another regarding CEC of the CSF samples (Lin's concordance coefficient $r_c = 0.71$–0.76), and were best predicted by models consisting of, correspondingly, CSF phospholipid ($R^2 = 0.87$, $p < 0.0001$), CSF apolipoprotein A-I and clusterin ($R^2 = 0.90$, $p < 0.0001$), and CSF clusterin ($R^2 = 0.62$, $p = 0.0005$).

Conclusion: Characteristics of the CSF CEC metrics suggest a potential for independent association with AD and provision of fresh insight into the role of cholesterol in AD pathogenesis.

Keywords: ABCA1, ABCG1, Alzheimer's disease biomarkers, apolipoprotein A-I, apolipoprotein E, cell cholesterol efflux, clusterin (apolipoprotein J), SR-BI

INTRODUCTION

Basic studies have shown that intracellular cholesterol promotes pathogenic metabolism of amyloid-β (Aβ) and tau and exacerbates Aβ cytotoxicity [1–4]. For example, a recent investigation using human induced pluripotent stem cell-derived neurons found that intraneuronal accumulation of cholesteryl ester, a storage metabolite of cholesterol, stimulates Aβ secretion and raises the level of phosphorylated tau [5]. Neuropathological studies have detected no difference between Alzheimer's disease (AD) individuals and controls in the cholesterol content in the cerebral cortex and hippocampus but found increased cholesteryl ester in AD subjects in the entorhinal cortex [6–8]. This suggests that neural tissues may preserve normal levels of free cholesterol by converting its excess to cholesteryl ester, which may be pathogenic. Corroborating and extending the findings from basic research and neuropathology, gene-set and pathway enrichment analyses of the variants associated with AD in genome-wide association studies have implicated intracellular cholesterol metabolism and cell cholesterol efflux to high-density lipoprotein (HDL) in the disease pathogenesis independently of the effect of apolipoprotein E (apo E) isoforms [9, 10]. Variants at the loci encoding secreted exchangeable apolipoproteins and adenosine triphosphate binding cassette transporter subfamily A member 1 (ABCA1), which together comprise an important cell cholesterol efflux pathway to HDL [11], are major drivers of the association between AD and cholesterol metabolism [9, 10]. The implication of this genetic finding is that efflux to extracellular acceptors may represent a benign means for disposal of cholesterol which otherwise could undergo esterification. However, strong support from epidemiology for cholesterol involvement in AD is lacking. Early epidemiological studies found an association between total serum cholesterol at midlife and late life AD [12], but the most recent, large prospective investigation did not report an association between serum cholesterol and AD [13]. There is also no consistent association between cerebrospinal fluid (CSF) cholesterol and AD [14]. It is likely that extracellular pools of cholesterol do not reflect intracellular cholesterol levels well.

Direct measurement of intracellular cholesterol concentrations in the brain of living people is impossible, but the field of atherosclerosis research suggests an approach to access intraneural cholesterol indirectly. Intracellular cholesterol in macrophages affects atherosclerosis but is likewise not amenable to measurement in living subjects [15]. An assay has been devised to quantify the capacity of human HDL, the main acceptor of cell cholesterol in plasma and other body fluids, to take cholesterol from macrophages *ex vivo*: when HDL cholesterol efflux capacity (CEC) from macrophages is low, macrophage cholesterol must be high, because macrophages cannot release it to HDL [16]. HDL CEC from macrophages correlates with atherosclerotic cardiovascular disease (ASCVD) inversely and independently of plasma HDL cholesterol concentration and thus demonstrates the importance of macrophage cholesterol and cholesterol efflux

from macrophages in this disorder [17, 18]. An analogous assay to access intracellular cholesterol and cholesterol efflux in the context of AD will measure the ability of CSF HDL [19] to take cholesterol from neural cells: low CSF HDL CEC would be indicative of high intraneural cholesterol levels.

Here, we draw on the extensive experience in the field of atherosclerosis to develop assays to measure CSF HDL CEC (in the following we refer to the metric only as CSF CEC for simplicity's sake) from the neural cell types in which intracellular cholesterol levels are most likely to affect AD pathology. We show that CSF CECs from neurons, microglia, and astrocytes substantially vary from CSF cholesterol and apolipoprotein concentrations and from one another and are likely to independently associate with AD and provide new insight into the role of cholesterol in AD pathogenesis.

MATERIALS AND METHODS

Cell culture

SH-SY5Y, A172, and J774 cells were obtained from the American Type Culture Collection; N9 cells were a kind gift of Dr. Oleg Butovsky [20]. SH-SY5Y cells were maintained in DMEM/4.5 g/L D-glucose/4 mM L-glutamine/110 mg/L sodium pyruvate, and A172, N9, and J774 cells were maintained in RPMI 1640/2 mM L-glutamine (both media from Life Technologies), supplemented with 10% FBS at 37°C in 5% CO_2.

Immunoblotting

The following antibodies were used: rabbit polyclonal anti-ABCA1 (NB400-105, Novus Biologicals), rabbit monoclonal anti-ABC transporter subfamily G member 1 (ABCG1; EP1366Y, Abcam), rabbit polyclonal anti-ABC transporter subfamily G member 4 (ABCG4; NBP2-15229, Novus Biologicals), rabbit polyclonal anti-scavenger receptor class B type I (SR-BI; NB400-113, Novus Biologicals), mouse monoclonal anti-GAPDH (NB300-221, Novus Biologicals), rabbit polyclonal anti-apolipoprotein B (apo B; ab20737, Abcam), goat polyclonal anti-human apo E and rabbit polyclonal anti-mouse apo E (NB100-1530 and NB100-2040, respectively, both from Novus Biologicals).

Cell cholesterol efflux to purified acceptors

Cell cholesterol efflux assays were conducted as previously described [21]. SH-SY5Y, N9, A172, and J774 cells were seeded in 24-well plates at 1/10 dilution from confluent tissue culture flasks, allowed to grow for 24 h in the maintenance medium/10% FBS and incubated with 2 μCi/mL [1,2-^{3}H(N)]cholesterol (Perkin Elmer) in DMEM/2.5% FBS or RPMI/2.5% FBS, depending on the cell type (see above), for 24 h. The human SH-SY5Y and A172 cells were then treated with 2 μM liver X receptor (LXR) agonist T0901317 (Tocris Bioscience) or corresponding vehicle, while the mouse N9 and J774 cells were treated with 0.3 mM 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate (8-CPT-cAMP; Sigma-Aldrich) or corresponding vehicle, in FBS-free cell type-appropriate medium/0.2% BSA for 18 h. To measure efflux, the cells were exposed to FBS-free cell type-appropriate medium/± 2 μM T0901317 or ± 0.3 mM 8-CPT-cAMP containing 10 μg/mL apolipoprotein A-I (apo A-I) or 50 μg/mL HDL or lacking cholesterol acceptors for 4 h. Efflux medium was filtered using 0.45 μm pore-size filter plates to eliminate floating cells. Cell lipids were extracted with hexane/isopropanol (3 : 2, v/v); the solvent was evaporated. The cell extracts and aliquots of the efflux media were read in a scintillation counter. Cholesterol efflux was expressed as the percentage of [^{3}H]cholesterol counts in the efflux medium from the total of [^{3}H]cholesterol counts in the medium and cells. Apo A-I and HDL were purified from human apheresis plasma.

CSF samples and pooled reference CSF

Twenty-two coded diagnostic remnant samples were obtained from a clinical laboratory at the Hospital of the University of Pennsylvania. There are no demographic or clinical data for these samples. Six diagnostic remnant samples were purchased (Discovery Life Sciences, Los Osos, CA). These samples were from a European ancestry female, 81 years of age, CSF protein 29 mg/dL (normal range of CSF protein 15–45 mg/dL); a European ancestry female, 68, CSF protein 36 mg/dL; an African ancestry female, 50, CSF protein 34 mg/dL; a European ancestry male, 76, CSF protein 44 mg/dL; a European ancestry male, 63, CSF protein 37 mg/dL; an African ancestry male, 57, CSF glucose 92 mg/dL (normal CSF glucose range 40–80 mg/dL). The commercial samples were used to prepare a reference CSF sample: 1 mL frac-

tions of the 6 samples were combined, mixed by inverting, divided into 75 μL aliquots and further stored at –80°C. The remainder of commercial samples and the University of Pennsylvania samples were aliquoted into 30–75 μL portions and stored at –80°C. CSF CEC was measured in 18 samples from the clinical laboratory and four commercial samples. Two samples from the clinical laboratory were found to be contaminated with apo B/blood, and four samples were used up for method optimization. Apo B was measured in 51 CSF samples from cognitively healthy and 43 AD individuals from a biobank at the Center for Neurodegenerative Disease Research at the University of Pennsylvania. CSF CEC from N9 cells was also measured in the 51 samples from cognitively healthy subjects. Use of the CSF samples and study design were approved by the Perelman School of Medicine Institutional Review Board (protocol #82361).

CSF cholesterol efflux capacity

SH-SY5Y (12.8×10^4 cells per well), A172 (3.75×10^4 cells per well), N9 (6×10^4 cells per well), and J774 (6×10^4 cells per well) cells were seeded in 96-well plates in the maintenance medium/10% FBS (75 μL/well), allowed to attach for 6 h and labelled with 2 μCi/mL [1,2-^{3}H(N)]cholesterol in cell type-appropriate medium/2.5% FBS for 18 h overnight. On day 2, SH-SY5Y and A172 cells were treated with 2 μM T0901317, and N9 and J774 cells were treated with 0.3 mM 8-CPT-cAMP, in FBS-free cell type-appropriate medium/0.2% BSA for 6 h. To measure CEC, the cells were exposed to 44% CSF in FBS-free MEM-HEPES/+2 μM T0901317 or 0.3 mM 8-CPT-cAMP (33 μL of a CSF sample + 42 μL of MEM-HEPES) for 2.5 h at 37°C in ambient CO_2. In the assay optimization experiments, the percent CSF in the efflux medium and duration of efflux were varied as indicated. Cell medium was centrifuged at 10,000 rpm in a table-top centrifuge for 3 min to remove floating cells and read in a scintillation counter. Cell lipids were extracted with isopropanol (200 μL/well) overnight and read in a scintillation counter.

CSF samples were assayed in duplicates. When duplicates deviated from the duplicate average by > 10%, then the CSF sample was assayed again. Each 96-well plate contained two wells for cholesterol efflux to medium without CSF (background control) and two wells for cholesterol efflux to the reference CSF sample. Cholesterol efflux to CSF samples, the reference CSF and medium without CSF was calculated as the percentage of [^{3}H]cholesterol counts in the efflux medium from the total of counts in the medium and cells. Background efflux was subtracted from sample and reference CSF sample efflux. CEC was calculated as a unitless ratio of cholesterol efflux to sample CSF divided by cholesterol efflux to the reference CSF.

CSF apolipoprotein, phospholipid, and cholesterol measurements

CSF apolipoproteins were measured using the following kits: Human Apolipoprotein B ELISA Kit (ab108807, Abcam), Human Apolipoprotein A-I Quantikine ELISA Kit (DAPA10, R&D Systems), Human Apolipoprotein E ELISA Kit (ab108813, Abcam), Human Clusterin Quantikine ELISA Kit (DCLU00, R&D Systems). CSF cholesterol and phospholipid were measured with an Amplex® Red Cholesterol Assay Kit (Thermo Fischer Scientific) and a Phospholipid Assay Kit (Sigma-Aldrich), respectively.

Statistical analysis

Cholesterol efflux to purified acceptors was analyzed by *t* test. Linear regression was used to analyze changes in cholesterol efflux in response to increasing CSF amounts and efflux durations. CSF CEC, cholesterol, phospholipid, apo A-I, apo E, and clusterin values were plotted as violin plots and frequency distributions and visually inspected. The standard deviation (SD) to mean ratio (ratios >0.25 indicating log-normal distributions) and Shapiro-Wilk normality test were further used to determine distribution of the values. Scatter plots were inspected for the presence of outliers, data range, and shape of the relationship. CSF CEC, cholesterol, apo A-I, and clusterin values were log-transformed. Pearson's correlation coefficients (r) were calculated to assess association of CSF CEC with CSF cholesterol, apo A-I, apo E, and clusterin. r values in the range 0.6–0.8 were considered indicative of moderate to moderately strong association [22]. Two-tailed p values were calculated. Descriptive statistics, *t* test, linear regression and Pearson's coefficient calculations, and data graphing were conducted using GraphPad Prism 8.3.0.

Lin's concordance correlation coefficient (r_c) is a modification of Pearson's correlation coefficient to

assess not only linearity of the relationship between two variables but also how much the best-fit line deviates from the 45-degree line through the origin, i.e., the line representing perfect agreement [23]. Lin's statistic is a stringent measure of the relationship between two variables ($r_c < 0.90$ - poor agreement, $r_c = 0.90–0.95$ - good agreement). Lin's coefficients and 95% confidence intervals (CI) were calculated on log-transformed CEC values using an Excel implementation following formulas on Real-Statistics.com (https://www.real-statistics.com/reliability/lins-concordance-correlation-coefficient/). Stepwise multivariate regressions were performed (using SAS/STAT® 9.4) to evaluate the prediction of CSF CEC values based on CSF apolipoprotein and phospholipid concentrations; the multivariate regression models went through stepwise variable selection by keeping only the statistically significant independent variables in the final model.

RESULTS

Selection and characterization of neural cells for use in the CSF CEC assay as the cholesterol source

The assay to measure HDL CEC from macrophages in the context of ASCVD employs macrophage cholesterol source cells (usually J774 immortalized macrophages) representing the primary cells with ASCVD-relevant cholesterol efflux (i.e., primary macrophages). Cholesterol source cells must express cholesterol efflux pathways of the corresponding primary cells and provide consistent cholesterol efflux through those pathways for the duration of the assay and from experiment to experiment but do not need to fully recapitulate intracellular cholesterol metabolism of the primary counterparts. Strong cases can be made that intracellular cholesterol in neurons, microglia, and astrocytes affects AD [1–5]. Therefore, we undertook to develop three versions of the CSF CEC assay, one for each of the neural cell types with potentially AD-relevant intracellular cholesterol levels and cholesterol efflux. We surveyed the literature to identify immortalized, commonly used, readily available and phenotypically stable neuronal, microglial, and astrocytic cell lines to be used as the cholesterol source cells. Another requirement was that the cells need not further differentiate to the target cell type, as this may introduce variability into the assay [24]. Preference was given to human over mouse lines, but a human microglial cell line matching the requirements could not be identified. The following cell lines were selected: human neuroblastoma SH-SY5Y cells, mouse microglial N9 cells, and human astroglial A172 cells [20, 25–27]. A CSF CEC assay using J774 cells as the cholesterol source was also developed for comparison.

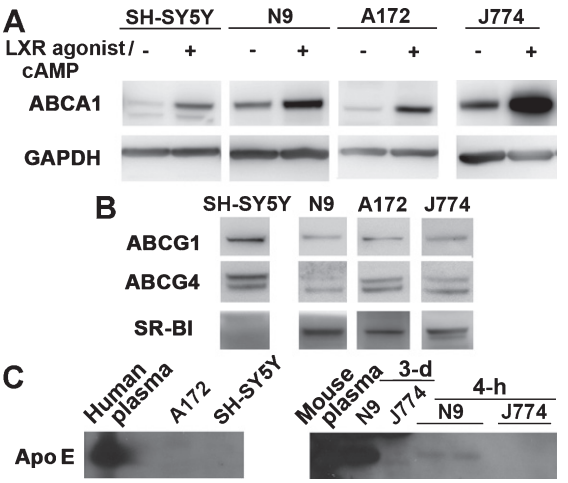

Fig. 1. Expression of cell cholesterol efflux mediator proteins and apo E secretion in SH-SY5Y, N9, A172, and J774 cells. A) Treatment with an LXR agonist (human SH-SY5Y and A172 cells) or a cAMP analog (mouse N9 and J774 cells; the mouse *Abca1* promoter contains a cAMP-response element, which is mutated in the human *ABCA1* promoter) upregulated expressed of ABCA1 in all of the cell types. B) N9, A172, and J774 cells expressed ABCG1, ABCG4, and SR-BI; SH-SY5Y cells expressed the first two proteins but did not express SR-BI. C) Apo E could not be detected in 3-day conditioned SH-SY5Y, A172, and J774 cell media. Apo E could be detected in 3-day and 4-hour N9 conditioned media.

Immortalized cells frequently lose expression of cholesterol efflux genes [28]. The selected cell lines and J774 cells were characterized for expression of cholesterol efflux mediators and cholesterol efflux by the ABCA1-mediated pathway and desorption-diffusion and direct transfer mechanisms [11]. N9, A172, and J774 cells expressed all of the major cholesterol efflux mediator proteins: ABCA1, ABCG1, ABCG4, and SR-BI (Fig. 1A, B). SH-SY5Y cells did not express SR-BI (Fig. 1B), in agreement with published reports that SR-BI is not expressed in primary neurons [29]. ABCA1 could be detected in unstimulated cells; treatment with an LXR agonist of the human cells or with a cAMP analog of the mouse cells dramatically increased ABCA1 expression (Fig. 1A). Apolipoprotein secreted by the cholesterol source cells can skew CEC measurement. SH-SY5Y, A172, and J774 cells did not secrete apo E, while N9 cells strongly secreted it (Fig. 1C).

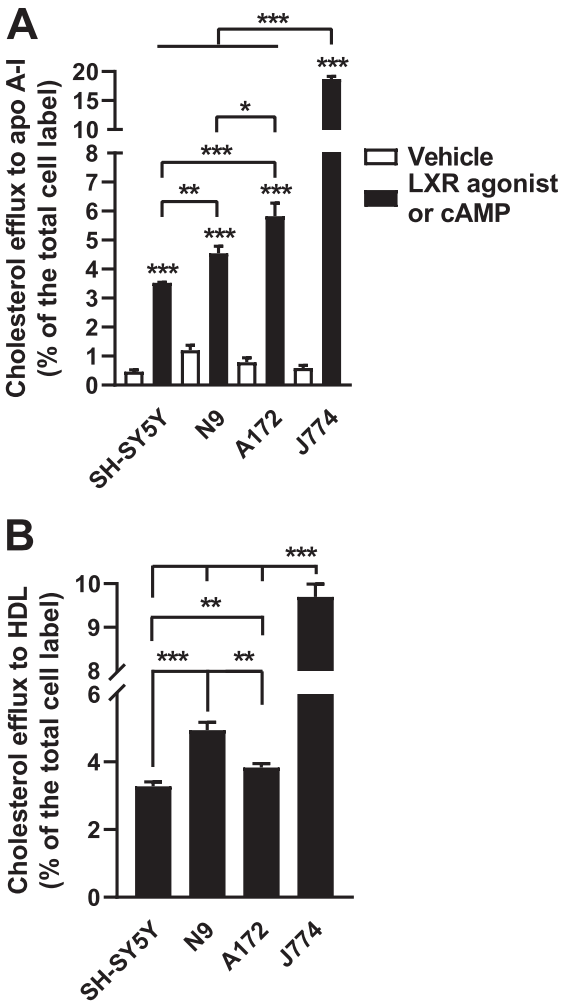

Fig. 2. ABCA1-mediated cholesterol efflux to apo A-I and desorption-diffusion/direct transfer cholesterol efflux to HDL from SH-SY5Y, N9, A172, and J774 cells. A) ABCA1-mediated cell cholesterol efflux to a saturating concentration of purified apo A-I without and with a treatment with an LXR agonist or cAMP analog. B) Cholesterol efflux by desorption-diffusion and direct transfer to HDL. Statistics: mean ± SD, Student's *t* test for unpaired samples ($^{*}p < 0.5$, $^{**}p < 0.01$, $^{***}p < 0.001$).

Without cell treatment to upregulate ABCA1 expression, cholesterol efflux to a saturating concentration of apo A-I (which accepts cholesterol specifically by the ABCA1-mediated pathway) was very low in all of the cell types (average ± mean percent of the radiolabeled intracellular cholesterol released to apo A-I-containing media: 0.47 ± 0.06% for SH-SY5Y, 1.20 ± 0.18% for N9, 0.79 ± 0.16% for A172, and 0.60 ± 0.09% for J774 cells; Fig. 2A). An LXR agonist/cAMP analog treatment increased cholesterol efflux to apo A-I by ~7 fold for SH-SY5Y and A172, ~4 fold for N9, and ~32 fold for J774 cells to 3.5 ± 0.2% for SH-SY5Y, 5.8 ± 0.5% for A172, 4.5 ± 0.2% for N9, and 18.7 ± 0.4% for J774 cells. The order of strength of ABCA1-mediated cholesterol efflux was J774≫A172 > N9 > SH-SY5Y.

ABCG1, ABCG4, and SR-BI promote cell cholesterol efflux by increasing the rate of cholesterol desorption from the plasma membrane, which is the rate-limiting step in diffusional cholesterol efflux from the cell surface to extracellular acceptors [11, 30]. SR-BI also mediates direct transfer of cholesterol from the plasma membrane to HDL by tethering HDL particles to the cell surface [11]. We used plasma HDL as a convenient extracellular cholesterol acceptor to assess cholesterol efflux from the cells by unmediated and ABCG1-, ABCG4-, and SR-BI-mediated desorption-diffusion and SR-BI-mediated direct transfer. The cells were not treated to upregulate ABCA1 expression, and efflux to medium without HDL was subtracted from efflux to HDL. Cholesterol efflux to HDL was 3.3 ± 0.1% of the radiolabeled intracellular cholesterol for SH-SY5Y, 3.8 ± 0.10% for A172, 4.9 ± 0.20% for N9, and 9.7 ± 0.24% for J774 cells (Fig. 2B). The order of strength of desorption-diffusion/direct transfer efflux was J774»N9 > A172~SH-SY5Y. These results show that the selected neural cells express all of the major cholesterol efflux mediators (except for SR-BI in neuronal SH-SY5Y cells) [11, 31], increase ABCA1 expression via the LXR/cAMP transcriptional regulation and efflux cholesterol by the ABCA1 pathway and desorption-diffusion/direct transfer mechanisms but release much less cholesterol as the percentage of intracellular cholesterol pool in comparison with J774 macrophages.

Adaptation and optimization of the HDL CEC assay to measure CSF CEC

In the assay for HDL CEC from macrophages, J774 cells are incubated with radiolabeled cholesterol, treated with a cAMP analog to upregulate ABCA1 expression, and then exposed to plasma HDL [16]. The cAMP treatment step is included because ABCA1 expression and ABCA1-mediated cholesterol efflux in cells cultured in regular (i.e., low cholesterol) growth medium are very low (Fig. 1A; Fig. 2A) and are not representative of high ABCA1 expression and strong ABCA1-mediated cholesterol efflux that may be present in cholesterol-rich macrophages residing in coronary atherosclerotic lesions *in vivo* [32]. High intracellular cholesterol rapidly upregulates ABCA1

expression in macrophages via LXR [33]. The LXR-ABCA1 signaling pathway also functions in primary neurons, astrocytes, and microglia, probably in an analogous way to upregulate ABCA1-mediated cholesterol efflux in response to high intracellular cholesterol levels [34]. We reasoned that rising intracellular cholesterol in neural cells *in vivo* (when the desorption-diffusion/direct transfer pathways of cholesterol efflux are weak) will trigger upregulation of ABCA1 expression via LXR and engage ABCA1-mediated efflux. If ABCA1 expression in the cholesterol source cells were to remain at the low level seen in untreated cells (Fig. 1A, B; Fig. 2A), then some CSF would be falsely classified as having poor CEC, when in fact it could have high CEC because of strong cholesterol efflux by the ABCA1-mediated pathway [35]. Also, single-cell RNA sequencing studies have found strong ABCA1 expression in the granule neurons of the dentate gyrus, a part of the hippocampus, in the mouse and in human inhibitory neurons, microglia, and astrocytes in the prefrontal cortex [36, 37]. ABCA1 expression is further increased in inhibitory neurons and astrocytes of the prefrontal cortex in AD subjects [37]. For the above reason and to bring ABCA1 expression to a high, physiologically relevant level seen in neural cells in single-cell transcriptome studies, we decided to include an LXR (or cAMP for the mouse cells) treatment step in the CSF CEC assay. The LXR/cAMP treatment will induce changes in the intracellular cholesterol metabolism in the cholesterol source cells, but except for elevated ABCA1-mediated cholesterol efflux, which is desirable, none of those changes will impact CEC measurements.

To optimize the CSF CEC assay, we measured cholesterol efflux at different CSF amounts and efflux durations using several CSF samples and J774 and SH-SY5Y cells. Cholesterol efflux rose linearly ($R^2 = 0.95$) as the CSF amount was increased from 16.5 μL to 66 μL per well (i.e., a rise from 22% to 88% CSF in the total 75 μL volume of cell medium and CSF) even when weak (i.e., SH-SY5Y) cholesterol source cells were employed to measure cholesterol efflux to a strong cholesterol acceptor CSF (Fig. 3A, B). Interestingly, cholesterol efflux to a weak cholesterol acceptor CSF was essentially the same regardless of whether strong (i.e., J774) or weak cholesterol source cells were employed, while cholesterol efflux to the strong cholesterol acceptor CSF

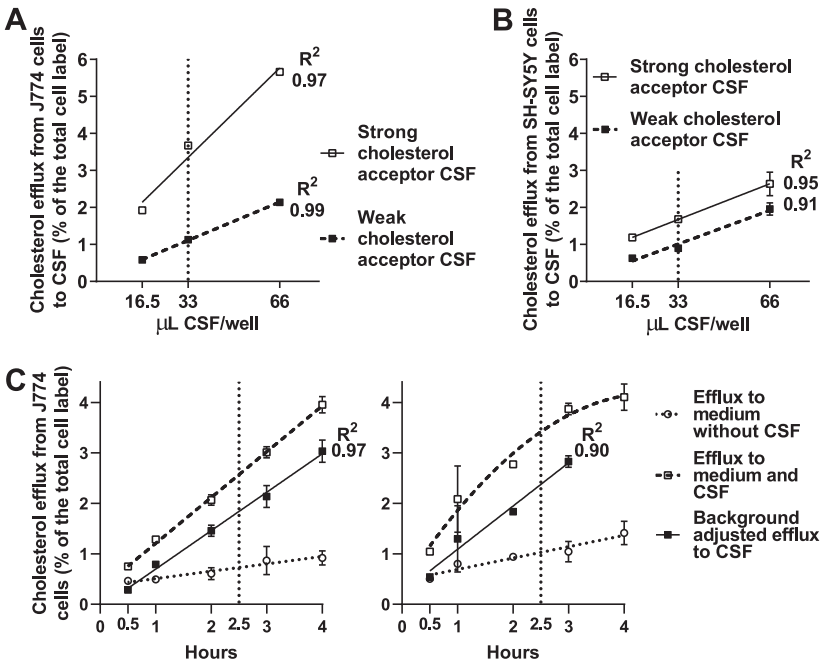

Fig. 3. Optimization of the CSF amount and efflux duration for the CSF CEC assays. A, B) Linearity of the relationship between CSF volumes and cholesterol efflux for a strong and a weak cholesterol acceptor CSF in cholesterol efflux assays employing strong (i.e., J774) or weak (i.e., SH-SY5Y) cholesterol source cells. C) Linearity of the relationship between efflux duration and cholesterol efflux for two CSF samples in cholesterol efflux assays employing strong cholesterol source cells. Dashed lines indicated the selected CSF volume and efflux duration.

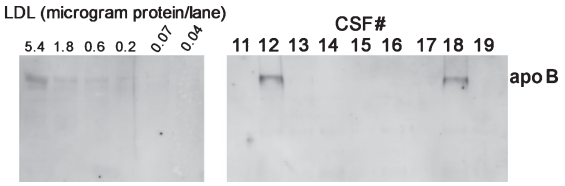

Fig. 4. Screening CSF samples using calibrated apo B western immunoblotting. The anti-apo B-100 antibody was sensitive to as little as 0.07 μg of LDL protein per lane (left panel). Apo B-100 was detected (>180 μg/mL LDL protein) in two CSF samples (right panel). 10 μL of CSF were loaded per lane.

was much higher with the strong cholesterol source cells (Fig. 3A, B). We selected 33 μL (44%) as the CSF amount for the CSF CEC assays. Cholesterol efflux rose linearly ($R^2 \geq 0.90$) for all of the tested CSF samples as the duration of efflux was increased from 0.5 h to 3 h (with the strong cholesterol source cells), but increases were not linear or did not occur at longer efflux durations with some of the CSF samples (Fig. 3C). We selected 2.5 h as the efflux duration for the CSF CEC assays to keep cholesterol efflux in the linear range and to limit the impact of apo E secretion by N9 cells (see Materials and Methods for a detailed protocol).

Screening CSF samples for apo B/blood contamination

Plasma contains apo B-lipoprotein (e.g., low-density lipoprotein, LDL), which is a major source of cholesterol influx into cells. To eliminate this confounder, plasma is treated to precipitate apo B-lipoprotein, leaving HDL and some minor, non-lipoprotein cholesterol acceptors in the apo B-depleted fluid [38]. CSF of cognitively healthy and AD individuals contains very small amounts of apo B-lipoprotein [19, 39–41] and does not require apo B-lipoprotein depletion. However, CSF samples are frequently contaminated with blood [42]. Contaminating blood HDL and apo B-lipoprotein may skew CSF CEC measurements. We screened the CSF that were to be used for CSF CEC measurement by a calibrated apo B western immunoblotting to identify and eliminate from further investigation blood contaminated samples. Two samples (~7% of this sample group) were found to contain > 180 μg/mL LDL protein and were not further used; the remaining samples had no detectable apo B (Fig. 4).

Frequent contamination of banked CSF with apo B/blood

To further explore how frequently banked CSF is contaminated with blood, we measured apo B in 51 CSF samples from cognitively healthy and 43 CSF samples from AD individuals in a biobank at the Perelman School of Medicine using a sensitive apo B ELISA. CSF CEC from N9 cells was also measured in the 51 samples from cognitively healthy subjects. One approach to screening for CSF blood contamination is to exclude CSF samples with the serum/plasma apo B to CSF apo B concentration ratio > 6000 [40, 43]. However, serum/plasma samples are often not available for the individuals who donated CSF, or serum/plasma and CSF were collected far apart in time. We took a different approach. We looked for the threshold concentration value of CSF apo B, so that after excluding the CSF samples with apo B concentrations above this value, the remaining CSF samples had the reported normal apo B concentrations, and CEC values of the remaining samples did not significantly correlate with the sample apo B concentrations. Such threshold value for the samples from cognitively healthy individuals was 140 μg/mL. After excluding the samples with apo B concentrations > 140 μg/mL, the average apo B concentration in the remaining CSF samples was 0.10 ± 0.03 μg/mL, which is the same as the reported apo B concentration (0.11 ± 0.06 μg/mL) in contamination-free samples [40]. There was a significant correlation (Pearson's $r = 0.51$, $p < 0.0001$) between CSF CEC values and apo B concentrations in the complete collection of samples from cognitively healthy subjects, but after the exclusion of the samples with apo B > 140 μg/mL, the correlation was no longer significant ($r = 0.29$, $p = 0.11$). CSF CEC of the samples with apo B < 140 μg/mL was significantly lower than CSF CEC of the samples with apo B > 140 μg/mL (0.97 ± 0.23 versus 1.4 ± 0.35, $p < 0.0001$). Apo B contamination at the level > 140 μg/mL was present in 39% of the CSF samples from cognitively healthy and 47% of the CSF samples from AD individuals. After the exclusion of CSF samples from AD individuals with apo B concentrations > 140 μg/mL, the average apo B concertation in the remaining samples was 0.09 ± 0.03 μg/mL. These results confirm widespread contamination of banked CSF with blood and demonstrate the effect of blood contamination on CSF CEC values.

Descriptive statistics of CSF CEC from SH-SY5Y, N9, A172, and J774 cells

CSF CEC from SH-SY5Y, N9, A172, and J774 cells was measured in the same 22 CSF samples. This was remnant CSF from a clinical testing laboratory. No demographic, clinical, or genetic data are available for the CSF donors. CSF CEC values were distributed lognormally (Fig. 5). The ratios of SD to mean for the four metrics were between 0.44 and 0.49. Table 1 presents descriptive statistics of CSF CEC.

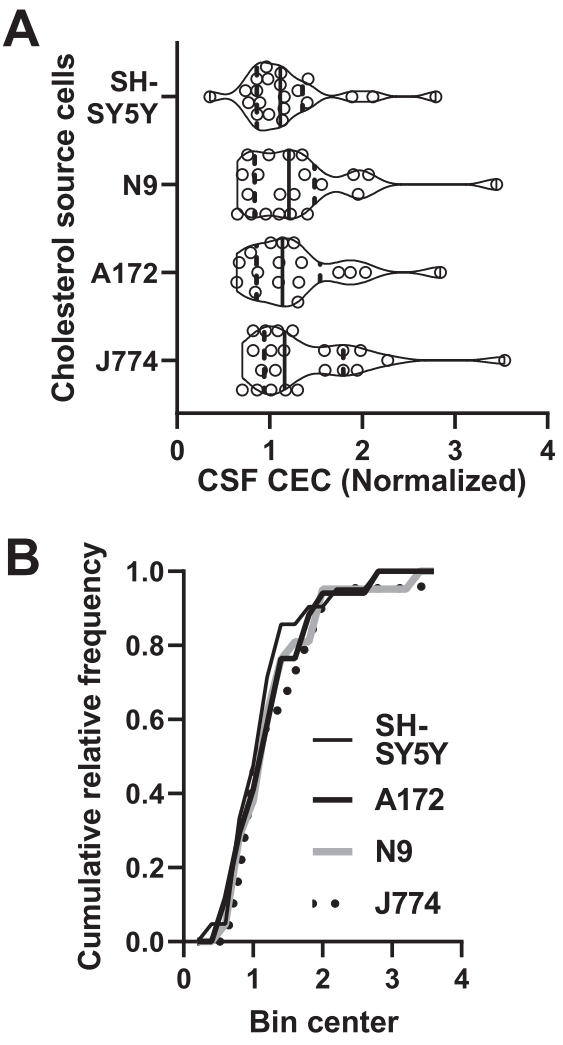

Fig. 5. Distribution of the CSF CEC values. A) A violin plot. Median – solid line; 25% and 75% quartiles – dashed lines. B) A cumulative relative frequency plot of CSF CEC.

Correlation of CSF CEC with CSF cholesterol and apolipoprotein concentrations

If CSF CEC is very tightly correlated with CSF cholesterol or apolipoprotein concentrations, then it is unlikely to give a better prediction of AD than what the cholesterol and apolipoprotein concentration biomarkers already provide. We measured cholesterol, apo A-I, apo E, and clusterin (also called apolipoprotein J) concentrations in the 22 CSF samples (Table 2). Pearson's correlation coefficients were calculated on log-transformed values of CSF CEC and concentrations of cholesterol, apo A-I, and clusterin. Apo E concentrations were not log-transformed for the analysis. CSF CECs from N9 and J774 cells were significantly correlated with CSF cholesterol, apo A-I, and clusterin (Pearson's correlation coefficient $r = 0.82$–0.86 for CSF CEC from N9 cells and $r = 0.79$–0.90 for CSF CEC from J774 cells; Fig. 6, Table 3). CSF CEC from SH-SY5Y cells was more correlated with CSF cholesterol and clusterin ($r \approx 0.8$) but less correlated with CSF apo A-I ($r = 0.67$). CSF CEC from A172 cells was the least correlated of the four metrics with CSF cholesterol, apo A-I, or clusterin ($r \approx 0.6$–0.7). CSF apo E concentrations were poorly correlated with CSF CEC. r coefficients in the range 0.6–0.8 indicate moderate to moderately strong correlation [22]. These results suggest that the CSF CEC metrics vary independently from CSF cholesterol and lipoprotein concentrations to an extent and may associate with AD better than the concentration biomarkers.

Poor agreement among the CSF CEC metrics in the assessment of CSF quality as cholesterol acceptor

It is important to determine whether CSF CECs from SH-SY5Y, N9, and A172 cells are largely inter-

Table 1
Descriptive statistics of CSF CEC ($n = 22$)

	CSF CEC from SH-SY5Y cells (unitless)	CSF CEC from N9 cells (unitless)	CSF CEC from A172 cells (unitless)	CSF CEC from J774 cells (unitless)
Mean ± SD	1.19 ± 0.53	1.31 ± 0.64	1.29 ± 0.57	1.39 ± 0.65
Median (interquartile range)	1.12 (0.86 – 1.36)	1.21 (0.84 – 1.49)	1.14 (0.86 – 1.55)	1.16 (0.94 – 1.79)
Geometric mean ± geometric SD	1.09 ± 1.53	1.20 ± 1.51	1.19 ± 1.49	1.28 ± 1.49

CSF CEC, cerebrospinal fluid cholesterol efflux capacity.

Table 2
Descriptive statistics of CSF cholesterol, apo A-I, apo E, and clusterin concentrations ($n = 22$)

	Cholesterol (μg/mL)	Apo A-I (μg/mL)	Apo E (μg/mL)	Clusterin (μg/mL)	Phospholipid (μg/mL)
Mean ± SD	2.79 ± 1.28	4.32 ± 1.94	8.66 ± 2.15	6.76 ± 3.37	2.80 ± 1.10
Median (interquartile range)	2.52 (1.53 – 4.34)	3.97 (2.66 – 5.34)	7.91 (7.30 – 10.97)	6.27 (3.98 – 8.76)	2.76 (1.97 – 3.44)
Geometric mean ± geometric SD	2.53 ± 1.58	3.89 ± 1.62	8.42 ± 1.28	6.06 ± 1.62	2.51 ± 1.76

Apo A-I, apolipoprotein A-I; apo E, apolipoprotein E; CSF CEC, cerebrospinal fluid cholesterol efflux capacity.

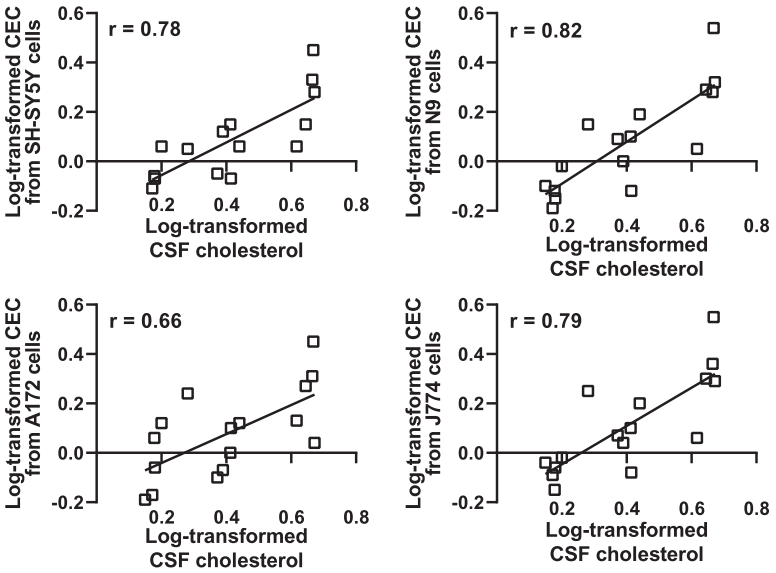

Fig. 6. Correlation between CSF CEC from SH-SY5Y, N9, A172, and J744 cells and CSF cholesterol. Pearson's *r* values are shown.

Table 3
Association of CSF CEC with CSF concentrations of cholesterol and major CSF apolipoproteins ($n = 22$)

	CSF CEC from SH-SY5Y cells	CSF CEC from N9 cells	CSF CEC from A172 cells	CSF CEC from J774 cells
Cholesterol	0.78 (0.47–0.92)***	0.82 (0.55–0.94)***	0.66 (0.24–0.87)**	0.79 (0.47–0.92)***
Apo A-I	0.67 (0.31–0.86)**	0.83 (0.62–0.93)***	0.58 (0.14–0.83)*	0.79 (0.54–0.90)***
Apo E	0.55 (0.03–0.83)*	0.53 (0.02–0.82)*	0.59 (0.11–0.84)*	0.50 (0.01–0.81)^{ns}
Clusterin	0.81 (0.49–0.93)***	0.86 (0.61–0.95)***	0.72 (0.33–0.90)**	0.90 (0.73–0.97)***

Apo A-I, apolipoprotein A-I; apo E, apolipoprotein E; CSF CEC, cerebrospinal fluid cholesterol efflux capacity. Pearson's correlation coefficient *r* (95% CI); $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; ns – not significant.

changeable measures of the same property of CSF or are distinct measures of different properties of CSF. There was no significant difference among the CSF CEC metrics by *t* test. Visual inspection of the data suggested, however, that there was disagreement in the ordering of CSF samples by quartile: some samples were in one quartile by one CSF CEC measure and in a different quartile by another CSF CEC measure. Lin's concordance correlation coefficients (r_c), a statistic for method agreement, were calculated for each pair of the CEC metrics (Table 4). CSF CECs from N9 and J774 cells were in good agreement regarding CSF quality as cholesterol acceptor ($r_c = 0.94$). CSF CECs from SH-SY5Y and A172 cells were in poor agreement with each other and with CSF CEC from N9 cells ($r_c = 0.71$–0.76). These results suggest that CSF CEC from N9 cells and CSF CEC from J774 cells measure essentially the same property of CSF and are interchangeable, while CSF CECs from SH-SY5Y, N9, and A172 cells measure different properties of CSF and are potentially independent biomarkers of AD.

Predictive models of CSF CEC based on CSF concentrations of cholesterol acceptors

Stepwise multiple linear regressions were conducted to predict CSF CEC based on CSF

Table 4
Agreement among CSF CECs from SH-SY5Y, N9, A172, and J774 cells in the assessment of CSF quality as cholesterol acceptor

	CSF CEC from SH-SY5Y cells	CSF CEC from N9 cells	CSF CEC from A172 cells	CSF CEC from J774 cells
CSF CEC from SH-SY5Y cells		0.76 (0.50–0.90)	0.74 (0.43–0.89)	0.71 (0.43–0.86)
CSF CEC from N9 cells	0.76 (0.50–0.90)		0.75 (0.45–0.90)	0.94 (0.86–0.98)
CSF CEC from A172 cells	0.74 (0.43–0.89)	0.75 (0.45–0.90)		0.76 (0.46–0.90)
CSF CEC from J774 cells	0.71 (0.43–0.86)	0.94 (0.86–0.98)	0.76 (0.46–0.90)	

CSF CEC, cerebrospinal fluid cholesterol efflux capacity. Lin's concordance correlation coefficient r_c (95% CI).

concentrations of apo A-I, apo E, clusterin, and phospholipid (Table 2 contains descriptive statistics for CSF phospholipid). The best-fitting model predicting CSF CEC from SH-SY5Y cells consisted of CSF phospholipid ($R^2 = 0.87$, $p < 0.0001$). The best-fitting model for predicting CSF CEC from N9 cells included apo A-I and clusterin ($R^2 = 0.90$, $p < 0.0001$), added in step 1 and 2, respectively. Clusterin comprised the best-fitting model for predicting CSF CEC from A172 cells ($R^2 = 0.62$, $p = 0.0005$). Addition of other variables did not significantly improve prediction. These results suggest that neurons, microglia and astrocytes rely on different cholesterol acceptors in CSF for intracellular cholesterol efflux.

DISCUSSION

Inability to access intracellular cholesterol in neural tissues in epidemiological studies hampers research into the role of cholesterol in AD. To address this impediment, we established CSF CEC assays that access intracellular cholesterol in neural cells in the context of AD. CSF CEC is the normalized percentage of intracellular cholesterol that is released from standard neural cholesterol source cells to a volume of CSF in a unit of time. The cholesterol source cells represent the primary cells in which intracellular cholesterol accumulation and cholesterol efflux are likely to affect AD pathogenesis. Intracellular cholesterol levels in neurons, microglia, and astrocytes bear on AD pathogenesis in some way [1–5]. Therefore, three related assays were developed to measure CSF CEC from neurons, microglia, and astrocytes. Human neuroblastoma SH-SY5Y, mouse microglial N9 and human astroglial A172 cells were chosen to represent the relevant cell types and to be used as the cholesterol source cells [20, 25–27]. The fourth assay using J774 macrophage cells as the cholesterol source was developed for comparison. CSF CECs from SH-SY5Y, N9, and A172 cells were moderately to moderately strongly correlated with CSF concentrations of cholesterol, apo A-I, and clusterin. HDL CEC from macrophages correlates with plasma HDL cholesterol and apo A-I concentrations with similar strength in some studies and still independently associates with ASCVD and other disorders [18, 44, 45]. CSF CECs from N9 microglia and J774 macrophages agreed very well in appraising CEC of CSF samples, indicating that these cell types are very similar with respect to the usage of cholesterol acceptors in CSF, which is not surprising given the similarity of cell function. CSF CECs from SH-SY5Y, N9, and A172 cells agreed poorly with one another in appraising CEC of CSF samples and furthermore were predicted by models consisting of different CSF cholesterol acceptors in multiple linear regression analysis. These findings indicate that neurons, microglia, and astrocytes use different cholesterol acceptors in CSF and that CSF CECs from these cell types are distinct metrics, one or more of which may independently predict AD and provide fresh insight into the role of cholesterol in AD pathogenesis in epidemiological studies.

Four points arising from the present work require further emphasis. First, because cell types differ in the usage of cholesterol efflux pathways and extracellular cholesterol acceptors, it is critical that the cholesterol source cells in the CSF CEC assay express the same cholesterol efflux pathways as the primary cells whose intracellular cholesterol levels and cholesterol efflux the assay is designed to access. This is achieved by employing cholesterol source cells that are of the same type as the relevant primary cells and by pharmacologically activating certain efflux pathways (i.e., activating ABCA1-mediated efflux by using an LXR agonist/cAMP analog). Intracellular changes in cholesterol metabolism in the cholesterol source cells that do not affect cholesterol efflux also do not bear on CSF CEC measurements. Second, we verified previously published observations that banked CSF is frequently contaminated with blood [42] and demonstrated the impact of blood contamination on CSF CEC values. It is important that CSF samples are screened for contamination before mea-

suring CEC. Because contamination is widespread, while CSF samples are scarce, it may be necessary to treat CSF to deplete contaminating apo B-lipoprotein (although this will not address contaminating plasma HDL). Apo B depletion from plasma does not change cholesterol efflux to plasma HDL [46]. This needs to be shown for apo B depletion from CSF. Third, CSF CEC from neurons, microglia, and astrocytes were highly correlated with CSF clusterin, and linear regression models predicting CSF CEC from microglia and astrocytes included clusterin. In comparison with apo A-I and apo E, CSF clusterin is poorly lipidated [47], and this may account for its large contribution to cholesterol uptake. *CLU*, the gene encoding clusterin, is associated with AD in genome-wide association studies [9, 10]. It is plausible that the AD-associated *CLU* variants affect AD pathology by modulating clusterin involvement in cholesterol efflux. Fourth, we developed CSF CEC assays for three neural cell types, because studies *in vitro* and in mouse AD models suggest that intracellular cholesterol in those three cell types affects AD pathogenesis. All three metrics should be measured on the same CSF samples at least in the initial epidemiological investigations to determine whether intracellular cholesterol in the same cell types also affects AD pathogenesis in humans. CSF CEC from the cell types with AD-relevant cholesterol metabolism will associate with AD independently of the other CSF CEC metrics and CSF cholesterol and apolipoprotein concentrations.

We propose to measure CSF CEC as a proxy for intracellular cholesterol in neural tissues, whereby low CSF CEC indicates high intracellular cholesterol. Applicability of CSF CEC for this purpose rests on the hypothesis that cholesterol efflux to HDL has the same high significance to intracellular cholesterol levels in neural cells as it does in macrophages, i.e., when it falters, intracellular cholesterol levels in those cell types sharply rise. Certain types of neurons (e.g., hippocampal pyramidal cells and interneurons; [48]) strongly express cytochrome P450 family 46 subfamily A member 1 (CYP46A1), a cholesterol 24(*S*)-hydroxylase. In young mice and rats, catabolism of cholesterol to 24(*S*)-hydroxycholesterol, which readily diffuses through the blood-brain barrier into the systemic circulation, accounts for half of all cholesterol disposal from the brain [49, 50]. Catabolism to 24(*S*)-hydroxycholesterol is also a major means of brain cholesterol disposal in the human [51]. Glial cells do not express CYP46A1 and have no presently known alternatives to efflux to extracellular acceptors for cholesterol elimination [52, 53]. Cholesterol removal via HDL from glial cells likely accounts for the other half of the cholesterol disposal from the brain [52]. Thus, CSF CECs from microglia and astrocytes are more likely than CSF CEC from neurons to reflect intracellular cholesterol levels in the corresponding cell types.

Yassine et al. [54] and Marchi et al. [55] have developed assays to measure cholesterol efflux pathway-specific CSF CECs using heterologous cells or J774 macrophages as the cholesterol source cells. Yassine et al. used baby hamster kidney 21 (BHK-21) cells expressing human ABCA1 in an inducible manner as the cholesterol source cells and found that CSF CEC for the ABCA1-mediated pathway is significantly reduced in individuals with mild cognitive impairment and AD. Marchi et al. used J774 and Chinese hamster ovary (CHO) cells stably expressing human ABCG1 as the cholesterol source cells and found that CSF CECs for the ABCA1- and ABCG1-mediated pathways are significantly reduced in AD individuals in comparison with cognitively healthy and non-AD dementia subjects. These studies have two drawbacks: usage of heterologous cells as the cholesterol source cells and inability of pathway-specific CSF CEC metrics to reveal in which cells types these pathways are relevant to AD. ABCA1 pathway-specific HDL CECs measured using J774 cells and macrophage cells RAW 264.7 are highly correlated (Pearson's $r = 0.92$ [38]); while ABCA1 pathway-specific HDL CECs measured using BHK-ABCA1 and J774 cells are poorly correlated ($r = 0.56$ [56]). This is likely because other cholesterol efflux pathways affect ABCA1-mediated efflux, and BHK cells differ substantially from J774 and RAW 264.7 cells in the expression of cholesterol efflux pathways. Nonetheless, these and our own preliminary observations that CSF CEC from microglia is reduced in mild cognitive impairment individuals [57] offer a lot of promise for the CSF CEC metrics as a new tool to probe AD pathogenesis.

The most significant limitation of this study is the lack of demographic, clinical, and genetic data for the 22-subject cohort. Furthermore, the CSF samples came from clinical testing laboratories and were selected mostly at random. Most of the individuals referred for CSF testing must have had a condition. It could be that some conditions raise CSF and others lower it. There is also sex dimorphism in CSF CEC [57]. These factors could contribute to a greater spread of CSF CEC values and, because Pearson's correlation coefficient is very sensitive to

data spread, would lead to an overestimate of the association between CSF CEC and CSF cholesterol and lipoprotein.

In summary, we devised CSF CEC assays to address the role of intracellular cholesterol and cholesterol efflux in neurons, microglia, and astrocytes in the pathogenesis of AD. Key features of the CSF CEC assay design are: cholesterol source cells are of the same type as the primary cells whose intracellular cholesterol the assays access; cholesterol source cells are treated to activate ABCA1-mediated cholesterol efflux; CSF samples are screened for apo B/blood contamination before measuring CSF CEC. CSF CECs from neurons, microglia, and astrocytes measured in a small cohort were correlated with CSF cholesterol and apolipoprotein concentrations but not too tightly, were in poor agreement regarding CEC values of individual CSF samples and were predicted based on models that included different CSF cholesterol acceptors. These characteristics of the new metrics signal a potential for independent association with AD and provision of fresh insight into AD pathology.

ACKNOWLEDGMENTS

This work was supported by the National Institute on Aging (grant number RF1 AG051550 to RKD and MAK) and by the National Institute on Aging Alzheimer's Disease Neuroimaging Initiative 3 (grant number U19 AG024904). NNL was supported by a Scientist Development Grant (grant number 14SDG20230024) from the American Heart Association. EC was supported by a scholarship from SIF (Italian Society of Pharmacology).

Authors' disclosures available online (https://www.j-alz.com/manuscript-disclosures/19-1246r1).

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