Aims: The serum ratios of the brain-specific oxysterol 24S-hydroxycholesterol (24S-OHC) to cholesterol and to 27-OHC reflect brain cholesterol turnover. We studied the effect of proprotein convertase subtilisin/kexin type 9 monoclonal antibodies (PCSK9ab) that enhance low-density lipoprotein receptor activity on serum cholesterol and oxysterol concentrations.

Methods: Twenty-eight hypercholesterolaemic patients (15 males and 13 females) responding insufficiently to maximally tolerated statin and/or ezetimibe therapy were additionally subcutaneously treated biweekly with either the PCSK9ab alirocumab (150 mg, n = 13) or evolocumab (140 mg, n = 15). Fasting serum cholesterol was measured by gas chromatography and the oxysterols 24S-OHC and 27-OHC using gas chromatography–mass spectrometry before, after 1-month (n = 28) and after 3-month (n = 13) treatment.

Results: As expected, PCSK9ab treatment lowered serum cholesterol and oxysterol levels after 1 month. The serum ratio of 24S-OHC to cholesterol increased after 1 month by 17 ± 28% (mean ± standard deviation; 95% confidence interval [CI]: 5.8 to 28%; P < .01) and 24S-OHC to 27-OHC by 15 ± 39% (95% CI: 0.2 to 30%; P < .01). Within 3 months, 24S-OHC to cholesterol increased by 2.8 μg g⁻¹ mo⁻¹ (95% CI: 2.1 to 3.6; P < .01) and 24S-OHC to 27-OHC by 0.019 mo⁻¹ (95% CI: 0.007 to 0.032; P < .01).

Conclusion: The serum ratios of 24S-OHC to cholesterol and to 27-OHC increased after treatment with PCSK9ab. We hypothesize that this is caused by a reduced entrance of 27-OHC into the brain, increased synthesis of brain cholesterol, increased production of 24S-OHC and its secretion across the blood–brain barrier.

KEYWORDS
brain cholesterol metabolism, cholesterol, cytochrome P450, low-density lipoprotein receptor, oxysterols, PCSK9 inhibitor
1 | INTRODUCTION

Most pronounced elevated serum total cholesterol and low-density lipoprotein (LDL)–cholesterol levels lead to atherosclerotic cardiovascular disease and its complications and are observed in familial hypercholesterolaemia (FH) caused by impaired LDL receptor (LDLR) activity. This may be the result of a genetic mutation in the LDLR protein or by excessive activity of proprotein convertase subtilisin/kexin type 9 (PCSK9), an enzyme catalysing the breakdown of LDLRs leading to a reduced number of LDLRs at the cell surface.1–3 FH patients often do not react sufficiently on maximally dosed statin and ezetimibe treatment. These patients are nowadays treated with a combination of anti-PCSK9 monoclonal antibodies and maximally dosed statins with or without ezetimibe.4,5

Hypercholesterolaemia in the midlife period has been associated with impaired brain function at later age.6 In Alzheimer disease (AD), it is believed that cognitive impairment is associated with cholesterol metabolism alteration, which could involve PCSK9. In a genetic and proteomic multicohort study Picard and colleagues found that PCSK9 plays an active role in the pathophysiology of late onset AD both in the presymptomatic and symptomatic phases of the disease.7 PCSK9 also appears to regulate the development of glucose intolerance, insulin resistance, abdominal obesity, inflammation and hypertension, conditions that have been identified as risk factors for AD.8 Recently it was found that PCSK9 is elevated in the cerebrospinal fluid (CSF) of individuals with alcohol use disorders. Therefore, inhibition of PCSK9 is discussed as a new therapeutic target for alcoholic liver disease.9

Cholesterol metabolism in the central nervous system (CNS) is clearly uncoupled from cholesterol in the other, extrahepatic and hepatic, tissues by a protective blood–brain barrier.10 While brain cholesterol cannot be measured directly in vivo, the predominant metabolite of brain cholesterol, 24S-hydroxycholesterol (24-OHC), can be measured in the blood.11–13 24S-OHC is formed from cholesterol by the cytochrome P450 enzyme (CYP) 46A1 in neurons and enables the major route of cholesterol elimination from the brain.14,15 In contrast to 24S-OHC, another oxysterol, 27-hydroxycholesterol (27-OHC), is ubiquitously synthesized in hepatic and extrahepatic tissues, except the brain, and partially secreted into the circulation and transported into the brain. It is suggested that 27-OHC in the brain plays a role by downregulating CNS cholesterol synthesis and, as a consequence, 24S-OHC production.16 Interestingly, patients with AD have an accumulation of 27-OHC in the brain.17 Serum oxysterols are transported in lipoprotein compartments in similar distribution as its substrate cholesterol itself. Therefore, the serum concentrations of cholesterol and oxysterols are highly correlated. Concentrations of 24S-OH in CSF and its ratio to cholesterol (R ratio) to 27-OHC (24S-OHC/27-OHC) in serum or plasma are considered biomarkers for CNS cholesterol metabolism and its implications in the aetiology of neurodegeneration.18–20

So far, the influence of PCSK9 inhibitory treatment on serum levels of R ratio or 24S-OHC/27-OHC as markers for brain cholesterol metabolism has not been studied. Therefore, we assessed the effect of PCSK9 inhibition in hypercholesterolaemic patients as add-on therapy to oral lipid-lowering treatments on serum concentrations of 24S-OHC and 27-OHC. Our hypothesis was that PCSK9 inhibition leads to upregulated LDLR activity, reduced total serum cholesterol and 27-OHC concentrations, reduced 27-OHC transfer into the brain, reduced suppression of brain cholesterol synthesis followed by an increased balancing production of 24S-OHC. As a consequence, the transfer of 24S-OHC into the circulation increases, which may lead to an increased serum ratio of 24S-OHC to cholesterol (R ratio) or to 27-OHC (24S-OHC/27-OHC), presumably indicating an increased cholesterol turnover in the CNS.

2 | METHODS

2.1 | Study enrolment and design

Within this observational trial hypercholesterolaemic patients responding insufficiently to maximally tolerated statin (n = 4, rosuvastatin n = 1 and atorvastatin n = 3) and/or ezetimibe (eze) therapy (n = 17; rosuvastatin/eze n = 3, simvastatin/eze n = 3, atorvastatin/eze n = 11; ezetimibe alone n = 7) received subcutaneously once every 2 weeks the anti-PCSK9 monoclonal antibodies alirocumab (Praluent, 150 mg, n = 13) or evolocumab (Repatha, 140 mg, n = 15). The pretreatment medication with statin and/or ezetimibe was continued during the study. Additional to the statin and ezetimibe combination therapy 3 patients received cholestagel and 1 patient received fenofibrate. The patients were recruited in 2016 at Klinik für Innere Medizin III (Kardiologie, Angiologie und Internistische Intensivmedizin), Universitätsklinikum des Saarlandes, Homburg, Germany and Medizinische Klinik IV—Campus Großhadern, Klinikum der Universität München, Munich, Germany. Inclusion criteria were age ≥ 18 years, prescription of anti-PCSK9 monoclonal antibodies and ability to understand the purpose of the study. Patients receiving alirocumab or evolocumab according to current guidelines were eligible for inclusion into the study. At the first visit, a standard questionnaire for demographic and clinical data was filled out and clinical investigations were performed. Before and after 4 and 12 weeks of treatment with PCSK9 inhibitors blood samples were taken 5–7 days after the last injection for enzymatic lipoprotein, chromatographic total cholesterol and oxysterol analysis as well as for safety laboratory analyses. Samples were taken in the morning after a 10-hour fasting state. Hypertension, cardiovascular disease (coronary artery disease, cerebral artery disease and/or peripheral artery disease) and diabetes mellitus were diagnosed based on medical records.

Written informed consent was obtained from each patient prior to inclusion in the study. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki, and the study protocol has been approved by the institutions’ ethics committees on research on humans (162/15—Homburg and 17–780 Munich).

This work was presented at the ENOR conference in Bologna in September 2018.
2.2 | Laboratory analysis

After centrifugation serum samples were kept frozen at −80 °C until analyses. Serum samples for routine blood investigations were analysed in the respective central laboratories of the 2 university study centres. Serum total cholesterol and oxysterol analysis was performed 4 weeks after the last sampling at the laboratory for Special Lipid Diagnostics at the Institute of Clinical Chemistry and Clinical Pharmacology, University of Bonn, Germany.

2.3 | Assessment of serum cholesterol and oxysterol chromatographic analysis

Serum concentrations of cholesterol were quantified using gas chromatography (GC)–flame ionization detection (FID) with 5α-cholestane as internal standard. The oxysterols 24S-OHC and 27-OHC were quantified by an highly specific and sensitive isotope dilution GC–mass spectrometry selected ion-monitoring (GC–MS–SIM) methodology using the corresponding deuterium-labelled oxysterols as internal standards. The assay coefficients of variation for the analytical method to determine serum concentration of cholesterol, 24S-OHC and 27-OHC were 2.7, 1.1 and 1.5%, respectively. According to Fakheri and Javitt, the preferred nomenclature for 27-hydroxylation and 27-OHC should be (25R)26-hydroxylation and (25R)26-OHC. Here, we prefer to use 27-OHC to be consistent with our previous publications.

2.4 | Data and statistical analysis

All subjects were studied before and after 1 month of biweekly subcutaneous injection of PCSK9 inhibitors. Data are presented as mean (standard deviation). In a subgroup of subjects samples were also collected after 3 months. In this subgroup data are presented as mean (standard error method).

Statistical significance of mean changes was tested against zero first to get the 95% CI values applying the 2-sided paired t-test for all cases. To get a robust result P-values were confirmed using Wilcoxon matched-pairs signed rank test. The mean absolute change of a parameter was calculated as the mean of all individual changes. The percent change of a parameter was calculated as the mean of all individual changes divided by the value of the parameter before treatment. To test the different effects of treatment, pretreatment and sex, the Mann–Whitney U-test was used.

Correlations between serum concentrations of and changes in serum concentrations of cholesterol and oxysterols and its ratios were estimated and tested using the Spearman rank correlation test.

Pairwise comparison of the ratios of 24S-OHC and 27-OHC to cholesterol (R_24SOHC and R_27-OHC) and 24S-OHC/27-OHC within a treatment period of 3 months (n = 13). All statistical tests were performed using Graphpad Prism (Graphpad software, version 8.00, San Diego, CA, USA). Stata 14.2 (StataCorp, College Station, TX, USA) was applied for mixed linear modelling and trend calculations. The α-level was set at .05.

3 | RESULTS

The patients’ characteristics are shown in Table 1, indicating 28 subjects (15 males, 13 females) being treated with alirocumab (n = 13) and evolocumab (n = 15). Seventeen subjects were simultaneously treated with statin and ezetimibe, whereas 11 subjects were treated with statin (n = 4) or ezetimibe (n = 7). The subgroup of subjects that was followed 3 months consisted of 13 subjects (7 males, 6 females) being treated with alirocumab (n = 8) and evolocumab (n = 5).

3.1 | Total study group

3.1.1 | Serum cholesterol and oxysterol concentrations and the ratios of 24S-OHC to cholesterol and to 27-OHC

In the total study group, biweekly subcutaneous applied PCSK9 inhibitors lowered serum total cholesterol after 1 month by −37% ± 12% (95% confidence interval [CI]: −42 to −33%; P < .01) and LDL-cholesterol by −52% ± 17% (95% CI: −59 to −46%; P < .01; Table 2). Serum concentrations of cholesterol, measured by GC–FID, decreased statistically significantly by −41% ± 15% (95% CI: −41 to −29%; P < .01), 24S-OHC by −26% ± 13% (95% CI: −32 to −21%; P < .01) and 27-OHC by −21% ± 16% (95% CI: −26 to −16%; P < .01) within a treatment period of 3 months (n = 28). All statistical tests were confirmed using Wilcoxon signed-rank test. The mean absolute change of a parameter was calculated as the mean of all individual changes divided by the value of the parameter before treatment.

### Table 1: Patient demographics and subgroup composition at baseline

| Characteristic                  | All patients n = 28 |
|--------------------------------|---------------------|
| Age (y), mean (SD)             | 55.8 (11.3)         |
| Sex                            |                     |
| Female, n (%)                  | 15 (53.6)           |
| Male, n (%)                    | 13 (46.4)           |
| BMI (kg/m²), mean (SD)         | 28.7 (5.3)          |
| PCSK9 inhibitors                |                     |
| Evolocumab (Repatha, 140 mg³)  | 15 (53.6)           |
| Alirocumab (Praluent, 150 mg³) | 13 (46.4)           |
| Pretreatment with statin and/or ezetimibe⁴ |     |
| Statin or ezetimibe, n (%)     | 11 (39.3)           |
| Statin and ezetimibe, n (%)    | 17 (60.7)           |

³biweekly subcutaneous injection.
⁴the initialized lipid lowering therapy by statins (rosuvastatin, simvastatin, atorvastatin) and/or ezetimibe was continued under PCSK9 inhibition.
SD, standard deviation; BMI, Body mass index; PCSK9, proprotein convertase subtilisin/kexin type 9.
27-OHC by \( -33\% \pm 16\% \) (95% CI: \(-39\% \) to \(-27\% \); \( P < .01 \); Table 3). R\(_{24S-OHC}\) was statistically significant increased by 17% \pm 28% (95% CI: 5.7 to 28%, \( P < .01 \); Table 3). Of the 28 subjects, 21 (75%) exerted an increased serum R\(_{24S-OHC}\). After 1-month treatment 21 (75%) patients presented a statistical significant increase in serum 24S-OHC/27OHC by 15% \pm 39% (95% CI: 2.5 to 30%; \( P < .01 \)).

### 3.1.2 Correlation analysis

In the following consideration we merged the data before and after 1-month treatment as the correlations of the individually considered data revealed no statistical differences. Comparison of enzymatically and GC-FID determined serum cholesterol revealed a statistical significant 2-tailed Spearman correlation with \( R = 0.95 \) (95% CI: 0.91 to 0.97; \( P < .01 \)). Serum concentrations of 24S-OHC and 27-OHC correlated statistically significant with each other by \( R = 0.59 \) (95% CI: 0.38 to 0.74; \( P < .01 \)). Serum total cholesterol (GC-FID) correlated statistically significant with 24S-OHC (\( R = 0.80 \); 95% CI: 0.68 to 0.88; \( P < .01 \)) and with 27-OHC (\( R = 0.84 \), 95%CI: 0.73 to 0.90; \( P < .01 \)). The changes in cholesterol, 24S-OHC and 27-OHC concentrations correlated with their baseline concentrations before the initiation of the PCSK9 inhibitor treatment (Figure 1A). The Spearman \( R \) and 95% CI values are \( R = -0.51 \) (95% CI: \(-0.75\% \) to \(-0.16\%; \( P < .01 \)) for cholesterol, \( R = -0.65 \) (95% CI: \(-0.83\% \) to \(-0.35\%; \( P < .01 \)) for 24S-OHC and \( R = -0.46 \) and (95% CI: \(-0.72\% \) to \(-0.09\%; \( P < .01 \)) for 27-OHC.

Furthermore, the changes in serum concentrations of 24S-OH cholesterol correlated statistically significant with the changes in cholesterol with \( R = 0.73 \) (95% CI: 0.47 to 0.87; \( P < .01 \)) and the changes of 27OHC-cholesterol with the changes in cholesterol \( R = 0.88 \) (95% CI: 0.75 to 0.95; \( P < .01 \); Figure 1B).

The changes in serum concentrations of 24S-OHC correlate statistically significant with those of 27-OHC with \( R = 0.73 \) (95% CI: 0.48 to 0.87; \( P < .01 \); Figure 1C). A slope of 1.96 in the regression line indicates that the net decrease in 27-OHC is on average nearly double as high as in 24S-OHC.

The changes in R\(_{24S-OHC}\) and R\(_{27OHC}\) were highly correlated to each other with \( R = 0.59 \) (95% CI: 0.26 to 0.80; \( P < .01 \); Figure 1D).

### 3.1.3 Effects of the different PCSK9ab and combined or single statin/ezetimibe pretreatment

Alirocumab showed statistically significant lower changes in the serum concentrations of 24S-OHC compared with evolocumab treatment (Table 3). Percentage changes under alirocumab were \(-33\% \pm 13\% \) (95% CI: \(-40\% \) to \(-26\%; \( P < .01 \)), while the percentage changes during evolocumab therapy were \(-19\% \pm 10\% \) (95% CI: \(-26\% \) to \(-13\%; \( P < .01 \)). This difference of 14% of the percentage change was statistically significant (95% CI: 4.0 to 23%; \( P < .01 \)).

The combined pretreatment and add-on treatment with ezetimibe and statins showed statistically significant lower absolute changes after 1 month in the serum concentrations of cholesterol compared with statin or ezetimibe treatment alone (Table 3). Percentage changes in the combined treatment were \(-33\% \pm 17\% \) (95% CI: \(-42\% \) to \(-24\%; \( P < .01 \)), while those in the single treatment ezetimibe or statins were \(-38\% \pm 13\% \) (95% CI: \(-47\% \) to \(-29\%; \( P < .01 \)). However, this difference of the percentage change was not statistically significant (95% CI: \(-8\% \) to \(-18\%; \( P = .46 \)).

The combined pretreatment and add-on treatment with ezetimibe and statins showed statistically significant lower changes in the serum concentrations of 27-OHC compared with statin or ezetimibe treatment alone (Table 3). Percentage changes in the combined treatment were \(-23\% \pm 17\% \) (95% CI: \(-39\% \) to \(-22\%; \( P < .01 \)), while the percentage changes in the single therapy were \(-37\% \pm 15\% \) (95% CI: \(-47\% \) to \(-29\%; \( P < .01 \)). This difference of the percentage change was not statistically significant (95% CI: \(-10\% \) to 18%; \( P = .50 \)).

### 3.1.4 Three-month vs. 1-month treatment

The 3-month follow-up data (before, after 1 mo and after 3 mo) for the ratio of 24S-OHC and 27-OHC to cholesterol and for the ratio of 24S-OHC to 27-OHC are shown in Table 4. Pairwise comparison of the different sampling dates using a robust mixed linear model with patient as random factor revealed statistically significant mean changes for all compared data points only for R\(_{24S-OHC}\). Using the same robust mixed linear model within 3 months a slope of 2.8 \( \mu \)g \( g^{-1} \) mo\(^{-1} \) for R\(_{24S-OHC}\) and of 0.019 mo\(^{-1} \) for 24S-OHC/27-OHC (Table 4) was found. Over the whole period we did find a trend image for both parameters as presented in Figure 2.

### TABLE 2 Serum lipids (measured enzymatically) before and 1 month after subcutaneous application of alirocumab (Praluent, 150 mg, \( n = 13 \)) or evolocumab (Repatha, 140 mg, \( n = 15 \)) once every 2 weeks

| Lipid parameter       | Before | After 1 mo | Mean change | 95% CI               | \( P_{PCS9 inh} \) |
|-----------------------|--------|-----------|-------------|----------------------|-------------------|
| Total cholesterol (g L\(^{-1}\)) | 2.89 (1.52) | 1.83 (1.04) | \(-1.06 \) (0.63) | \(-1.30 \) to \(-0.82 \) | \(< .01 \) |
| LDL cholesterol (g L\(^{-1}\)) | 2.01 (1.09) | 1.06 (0.97) | \(-0.96 \) (0.33) | \(-1.09 \) to \(-0.83 \) | \(< .01 \) |
| HDL cholesterol (g L\(^{-1}\)) | 0.56 (0.16) | 0.55 (0.14) | \(-0.01 \) (0.08) | \(-0.04 \) to \(-0.02 \) | 0.61 |
| Triglyceride (g L\(^{-1}\)) | 1.64 (0.89) | 1.51 (0.71) | \(-0.13 \) (0.66) | \(-0.39 \) to \(-0.12 \) | 0.42 |

\(^{a}\)The Wilcoxon matched-pairs signed rank test \( P_{PCS9 inh} \) expresses the statistical significance of the effect of PCSK9 inhibition.

\(^{b}\)Mean (SD).
TABLE 3  Serum concentrations of cholesterol (measured by GC-FID), the oxysterols 24S- and 27-Hydroxycholesterol (24S- and 27-OHC; measured by GC–MS–SIM) as well as 24S-OHC to cholesterol (R_{24S-OHC}), 27-OHC to cholesterol (R_{27-OHC}) and the ratio 24S-OHC/27-OHC, before and 1 month after subcutaneous application of evolocumab (Repatha, 140 mg, n = 15) or alirocumab (Praluent, 150 mg, n = 13) once every 2 weeks. The patients (13 females and 15 males) are pretreated with statin or ezetimibe (n = 11) or statin and ezetimibe (n = 17) for 5 to 15 years. The mean (standard deviation) is expressed for each subgroup:

| Sterol | Subgroup | Category | Before | After 1 mo | Mean change | 95% CI | \( P_{\text{PCSK9 inh}} \) | \( P_{\text{A}} \) |
|--------|----------|----------|--------|------------|-------------|-------|-----------------|-------------|
| Cholesterol (g L\(^{-1}\)) | All | 2.89 (1.43) | 1.88 (0.97) | −1.01 (0.64) | −1.3 to −0.76 | <.01 | | |
| Evolocumab | A | 3.08 (1.81) | 1.98 (1.18) | −1.10 (0.76) | −1.5 to −0.69 | <.01 | | |
| Alirocumab | B | 2.66 (0.82) | 1.76 (0.68) | −0.89 (0.47) | −1.2 to −0.61 | <.01 | | |
| Statin or ezetimibe | A | 3.82 (1.90) | 2.42 (1.29) | −1.41 (0.77) | −1.9 to −0.89 | <.01 | | |
| Statin and ezetimibe | B | 2.28 (0.42) | 1.53 (0.47) | −0.75 (0.38) | −0.94 to −0.55 | <.01 | .01 |
| Females | A | 3.29 (1.94) | 2.29 (1.17) | −1.00 (0.85) | −1.5 to −0.49 | <.01 | | |
| Males | B | 2.53 (0.64) | 1.52 (0.59) | −1.01 (0.41) | −1.2 to −0.79 | <.01 | .37 |

| 24S-OHC (μg L\(^{-1}\)) | All | 117 (48) | 84 (30) | −33 (24) | −42 to −23 | <.01 | | |
| Evolocumab | A | 129 (58) | 86 (36) | −43 (28) | −59 to −28 | <.01 | | |
| Alirocumab | B | 103 (30) | 83 (24) | −20 (12) | −28 to −13 | <.01 | <.01 | |
| Statin or ezetimibe | A | 144 (64) | 101 (37) | −43 (31) | −64 to −22 | <.01 | | |
| Statin and ezetimibe | B | 100 (22) | 74 (20) | −26 (17) | −35 to −18 | <.01 | .16 | |
| Females | A | 133 (61) | 98 (32) | −35 (32) | −54 to −16 | <.01 | | |
| Males | B | 103 (27) | 72 (23) | −31 (17) | −40 to −22 | <.01 | .64 | |

| 27-OHC (μg L\(^{-1}\)) | All | 225 (105) | 146 (75) | −76 (55) | −98 to −55 | <.01 | | |
| Evolocumab | A | 242 (137) | 154 (82) | −88 (67) | −126 to −51 | <.01 | | |
| Alirocumab | B | 211 (59) | 149 (57) | −62 (35) | −83 to −41 | <.01 | .61 | |
| Statin or ezetimibe | A | 296 (136) | 188 (66) | −108 (67) | −154 to −63 | <.01 | | |
| Statin and ezetimibe | B | 183 (52) | 128 (47) | −55 (34) | −73 to −38 | <.01 | <.01 | |
| Females | A | 260 (142) | 176 (77) | −84 (72) | −127 to −40 | <.01 | | |
| Males | B | 200 (57) | 130 (58) | −70 (36) | −90 to −50 | <.01 | .75 | |

| R_{24S-OHC} (μg g\(^{-1}\)) | All | 42 (10) | 49 (16) | 7.1 (11) | 2.6 to 11 | <.01 | | |
| Evolocumab | A | 44 (13) | 48 (17) | 3.9 (6.3) | 0.45 to 7.3 | .05 | | |
| Alirocumab | B | 40 (6) | 50 (15) | 11 (15) | 2.1 to 20 | .01 | .06 | |
| Statin or ezetimibe | A | 38 (6) | 47 (18) | 9.0 (16) | −2.0 to 20 | .05 | | |
| Statin and ezetimibe | B | 45 (12) | 51 (15) | 5.9 (6.6) | 2.6 to 9.1 | <.01 | >.99 | |
| Females | A | 43 (14) | 47 (17) | 3.8 (5.8) | 0.27 to 7.3 | .05 | | |
| Males | B | 41 (6) | 51 (15) | 9.9 (14) | 2.1 to 18 | <.01 | .70 | |

| R_{27-OHC} (μg g\(^{-1}\)) | All | 80 (17) | 82 (20) | 2.3 (10) | −1.8 to 6.1 | .43 | | |
| Evolocumab | A | 79 (18) | 79 (18) | 0.2 (11) | −6.1 to 6.5 | .73 | | |
| Alirocumab | B | 81 (17) | 86 (21) | 4.2 (9.2) | −1.3 to 9.8 | .05 | .07 | |
| Statin or ezetimibe | A | 79 (12) | 79 (12) | 0.4 (9.7) | −6.1 to 6.9 | .82 | | |
| Statin and ezetimibe | B | 81 (21) | 84 (23) | 3.2 (11) | −2.2 to 9.1 | .41 | .82 | |
| Females | A | 80 (18) | 79 (17) | −1.8 (6.2) | −5.6 to 2.0 | .33 | | |
| Males | B | 80 (18) | 85 (22) | 5.4 (12) | −1.4 to 12 | .12 | .06 | |

| 24S-OHC/27-OHC | All | 0.55 (0.19) | 0.64 (0.29) | 0.09 (0.19) | 0.01 to 0.16 | <.01 | | |
| Evolocumab | A | 0.60 (0.22) | 0.65 (0.32) | 0.06 (0.11) | −0.01 to 0.12 | .05 | | |
| Alirocumab | B | 0.50 (0.12) | 0.63 (0.27) | 0.12 (0.25) | −0.03 to 0.27 | <.01 | .42 | |
| Statin or ezetimibe | A | 0.49 (0.10) | 0.62 (0.29) | 0.13 (0.27) | −0.06 to 0.31 | .02 | | |
| Statin and ezetimibe | B | 0.59 (0.22) | 0.65 (0.30) | 0.06 (0.11) | 0.005 to 0.11 | .02 | .22 | |
| Females | A | 0.57 (0.24) | 0.64 (0.33) | 0.07 (0.11) | 0.004 to 0.14 | <.01 | | |
| Males | B | 0.54 (0.13) | 0.64 (0.26) | 0.10 (0.24) | −0.04 to 0.23 | .03 | >.99 | |

The Wilcoxon matched-pairs signed rank test \( P_{\text{PCSK9 inh}} \) expresses the statistical significance of the effect of PCSK9 inhibition.

The Mann–Whitney \( P_{AB} \) expresses the statistical significance of the difference of the mean change between the actual categories A and B. Mean (standard deviation).

GC-FID, gas chromatography–flame ionization detection; GC–MS–SIM, gas chromatography–mass spectrometry–selection ion monitoring; 95% CI, 95% confidence interval of the mean change; SD, standard deviation.
DISCUSSION

R_{24S-OHC} increased statistically significant by 17% and 24S-OHC/27-OHC by 15% after 1-month treatment in all subjects. In a subgroup of patients, we found a statistically significant increase in R_{24S-OHC} with a slope of 2.8 μg g⁻¹ mo⁻¹ and in 24S-OHC/27-OHC with a slope of 0.019 mo⁻¹ within 3-month treatment with PCSK9ab.

The oxysterols 24S-OHC and 27-OHC are, in addition to 7a-OHC, the major enzymatically produced oxysterols transported by lipoproteins in blood.23 Their common fate is the catabolism to bile acids after uptake into the liver.15,24 Cholesterol in the CNS is exclusively formed locally and excess of cholesterol is metabolized into 24S-OHC.11 This oxysterol can cross the blood–brain-barrier entering the peripheral circulation. The origin of 27-OHC is formation from cholesterol by sterol 27-hydroxylase (CYP27A1) in peripheral hepatic and extrahepatic tissues, among others in macrophages.23,25,26 A fraction of serum 27-OHC crosses the blood–brain-barrier and enters the brain. Here it is thought to dose-dependently inhibit cholesterol synthesis in the astrocytes within the CNS.10

Our data show a number of remarkable correlations between serum cholesterol and oxysterols (Figure 1), its ratios and the changes between each other. These correlations confirm the concept that cholesterol and oxysterols in serum generally undergo the same fluxes that are affected by PCSK9 inhibitors. PCSK9 inhibition leads to enhancement of the LDLR capacity, enhanced tissue uptake and similarly lowered serum concentrations of all 3 sterols together with cholesterol itself. This suggests that 24S-OHC and 27-OHC are also transported into peripheral tissues via the LDL and HDL particles as used for cholesterol transport. Only the degrees of concentration reduction are different, the highest for cholesterol, the lowest for 24S-OHC. As a result, the R_{27-OHC} remains unaltered under treatment, whereas R_{24S-OHC} and the 24S-OHC/27-OHC ratio were statistically significant increased. Thus, the effect of PCSK9 inhibition on 24S-OHC appears different from the effect on cholesterol and 27-OHC. This is probably caused by the different origins being the brain or CNS for 24S-OHC and hepatic and extrahepatic tissues for cholesterol and 27-OHC outside the brain.27 A link between 24S- and 27-OHC may play an important role. It may be expected that a reduction of the serum concentration of 27-OHC due to PCSK9 inhibition leads to a reduced appearance and concentration of 27-OHC in the brain and therefore a reduced inhibition of cholesterol synthesis. Enhanced cholesterol synthesis in the brain results in an increased 24S-OH formation, increased transfer across the blood–brain barrier and thus an increased inflow of 24S-OHC into the blood. A number of factors must be taken into consideration when drawing conclusions from serum concentrations. In healthy normocholesterolaemic subjects, the serum concentration of 24S-OHC averages 80 ng/mL.11 Considering a total serum pool

FIGURE 1  Correlations between concentrations of cholesterol, oxysterols, ratios of oxysterols to cholesterol, its baseline levels and its changes during treatment. The Spearman R and P-values are given. (A) Dependence of the change in cholesterol and oxysterol concentrations on the baseline concentration (before treatment). Serum concentrations of cholesterol are given in 10² x g L⁻¹ and those of oxysterols in μg L⁻¹. (B) Correlations between the changes in serum concentration of cholesterol and oxysterols. (C) Correlation between the changes in serum concentrations of 24S-OHC and 27-OHC with R = 0.73 (95%CI: 0.48 to 0.87). (D) Correlation between the changes in R_{24-OHC} and R_{27-OHC}
of 3 L, the total serum pool accounts for about 240 μg. The daily flux of 24S-OHC entering the blood via the blood–brain barrier is expected to be in the range of 4–7 mg/day, which exceeds the serum pool size by 15- to 30-times.11 Similar values can be calculated for 27-OHC.28 Thus, the actual pool sizes are very small compared to the fluxes passing the serum compartment. The serum concentrations are not under control, they are established by the accidental presence of molecules moving between brain and liver and extrahepatic tissues and the liver. In this respect, it is important to discuss the lipoprotein fractions in which the oxysterols are transported through the blood compartment. 24S-OHC enters across the blood–brain barrier and is probably transported by HDL and delivered to the liver for metabolism.29

**Table 4** The 3-month follow-up data for the ratio of 24S-hydroxycholesterol (OHC) to cholesterol (R\(_{24S-OHC}\)), 27-OHC to cholesterol (R\(_{27-OHC}\)) and the 24S-OHC/27-OHC ratio in 13 patients (7 males and 6 females) treated with evolocumab (Repatha, 140 mg, \(n=5\)) or alirocumab (Praluent, 150 mg, \(n=8\)) once every 2 weeks.

|                      | R\(_{24S-OHC}\) (μg g\(^{-1}\)) | R\(_{27-OHC}\) (μg g\(^{-1}\)) | 24S-OHC/27-OHC |
|----------------------|---------------------------------|---------------------------------|----------------|
| **Before**           | 39 (1.6)*                       | 107 (5.4)                      | 0.38 (0.025)   |
| **1 mo after**       | 42 (2.1)                        | 111 (8.2)                      | 0.41 (0.038)   |
| **3 mo after**       | 47 (2.3)                        | 117 (11)                       | 0.44 (0.037)   |
| **1 mo after vs. before** |                                |                                 |                |
| Mean change          | 3.2 (1.4)                       | 4.0 (5.0)                      | 0.034 (0.018)  |
| 95%CI                | 0.49 to 5.9                     | −5.8 to 14                     | −0.0017 to 0.069|
| \(P\)                 | .02                             | .42                            | .06            |
| **3 mo after vs. before** |                                |                                 |                |
| Mean change          | 8.4 (1.1)                       | 10 (7.4)                       | 0.061 (0.019)  |
| 95%CI                | 6.3 to 11                       | −4.2 to 25                     | 0.022 to 0.10  |
| \(P\)                 | <.01                            | .16                            | <.01           |
| **3 mo after vs. 1 mo after** |                                |                                 |                |
| Mean change          | 5.3 (1.5)                       | 6.3 (4.5)                      | 0.027 (0.018)  |
| 95%CI                | 2.3 to 8.2                      | −2.4 to 15                     | −0.0078 to 0.062|
| \(P\)                 | <.01                            | .16                            | .13            |
| All over 3 mo        | (μg g\(^{-1}\) mo\(^{-1}\))    | (μg g\(^{-1}\) mo\(^{-1}\))    | mo\(^{-1}\)    |
| Slope per mo         | 2.8 (0.38)                      | 3.4 (2.4)                      | 0.019 (0.0064) |
| 95%CI                | 2.0 to 3.5                      | −1.2 to 8.0                    | 0.0067 to 0.032|
| \(P\)                 | <.01                            | .15                            | <.01           |

\(P_{PCSK9 \text{ inh pw}}\) expresses the statistical significance of the effect of PCSK9 inhibition pairwise comparing different sampling dates using a robust mixed linear model with patient as random factor. 
\(P_{PCSK9 \text{ 0–1-3}}\) expresses the statistical significance of the effect of PCSK9 inhibition within 3 months using a robust mixed linear model with patient as random factor.

*Mean (standard error \(\delta\)-method); 95%CI, 95% confidence interval.

**Figure 2** The courses of serum ratios of 24S-OHC to cholesterol (R\(_{24S-OHC}\); A) and 24S-OHC to 27-OHC (24S-OHC/27-OHC; B) during 3-month treatment are presented as graphs. Data points and progress (grey dots and grey lines) are given for each of the 13 patients. The dark black line shows the mean course surrounded by 95% confidence interval of the mean.
low-density lipoproteins (VLDL) secretion and transferred to LDL. Another fraction is converted to bile acids. The oxysterol 27-OHC is also partially produced in extrahepatic tissues, and a fraction is secreted into blood via HDL and delivered to the liver. Babiker et al. analysed oxysterol profiles in the serum lipoprotein fractions and showed that both 24S- and 27-OHC are equally distributed over mainly LDL and HDL (40% for each) and are present as esterified sterol. For 27-OHC, this may be explained by the 2 origins being hepatic and extrahepatic, for 24S-OHC, it must mean that esterified 24S-OHC is exchanged between HDL and LDL via the cholesterol ester transfer protein or that in the liver a large fraction of the extracted 24S-OHC is resecreted via VLDL. From previous studies on synthesis and absorption serum markers in PCSK9ab treated patients it may be expected that the cholesterol pools in hepatic and extrahepatic tissues are not drastically changed under treatment. Thus, no decreased or increased formation of 27-OHC may be expected. Little is known about hepatic metabolism of 24S-OHC. Norlin et al. provided evidence for 7α-hydroxylation in the human liver and conversion into bile acids. Björkhem et al. confirmed the evidence for hepatic 7α-hydroxylation, but also for conversion to 5-cholestene-3, 24, 27-triol and faecal excretion of 24S-OHC and the triol metabolite in conjugated forms via the bile. Potentially, a relatively stronger treatment effect on metabolism of 27-OHC (blocked formation and increased catabolism) compared to that of 24S-OHC (increased catabolism only) may partly explain the increased 24S-OHC/27-OHC ratio. Furthermore, the presented data may be influenced by the fact that the oxysterols are largely transported by HDL (40%), whereas cholesterol itself is mainly transported by the VLDL/LDL system. Since the PCSK9 inhibitors affect mainly LDL-cholesterol reduction, the effect on R24S-OHC and 24S-OHC/27-OHC, but also on R27-OHC may be determined by the sterol distribution over lipoproteins.

Increased catabolism of brain cholesterol by increased activity of the neuron-specific enzyme that converts cholesterol to 24S-OHC (CYP46A1) is the main target of actual approaches in the fight against cholesterol-related pathologies in neurodegenerative diseases. Overexpression of CYP46A1 in AD and Huntington disease (HD) mice corrected behavioural and neuropathological abnormalities of AD and HD. The authors revealed a new cholesterol-targeting therapeutic strategy in HD, and presumably in other neurodegenerative diseases where dysregulation of cholesterol homeostasis has been clearly identified. Pikuleva and colleagues developed a pure pharmacological path to enhance CYP46A1 activity using the well-known anti-human immunodeficiency virus medication efavirenz. More recently, they found that CYP46A1 activation by efavirenz leads to behavioural improvement without significant changes in amyloid plaque load in the brain of 5XFAD mice, a mouse model for AD. PCSK9 was initially identified with a potential involvement in brain development and apoptosis as neural apoptosis-regulated convertase 1, a protein that is upregulated in primary cerebellar neurons that undergo apoptosis induced by serum deprivation. In addition to LDLR, PCSK9 also degrades VLDLR, ApoER and ApoER2 (for a general review see). Concerning neuronal apoptosis, a proapoptotic activity of PCSK9 may occur through the upregulation of caspases or the reduction of the ApEr2 levels. More recently, studies have shown that PCSK9 may promote neuroinflammation. In AD, PCSK9 plays a direct role in the brain by lowering BACE1 expression. Genetic studies conducted in humans are not conclusive on the impact of PCSK9 mutations on AD. Interestingly Zimetti et al. demonstrated increased levels of CSF PCSK9 in AD patients with the highest levels in APOE4 e4 carriers. We observed elevated levels of 24S-OHC in the CSF of AD patients with a gene-dosage effect for APOE, suggesting a link between the AD risk factor APOE4 and the central cholesterol metabolism. Plasma cholesterol under PCSK9 inhibitor treatment reached very low levels (<0.30 g/L). Thus, the question arose of whether this drastic lowering in the periphery presents negative effects on brain cholesterol metabolism and cognitive functions. Lifelong exposure to extremely low levels of LDL-cholesterol in carriers of PCSK9 loss-of-function variants is not associated with neurocognitive effects. Under healthy conditions, the intact blood–brain barrier limits the access of both PCSK9 and of monoclonal antibodies from the periphery into the CNS such as alirocumab or evolocumab. In general, penetration of antibodies into the brain has been estimated to be extremely low, both in humans and animals. In an cardiac ischaemic/reperfusion rat model, leading to brain damage, PCSK9 inhibitor did not reduce PCSK9 levels in the brain of these animals, indicating that there was no local presence of this PCK9 inhibitor in the CNS. Treatment with evolocumab or placebo for 1.6 years in a total of 1204 subjects with a mean age of 65 years revealed no association with adverse cognitive effects. The 5-year extension of the FOURIER trial will provide further findings on neurocognitive function (https://clinicaltrials.gov/ct2/show/NCT02867813). Perhaps a good chance to follow changes in R24S-OHC and 24S-OHC/27-OHC in the plasma of participants of this study.

Our study has certain limitations. At first, the number of subjects is small and highly diverse in sex, pretreatment and anti-PCSK9 antibody agent. The 17% increase of R24S-OHC and 15% increase of 24S-OHC/27-OHC after 1-month treatment in all subjects was relatively small but statistically significant. This can be explained by the fact that, despite the large diversity, 21 of 28 subjects (75%) presented an increased R24S-OHC and 24S-OHC/27-OHC. Across the whole period of 3 months, the increases for R24S-OHC were more prominent than for 24S-OHC/27-OHC. In total, the increases can be solely ascribed to the PCSK9 inhibition. Clearly, larger and longer lasting population studies are necessary. Based on our results for the ratios of oxysterols to cholesterol or to each other (Table 3), these studies do not need to select patients on sex, pretreatment and PCSK9 inhibiting agent. One missing link in the information is the conversion of 27-OHC into 7α-hydroxy-3-oxo-4-cholesten-2-one in the brain. The degree of conversion may affect the degree of inhibition of brain cholesterol synthesis by 27-OHC. The bile acid precursor 7α-hydroxy-3-oxo-4-cholesten-2-one is known to pass the blood–brain barrier and to be transported back from the brain to blood into the lipoprotein-free fraction. Measurement of serum concentrations of 7α-hydroxy-3-oxo-4-cholesten-2-one may create additional
information on brain cholesterol metabolism. Measuring the arteriovenous difference of oxysterols between the internal jugular vein and an artery in healthy human volunteers we could demonstrate a significant flux of the oxysterol 24S-OHC from the brain into the circulation.\textsuperscript{11} A similar flux rate, however, in the opposite direction, was found for 27-OHC.\textsuperscript{27,28} It would be of high importance to perform these investigations in patients during PCSK9 treatment. The major metabolism of 27-OHC and 24S-OHC is directed to bile acid synthesis. Quantitation of these transformations cannot be simply measured. Thus, the question, whether PCSK9 inhibition leads to similarly increased catabolism, remains unanswered. Follow-up studies may include the additional analysis of cholesterol and oxysterols in cerebrospinal fluid. This may give more direct information on brain cholesterol metabolism.

5 | CONCLUSION

The results support the hypothesis that excretion of 24S-OHC from the brain is increased under anti-PCSK9 antibody treatment as indicated by an increasing ratio of 24S-OHC to cholesterol and to 27-OHC in the circulating serum.

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CONTRIBUTORS
B.B., K.P. and U.L. participated in research design and provided the human samples. D.L. and A.K. performed the GC-FID and GC-MS-SIM analyses and performed data analysis together with F.S. D.L., F.S., B.B., A.K., K.P. and U.L. wrote or contributed to the writing of the manuscript.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

ORCID
Dieter Lütjohann \(https://orcid.org/0000-0002-7941-8308\)

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