

# PCSK9 is Present in Human Cerebrospinal Fluid and is Maintained at Remarkably Constant Concentrations Throughout the Course of the Day

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**Abstract** Proprotein convertase subtilisin kexin type 9 (PCSK9) is a key regulator of serum low density lipoprotein cholesterol levels. PCSK9 is secreted by the liver and binds the hepatic low density lipoprotein receptor, causing its subsequent degradation. PCSK9 has also been shown to regulate the levels of additional membrane-bound proteins *in vitro*, including very low-density lipoprotein receptor, apolipoprotein E receptor 2, and beta-site amyloid precursor protein-cleaving enzyme 1, which are highly expressed in central nervous system (CNS) and have been implicated in Alzheimer's disease. Previous studies have demonstrated that human circulating PCSK9 displays a diurnal rhythm. Currently, little is known about PCSK9 levels in human cerebrospinal fluid (CSF). In the present study, we measured PCSK9 concentrations in both serum and CSF collected from healthy human subjects at multiple time points throughout the day. While PCSK9 in serum manifested a distinct diurnal pattern, CSF PCSK9 levels were remarkably constant throughout the course of the day and were also consistently lower than corresponding serum PCSK9 concentrations. Our results indicate that regulation of PCSK9 in human CSF may be different than for plasma PCSK9, suggesting that further study of the role of PCSK9 in the CNS is warranted.

**Keywords** PCSK9 · LDL-cholesterol · LDL receptor · Cerebrospinal fluid · Diurnal variation

## Abbreviations

AD	Alzheimer's disease
ApoA1	Apolipoprotein A1
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
ApoER2	Apolipoprotein E receptor 2
BACE1	Beta-site amyloid precursor protein (APP)-cleaving enzyme
CHD	Coronary heart disease
CNS	Central nervous system
CSF	Cerebrospinal fluid
IDOL	Inducible degrader of LDLR
IP	Immunoprecipitation
LDL	Low density lipoprotein
LDL-C	Low density lipoprotein cholesterol
LDLR	Low density lipoprotein receptor
PCSK9	Proprotein convertase subtilisin kexin type 9
VLDLR	Very low-density lipoprotein receptor
WB	Western blotting

## Introduction

Proprotein convertase subtilisin kexin type 9 (PCSK9) has been recognized as a key regulator of serum low density lipoprotein cholesterol (LDL-C) levels [1–4]. PCSK9 is a protease that is synthesized in the liver and secreted into the circulation upon self-cleavage of its pro-domain. PCSK9 then binds the hepatic low-density lipoprotein receptor (LDLR), ultimately leading to its degradation [5–10]. The mechanism by which PCSK9 degrades the LDLR is complex and is still not fully elucidated. It has been observed that PCSK9 does not have to be enzymatically active for it to cause the degradation of the LDLR. Instead,

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PCSK9 binds to the LDLR and subsequently targets it for lysosomal degradation within the hepatocyte [11–13]. This concept of how PCSK9 acts to decrease hepatic LDLR levels is supported by recent findings that disruption of the binding of PCSK9 to the LDLR using anti-PCSK9 antibodies results in preserved LDLR and decreased LDL-C [14–16].

In humans, gain-of-function mutations in PCSK9 result in severe familial hypercholesterolemia and an accompanying increased cardiovascular risk [17–20]. Conversely, loss-of-function mutations in PCSK9 are associated with a significant reduction in circulating LDL-C levels and protection against coronary heart disease (CHD) [21–23]. Approximately 3 % of African-Americans are heterozygous for such mutations [21]. Of note, a compound heterozygote for PCSK9 loss-of-function mutations has been described. The subject, a healthy 32-year-old female at the time the observation was made, had a serum LDL-C level of 14 mg/dL with no known detrimental impact [23].

In addition to its role in regulating LDLR levels, there have been recent reports suggesting that PCSK9 regulates cellular levels of two other LDLR family members, very low-density lipoprotein receptor (VLDLR) and apolipoprotein E receptor 2 (ApoER2). It is unclear, however, whether PCSK9 also regulates the levels of VLDLR and ApoER2 *in vivo*, possibly affecting a wide range of neuronal functions [24–27]. Overexpression of VLDLR or ApoER2 was reported to result in increased binding of PCSK9 to CHO-A7 cells. Furthermore, in the same study, co-overexpression of VLDLR or ApoER2 and PCSK9 increased the degradation of these receptors [24]. A recent report showed that while PCSK9 directly bound to recombinant LDLR, VLDLR, and ApoER2 protein *in vitro*, changes in PCSK9 expression did not alter the level of these receptors in mouse brain [25]. It has also been shown that overexpression of PCSK9 decreased cellular levels of the  $\beta$ -site amyloid precursor protein (APP)-cleaving enzyme 1 (BACE1), a membrane protease responsible for the production of  $\beta$ -amyloid peptides (A $\beta$ ) that accumulate in senile plaques of Alzheimer's disease (AD) brains [31]. One study, however, indicated that the lack or overexpression of PCSK9 in the mouse CNS did not significantly alter BACE1 levels or APP processing to A $\beta$  [25]. Because VLDLR, ApoER2, and BACE1 are all highly expressed in the central nervous system (CNS) and have been implicated in AD [33–35], we sought to investigate the potential presence of PCSK9 in human cerebrospinal fluid (CSF).

It has previously been reported that circulating human PCSK9 is under dynamic control such that its regulation may contribute to the relative stability of observed LDL-C levels [36]. It is still unclear, however, to what extent PCSK9 is present in human CSF or whether CSF concentrations are subject to the same diurnal changes described

for circulating PCSK9. In the present study, we have attempted to address these questions by measuring matched serum and CSF PCSK9 levels in 12 healthy subjects from which multiple serum and CSF samples were collected throughout the course of a day. Our data from these subjects confirm that serum PCSK9 levels exhibit marked diurnal variation. Interestingly, our results demonstrate that PCSK9 is present in human CSF and that CSF PCSK9 concentrations remain constant throughout the day, suggesting that the regulation of CSF PCSK9 may be different from that of circulating PCSK9.

## Materials and Methods

### Matched Serum and CSF Samples

Human serum and CSF samples were obtained from a diurnal variation study in healthy subjects who gave permission for their serum and CSF samples to be banked for future exploratory analysis. After obtaining protocol approval from an Institutional Review Board and the proper informed consent from each subject, samples were collected, banked, and de-identified to protect participants' privacy. The study was a single site, single period methodology study in healthy subjects, aged 18–65 years, inclusive, with all 12 subjects enrolled completing the full study. Multiple CSF samples were obtained from subjects via an indwelling intrathecal catheter over a period of approximately 24 h. Concomitant blood samples were collected. Subjects were admitted to the clinical research unit in the late afternoon of day  $-1$  at approximately 5:00 PM and underwent appropriate admission procedures.

All subjects received the same standardized evening meal at approximately 6:00 PM on day  $-1$  and day 1. Subjects received a light snack at approximately 10:00 PM and were fasted (with water allowed) until the 6 h CSF sample had been obtained on day 1. Subjects went to bed at 10:30 PM, and lights were turned out at 11:00 PM. On day 1, CSF samples (approximately 4 mL per sample) were taken via an indwelling intrathecal catheter. During this time, subjects were kept in a standardized environment (temperature 20–23 °C). The catheter was inserted into the disc space between either the L3 and L4 or L4 and L5 vertebrae at approximately 8:00 AM ( $\pm 1$  h) on the morning of the study (Day 1). While the catheter was in place subjects remained in bed (including meals). The first sample of CSF was taken immediately after insertion of the catheter. The time of this sample was recorded and designated as '0 h'. Subsequent CSF samples were then collected at times listed in the study schedule. Blood samples were obtained at the same time points. During the study,

CSF sampling took priority, blood samples were taken prior to or after the CSF sample at the discretion of the investigator. All serum and CSF samples were shipped on dry ice and stored at  $-70^{\circ}\text{C}$  prior to analysis.

#### PCSK9 MesoScale Discovery (MSD) Assay

PCSK9 levels in the serum and CSF samples were measured using our recently described PCSK9 dual monoclonal antibody sandwich ELISA [37] with minor modifications. Briefly, a streptavidin-MSD (SA-MSD, MesoScale Discovery, Rockville, MD) plate was blocked with TBS +1 % BSA and coated with biotinylated anti-PCSK9 monoclonal antibody at a concentration of 1  $\mu\text{g}/\text{mL}$  in TBST +0.1 % BSA for 1 h at room temperature. The wells were aspirated and washed three times with TBST. Next, 100  $\mu\text{L}$  of recombinant PCSK9 standards (varying concentrations of recombinant protein in assay buffer) were added to the wells as a standard curve. Afterward, serum samples were diluted 1:50 and CSF samples were diluted 1:4 in assay buffer (50 mmol/L HEPES, pH 7.40, 150 mmol/L NaCl, 10 mL/L Triton X-100, 5 mmol/L EDTA, 5 mmol/L EGTA) and added to their respective wells. The SA-MSD plate was incubated for 2 h at room temperature. Following aspiration, wells were washed three times with TBST and 100  $\mu\text{L}$  of ruthenium-labeled anti-PCSK9 antibody at a concentration of 1  $\mu\text{g}/\text{mL}$  in assay buffer +0.1 % BSA were added to the wells for an additional 1 h incubation at room temperature. Following aspiration, wells were washed three times with TBST. After the last aspiration of TBST, 150  $\mu\text{L}$  of 2 $\times$  MSD Read Buffer were added to the wells for analysis on the SECTOR Imager 6000 (MSD, Rockville, MD). For each plate, the Discovery Workbench assay analysis software was used for fitting of the calibration curves. The PCSK9 MSD assay was found to have a sensitivity of 0.025 ng/mL, easily permitting measurements of the relatively lower levels of PCSK9 present in CSF.

#### Immunoprecipitation and Western Blotting (IP–WB) of PCSK9

Analysis of PCSK9 protein levels in serum samples by IP–WB was performed as previously described with minor modifications [39]. For each IP of serum, 20  $\mu\text{L}$  of serum were added to 980  $\mu\text{L}$  of IP buffer (50 mmol/L HEPES, pH 7.40, 150 mmol/L NaCl, 10 mL/L Triton X-100, 5 mmol/L EDTA, 5 mmol/L EGTA). For each IP of CSF, 100  $\mu\text{L}$  of CSF were added per 900  $\mu\text{L}$  of IP buffer. Next, PCSK9 was immunoprecipitated overnight with 1  $\mu\text{g}$  of anti-PCSK9 monoclonal antibody covalently coupled to tris-acryl beads (Pierce, Rockford IL). Afterward, beads were washed twice with IP buffer, and 30  $\mu\text{L}$  of 2 $\times$  sample

buffer (100 mmol/L Tris, pH 6.80, 40 g/L SDS, 200 mL/L glycerol, 20 mg/L bromophenol blue, 15 g/L dithiothreitol) were added to each tube. Samples were vortexed, boiled for 5 min, vortexed again and briefly centrifuged. Twenty microliters of each lysate was separated by SDS-PAGE and transferred to a nitrocellulose membrane that was blocked with Odyssey blocking buffer (LI-COR, Lincoln, NE). Membranes were incubated overnight at  $4^{\circ}\text{C}$  with polyclonal sheep anti-human PCSK9 Antibody (R&D Systems, Minneapolis, MN) in Odyssey blocking buffer containing 0.1 % (v/v) Tween 20 and washed prior to incubation with Alexa Fluor 680 donkey anti-sheep secondary antibody (Life Technologies, Grand Island NY) for 1 h at room temperature. Membranes were analyzed using an Odyssey Infrared Imaging System (LI-COR; Millennium Science, Surrey Hills, Australia), with signal intensity determined using LI-COR imaging software and exported to Microsoft Excel for graphical representation.

#### Immunoassays for ApoA1, ApoB, and BACE1

Due to volume limitations, samples were pooled in groups of three patients for each time point (resulting in 4 samples per time point) prior to analysis of ApoA1, ApoB, and BACE1 levels. Serum and CSF apoA1 and ApoB100 levels were determined with the Human ApoA1 and ApoB100 ELISA kits (Mabtech, Inc. Cincinnati, OH) according to the manufacturer's instructions. For ApoB measurements, serum samples were diluted 1:40,000 and CSF samples were diluted 1:4. CSF BACE1 levels were determined with a human BACE1 ELISA kit (Cloud-Clone Corp. Houston, TX) according to the manufacturer's instructions.

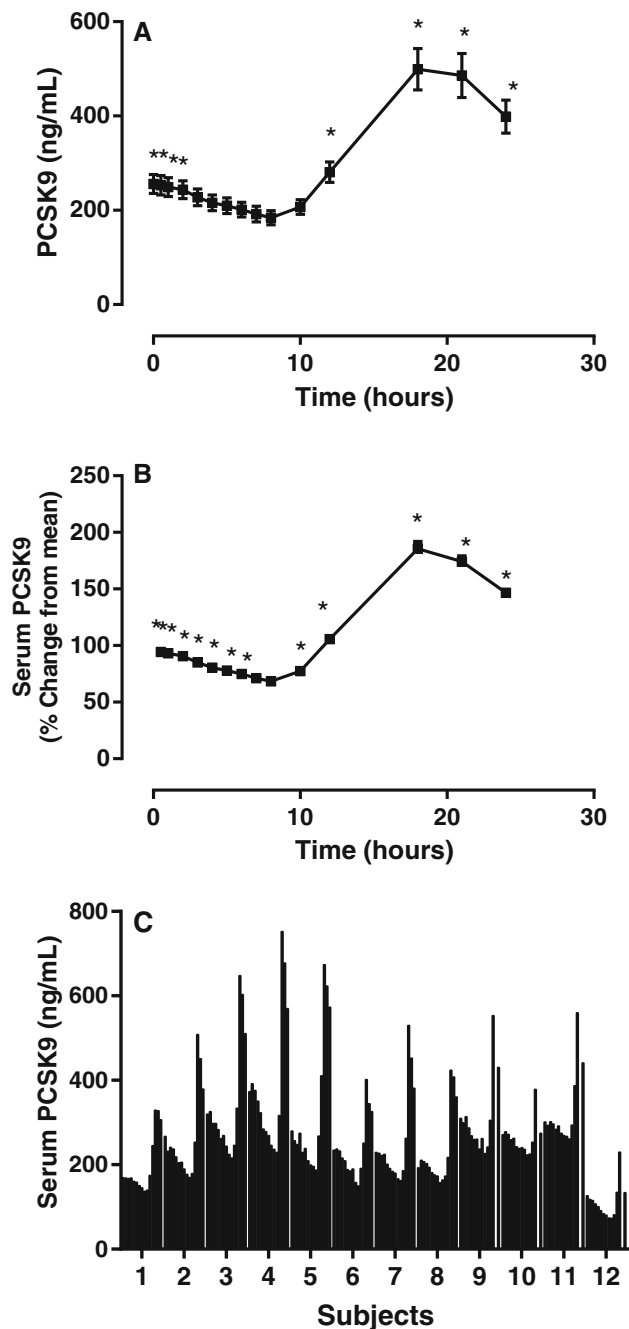
#### Data Analysis

GraphPad Prism Software program (GraphPad Software Inc. La Jolla, CA) was used for fitting of the calibration curves for the PCSK9, ApoA1, ApoB, and BACE1 ELISA data. Statistical analysis was performed using the same program. Data were analyzed by one-way analysis of variance followed by comparisons between the means using the least significant difference test. All data were expressed as the means  $\pm$  SEM. For Figs. 2, 5, 6c, and 7d, statistical analysis was performed to calculate Spearman correlation coefficients. In each case, a  $p$  value of  $<0.05$  was considered to indicate statistical significance.

## Results

We first investigated the diurnal variation of serum PCSK9 levels in 12 healthy subjects who had serum samples obtained at 15 time points over a 24-h period, from

approximately 8:00 AM ( $\pm 1$  h) on day 1 to 8:00 AM on day 2 ( $\pm 1$  h). Figure 1a shows the results of this experiment. In the serum, PCSK9 concentrations displayed a



**Fig. 1** Diurnal variation of PCSK9 in human serum. **a** Twelve healthy subjects had an initial blood sample drawn at 8:00 AM ( $\pm 1$  h). Subsequent samples were obtained at times indicated, with the final sample collected at 8:00 AM ( $\pm 1$  h) the next day. Serum PCSK9 levels are shown as the means  $\pm$  SEM ( $*p < 0.05$  versus 8 h/4:00 PM time point). **b** Data from Fig. 1a are shown as the percentage change from the mean of all samples from each individual (with the mean set at 100 %). Results are shown as the means  $\pm$  SEM ( $*p < 0.05$  versus 8 h/4:00 PM time point). **c** Intra-subject variability in serum PCSK9 levels is shown over the 24-h period for each subject

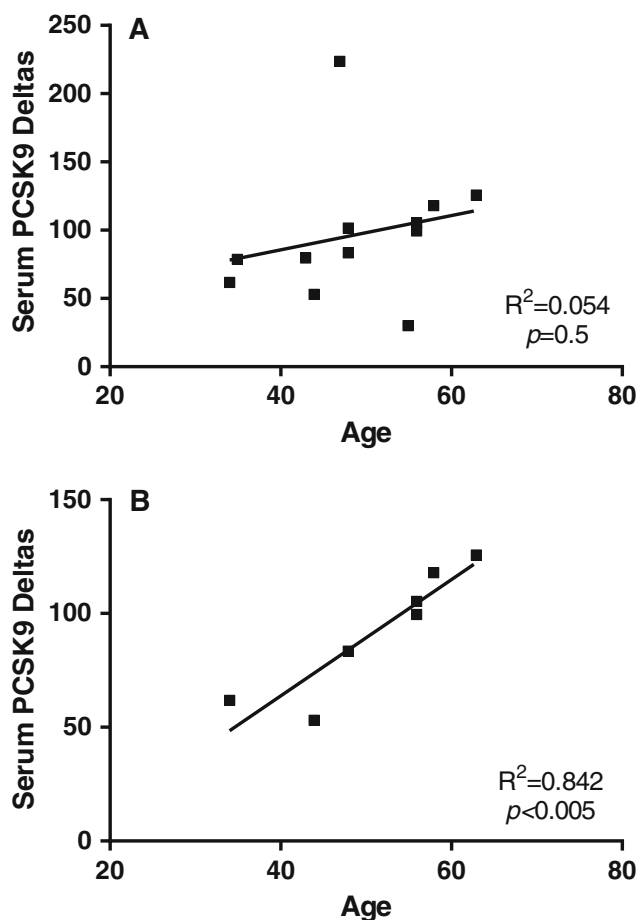
marked diurnal rhythm, with concentrations declining gradually starting in the late morning hours and reaching their lowest point during the mid-afternoon hours (approximately 4:00 PM).

After reaching this low point, PCSK9 levels steadily increased throughout the evening to peak at around midnight, before returning close to baseline by 8:00 AM the following day. The fact that the 8:00 AM serum PCSK9 levels on day 2 were higher than at 8:00 AM on day 1 was not surprising, given the relatively rapid decline in levels noted in the early morning hours and that either 8:00 AM blood draw could have been within  $\pm 1$  h. The overall trend of these diurnal changes of PCSK9 in serum was similar to the results we and others previously reported [36, 40], although the amplitude of the changes was somewhat larger than what has been previously described.

Figure 1b shows that when PCSK9 concentrations for each individual subject were expressed as a percentage of each subject's mean PCSK9 level, levels in serum started to change significantly as little as 2 h after the initial blood draw. Remarkably, the PCSK9 peak increases were almost 3-fold greater than those observed at the low point in the mid-afternoon. The intra-subject variability of serum PCSK9 levels is shown in Fig. 1c. These data show that each individual subject manifested a strikingly similar pattern of PCSK9 diurnal rhythm. In fact, there was no subject who did not display this rhythm.

In addition, we further investigated if the intra-subject variability correlated with age and gender. The delta differences of serum PCSK9 values from the 18-h time point were calculated against the serum PCSK9 values at 8-h time point for each individual subject. No significant correlations were found between serum PCSK9 deltas and age when all 12 subjects (7 males, 5 females) were analyzed (Fig. 2a). Interestingly, serum PCSK9 delta values exhibited significant correlation with age at 8 and 18-h time points in male subjects ( $p < 0.005$ ) as shown in Fig. 2b. It should be noted, however, that the number of male subjects analyzed was small ( $n = 7$ ), and that this conclusion should be taken in context.

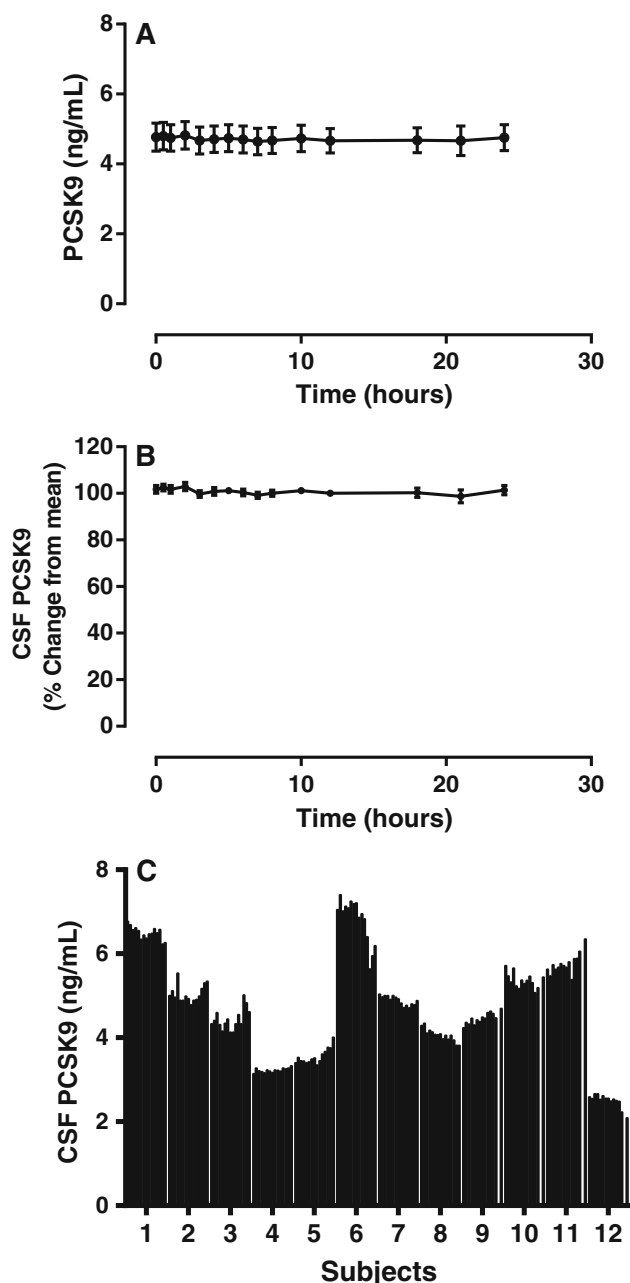
We next measured PCSK9 concentrations in CSF obtained from the same 12 subjects at the same time points when blood samples were collected. Interestingly, as Fig. 3a demonstrates, the CSF samples did not display the diurnal variation that was observed for serum PCSK9 concentrations. In contrast to the diurnal pattern observed in serum, CSF PCSK9 concentrations remained almost constant throughout the day. Furthermore, it was noted that CSF levels of PCSK9 were lower than for serum, with the mean CSF PCSK9 level being 5 ng/mL versus 289 ng/mL for serum. Overall, the serum/CSF PCSK9 ratio was about 60. As Fig. 3b demonstrates, when CSF PCSK9 concentrations for each individual subject were expressed as a



**Fig. 2** Relationship between serum PCSK9 levels and demographic parameters. **a** Serum PCSK9 concentrations at 8 and 18 h time points were calculated as deltas against each other. The serum PCSK9 delta values from all 12 healthy subjects (7 males, 5 females) were plotted against age. There was no significant correlation between serum PCSK9 deltas and age ( $R^2 = 0.054$ ,  $p = 0.5$ ). **b** The serum PCSK9 delta values described in Fig. 2a were plotted against age for male subjects only. There was a significant correlation between serum PCSK9 delta values and age for the seven male subjects ( $R^2 = 0.842$ ,  $p < 0.005$ )

percentage of each subject's mean CSF PCSK9 level, the concentration of PCSK9 in CSF was remarkably constant throughout the day, with changes being well less than 5 % from the mean. The intra-subject variability of PCSK9 levels in CSF is shown in Fig. 3c. These data show that each individual subject manifested a similar pattern of constant CSF PCSK9 concentrations, and that there was no subject who did not display this constant pattern. We also calculated the CSF PCSK9 delta values as described above and observed no correlation with age or gender.

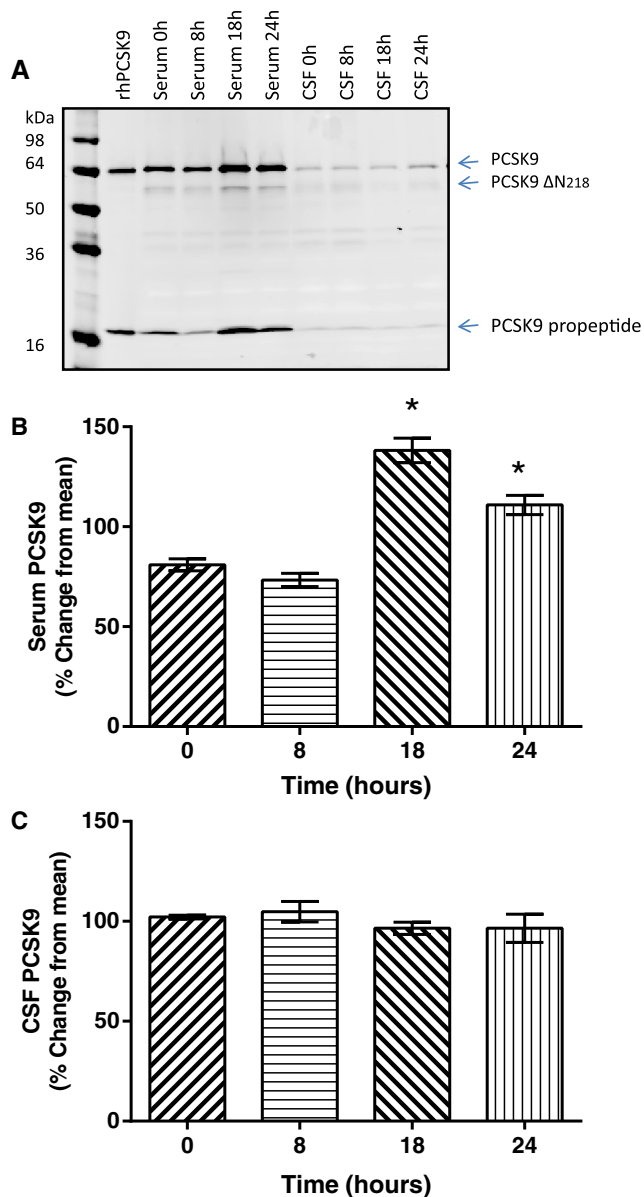
In light of these results showing the diurnal variation of serum PCSK9 as well as the constant nature of CSF PCSK9 levels as measured by our sandwich ELISA method, we sought to further characterize CSF PCSK9 protein and compare it to serum PCSK9 protein. Considering a recent report of a furin breakdown product of PCSK9 protein



**Fig. 3** PCSK9 levels in CSF. **a** Twelve healthy subjects had an initial CSF sample drawn at 8:00 AM ( $\pm 1$  h). Subsequent samples were obtained at times indicated, with the final sample collected at 8:00 AM ( $\pm 1$  h) the next day. Levels of PCSK9 in CSF are shown as the means  $\pm$  SEM. **b** Data from Fig. 2a are shown as the percentage change from the mean of all samples from each individual (with the mean set at 100 %). Results are shown as the means  $\pm$  SEM. **c** Intra-subject variability in CSF PCSK9 levels is shown over the 24-h period for each subject

present in plasma migrating as a band below the intact PCSK9 band [41], we investigated the PCSK9 isoforms present in serum and CSF by performing immunoprecipitation and Western Blotting (IP–WB) analyses of serum and CSF samples representative of time points during

which the greatest diurnal variation was observed for serum PCSK9 concentrations. Representative Western blotting results from these experiments are shown in Fig. 4a, in which samples collected at 0, 8, 18, and 24 h were analyzed. As shown in the figure, the intact PCSK9 protein and propeptide bands that co-migrated with the



**Fig. 4** Characterization of serum and CSF PCSK9. **a** Serum and CSF PCSK9 protein levels from three subjects were analyzed via immunoprecipitation and Western blotting. The predominant band represents intact PCSK9 protein. The band immediately below is consistent with the furin cleavage product of PCSK9, while the lowest band is consistent with PCSK9 propeptide. Results shown are representative of all three subjects analyzed. **b** Serum PCSK9 protein levels obtained as described in Fig. 3a were quantitated, with results shown as the means  $\pm$  SEM (\* $p$  < 0.05 versus 8 h/4:00 PM time point,  $n$  = 3). **c** CSF PCSK9 protein levels obtained as described in Fig. 3a were quantitated, with results shown as the means  $\pm$  SEM

recombinant PCSK9 protein standard confirmed the diurnal variation observed in serum samples using the sandwich ELISA, as well as the lack of diurnal variation in the matching CSF samples.

Also noted during the Western blotting, was the likely furin breakdown product of PCSK9 protein, which migrated just below the intact PCSK9 band both in the serum and CSF samples. Figure 4b represents the serum PCSK9 percentage change from the mean for three individual subjects at four time points as determined by IP and WB. The data confirm that serum PCSK9 protein levels increased significantly at the 18 and 24 h time points. In comparison, CSF PCSK9 protein levels showed no significant changes over the same time intervals (Fig. 4c).

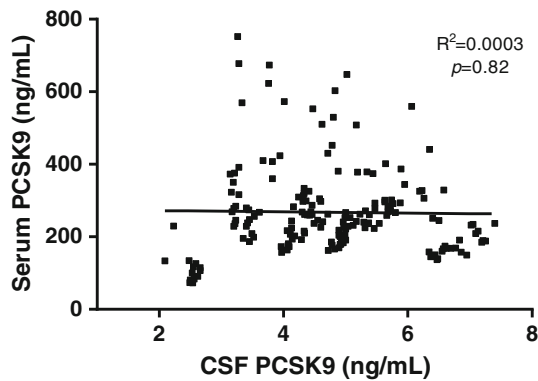
In light of these data, we examined the correlation of serum PCSK9 levels with CSF PCSK9 levels from the 12 healthy subjects for all sets of matched serum and CSF samples obtained throughout the study. As Fig. 5 demonstrates, there was no significant correlation observed between serum and CSF PCSK9 levels, further confirming that regulation of PCSK9 protein in the CSF appears to be different than for PCSK9 protein circulating in plasma.

We also measured the BACE1 concentrations in the CSF samples. As Fig. 6a demonstrates, BACE1 concentrations in CSF varied from 3.5 to 10 ng/mL and did not display diurnal variation. We next examined the correlation between BACE1 and PCSK9 (Fig. 6b) in CSF. As Fig. 6c demonstrates, there was no significant correlation observed between BACE1 and PCSK9 levels in CSF.

We further investigated whether ApoA1 and ApoB exhibited diurnal variation in serum or CSF in order to better confirm our findings for PCSK9. As Fig. 7a, b demonstrate, in contrast to the serum PCSK9 diurnal pattern, ApoA1 concentrations in serum and CSF remained almost constant throughout the day. It was also noted that ApoA1 levels in CSF were much lower than for serum, with the mean CSF ApoA1 level being 2.6  $\mu$ g/mL versus 1.6 mg/mL for serum. Overall, the serum/CSF ApoA1 ratio was about 600. As Fig. 6c demonstrates, ApoB concentrations in serum remained almost constant throughout the day. The mean serum ApoB level was 1.3 mg/mL. As expected, no ApoB was detected in the CSF samples. Finally, we examined the relationship between serum and CSF ApoA1 levels. As Fig. 7d demonstrates, there was no correlation observed between serum and CSF ApoA1 levels.

## Discussion

Our results demonstrate that serum PCSK9 concentrations in healthy human subjects exhibit a marked diurnal variation, with values being lowest during the mid-afternoon



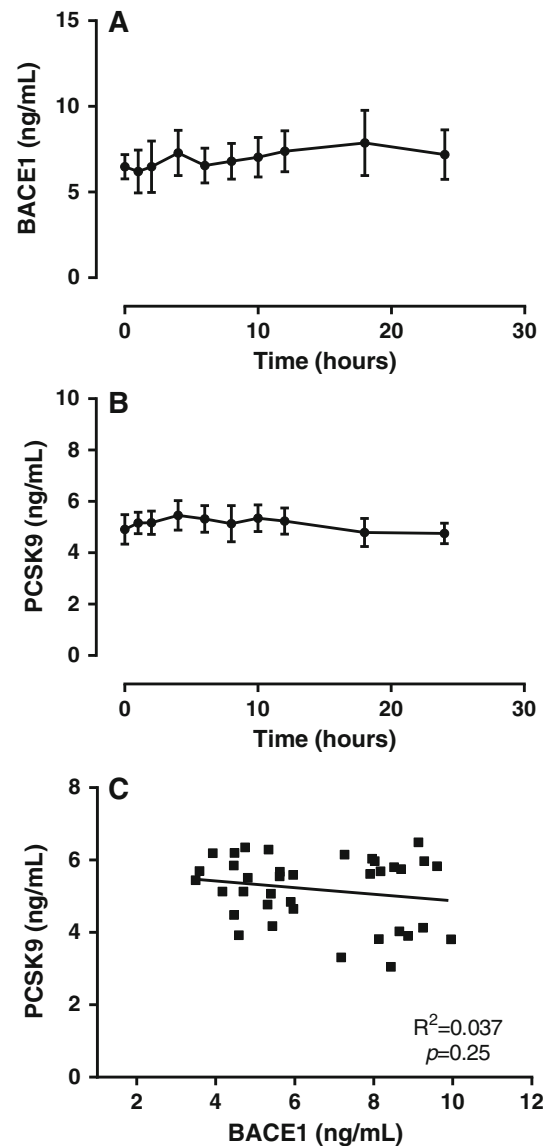
**Fig. 5** Relationship between serum and CSF PCSK9 levels. Serum and CSF PCSK9 concentrations measured as described in Figs. 1 and 3 were plotted against each other. There was no significant correlation between serum and CSF PCSK9 levels

hours, steadily increasing throughout the rest of the day to reach a peak around the late evening/early morning hours, and eventually returning to near where they began the previous day. In the present study, we have thus confirmed previous reports regarding the diurnal changes in human serum PCSK9 concentrations [36, 40] and have further extended these observations by frequent sampling of large numbers of healthy subjects.

The average PCSK9 peak level in serum was found to be almost three times higher than the average trough level in the study, suggesting that the extent of PCSK9 diurnal variation may be even greater than what has been previously described [36, 40]. In addition, we observed that serum PCSK9 levels from each individual subject exhibited almost exactly the same diurnal pattern, although the exact amplitude of the variation and the baseline PCSK9 levels varied from subject to subject. Interestingly, serum PCSK9 deltas correlated with age in male subjects.

In previous reports, the dynamic regulation of circulating PCSK9 has demonstrated the same diurnal variation as hepatic cholesterol synthesis, while LDL-C levels remained relatively stable [36]. One explanation for the diurnal variation of serum PCSK9 may be the dynamic changes that occur in the regulatory pool of hepatic cholesterol content during the day. Cholesterol synthesis and serum PCSK9 levels tend to reach their peaks late at night, presumably because of a nadir in hepatic cholesterol.

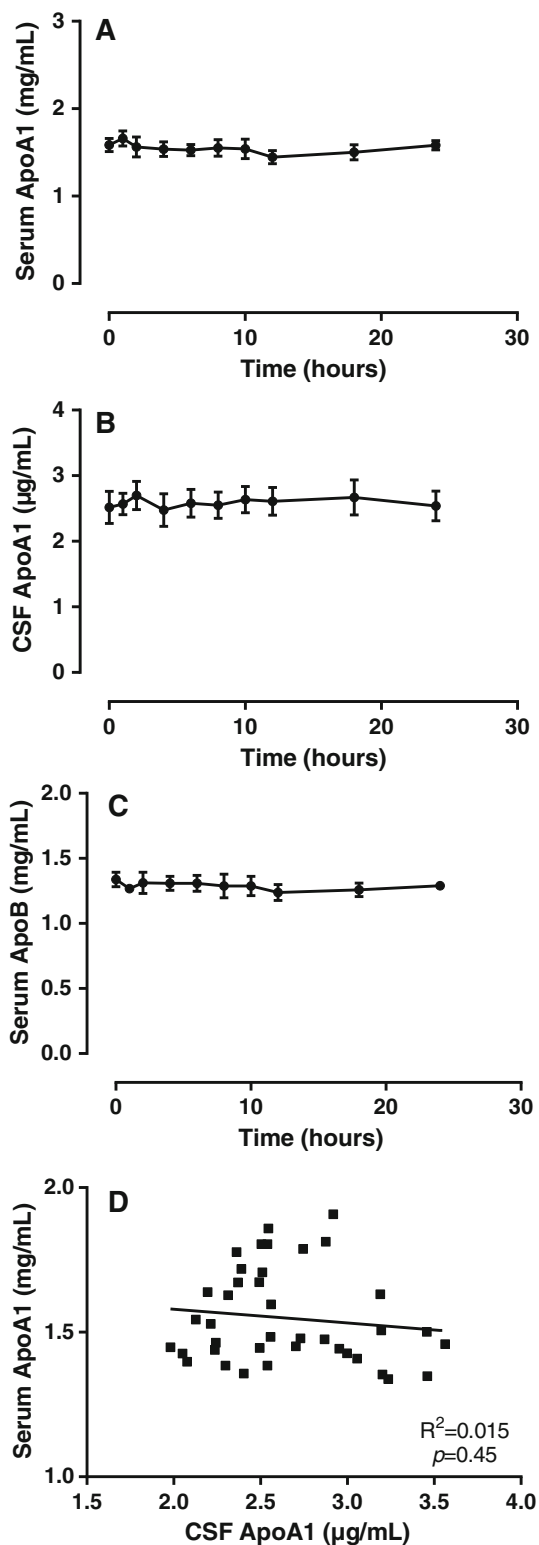
Our present study shows for the first time that CSF PCSK9 concentrations do not display the diurnal variation observed for serum PCSK9. In contrast to serum PCSK9 levels, concentrations of PCSK9 in the CSF remained constant over a 24-h period. Moreover, we observed that PCSK9 concentrations in CSF were much lower than that in serum. The average CSF PCSK9 concentration was approximately 5 ng/mL throughout the day, while mean serum PCSK9 concentrations varied from 183 ng/mL in the late afternoon to



**Fig. 6** BACE1 and PCSK9 levels in human CSF. **a** CSF BACE1 levels from healthy subjects (three subjects pooled per time point) are shown as the means  $\pm$  SEM. **b** CSF PCSK9 levels from healthy subjects (three subjects pooled per time point) are shown as the means  $\pm$  SEM. **c** CSF BACE1 and PCSK9 concentrations measured as described in Fig. 6a, b were plotted against each other. There was no significant correlation between BACE1 and PCSK9 levels in CSF

552 ng/mL during the early morning hours. There were no correlations observed between CSF PCSK9 deltas and age.

At the current time, the exact role of PCSK9 in CSF as well as the effect of inhibiting PCSK9 in the CSF is unclear. In the systemic circulation, several studies have suggested that disruption of the binding of PCSK9 to the LDLR using an anti-PCSK9 antibody results in preserved LDLR and decreased LDL-C [14, 15]. As a result, it has been suggested that inhibition of circulating PCSK9 may represent a novel approach to lowering LDL-C levels.



**Fig. 7** ApoA1 and ApoB levels in human serum and CSF. **a** Serum ApoA1 levels from healthy subjects (three subjects pooled per time point) are shown as the means  $\pm$  SEM. **b** CSF ApoA1 levels from healthy subjects (three subjects pooled per time point) are shown as the means  $\pm$  SEM. **c** Serum ApoB levels from healthy subjects (three subjects pooled per time point) are shown as the means  $\pm$  SEM. **d** Serum and CSF ApoA1 concentrations measured in Fig. 6a, b were plotted against each other. There was no significant correlation between serum and CSF ApoA1 levels

neuronal apoptosis via modulation of ApoER2 levels, and its related anti-apoptotic signaling pathway [26]. In addition, another report indicated that PCSK9 reduces LDLR levels in mouse brain *in vivo* during development and after ischemic stroke [27], but the effect on ApoER2 and VLDLR remains unexplored. There have been inconsistent data regarding the interaction between PCSK9 and LDLR family members such as VLDLR and ApoER2 [24, 25]. PCSK9 can directly bind to LDLR, VLDLR, and ApoER2, but there were no differences in the steady-state levels of these receptors in the brain of PCSK9 knockout and PCSK9 transgenic mice when compared with wild type mice, suggesting that PCSK9 may not be a physiological regulator of these receptors in the CNS [25]. Inducible degrader of the LDLR (IDOL) has recently been identified as an E3 ubiquitin ligase that modulates cholesterol levels by regulating the stability of LDLR via the LXR–IDOL pathway [28]. VLDLR and ApoER2 also have been identified as IDOL targets [29]. A recent study has shown that VLDLR is regulated in hippocampal neurons by Reelin, which decreased VLDLR levels largely via induction of IDOL [30]. Based on these recent studies, the LXR–IDOL pathway may play a critical role in the CNS to balance the LDLR, VLDLR, and ApoER2 levels, especially since PCSK9 levels in CSF are so low compared to those in serum.

A recent study also suggested that PCSK9 can modulate BACE1 protein levels in the CNS [31]. BACE1 is the major  $\beta$ -secretase that cleaves amyloid precursor protein (APP) to generate a soluble fragment (sAPP $\beta$ ) and a membrane stalk ( $\beta$ -CTF). Subsequent intra-membrane cleavage of the  $\beta$ -CTF by the  $\gamma$ -secretase complex releases the amyloidogenic A $\beta$ 40 and A $\beta$ 42 peptides, which are believed to play a major role in AD pathology. Jonas and co-workers [31] reported that the overexpression of PCSK9 in cell culture decreased BACE1 protein levels, and conversely that siRNA down-regulation of PCSK9 increased BACE1 protein levels *in vitro*. A separate recent study, however, indicated that PCSK9 did not significantly alter BACE1 levels or APP processing to A $\beta$  in the adult mouse brain [25]. An additional study measured the soluble form of BACE1 levels by both Western blot and ELISA in human CSF and found significant increased levels in Mild Cognitive Impairment (MCI), which could be an early

In the CNS, the role of PCSK9 is less clear. In neurons, VLDLR and ApoER2 are involved in multiple cell signaling cascades through several extra- and intracellular binding partners including Reelin and ApoE, among others [42]. Recently, it has been shown that PCSK9 potentiates



stage of AD [32]. In the present study, however, we demonstrated that the BACE1 protein levels in CSF do not display diurnal variation and that no correlation existed between BACE1 and PCSK9 levels in human CSF. Therefore, our data may suggest that PCSK9 levels in the CSF are too low to significantly impact BACE1 levels.

The implications of our current findings that CSF PCSK9 concentrations are maintained at a constant level, despite significant diurnal variation of PCSK9 protein levels in the peripheral circulation, are not completely clear. A recent study reported that in young, healthy, human subjects, CSF A $\beta$  displayed diurnal variation, however, the CSF diurnal changes decreased in both amyloid negative and amyloid positive older subjects [43].

In addition, transporters likely limit the transport of serum proteins to the CNS. CSF ApoA1 is believed to originate from the translocation of plasma ApoA1 across the blood–brain barrier [44, 45]. In the present study, we measured both serum and CSF ApoA1 levels and the results showed that neither serum nor CSF ApoA1 displayed diurnal variations, and no correlation was observed between serum and CSF ApoA1. The serum/CSF ratio in ApoA1 was about 600, which was much higher than the PCSK9 serum/CSF ratio of 60. This indicates that transport may not entirely explain the presence of PCSK9 in CSF, and further studies are needed to determine if a potential transporter is involved in PCSK9 transport from serum to CSF. We also measured both serum and CSF ApoB levels. The results showed that no ApoB diurnal variation is displayed in serum and that no ApoB was detected in CSF, thus minimizing the likelihood of potential blood contamination of the CSF samples. It should be pointed out, however, that our current study did not reproduce previously reported diurnal variation of ApoA1 in serum [46, 47]. There may be several different reasons for this, including the effects of different study designs, subject selection and inclusion, and timing of type of caloric intake during the study. Therefore, our results showing lack of fluctuation in CSF ApoA1 do not conclusively support potential differential regulation of serum and CSF PCSK9.

The fact that the CSF PCSK9 levels were observed to be so constant in our human subjects, even in light of the up to threefold variation of serum PCSK9 concentrations over the course of the day, suggests that regulation of CSF PCSK9 levels may be under different control than for peripheral circulating levels. One may speculate that our findings are interesting given that receptors for ApoE in the CNS, which include the LDLR, VLDLR, and ApoER2, may modulate ApoE mediated cholesterol uptake, which in turn may be connected to A $\beta$  metabolism and toxicity [48, 49]. At the current time, however, this remains speculation, especially since PCSK9 levels in CSF are so low compared to serum and do not appear to correlate with CSF BACE1 concentrations.

The exact role of PCSK9 in CSF, therefore, remains to be elucidated. Nevertheless, the fact that PCSK9 in the CSF might possibly act to alter the protein levels of one or more of these receptors suggests that further studies on the role and regulation of PCSK9 in the CSF are warranted.

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