From THE DIVISION OF CLINICAL CHEMISTRY
DEPARTMENT OF LABORATORY MEDICINE
Karolinska Institutet, Stockholm, Sweden

FLUX OF CHOLESTEROL, OXYSTEROLS AND PLANT STEROLS ACROSS THE BLOOD-BRAIN BARRIER AND METABOLIC CONSEQUENCES

Ahmed Abdalla Abdelwahid Saeed
احمد عبدالله عبدالواحد سعيد

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Flux of cholesterol, oxysterols and plant sterols across the blood-brain barrier and metabolic consequences

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By

Ahmed Abdalla Abdelwahid Saeed M.D., M.Sc.

Principal Supervisor:
Professor Ingemar Björkhem
Karolinska Institutet
Department of Laboratory Medicine
Division of Clinical Chemistry

Co-supervisor(s):
Associate Professor Guillem Genové
Karolinska Institutet
Department of Medical Biochemistry and Biophysics
Division of Vascular Biology

Associate Professor Angel Cedazo Minguez
Karolinska Institutet
Department of Neurobiology, Care Sciences and Society (NVS)
Division of Neurogeriatrics

Lecturer Maura Heverin
Karolinska Institutet
Department of Laboratory Medicine
Division of Clinical Chemistry

Opponent:
Professor Agneta Mode
Karolinska Institutet
Department of Biosciences and Nutrition

Examination Board:
Associate Professor Sven Gustafsson
Karolinska Institutet
Department of Molecular Medicine and Surgery

Professor Sten Fredrikson
Karolinska Institutet
Department of Clinical Neuroscience
Division of Neurology

Associate Professor Anna-Lena Ström
Stockholm University
Department of Neurochemistry

Sal R64, Karolinska University Hospital Huddinge 9.30 Thursday 27th October 2016

"Only a life lived for others is a life worthwhile"

Albert Einstein

To the man who has always lived for others and still does

My father

Abdalla Abdelwahid Saeed

ABSTRACT

Brain is the cholesterol-richest organ and contains one fourth of the total body cholesterol. The majority of brain cholesterol is present in myelin that forms myelin sheathes around neuronal axons. This large pool of cholesterol present in brain is separated from body cholesterol by the blood brain barrier (BBB). This barrier prevents cholesterol, among many other substances, to flux from the circulation to the brain. BBB is composed of specific cellular and molecular components that allows it to perform its function.

Oxysterols are oxygenated cholesterol derivatives that play significant regulatory roles. 24S-hydroxycholesterol (24-OHC) and 27-hydroxycholesterol (27-OHC) are two important oxysterols. Unlike cholesterol they have the ability to cross BBB. 24-OHC is produced exclusively in brain and it fluxes out to the circulation. 27-OHC is produced by all tissues including neural tissues but there is a net flux from circulation to the brain. These two oxysterols have been implicated to be important in some neurodegenerative diseases such as Alzheimer's disease where 24-OHC is thought to be protective and 27-OHC is blamed for some negative effects.

27-OHC is effeciently metabolized in the brain by a number of enzymes to produce 7α-hydroxy-3-oxo-4-cholestenoic acid (7-Hoca). This steroid acid has also the ability to cross the BBB to flux back to the circulation. Part of 7-Hoca fluxes to CSF where it represents the most abundant cholesterol metabolite. High levels of this compound were found in chronic subdural hematoma (CSH) samples.

Plant sterols are structurally similar to cholesterol. They are synthesized by plant cells only while animals obtain them from diet. Plant sterols are able to cross the BBB from blood to settle in the brain.

Disturbances in cholesterol metabolism, both cerebral and extracerebral, have been linked to neurodegenerative diseases such as Alzheimer's disease, Huntington’s disease and Parkinson’s diseases. BBB may also be affected in such diseases. Consequences of BBB disruption on cholesterol metabolism have never been studied previously.

In **Paper I** and **Paper II**, the levels of 7-Hoca in different neurological diseases were measured with a newly developed assay based on isotope dilution mass spectrometry. The level of this compound in patients with Alzheimer’s disease and vascular dementia were similar to controls. 7-Hoca was, however, elevated in a group of patients with different conditions involving BBB dysfunction. The possibility is discussed that 7-Hoca in CSF can be used as a diagnostic marker for conditions with a dysfunctional BBB.

Two alternative mechanisms for the elevated levels of 7-Hoca are suggested. There may be a primary increase in the flux of 27-OHC across the disrupted barrier followed by increased formation of 7-Hoca. The other possibility is a primary increase in the flux of albumin across the disrupted BBB followed by binding of 7-Hoca from the brain to the albumin.

Investigations were made to elucidate the mechanism behind of 7-Hoca accumulation in CSH. A very efficient binding of 7-Hoca to albumin could be demonstrated in two model experiments in-vitro. A high ratio between 7-Hoca and albumin could be demonstrated in the CSH from patients. The results suggest that the high affinity between 7-Hoca and albumin is the driving force for the accumulation of 7-Hoca in CSH.

In **Paper III** and **Paper VI**, characterization of a mouse model with BBB deficiency was performed. This mouse model (*Pdgfb*$^{ret/ret}$) lacks pericyte which is an essential component of BBB and therefore it ends up with a leaking BBB. Diet treatments were applied and brain, liver, plasma and other organs were inspected. Results show that plasma cholesterol reaches brain parenchyma in those animals while more 24-OHC fluxes through the defective BBB to the circulation. This effect leads to increased cholesterol synthesis in the brain. A theoretical model for regulation of cholesterol synthesis in a brain with BBB disruption is suggested. Plant sterols levels were found to be significantly increased in the brain of BBB deficient mice. Flux of two plant sterols across the BBB was studied in more detail. *Pdgfb*$^{ret/ret}$ and control mice were fed diet mixed with deuterium labeled campesterol and sitosterol. Results show a time-dependent accumulation of these sterols in brain. More plant sterols were shown to pass across the defective barrier than across the normal one. Campesterol was found to cross both normal and disrupted barrier more effectively than sitosterol.

To summarize the present thesis illustrates the capacity of some specific cholesterol metabolites and plant sterols to pass the BBB and in the former case also membranes surrounding subdural hematomas. It also demonstrates presence of a cross-talk between the isolated pool of cholesterol in the brain and extracerebral pools of cholesterol. Furthermore it emphasizes the role of albumin binding for the flux of a steroid acid (7-Hoca) in the brain. The investigations also support the contention that 24-OHC is of importance for the regulation of cholesterol synthesis in the brain.

LIST OF SCIENTIFIC PAPERS

I. **SAEED, A.**, FLORIS, F., ANDERSSON, U., PIKULEVA, I., LOVGREN-SANDBLOM, A., BJERKE, M., PAUCAR, M., WALLIN, A., SVENNINGSSON, P. & BJORKHEM, I. 2014. **7alpha-hydroxy-3-oxo-4-cholestenoic acid in cerebrospinal fluid reflects the integrity of the blood-brain barrier.** J Lipid Res, 55, 313-8.

II. **SAEED, A. A.**, EDSTRÖM, E., PIKULEVA, I., EGGERTSEN, G. & BJORKHEM, I. 2016. **On the importance of albumin binding for the flux of 7 alpha hydroxy-3-oxo-4-cholestenoic acid in the brain.** *Manuscript.*

III. **SAEED, A. A.**, GENOVE, G., LI, T., LUTJOHANN, D., OLIN, M., MAST, N., PIKULEVA, I. A., CRICK, P., WANG, Y., GRIFFITHS, W., BETSHOLTZ, C. & BJORKHEM, I. 2014. **Effects of a disrupted blood-brain barrier on cholesterol homeostasis in the brain.** J Biol Chem, 289, 23712-22.

IV. **SAEED, A. A.**, GENOVE, G., LI, T., HULSHORST, F., BETSHOLTZ, C., BJORKHEM, I. & LUTJOHANN, D. 2015. **Increased flux of the plant sterols campesterol and sitosterol across a disrupted blood brain barrier.** Steroids, 99, 183-8.

OTHER PUBLICATIONS NOT INCLUDED IN THE THESIS

I. MAIOLI, S., BÅVNER, A., ALI, Z., HEVERIN, M., ISMAIL, M.-A.-M., PUERTA, E., OLIN, M., **SAEED, A.**, SHAFAATI, M., PARINI, P., CEDAZO-MINGUEZ, A. & BJÖRKHEM, I. 2013. **Is It Possible to Improve Memory Function by Upregulation of the Cholesterol 24S-Hydroxylase (CYP46A1) in the Brain?** PLoS ONE, 8, e68534.

II. PAUCAR, M., ALMQVIST, H., **SAEED, A.**, BERGENDAL, G., YGGE, J., HOLMIN, S., BJORKHEM, I. & SVENNINGSSON, P. 2016. **Progressive brain calcifications and signs in a family with the L9R mutation in the PDGFB gene.** Neurol Genet, 2, e84.

CONTENTS

LIST OF ABBREVIATIONS

7-Hoca	7α-hydroxy-3-oxo-4-cholestenoic acid
24-OHC	24S-hydroxycholesterol
27-OHC	27-hydroxycholesterol
Aβ	Amyloid beta
ABCA	ATP-binding cassette transporter subfamily A
ABCG	ATP-binding cassette transporter subfamily G
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
BBB	Blood-brain barrier
cDNA	Complementary DNA
CNS	Central nervous system
CSF	Cerebrospinal fluid
CSH	Chronic subdural hematoma
CTX	Cerebrotendinous xanthomatosis
CYP	Cytochrome P450
CYP7A1	Cholesterol 7alpha-hydroxylase
CYP7B1	Oxysterol 7alpha-hydroxylase
CYP27A1	Sterol 27-hydroxylase
CYP46	Cholesterol 24-hydroxylase
D_2O	Deuterium water (heavy water)
d4-7-Hoca	Deuterium labeled 7α-hydroxy-3-oxo-4-cholestenoic acid
d6-sterol	Deuterium labeled sterol
DNA	Deoxyribonucleic acid
FSR	Fractional synthetic rate
GC-MS	Gas chromatography–mass spectrometry
HDL	High-density lipoprotein

HMG CoA	3-hydroxy-3-methyl-glutaryl-coenzyme A
HSD3B7	hydroxy-delta-5-steroid dehydrogenase
IUPAC	International Union of Pure and Applied Chemistry
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LRP	Low-density lipoprotein receptor-related protein
LTP	Long-term potentiation
LXR	liver X receptor
mRNA	Messenger RNA
NADPH	Nicotinamide adenine dinucleotide phosphate
NPC	Niemann-Pick type C protein
PDGFB	Platelet-derived growth factor subunit B
Pdgfb$^{ret/ret}$	PDGF-B retention-motif knockout mouse model
SREBP	Sterol regulatory element binding protein
SPG5	Hereditary spastic paraplegia type 5
SR-B	Scavenger receptor class B
TMS	Tetramethylsilane
VLDL	Very low density lipoprotein
VD	Vascular dementia

1 INTRODUCTION

1.1 GENERAL OVERVIEW

This thesis, as its title indicates, is a study of the flux of cholesterol and five other related compounds between blood and brain. Figure 1 shows the structures of these compounds. Cholesterol is a well known lipid that is linked to cardiovascular diseases and many other diseases. However, it is also essential for human and animal life. Cholesterol is responsible for many vital functions such as cellular membrane fluidity, bile acid synthesis and steroid hormone formation besides many other necessary biological functions. Structurally, it is composed of 27 carbon atoms that form 4 rings and a hydrocarbon tail. It is also characterized by a hydroxyl group at C3 that makes it an alcohol. One additional important chemical feature of cholesterol is the double bond between C5 and C6.

24-hydroxycholesterol (24-OHC) and 27-hydroxycholesterol (27-OHC) are two molecules that are also studied in this work. They belong to a group called oxysterols which are defined as oxygenated derivatives of cholesterol i.e. cholesterol with extra oxygen atom or atoms. These two molecules are produced from cholesterol by action of specific enzymes. They have different effects on the central nervous system (CNS) that are discussed in this thesis. Their structures are identical to cholesterol except an additional hydroxyl group at C24 and C27 in 24-OHC and 27-OHC respectively.

Another molecule related to cholesterol and oxysterols is 7α-hydroxy-3-oxo-4-cholestenoic acid (7-Hoca). Its basic structure is that of cholesterol but with four differences: further oxidation of the hydroxyl group at C3, isomerization of the double bond to be located between C4 and C5, a hydroxyl group at C7 and a terminal carboxyl group which makes the compoud acidic. 7-Hoca can be produced from cholesterol by different pathways both in the brain and in the liver. The possibility to use this cholestenoic acid as a marker for different of neurological diseases is investigated in this work.

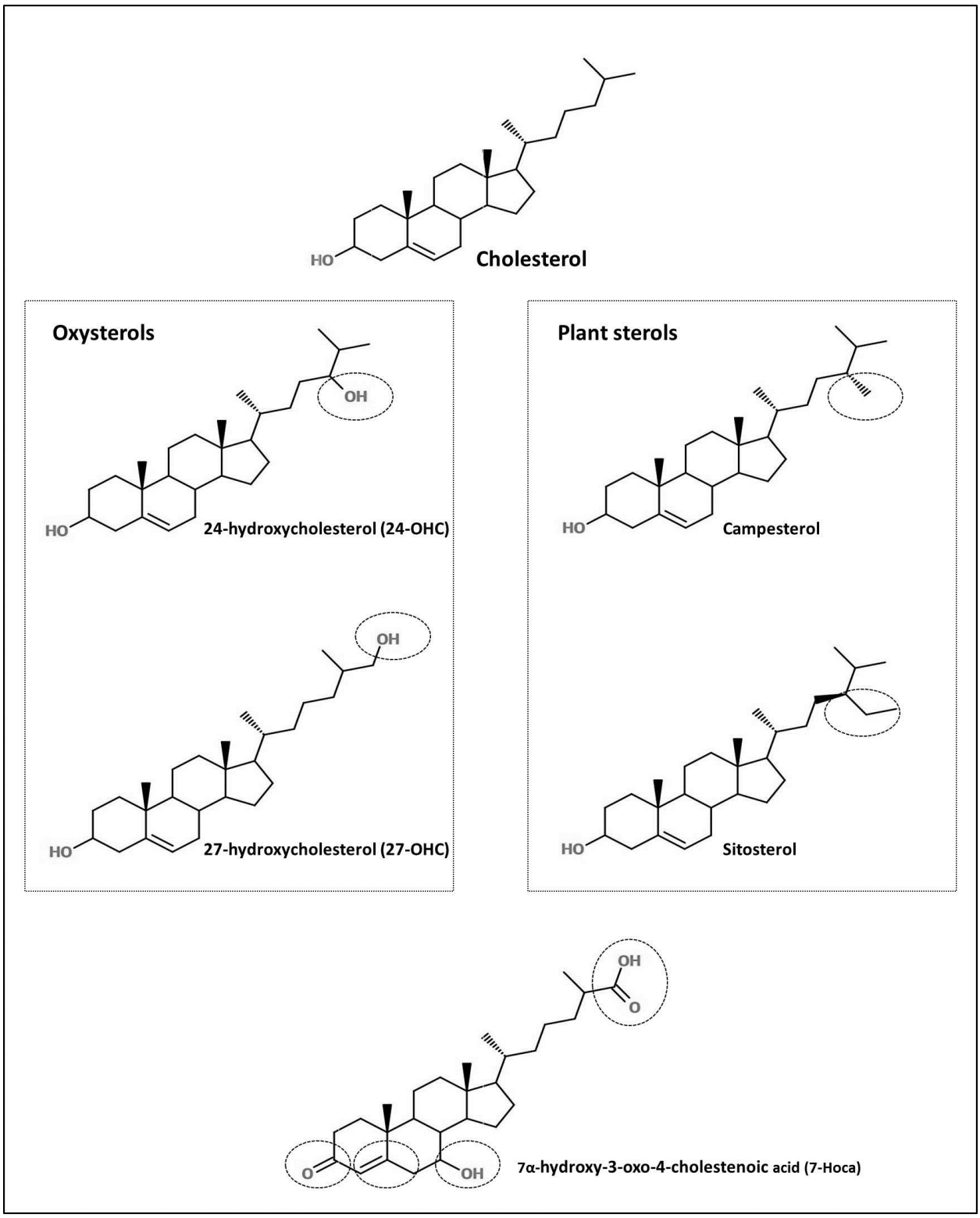

Figure 1. Chemical structures of cholesterol, 24-OHC, 27-OHC, campesterol, sitosterol and 7-Hoca. Structural differences between cholesterol and the other compounds are encircled.

The last two cholesterol-related molecules studied here are campesterol and sitosterol. They are the two most common members of a group of sterols called plant sterols or phytosterols.

Plant sterols are described as "the cholesterol of the plant kingdom". Similar to cholesterol in animals, they play important structural and physiological roles in plants. Humans and other animals can not synthesize these sterols but they obtain them from the diet. Campesterol and sitosterol are very similar to cholesterol in their structures. The only difference is an extra methyl group in campesterol and an extra ethyl group in sitosterol at C24.

In the current work, the flux of these 6 sterols (cholesterol and its 5 relatives) across the blood-brain barrier (BBB) is investigated. The BBB is exactly as its name describes, a barrier that separates brain and CNS from blood i.e. from the rest of the body. This barrier protects the brain from being exposed to substances carried in the blood that may be toxic or harmful to brain cells. Some compounds can cross this barrier but many other can not. Cholesterol is unable to cross the BBB but its other aforementioned five relatives can cross it more or less effecient. Many studies have linked plasma cholesterol to neurological diseases. Therefore it is of interest to understand how cholesterol can possess such effects without being able to cross the BBB. Hence, this thesis presents results of several studies done on cholesterol homeostasis in brain and the role of the BBB.

1.2 CHOLESTEROL

1.2.1 History of discovery

The first known scientific contact with cholesterol occured in the middle of the 18th century. The french chemist *De Fourcroy* mentioned the previous work of *Franciou Poulletier* in his article about the nature of substances in gallstone. The article was puplished in *Annales de Chimie et de Physique* in 1789, about 30 years after *Poulletier* breakthrough experiment. In 1758 the latter french doctor and chemist extracted the alcohol-soluble part of human gallstones to isolate what we now know as cholesterol. *Poulletier* described the extracted substance at that time as "lamellated brilliant substance quite similar to boric acid" (Dam, 1958). *De Fourcroy* prepared larger amount of the substance and continued studying its nature. He stated that the compound was similar to spermaceti, a waxy substance extracted from spermwhales. About 25 years later, *Chevreul*, a french chemist, proved that the substace had a different melting point than spermiaceti and therefore he ruled out *De Fourcroy*'s suggestion. *Chevreul* also found the substance to be present in human and animal bile. He was the one who gave the compound the name *cholestriene*. The name means solid bile and is derived from Greek (chole: bile and steros: solid) (Dam, 1958). By the middle of the 19th

century, other scientists found cholesterol, or cholesterien at that time, in materials other than gallstones. It was discovered in blood (1838), brain (1834), atheramotous artery (1843) and in egg yolk (1846). The latter was discovered by the French biochemist *Theodore Gobley* and led to the awareness that cholesterol is an essential component of animal cells. In 1859, the french chemist and politicain *Marcellin Berthelot* proved that cholestrien is in fact an alcohol and suceeded to make esters of it. This was the beginning of replacing the old name cholesterin with cholesterol. The new term i.e. cholesterol had become dominant in English and French literature at the beginning of the 20^{th} century, about 150 years after its initial discovery by *Poulletier*. German literature, however, has kept using the older name, cholestrien (Dam, 1958).

The correct chemical formula of cholesterol could not be revealed until 1888 when the Austerian botanist *Friedrich Reinitzer* published his work. He used an elemental analysis method to analyze carbon and hydrogen contents. The formula published by *Rienitzer* is the formula we know today i.e. $C_{27}H_{46}O$ (Li, 2009). Despite having the correct formula, it took another 50 years to identify the correct four-ring structure. Many scientists participated in this accumulative work yet it is worth to mention the two Germans: *Weiland* and *Windaus*. Their contribution was vital and they were awarded with two consecutive Noble Prizes in chemistry in 1927 and 1928. The structure was not however established definitively until 1945 when the British biochemist *Dorothy Hodgkin* used X-ray crystallography to accomplish that (Li, 2009). She indeed deserved the Nobel Prize in Chemistry that she got in 1964 for using this new technique to make many discoveries among them the solution to this 200 year old puzzle. Cholesterol was described by the two Noble Prize winners *Brown and Goldstein* in their Noble lecture in 1985 as "the most highly decorated small molecule in biology". Thirteen Nobel Prizes have been awarded to scientists who devoted major parts of their careers to cholesterol" (Brown and Goldstein, 1986). They said that the molecule "has exerted a hypnotic fascination for scientists from the most diverse domains of science and medicine". Cholesterol has certainly persuaded the scientific curiosity in organic chemists, biochemists, botanists, physiologists, cell biologists and physicians for centuries.

1.2.2 Synthesis and regulation

Cholesterol has many structural and physiological roles. It is an essential component of the cell bilayer membranes and is important for its properties. Cholesterol is also a precursor of

steroid hormones and bile acids as well as being an essential component in myelin in central nervous system (CNS).

The daily turnover of cholesterol is around 1.2 g in the average adult human. This represents less than one percent of the total content of cholesterol in the body, which is around 140 g (Turley and Dietschy, 2003). Around one third of the daily cholesterol turnover is replaced by dietary cholesterol, while the rest is compensated for by de novo synthesis. Cholesterol synthesis takes place in all nucleated cells in the body, particularly in hepatocytes and enterocytes (Morgan et al., 2016). The synthesis process involves a long series of enzymatic reactions that consume a large amount of energy. It can be divided into 6 stages that are shown in figure 2. This synthetic process is well regulated. HMG CoA reductase that converts HMG CoA into mevalonate catalyzes the rate limiting step of this pathway. Statins are a group of drugs that inhibit this enzyme and lead to significant reduction in cholesterol synthesis. An increased level of cholesterol leads to a negative feedback inhibition on its own synthesis. This is mediated by Sterol regulatory element binding protein (SREBP) which is an endoplasmic reticulum integral protein. It functions as a transcription factor that upregulates the expression of many genes involved in cholesterol homeostasis. Several hormones are also involved in regulation of cholesterol synthesis, most importantly: insulin, glucagon and glucocorticoids (Ness and Chambers, 2000).

Cholesterol obtained from the diet is absorbed by enterocytes in the small intestine. Bile acids are essential in this process as they form micelles that incorporate free cholesterol and other lipids to facilitate their absorption. Approximately half of the ingested cholesterol is absorbed (Gylling and Simonen, 2015). In the enterocyte, cholesterol is esterified and packaged with triglycerides into chylomicrons that are ultimately delivered to the liver. The liver, where a substantial part of cholesterol synthesis takes place, plays a central role in cholesterol metabolism as it is responsible for distribution, excretion and regulation of the level of this compound. Very low density lipoprotein (VLDL) composed mainly of triglycerides with some cholesterol is formed by the liver and then exported to the blood stream. The action of lipoprotein lipase, that is found in endothelial cells lining capillaries, remodels VLDL by hydrolyzing the triglycerides component to ultimately produce low density lipoprotein (LDL). LDL is the main fraction responsible for delivering cholesterol to extrahepatic tissues.

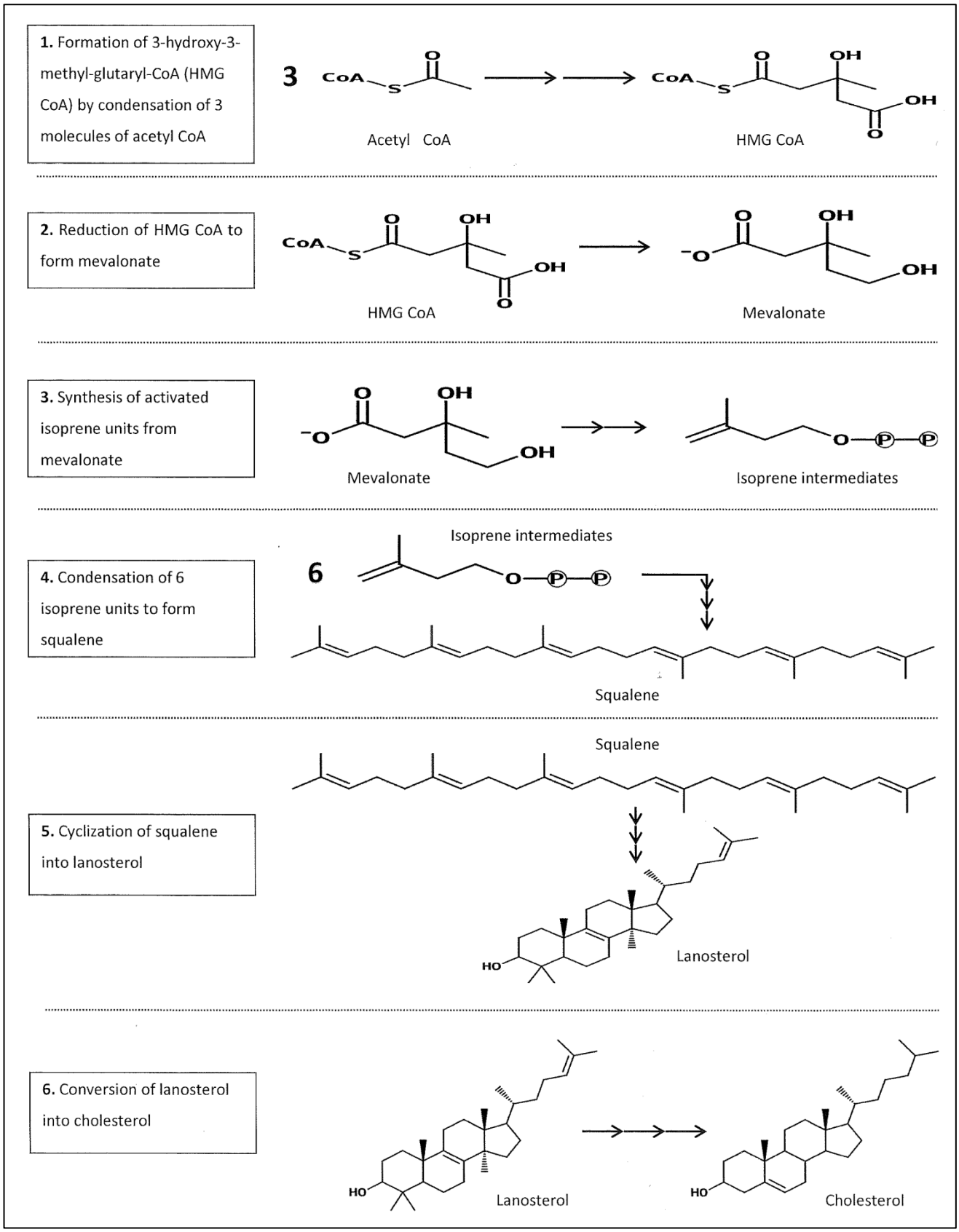

Figure 2. Outlines of cholesterol synthesis

Entry of these particles into the cells is governed by LDL receptors (LDLR). High levels of LDL in blood accelerates atherosclerosis and therefore it is considered as a causative factor for ischemic heart disease and cerebrovascular incidents. Smaller cholesterol carrying particles named high density lipoprotein (HDL) are, in contrast to LDL, protective against atherosclerosis. They are responsible for extracting cholesterol from extrahepatic tissues and transporting it back to the liver, a process called reverse cholesterol transport. Cells from different tissues transfer their cholesterol to HDL particles through the ATP-binding cassette subfamily A member 1 (ABCA1) transporter. Upon reaching the hepatocytes, HDL cholesterol is taken up by scavenger receptor class B member 1 (SR-B1). Liver can eliminate cholesterol by directly secreting it into bile via ATP-binding cassette subfamily G5/G8 (ABCG5/G8) receptor. The other route for cholesterol elimnation is to convert it into bile acids. Bile acid synthesis is a long energy consuming biochemical process involving many reactions catalyzed by different enzymes. The first and rate-limiting step in in this process is the conversion of cholesterol to 7α-hydroxycholesterol by the enzyme CYP7A1. Cholic acid and chenodeoxycholic acid are the main final products of this pathway.

The expression of many enzymes and transporters involved in cholesterol metabolism, and lipid metabolism in general, are regulated by liver X recptors (LXR). LXR are nuclear receptors that are widely expressed in different tissues. Two isoforms of these receptors have been identified, LXRα and LXRβ. The former is expressed in tissues with active lipid metabolism such as liver, intestine and macrophages while LXRβ is expressed in almost all organs. LXR activation increases transcription of major players in cholesterol metabolism such as SREBP-1c, ABCA1, ABCG5/8, ApoE, CYP7A1 and lipoprotein lipase. Oxysterols, the oxygenated cholesterol derivatives, are regarded as the ligands for these receptors.

1.2.3 Cholesterol in brain

The possibility that cholesterol plays a critical role in brain was speculated on as early as the middle of the 19th century. *Couerbe*, a french chemist who studied brain extensively, identified cholesterol as a normal constituent of brain. He also described it as a key element (Bjorkhem and Meaney, 2004). Although brain weight is not more than 2% of total body weight, it accounts for about 20-25% of total body cholesterol (Dietschy and Turley, 2001). Around two thirds of brain cholesterol is found in the myelin sheathes produced by oligodendrocytes. Myelin is composed from cholesterol, phosophlipids and

glycosphingolipids. Its main function is to provide isolation for neuronal axons so neuronal electrical signals are transmitted efficiently. The remaining third of brain cholesterol is found in neuronal and astrocytic cellular membranes. It represents an essential component of the lipid bilayer membrane . Allmost all cholesterol present in brain is unesterified, i.e found as free cholesterol (Bjorkhem and Meaney, 2004).

Brain cholesterol turnover is very low compared to extracerebral cholesterol. In mouse, for example, the total daily cholesterol turnover is about 130 mg cholesterol per kg body weight and only 1.4 mg/kg/day is the brain share in this amount (Dietschy and Turley, 2001). In humans and larger animals the rate of turnover is lower but the proportion remains the same with cerebral cholesterol turnover always representing about 1% of total turnover. Brain, which has the highest cholesterol content than any other tissue, does not share its cholesterol with other body parts i.e with circulation. Cholesterol in brain and in other tissues can be regarded as two distinctive pools separated by the BBB. One of the first observations that led to such a conclusion was made by *Bloch et al.* when they administered deuterium labeled cholesterol to an adult dog to study cholesterol conversion to cholic acid. They noticed that deuteriated cholesterol could be recovered from all organs they studied with exception of brain and spinal cord. They suggested a lack of sterol metabolic interchange between CNS and blood (Bloch et al., 1943). Previous studies by *Waelsch et al.* used deuterium water to demonstrate the low rate of cholesterol synthesis in brain (Waelsch et al., 1940). They described the brain as the most inert organ among all tissues as it does not regenerate and does not exchange with dietery cholesterol. The extent of exchange between brain and plasma cholesterol however remained debatable for while. In the middle of the ninties, *Juervics et al.* used tritium water to show clearly that all cholesterol in myelin is produced locally. They showed that neither dietary cholesterol nor cholesterol donated by other organs participate in this process (Jurevics and Morell, 1995). It is now well established that nearly all cholesterol found in CNS is generated by de novo in situ synthesis.

Cholesterol synthesis takes place mainly in astrocytes and to a lesser extent in neurons. In astrocytes, the synthesised cholesterol is associated with ApoE and exported by ABCA1 transporter to the extracellular space. Cholesterol is then taken up by neurons through LRP1/LDLR. In neurons, cholesterol is handled in the endosomes/lysosomes to be converted

into free cholesterol and then exported from there by NPC1 and NPC2 to cellular membranes and other organelles (Zhang and Liu, 2015).

Oxysterols play important roles in cholesterol synthesis regulation in the brain as they are inhibitors of cholesterol synthesis and act as natural ligands for LXR. Expression of SREBP1, ABCA1 and ApoE, three important players in cholesterol synthesis and trafficking, are modulated by LXR. Both LXR types, α and β, are expressed in brain but the latter is dominating. Mice with a deficiency of both receptors show several CNS defects related to lipid metabolism disturbances.

1.2.4 Cholesterol and neurological diseases

One fourth of body cholesterol is present in the brain so it is not surprising that normal cholesterol metabolism is requied for normal brain functions. Cholesterol metabolism defects have been implicated in several neurodegenerative diseases.

There are many described inborn errors for cholesterol metabolism that lead to CNS disturbances. Smith–Lemli–Opitz syndrome is among the most common in this category. It is caused by defeciency of the enzyme 7-dehydrocholesterol reductase that converts 7-dehydrocholesterol to cholesterol. The deficiency leads to low cholesterol levels and accumulation of 7-dehydrocholesterol. Abnormal development and intellectual difficulties affect patients with this syndrom in addition to emotional and sleep disorders (Petrov et al., 2016). Desmosterolosis, caused by 3β-hydroxysterol-24-reductase defeciency, and Lathosterolosis caused by 3β-hydroxysteroid-5-desaturase deficiency, are two other examples of inborn errors in the cholesterol synthesis pathway (Herman and Kratz, 2012).

Neurodegeneration can also occur with inherited errors affecting transporters and associated protein involved in cholesterol metabolism like NPC and ApoE. Niemann–Pick type C disease is an autosomal recessive disease that leads to defective NPC proteins (NPC1 in 95% of the cases) (Petrov et al., 2016). Due to the defect, cholesterol is not liberated from the lysosomes/endosomes in the affected neurons. This results in a serious reduction of the cholesterol provided to distal axons which is manifested as severe neurodegeneration (Rosenbaum and Maxfield, 2011).

The nature of ApoE, which is important in trafficking cholesterol between astrocytes and neurons, is of major importance in Alzheimer's disease (AD). There are 3 common forms (ApoE2, ApoE3 and ApoE4) that differs from each other by single or double amino acids (Michaelson, 2014). Genetic epidemiological data show that having one allele of ApoE4 increases AD risk with 2-3 fold and several fold more upon having two alleles. On the other hand, ApoE2 appears to be protective against AD. Patients with ApoE4 demonstrate more pronounced brain inflammation and increased Aβ deposition. ApoE4 is thought to affect cerebrovascular, neuronal and inflammatory systems to possess these effects (Michaelson, 2014).

Cholesterol metabolism is also affected in Huntington's disease. This autosomal dominant disease leads to the production of a defective protein, Huntingtin, which downregulates SREBP. This results in lower synthesis and transport of cholesterol. The cholesterol level is reduced at synapses and myelin sheathes which leads to neurodegeneration and ultimately is manifested as severe dementia.

Disturbances in cerebral cholesterol metabolism have also been linked to some other neurodegenerative disorders such as Parkinson's disease (Segatto et al., 2014) and multiple sclerosis (Zhornitsky et al., 2016). The association between cholesterol and the pathogenesis of these two conditions is not yet clear.

1.3 24S-HYDROXYCHOLESTEROL

1.3.1 Discovery and early researches

24-hydroxycholesterol (24-OHC) was described for the first time by two American chemists in the forties. They synthesized the compound starting from the bile acid 3βhydroxy-5-cholestenoic acid through 24-ketocholesterol (Riegel and Kaye, 1944). In 1953, two Italian scientists published their discovery of a new sterol isolated from horse brain. The sterol was similar to cholesterol but with an extra hydroxyl group at position 24. They first called it cerebrostenediol (Ercoli et al., 1953) then shortly changed the name to cerebrosterol (Ercoli and Deruggieri, 1953). Shortly after, the compound was identified in extract from human brain (Di Frisco et al., 1953). It had also been isolated from brain of different animal species including cattle and rabbit with a general conclusion that it presents universally in all

mammals (Dhar et al., 1973). Chromatographic studies revealed that human brain contains only one epimer of 24-OHC (Van Lier and Smith, 1970). 24-OHC was then isolated from different human brain parts including cortex, white matter, midbrain, pons and cerebellum (Smith et al., 1972). In 1973 *Smith* and his group reported biosynthesis of 24-OHC from radiolabeled cholesterol in cortical microsomes obtained from bovine brain. They described a sterol 24-hydroxylase system that requires both oxygen and NADPH (Dhar et al., 1973). A following study on rat brain showed that there is a several fold difference between the levels of 24-OHC in immature rat brain and in the mature brains (Lin and Smith, 1974). Another study was performed on developing rat brain aimed to study the metabolic fate of 24-OHC through injecting tritium labeled 24-OHC intracerebrally. The study suggested that 24-OHC is catabolized enzymatically in the subcellular fraction (Lin and Smith, 1974). 24-OHC had also been reported to be formed by rat liver mitochondria (Aringer et al., 1976) though the significance of such formation was not revealed. Apart from brain and liver, 24-OHC could be identified also in bovine adrenals (Prasad et al., 1984). In human, the compound was detected in several secretions from fetuses and infants: in meconium (Eneroth and Gustafsson, 1969), in feces (Gustafsson and Sjovall, 1969) and in urine from infants with biliary atresia (Makino et al., 1971).

1.3.2 Production and metabolism

In 1996, *Lütjohann et al.* studied possible mechanisms of elimination of brain cholesterol across the BBB. They measured the difference in 24-OHC levels between the internal jugular vein and the brachial artery in eight healthy volunteers. They found that the levels in the vein were significantly higher than in the artery indicating that brain is the main source of this oxysterol (Lütjohann et al., 1996). The same group confirmed this potential elimination mechanism in their next study. They measured conversion of cholesterol to 24-OHC in-vivo and in-vitro and got similar results (about 0.02% of the cholesterol pool/h). They also suggested a 24-hydroxylase enzyme belonging to the CYP450 family to be responsible for this reaction (Bjorkhem et al., 1997). In further research they calculated the daily efflux of 24-OHC from the human brain to be about 6 mg. This amount is transported by the circulation to liver where it is metabolized (Björkhem et al., 1998). About 50% of 24-OHC taken up by the liver is converted to bile while the other half is conjugated and excreted in the bile (as it is or as 27-hydroxylated metabolites) (Björkhem et al., 2001).

The production of 24-OHC from cholesterol is catalyzed by CYP46A1. This enzyme belongs to the CYP450 family and it uses oxygen and NADPH to hydroxylate cholesterol at position C24. *Lund et al.* isolated cDNA that encodes this enzyme from mouse liver. They screened for mRNA levels in different tissues and observed that it was almost exclusively expressed in the brain. Although low levels of mRNA could be seen in murine liver and testis, no corresponding protein was expressed in these organs. They also reported wide expression of the enzyme in different areas of the brain with higher levels in areaa rich with grey matter (Lund et al., 1999). Their data also show that the enzyme is expressed only in neurons with almost no expression in glial cells. In humans, the expression of the enzyme was shown to be steady from the first year of life through to adulthood. A previous report showed higher levels of 24-OHC at an early stage of life with a decline with age (Lütjohann et al., 1996). This was attributed to the age-dependant ratio between liver and brain volumes which means that the capacity of liver to metabolize the 24-OHC produced by brain, rather than CYP46 expression, is responsible for the elevated levels of this sterols seen in early years of life (Bretillon et al., 2000). Molecular studies showed that CYP46A1 expression does not respond to a number of transcriptional regulatory axes. Substrate availability also does not have a significant effect on transcription of the gene. Oxidative stress was the only factor that was shown to increase CYP46A1 transcriptional activity (Ohyama et al., 2006). Presence of epigenetic regulation of CYP46A1 was suggested to be possible from results showing that expression can be affected by a histone deacetylase inhibitor (Shafaati et al., 2009).

In-vitro studies showed that 24-OHC is an efficient LXR activator (Janowski et al., 1999) that leads to increased expression of a number of LXR target genes (e.g. ABCA1 and SREBP1). Activation of these genes stimulates cholesterol synthesis and therefore it was assumed that higher levels of 24-OHC lead to increased synthesis of cholesterol. On the other hand it is well established that side-chain oxidized oxysterols are effective inhibitors of cholesterol synthesis. In accordance with the latter effect, addition of 24-OHC to neuronal cells was shown to downregulate cholesterol synthesis (Wang et al., 2008). In-vivo experiments showed the failure of increased 24-OHC to activate LXR. Mice with CYP46A1 overexpression have thus 2-fold increased levels of 24-OHC in brain and yet no difference in expression of LXR target genes (Shafaati et al., 2011). Recently the mechanism responsible for the lack of effect on the LXR target genes in this mouse model was clarified. The overexpression of CYP46A1 leads to consumption of cholesterol with a subsequent increase in cholesterol synthesis as well as increased prenylation of sGTPase in neuronal cells. The

latter prenylation was shown to lead to a general decrease in the expression of LXR target genes (Moutinho et al., 2015)

Ali et al. suggested a theoretical model for cholesterol metabolism in the brain in which 24-OHC is important as an inhibitor of cholesterol synthesis (Ali et al., 2013). Increased production of 24-OHC leads to consumption of brain cholesterol which in turn leads to a compensatory increase in cholesterol synthesis (Shafaati et al., 2011). The latter positive effect on cholesterol synthesis is however balanced by the inhibitory effect of 24-OHC on this synthesis.

1.3.3 Effect on memory function

Lütjohann et al. showed that the levels of plasma 24-OHC are significantly higher in patients with early AD and vascular dementia compared to controls. It was suggested that higher levels of this oxysterol in plasma may reflect a state of neurodegeneration (Lutjohann et al., 2000). Further studies on brain samples from advanced AD patients showed that they contain significantly lower levels of 24-OHC compared to controls (Heverin et al., 2004). In-vitro studies investigating the role of 24-OHC in AD pathology revealed that this oxysterol has a protective function. 24-OHC increases α-secretase activity which leads to the production of the more favorable soluble nonpathogenic products of amyloid (Famer et al., 2007). Such findings could be also seen under in-vivo conditions, in an animal model for AD. Cyp46a1 overexpression in APP23 mice, a model with human APP overexpression and with the Swedish double mutation, decreased Aβ peptide accumulation and improved spatial memory (Hudry et al., 2010). On the other hand, the reverse situation was seen when production of 24-OHC was hindered. Targeting Cyp46a1 mRNA in wild type mice led to decreased expression of the enzyme, lower 24-OHC and higher cholesterol levels in hippocampus. These changes were accompanied by increased Aβ peptide, apoptotic neuronal death, hippocampal atrophy and subsequent cognitive deficits (Djelti et al., 2015).

The beneficial effect of 24-OHC on memory becomes more apparent on comparing two mouse models, one with human CYP46A1 overexpression and the other with the gene being knocked out. Older mice with over production of 24-OHC showed improved spatial memory and increased expression of a number of synaptic proteins (Maioli et al., 2013). On the other

hand, Cyp46a1 knock out mice revealed deficiencies in spatial, associative, and motor learning (Kotti et al., 2006). Hippocampal sections from these mice showed impaired long-term potentiation (LTP) indicating subnormal synaptic activity. Interestingly, LTP in knock out mice could be restored to wild type levels by treatment with geranylgeraniol. This compound is a nonsterol isoprenoid that is generated as a byproduct of the mevalonate pathway, the first few steps of cholesterol synthesis. Geranylgeraniol is covalently linked to many proteins that play important role in synapses formation and signal transduction (Kotti et al., 2006). Therefore it was postulated that the activity of cholesterol synthesis, the mevalonate pathway in particular, rather than cholesterol levels is the crucial factor for memory function in the brain. *Maioli et al.* used this hypothesis to explain the improved memory and the higher levels of synaptic proteins demonstrated in their CYP46A1 model. In those animals, the high activity of the enzyme led to higher cholesterol catabolism in the brain with a subsequent compensatory increase in cholesterol synthesis. The latter effect was evident by increased cholesterol precursors (Maioli et al., 2013). The contrast situation is observed in Cyp46a1 knock out mice where cholesterol synthesis in brain is reduced by 40% (Lund et al., 2003). It is noteworthy that cholesterol levels in the brain are not changed in either of these two animal models. According to the above studies the rate of cholesterol synthesis, and hence the level of geranylgeraniol produced, is important for memory function.

1.4 27-HYDROXYCHOLESTEROL

1.4.1 Discovery and early research

27-Hydroxycholesterol (27-OHC) was known in the past as 26-hydroxycholesterol. It was first discovered by *Fredrickson* in 1956 as a product after incubation of radioactive cholesterol with mouse liver mitochondria (Fredrickson, 1956). *Danielsson* confirmed the finding and showed that this hydroxycholesterol is metabolized into chenodeoxycholic acid in rat liver (Danielsson, 1961). The metabolic pathway of such conversion was suggested a few years later (Mitropoulos and Myant, 1967).

27-OHC was reported to be present in atheromatous plaques (Brooks et al., 1966) and in the intima of aorta (Van Lier and Smith, 1967). *Gustafsson* and his collaborators could identify this compound in stool samples from infants under one year of age (Gustafsson and Sjovall, 1969) and in meconium from newborns (Eneroth and Gustafsson, 1969) among other

hydroxycholesterols. 27-OHC was also identified in urine samples from infants with biliary atresia (Makino et al., 1971) and in both the urine and plasma in patients with cholestasis (Summerfield et al., 1976).

The metabolic fate of 27-OHC in humans was studied by *Andersson et al.* by giving tritium labeled 27-OHC to patients with external biliary drainage. They demonstrated that 78% of 27-OHC is converted into bile acids with a greater proportion being converted in chenodeoxycholic acid (Anderson et al., 1972). *Björkhem* and *Gustafsson* reported 27-hydroxylase activity in rat liver microsomes and mitochondria that is able to 27-hydroxylate a large number of sterols that are involved in bile acid synthesis including cholesterol. They postulated that a cytochrome P-450 is involved in this 26-hydroxylation reaction (Bjorkhem and Gustafsson, 1973). This postulation was confirmed many years later when rabbit mitochondrial sterol 27-hydroxylase was characterized by protein sequencing and cDNA sequence analysis (Andersson et al., 1989). The same study found that mRNA of sterol 27-hydroxylase is present in many extrahepatic organs which supports an earlier finding of enzyme activity in human fibroblasts (Skrede et al., 1986).

Several reports then proposed an "alternative pathway" for bile acid synthesis starting by conversion of cholesterol to 27-OHC rather than the classical pathway where cholesterol first is converted into 7α-hydroxycholesterol (Swell et al., 1980, Vlahcevic et al., 1980). *Kok et al.* found that chenodeoxycholic acid and lithocholic acid are the primary bile acids produced through this alternative pathway in hamster (Kok et al., 1981). This pathway is reported to be disrupted in patients with cerebrotendinous xanthomatosis (CTX) due to the lack of 27-hydroxylase activity in liver mitochondria (Oftebro et al., 1980).

27-OHC was detected in the sera of normal adults using isotope dilution mass spectrometry. It was reported to be distributed between both HDL and LDL (Javitt et al., 1981). Markedly reduced levels were seen in patients with CTX (Javitt et al., 1982) supporting the earlier finding of *Oftebro* and his colleagues demonstrating lack of the sterol 27-hydroxylase in these patients. In-vitro studies conducted by *Esterman et al.* using Chinese hamster ovary cell cultures showed that 27-OHC is a potent inhibitor of HMG CoA reductase (Esterman et al., 1983). In another in-vitro experiment, 27-OHC was found to reduce LDL binding sites in

cultured human fibroblasts leading to inhibition of binding, uptake and degradation of LDL (Lorenzo et al., 1987).

A positive correlation between serum levels of 27-OHC and cholesterol has been reported (Harik-Khan and Holmes, 1990). However, the same study found that the majority of the studied patients with proven atherosclerosis have normal or low levels of 27-OHC. Therefore it was concluded that high serum levels of 27-OHC is not of major importance in atherosclerosis development (Harik-Khan and Holmes, 1990). *Jimi et al.*, nevertheless, reported a cytotoxic effect of 27-OHC on cultured bovine endothelial cells and smooth muscle cells. They used however 10-fold the physiological serum level of 27-OHC (Jimi et al., 1990). *Zhou et al.* demonstrated this cytotoxic effect on cultured human arterial cells also when using a physiological dose of 27-OHC (Zhou et al., 1993).

1.4.2 Nomenclature

Up till the early nineties, 27-OHC was known in the literature as 26-hydroxycholesterol. In 1989 *Andersson et al.* identified the gene responsible for the hydroxylase activity in rabbit that results in the formation of 27-OHC among other products (Andersson et al., 1989). The gene belongs to the cytochrome P-450 family and the name CYP26 was recommended for nomenclature initially (Nebert et al., 1989). After revision of the exact site of hydroxylation, the name was changed into CYP27 in the updated nomenclature list (Nebert et al., 1991). In 1991, *Cali* and *Russel* published their work on the sterol 27-hydroxylase gene isolated from human liver. They used the updated nomenclature calling the gene (and the enzyme) CYP27 (Cali and Russell, 1991). The trend of using the name 27-OHC instead of 26-hydroxycholesterol started with this article according to *Fakheri* and *Javitt*. The latter authors refuted this change and claimed that according to IUPAC nomenclature roles the molecule should be named 26-hydroxycholesterol (Fakheri and Javitt, 2012). Nevertheless, the majority of the scientific community have now adopted the name 27-OHC and use it in their works.

1.4.3 27-OHC in brain

Heverin et al. measured the levels 27-OHC in the internal jugular vein and in the brachial artery in male volunteers and found that there is a net flux of 5 mg/day of 27-OHC from circulation to brain. They also studied brain autopsy from rats given deuterium labeled 27-OHC to reveal that significantly higher amount of the fluxed 27-OHC is present in the white matter (Heverin et al., 2005).

The level of 27-OHC in CSF was shown to be increased in patients with neurodegenerative diseases (Bjorkhem et al., 2013). This finding can in part be attributed to the disruption in the BBB that may accompany some neurodegenerative diseases. It can also be explained by the loss of neuronal cells expressing CYP7B1 enzyme that metabolizes 27-OHC (Leoni and Caccia, 2011). *Meaney et al.* showed that this enzyme along with two others are responsible for the rapid and efficient metabolism of 27-OHC in brain. The product of this process is 7α-hydroxy-3-oxo-4-cholestenoic acid (7-Hoca) that fluxes back into the circulation (Meaney et al., 2007). The efficiency of mtabolism of 27-OHC by the brain can be seen as a detoxification process in light of the reported negative impact caused by this oxysterol (Bjorkhem et al., 2009). In-vitro studies showed that 27-OHC increasesd Aβ precursor proteins, Aβ and phosphorylated tau which are the hallmarks of AD (Marwarha and Ghribi, 2015). Arc protein which is of major importance in memory function is affected and reduced by high cholesterol diet. Since cholesterol cannot pass the BBB, many investigations were carried out to elucidate the mechanism by which cholesterol causes this effect. A recent study has shown that 27-OHC is most probably mediating this process. Cyp27a1 knock out mice on high cholesterol diet had normal levels of the "memory protein" Arc in hippocampus in contrast to wild type. The spatial learning (assessed by Morris water maze test) in the mice lacking 27-OHC in serum and brain was not affected contrary to wild type mice (Heverin et al., 2015). In accordance with this, an extended unpublished study by the same group and their collaborators showed that CYP27A1 overexpressing mice with high levels of 27-OHC in brain have reduced levels of Arc. Data also show that these animals have reduction in glucose uptake in brain and reduced capacity for spatial learning (Ismail et al., unpublished).

Defects in 27-OHC synthesis or metabolism may occur and cerebrotendinous xanthomatosis (CTX) and hereditary spastic paraplegia type 5 (SPG5) are two examples. The inherited error in CTX is autosomal recessive. The defective enzyme in this disease is CYP27A1 which

catalyzes conversion of cholesterol into 27-OHC. Cholesterol and cholestanol are accumulated which leads to formation of xanthomas in brain and tendons. Several neurological defects are associated with the disease including mental and intellectual difficulties and chronic dementia (Bjorkhem, 2013). In SPG5, 27-OHC metabolism is impaired due to lack of CYP7B1 enzyme. This enzyme is a key enzyme for the alternative pathway for bile acid synthesis that converts 27-OHC ultimately to bile acids. The autosomal recessive lacking of this enzyme in SPG5 leads to accumulation of 27-OHC in plasma and CSF. Patients with this disease get progressive spasticity and weakness of the lower limbs beside many other neurological and systemic symptoms. Cholesterol lowering treatment such as statins and ezetimibe aimed to reduce 27-OHC are being investigated as possible therapy for this condition (Mignarri et al., 2015). A recent study showed that two cholestenoic acids present in CSF are LXR ligands. They are 27-OHC metabolites through pathways involving both CYP27A1 and CYP7B1. The first is 3β,7α-dihydroxy cholestenoic acid which was shown to promote neuronal survival. The second one is 3β- cholestenoic acid which causes cell death. Interestingly, a low level was found of the former in CSF from patients with SPG5 with higher levels of the latter cholestenoic acid. These findings may at least in part explain the mechanism by which 27-OHC negatively affects the CNS (Theofilopoulos et al., 2014).

1.5 7ALPHA-HYDROXY-3-OXO-4-CHOLESTENOIC ACID:

1.5.1 Discovery and early researches

7α-hydroxy-3-oxo-4-cholestenoic acid (7-Hoca) was first discovered in 1988 by *Magnus Axelson, Jan Sjövall* and their team during their studies on unconjugated bile in the plasma (Axelson et al., 1988). Using HPLC they determined the mean of plasma levels of 7-Hoca in healthy volunteers to be about 80 ng/ml (Axelson et al., 1988). In some later studies lower levels have been reported (Heverin, 2005). The same group continued studying 7-Hoca and other cholestenoic acids in relation to different diseases and conditions. They found that plasma 7-Hoca levels are elevated in liver cirrhosis, both primary biliary cirrhosis and alcohol liver cirrhosis. They attributed that to the decreased clearance of the compound by the damaged liver (Axelson et al., 1989b). In a following study, they demonstrated higher levels of 7-Hoca in patients with ileal resection and in patients treated with cholestyramin. They concluded that plasma levels of 7-Hoca can be used as a marker for the activity of cholesterol 7α-hydroxylase, the enzyme that catalyzes the rate limiting step of the classical pathway of bile acid synthesis (Axelson et al., 1989a). *Axelson* and *Sjövall* also correlated different bile

acid intermediates in patients and controls to 7-Hoca. They concluded that 7-Hoca is an intermediate in the alternative pathway of bile acid synthesis. They also proposed that it can be synthesized from 3β,7α-dihydroxy-5-cholestenoic acid or from 7α-hydroxy-4-cholesten-3-one depending on the rate of bile acid synthesis (Axelson and Sjovall, 1990b). In their next study, *Axelson* and *Sjövall* compared the levels of 7-Hoca, 7α-Hydroxy-4-cholesten-3-one and 3β,7α-dihydroxy-5-cholestenoic acid in controls and in patients with disturbances in bile acid metabolism. Based on correlation analysis they found that the precursor of 7-Hoca differs depending on the activity of 7α-hydroxylase (Axelson and Sjovall, 1990a). They also studied the metabolism of radiolabeled cholesterol by pig mitochondria. Results showed that cholesterol is metabolized into five intermediates with 7-Hoca as the end product. They proposed, based on this experiment, different pathways for 7-Hoca formation from cholesterol, for example via 27-OHC or 3βhydroxy-5-cholestenoic acid (Axelson et al., 1992). Similar results were also obtained one year earlier in cultured human hepatoblastoma cells (Axelson et al., 1991) and one year later in human liver microsomes and mitochondria (Shoda et al., 1993). *Lund et al.* measured the arterial-hepatic venous difference of 7-Hoca levels in six healthy volunteers. The result showed that there is a net uptake of this compound by the liver of about 12 mg/24h (Lund et al., 1996). This is consistent with extrahepatic sources for this steroid acid. The same study reported that all circulating 27-oxygenated products, of which 7-hoca constitutes about 50%, are responsible for about 4% of total bile production (Lund et al., 1996). Data obtained from a healthy volunteer who ingested deuterium labeled cholesterol suggested that the origin of plasma 7-Hoca is mainly extrahepatic (Meaney et al., 2003). They also concluded that under normal conditions the majority of circulating 7-Hoca is derived from 7α-hydroxy-4-cholesten-3-one (Meaney et al., 2003).

1.5.2 7-Hoca in chronic subdural hematoma

A Japanese group discovered in 1992 that chronic subdural haematomas (CSH) contain high level of 7-Hoca (Nagata et al., 1992). The levels were between 5 to 20 times higher than plasma levels. Interestingly, 7-Hoca could not be detected in subdural hygroma, which resembles CSH but lacks the capsule. These findings indicated an extrahepatic synthesis of this compound. Another study confirmed the high levels of 7-Hoca in CSH and showed that it is accompanied by extremely low level of 7α-hydroxy-4-cholesten-3-one (Nagata et al., 1993). The levels of other potential precursors of 7-Hoca such as 3β,7α-dihydroxy-5-cholestenoic acid were found to be similar to plasma levels. Cholic acid and

chenodeoxycholic acid were about 3 fold higher in CSH than in plasma. The authors suggested that 7-Hoca is produced from 7α-hydroxy-4-cholesten-3-one in CSH as a part of a concentration process of polar bile acids in CSH. Yet the exact mechanism remained unclear. The same Japanese group also reported higher levels of 7-Hoca in CSF after subarachnoid haemorrhage (Nagata et al., 1995). The levels were about 5 times higher than plasma levels in the first day after the incident of haemorrahge. The levels dropped sharply during the second day and become around normal plasma level (126 ± 59 ng/ml) (Nagata et al., 1995). The mechanism that lies behind this finding could not be established and has been further studied in this work.

1.5.3. 7-Hoca in brain

Using radiolabeled 27-OHC, Zhang et al. reported the production of trace amounts of 7-Hoca by cultured rat astrocytes (Zhang et al., 1997). They proposed that conversion of 27-OHC into 7-Hoca in the brain may be part of the explanation for the higher level of 7-HOCA in CSH. Ten years later, the metabolism of 27-OHC into 7-Hoca was reported to be a novel mechanism for elimination of 27-OHC, and cholesterol, from the brain across the BBB. *Meaney et al.* described the series of metabolic reaction involved in this process (Meaney et al., 2007). Three enzymes expressed in the neuronal cells are responsible for these reactions: sterol 27-hydroxylase (CYP27A), oxysterol 7α hydroxylase (CYP7B1) and 3β-hydroxy-C27-steroid dehydrogenase/isomerase (HSD3B7). The authors also demonstrated the efficient ability of 7-Hoca to cross the BBB in an in-vitro model. In addition, they measured the flux of 7-Hoca out of the brain in healthy volunteers by comparing its level in the jugular vein to that in the brachial artery. They reported a small but significant increase in jugular vein levels. They estimated this efflux of 7-Hoca to be about 2 mg/day one third of that of 24S-OHC (Meaney et al., 2007).

In CSF, 7-Hoca was found to be the dominating cholesterol metabolite (Ogundare et al., 2010). Given this fact, it was suspected that the level of this metabolite could be of interest in connection with different neurological diseases, possibly reflecting pathogenetic mechanisms. 7-Hoca was also regarded to be important to further investigate the mechanism by which this metabolite accumulates in CSH and in CSF in connection with subarachnoid bleeding.

It is worthy to note that the reported plasma level of 7-Hoca differed considerably between the work by Meaney et al. (Meaney et al., 2007) and the work by Nagata et al. (Nagata et al., 1993, Nagata et al., 1995, Nagata et al., 1992). These variations are propably explained by the diversity of quantification methods and the different natures of internal standards used. Part of the difference may also be due to the different population studied.

1.6 PLANT STEROLS:

1.6.1 History of discovery

In 1941 *Fernholz* and *MacPhillamy* reported their discovery, a new phytosterol that was not previously known. Since it was first extracted from rapes oil (*Brassica cumpestris*), they called it campesterol (Fernholz and MacPhillamy, 1941). This discovery took place more than 40 years after identification of sitosterol by *Burian* in 1897. *Burian* used oil extracts of wheat and rye germ to precipitate crystals to which the name sitosterol was given (sito is a Greek prefix that means grain) (Anderson and Shriner, 1926). This extracted sitosterol was discovered to be a mixture of three isomers: α-sitosterol, β-sitosterol and γ-sitosterol (Wallis and Fernholz, 1936). It took many years to separate these components and to recognize that β-sitosterol is the most abundant one (Rosenheim and Webster, 1941). The first contact with plant sterols occurred however around 30 years before identification of sitosterol. In 1862, German biochemist *Benecke* published his work in which he claimed that cholesterol does occur in plant kingdom. He based his conclusion on his experimental results where he succeeded to extract (cholesterien) from peas (Needham, 1933). His discovery triggered a lot of research aimed to compare the cholesterol he found in plants with that found in animals. Less than two decades later, *Hesse*, another German scientist, extracted this claimed cholesterol from Calabar beans (*Physostigma venenosum*). He showed in his work published in 1878 that there are certain physical differences that separate the sterol present in plants from the known cholesterin (Arnqvist, 2007). He called the former *phytosterien* (phyto is a Greek word for plant). As what happened with cholesterin, the phytosterien was later changed to phytosterol (Needham, 1933). However, in contrast to cholesterol which has one universal form in all animals, phytosterol proved to be a mixture of different compounds. The product extracted by *Hesse* was actually stigmasterol, as indicated by the plant name. It was identified in 1906 by Windaus an Hauth (Dam, 1958).

More than ten phytosterols have been discovered and described. Sitosterol, campesterol and stigmasterol are the most common naturally occurring sterols in plant kingdom. Two other sterols to be mentioned are: ergosterol and brassicasterol. Ergosterol is synthesized mainly by fungi and brassicasterol is found chiefly in algae (Toshitake et al., 1992).

1.6.2 Absorption and relation to cardiovascular health

Similar to cholesterol, dietary acquired phytosterols are absorbed by the small intestine with the help of bile acids micelles. Absorption is however far less efficient than cholesterol as only 2-5% of the ingested plant sterols are absorbed (Gylling and Simonen, 2015). The rate of absorption also varies considerably between different phytosterols. The majority of the absorbed amount is secreted back again into intestinal lumen by ABCG5/8 leaving around 1% that reaches the circulation (Rocha et al., 2011). Again like cholesterol, phytosterols are esterified in enterocytes and packed in chylomicrons to reach the liver. In liver, ABCG5/8 transporters secret a large amount of the plant sterols into bile. The rest leaves the liver to circulate in blood carried in lipoproteins. More than 70% of phytosterols are carried in the LDL fraction (Gylling and Simonen, 2015) by which they are delivered to peripheral tissues. The majority of the remaining fraction is carried by HDL particles which transport these sterols back to liver to be excreted.

Phytosterols have been known to reduce LDL cholesterol since the fifties (Genser et al., 2012). There are many postulated mechanisms for this action among them reducing cholesterol absorption by competition at the enterocytic absorption sites. A dose of 2 g of phytosterols was shown to reduce cholesterol absorption by up to 40% (Rocha et al., 2011). Other mechanisms include competition with cholesterol for esterification in enterocytes and increased expression of ABCA1 leading to more cholesterol secretion back into the intestinal lumen. Other studies showed several effects of plant sterols on LXR, HMG CoA reductase and LDL that may be responsible for their LDL lowering effect (Calpe-Berdiel et al., 2009). This property of phytosterols promoted them as natural protectors against cardiovascular diseases. Over the past decades, the food industry has started to use phytosterol-rich substances as a base for new products or as food additives. However, benefits of this common practice were questionable and debatable. Many systematic reviews claimed that plant sterol consumption, despite its LDL lowering effect, does not decrease the risk of cardiovascular disease neither reduce the mortality. Additionally, plant sterols themselves were suspected to

be involved in atherogenesis (Assmann et al., 2006). One metanalysis of several large studies over the last 60 years could not detect any association between sitosterol and campesterol levels in plasma and the risk of cardiovascular disease (Genser et al., 2012).

1.6.3 Plant sterols in brain and their relation to neurological diseases

Sitosterol was the first phytosterol to be detected in mammalian brain and was isolated as sitosterol-containing sulfolipids from bovine brain (Prasad et al., 1985). The significance of this finding was difficult to comperhend since it was generally assumed that the BBB prevents flux of these sterols into the brain. Around two decades later, *Lütjohann et al.* reported that mice carrying the Swedish mutation (APP23) have a 2-3 fold increased brain campesterol and sitosterol compared to controls. This was observed in older animals (12-15 months old) and the integrity of the BBB was claimed to be the reason (Lütjohann et al., 2002). One year later, a study showed increased campesterol and sitosterol levels in the brain of Abcg5/Abcg8-deficient mice. These animals have increased phytosterols level in plasma as a consequence of decreased secretion by liver and intestine. The levels of phytosterols detected in their brain were low and they were in free form in contrast to the esterified form present in plasma (Yu et al., 2004). *Jansen et al*. were the first to report dietary plant sterol accumulation in brain. This accumulation occurred in wild type mice on a phytosterol enriched diet and also occurred in mice lacking Abcg5 or Abcg8 transporters resulting in high plasma phytosterols levels (Jansen et al., 2006). Accumulation of plant sterols was also observed in brain of Watanabe rabbits, a model for familial hypercholesterolemia (Fricke et al., 2007). Long term feeding experiments were performed to evaluate this accumulation in mouse brain. *Vanmierlo et al*. fed wild type mice a plant sterol enriched diet for 6 weeks, followed by a plant stanol ester enriched diet for 6 months. The latter diet aimed to deplete sterols absorption. Compared to controls on chow diet, significantly higher amounts of plant sterols were found in plasma liver and brain after 6 weeks of feeding. After shifting to the other diet for 6 months, plant sterols were almost totally eliminated from plasma and liver. However, brain levels were similar to those obtained after 6 weeks. The authors concluded that phytosterols accumulation in brain is concentration-dependent, stable and irreversible (Vanmierlo et al., 2012).

The mechanism by which plant sterols can cross the BBB is not yet clear. Since mice deficient in both Abcg5 and Abcg8 were accumulating phytosterols in brain, it is unlikely for

these two transporters to be involved in the transfer (Jansen et al., 2006). Based on the finding that the majority of plant sterols are carried in the HDL fraction, it was suggested that SR-B1 may play an important role. These HDL receptors are expressed by the apical endothelial cells of the BBB and could be involved in phytosterol transfer from blood into brain parenchyma (Vanmierlo et al., 2015).

The use of plant sterol additives in food has been increased over the past decades due to their assumed benefit on cardiovascular health. Although such benefits are still questionable, the side effect of this trend is more accumulation of phytosterols in brain. The importance of such accumulation is debatable. *Vanmierlo et al.* studied older mice with Abcg5 deficiency with high plasma phytosterols levels. The brain of these animals contain about 6 fold higher campesterol and about 12 fold higher sitosterol than control mice. Despite these levels, mice were normal with respect to memory, mood and anxiety-related behavior. Brain cholesterol homeostasis, however, was affected with lower cholesterol, lower 24-OHC and slightly higher 27-OHC in the brain of these mice as compared to controls (Vanmierlo et al., 2011).

Phytosterols have also been linked to amyotrophic lateral sclerosis (ALS), which is the most common form of motor neuron disease. In-vitro studies showed a neurotoxic effect of glycosylated sitosterol (β-sitosterol-β-d-glucoside) present in cycad seeds. Cycad is a plant found in tropical and subtropical areas where its seeds are used to make flour. Many studies have linked consumption of cycad seeds with ALS but the exact causative agent remains uncertain (Vanmierlo et al., 2015). Wild type mice fed β-sitosterol-β-d-glucoside for 10 weeks showed progressive spinal motor neuron loss that continued even after cessation of the compound. It was concluded that this glycosylated phytosterol is a potential causative factor for ALS and the ALS-Parkinsonism dementia complex. The latter is a heterogeneous form of the disease where loss of motor neurons is combined with Parkinsonism and dementia (Tabata et al., 2008). The mechanism by which glycosylated sitosterol possesses these harmful effects is not clear. It is speculated that it may occur through LXRβ disabling or through hindering mitochondrial function (Vanmierlo et al., 2015).

Many studies suggest beneficial roles for plant sterols in some neurodegenerative diseases. For example, a mixture of plant sterols given to mice with experimental autoimmune encephalomyelitis, a model for multiple sclerosis, were shown to be protective. Phytosterols seem to delay the onset of the inflammation, decrease its severity and reduce demyelination

(Valerio et al., 2011). With regard to Alzheimer's disease, many in-vitro studies showed that phytosterols reduce Aβ generation (Vanmierlo et al., 2015).

1.7 BLOOD-BRAIN BARRIER

1.7.1 History

In the end of the 19th century, *Paul Ehrlich* documented the first observation that later led to the discovery of the BBB. Paul Ehrlich was a physician, scientist and Nobel laureate. He observed that parenteral administration of dyes to an experimental animal results in staining of all body parts except the brain (Moody, 2006). He attributed this finding to the low affinity of brain tissue to the dye. *Max Lewandowsky*, a German neurologist, had however a different explanation. Based on his studies on neurotoxins he observed that many toxins become several fold more efficient if injected intrathecally rather than parentally. He hypothesized in his article that was published in 1900 that brain capillaries have the ability to block the passing of certain substances and allowing others. This appealing hypothesis was rejected by *Ehrlich* who did not accept the idea that the vascular endothelium may have different functions in different organs (Saunders et al., 2014). Nevertheless, *Edwin Goldman*, a German surgeon and a pupil of *Ehrlich*, carried out an experiment that revealed the truth. He injected trypan blue dye directly into the intrathecal space of a rat. The dye was confined to the intrathecal space so that the brain and spinal cord were the only organs to be stained. *Goldmann*'s results that were published in 1913, disproved *Ehrlich*'s explanation and supported *Lewandowsky* hypothesis about the presence of "a barrier" between the brain and the circulation. The actual term "barrier" appeared a couple of years later in 1918 when the two French scientists *Stern* and *Gautier* introduced it (Saunders et al., 2014). They used it recurrently in their publications that were dedicated to study the transfer of various substances across the BBB. The physical existence of the barrier and its nature continued to be a debatable issue for about 50 years. The main reason for this was that scientists could not observe differences between endothelial cells that line brain capillaries and those found in other body parts. In 1967, two American scientists from Harvard, *Reese* and *Karnovsky*, showed the world the actual barrier for the first time. They administered Horseradish peroxidase parentally to mice and compared tissue staining under electron microscopy. They showed that in tissues other than brain, the stain managed to pass from the capillary lumen to the basement membrane through intracellular spaces. This was not the case in the brain where the dye stopped completely at narrow points between brain endothelial cells. They called

these points “tight junctions” (Reese and Karnovsky, 1967). *Reese* and *Karnovsky*’s work proved that there are differences in function and structure between brain capillaries and other capillaries. Their findings established the BBB concept as a solid fact and took BBB-related research to more advanced levels.

1.7.2 Components of the BBB

Over years of research knowledge has accumulated about the BBB. In general, components of the BBB can be divided into cellular and molecular components. Cellular components represent the structural parts of which the BBB consists while molecular compounds are the specialized molecules and micro-structures that enable the BBB to perform its biological functions.

1.7.2.1 Cellular components

The cellular components of the BBB are similar to microvascular units elsewhere in the body, yet with some distinctive features (figure 3). The endothelial lining of CNS capillaries is the first part of the barrier. These cells are thinner and more active metabolically than other endothelial cells. They possess their barrier function by preventing paracellular diffusion, which is stopped by tight junctions. They also have a very low rate of transcytosis which minimizes transcellular transfer (Daneman and Prat, 2015). In addition, CNS endothelial cells prevent leucocytes passing through them due to their very low expression of Leukocytes Adhesion Molecules. The expression of this factor increases with inflammation allowing neutrophils and macrophages accessing neural tissues (Blanchette and Daneman, 2015).

The basement membrane lies on top of the endothelial cells. It is the extracellular matrix that is secreted by endothelial cells and pericytes. It consists mainly of collagens, glycoprotein and proteoglycan. The basement membrane of neurovascular unites serves both as physical and chemical barrier. In addition it is involved in many signaling processes (Daneman and Prat, 2015).

Pericytes are embedded in the basement membrane. They are supporting cells with extended cellular processes reaching the endothelial cells. These cells have several functions including angiogenesis regulation, secretion of basement membrane and regulation of capillary

diameter, and hence blood flow, through contraction. Pericytes in CNS are distinctive from those in other organs by being originated embryologically from the neural crest instead of the mesoderm. In addition, the CNS microvascular units have extensive pericytes coverage so there are more pericytes around endothelial cells than in other organs. Pericytes have long been considered as an important part of the BBB but their exact role was not elucidated until recently.

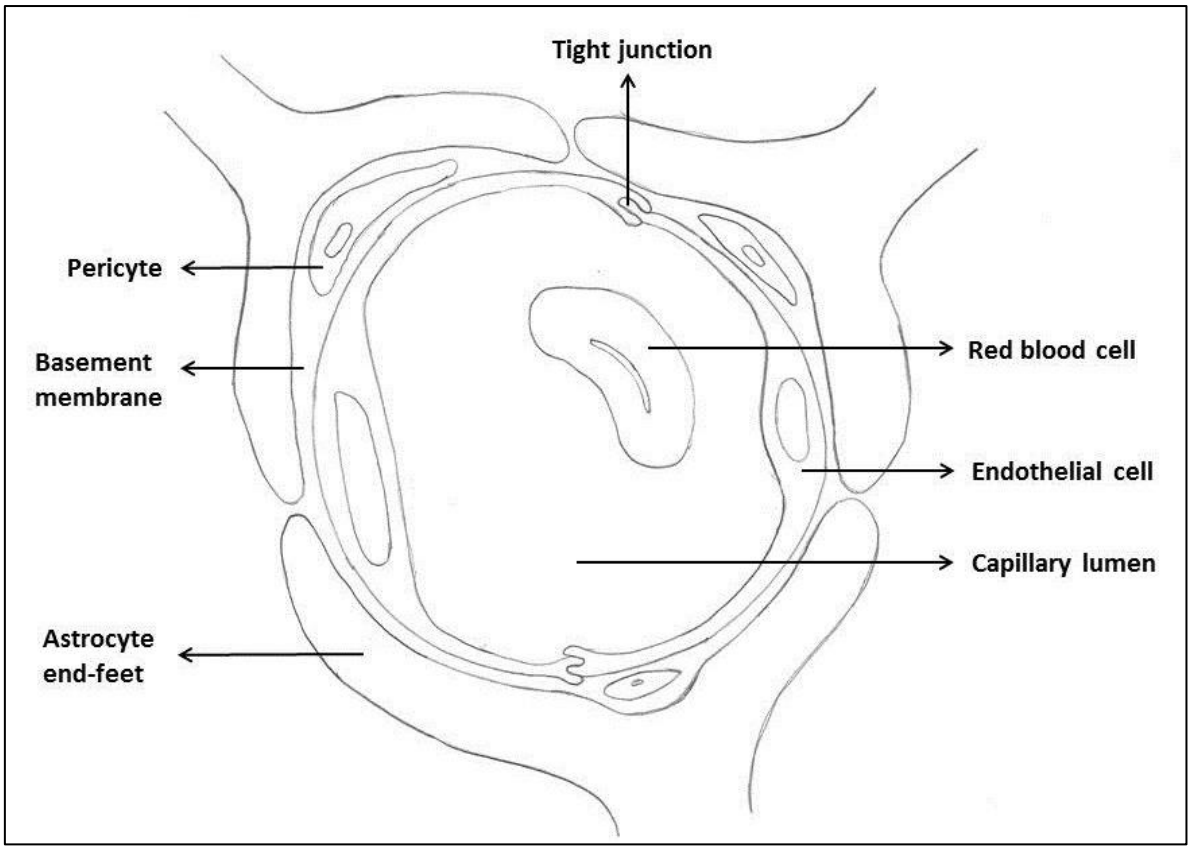

Figure 3. Cellular components of the BBB. Tight junctions, which are one of the molecular components, are also shown.

In 2010, *Armulik et al.* published the characterization of a mouse model with pericyte deficiency. The model was created by knocking out the retention motif of platelets-derived growth factor β gene (*Pdgfb*$^{ret/ret}$). This modification results in failure of the endothelial-secreted PDGFβ to be retained locally so it loses its functionality. Since PDGFβ is important for incorporation of pericytes around endothelial cells, pericytes in this mouse model with disrupted PDGFβ fail to migrate. Therefore these mice have around 70% reduction in pericytes coverage compared to the controls. The result of this dramatic loss of pericytes is a

leaking BBB. Parenteral administration of dyes and other molecular tracers, that cannot normally cross BBB, all ended up in the brain. This study provided the first in-vivo evidence of pericytes' crucial role in BBB function. The authors suggested two mechanisms by which pericytes regulate BBB: by regulating BBB-specific gene expression patterns in endothelial cells and by inducing polarization of astrocyte end-feet surrounding CNS blood vessels (Armulik et al., 2010). This mouse model with pericyte deficiency (*Pdgfb*$^{ret/ret}$) was the main tool used to study the consequences of BBB disruption on cholesterol homeostasis in this thesis.

Astrocytes are the last layer of the CNS microvascular unit. They are a specialized type of glial cells characterized by numerous long processes ending with final parts called *end-feet*. These end-feet surround the capillary vessels and ensheath them. Astrocytes possess a physical barrier in addition to their biochemical role. They also regulate capillary blood flow by sending signals to smooth muscle cells and pericytes. It is believed that astrocytes play an important role in initiating the barrier function of the BBB. This was concluded from studies demonstrating that transfer of astrocytes to non-CNS capillaries induce a barrier function in them. The same results were also obtained in endothelial cells cultured in-vitro with astrocytes (Moody, 2006).

1.7.2.2 Molecular components:

One of the distinctive features that characterize CNS endothelial cells is the tight junctions. They are formed by a group of transmembranous proteins that link adjacent endothelial cells together. This linkage leads to narrowing of the intercellular spaces preventing transcellular transfer of cells and compounds. Examples of these transmembranous proteins include: claudins, occludins and junction adhesive molecules (JAM) (Daneman and Prat, 2015).

Transporters are highly expressed in endothelial cells and are crucial for BBB function. They carry out active transport of many selective substances across BBB with or against their concentration gradients. There are many transporters dedicated to transport nutrients, for example GLUT1 which is responsible for transporting glucose from the vascular lumen. Some transporters are receptor mediated e.g. Ager, which is important in amyloid elimination from brain through the barrier (Moody, 2006). The BBB also contains many efflux

transporters. These transporters use ATP to transport a wide range of substances, mainly xenobiotics, from the brain to the blood against a concentration gradient. This class of transporters, which includes e.g. Mdr1, MRP and BCRP, is a hot research topic in pharmacology. Many drugs designed to treat CNS conditions face the challenge of bypassing those transporters to reach a therapeutic concentration in the CNS. Nevertheless, those receptors provide a necessary protecting barrier function against many toxins and unwanted chemicals (Daneman and Prat, 2015).

1.7.3 BBB and neurodegenerative diseases

BBB dysfunction occurs in a variety of neurological disorders. The compromising of the BBB is secondary to the primary pathology in most of these diseases. Disruption of the barrier occurs mainly due to inflammation. Inflammatory cytokines disrupt junctional proteins and enable inflammatory cells to reach brain tissue. BBB function can also be disrupted by hypoxia which increases its permeability. Hypoxia disrupts tight junctions, increases transcytosis and disorganizes transporters and channel functions. The following ischemia in the affected brain tissue may induce inflammation which in its turn disrupts the barrier function even more (Daneman and Prat, 2015).

Dysfunction of endothelial cells in the brain capillaries may contribute to the development of neurodegenerative diseases. Oxidative stress could be important as it is observed that many protective transporter functions decrease with age. Rage is a receptor-transporter system that can binds to many ligands and activates many pathways. Disturbances in Rage have been linked to many inflammatory and neurodegenerative disorders like Huntington's disease (Serratos et al., 2015).

BBB disruption does not occur in all cases of neurodegeneration. *Erickson* and *Banks* made a meta analysis of a number of published studies that investigate BBB dysfunction in AD by measuring CSF/serum albumin ratio, a marker of BBB disruption. They found that results were not univocal as many studies reported no difference between AD and controls (Erickson and Banks, 2013). Thus other factors than the neurodegeneration itself may be important in BBB dysfunction seen in neurodegenerative diseases.

2 AIMS

2.1 GENERAL AIMS

The general aims of this work is to elucidate the role of the BBB in brain cholesterol homeostasis and to study the flux of cholesterol metabolites and plant sterols across the barrier and in the brain.

2.2 SPECIFIC AIMS

Paper 1 To study the relationship between levels of 7-Hoca in CSF and the status of the BBB

Paper 2 To investigate the mechanism by which 7-Hoca gets accumulated in chronic subdural hematoma

Paper 3 To study cholesterol homeostasis in brain in mouse model with disrupted BBB (*Pdgfb*$^{ret/ret}$)

Paper 4 To study plant sterols flux across the BBB in the *Pdgfb*$^{ret/ret}$ mouse model.

3 MATERIALS AND METHODS

3.1 PAPER I AND PAPER II: STUDIES ON 7-HOCA FLUX IN THE BRAIN

3.1.1 Synthesis of deuterium labeled 7-Hoca (d4-7-Hoca) and unlabeled 7-Hoca

Deuterium labeled 7-Hoca was synthesized to be used as internal standard in assays aimed to quantify 7-Hoca in CSF, CSH and blood. Synthesis started with oxidation of 1mg d7-7α-hydroxycholesterol (Avanti Polar Lipids) with cholesterol oxidase (Cellulomonas species, Sigma C-5421) under the conditions recommended by Sigma. The reaction yielded d7-7α-hydroxy-4-cholesten-3-one which was quantified and used directly without purification in the next reaction. Sixteen μg of d7-7α-Hydroxy-4-cholesten-3-one was further oxidized by a reconstituted human CYP27A1 system consisting of CYP27A1, adrenodoxin, and adrenodoxin reductase (Meaney et al., 2007). The material was extracted by chloroform or diethyl ether and quantified. The yield of this reaction ranged between 30 and 75%.
Unlabeled 7-Hoca was produced in the same way starting with 7α-hydroxy-4-cholesten-3-one and quantitated by spectrophotometry and GC-MS.

3.1.2 Patients

CSF was collected from 54 subjects (25 females and 29 males, age range 23-77 years) and used in the study described in paper I. They were divided into four groups:

3.1.2.1 Headache controls

This group consisted of 8 subjects referred to the neurological clinic because of headache, who were investigated with a CT-scan of the brain, plasma analysis, and a lumbar puncture. All these patients had a normal CSF/serum albumin ratio (<10), and therefore were used as a control group.

3.1.2.2 Alzheimer's disease group

This group consisted of 11 subjects. The patients were diagnosed as having Alzheimer's disease based on the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer´s Disease and Related Disorders Association (NINCDS-ADRDA) criteria (McKhann et al., 1984).

3.1.2.3 Patients with vascular dementia

This group consisted of 13 subjects. The diagnostic criteria used were those of *Erkinjuntti et al.* (Erkinjuntti et al., 2000). Three of the patients had a slightly increased CSF/serum albumin ratio (11–13, normally <10).

3.1.2.4 Patients with BBB dysfunction

This group consisted of 22 patients who had a defective BBB function defined as an increased CSF/serum albumin ratio (14–160, normally <10). Diagnoses in this group included Guillain-Barré's disease (n = 5), meningitis (n = 9), encephalitis (n = 2), polyneuropathy (n = 1), pareses (n = 2), and miscellaneous (n = 3).

Hematoma and blood samples collected from patients operated on to remove CSH were used in the study in paper VI. Twenty sex patients were involved in the study (11 females and 15 males, age range 39-93 years, median 75 years).

3.1.3 In-vitro experiments with CSF

Albumin and artificial hematomas were incubated in CSF in order to mimic the situation in CSH. An albumin solution was made by dissolving bovine albumin (sigma) in normal saline to a final concentration of 40 mg/ml. Two ml of albumin solution were sealed in dialysis membrane (Medicell International Ltd) and incubated in 50 ml of a CSF pool with magnetic stirring at 37C for 48h. Hemolyzed blood was prepared according to the method described by *Moore et al.* by snap-freezing a few milliliters of a blood pool in liquid nitrogen (Moore et al., 2015). Two milliliters of the hemolyzed blood were sealed in a dialysis membrane and incubated in CSF in the same way described for the albumin solution. The pools of CSF and blood were obtained from anonymous donors (waste materials from the routine laboratory).

2.1.4 Albumin determination

Albumin was determined in hematoma, blood and CSF samples using Tina-quant Albumin Gen.2 kit (Roche).

3.1.5 Assay of 7-Hoca

D4-7-Hoca (30-60 ng) dissolved in ethanol was used as internal standard. It was added to 1 ml CSF and 5 ml ether in a separation funnel. After addition of 20 µl of 0.1 M HCl, extraction was performed and the ether phase was collected while the water phase was saved for the subsequent extraction. The extraction was repeated, and the ether phases were pooled and washed with water until neutral. The ether was evaporated under nitrogen. Then the material was converted into methyl ester with TMS -diazomethane (Aldrich) by adding 100 µl of methanol, 400 µl of toluene, and 40 µl of hexane solution containing 2 M of TMS-diazomethane directly to a glass tube. The mixture was vortexed for 10 s and agitated for 5 min. The solvent and reagents were then removed under a stream of nitrogen.

For hematoma and blood samples, internal standard was added to 0.5 ml of the sample in a separation funnel. Extraction was done twice with 4 ml of chloroform. The solvent was evaporated under nitrogen before resuspending in another 1 ml of chloroform. The latter mixture was subjected to solid phase extraction using a NH2 column (Bond Elut, Agilent Technology). A chloroform isopropanol mixture (2:1) was used to elute oxysterols and 2% acetic acid in ether to elute 7-Hoca. The eluted solution was dryed under nitrogen and then methylated in the same way described for CSF.

After methylation, the material was converted into TMS ether. TMS reagent (pyridine/hexamethyldisilazane/chlorotrimethylsilane, 3:2:1, v/v/v) was added to the dried extracts and the sealed tube was treated at 60°C for 30 min. The solvent and reagents were removed under a stream of nitrogen until complete dryness. The residue was then dissolved in 20-70 µl of hexane and transferred to a glass vial, suitable for Gas chromatography–mass spectrometry (GC-MS) injection.

GC-MS was performed on a Hewlett Packard 6890 Plus series gas chromatograph equipped with an HP-5MS capillary column (30 m × 0.25 mm, 0.25 µm phase thickness). The chromatograph was connected to an HP 5963 mass selective detector and to CTC Analytics Pal system automatic injector. The oven temperature program was as follows: 180°C for 1 min, 20°C/min to 250°C, and then 4°C/min to 300°C where the temperature was kept for 8 min. Helium was used as a carrier gas. The gas chromatograph was operated in the constant

flow mode, with the flow rate set to 0.8 ml He/min. The injector was operated in the splitless mode and was kept at 270°C, and the detector transfer line was kept at 280°C. The samples were analyzed with GC-MS using single ion monitoring, following the ion *m/z* 426 (for the derivative of 7-Hoca) and the ion *m/z* 430 (for d4-7-Hoca).

2.1.6 Ethical considerations

With exception of the CSF samples from anonymous patients used in paper II, all other patients gave their informed consent to participate in these studies. All the investigations of the patients and the analyses of their CSF, hematoma and blood were approved by the ethic committees of the respective institutions.

3.2 PAPER III AND PAPER IV: STUDIES ON A MOUSE MODEL WITH DISRUPTED BBB

3.2.1 Animals

The studies were performed with transgenic mice (*Pdgfb*$^{ret/ret}$) lacking the PDGFB retention motif and their littermates as controls (*Pdgfb*$^{ret/+}$). The homozygote mutant mice were shown to have leaking BBB due to great reduction in CNS pericytes coverage. The heterozygote controls are shown to have normal BBB function (Armulik et al., 2010). The mice were housed under standard conditions in an enriched environment with 12 h light/dark cycle. The study was performed on 10 week old male mice. To harvest the tissue, the mice were euthanized by carbon dioxide and blood was collected by heart puncture. The animals were then infused using ringer lactate to flush blood out from the organs. The two halves of the brain, liver, lungs, proximal intestine, testis and adrenals were collected. The tissue samples were immediately snap-frozen in liquid nitrogen and then stored at −80 °C until analysis. Serum was separated from the whole blood and stored at −20 °C.

3.2.2 Diets

Four different types of diets were used in the studies: chow diet, 1% cholesterol diet, chow diet labeled with 0.3% d6-cholesterol and chow diet labeled with 0.3% deuterium labeled phytosterols (55% d6-campesterol and 45% d6-sitosterol).

3.2.3 Lipid extraction and analysis

Lipids were extracted according to the Folch method. Different volumes of Folch solution (chloroform/methanol, 2:1 (v/v)) were added to half of a brain and other different organs according to their weights. After 24 h at room temperature, extracts were transferred to new vials and evaporated under argon. After evaporation, the extracts were redissolved in appropriate volumes of Folch and stored at −20 °C until required.

Specific volumes of the extracts (with or without internal standard) were dried and then hydrolyzed with 1 ml of 1 M NaOH in ethanol at 60 °C for 1 h. Water, 0.5 ml, was added before extraction with 3 ml of cyclohexane. The tubes were vortexed for 1 min and then centrifuged. The organic phase was collected and extraction was repeated again with another 3 ml of cyclohexane. The organic phase was evaporated under nitrogen. The dried residue was silylated by treating with pyridine/hexamethyldisililazane/chlorotrimethylsilane, 3:2:1, v/v/v) at 60 °C for 30 min. After evaporation of the solvent under nitrogen, 70 µl of hexane was added and then transferred into special glass viable for GC–MS analysis.

Sterols and oxysterols were measured by GC-MS using deuterium-labeled internal standards as described previously (Dzeletovic et al., 1995, Acimovic et al., 2009).

Separation and analysis of 24(*R*)- and 24(*S*)-hydroxycholesterols were done in Professor Griffith's laboratory in Swansea, UK. They were analyzed as their Girard P hydrazone derivatives by liquid chromatography-mass spectrometry (LC-MS*n*) as described (Griffiths et al., 2013). The two isomers separate chromatographically on the reversed phase LC column.

3.2.4 RNA Preparation and Real-time PCR

Total RNA was prepared using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RNA (1 µg) was transcribed into cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA). The cDNA obtained was diluted 1:10 in RNase-free water. Real-time PCR was performed with 5 µl of cDNA and 12.5 µl of SYBR Green Mastermix (Applied Biosystems). Forward and reverse primers for 12 genes, including *Hprt* as a housekeeping gene, were used.

3.2.5 Western Blots

Microsomes prepared from brains of the *Pdgfb*$^{ret/ret}$ mice and their controls were subjected to electrophoresis (20–25 μg/lane) on a 10% SDS or BisTris polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were incubated for 2 h at room temperature in blocking buffer (5% milk in phosphate-buffered saline, 0.05% Tween) followed by incubation overnight at 4 °C with an anti-CYP46 antibody (a generous gift from Prof. D. Russell, University of Texas Southwestern Medical Center, Dallas, TX) or an antibody directed toward Abca1 (anti-Abca1 antibody ab 18180 from Abcam). Goat anti-rabbit IgG or goat anti-mouse IgG coupled with horseradish peroxidase (Pierce) was used as a secondary antibody with incubation at room temperature for 2 h. The membranes were incubated in Super Signal West Dura extended duration substrate (product number 34075 (Pierce) according to the manufacturer's instructions. The signal (around 50 kDa for CYP46 and 250 kDa for ABCA1) was determined using Universal hood II equipment (Bio-Rad).

3.2.6 Experiments with Deuterated Water and Calculation of Fractional Synthetic Rate

The mice were exposed to drinking water containing 10% deuterium water for a period of 11 days before sacrifice. In addition to measurements of deuterium content in brain cholesterol, the deuterium content was measured in body fluid (serum) of the mice as described (Shah et al., 2010). From the deuterium enrichment in cholesterol and body water, the fractional synthetic rate could be calculated (Diraison et al., 1996).

3.2.7 Statistics

Gene expression data are expressed as mean ± range as described by *Livak et al.* (Livak and Schmittgen, 2001). Sterol determinations are expressed as means ± S.E. Statistical comparisons were performed using the unpaired Student's *t* test.

3.2.8 Ethical Considerations

All experimental procedures in this study were in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and the European Communities Council Directive of 24 November 1986 (86/609/EEC) and approved by the relevant Research Animal Ethics Committees.

4 RESULTS AND DISCUSSION

The aim of this section is to highlight the most important findings and to discuss their signicance. Detailed results and further discussion are found in the corresponding articles.

4.1 PAPER I AND PAPER II: STUDIES ON 7-HOCA FLUX IN THE BRAIN

4.1.1 Assay:

The assay developed in this project to quantify 7-Hoca is simple and accurate. In previous methods aimed to quantify 7-Hoca in plasma or hematoma (Axelson et al., 1988, Nagata et al., 1992), two chromatographic steps were needed compared to one single step of solid phase extraction in the protocol developed in this study. *Ogundare et al*. still used double chromatographic steps to quantify 7-Hoca in CSF samples (Ogundare et al., 2010) while these steps are completely omitted in this new method because CSF has low fat and protein contents. CSF samples are extracted with ether, methylated and derivatized directly. This simplified assay reduces the cost and time needed to analyze 7-Hoca.

The synthesized internal standard used in the method (d4-7-Hoca) can be described as "the ideal standard". This is because it has the same structure as the target compound with addition of four extra neutrons. The target compound, 7-Hoca, could be detected with the ion m/z 426 while the internal standard, d4-7-Hoca, was detected with m/z 430 with about 0.1 min difference in retention time. Previous internal standards used to quantify 7-Hoca could not provide the high accuracy granted by this assay. 7-Hoca is labile and can readily lose the 7α-hydroxyl group to form 3-oxo-cholesta-4,6-dienoic acid. The latter compound has the same ion as 7-Hoca (m/z 426) and can reach up to 30% of original 7-Hoca. Therefore it represents a disruption factor leading to underestimated results. Another important attribute of d4-7-Hoca is that it deteriorates during analysis at the same rate as 7-Hoca. Thus the values obtained with this internal standard are closer to the true values.

4.1.2 Analyses of CSF from controls and patients:

Figure 4 shows the levels of 7-Hoca in controls, patients with Alzheimer's disease (AD), patients with vascular dementia (VD) and patients with BBB dysfunction (BBB defect) due to different causes. The mean value of the control group was found to be 15±3 ng/ml. *Nagata et al.* reported increased 7-Hoca in CSF following subarachnoid hemorrhage but they could not detect any level in normal CSF (Nagata et al., 1995). Their analytical system based on HPLC supplied with UV detector may be less sensitive than the LC-MS method used by *Ogundare et al.* The latter group was first to report that 7-Hoca is the most abundant cholesterol metabolite in normal CSF (Ogundare et al., 2010). However, the level they reported (7±3 ng/ml) is about half of that found out in this study. This may be explained by that the optimal internal standard used in the current study.

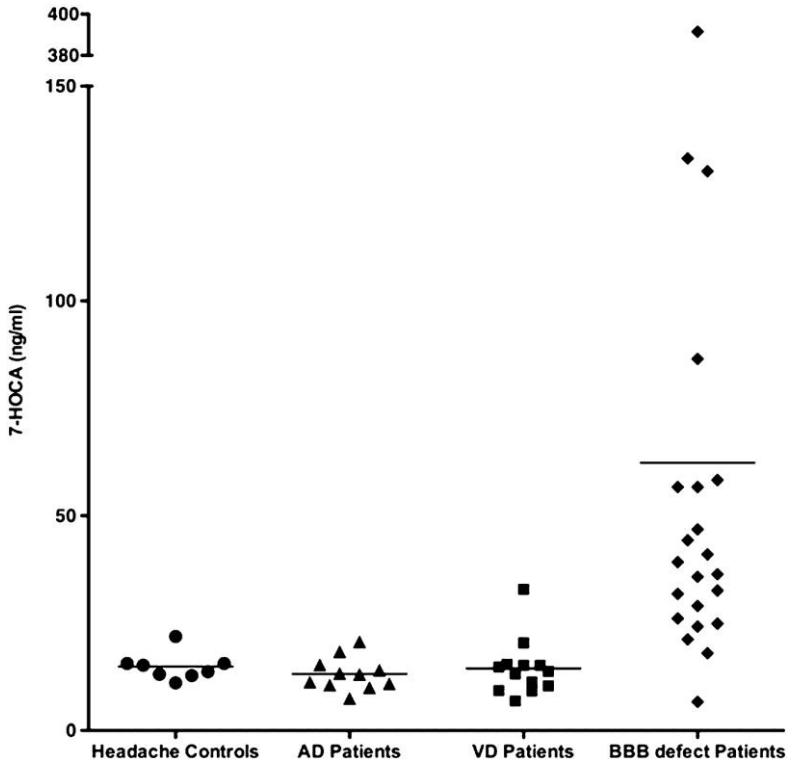

Figure 4. Levels of 7-Hoca in patients with headache (controls) (n = 8), Alzheimer's disease (n = 11), vascular dementia (n = 13), and BBB defects (n = 22).

The AD patients group and VD patients group had similar values (13±4 and 14±7 ng/ml respectively). Both don't differ significantly from the control group. It is worth noting that all subjects in the three groups (with exception of the last group) have a normal CSF/serum

albumin ratio. This is the current parameter used by physicians to evaluate the status of the BBB. Its principle is very simple and direct, albumin present in CSF is derived from plasma so its level there reflects BBB status (Erickson and Banks, 2013). A normal functioning BBB has a CSF/serum albumin ratio up to 10 while it is increased in conditions leading to BBB dysfunction. The last group is a group of patients diagnosed with different neurological illnesses resulting in BBB disruption with high CSF/serum albumin ratio. In this group, almost all 7-Hoca levels in CSF were above the mean of the control group. Some of the patients had extremely high levels of 7-Hoca.

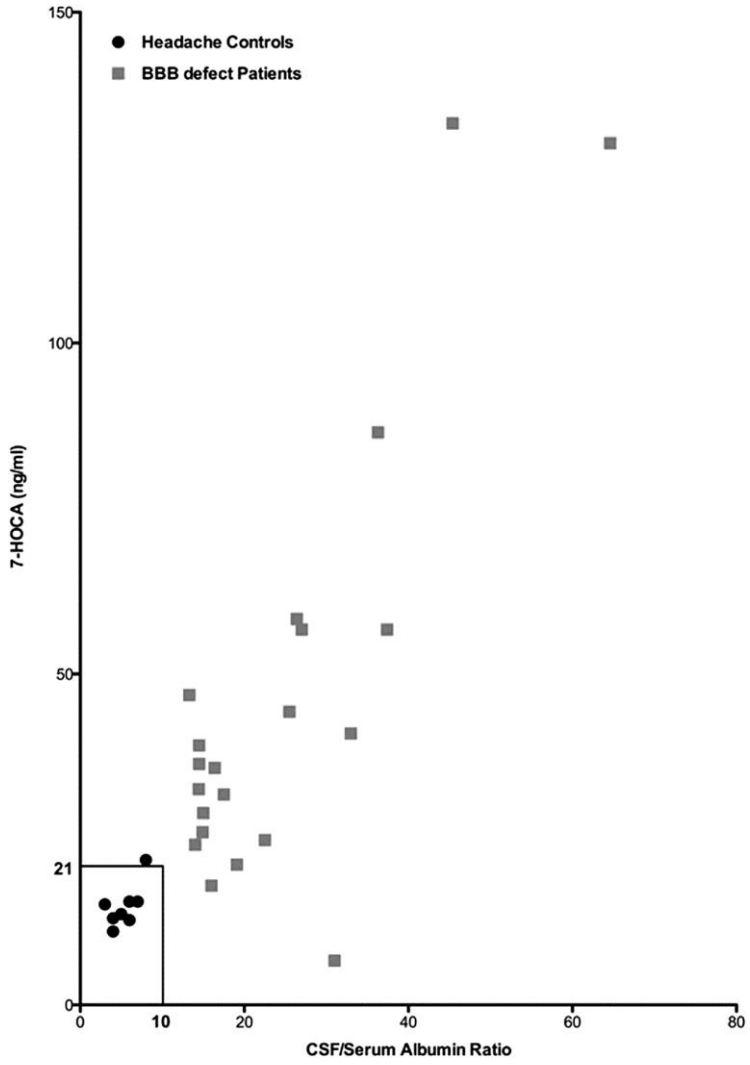

Figure 5. Correlation between CSF/serum albumin ratio and level of 7-Hoca in CSF in control patients and 21 patients with a BBB defect. The patient with the highest level of 7-Hoca and CSF/serum albumin ratio was excluded and the upper normal levels for the CSF/serum albumin ratio (normal 10) and 7-Hoca (normal 21) are indicated.

No particular pattern could be observed with any certain disease. For example, the values of patients with meningitis or encephalitis ranged between 33 to 392 ng/ml while they were from 24 to 47 ng/ml in the case of patients with Guillain-Barré's disease. Nevertheless, a clear positive correlation relationship could be seen between the levels of 7-Hoca in the CSF and CSF/serum albumin ratio in those patients. Figure 5 shows this linear relationship between these two factors in controls and BBB defect groups. The figure also shows the proposed window for normal values. Twenty one ng/ml was set as the upper limit for normal value as it is equal to mean + 2SD.

27-OHC is produced in the majority of cell types and it is the most abundant oxysterol in plasma. Its level is however very low in brain because of its rapid metabolism to 7-Hoca (Meaney et al., 2007). This process requires a set of 3 enzymes among them CYP7B1 which is located in neuronal cells. It has been shown that more 27-OHC enters the brain under conditions where the BBB is disrupted. This can be expected to lead to increased metabolism to produce 7-Hoca that flows back to the circulation and to the CSF. This suggested mechanism may explain the high levels of this steroid acid in CSF. On the other hand, normal levels of 7-Hoca in AD patients and VD patients could be explained by the loss of neuronal cells expressing the required enzymes, especially CYP7B1. This represents a logical explanation since the BBB may be affected in these conditions (Daneman and Prat, 2015). Figure 6 summarizes the synthesis and flux of 7-Hoca.

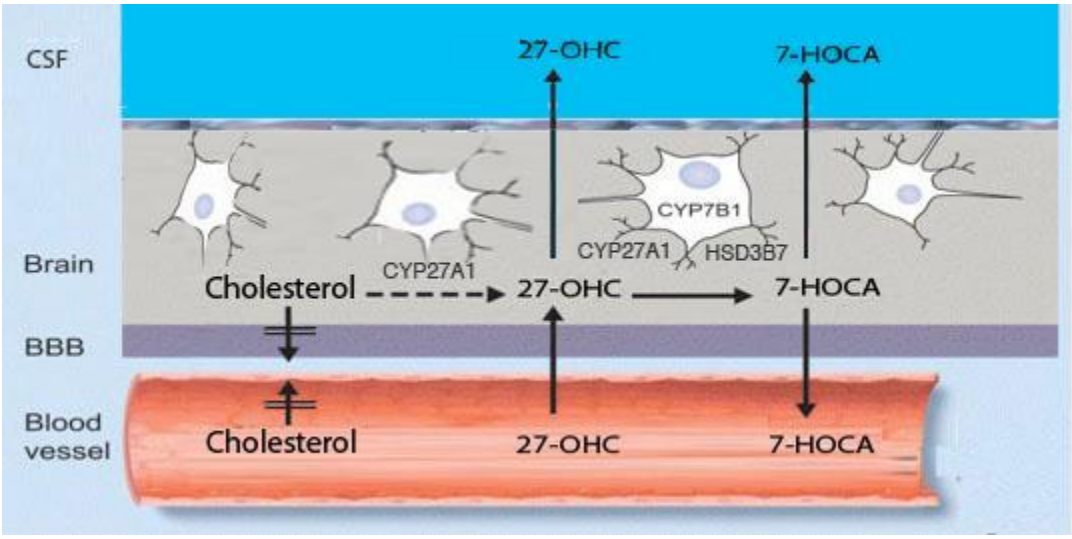

Figure 6. Flux of 27-OHC from the circulation into the brain and from the brain into CSF and flux of 7-HOCA from the brain into the circulation and CSF.

4.1.3 7-Hoca in hematoma and blood from patients with CSH

Table 1 shows 7-Hoca and albumin levels beside 7-Hoca/albumin ratios in hematoma and blood samples. These results confirm previous reports (Nagata et al., 1992, Nagata et al., 1993) that the levels of 7-Hoca are two to several fold higher in subdural hematomas than in peripheral blood. Albumin levels were however not measured in the studies by *Nagata et al.*

	Hematoma			Peripheral blood		
Patient no.	7-Hoca (ng/ml)	Albumin (mg/ml)	7-Hoca/Albumin (ng/mg)	7-Hoca (ng/ml)	Albumin (mg/ml)	7-Hoca/Albumin (ng/mg)
Patient 1	**181**	**20,6**	**8,8**	**27**	**18,0**	**1,5**
Patient 2	**93**	**18,0**	**5,2**	**23**	**20,5**	**1,1**
Patient 3	**196**	**21,4**	**9,2**	**22**	**21,2**	**1,0**
Patient 4	**69**	**10,1**	**6,8**	**32**	**19,4**	**1,6**
Patient 5	**235**	**23,1**	**10,2**	**72**	**20,4**	**3,5**
Patient 6	**143**	**27,6**	**5,2**	**23**	**23,8**	**1,0**
Patient 7	**84**	**27,9**	**3,0**	**16**	**18,0**	**0,9**
Patient 8	**104**	**33,9**	**3,1**	**21**	**22,1**	**1,0**
Patient 9	**87**	**16,8**	**5,2**	**17**	**17,7**	**1,0**
Patient 10	**244**	**30,7**	**7,9**	**15**	**17,7**	**0,9**
Patient 11	**136**	**21,7**	**6,3**	**19**	**22,2**	**0,9**
Patient 12	**129**	**29,4**	**4,4**	**35**	**18,0**	**1,9**
Patient 13	**259**	**20,5**	**12,7**	**23**	**20,5**	**1,1**
Patient 14	**173**	**25,3**	**6,8**	**23**	**14,6**	**1,6**
Patient 15	**372**	**30,7**	**12,1**	**19**	**11,9**	**1,6**
Patient 16	**122**	**16,9**	**7,2**	**18**	**18,4**	**1,0**
Patient 17	**123**	**25,1**	**4,9**	**31**	**16,2**	**1,9**
Patient 18	**98**	**9,1**	**10,7**	**18**	**15,2**	**1,2**
Patient 19	**117**	**20,4**	**5,7**	**24**	**18,7**	**1,3**
Patient 20	**166**	**29,3**	**5,7**	**22**	**16,5**	**1,3**
Mean	**157**	**23**	**7,1**	**25**	**18,6**	**1,4**

Table 1. Levels of 7-Hoca, albumin and 7-Hoca/albumin ratio obtained from analysis of hematoma and peripheral blood samples collected from 20 patients with CSH.

Electrophoresis analysis of hematoma samples revealed that albumin and hemoglobin are the dominant proteins. Extraction of and anlysis of the albumin band showed that about 90% of 7-Hoca is located there. The structure of 7-Hoca is similar to bile acids and therefore it is logical to assume that it should be bound to albumin in the hematomas. As shown in the tables the ratio between 7-Hoca and albumin was 5 times higher in the hematomas than in the blood of these 20 patients.

Significant amounts of 3-oxo-cholesta-4,6-dienoic acid, which is a degradation product of 7-Hoca, were detected in hematoma samples. This indicates that the original levels of 7-Hoca are higher than the values obtained. It is unlikely that the decomposition to 3-oxo-cholesta-4,6-dienoic acid took place during analysis because the internal standard showed no decomposition. Approximate levels of 3-oxo-cholesta-4,6-dienoic acid were measured using the same standard curve of 7-Hoca with correction. Its levels show a high degree of positive correlation with 7-Hoca levels. Interestingly, this compound was totally absent in the blood samples obtained from the same patients. This may reflect that the presence of favoring conditions for decomposition of 7-Hoca to 3-oxo-cholesta-4,6-dienoic acid in hematoma but not in peripheral blood.

4.1.4 Incubation of albumin and artificial hematoma in CSF

In-vitro experiments were performed to elucidate the role of albumin in 7-Hoca accumulation in CSH. The first experiment comprised incubation of pure albumin solution in a dialysis bag exposed to CSF. Results are shown in table 2.

	7-Hoca (ng/ml)
Albumin solution	**0**
Incubated Albumin	**18**
CSF pool	**9**
CSF remnant	**8**

Table 2. Levels of 7-Hoca obtained from the experiment of incubation of albumin solution in CSF.

Data shows that after 48h of incubation, the concentration of 7-Hoca in the albumin solution increased from 0 ng/ml to 18 ng/ml. The latter figure is double the concentration of 7-Hoca in the CSF pool at the beginning of the experiment. This indicates that the transfer of 7-Hoca from CSF into the albumin solution is not a mere result of equilibration but a true concentration process.

In the other in-vitro experiment, albumin was replaced by hemolyzed blood to simulate hematoma. Results, which are displayed in table 3, show a 27% increase in the concentration of 7-Hoca in the hemolyzed bold after 48h incubation. There was, however, a 110% increase in the volume of the incubated blood due to difference in osmotic pressure. The total amount of 7-Hoca, corrected for the volume, increased almost 3-fold. The total albumin had not changed however which also creates a 3-fold difference in 7-Hoca/albumin ratio between hemolyzed and incubated blood. There was a corresponding decrease in the amount of 7-Hoca in the CSF-pool explained by migration of this compound to the artificial hematoma. It is noteworthy that the ratio 7-Hoca/albumin in the incubated hemolyzed blood was close to that of hematomas from many of the patients.

	Volume	7-Hoca	Total 7-Hoca	Albumin	Total albumin	7-Hoca/Albumin
	(ml)	(ng/ml)	(ng)	(mg/ml)	(mg)	(ng/mg)
Hemolyzed blood	**2**	**81**	**163**	**19,7**	**39**	**4,1**
Incubated blood	**4,2**	**111**	**467**	**9,7**	**41**	**11,5**
CSF pool	**50**	**10**	**481**	**0,3**	**17**	**28,0**
CSF remnant	**38**	**8**	**310**	**0,4**	**20**	**19,9**

Table 3. Results of the experiment of incubation of artificial hematoma in CSF. Volumes are measured at the beginning of the experiment for hemolyzed blood and CSF pool and at the end of the experiment for the incubated blood and CSF remnant. Total amounts of 7-Hoca and albumin are calculated by multiplying the concentration by the volume. 7-Hoca/Albumin ratio is calculated by dividing 7-Hoca concentration by albumin concentration CSF.

Under the in-vitro conditions used in the experiment shown in Table 3, volume changes occurred due to the osmotic conditions. Under in-vivo conditions such volume changes are likely to be compensated for. Such compensatory mechanisms are however unlikely to change the ratio between 7-Hoca and albumin.

Meaney et al. showed that there is a net efflux of 7-Hoca from the brain into the circulation of about 2 mg/24h. (Meaney et al., 2007). Since the levels of 7-Hoca are 5-10-fold higher in the circulation than in the CSF this flux occurs against a concentration gradient. We suggest that this flux can be explained by the difference in the 7-Hoca/albumin ratio which is at least 7 times higher in CSF than in blood (Table 3). Thus the high affinity of 7-Hoca to albumin may drive the compound to flux towards compartments with a lower 7-Hoca/albumin ratio.

The current data also provides an alternative mechanism for the elevated levels of 7-Hoca in patients with a disrupted BBB found in the first study. BBB defects lead to increased flux of albumin from circulation to the brain, evident by increased CSF/serum albumin ratio. The presence of more albumin in the CSF will increase the flux of 7-Hoca from the brain to the CSF to bind to albumin. It is difficult to evaluate, based on the current studies, if the accumulation of 7-Hoca in CSF of these patients is a result of increased flux of either 27-OH or albumin. Nevertheless, both explanations are valid and can reasonably explain the situation.

4.2 PAPER III AND PAPER IV: STUDIES ON A MOUSE MODEL WITH DISRUPTED BBB (*Pdgfb*$^{ret/ret}$)

4.2.1 Evidence for influx of cholesterol from the circulation into the brain across disrupted BBB

Mutant (*Pdgfb*$^{ret/ret}$) and control (*Pdgfb*$^{ret/+}$) mice were treated with a diet containing 0.3% d6-cholesterol for different periods. After 20 days of feeding, the enrichment of this labeled cholesterol in serum was 45% in both mutant and control mice. It means that 45% of the total cholesterol pool in serum was replaced by d6-cholesterol. The enrichment in liver, lung and adrenal was about the same. Brain however showed extremely little enrichment, 0.7% in the control and 2.6% in the mutant mice. Figure 7 shows the enrichment of brain cholesterol with

d6-cholesterol after 10, 20 and 40 days of feeding. In control mice with an intact BBB the enrichment increased from 0.5% after 10 days to 0.7% after 20 days. It is well established that plasma cholesterol cannot cross an intact BBB (Dietschy and Turley, 2001, Bjorkhem and Meaney, 2004). Therefore it is plausible to assume that this tiny fraction of d6-cholesterol that was recovered from brain of controls is a contamination from blood. This assumption is supported by the tiny and insignificant increase in the enrichment (from 0.6% to 0.7%). On the other hand, enrichment of d6-cholesterol in mutant brain increased by more than 40% (from 1.5% to 2.6%) in the same period.

Longer feeding time led to more incorporation of this labeled cholesterol from plasma to brain across the defective BBB. After 40 days of feeding 7.3% of total brain cholesterol in the mutant was d6-cholesterol i.e. derived from plasma cholesterol. The enrichment with the labeled cholesterol in serum after 40 days of feeding was about 70%. When the enrichment in brain was corrected to that of serum, it could be calculated that 10% of the cholesterol pool in the brain was derived from serum in the mutant mouse.

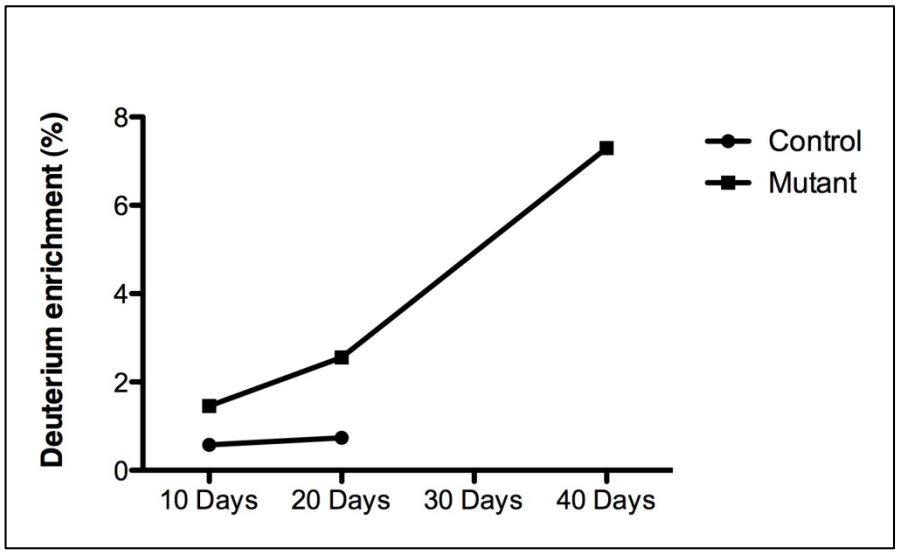

Figure 7. Enrichment of d6-cholesterol in cholesterol isolated from the brains of three mutants and three control mice treated with 0.3% d6-cholesterol for 10, 20, or 40 days, respectively.

Enrichment of different parts of the brain with labeled cholesterol after 40 days of feeding was measured. The highest enrichment was observed in cortex (9.4%) while the lowest one was seen in cerebellum (3.7%). The pituitary has an enrichment of 47% which is closer to the other organs than to the brain. This however is easily explained by the fact that the pituitary lacks BBB.

4.2.2 Evidence for increased 24-OHC leaking from the brain to the circulation through a disrupted BBB

Figure 8 shows the levels of cholesterol, 24-OHC and 27-OHC in the brains of mutants and controls. No differences were seen in cholesterol and 27-OHC levels. Levels of 24-OHC however were significantly reduced in the mutants (about 20% reduction). The results remained the same after correlation to protein or cholesterol instead of weight. There are two possible explanations, it could be attributed to decreased production or increased elimination.

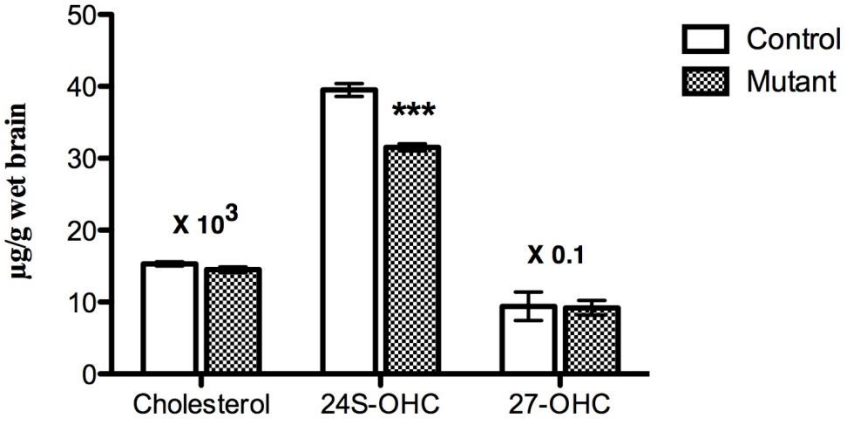

Figure 8. Levels of cholesterol, 24-OHC and 27-OHC in the brain of the *Pdgfb*$^{ret/ret}$ mice and the controls (n=6)

To investigate the first possibility, gene expression of Cyp46, the enzyme that catalyzes production of 24-OHC from cholesterol, was estimated by real time PCR. Protein expression of the enzyme was also assessed by western blotting. Data obtained from these experiments showed that there was no difference between controls and mutants neither in mRNA nor protein levels which indicates that there is no difference in the capacity to produce this

oxysterol. The other possibility of increased elimination of this oxysterol from brain to circulation appeared more plausible. It was challenged however with the results that showed no significant differences in levels of 24-OHC between mutant and controls in serum. Unlike 24-OHC, cholesterol and 27-OHC were significantly lower in mutants' sera. Searching for the lost 24-OHC brought differences between man and mouse into consideration. In man, all 24-hydroxycholesterol is the 24S- steroisomer which exclusively originates from the brain. In mouse however, there is some sterol 24-hydroxlase activity in liver that generates 24-hydroxycholesterol. This 24-hydroxycholesterol originating from liver is the 24R-isomer rather than the 24S- isomer produced in brain. The GC-MS method used here to analyze levels of 24-OHC cannot differentiate between 24S- and 24R- steroisomer. They could however be analyzed as their Gerard P hydrozone derivative using LC-MS. Results showed significantly higher levels of 24S-hydroxycholesterol in the circulation of the mutants than in the controls. This piece of data provides the required evidence to state that 24-OHC leaks increasingly in mutant mice from brain to circulation across the defective BBB.

4.2.3 Evidence for increased cholesterol synthesis in brain of the mutants

Cholesterol precursors are a group of sterols that are intermediates in the long process of cholesterol synthesis. Their levels reflect the rate of cholesterol synthesis. Analyses of 6 of these sterols revealed that 4 of them were increased in the brain of mutant mice which is consistent with increased cholesterol synthesis in this organ. Moreover, relative gene expression of HMG CoA synthase, an important enzyme in cholesterol synthesis, was significantly higher in the brain of the mutant mice. Relative expression of HMG CoA reductase that catalyzes the rate limiting step of cholesterol synthesis was similar in both controls and mutants. This however did not affect the conclusion as additional evidence came from a feeding experiment with 1% cholesterol diet. After 10 days on this diet, both cholesterol precursors and mRNA of HMG CoA synthase were normalized and the difference between controls and mutant mice had disappeared. The higher cholesterol diet would be expected to lead to higher cholesterol in liver and serum. Therefore more cholesterol will find its way from serum to brain through the broken barrier in the mutant mice. This cholesterol will possess an inhibitory effect on cholesterol synthesis and bring it back to control levels.

The strongest evidence of an increased rate of cholesterol synthesis in *Pdgfb*$^{ret/ret}$ mice came from the deuterium water (D_2O) experiment. In-vivo cholesterol fractional synthetic rate

(FSR) was measured in mice that were allowed to drink D_2O over 11 days. Data showed that the calculated FSR was 0.29 ± 0.03% in controls and 0.47 ± 0.05% in mutants ($p < 0.05$, student's *t* test). This means that cholesterol synthesis in mutant brain was 62% higher that in controls.

Ali et al. suggested a theoretical model for the regulation of cholesterol synthesis in the brain (Ali et al., 2013). The model was based on studies done on mice with overexpression or lacking one of the genes: CYP46A1, CYP27A1 or CYP7B1. They suggested that both 24-OHC and 27-OHC have an inhibitory effect on cholesterol synthesis in-vivo. The results obtained in the current study support their model. The most logical reason for the increased cholesterol synthesis in mutant brain is thus the loss of 24-OHC through the disrupted BBB. This oxysterol has been shown to be a potent inhibitor for cholesterol synthesis so its loss would be expected to have increase the syntheis. The effect of low 24-OHC in mutant mice brain is thus dominating over the inhibitory effect of cholesterol and 27-OHC entering from plasma. Figure 9 summarizes brain cholesterol homeostasis with intact and defective BBB.

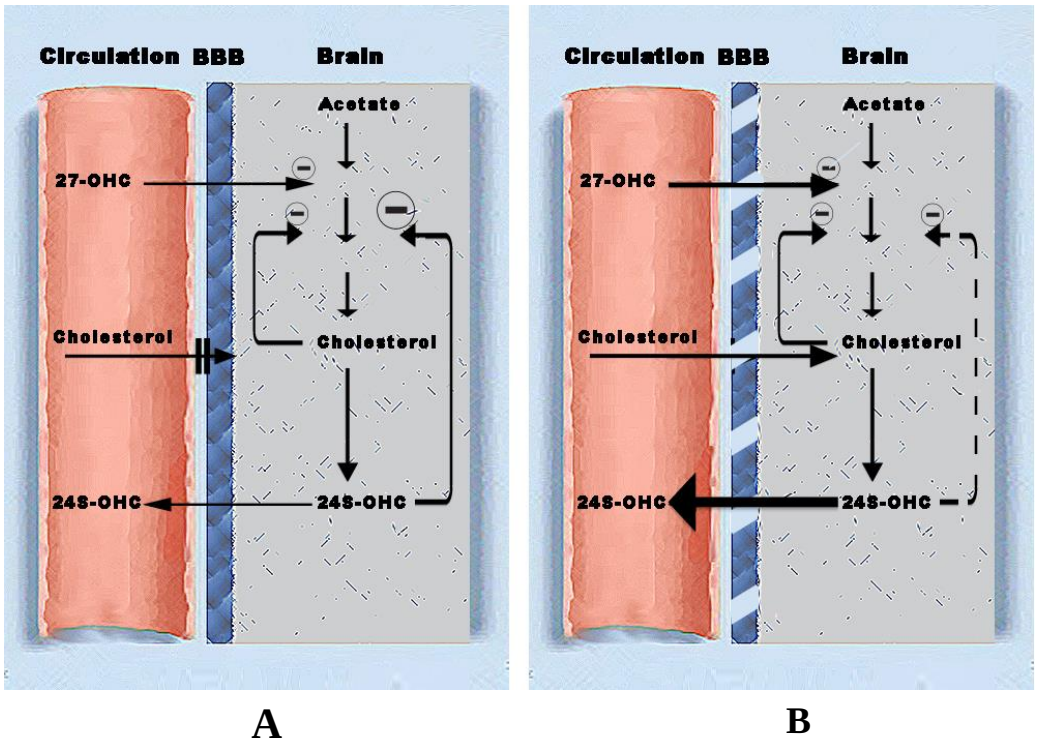

Figure 9. Theoretical model for the mechanism of regulation of cholesterol homeostasis in the brain with intact (A) and disrupted (B) BBB.

The increased rate of cholesterol synthesis with BBB disruption brings into question its consequences on memory function. It was shown that old mice overexpressing CYP46A1 showed improved memory (Maioli et al., 2013) while mice lacking the enzyme show the reverse (Kotti et al., 2006). This effect on the memory can be explained, to some extent, by the rate of cholesterol synthesis in both animal models. CYP46A1 overexpressing mice have a higher rate of cholesterol synthesis in brain while Cyp46A1 knocked out mice have 40% reduction of this rate. The rate of cholesterol synthesis is regarded to be important for memory as it leads to the generation of geranylgeraniol. This compound is a nonsterol isoprenoid that is important for synaptic formation and signal transduction. Addition of geranylgeraniol was shown to restore the impaired long term potentiation to normal levels in hippocampal slides from Cyp46a1 knock out mice, reflecting its necessity for memory and learning function. One could speculate that impaired BBB leads to increased rate of cholesterol synthesis with possible positive effects on memory function. Such a possibility should be investigated in the future.

The relevance of this study on this animal model (*Pdgfb*$^{ret/ret}$) to the human situation is difficult to evaluate. Autosomal dominant defects in the PDGFB gene cause a disease called Primary familial brain calcification (Paucar et al., 2016). The disease is rare, affecting only a few number of families worldwide and is characterized by progressive calcification in brain and some other neurological symptoms. *Pdgfb*$^{ret/ret}$ mice were also found to have similar pathology of brain calcification (Keller et al., 2013). Unlike the situation in the mice, we could not find evidence for signs of BBB disruption in patients with primary familial brain calcification (Paucar et al., 2016).

4.2.4 Evidence for increased plant sterols accumulation in the brain of the mutants

Plant sterols are the cholesterol analogues of the plant kingdom. They cannot be synthesized by mammalian cells. Therefore all plant sterols found in animal tissue must ultimately originate from a dietary plant source. Phytosterols are absorbed from the gastrointestinal tract, reach blood and are distributed among tissues. Unlike cholesterol, they have the ability to cross the BBB to some extent. Accumulation of these phytosterols inside the brain occurs throughout animal life. We measured campesterol and sitosterol levels in the brain of control

and mutant mice. Data revealed that mutant mice with a defective BBB had significantly higher levels of these sterols in brain. The levels of campesterol in particular were more than double than that found in the control mice. It is concluded that the defective barrier leads to higher plant sterols influx from circulation into brain.

To study the subject in more detail, deuterium labeled phytosterols were synthesized by our collaborator using a newly developed method. The method is relatively simple with an overall yield of 35%. Synthesized d6-phytosterol were mixed with chow diet and fed to animals for 10, 20 and 40 days. Figure 10 shows the enrichment of different organs with d6-campesterol and d6-sitosterol after 40 days of feeding. With exception of the brain, all tissues had enrichment similar to that of plasma. These levels were approximately 80% for d6-campesterol and approximately 60% for d6-sitosterol. No difference was seen between control and mutant mice regarding enrichment in the extra cerebral organs.

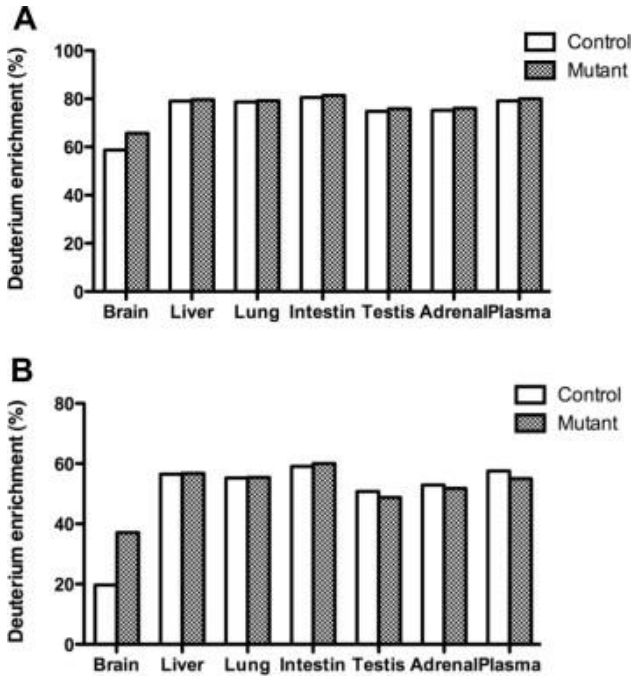

Figure 10. Enrichment of d6-campesterol in campesterol (A) and d6-sitosterol in sitosterol (B) isolated from plasma and different organs in a control mouse and a mutant mouse fed with diet containing 0.3% deuterium-labeled phytosterols for 40 days.

The enrichment of labeled plant sterols in the brain however, differed significantly between controls and mutants. This difference was most prominent with sitosterol with a double enrichment of d6-sitosterol as compared with the control. Enrichment with labeled sitosterol remained however lower in both control and mutant compared to campesterol. After 40 days of feeding on labeled diet, enrichment with d6-campesterol reached 58% in the control and 64% in the mutant. The narrow difference could be explained by the fact that the initial amount of campesterol in mutant brain was two-fold higher than that of control's.

Brain enrichment with d6-phytosterols had the same pattern at all three selected time points (10, 20 and 40 days). At all points in time, mutants had always higher enrichment than controls. Both mouse models had always higher enrichment of labeled campesterol than sitosterol.

To get more information about accumulation of these labeled sterols in brain, the absolute levels of d6-campsterol and d6-sitosterol were quantified. This was done by measuring the unlabeled fraction of both plant sterols and using the calculated ratio to find the concentration of the labeled fraction. Figure 11 shows the absolute concentration of d6-phytosterols in the mutant and control mice. It was observed that the control brain had 10-fold more deuterium labeled campesterol than sitosterol. The difference in mutant mice was found to be even greater. This difference reflects the situation in plasma, where there is about 10-fold difference between the two phytosterols. Levels in the plasma in their turn reflect the absorption magnitude of the sterols where campesterol is more efficiently absorbed than sitosterol.

Accumulation of d6-campesterol and d6-sitosterol during the days of feeding appeared to be linear in the controls, i.e. the longer the exposure the greater the accumulation. In the mutant nonetheless a levelling-off effect took place after 20 days. The levels of both d6-campesterol and d6-sitosterol at 20 and 40 days were thus almost equal. This finding points toward a saturation effect or a possible eliminating mechanism allowing plant sterol to be removed through the defective BBB back to circulation.

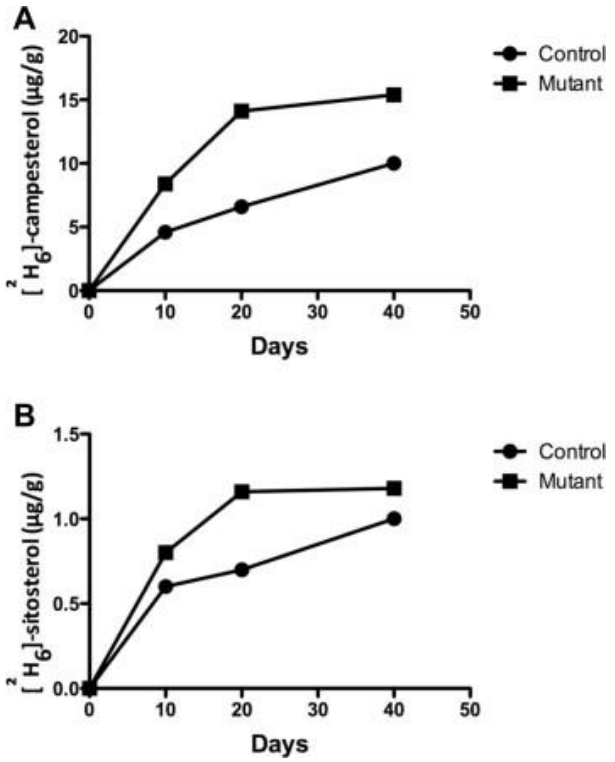

Figure 11. Absolute levels of d6-campesterol (A) and d6-sitosterol (B) in the brain of three *Pdgfb*$^{ret/ret}$ mice and three controls fed with the d6-phytosterol labeled diet for 10, 20 or 40 days, respectively.

To investigate this possibility, another experiment was set up in which a control mouse and a mutant mouse were put on the labeled diet for 80 days. It is anticipated that if such effect really existed in the mutant mouse, they would end up having lower levels of labeled phytosterols than the controls. This was not the case however. The mutant mouse had 5-fold higher d6-campesterol and 4-fold more d6-sitosterol than the control mouse. This confirms the continuous accumulation of plant sterols through BBB and that the defective barrier increases the magnitude of this accumulation. This experiment also highlights the risk of doing experiments with only one individual at each time point. Our main justification to carry out such an experiment with a few animals was the very high cost of the deuterium labeled materials.

5 CONCLUSIONS

Paper I Levels of 7-Hoca in the CSF can be used as a marker for BBB integrity. Higher levels of this cholestenoic acid indicate disruption of the BBB. One explanation for this is an increased flux of 27-OHC that fluxes from the blood through the disrupted BBB. This oxysterol is metabolized into 7-Hoca in the brain which then reaches the CSF. Another explanation is that a primary increase in the flux of albumin across the disrupted BBB leads to increased levels of albumin in CSF with increased binding of 7-Hoca. The strong relation between 7-Hoca and albumin is noteworthy.

Paper II Analysis of hematoma samples obtained from 20 patients with CSH confirmed the previously reported finding of presence of high levels of 7-Hoca. Albumin was found to be the dominant protein in hematoma similar to the situation in the peripheral blood. In-vitro experiments showed that albumin, as pure solution or in hemolyzed blood, attracts 7-Hoca from CSF surroundings. The ratio of 7-Hoca to albumin (7-Hoca/albumin ratio) was regarded to be the decisive factor controlling flux of 7-Hoca. The difference in this ratio between plasma and CSF may explain the accumulation of 7-Hoca in CSH and the normal continuous flux of 7-Hoca from brain to the circulation against a concentration gradient.

Paper III Studies on *Pdgfb*$^{ret/ret}$, a mouse model with disrupted BBB, revealed that plasma cholesterol can pass through defective BBB to reach brain. On the other hand, more 24-OHC leaks out from the brain to the circulation. Since 24-OHC is potent inhibitor for cholesterol synthesis, its loss through the disrupted barrier leads to increased cholesterol synthesis. Defects in BBB also leads to more accumulation of plant sterols in the brain.

Paper IV Higher fluxes of both campesterol and sitosterol occur with disruption of BBB. Campesterol crosses intact and disrupted barrier more efficiently that sitosterol. The accumulation of these two plant sterols is linear and time dependent.

A summery of the major findings is illustrated in Figure 12.

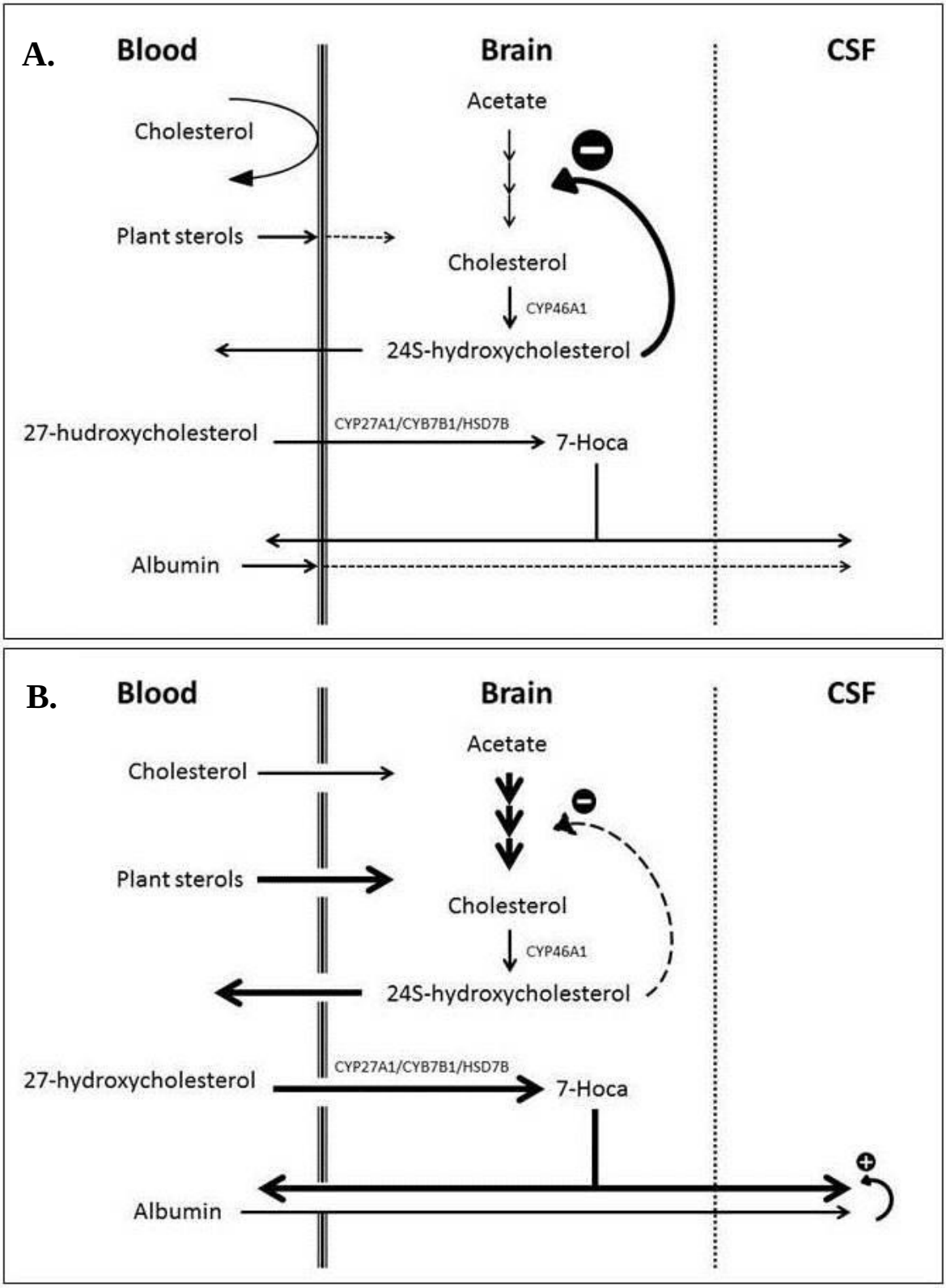

Figure 12. Flux of cholesterol, cholesterol metabolites and plant sterols across intact (A) and disrupted (B) BBB.

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