# Sex-difference in flux of 27-hydroxycholesterol in the brain

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**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.

Footnote

According to Fakheri and Javitt (2012) the preferred nomenclature for 27-hydroxylation and 27-hydroxycholesterol should be (25R)26-hydroxylation and (25R)26-hydroxycholesterol. Here we prefer to use 27-hydroxycholesterol (27OH) to fit with our previous publications.

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**Abstract**

**Background and purpose**

The CSF/plasma albumin ratio (QAlb) is believed to reflect the integrity of the blood-brain barrier (BBB). Recently we reported that QAlb is lower in females. This may be important for uptake of neurotoxic 27-hydroxycholesterol (27OH) by the brain in particular since plasma levels of 27OH are higher in males. We studied sex differences in the relation between CSF and plasma levels of 27OH and its major metabolite 7 alpha hydroxy-3-oxo-4-cholestenoic acid (7Hoca) with QAlb. We tested the possibility of sex differences in the brain metabolism of 27OH and if its flux into the brain has a disruptive effect on the BBB.

**Experimental approach**

In retrospect we have gone through our previous studies looking for sex differences in CSF levels of oxysterols and their relation to QAlb. We utilized an in vitro model for the BBB with primary cultured brain endothelial cells to test if 27OH has a disruptive effect on this barrier. We measured mRNA and protein levels of CYP7B1 in autopsy brain samples

**Key results**

The correlation between CSF-levels of 27OH and QAlb was higher in males while, with 7Hoca, the correlation was higher in females. No significant sex difference in the expression of *CYP7B1* mRNA in brain autopsy samples. A correlation was found between plasma levels of 27OH and QAlb. No support was obtained for the hypothesis that plasma levels of 27OH have a disruptive effect on the BBB.

**Conclusions and implications**

The sex differences are discussed in relation to negative effects of 27OH on different brain functions.

**Abbreviations**

27OH, 27-hydroxycholesterol; CSF, Cerebrospinal fluid; BBB, blood-brain barrier; 7 Hoca, metabolite 7 alpha hydroxy-3-oxo-4-cholestenoic acid; CYP7B1, Cytochrome P450 Family 7 Subfamily B Member 1; CYP27, Sterol 27-hydroxylase; AD, Alzheimer´s disease; DSM-IV and NINCDS-ADRDA, National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association; Ct, threshold cycle; pBCEC, porcine brain capillary endothelial cells; TEER, transendothelial electrical resistance.

**Key words**

27-hydroxycholesterol, sex differences, neurodegeneration, blood-brain barrier, CYP7B1

**Bullet point summary**

*What is already known?*

- Higher plasma levels of neurotoxic 27OH in males than in females
- Higher QAlb in males than in females consistent with a more permeable BBB in males

*What this study adds*

- Significant sex differences in the relation between QAlb and CSF-levels of 27OH and its metabolite 7Hoca.
- No support for a direct disruptive effect of circulating 27OH on the BBB

*Clinical significance*

- We discuss whether the lower flux of 27OH into the female brain may reduce neurodegeneration

**Introduction**

Hypercholesterolemia in mid-life is regarded to be a risk factor for Alzheimer´s disease (AD) in spite of the fact that lipoprotein-bound cholesterol does not pass the BBB (**Kivipelto et al., 2001)**. 27OH is a major metabolite of cholesterol and there is a high correlation between levels of this oxysterol and cholesterol in the circulation. In contrast to cholesterol itself, 27OH can pass the BBB and there is a continuous uptake of this oxysterol in the brain **(Heverin et al., 2005a)**. Given the negative effects of 27OH under both *in vitro* conditions and *in vivo* experiments with different mouse models, the possibility has been discussed that the negative effects of hypercholesterolemia are mediated by 27OH **(Björkhem et al., 2009; Loera-Valencia et al., 2019)**. The negative effects of 27OH include memory defects **(Ismael et al., 2017; Heverin et al., 2015b)** , reduced production of the "memory protein" Arc in hippocampus **(Ismael et al., 2017; Mateos et al., 2009a)**, reduced uptake of glucose by the brain **(Ismael et al., 2017)**, decrease in key synaptic protein levels **(Ismael et al., 2017)**, decrease in dendritic spine density **(Merino-Serrais et al., 2019)**, worsening of amyloid pathologies **(Famer et al., 2007; Marwarha et al., 2013)** and effects on the renin-angiotensinogen system in the brain **(Mateos et al., 2011b)** . It can be assumed that high levels of cholesterol are associated with increased flux of 27OH across the blood-brain barrier. We have shown that this flux is dependent upon the permeability of the BBB and that disruption of the barrier due to different diseases leads to a higher influx of 27OH **(Leoni et al., 2013a)**.

The ratio between albumin in plasma and CSF is regarded to be a marker for the integrity of the BBB. Recently, we showed that there is significant and consistent sex difference in this ratio (20-30%) in patients and healthy subjects from birth up to 90 years-old **(Parrado-Fernandez et al., 2018)**. In addition to this there is a highly significant sex difference in the level of 27OH in the circulation, with levels about 30% higher in males than in females **(Dzeletovic et al., 1995)**. Thus, it may be concluded that the female brain is less exposed to 27OH than the male brain.

In the present work we have got additional support for the contention that there are significant sex differences in the flux of 27OH and its final metabolite 7Hoca in the human brain. We have also studied the possibility that the higher levels of 27OH in the male circulation may have a direct disruptive effect on the BBB. We speculate that the lower exposure of the female brain to 27OH may reduce the negative effect of hypercholesterolemia on neurodegeneration in this sex. This work was presented at the ENOR-conference in Edinburgh in September 2019. The present manuscript complies with the recommendations by BJP.

## *Materials and methods*

### *Design of the studies and ethical aspects*

All the different clinical studies are reported in detail in the articles referred to below and were re-invested here only with respect to sex differences. All these studies were approved by the ethical committee at Karolinska University Hospital Huddinge and informed consent was obtained from all the subjects involved.

### *Statistical evaluations*

It should be emphasized that all the studies were retrospect and primarily designed to study relation between levels of oxysterols in cerebrospinal fluid and circulation in different populations of patients rather than to document sex differences. Because of this the size of the groups of males and females could not be equal and varied in the different studies. All the analyses were performed blinded with respect to the identity (including sex) of the subject investigated.

In accordance with the recommendations of BJP no statistical evaluations were performed with $n < 5$. All the patient data concerned with CSF and plasma levels were found to have a Gaussian distribution as shown by the Komolgorov Smirnov analysis and thus parametric tests could be used. We used One-way ANOVA followed by Tukey´s test for multiple comparison and Student´s t-test for group comparisons and Pearsons test for correlation. The level of significance was set to $P = 0.05$.

All the outliers were included in all material presented except for Fig. 2. If the two female outliers were included the coefficient of correlation increased from 0-90 to 0.98 (which should be compared to the correlation coefficient of 0.38 in males).

### *Patients and control subjects*

1. Patients and control subjects with headache without objective findings, 128 females (age 46+/- 17 years) and 116 males (age 52+/-18 years). Subjects included in previous studies by Leoni **Leoni et al., 2013a; Leoni et al., 2004b; Leoni et al., 2005c; Leoni et al., 2006d; Shafaati et al., 2007)**

2. Control subjects with headache but without objective findings (subgroup included in the above heterogeneous group), 79 females (age 79 +/- 15 years) and 35 males age (44+/- 15 years). Subjects included in previous studies by Leoni **(Leoni et al., 2013a; Leoni et al., 2004b; Leoni et al., 2005c; Leoni et al., 2006d; Shafaati et al., 2007)** .
3. Patients and control subjects with a defect in the BBB (13 males and 13 females). A defective BBB function was defined as an increased CSF/serum albumin ratio (14–160, normally <10). Diagnoses in this group included Guillain-Barre's disease, meningitis, encephalitis, polyneuropathy, pareses and miscellaneous. These subjects were included in the previous study by Saeed **(Saeed et al., 2014a)**.
4. Patients with different degrees of neurodegeneration diagnosed according to DSM-IV and NINCDS-ADRDA criteria (28 males and 38 females). Subgroup of subjects included in previous studies conducted by Gil-Bea **(Gil-Bea et al., 2010)**.

Assay of oxysterols

24S-Hydroxycholesterol and 27-hydroxycholesterol in plasma and cerebrospinal fluid were measured by isotope dilution mass spectrometry after hydrolysis as previously **(Leoni et. al., 2013a)**. Also 7 alpha hydroxy-3-oxo-4-cholestenoic acid was measured by isotope dilution mass spectrometry as described **(Saeed et al.,2014a).**

*Human brain samples*

Fresh frozen human brain specimens of frontal cortex and hippocampus were obtained from Karolinska Institutet Brain Bank (Sweden) and the Harvard Brain Tissue Resource Center (MA, USA).

*Relative quantification in real time*

Total RNA was isolated using the RNeasy® mini kit (Qiagen) including DNase treatment (RNase-Free DNase Set, Qiagen). RNA samples were run on agarose gels to confirm no genomic DNA contamination and the purity was assessed by RNA 260/280 ratio. Total RNA (500 ng) was then reverse transcribed using High Capacity cDNA Reverse Transcription kit (Applied Biosystems). To perform real time RT-qPCR assays, we used the relative standard method supplied by Applied Biosystem (Applied Biosystems, 2004). Serial dilutions of a reverse transcription product (cDNA) from human cell line were carried out to obtain standards curves containing 100, 50, 5, 0.5, 0.05 ng/µl, and run by duplicate in the same PCR plate as the experimental samples. Relative quantities of mRNA were determined by comparing the threshold cycle (Ct) of each sample with that of a standard curve run on the same PCR plate. Thermocycling and fluorescence detection was performed according to the TaqMan® Gene Expression Assays Protocol using an ABI PRISM® 7000 Sequence Detection System with a total volume of 20 µl in each well containing 10 µl of PCR Master Mix (Applied Biosystems), 2 µl of cDNA (corresponding to 15 ng of total RNA), 1 µl of each TaqMan® Gene Expression Assays primer (Applied Biosystems), and 7 µl molecular grade RNase-free water (Dharmacon GE Lifesciences, B-003000-WB-100). Relative quantities of target genes were

adjusted to relative quantities of RPLPO and normalized to controls conditions (set at 100%). Values are reported as the mean ± SEM (standard error of the mean).

*Western blotting*

Human samples for western blot analysis were prepared from 100 mg frozen human tissue. Tissue was homogenized using a pestle motor homogenizer (Sigma-Aldrich) at + 4°C in 1% TritonTM X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 50 mM Tris; pH 7.5, supplemented with phosphatase and protease inhibitors (Sigma-Aldrich). Then homogenates were sonicated and centrifuged for 10 min at 10,000 rpm to remove cell debris. Protein content was measured using the Pierce BCA kit (Thermo Scientific) according to the manufacturer's instructions. Same amount of protein (50 ug) was prepared in 5x Laemmli sample buffer (10% SDS, 325 mM Tris-Cl pH 6,8, 20% glycerol, 500 mM DTT, 0,4% bromophenol blue) and loaded into 10% sodium dodecyl sulphate (SDS) polyacrylamide gels. Precision Plus Protein Dual Xtra Standard (Bio-Rad) was used as molecular weight marker. The samples were then subjected to electrophoresis (120 mV, 1h 30 min) in a MiniProtean system (Bio-Rad). Proteins were transferred to nitrocellulose membranes (GE healthcare) (100 mV, 1 h) and blocked in 5% non-fat milk for 1 h at room temperature. Afterwards the membranes were incubated overnight at 4°C with the primary antibodies CYP7B1 (Ab77157, mouse mAB, Isotype IgG2a, 1:1000, Abcam UK) and GAPDH (1D4, mouse mAb, Isotype IgG1, 1:2000, Enzo Sweden). Then, membranes were washed with tris-buffered saline pH 7,4 (50 mM Tris-Cl, 150 mM NaCl) containing 0,1% Tween to eliminate unbound antibody followed by incubations with secondary antibodies IRDye 800CW goat anti-mouse IgG (1:10 000)[ AB_2687825] and IRDye 680RD goat anti-mouse IgG (1:10 000)[ AB_2651128] (LI-COR Biotechnology – GmbH, UK). Signal detection was performed using an Odyssey imaging system (for the fluorescent secondary antibodies. Quantification of the bands was performed with the ImageStudioLite app and calculated from the band intensity minus the surrounding background. Results were expressed as percentages of the values obtained from the appropriate controls and normalized by GAPDH expression.

*Isolation and culture of porcine brain capillary endothelial cells (pBCEC)*

Brains from freshly slaughtered pigs (about 6 months old) were obtained from the local slaughterhouse and pBCEC were isolated as first described by Franke et al. **(Franke et al., 2000)** with minor modifications **(Kober et al., 2017)**. In brief, meninges and secretory areas were removed from hemispheres and pBCEC were isolated from the remaining cerebral cortex by sequential enzymatic digestion and centrifugation steps as described. Porcine BCEC were plated on collagen coated (60 µg/ml) 75 $cm^2$ culture flasks in M199 medium (containing 1% penicillin/streptomycin, 1% gentamicin, 1 mM L-glutamine and 10% horse serum). After 24 h, cells were rinsed twice with PBS to remove cell debris and non-adherent cells and were grown in fresh M199 medium (containing 1% penicillin/streptomycine, 1 mM L-glutamine and 10% horse serum) until confluent. All cell culture incubations were performed at 37 °C, 95% humidity, and 5% CO2.

*Transwell experiments*

To establish polarized pBCEC cultures **(Kober et al., 2017)** cells were trypsinized and plated onto collagen coated (120 µg/ml) 12-well Transwell filter plates (0.4 µM pore size, Corning) at a density of 40,000 cells/cm$^2$. Cells were grown for 2–3 days depending on the transendothelial electrical resistance (TEER; 50 Ω/cm$^2$). The tightness of the transwell culture was assessed by measuring TEER using an EndOhm tissue resistance measurement chamber and EndOhm ohmmeter (World Precision Instruments, Florida). TEER of collagen-coated, cell-free filters were used as blanks. Tight junction formation was induced (overnight) by adding DMEM/Ham's F-12 medium containing 550 nM hydrocortisone, 1% penicillin/streptomycin, and 0.7 mM L-glutamine. Establishment of intact tight junctions was indicated by TEER rising above 150 Ω/cm$^2$. 27OH (0.055 µM and 0.5 µM), 24OH (0.5 µM), 7betaOH (0.5 µM), or LPS (5 µM, known to increase BBB permeability, was used as positive control) were added to apical media containing 1% BSA. TEER were measured at indicated time points for up to 24 h. 6 independent experiments with the above additions and use of aliquots from the same cell culture were performed (Fig.5). In addition, 2 separate independent experiments with other cell cultures were performed with n=3

***Results***

*Measurements of QAlb and level of 27hydroxycholesterol in CSF in different groups of patients and controls*

In previous work we measured QAlb and levels of 27OH in cerebrospinal fluid in 128 females and 116 males with different neurological diseases and with headache without objective findings **Leoni et al., 2013a; Leoni et al., 2004b; Leoni et al., 2005c; Leoni et al., 2006d; Shafaati et al., 2007)** .

As expected, **(Parrado-Fernandez et al., 2018)**, QAlb was significantly lower in females than in males (7.8 +/- 5.7 vs 13.3+/-12.3, p<0.001) in these populations.

The levels of 27-hydroxycholesterol in CSF were slightly but significantly lower in females than in males (1.6+/-1.2 vs 2.0+/-1.3 ng/ml (p<0.005).

There was a highly significant correlation between levels of 27OH in CSF and QAlb in both females and males (R = 0.46 and 0.59, respectively, p < 0.0001 in both cases).

The above populations are heterogenous with several different diagnoses that could affect the integrity of the blood-brain barrier by different mechanisms. Among the above subjects 79 females and 35 males were regarded as controls (headache without neurological findings). Also, in this homogenous subpopulation there was a significantly lower QAlb in females than in males (5.6 +/-1.6 vs 6.7+/- 1.7, p <0.001).

Also, in this subgroup there was a highly significant correlation between levels of 27OH in CSF and QAlb in both females (R=0.42, p<0.001) and males (R=0.67, p<0.001) (**Fig. 1A and B**). Altogether, in the above studies the correlation between 27OH in CSF and QAlb was higher in males than in females.

In a separate study on a group of patients with different degrees of neurodegeneration there was no significant correlation between 27OH in CSF and QAlb in females but a highly significant such correlation in males (p= 0.0015) (**Fig. 1C and D**). In a separate group of patients with cognitive impairment we observed that the correlation between QAlb and 27OH was lower (R=0.43, p=0.07 in females compared to males (R=0.87, p<0.0001).

The final brain metabolite of 27OH is 7 alpha hydroxy-3-oxo-4-cholestenoic acid (7Hoca) **(Saeed et al., 2014b)**. We have measured this metabolite in CSF from patients with different neurological diseases and found a significant correlation between the levels of this metabolite in CSF and QAlb **(Saeed et al., 2014a)**. This correlation was highest in patients with a defect blood-brain barrier. In a retrospective comparison between males and females in that study we found that the degree of correlation between the two parameters was markedly different in the two sexes with a much higher correlation in females than in males (**Fig 2A and 2B**). There was no significant difference in the level of this metabolite in control subjects (10 males and 10 females 11+/- 6 and 12+/- 3 ng/ml, respectively (mean +/- SD).

*Measurements of QAlb and levels of 27-hydroxycholesterol in plasma*

We measured levels of 27-hydroxycholesterol in the circulation and compared with QAlb in patients with different degrees of neurodegeneration. A significant (p<<0.01) correlation was found (results not shown). This study was repeated with the control patients. As shown in Fig. 3A and 3B there was a significant correlation between the two parameters in both females (R=0.52, p<0.001) and males (R=0.85, p<0.0001) with a higher correlation in males.

No significant correlation was observed between QAlb and plasma levels of 24S-hydroxycholesterol (R=0.1, p=0.53 in females and R=0.29, p=0.29 in males) (**Fig. 3C and D**). Also, there was no significant correlation between QAlb and plasma levels of cholesterol (R=0.12, p=0.44 in females and R=0.28, p=0.32 in males). Data not shown.

*Measurements of CYP7B1 in hippocampus and frontal cortex of autopsy brain samples from males and females*

Relative mRNA and protein expression levels for *CYP7B1* in hippocampus and frontal cortex from control subjects showed no significant sex differences. As shown in **Fig. 4**, when the study was repeated with samples from a heterogenous group of subjects including both controls and patients with neurodegeneration, there was a tendency to higher mRNA and protein levels in females both in frontal cortex and hippocampus. This difference did not, however, reach significance (p>0.05).

*Experiments with an in vitro model for the BBB*

Given the correlation between levels of 27-hydroxycholesterol in the circulation and QAlb we tested the possibility that physiological levels of 27-hydroxycholesterol may have a direct disruptive effect on the BBB. TEER values of brain endothelial cells growing on well cell culture inserts were recorded after hydrocortisone induction (**Fig. 5A**) followed by registration of TEER changes after exposure to 0.1% BSA for different time intervals (**Fig.**

**5B**). The TEER values were then recorded after exposure to 27-hydroxycholesterol levels (0.5 and 0.05 µM) during 25h (**Figs. 5C and 5D**). For reason of comparison also 7 beta hydroxycholesterol (0.5 µM) and 24S-hydroxycholesterol (0.5 µM) were tested in the system. A positive control, LPS, 5 µg/ml was also included. The positive control had a significant effect on the permeability as evaluated by the change in TEER value (**Figs. 5C and 5D**). There was not, however, any significant effect of the different steroids on the TEER value. The experiment shown in Fig. 5C and 5D with 6 replicates was repeated with different cell cultures with n=3 showing the same pattern (results not shown).

***Discussion***

As shown previously in healthy subjects and in anonymized patients there is a clear sex difference in the degree of integrity of the blood-brain barrier as evaluated from QAlb measurements **(Parrado-Fernandez et al., 2018)**. In a previous work we showed that a leaking blood-brain barrier due to pericyte deficiency increases both influx and efflux of steroids across the blood brain barrier **(Saeed et al., 2014b)**. It seems likely that this integrity is affecting flux of steroids in both directions. If both the influx of 27-hydroxycholesterol and the efflux of the metabolite 7Hoca are reduced in the brain of females as compared to males, there will be a tendency to retention of both 27-hydroxycholesterol and 7Hoca in the brain of females. This may be the explanation for our surprising finding that despite the lower influx of 27-hydroxycholesterol in the female brain there was little of no difference in the levels of this oxysterol in CSF from males and females. A relative retention of the metabolite 7Hoca in the brain of females will be dependent upon the influx of 27-hydroxycholesterol and may explain the higher correlation between QAlb and CSF-level of 7Hoca in females.

The rate of metabolism of 27-hydroxycholesterol in the brain may also be of importance. If this metabolism is higher in females than in males it would tend to increase a relative accumulation of 7Hoca in the brain of females. The rate-limiting step in the conversion of 27OH into 7Hoca has been found to be *CYP7B1* **(Meaney et al., 2007)**. The levels of *CYP7B1* mRNA levels in autopsy samples of brain were however similar in males and females.

**Fig. 6** illustrates a possible mechanism behind the sex differences in the flux of 27-hydroxycholesterol and 7Hoca in the brain. The higher influx of 27-hydroxycholesterol in the male brain may be compensated for by a higher efflux of the final metabolite 7Hoca. In the female brain the lower influx may be balanced by a lower efflux resulting in similar levels of oxysterols in the brain of the two sexes. We have previously found that the levels of 7Hoca in CSF from males and females (control subjects) are similar **(Saeed et al., 2014a)**.

We found a significant correlation between 27-hydroxycholesterol in the circulation and QAlb (**Fig. 3**). It is known that sidechain oxidized oxysterols may affect the properties of biomembranes and increase their permeability **(Bielska et al., 2014)**. In view of this the possibility must be considered that 27-hydroxycholesterol in the circulation may affect the integrity of the blood-brain barrier and that the lower integrity of the blood-brain barrier in males may be related to this. In order to test this possibility, we used an *in vitro* model for

the blood-brain barrier with cultured brain endothelial cells. Physiological level of 27OH (0.5 µM) had no significant effect on the permeability of the barrier used. In another *in vitro* model using endothelial cells of another origin, a high and unphysiological level of 27OH, 10 µM, was shown to increase the permeability of the membrane **(Dias et al., 2018)**. From our study It may be concluded that physiological levels of 27OH in the circulation are not likely to affect the integrity of the blood-brain barrier. Further support for this contention is obtained from patients with the hereditary spastic paresis of type SPG5 with a mutation in the *CYP7B1* gene. These patients with levels of 27OH up to 3 µM were recently reported to have normal QAlb **(Schöls et al., 2017)**. We have used a mouse model with overexpressed CYP27 with high levels of 27-hydroxycholesterol in the circulation **(Meir et al., 2002)**. If the high levels of 27OH causes a disruption of the BBB, higher levels of albumin would be expected in the brain of this transgenic mouse model. This was not found, however (unpublished observation). The data presented here in combination with the data from patients with a mutation in the *CYP27* gene and observations in mice with overexpression of CYP27 give strong support for the contention that 27OH in the circulation does not have a direct disruptive effect on the BBB.

At present we have no explanation for the significant correlation between QAlb and level of 27OH in the circulation. The possibility must be considered that there is an unknown circulatory factor with a level correlated to level of 27OH which has a regulatory effect on the permeability of the BBB. According to our studies this regulatory factor cannot be identical to cholesterol or 24S-hydroxycholesterol. If such a factor exists, its link to 27OH may be different in patients with SPG5.

We do not know if the negative effects of 27OH on the brain are related to the magnitude of the flux of the oxysterol into the brain or the actual concentration of it in the brain. The flux of the oxysterol into the brain is higher in males than in females whereas the concentration of the oxysterol appears to be similar in the brain of the two sexes. If the magnitude of the flux is most important and given the close relation between 27OH and cholesterol in the circulation, hypercholesterolemia would be expected to be associated with more negative effects in males than in females. In a recent Chinese cross-sectional study, the relation between serum lipids and cognitive function in 1762 participants was studied **(Zhao et al., 2019)** High total cholesterol levels in the circulation were positively associated with cognitive impairment in elderly males but not in females. In a French study on 2737 men and 4118 women a hypercholesterolemic pattern with high total cholesterol was associated with increased risk of cognitive decline in men but not in women **(Ancelin et al., 2014)**. These studies agree with the hypothesis that hypercholesterolemia is a more serious risk factor for neurodegeneration in males than in females. More studies in different populations are required, however, before definitive conclusions can be drawn.

**Author contributions**

Conception and design of the work: IB, AC, CP, VL

Analysis of CSF and plasma: VL, CP, AS

In vitro measurements: CP, AM, PR, AM, CB, PB

Transwell experiments: CG, UP

Drafting: IB, AC, CP, VL

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**Conflicts of interest**

The authors declare no conflict of interest

**Declaration of transparency and scientific rigour**

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Natural Products Research, Design and Analysis, and Immunoblotting and Immunochemistry, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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# Figure 1

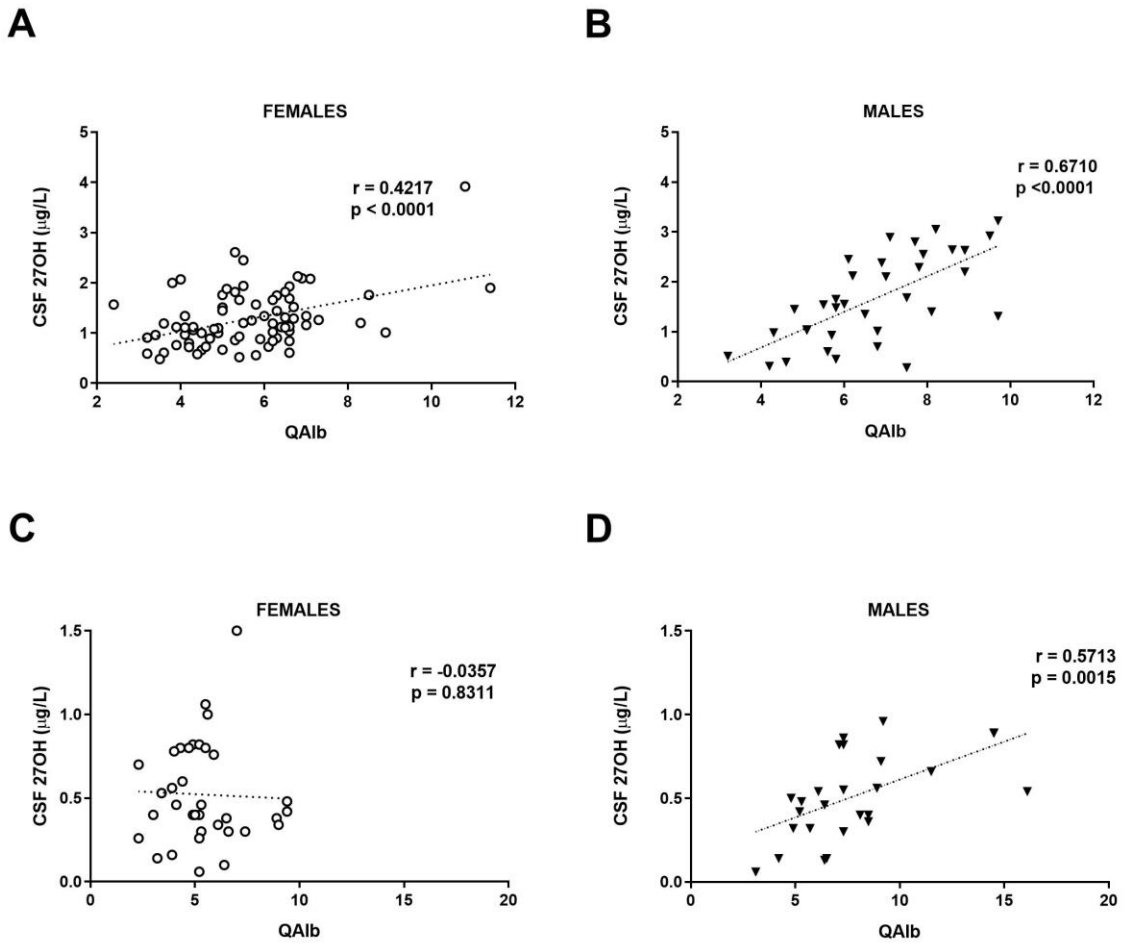


**Figure 1. Correlation between CSF/Serum Albumin Ratio and 27OH in CSF**. Control subjects with headache but without objective findings, 35 males (**A**) and 79 females (**B**). Patients with different degrees of neurodegeneration diagnosed according to DSM-IV and NINCDS-ADRDA criteria, 28 males (**C**) and 38 females (**D**).

# Figure 2

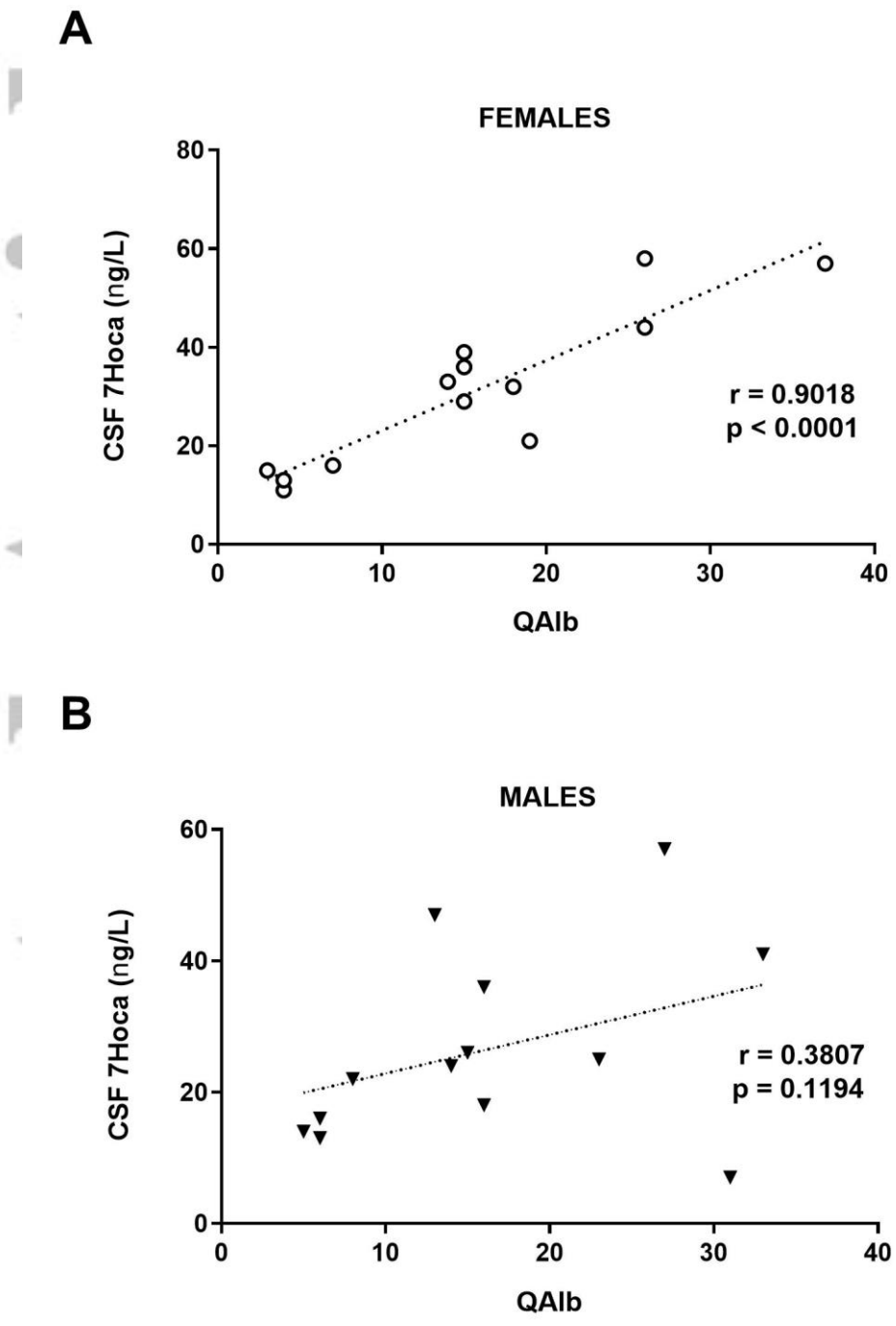


**Figure 2. Correlation between CSF/Serum Albumin Ratio and 7Hoca in CSF**. Patients and control subjects with a defect in the BBB, 13 males (**A**) and 13 females (**B**).

## Figure 3

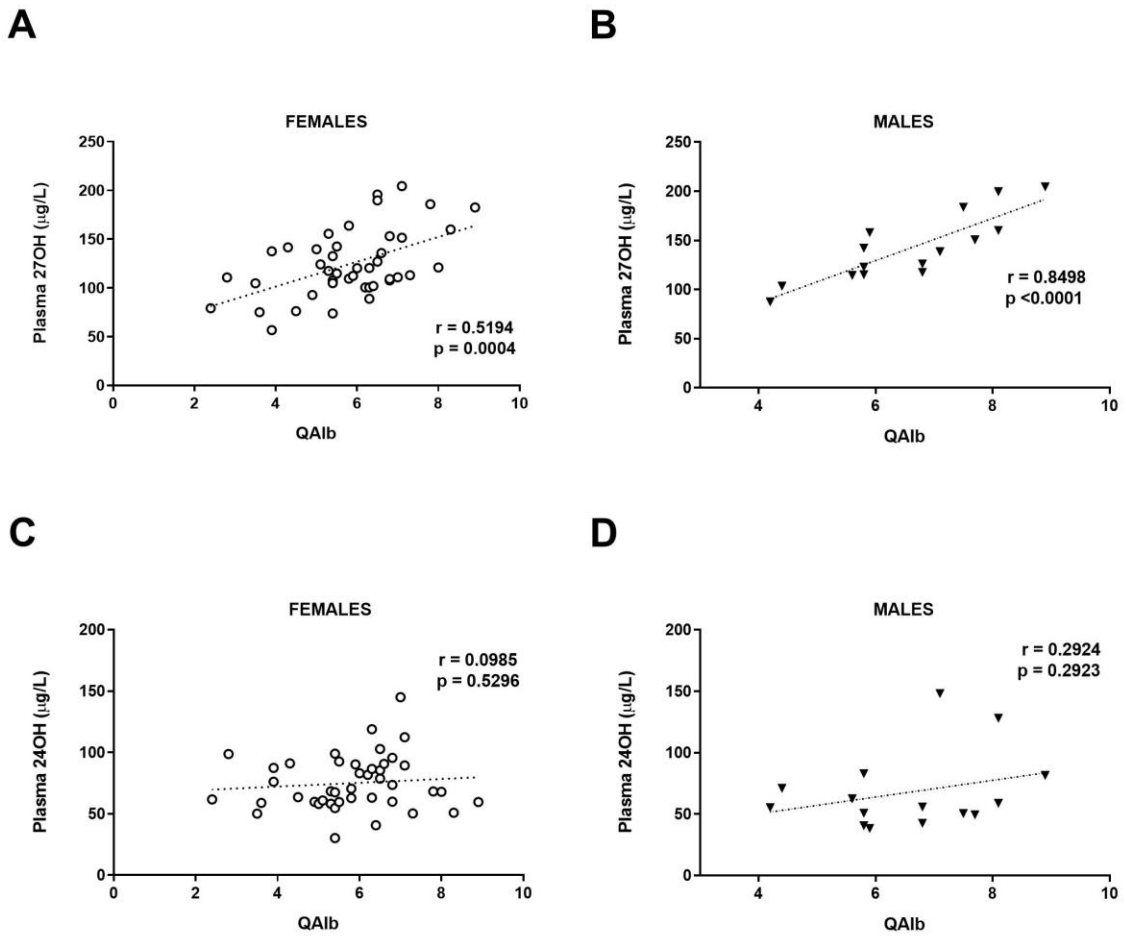


**Figure 3. Correlation between CSF/Serum Albumin Ratio and oxysterols in plasma.** Control subjects with headache but without objective findings, 35 males and 48 females. Correlation studies between plasma 27OH and QAlb (**A, B**). Correlation studies between plasma 24OH and QAlb (**C, D**)

## Figure 4

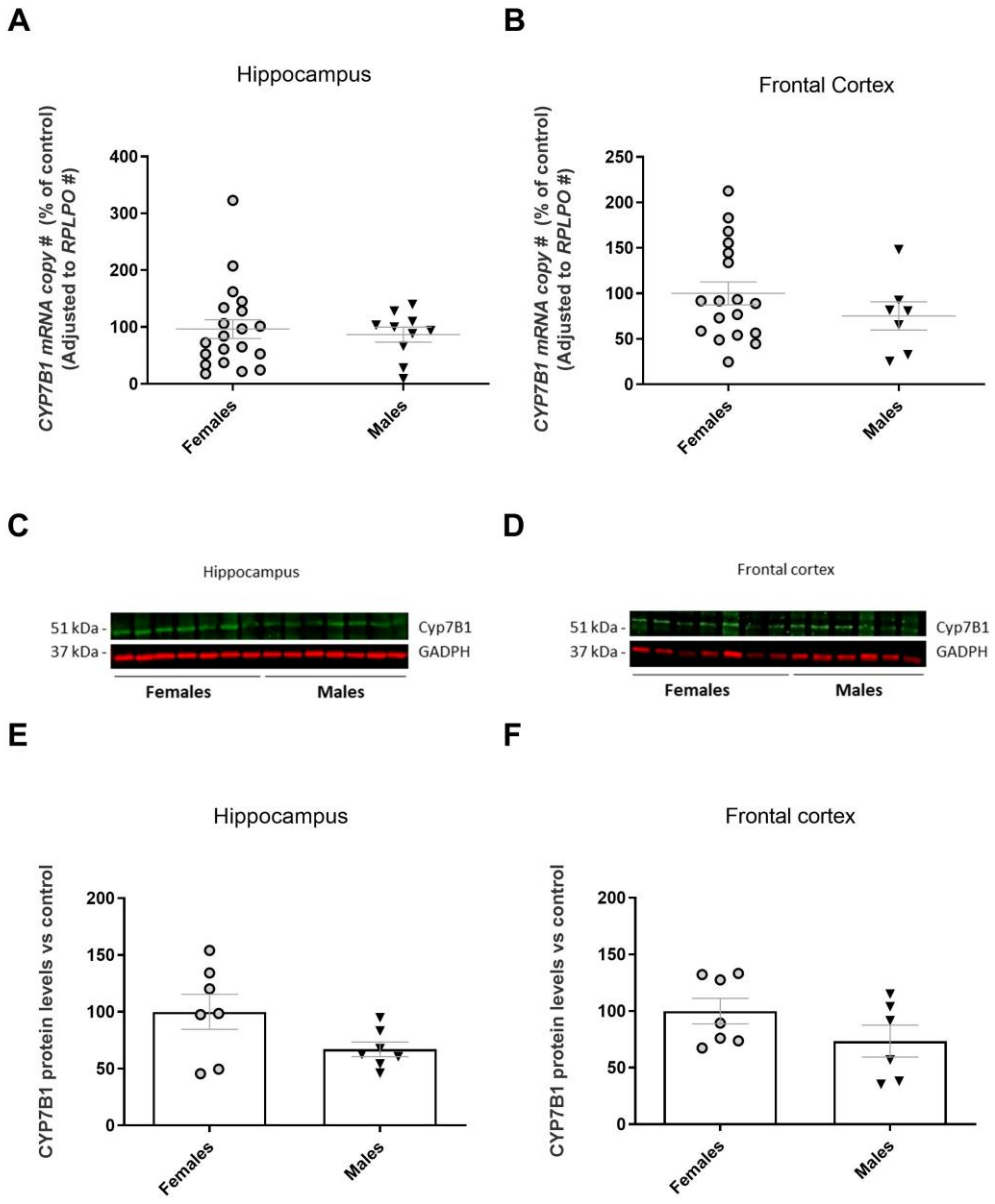


**Figure 4. *CYP7B1* mRNA expression and protein levels in human brain.** Real time RT-qPCR analysis of *CYP7B1* mRNA from human hippocampus (females N=23 and males n=7) (A) and frontal cortex (Females n=18, males n=7) (B). Representative western blot images of CYP7B1 from human hippocampal homogenates (females N=7 and males N=7) (C) and frontal cortex homogenates (Females=7 and males N=6) (D). Respective histograms showing the quantification of the immunoreactivity measurements with data normalized against loading control (GAPDH) and expressed as a percentage of female group (E and F). Student t-test was assessed. Statistical differences were not detectable. Data are represented as mean ± SEM.

## Figure 5

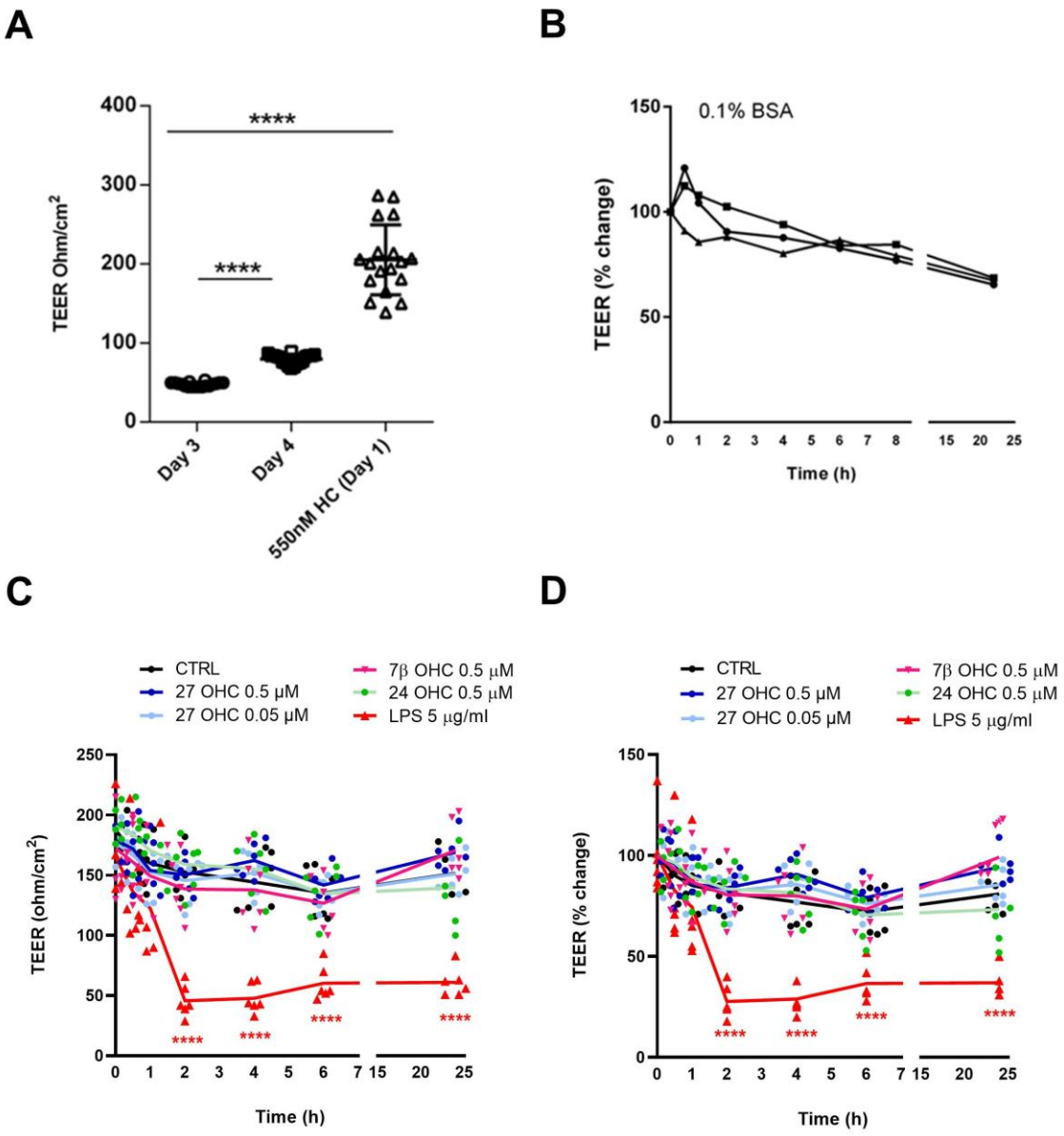


**Figure 5. Effect of 24OH, 27OH, 7βOH and LPS on the blood-brain barrier integrity**. BBB permeability in vitro was assessed by measuring TEER values of pBCEC grown on 12 well trans-well cell culture inserts. (A) TEER (ohm/cm²) was recorded on day 3, day 4 and a day after hydrocortisone-mediated induction (550 nM) of tight junction formation. Statistical analyses were done using unpaired t-test, 2 tailed. ****p<0.0001. (B) Percentage change in TEER values at 0h, 0.5h, 1h, 2h, 4h, 6h, 8h and 22h after 0.1% BSA treatments. (C) TEER values (ohm/cm²) and (D) percentage change in TEER values at 0h, 0.5h, 1h, 2h, 4h, 6h, 8h and 22h after vehicle (ethanol), 24OH (0.5 µM), 27OH (0.055 µM and 0.5 µM), 7βOH (0.5 µM) and LPS (0.5 µg/ml) treatment. Means ±SD, n=6. Shown is one experiment with n=6. The experiment was repeated twice with different cell cultures and n=3 showing the same pattern.

## Figure 6

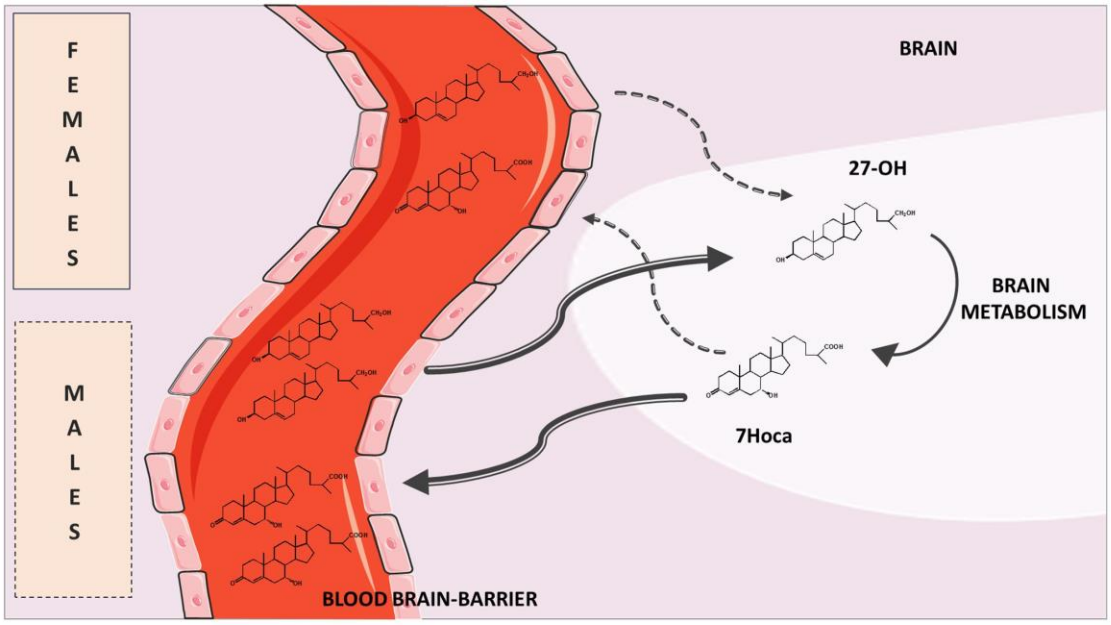


**Figure 6. Schematic overview of the proposed mechanism behind the sex differences in the flux of 27-hydroxycholesterol and 7Hoca in the brain.** In males, higher influx of 27OH into the brain in relation to metabolism results in higher efflux of its metabolite 7Hoca. In the female brain the lower influx may be balanced by a lower efflux resulting in similar levels of oxysterols in the brain of the two sexes. Dotted arrows indicate lower flux.