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On the possible use of oxysterols for the diagnosis and evaluation of patients with neurological and neurodegenerative diseases

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“[illegible]”
“Whom are looking for gold should dig so much and find so little”

Eraclitos

“Philosophy is knowledge in its totality.
For science the effective divorce from totality means death”.

Karl Jaspers

“The fruit of healing grows on the tree of knowledge”.

Rudolf Virchow

To Jolanda

climbing together the
mountains of our life

ABSTRACT

Oxysterols are monooxygenated derivatives of cholesterol with a unique ability to pass lipophilic membranes and are involved in a number of elimination pathways for cholesterol.
One of the major oxysterols in the circulation, 24S-hydroxycholesterol, is formed exclusively in the brain, mainly in neuronal cells. There is a continuous flux of this oxysterol from the brain over the blood-brain barrier (BBB) into the circulation, and the levels of 24S-hydroxycholesterol may thus reflect cholesterol homeostasis in the brain. Another major oxysterol in the circulation, 27-hydroxycholesterol is formed by most cells in the body. The levels of this oxysterol are relatively low in the brain, and the possibility must be considered that part of it may originate from the circulation.

In the present thesis the following hypotheses were tested: 1) Patients with multiple sclerosis (MS) have increased flux of 24S-hydroxycholesterol during active periods of the disease and may then have increased plasma levels of this oxysterol; 2) the plasma levels of 24S-hydroxycholesterol in circulation are correlated with the degree of brain atrophy; 3) Patients with neuronal damage and/or demyelination have an increased flux of 24S-hydroxycholesterol from the brain into the cerebrospinal fluid (CSF); 4) A defect BBB causes increased flux of 27-hydroxycholesterol from the circulation into the CSF; 5) Patients with MS may have increased CSF-levels of 7-oxocholesterol, an oxysterol formed from cholesterol as a consequence of lipid peroxidation and oxidative stress.

Isotope dilution – mass spectrometry was used to assay the different oxysterols in CSF and plasma from more than 300 patients with different neurological and geriatric diseases. The amount of the disease burden and the degree of brain atrophy was evaluated in some of the MS patients by Magnetic Resonance Imaging (MRI).
The 95% confidence interval for 24S- and 27-hydroxycholesterol in CSF from a normal population was found to be 0.4-2.5 μg/L and 0.2-1 μg/L, respectively.

In the course of the investigations it was found that: 1) plasma levels of 24S-hydroxycholesterol are significantly decreased in MS patients with advanced disease, and are likely to reflect the degree of brain atrophy; 2) neuronal damage or demyelination are associated with increased levels of 24S-hydroxycholesterol in CSF; 3) most of the 27-hydroxycholesterol in CSF originates from the circulation and a compromised BBB is associated with increased CSF levels of this oxysterol; 4) patients with MS have slightly increased levels of 7-oxocholesterol in CSF, possibly reflecting an increased lipid peroxidation.

The high proportion of the neurological patients with increased CSF levels of one or both of the two side-chain oxidized oxysterols is consistent with the contention that a normal level of these two oxysterols has a high negative predictive value.

In conclusion, the determination of both plasma and CSF oxysterol levels may offer important information for a better understanding of the different processes active in central nervous system diseases.

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CONTENTS

LIST OF ABBREVIATIONS

24OHC	24S-hydroxycholesterol
24OHC/chol ratio	24S-hydroxycholesterol:cholesterol ratio
27OHC	27-hydroxycholesterol
7□OHC	7□-hydroxycholesterol
7□OHC	7□-hydroxycholesterol
7oxo	7-oxocholesterol
A□	Amyloid beta
ABAP	2,2-Azobis (2-Amidinopropane) hydrochloride
ABC-transporter	ATP Binding Cassette-transporter
ACAT	acylCoA:cholesterol acyltransferase
AD	Alzheimer's disease
ADP	Adenosine diphosphate
ApoA	Apolipoprotein A
ApoA1	Apolipoprotein A1
ApoB	Apolipoprotein B
ApoC	Apolipoprotein C
ApoE	Apolipoprotein A
APP	Amyloid Precursor Protein
ATP	Adenosine triphosphate
BBB	Blood brain barrier
BPF	Brain Parenchymal Fraction
CD11a	Cluster of definition 11a
CETP	Cholesterol Ester Transfer Protein
CNS	Central Nervous System
CoA	Coenzyme A
CSF	Cerebrospinal fluid
$CuSO_4$	Copper Sulphate
CYP	Cytocrome P-450
CYP27A1	Sterol 27-hydroxylase
CYP46A1	Cholesterol 24-hydroxylase
CYP7A1	Cholesterol 7□-hydroxylases
DNA	Deoxyribonucleic acid
EDSS	Kurtzke Expanded Disability Status Scale
ELISA	Enzyme Linked Immuno-Sorbent Assay
GC	Gas chromatography
GC-MS	Gas chromatography mass spectrometry
GMF	Gray Matter Fraction
HDL	High Density Lipoproteins
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HPLC	High pressure liquid chromatography
IGF-I	Insulin-like Growth Factor I
IgG	Immunoglobulin G
IL-1	Interleukin 1
IL-13	Interleukin-13

IL-4	Interleukin-4
INF	Interferon beta
INF_	Interferon gamma
LDL	Low Density Lipoproteins
LDLR	LDL-Receptor
LPO	Lipid Peroxidation
LRP1	LDL receptor related protein 1
LXR	Liver X Receptor
mRNA	Messenger RNA
MS	Multiple Sclerosis
NADPH	Nicotinamide adenine dinucleotide phosphate reduced
NRP	Not Reactive Product
OHCs	Oxysterols
PARP	Poly (ADP-ribose)-polymerase-1
PP-MS	Primary Progressive Multiple Sclerosis
PUFA	Polyunsaturated Fatty Acid
QAlb	$Albumin_{CSF}$:$Albumin_{serum}$ quotient
RNA	Ribonucleic acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RR-MS	Relapsing Remitting MS
SP-MS	Secondary Progressive MS
SREBP	Sterol Response Element Binding Protein
TGF	Transforming Growth Factor
TIMP-2	Tissue Inhibitor MetalloProtreasis-2
TNF	Tumour Necrosis Factor
VEGF	Vascular endothelial growth factor
VLDL	Very Low Density Lipoproteins
WMF	White Matter Fraction

INTRODUCTION

Cholesterol

Cholesterol is present in all vertebrate cells where it has several important functions. Being a structural element in plasma membranes it supports the structure and function of lipid bilayers, and is regarded to be the most important "fluidity buffer" of the membrane. Secondarily it is a precursor of bile acids, steroid hormones and vitamin D. While cholesterol is involved in many cellular processes, a strong indicator of its importance is that it is the only major lipid of mammals not used in energy generation.

De novo synthesis and uptake from circulating lipoproteins cover the cholesterol needs of the cells. Almost all the mammalian cells are able to synthesize cholesterol and express the sophisticated and energy demanding enzymatic machinery required for the de novo synthesis.

In general, the cells in the body are able to release and take up cholesterol to maintain their cholesterol homeostasis: some are able to produce an excess to provide other cells, some others need exogenous cholesterol because of limited synthetic capacity.

Excess cholesterol may be toxic for the cells and a number of strategies have evolved either to export it or to store it in an esterified form. The exogenous cell supply is covered via the Low Density Lipoproteins (LDL) cycle and most of the excess is exported by the High Density Lipoprotein (HDL) mechanism (reverse cholesterol transport), mediated by members of the ATP-Binding Cassette (ABC)-transporter family.

Under normal conditions, about the 60% of the body's cholesterol is synthesized (about 700 mg/day) and the remaining is provided by the diet. The liver accounts for approximately 10% of total synthesis in humans, as does the small intestines.

The biosynthesis of cholesterol may be divided into five stages 1) synthesis of mevalonate from acetyl-coenzymeA (CoA); 2) synthesis of isoprenoid units from mevalonate by loss of CO_2; 3) condensation of six isoprenoids units to form squalene; 4) cyclization of squalene to give the parental steroid, lanosterol; 5) formation of cholesterol by rearranging the lanosterol molecule. The most important rate limiting step is the conversion of the 3-hydroxy-3-methylglutarylcoenzyme A (HMG-CoA) into mevalonate, catalysed by the microsomal HMG-CoA reductase (1). The activity of the enzyme is regulated by a negative feedback mechanism both at the protein level and at the transcriptional level. To some extent the latter effects may be mediated by oxysterols and bile acids.

Cholesterol is insoluble in water and is transported in the circulation associated with lipoproteins. The cholesterol is taken from the intestinal lumen and transported to the liver via chylomicrons. These can be esterified, converted into bile acids or secreted into bile or collected in Very Low Density Lipoproteins (VLDL) to be transported to the extrahepatic tissues. VLDL can be remodelled by the action of lipoprotein lipase that removes triacyl-glycerol, transferring Apolipoprotein A (ApoA) and Apolipoprotein C (ApoC) from VLDL to HDL and getting cholesterol esters back. The product of these remodelling is LDL which supplies the peripheral tissue with cholesterol. The cellular LDL intake is highly regulated via the LDL receptors (LDLR) and Apolipoprotein B (ApoB). The influx of cholesterol inhibits HMG-CoA reductase and cholesterol synthesis and stimulates the cholesterol esterification by acylCoA:cholesterol acyltransferase (ACAT).

The reverse cholesterol transport, whereby cells from different organs eliminate excess cholesterol through the liver, is mediated by HDL. The HDL particles contain ApoA1 and acquire cholesterol directly from the plasma membrane. This transfer is mediated by members of the ABC-transporter family.

About 1 g of cholesterol is eliminated from the body every day. Approximately half of this is excreted into the faces after conversion into bile acids; the remainder is excreted as unmetabolised cholesterol. The bile acids formed have an important role in the solubilisation and absorption of fats, cholesterol, vitamins and drugs. Approximately 95% of the bile acids are reabsorbed from the intestine and reach the liver via the portal vein (enterohepatic cycling).

There are two different major pathways in bile acid synthesis. The neutral pathway is initiated by the rate-limiting enzyme cholesterol 7-hydroxylase, whereas the alternative or acidic pathway is initiated by the enzyme sterol 27-hydroxylase. Under normal conditions the neutral pathway dominates in healthy adult humans.

Cholesterol and the Brain

Cholesterol represents as much as 2-3% of the wet weight of the brain and about 25% of the whole body cholesterol is located here (2).

Since the blood-brain barrier (BBB) efficiently prevents cholesterol uptake from the circulation into the brain, de novo synthesis is responsible for almost all cholesterol present in this organ. The BBB prevents diffusion of the large molecules at the levels of tight junction between adjacent capillary endothelial cells. Interestingly, it has been shown that brain endothelial cells have the potential to take up LDL-cholesterol through luminal LDLRs and translocate this LDL across the cells (3, 4).

The majority of cholesterol present in the Central Nervous System (CNS) is believed to reside in 2 pools: one is represented by the myelin sheaths (oligodendroglia) and the other by the plasma membranes of astrocytes and neurons (5).

About 70% of the brain cholesterol is associated with myelin. In view of the fact that about half of the white matter may be composed of myelin, the brain is by far the most cholesterol rich organ in the body (6).

Nervous tissues are able to synthesize cholesterol and the synthesis rate of cholesterol increases during brain development, but declines to a very low level in the adult state. The latter can be explained by an efficient recycling of brain cholesterol. As a consequence, cholesterol has an extremely long half-life in the adult human brain, estimated to be at least 5 years (7).

The HMG-CoA synthase has been found to be expressed in some neuronal populations (8), whereas the expression of other key enzymes in the cholesterol synthesis in neurons has not been investigated (9-12). Cholesterol synthesis has been found in several cultured neuronal cells derived from embryo or newborn animals (13-17). According to a recent paper the neurons require glia-derived cholesterol to form synapses (17).

Astrocytes synthesise about 2-3 fold more cholesterol than neuronal cells and fibroblasts. Oligodendrocytes, the cell responsible for myelinisation, have an even higher capacity for cholesterol synthesis than astrocytes (14). It has been hypothesised (18,19) that during postnatal development the neurons may down-regulate their own cholesterol synthesis and rely on delivery of cholesterol from astrocytes, which differentiate postnatally and release cholesterol rich lipoproteins. The observation that neurons do not have enough cholesterol to support massive synaptogenesis in the absence of glia cells (20) indicates that neuronal cells are dependent upon exogenous cholesterol. “Outsourcing” of cholesterol synthesis may allow neurons to focus on generation of electrical activity rather than dispense energy on costly cholesterol synthesis, particularly in presynaptic terminals and dendritic spines, which are distant from the soma (18-20).

Apart from de novo synthesis, cells can acquire cholesterol by lipoprotein uptake (also in the CNS). The classic pathway described above is via LDLR and LDL particles.

Several experimental findings suggest that the LDL receptor and the LDL receptor related protein 1 (LRP1) are involved in lipoprotein endocytosis in neuronal cells lines (21-23) and in cultures of CNS neurons (23) supporting the idea that at least cultured neuronal cells may acquire cholesterol by lipoprotein endocytosis.

The cells eliminates most of the excess of cellular cholesterol via reverse cholesterol transport. In the case of neurons, there is no evidence to suggest that the neurons provide other cells with cholesterol. Furthermore, Apolipoprotein E (ApoE), supposed to replace the ApoB in the brain, is not expressed by neurons but by astrocytes (24,25). So, the neurons appear to have two different mechanisms for protection from cholesterol overload. One is by disposing of cholesterol excess by ApoA1 HDL-like particles, in similarity with the reverse cholesterol transport. Whereas this mechanism is still not characterised in the brain, the neuronal cells in rodent CNS express ABCA1-transporter (26,27). Moreover, ApoA1 was found to be expressed in the spinal cord of adults rats (28). There is also some excretion of ApoE-bound cholesterol via CSF (29). Based on the CSF cholesterol content and the rate of CSF renewal, it has been estimated that 1-2 mg/day cholesterol may be eliminated in this way.

The quantitatively most important mechanism to regulate cholesterol in the neuronal cells is however the conversion of cholesterol into 24S-hydroxycholesterol, that, in contrast to cholesterol, is able to passes the BBB (7,30-32) (cf. below).

ApoE, one of the major apolipoproteins expressed in plasma, is regarded to be the most important transport protein for cholesterol in the brain. It is also a ligand for all the members of the LDLR family. Within the CNS, the ApoE is a spherical or discoidal particle with a size similar to HDL. Astrocytes are believed to have the highest capacity to produce ApoE (6, 25) and in culture these cells are able to secrete ApoE together with cholesterol into the culture medium (33).

The ApoE secreted by glial cells serves on one hand to supply the neurons with the cholesterol necessary for the formation of synapses. On the other hand ApoE may also interfere with other important functions regulated by the same receptors, as the LDLR-family.

Because ApoE and cholesterol rich particles are present in CSF (34), it is evident that there is some circulation of lipoproteins within the brain (24) and that ApoE could be a major agent in this cholesterol redistribution network.

The allelic composition of ApoE affects the efficiency of the cholesterol transport and presence of ApoE4, is a major risk factor for Alzheimer's disease (AD), accounting for about 40-60% of the genetic variation of the disease (35,36) (cf. below). It is also a significant risk factor in other forms of neuronal damage, including poor recovery from head injury (37) and other central nervous system stresses (38). It has been hypothesized that ApoE plays a key role in the normal maintenance and remodelling (plasticity) of neurons, as well as repair in response to injury, and that ApoE4 is much less effective in these processes than ApoE3 or ApoE2 (39). The mechanisms by which ApoE4 exerts its effects in neurodegeneration and neuronal repair are still largely unknown.

Efflux of cholesterol from the brain

As discussed above, the reutilisation and recycling of cholesterol between different cells and locations in the brain is an important regulatory factor for brain cholesterol homeostasis. In addition there appears to be a need for excretion of some cholesterol from the brain to maintain a proper homeostasis. Enzymatic conversion of the cholesterol into the metabolite 24S-hydroxycholesterol, which is able to traverse the BBB, has recently been demonstrated to be the most important excretion mechanism (30-32).

Measurement of the arteriovenous concentration difference over the human brain showed that the flux of 24S-hydroxycholesterol from the brain into the circulation corresponds to 6-7 mg/day (7). There is a corresponding uptake of the oxysterols in the liver (7), suggesting that the brain is the unique source of 24S-hydroxycholesterol in humans. The cholesterol 24-hydroxylase or CYP46A1, the cytocrome P-450 enzyme responsible for the 24S-hydroxylation of cholesterol, was found to be localized to neuronal cells, indicating that the excretion of the cholesterol from the brain is initiated in the neurons rather than in the glia cells. However, in the brain of patients diseased with advanced AD some cholesterol 24-hydroxylase immunoreactivity was also detected in glial cells (40).

In view of the fact that almost all 24S-hydroxycholesterol present in human circulation is of cerebral origin, it may be used as a surrogate marker for brain cholesterol homeostasis. It has been shown that the levels of this oxysterol in the circulation reflects the balance between cerebral production and hepatic metabolism (41). The cerebral production appears to be constant, whereas the hepatic metabolism varies with age. Since the ratio between the brain and the liver is about 1:4 in newborns, but about 1:1 in adults, the plasma levels of 24S-hydroxycholesterol are high during the first decade of life but relatively constant after the second decade. There is a tendency towards slightly increased levels of the oxysterols in the circulation after the sixth decade probably due to a decreased metabolic capacity of the liver.

Since the number of active neuronal cells is likely to be important for the flux of 24S-hydroxycholesterol from the brain, it is not surprising that different neurological diseases have been reported to affect the levels of 24S-hydroxycholesterol in the circulation (42-45). The 24S-hydroxycholesterol is transported in the circulation by the same lipoproteins as cholesterol (45), and changes in lipoprotein content may therefore

also affect the levels of 24S-hydroxycholesterol (41). This relation must to be taken in account when using 24S-hydroxycholesterol as a marker for brain cholesterol homeostasis. Under conditions with changes in plasma cholesterol levels, the ratio between 24S-hydroxycholesterol and cholesterol (24OHC/chol ratio) may thus better reflect brain cholesterol homeostasis than the absolute levels.

Neurodegeneration, with loss of neurons, would be expected to lead to reduced levels of 24-hydroxylase with subsequent reduction in the formation of 24S-hydroxycholesterol and a lower 24S-hydroxycholesterol efflux from the brain to the circulation.

In the case of neurodegenerative diseases associated with progressive brain atrophy, such as AD, the levels of 24S-hydroxycholesterol were found to be consistently lower than in the controls (42). This decrease is likely to reflect the lower number of cholesterol 24-hydroxylase containing neuronal cells in AD. These results are consistent with the previous observation that patients with advanced AD have significantly reduced plasma levels of 24S-hydroxycholesterol as compared with healthy controls (43).

Cholesterol homeostasis in Alzheimer's disease

In the last few years, several independent findings have accumulated to suggest a possible link between cholesterol and AD.

Hypercholesterolemia has long been recognised as an important risk factor for developing of AD (cf above). ApoE4 genotype is the greatest known risk factor for development of late-onset Alzheimer disease (46,47).

In addition to its reduced capacity for lipid transport ApoE4 may have a lower antioxidant effect than ApoE3 (48). AD appears to be associated with increased

production of reactive oxygen and nitrogen species able to oxidise lipids. Loss of protective antioxidant factors may exacerbate any damage due to oxidant stress.

Experimentally induced hypercholesterolemia in rabbits leads to accumulation of pathogenic amyloid beta peptide (A□) in brain (49). As the BBB efficiently prevents the passage of peripheral cholesterol into the brain, the mechanism behind this accumulation remains obscure. In cell cultures, increased and decreased cholesterol levels promote and inhibit the formation of A□ from Amyloid Precursor Protein (APP), respectively (50, 51).

Very recently, epidemiological studies have reported a significant (40-70%) reduction in the risk of AD associated with statins use, a class of HMG-CoA reductase inhibitors (52-54). As in atherosclerosis, both cholesterol and cholesterol-independent effects could explain the benefit of statins in AD. In neuronal and peripheral cell lines, statins seem to promote a favourable shift in APP processing toward secretion of the neurotrophic APPs peptide and away from A□ production (55,56). Depletion of membrane cholesterol by statins produces an increase in the activity of enzymes with □-secretase activity, and or reciprocal decrease in □-secretase-mediated APP proteolysis.

The above observations suggest that cholesterol efflux from neurons is an important aspect of neuronal maintenance.

Neuronal injury may result from A□-induced injury, deprivation of oxygen, acute head trauma, oxidative stress, or any other insult that requires a repair response. Since AD manifests symptoms over decades, the relative inability of apoE4 to respond effectively to chronic insults provides, in addition to its nonlipid-related effects, a potential explanation for the strong association of apoE4 with AD.

Oxysterols

Oxysterols are mono-oxygenated derivatives of cholesterol (or precursors to cholesterol) that are important as intermediates and end products in cholesterol excretion pathways. The rapid degradation and excretion of oxysterols is facilitated by their physical properties, allowing them to pass lipophilic membranes and to be redistributed in the body at a much faster rate than cholesterol itself (57). The introduction of an oxygen function in the cholesterol molecule makes it much more polar, drastically reduces its half-life and directs it to excretion or to oxidation to water-soluble bile acids.

Oxysterols are typically found in conjunction with cholesterol in almost all biological locations, though at significantly lower concentrations (10-100 thousand fold less). As they may be formed by spontaneous or enzyme mediated processes they tend to be unevenly distributed across different tissues.

The spontaneous oxidation of cholesterol is a well-recognised phenomenon generally termed auto-oxidation (58). This process is a non-enzymatic reaction that can be initiated by diverse compounds including various radical species, lipid peroxides and divalent metal cations (such as Cu^{2+}). Due to its structure cholesterol is vulnerable to auto-oxidation at several positions, in particular the allylic position at C-7, which is susceptible to attack by peroxy radicals. The primary products of an oxidative attack are the epimeric C-7 peroxy radicals, with subsequent formation of epimeric cholesterol-7-hydroxyperoxides. These compounds then undergo rapid thermal decomposition to yield a mixture of 7α-hydroxy-, 7β-hydroxy- and 7-oxo-cholesterol (58). In addition to these auto-oxidative processes some oxysterols are formed by the action of specific enzymes, most of which belong to the cytochrome P-450 superfamily (59). The products of such enzymatic reactions represent the majority of oxysterols

present in the circulation: the cholesterol 7α-hydroxylases or CYP7A1 is responsible for the production of 7α-hydroxycholesterol, the sterol 27-hydroxylase or CYP27A1 for the 27-hydroxycholesterol, the cholesterol 4-hydroxylase or CYP46A1 for 24S-hydroxycholesterol, the cytocrome P450 3A4 or CYP3A4 for 4β-hydroxycholesterol and finally the sterol 25-hydroxylase (not a cytocrome P450 enzyme) for 25-hydroxycholesterol.

Oxysterol properties

Cholesterol orders and condenses fluid-state acyl chains and prevents the hydrocarbon chains of phospholipids from aggregating as temperature decreases, inhibiting or even obliterating temperature-induced phase changes, resulting in a decrease of membrane fluidity.

The preferred bilayer orientation is such that the 3β-hydroxyl group interacts with the polar head group of phospholipids, with the rest of the molecule oriented roughly perpendicular to the plane of the membrane.

The addition of a polar group distal to the 3β-hydroxyl group, i.e. on the sterol side chain, leads to a significant alteration of the sterol configuration and interaction with the acyl-chains: it becomes energetically more favourable for the oxysterols to leave the membrane and translocate through the aqueous compartment to a more suitable acceptor. As a consequence these oxysterols are capable of movies between lipophilic compartments several orders of magnitude faster than cholesterol. As a consequence, the formation of side-chain 24- and 27-hydroxycholesterol are important for the removal of cholesterol from peripheral tissues (60,61).

About 30 years ago, Kandutsch presented his “oxysterols hypothesis” (62,63). This hypothesis suggests that most of the regulatory effects of cholesterol on its own

synthesis are due to oxysterols rather than to cholesterol itself. Indeed the ability of oxysterols to interact with the liver X receptor (LXR) subfamily of orphan receptors and the fact that several LXR-responsive genes are involved in the lipid homeostasis (ABCA1, CETP, SREBP-1c, apoE) are consistent with a role of oxysterols as regulators.

A large corpus of literature suggests that oxysterols are cytotoxic towards cells both in vivo and in vivo. The results of this type of studies should be interpreted carefully in view of the fact that the toxic effects described in vitro depend very much on the conditions under which exposure is conducted, the cell type and the species. In most studies supraphysiological concentrations of oxysterols have been used (typically mg/L up to 1 g/L) in a poorly defined lipid matrix. The concentration of oxysterols required to achieve detectable toxicity is higher in the presence of serum lipoproteins than in serum free media, due to the capacity of lipoproteins to absorb oxysterols and/or direct antagonism of oxysterols toxicity by cholesterol) (64). It should be pointed put that cholesterol may antagonize the effect of oxysterols, and normally the excess of cholesterol in relation to oxysterols is at magnitude of 10^4 or even more (65).

24S-Hydroxycholesterol

24S-hydroxycholesterol is almost exclusively formed in the brain, where it is present in greater amounts than in any other organ (4-15 ng/mg wet weight). In accordance with this the brain is the only organ with detectable levels of 24-hydroxylase mRNA (66). As discussed above, there is a daily flux of about 6-7 mg of this oxysterol from the brain into the circulation, with the majority of this efflux occurring as direct transport across the BBB (31-32). It has been estimated that about 1% of the 24S-hydroxycholesterol produced by the brain is transported to the circulation via passage through the CSF.

Analysis of RNA isolated from different regions of the human brain indicated that the cholesterol 24-hydroxylase mRNA was broadly distributed, with somewhat higher levels in zones rich in grey matter (e.g., putamen, cerebral cortex, caudate nucleus, amygdala) and lower levels in regions rich in white matter (e.g., corpus callosum).

The mRNA hybridization and immunohistochemically analysis in murine brain revealed abundant cholesterol 24-hydroxylase mRNA in neurons of the cerebral cortex, hippocampus, dentate gyrus, amygdale, putamen and thalamus. The cholesterol 24-hydroxylase protein was found to be localized to neurons in all six layers of the cortex, with the highest levels in pyramidal cells of cortical layers II, III, V and VI. Lower amounts of cholesterol 24-hydroxylase protein were detected in cortical layer IV, which is rich in nonpyramidal cells The enzyme has been found to have a perinuclear distribution within the nerve cell body consistent with a microsomal localization (66).

There is also a significant positive correlation between the levels of cholesterol 24S-hydroxylase protein nad 24S-hydroxycholesterol in the different areas of a rat brain (O'Shea S, Meaney S, Leoni V, unpubblished).

27-Hydroxycholesterol

27-Hydroxycholesterol is formed from cholesterol in most extrahepatic organs by sterol 27-hydroxylase, a cytocrome P450 species located in the inner membranes of mitochondria. It plays an important role in the acidic or alternate pathway of bile acid synthesis, and hence cholesterol elimination. At high levels of the enzyme, 27-hydroxycholesterol may be further oxidized into 7α-hydroxy-3-oxo-4-cholestenoic acid.

The 27-hydroxylase is also involved in a mechanism for reverse cholesterol transport by extrahepatic cells. In contrast to cholesterol, 27-hydroxycholesterol, as well as 7α-

hydroxy-3-oxo-4-cholestenoic acid, is able to cross the plasma membranes to be taken up by various lipoproteins.

The production of 27-hydroxycholesterol represents an important mechanism for the daily elimination of cholesterol by the body. There is a net flux of that compound from extrahepatic sources to the liver and the liver takes up about 20 mg/day of 27-oxygenated compounds. This uptake corresponds to about 4% of the bile acid production under normal conditions (67).

Patients with advanced atherosclerosis tend to have higher and more heterogeneous levels of 27-oxygenated products in the circulation than healthy subjects.

27-hydroxylase as well as 27-hydroxycholesterol are present also in the brain (68). Measurement of the arteriovenous concentration difference of this oxysterol over the brain did not show any significant efflux of 27-hydroxycholesterol from the brain (31). Very recently some evidence was obtained for a net uptake of this steroid by the brain from the circulation (Heverin M et al., unpublished).

Enzymes are present in the brain which are capable to convert 27-hydroxycholesterol into a steroidal acid. It has been shown in vitro that rat glial cells and neurons are able to convert 27-hydroxycholesterol into 7□-hydroxy-3-oxo-4-cholestenoic acid (69). Interestingly the latter acid has been found in subdural haematomas, at a concentration exceeding 5 times the concentration found in plasma (70).

In a recent study (71) the sterol composition of several different brain areas of diseased patients with AD were compared with age- and gender matched controls. The levels of 27-hydroxycholesterol were found to be increased in all four areas of the AD brain in comparison to the controls. Moreover, also in the case of aged transgenic APP mice the brain levels 27-hydroxycholesterol were found to be higher than in controls (71).

A very recent immunocytochemical study reported that 27-hydroxylase is expressed in neurons as well as in some astrocytes and oligodendrocytes in the normal human brain. In AD, sterol 27-hydroxylase expression was found to be decreased in neurons and increased in oligodendrocytes (72).

7-oxygenated cholesterol species

7oxo-, 7□-hydroxy- and 7□-hydroxy-cholesterol are formed by auto-oxidation of cholesterol. 7□-hydroxycholesterol may also be formed by activity of the cholesterol 7□-hydroxylase (uniquely expressed in the liver, the main regulatory step in the bile acids synthesis): the plasma levels of 7□-hydroxycholesterol can be used as an index of bile acid synthesis (73). It has been suggested previously that 7_-hydroxycholesterol may be a sensitive indicator of oxidative stress *in vivo* (74).

Cytotoxic and pro-apoptotic effects have been reported for 7-oxygenated oxysterols, although most or all of these studies have utilised unphysiological levels of the oxysterols (64).

Very recently another group (75) reported extremely high levels of 7-oxocholesterol in CSF samples of MS patients. These levels were in the order of 5-10mg/L, about 1000 to 5000 times higher than the levels of 24- and 27-hydroxycholesterol found in the present research. According to the publication the 7-oxocholesterol rapidly enters the nucleus and activates poly (ADP-ribose)-polymerase (PARP)-1, followed by the expression of migration-regulating integrins CD11a and intercellular adhesion molecule 1. This was found to occur in vitro at levels of 7-oxocholesterol similar to those found in the CSF samples. The high levels of 7-oxocholesterol were also found to induce neuronal damage mediated by activated microglial cells and this mechanism

was thought to be responsible for the perpetuating the neuronal damage, a key factor in the pathogenesis of MS.

Oxidative stress and lipid peroxidation

The term oxidative stress is used to denote an imbalance between the production of oxidants and the respective defence systems of an organism (76). Oxidants are represented by reactive oxygen species (ROS), reactive nitrogen (RNS) species, sulfur-centered radicals and various others compounds. The most important reactive species mediating oxidative stress in biological systems are thought to be radicals. These are molecules with one or more unpaired electrons, which are usually highly reactive towards biomolecules. There are other reactive molecules, in particular those derived from oxygen, which are not radicals but still highly reactive, eg. hydrogen peroxide (H_2O_2) and singlet oxygen ($^1\Delta O_2$). The radicals are able to propagate the reaction leading to extensive damage. Oxidants can be generated in numerous ways, such as by ionizing radiation, by chemical reactions, enzymatically, through redox-catalysis involving free transition metal ions, or metal ions bound to enzymes. Cellular sources of oxidative stress are: a) the formation of reactive oxygen species by incomplete reduction of oxygen in the respiratory chain of mitochondria; and b) the host defence systems, which includes the "oxidative burst" mediated by NADPH oxidase (producing superoxide radical) and myeloperoxidase (leading to the formation of hypochlorous acid). Another important molecule, nitric oxide, synthesized by a group of enzymes termed nitric oxide synthases, which transform L-arginine into $NO^{\bullet}$ and L-citrulline, is rather unreactive by itself. In the presence of oxygen or superoxide, however, $NO^{\bullet}$ is converted into more reactive species, such as nitrogen dioxide and peroxynitrite.

Lipid peroxidation (LPO) reactions are generally free radical-driven chain reactions in which one radical can induce the oxidation of a comparatively large number of substrate molecules, which are represented by polyunsaturated fatty acids (PUFAs). LPO is one of the major outcomes of free radical injury to tissue. Such a chain reaction is initiated by the abstraction of a hydrogen atom from a reactive methylene group (LH) of a PUFA residue; monounsaturated and saturated fatty acids are much less reactive and do not usually participate in LPO. This initiation is usually performed by a radical (R$^{\bullet}$) of sufficient reactivity:

$$LH + R^{\bullet} \rightarrow L^{\bullet} + RH \qquad (1)$$

Molecular oxygen rapidly adds to the carbon-centered radical (L$^{\bullet}$) formed in this process, yielding a lipid peroxyl radical (LOO$^{\bullet}$):

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet} \qquad (2)$$

which can abstract a hydrogen from another PUFA, analogously to reaction 1

$$LH + LOO^{\bullet} \rightarrow L^{\bullet} + LOOH \qquad (3)$$

This reaction is termed propagation, implying that one initiating hit can result in the conversion of numerous PUFA to lipid hydroperoxides. Lipid hydroperoxide (LOOH) is the first, comparatively stable, product of the lipid peroxidation reaction. Under conditions where LPO is continuously initiated, a *termination* reaction limits the extent of LPO, yielding non-radical products (NRP), and destroying two radicals at once:

$$LOO^{\bullet} + LOO^{\bullet} \rightarrow NRP \qquad (4)$$

In the presence of transition metal ions, LOOH can give rise to the generation of radicals capable of (re-)initiating LPO by redox-cycling these metal ions:

$$LOOH + Me^{n+} \rightarrow LO^{\bullet} + Me^{(n-1)+} \qquad (5)$$

$$LOOH+Me^{(n-1)+} \rightarrow LOO^{\bullet}+Me^{n+} \qquad (6)$$

Lipid hydroperoxides in the presence or absence of catalytic metal ions also give rise to a large variety of products, including short and long chain aldehydes and phospholipid and cholesterol ester core aldehydes, many of which can be used to assess the degree of LPO in a system.

Oxysterols as markers for oxidative stress, lipid oxidation and atherosclerosis

The oxidation of the cholesterol molecule secondary to LPO is responsible for the formation of some oxysterols in biological matrices (58). Oxidative modification of cholesterol in LDL was found to result in the formation of 7- and 5,6-oxygenated species (77,78). The 7-oxygenated sterols are derived from the reduction or dehydration of the 7-hydroperoxycholesterols whereas the 5,6-epoxides are believed to be formed by the reaction of 7-peroxyl radicals with the C-5 double bound in cholesterol (58). It has been suggested that these oxysterols could be used as in vivo lipoprotein oxidation markers. The major difficulties associated with this are however that during analysis and workup there is a great risk of auto-oxidation of the parent cholesterol during workup procedures. This makes it difficult to draw accurate conclusions from the results of many old studies.

The primary 7-oxygenated product of cholesterol, 7-hydroperoxycholesterol, seems to be the most cytotoxic oxygenated lipid present in oxidized LDL (79). 7-hydroperoxycholesterol decomposes rapidly to form 7α-, 7β-hydroxycholesterol and 7-oxocholesterol, all of which may be found in relatively high concentrations in foam cells and fatty streaks (64). It has been suggested however that the levels of 7β-hydroxycholesterol might reflect “oxidative stress” in vivo (74).

The levels of the 27-hydroxycholesterol in the circulation are likely to reflect the antiatherogenic defence in some of the patients at some stage of the disease (80).

The blood-brain barrier

The term blood-brain barrier (BBB) is commonly used to describe a whole range of mechanisms that regulate and protect the internal environment in the brain (81-85). More specifically this term is used to indicate a relative restriction of the entry of plasma protein into the brain. This barrier acts as a diffusion restraint to an extent that depends on molecular size and lipid solubility. Contributing to this selectivity are the tight junctions between the endothelial cells (blood-brain barrier) of brain capillaries and the tight junctions at apices of epithelial cells of the choroid plexuses (blood-CSF barrier). The basal membranes of these endothelial cells are intimately associated with the foot processes of adjacent astrocytes (86). The capillaries of the brain have apparently no fenestrae and show little vescicular transport. Adjacent cells are so tightly adherent that there is little or no paracellular molecular diffusion (87, 88). The endothelial cells of these capillaries form a strong resistance barrier where the exchange of molecules, between plasma and CNS takes place across the two parallel plasma membranes covering each surface of the capillary endothelial cell (86). These membranes are freely permeable to water and solute and contain a number of transport proteins. Furthermore these bilayers are also permeable to a number of more hydrophobic molecules, including sterols, that are able to diffuse across the BBB as a consequence of existing activity gradients. The rate of the flux depends on the degree of solubility that a certain molecule can achieve in the blood or extracellular fluid as well as on its passive permeability coefficient (89-91). Importantly, when a sterol like cholesterol is hydroxylated, there is an increase in the aqueous solubility but a

reduction in its passive permeability. Thus, the net effect of the hydroxylation is thus a greatly increased rate of passive diffusion of the molecule across the BBB.

The role of the BBB is to protect the central nervous system (CNS) from the compositional fluctuations that may occur in the circulation.

Many diseases and injuries of the CNS such as stroke, tumours, autoimmunity, infection and traumatic brain injury are accompanied by BBB disruption, resulting in secondary damage to neurons (92).

A broad array of cytokines, which serve as extracellular signals in a variety of physiological and pathological conditions, influence intercellular associations and permeability across endothelial cells and the function of the tight junctions and the BBB. The effects of the proinflammatory cytokines IFN-_ and TNF on intercellular junctions of epithelial and endothelial cells have been investigated broadly. The initial studies addressing the influence of IFN-_ on tight junctions found an increase in paracellular permeability (93). In concert with TNF, IFN-_ enhanced paracellular permeability of microvascular endothelial cells (94). TNF alone has been documented to increase intercellular gaps. Many other cytokines have been investigated with respect to epithelial/endothelial barrier function. TGF-□ has been reported to promote retinal endothelial permeability by increasing gelatinase B followed by a redistribution and reduced immunoreactivity of occludin (95). Other cytokines such as IL-1, IL-4, IL-13, TGF-□, VEGF, IGF-I and –II have been documented to decrease the barrier properties of epithelial / endothelial cells.

Proteolysis leads to partial disappearance of structural elements of the tight junctions, prevented by a metalloproteinase inhibitor (96), indicating the involvement of proteolysis in permeability regulation. Experimentally, opening of the BBB can occur

after intracerebral injection of activated gelatinase A. Inhibition of gelatinase A activity by TIMP-2 (Tissue Inhibitor of MetalloProteinsase), a natural inhibitor of metalloproteinases was found to block that opening (97).

The cerebrospinal fluid

The cerebrospinal fluid (CSF) is a colloidal solution with characteristics similar to plasma. The bulk of CSF is secreted by the choroid plexus (a leaf-like structure with high density of fenestrated capillaries and venules covered by specialized ependimal cells), localized in the lateral, third, and fourth ventricles. It flows in the subarachnoidal space and subarachnoidal cisterns. The bulk is removed through the arachnoid villi in the dural venous sinuses and there are about 4-7 CSF changing per day. The volume of CSF in adult humans is about 140-170 mL. The CSF acts as a hydraulic shock-absorber and as a sink for the brain.

The extracellular fluid within the brain communicates directly with the CSF. The composition of CSF is thus reflecting the extracellular environments in the brain and the spinal chord. The main differences to plasma are the concentration of Na^+, Cl^-, glucose and protein (0.1-0.4 g/L, predominantly albumin).

Laboratory investigation of CSF

The CSF analysis is an important diagnostic tool reflecting both biochemical and cytological features of the brain and the barrier system.

The CSF is collected by lumbar puncture, withdrawn from the lumbar theca and is normally examined for pressure, colour and a variety of cellular and noncellular components (98). Routine CSF examination should also include the cell count and leukocyte differentiation; calculation of the $Albumin_{CSF}$:$Albumin_{serum}$ ratio (or Q_{Alb}) to evaluate the integrity of the blood brain barrier; demonstration of intrathecal

Immunoglobulin G (IgG) synthesis by quantitative methods (IgG index: IgG_{CSF}/ IgG_{serum}) as well as qualitative methods (demonstration of oligoclonal bands by means of isoelectrofocusing and specific immunofixation); virological tests (specifically Ig synthesis, PCR, cultural isolation).

The $Albumin_{CSF}$:$Albumin_{serum}$ ratio is a widely accepted indicator of the blood-CSF barrier dysfunction, including CSF flow rate (99).

Multiple Sclerosis

MS is the most common inflammatory disease of the CNS. It affects between 250,000 and 350,000 people in the United States (100) and remains a major cause of disability in both young and old populations. Although MS has been studied for centuries, its aetiology has remained elusive. Until recently, no effective therapy for reducing attacks and slowing the disease process was available. Much progress has been made over the past decade in elucidating the pathogenesis of MS and improving patient outcomes (101).

MS is now generally believed to be an immune-mediated disorder that occurs in genetically susceptible people. The pathological hallmark of chronic MS is the demyelinated plaque: a well-demarcated hypocellular area characterized by loss of myelin, relative preservation of axons in the early phase, formation of astrocytic scars, focal BBB breakdown. Lesions have a predilection for the optic nerves, periventricular white matter, brain stem, cerebellum, and spinal cord white matter, and they often surround one or several medium-sized vessels (101).

MS has long been regarded as an autoimmune demyelinating disorder of the CNS caused by T cells autoreactive for myelin proteins. However, recent data suggest that the disorder may start with oligodendrocyte apoptosis. Axonal degeneration also occurs

and was reported in early descriptions of MS plaques. The estimated axon loss in chronic spinal-cord lesions in patients with disease of long duration is 58–68%. Although acute axonal damage happens in all lesion types, it is most pronounced in early lesions (102).

Axonal damage and degeneration are thought to occur in MS by several possible mechanisms, such as BBB dysfunction, local inflammation, axonal injury, iron deposition, and distal effects of Wallerian degeneration (103-107). T cells, especially the $CD8^+$, microglia and macrophages are thought to be involved in these process, but it might also be an indirect effect of demyelination over a long period, resulting from redistribution of sodium channels or absence of oligodendrocyte enzymes and myelin proteins (102).

Axonal transection is commonly found in the brains of patients with MS in both lesions and in normal-appearing white matter (108). Global degeneration of white matter may be a result of Wallerian degeneration in MS brains secondary to axonal transection at sites of inflammation and in normal-appearing white matter (107-109). Disconnection through white matter lesions may also be a cause of reported widespread hypometabolism in deep and cortical grey matter structures, contributing to grey matter atrophy (110). Substantial neuronal loss and volume loss has also been demonstrated in grey matter (103). Neuronal injury and degeneration may also occur in grey matter through an independent process such as pathologic iron deposition (111).

The presenting of symptoms and their characteristic progression may suggest the diagnosis. The Relapsing-Remitting MS (RR-MS) is present in 80% of the patients. Clinical manifestations begin typically in the second or third decades of life, evolve over a period of several days, stabilize and often improve spontaneously in response to

corticosteroids. About 20% of affected patients have Primary Progressive MS (PP-MS), which is characterized by a gradual progressive clinical course and a similar incidence in the two sexes. Secondary Progressive MS (SP-MS) involves persistent signs of central nervous system dysfunction, which may develop after a relapse, with progression between relapses.

Accumulating data indicates that oxidative stress plays an important role in the pathogenesis of MS. ROS, generated in excess primarily by macrophages, have been implicated as mediators of demyelination and axonal damage in both MS patients and in animal model with autoimmune encephalomyelitis. ROS cause damage to cardinal cellular components such as lipids, proteins and nucleic acids (e.g. RNA, DNA), resulting in cell death by necrosis or apoptosis (112)

Magnetic Resonance Imaging in MS

Magnetic Resonance Imaging (MRI) is a highly sensitive technique for the detection of brain and spinal cord involvement in MS. MRI provides objective and sensitive measures of disease pathology and is now routinely used in clinical practice as a surrogate marker in the diagnosis and evaluation of MS (109, 113–115). The new diagnostic criteria for MS underline the importance of MRI to recognise the dissemination of lesions in space and/or in time (113).

Furthermore the MRI is the most sensitive method for predicting the development of clinically definite MS in patients with suspected MS (116).

The lesions revealed by conventional MRI include hyperintense lesions detected on T2-weighted images, hypointense lesions on T1-weighted images, and Gadolinium (Gd) enhanced foci on post-contrast images. Decreased brain volume or brain atrophy, a clinically relevant measure of disease activity, can also be measured using MRI (117).

In general, MRI supplies information about the number of active lesions (disease activity) as well as the total number and size of lesions (burden of disease) in a patient with MS.

1) T2-Weighted Sequences: MS lesions typically appear bright or hyperintense. The lesions are seen predominantly in white matter but also in gray matter (114, 118). The T2-wighted lesions are characterised by a low disease-specificity: a number of disorders besides MS may cause small white-matter hyperintensities on T2 imaging, including vascular, infectious, and other inflammatory disorders: those have to be considered in the differential diagnosis of MS.

T2 hyperintense lesions in MS may reflect edema, demyelination, remyelination, axonal loss, and gliosis (119).

2) Gadolinium (Gd) Enhancement. Gd is used as contrast agents to increase the sensitivity of conventional T1-weighted spin-echo images. Enhancing agents do not normally traverse the BBB: enhancement of MS lesions in presence of Gd is suggestive of acute inflammatory changes that increase the permeability of the BBB (120). The transient (2–6 weeks) nature of Gd enhancement makes serial Gd-enhanced MRI a useful marker for monitoring active disease and treatment effects (121).

3) T1-Weighted Sequences. About 80% of Gd-enhancing lesions appear hypointense on the corresponding un-enhanced T1-weighted images (122). Once contrast enhancement ends, these areas may become iso-intense to the normal appearing white matter on T1-weighted images, whereas about half of these areas develop into persistent hypointense lesions ("black holes") over time (123).

Non persistent hypointense lesions suggest reversible edema or demyelination, whereas chronic areas of hypointensity, termed persistent T1 black holes, may indicate areas of more severe tissue destruction such as axonal loss and severe demyelination (124).

Measurements of T1 lesion load correlate with clinical disability better than do measurements of T2 lesion load (125).

4) MRI Brain Atrophy. Axonal loss and neuronal degeneration cannot be assessed directly in vivo: however a macroscopic loss in tissue volume concomitant of these losses, namely brain or spinal cord atrophy, can be evaluated using MRI.

The extent of brain atrophy in MS patients has been shown to correlate with clinical outcome, such as: physical disability, cognitive dysfunction and depression. Brain atrophy can occur during early stages of MS, in relapsing remitting (126-128), progressive (129) and both (130-132) clinical subtypes of MS.

Alzheimer's Disease

The AD is a progressive neurodegenerative disease characterised by extracellular neuritic plaques composed of the A□, intracellular neurofibrillary tangles of hyperphosphorylated tau and neuronal cell loss. The process starts with a progressive intra- and extra-cellular accumulation of A□ in cerebral cortex followed by a microglial and astrocyte activation with oxidative injury. Finally the combination on neuronal and synaptic dysfunction and neuronal death leads to dementia (133-135).

Accumulated epidemiological and experimental data suggest cholesterol homeostasis to be important in the development of AD (see also above).

In the case of AD, the annual rate of global brain atrophy is 2-3% as compared with 0.2-0.5% in healthy controls (136). Pathological studies in AD have shown prominent early involvement of medial temporal lobe structures, especially the entorhineal cortex and hippocampus (137). The progressive extensive atrophy is the result of the neurodegenerative processes.

Actually there is accumulating evidence indicating that oxidative stress may play a major pivotal role in the early pathogenesis of the disease. Activated glial cells in

inflammatory reactions produce ROS with consequent damage of lipids and proteins of the neuronal plasma membrane (138). A is aggregated in the presence of free radicals and may amplify the generation of ROS generation. Moreover the AD brain has increased concentrations of metals, including iron (139), aluminium (140) and copper that may catalyse the production of free radicals.

AIMS OF THE STUDY

It is evident from the above introduction that cholesterol metabolism within the brain is still unclear and that its relation to CNS diseases has to be clarified.

The physiological significance of the oxysterols, and especially their involvement in brain cholesterol homeostasis is still far from being well understood.

The aims of the present study:

1. To clarify whether or not plasma levels of 24S-hydroxycholesterol are affected in patients with MS. If such changes are observed, is it possible to relate them to changes in the brain observed by MRI ? (paper I and II)

2. To clarify the origin of 27-hydroxycholesterol in CSF. Is there a flux of this oxysterols from the circulation into the CSF ? (paper III)

3. To define the CSF levels of 24S- and 27-hydroxycholesterol levels in a normal population (papers III and IV)

4. To evaluate whether or not the levels of 24S- and 27-hydroxycholesterol in CSF can give diagnostic information (paper III and IV)

5. To measure the levels of 7-oxocholesterol in CSF both in MS- and control subjects with an accurate method. Are these levels more than thousand times higher than the levels of the other oxysterols as reported by Diestel et al.? (paper V).

MATERIALS AND METHODS

Materials

All the organic solvent were for GC or HPLC grade. [26,26,26,27,27,27-$^{2}H_6$]-cholesterol had an isotopic purity of 95% and a chemical purity of more than 99%.

Patients

Paper I

Blood and CSF were taken from 118 patients (34 males and 84 females) at different stages of the disease. The age ranged from 24-70 years. All the patients were diagnosed with definite MS according to the Poser criteria (141). Among the patients 65% were defined as RR-MS (n = 77), 3% were PP-MS and 13% were SP-MS.

Only 12 patients were receiving immuno-modulating drugs at the time of the sampling: 11 received IFN_-1b and 1 Copaxone®.

The plasma was rapidly separated after centrifugation and then stored together with CSF at -80°C for a maximum of 2 years. Storage for up to 4 years under such condition does not affect the levels of the steroids.

All the samples were matched with age related controls groups of 183 healthy volunteers of both sexes ranging in age between 21-70 years (41).

Patients were considered MRI-positive if cranial scans performed before or at the time of blood and CSF sampling were judged to be consistent with MS by a neuroradiologist. Only scans performed within two months before or two months after sampling were assessed with regard to gadolinium enhancement.

A spike in the level of 24S-hydroxycholesterl was defined as a level above the mean + 2SD for the corresponding control population.

In the whole population of MS patients, information about presence or absence of gadolinium-enhancing lesions was available in 48 cases only. Of these 48 cases, 19 patients had such lesions while 29 did not.

Paper II

Blood samples were collected from 56 MS patients: 37 with a relapsing-remitting (RR) disease course (mean age, 36.7 years, female:male 27:10), 19 with a primary progressive (PP) course (mean age, 43.5 years, female:male 8:11) and 23 healthy controls(mean age, 33.6 years, female:male 12:11), all recruited through the National Hospital for Neurology and Neurosurgery in London. None of these patients were included in any other of our studies.

Because RRMS and PPMS differ markedly clinically, radiologically, and histopathologically (142), patients of the two groups were considered separately. RRMS patients were diagnosed with MS according to the criteria of McDonald and co-workers (113); PPMS patients fulfilled the criteria proposed by Thompson and co-workers (143) for definite or probable PPMS. Plasma was rapidly separated by centrifugation and frozen for storage.

Paper III

Plasma and CSF samples were collected for routine diagnostic purposes by the Division of Neurology, Huddinge University Hospital. After determination of albumin and IgG levels, the excess sample material was frozen at –80°C for a maximal of three years before analysis. In a few cases the samples were stored for a short time at -20°C.

From the population of MS-patients of Paper I a subgroup (n = 49, mean age, 41 years , female:male, 33:15) was selected based on the fulfilment of the following criteria for inactive disease: i) "normal" $Albumin_{CSF}$:$Albumin_{plasma}$ ratio ($< 8 \times 10^{-3}$); ii) levels of

24S-hydroxycholesterol in the plasma (≤ 120 μg/L) and CSF levels(≤ 3.5 μg/L); iii) age ≤ 60 years; iv) absence of gadolinium-enhancing lesions on the MRI investigation immediately preceding or following plasma and CSF collection. This group was designated "Controls".

A second population of patients (n = 92, mean age, 50 years, female:male, 60:32) was selected primarily on the basis of an increased $Albumin_{CSF}$:$Albumin_{plasma}$ ratio, greater than 8 x 10^{-3}, which is strongly suggestive of a blood –brain and blood-CSF barrier dysfunction (98,99). These patients were collectively designated "BBB group". According to diagnosis upon release from the hospital, they were subgrouped as follows: demyelinating polyneuropathy (i.e. Guillain-Barré syndrome or chronic inflammatory demyelinating polyneuropathy, CIDP, n = 17) was designated "Polyneuropathy"; the subgroup of patients with headache of uncertain etiology and an increased $Albumin_{CSF}$:$Albumin_{plasma}$ ratio, (n = 12) was designated "Headache"; the subgroup of patients with confirmed subarachnoid haemorrhage (n = 6) was designated "SAH", while those with confirmed meningitis (n = 11) were designated "Meningitis". Patients with RR- (n = 20) and PP (n = 1) -MS and with an increased $Albumin_{CSF}$:$Albumin_{plasma}$ ratio comprised the group designated "Alb-MS". The BBB group also included patients with the following diagnoses: non-demyelinating polyneuropathies of heterogeneous origins (n = 10), motor neuron disease (n = 3), Bell's palsy (n = 4), fatigue syndrome (n = 1), Wegener's granulomatosis (n = 1), general myalgia (n = 1), vitamin-B12 deficiency (n = 1); cervical myelitis (n = 1), cranial mononeuropathy (n = 1).

A third group, also selected from the previously characterised MS population, was comprised of MS patients with an active disease ("MS-active") (n = 20, mean age 37 years, female:male = 13:7), as indicated by fulfilment of one or both of the following

criteria: i) presence of gadolinium enhancing lesion on MRI; ii) plasma levels of 24S-hydroxycholesterol greater than 2 SD above those of age-matched controls (cf. paper I).

Paper IV

255 patients were retrospectively studied at the division of Neurology and division of Geriatrics, Huddinge University Hospital. Plasma and CSF samples were collected for routine diagnostic purposes under varying conditions. 120 of these patients had been studied also in Paper II. After determination of albumin and IgG levels, the excess sample material was frozen at –80°C for a maximal of three years before analysis. In a few cases the samples were stored for a short time at -20°C.

The patients were grouped according to the following diagnoses:

1. Control patients ("Controls"). This group consisted of 42 females and 16 males with a mean age of 39 years with headache of uncertain background without any clinical or laboratory signs of CNS-diseases. The patients thus had normal $Albumin_{CSF}$:$Albumin_{plasma}$ ratio (98,99), and absence of blood cells in CSF. This group of patients was considered to be more suitable as controls than those used in Paper III.

2. Patients with Neuroborreliosis ("Borrelia"). Six females and 8 males were studied with a mean age of 54 years with clinical signs and serological evidence for infection with Borrelia Burgdorferi. Antibodies against Borrelia flagellar antigen in CSF and serum were determined using Lyme Neuroborreliosis ELISA kit from DAKO (Glostrup, Denmark). A positive antibody index in this capture ELISA indicates intrathecal production of antibodies to Borrelia. For the diagnosis of neuroborreliosis, clinical symptoms and positive IgM index was required.

3. Patients with viral meningitis ("Vmeningitis"). This group consisted of 6 males and 6 females with a mean age of 42 years. All the patients were diagnosed by recognition of clinical symptoms (meningitis, meningoencephalitis). The cytological examination

was compatible with the typical findings in case of aseptic meningitis. Titres of the specific antiviral antibodies were determined by ELISA in both CSF and plasma of each patient, followed by western blotting to demonstrate intrathecal synthesis of immunoglobulins. In all the patients also $Albumin_{CSF}$:$Albumin_{plasma}$ ratio, CSF- and plasma levels of both IgG and IgM were determined. 11/12 of these patients had been included in the previous study (cfr. Paper III)

4. Multiple Sclerosis Patients without Gadolinium-enhancing lesion ("MS-Gdnegative"). This group of patients consisted of 15 males and 41 females with a mean age of 39 years. The diagnosis of MS had been obtained with use of the Poser criteria (141). At MRI-investigation immediately preceding or following plasma and CSF collection all patients were negative with respect to gadolinium-enhancing lesions; 40/56 of the patients of these groups had also been included in the Paper III investigation.

5. Multiple Sclerosis Patients with Gadolinium-enhancing lesions ("MS-Gdpositive"). This group consisted of 25 females and 7 males with a mean age of 36 years. All the patients presented positive gadolinium-enhancing lesions on MRI; 14/20 of these patients had been included in the previous investigation (Paper I and III)

6. Patients with acute or chronic demyelinating polyneuropathies ("Demyelinating"). This group consisted of 5 females and 16 males with a mean age of 59 years. The patients in this group had Guillain-Barré syndrome (GBS) (n = 8) or "Chronic Inflammatory Demyelinating Polyradiculoneuropathy (CIDP) (n = 13). The patients all had an increased $Albumin_{CSF}$:$Albumin_{plasma}$ ratio and/or minor cytological alterations in the CSF analysis. 17/21 patients of this group had been included in the previous investigation (Paper III).

7. Patients with subarachnoid haemorrhage ("SAH"). This group of patients consisted of 4 females and 4 males with a mean age of 59 years. The CSF and plasma samples

were collected in the emergency room as a part of the diagnostic procedure in case of a suspected stroke. The diagnosis was confirmed a second time by CT-scanning; 6/8 of this group had been included in the previous investigation (Paper III)

8. Patients with Alzhemier disease ("Alzheimer"). This group of patients consisted of 30 females and 24 males with a mean age of 72 years. All the patients fulfilled the clinical criteria for "probable" AD (144,145).

Paper V

We retrospectively studied 53 patients at the Division of Neurology and the Division of Geriatrics, Huddinge University Hospital. None of these patients had been included in any of our previous studies. After normal analysis, excess CSF and parallel plasma samples were frozen at –80°C.

1. Control Patients: this group consisted of 20 females and 4 males with a mean age of 43 ± 3 years. These subjects presented with a headache of uncertain background without any clinical or laboratory signs of CNS-diseases. The patients thus had a normal $Albumin_{CSF}$:$Albumin_{plasma}$ ratio and an absence of blood in the CSF (98,99)

2. MS Patients: this group of patients consisted of 20 females and 9 males with a mean age of 41 ± 2 years. MS was diagnosed in accordance with Poser's criteria (141). On MRI investigation immediately proceeding or following CSF collection, only 5 of the patients had positive gadolinium-enhancing lesions whereas 7 were without such lesions. For the other 16 patients MRI scans had not been performed at the time of sampling.

Analysis of the oxysterols in CSF (Paper I-V)

The oxysterol levels both in CSF and in the oxidation induction experiments were determined by isotope dilution-mass spectrometry essentially as previously described (146) with minor modifications to optimize the analysis for CSF.

To a screw-capped vial sealed with Teflon-lined septum 500 □L of CSF was added together with 10 ng of [$^2H_6$] 7□-hydroxycholesterol, [$^2H_6$] 7□-hydroxycholesterol, [$^2H_6$] 7-oxocholesterol, [$^2H_3$] 24S-hydroxycholesterol, [$^2H_6$] 27-hydroxycholesterol (50 _L from a methanolic solution of 0.2 mg/L of each of the deuterated-oxysterols) as Internal Standards. To prevent autoxidation 10 _L of butylated hydroxytoluene (BHT) (5 g/L) and 20 _L of EDTA (10 g/L) were added to each vials and argon was flushed through the vial to remove air.

The alkaline hydrolysis was then allowed to proceed at room temperature (22°C) and magnetic stirring for 2 hours in the presence of ethanolic 0.35 M potassium hydroxide solution.

The lipid extract was obtained by chloroform (18 mL) extraction in separatory funnels with 6 mL 0.9% NaCl and 130 _L phosphoric acid (85%) at pH 7. The chloroform phase was evaporated at room temperature under reduced pressure using a rotary evaporator. Cholesterol was separated from oxysterols by means of solid-phase extraction using a 100 mg Isolute Silica cartridge (International Sorbent Technology, Mid Glamorgan, UK) conditioned with hexane and eluted with 0.5% 2-propanol in hexane. The oxysterols were finally eluted with 5mL 30% 2-propanol in hexane, evaporated under a gentle stream of argon and converted into trimethylsilyl ethers (pyridine:hexamethyldisilazane:trimethylchlorosilane 3:2:1 (v/v/v)).

Gas chromatography -mass spectrometry (GC-MS) analysis was performed on an Agilent Technologies HP 5890 series II combined with a 5972 mass selective detector. The GC was equipped with a DB-XLB (30 m x 0,25 mm id x 0,25 μm film; J&W, Palo

Alto, CA, USA) and injection was performed in the splitless mode using helium (1 mL/min) as a carrier gas. The temperature program was as follows: initial temperature of 150°C kept for 1 minute, followed by a rise of 20°C/min up to 260°C and immediate rise by 10°C/minute up to the end temperature of 280°C, kept for 15 minutes. The mass spectrometer was operated in the selected ion-monitoring mode, and 2 ions were detected at the same time. The ions used for the analysis (m/z) and typical retention times (in minutes) are reported in table 1.

Table 1. Steroids analysed, m/z ratio and retention time (min)

Steorid	m/z	Retention time (min)
[$^2H_6$]7□-hydroxycholesterol	462	14.55
7□-hydroxycholesterol	456	14.61
[$^2H_6$]7□-hydroxycholestrerol	462	15.98
7□-hydroxycholesterol	456	16.05
[$^2H_6$]7-oxocholesterol	478	18.83
7-oxocholesterol	472	18.96
[$^2H_3$]-24*S*-hydroxycholesterol	416	18.68
24S-hydroxycholesterol	413	18.43
[$^2H_6$] 27-hydroxycholesterol	462	19.64
27-hydroxycholesterol	456	19.74
Cholesterol	458	14.92
[$^2H_6$] cholesterol	464	14.86

Analysis of the oxysterols in plasma (Paper I-IV)

The oxysterol levels in plasma were analysed using a technique similar with to that above (cf 146). The amount of internal standard added to each samples was 200 ng (from a methanolic solution of 4 mg/L of each of the deuterated-oxysterols).

Analysis of cholesterol in CSF (paper I, V)

Cholesterol levels in CSF samples were analysed as described previously (147), with minor modifications. To a glass vial 50 □L of CSF were added together with 1 µg of [$^2H_6$] cholesterol (50 _L from a methanolic stock solution of 20 mg/L) as internal standard. To prevent autoxidation 10 _L of BHT (5 g/L) and 20 _L of EDTA (10 g/L) were added to each vial and argon was flushed through the vial to remove air. The alkaline hydrolysis was then allowed to proceed at room temperature (22°C) to mix with a magnetic stirring bar for one hour in presence of 1 ml of 99.9% ethanol and 0.25 mL of an aqueous 5.9 M KOH solution. After adding 1 ml of 0.9% NaCl and about 50 _L phosphoric acid (85%) a neutral pH (pH 7) was reached and the sterols and oxysterols were extracted by adding 2 mL chloroform and gently shaking at room temperature. The chloroform phase was evaporated under argon, then suspended in 0.5 ml of a methanol:water solution (80:20 v/v). Cholesterol was separated from other lipid compounds by means of solid-phase extraction using a 100 mg Isolute-MF C18 cartridge (International Sorbent Technology, Mid Glamorgan, UK), freshly conditioned consecutively first with 2 mL of methanol and thereafter with 2 mL of methanol:water 80:20 (v/v). The cholesterol was finally eluted from the C18 cartridge with 2.5 mL of methanol. The collected cholesterol fractions were evaporated under a gentle stream of argon. The residual cholesterol was converted into its trimethylsilyl ether and the gas chromatography -mass spectrometry (GC-MS) analysis was performed as described above.

MRI protocols (Paper II)

MRI scans performed within two months of blood sampling were available for 27 RRMS patients (20 women and 7 men) and for all 19 PPMS patients. Images were obtained using a GE Signa 1.5 Tesla scanner (General Electric Medical Systems,

Milwaukee, Wis, USA). In all patients, T2 lesion loads were determined from a proton density weighted fast spin echo sequence (28 axial slices; TE, 95 ms; TR, 2000 ms; ST, 5 mm); T1-weighted images were acquired using a conventional spin-echo sequence (28 axial slices; TE, 20 ms; TR, 540 ms; ST, 5 mm), both before and after intravenous administration of triple-dose Gd DTPA (0.3 mmol/kg; Magnevist, Schering AG, Berlin, Germany). Lesions were outlined using a semi-automated contouring technique according to previously described criteria (148), to enable calculation of lesion loads on, T2-weighted and T1-weighted scans, using Dispimage software (149).

The brain atrophy parameters were measured on the basis of a three dimensional segmentation algorithm of the t1-weighted scans. The Brain Parenchymal Fraction was calculated as the ratio of the brain parenchymal tissue volume to the total volume contained within the brain surface contour. The Grey Matter Fraction (GMF) was calculated as the ratio of the grey matter volume to the total brain volume (calculated as above). Also the White Matter Fraction (WMF) was calculated as the ratio of the volume corresponding to the White Matter to the total brain volume (150).

Infusion of hexadeuterated cholesterol into a healthy volunteer (Paper III)

In order to investigate a possible influx of 27-hydrpxycholesterol from the circulation into the CSF, material was used from a previously described experiment in which a healthy volunteer was infused with hexadeuterated cholesterol (151) was used. The experimental conditions for this infusion have been previously described (151). Briefly, phospholipid liposomes enriched with 10g of [26,26,26,27,27,27-$^{2}H_{6}$]-cholesterol were slowly infused into a healthy, normolipidemic male (age = 58, weight = 84 kg, Body Mass Index = 25). Infusion of the mixture did not produce any obvious adverse effects, and there was no effect on plasma levels of transaminases or alkaline phosphatase

during the infusions. A single collection of CSF was made by lumbar puncture three days after the start of the infusion, and analysed for deuterium enrichment as previously described (151).

Formation of oxysterols after lipid peroxidation of CSF (paper V)

In order to study the formation of the oxysterols during lipid peroxidation, we monitored the levels of the oxysterols in CSF in a time-dependent manner.

Two different models of LPO induction were chosen: 1) a metal ion-dependent mechanism, with Cu^{2+} (as Copper Sulfate $CuSO_4$) or 2) a non metal ion dependent mechanism, with 2,2.-Azobis (2-Amidinopropane) hydrochloride (ABAP).

Each experiment was run in duplicate.

CSF samples freshly collected for diagnostic purposes were pooled together to provide a sufficient quantity of sample for the experiments. The CSF was then diluted to a 1:1 ratio with NaCl 0.9% (w/v) and ABAP (Polyscience, Warrington, PA, USA) 2 or 5mM or CuSO4 250 □M were added to each test tube at time 0. The tubes were incubated for various period of time in air at 37°C. Then the peroxidation was stopped by addition of 50µl of a solution of BHT (5 g/L) and 50µl of EDTA (10 g/L). The samples were capped, vortexed and placed in the –20°C freezer until the oxysterol levels could be analyzed as described above.

Statistical calculations

Zero-order bivariate correlation analysis was performed using Sperman's correlation coefficient. Normality of the distribution was tested using Komolgorov-Smirnov test. Data of the groups was evaluated statistically using the two-tailed paired Student's t test, the Mann-Whitney rank-sum test, parametric and non-parametric Analysis of Variance (ANOVA).

Significance level was at $P<0.05$.

In Paper II, the natural logarithm transformed 24OHC/chol ratio levels in RR-, PP-MS patients and controls were compared by one-way analysis of variance with Scheffé's post hoc test; groups were stratified by age at sampling.

To assess the influence of MRI measures on 24S-hydroxycholesterol levels, multiple linear regression was performed, using, as independent variables, the three lesion-volume parameters and age at sampling (as a surrogate for the confounder disease duration); and, as the dependent variable, the 24OHC/chol ratio, doubly logarithmically transformed to fulfil the assumption of normally distributed residuals.

All analyses were performed using STATISTICA software (StatSoft Inc., Tulsa, Okla, USA).

Ethical aspects

All experiments involving the human volunteer, as well as those in which samples were obtained from patients suffering from neurological diseases were reviewed and approved by the Ethics Committee at Huddinge University Hospital.

RESULTS AND DISCUSSION

Plasma levels of 24S-hydroxycholesterol in multiple sclerosis patients and their relation to the disease processes (Paper I and II).

The results of our first investigation on MS patients are reported in table 2. Patients were divided in age decades; plasma levels of 24S-hydroxycholesterol and the 24OHC/chol ratio of both MS patients and Controls are reported. There was a tendency towards increased levels in the three youngest groups of patients (age < 50year). The difference between patients and controls was however not statistically significant (P>0.05, Students' *t*-test). In the two oldest groups of patients, the levels of 24OHC were lower than in the controls. This difference was statistically significant (P<0.05) only in the oldest group (7th decade, 61-70 age group) and not significant (P = 0.152) in the 51-60 age group.

Table 2: Plasma levels of 24S-hydroxycholesterol (24OHC) and the 24OHC/chol ratio in MS patients and Controls (Cntr).

Age	Number		24OHC (□g/L)			24OHC/chol ratio (□g/g)		
	Cntr	MS	Cntr	MS	P(<0.05)[b]	Cntr	MS	P(<0.05)[b]
21 - 30	37	27	78 ± 3	82 ± 4	0.653	44 ± 2	43 ± 2	0.728
31 - 40	30	42	76 ± 4	83 ± 4	0.218	43 ± 2	41 ± 2	0.637
41 - 50	47	34	76 ± 3	82 ± 4	0.249	37 ± 1	35 ± 2	0.532
51 - 60	49	18	85 ± 3	78 ± 5	0.152	38 ± 1	33 ± 2	0.03
61 - 70	20	6	108 ± 6	78 ± 5	0.020	45 ± 2	31 ± 2	<0.001

[a]values are expressed as mean ± SEM; [b]calculated by Student's t-test

All samples from patients with negative cranial MRI scans (n = 14) had normal levels of 24S-hydroxycholesterol.

Some of these patients had positive spinal MRI scans (n = 4).

Because of the fact that there is a covariation between levels of 24S-hydroxycholesterol and cholesterol in the circulation (41), it was considered to be of importance to compare the 24OHC/chol ratio in patients and in control subjects. This ratio was found

to be similar in the three youngest groups of patients, but significantly decreased ($P<0.05$ and $P<0.001$ respectively) in the two oldest groups of patients.

A significant inverse correlation between the Kurtzke Expanded Disability Status Scale (EDSS)-grade and the 24OHC/chol ratio ($P<0.05$) was also found.

According to current concepts, the presence of gadolinium-enhancing lesions may be used as a marker of disease activity, since gadolinium-enhancement occurs as a result of increased BBB permeability and corresponds to areas with ongoing inflammation (120). In our population of patients in paper I, 48 were evaluated with respect to the presence or absence of such changes. Among these patients gadolinium-enhancing lesions were found in 19 of the 48 patients studied. The former group of patients had levels of 24S-hydroxycholesterol (88 ± 6 μg/L) that were higher but not significantly different from those lacking gadolinium-enhanced lesions (81 ± 5 μg/L).

The results may be considered to be consistent with, but do not prove our initial hypothesis, that an acute inflammatory episode of demyelination in MS is associated with increased flux of 24S-hydroxycholesterol from the brain to the circulation. Such as increased flux was thought to be more likely to occur in young MS-patients at an early stage of the disease. In accordance with this hypothesis, the highest levels of the 24S-hydroxycholesterol were found in young patients with positive cranial MRI scans.

With the progression of the disease, there may be an increased loss of neuronal cells able to synthesize 24S-hydroxycholesterol: this should lead to reduced flux of 24S-hydroxycholesterol from the brain with lower level of the oxysterols in the circulation. In accordance with this the absolute as well as the cholesterol related levels of 24S-hydroxycholesterol were found to be reduced in the older (>50 years) patients with MS. This situation may be similar to that in AD patients.

In a more recent paper, Teunissen et al (44) confirmed our results. The 24OHC/chol ratio was found to be lower in PP-MS patients than in healthy controls and a significant

negative correlation with age was reported in the MS patients. The decreased levels in the MS patients older than 50 years were also confirmed. No correlation was found between 24OHC/chol ratio and EDSS grade in that study.

The pathophysiology of MS is heterogeneous, however, with lesions classically progressing through the following stages: 1) inflammation and demyelination; 2) edema and gliosis; 3) axonal loss and tissue degeneration (101). It appears likely that each of these processes could leave its characteristic mark on plasma levels of 24S-hydroxycholesterol.

Paper II reports a collaborative study on a group of English MS-patients with an MRI examination performed in parallel with the blood collection. In this study it was possible to relate the above stages with the peripheral levels of 24S-hydroxycholesterol. As surrogates for these histopathologically defined processes, we made use of the following magnetic resonance imaging (MRI) measures: 1) the total volume of gadolinium (Gd)-enhanced lesions (believed to be related with foci to BBB disruption (120), as a marker of ongoing inflammation measured at T_1-weighted brain scans); 2) the total volume of hypointense lesions (corresponding histopathologically to areas devoid of myelinated axons (122-125), as a marker of irreversible neuronal damage, measured at T_1-weighted brain scans); 3) the total volume of hyperintense lesions (corresponding to a spectrum of events, from demyelination and edema to gliosis and axonal loss (119), as a marker of cumulative disease burden at T_2-weighted brain scans). Finally plasma 24S-hydroxycholesterol was related to data on brain atrophy evaluated by MRI available in some of the patients.

The demographic, biochemical and MRI measures in MS patients and healthy controls are given in the table 3.

Table 3. Demographic, biochemical and MRI measures in MS patients and healthy controls

	RRMS (n=37)	PPMS (n=19)	Controls (n=23)
Age at sampling [years; mean (range)]	36.7 (24-56)	43.5 (23-58)	33.6 (25-53)
Gender, female:male	27:10	8:11	12:11
Duration at sampling [years; mean (range)]	1.9 (0.5-3.7)[a]	3.3 (2.0-5.0)	NA
EDSS score at sampling [median (range)]	1.25 (0-3.5)[b]	5.0 (3.5-7.0)	NA
Plasma 24OHC [µg/L; mean (IQ range)]	63.3 (54.3-73.0)	63.9 (51.5-69.5)	74.5 (61.7-89.6)
Plasma chol [g/L; mean (IQ range)]	1.70 (1.47-1.94)	1.82 (1.57-2.10)	1.73 (1.46-1.82)
Ratio 24OHC/chol [µg/g; mean (IQ range)]	37.3 (32.3-41.9)	35.8 (28.0-42.7)	45.6 (35.2-57.6)
Gd-lesion volume [mm^3; mean (range)]	520 (0-2210)[a]	190 (0-580)	NA
T_1-lesion volume [mm^3; mean (range)]	2970 (100-37570)[a]	4950 (0-31900)	NA
T_2--lesion volume [mm^3; mean (range)]	9900 (1120-47940)[a]	13850 (200-87410)	NA

RR=relapsing-remitting; PP=primary progressive; EDSS=Expanded Disability Status Scale; 24OHC=24S-hydroxycholesterol; chol=cholesterol; IQ=interquartile; Gd=gadolinium; NA=not applicable; [a]n=27; [b]n=30

In subjects younger than 35 years of age at sampling, the log-transformed 24OHC/chol ratio did not differ significantly (P = 0.288) between RRMS patients (n = 17), PPMS patients (n = 5) and controls (n = 16). In subjects aged 35 or older, however, the log-transformed 24OHC/chol ratio differed significantly between groups (P <0.001); it was significantly lower in both PPMS patients (n = 14; P<0.001) and RRMS patients (n = 20; P <0.001) than in controls (n = 7), but did not differ between the two disease-course subgroups.

In the case of 27-hydroxycholesterol, cholesterol and 27-hydroxycholesterol:cholesterol ratio, no statistically significant differences were found between Controls and PP- and RR-MS groups.

The results of the analysis by multiple linear regression of the influence of age at sampling and the three lesion-volumes measures on log-log-transformed 24OHC/chol ratios are presented in Table 2 of paper II.

As expected, in both RRMS and PPMS, age at sampling was negatively correlated with the ratio ($\beta = -0.35$ in RRMS, and $\beta = -0.76$ in PPMS); indeed, it was the only variable

significantly associated with the ratio in either model (P = 0.006 in PPMS). Regression coefficients for the volume of T_2-weighted hyperintense lesions were strikingly similar in the disease-course subgroups, (= 0.52 in RRMS, and = 0.50 in PPMS). These correlations are in accordance with the steady decreases in the 24OHC/chol ratio and suggest that the reduction of the ratio may reflect the total spatiotemporal burden of MS—i.e., the cumulative effects of its dissemination in the CNS and its duration in time.

The volume of Gd-enhanced lesions in PPMS and the volume T_1-weighted hypointense lesions in RRMS were positively correlated with the 24OHC/chol ratio, albeit nonsignificantly, (= 0.38) and (= 0.59), respectively. These findings suggest that intermittent increases in the ratio may correspond to the activation of spatially delimited processes that deviate from background pathophysiology—i.e., to focal degeneration in RRMS, and to focal inflammation in PPMS.

Considering all the MS patients together, not ranked with respect to age, a significant direct correlation was observed between the plasma 24OHC/chol ratio and the Gray Matter Fraction (GMF) (r = 0.361, P = 0.02).

A significant negative correlation was also found between the 24OHC/chol ratio and the White Matter Fraction (WMF) (r = -0.313, P < 0.05).

In case of RR patients (n = 9), there was a significant reverse correlation between the 24OHC/chol ratio and WMF (r = -0.705, P<0.03) (Figure 1A). A positive, but statistically not significant correlation was also found between the 24OHC/chol ratio and GMF (r = 0.62, P = 0.07) (Figure 1B).

In PP patients the patients were divided with respect to absence or presence of gadolinium enhancing lesions. In case of patients without gadolinium-enhancing lesions, a significant and positive correlation was found between plasma 24OH/chol

levels and both GMF (r = 0.641, P = 0.03) (Figure 1C) and Brain Parenchymal Fraction BPF (r = 0.6, P = 0.05) (Figure 1D). In the second group a strong and significant reverse correlation was found between the 24OHC/chol ratio and the (BPF) (r = -0.777; P< 0.05). The 24OHC/chol ratio levels were slightly increased in the second group as compared to the first group.

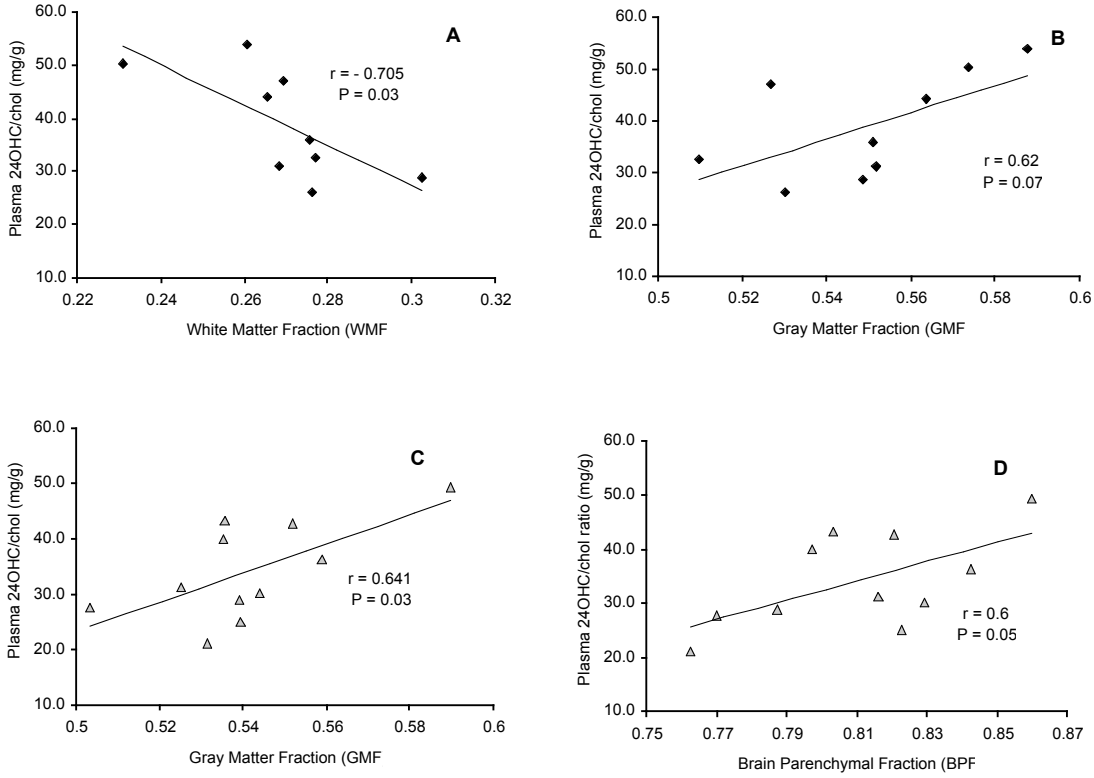

Figure 2. In case of RRMS patients a significant reverse correlation was found between the 24OHC/chol ratio and the White Matter Fraction (panel A). The 24OHC/chol ratio was also found positively correlated with the Gray Matter Fraction (GMF) (panel B). In case of PPMS patients the 24OHC/xchol ratio was found to be significantly correlated with the GMF (panel C) and the Brain Parenchymal Fraction (BPF) (panel D).

Considered all together, the above preliminary data are consistent with the hypothesis that neurodegeneration, with loss of neurons, leads to reduced levels of cholesterol 24S-hydroxylase with subsequent reduction in the formation of 24S-hydroxycholesterol and a reduced 24S-hydroxycholesterol efflux from the brain into the circulation.

In accordance with this, significant correlation between the degree of brain atrophy or the total spatiotemporal burden of the disease and plasma levels of 24S-hydroxyhcolesterol was found.

During phases of acute neuronal loss, the possibility must be considered that there may be an increased release of 24OHC from the affected brain areas. Instead, in MS patients with chronic disease the levels of 24OHC are decreased as a consequence of the reduced viable neuronal cells.

It may be speculated that increased levels of 24S-hydroxycholesterol could help to identify both RRMS patients in need of neuroprotective therapy, and PPMS patients in need of immunosuppression. In absence of superimposed inflammatory processes, the plasma levels of 24S-hydroxycholesterol might become a reliable biological marker for evaluation of brain atrophy during CNS-disease.

Further study with larger groups of patients are needed however to allow firm conclusion.

Evidence for entry of plasma 27-hydroxycholesterol into the CSF (paper III)

Evidence for a flux of 27-hydroxycholesterol from the circulation into the CSF in a healthy volunteer (Paper III)

Three days after the start of infusion of $^2H_6$-cholesterol, when the deuterium enrichment of plasma cholesterol was maximal (≈ 13%), a sample of CSF was collected and analysed in parallel with the plasma sample. There was no significant incorporation of deuterium in cholesterol or 24S-hydroxycholesterol. The observed deuterium enrichment of CSF 27-hydroxycholesterol was about 4%. A similar degree of enrichment was found in the free fraction of 27-hydroxycholesterol in a matching plasma sample (S. Meaney, unpublished observation). At this time point the deuterium enrichment of total plasma 27-hydroxycholesterol was about 3% (135).

Levels of 24S- and 27-hydroxycholesterol in plasma and CSF of patients with a compromised BBB (Paper III)

No statistically significant differences were found when plasma levels of 24S- and 27-hydroxycholesterol in the group with a defective BBB were compared with those in Controls (Student's *t* test); however, CSF levels of both oxysterols were significantly increased in the BBB group ($P<0.01$ and $P<0.001$, respectively). Furthermore, there was a highly significant correlation between 24S- and 27-hydroxycholesterol in CSF ($r = 0.74$; $P<0.001$). There was also a highly significant correlation between the levels of both oxysterols in CSF and the $Albumin_{CSF}$:$Albumin_{plasma}$ ratio ($r = 0.55$, $P<0.001$ and $r = 0.68$, $P<0.001$ for 27- and 24S-hydroxycholesterol, respectively).

Application of the Mann-Whitney rank-sum test, as required by the nonparametric distribution of the values, revealed a statistically significant increase ($P<0.001$) in the 27-hydroxycholesterol$_{CSF}$:24*S*-hydroxycholesterol$_{CSF}$ ratio in the patient group.

The finding that the 27-hydroxycholesterol$_{CSF}$:24S-hydroxycholesterol$_{CSF}$ ratio was increased about four-fold compared to that in the brain, and that it is four-fold lower than the corresponding ratio in the circulation, suggests that the bulk of the CSF 27-hydroxycholesterol originates from the circulating pool. In accordance with this a statistically significant correlation ($P<0.01$) between 27-hydroxycholesterol in the plasma and the CSF was observed in our patients. Furthermore, it was possible to demonstrate that, after infusion of hexadeuterated cholesterol into a healthy volunteer, the deuterium enrichment of total CSF 27-hydroxycholesterol was about equal to that of the free fraction of plasma 27-hydroxycholesterol. Taken together these results support the idea that the major portion of 27-hydroxycholesterol, but not CSF cholesterol or 24S-hydroxycholesterol, originates from the circulation.

By measuring the arterial-venous difference of 27-hydroxycholesterol over the brain in catheter experiments with healthy volunteers, evidence was recently obtained that there is a net uptake of this steroid by the brain from the circulation (Heverin M., unpublished).

A consequence of the extracerebral origin of CSF 27-hydroxycholesterol is that a defect in the blood-brain or blood-CSF barriers should lead to an increased entry of plasma 27-hydroxycholesterol into the CSF, with a resulting increase in the 27-hydroxycholesterol$_{CSF}$:24S-hydroxycholesterol$_{CSF}$ ratio. A clear increase was observed in the absolute concentration of 27-hydroxycholesterol in CSF from such patients, in concert with an increased Albumin$_{CSF}$:Albumin$_{plasma}$ ratio. Surprisingly, CSF levels of 24S-hydroxycholesterol were also increased in those subjects. If this increase corresponds to a direct leakage of plasma oxysterols into the CSF, the 27-hydroxycholesterol$_{CSF}$:24S-hydroxycholesterol$_{CSF}$ ratio would be expected to approach

that of plasma. However, in the majority of individuals with a defective BBB this was not the case, indicating that most of the CSF 24S-hydroxycholesterol originates from the brain.

The highly significant correlations observed between 27-, 24S-hydroxycholesterol and $Albumin_{CSF}$:$Albumin_{plasma}$ ratio suggest that the CSF-blood barrier dysfunction as well as the alteration of the CSF-flow rate (98,99) are important determinants of the CSF oxysterols levels. That the CSF-levels of 24S-hydroxycholesterol may also increase in patients with an intact blood-CSF barrier (i.e., not increased $Albumin_{CSF}$:$Albumin_{plasma}$ ratio) was illustrated here in the MS patients with a normal $Albumin_{CSF}$:$Albumin_{plasma}$ ratio ("*MS-active"*). We can speculate that CSF 24S-hydroxycholesterol levels are affected by: i) CSF-flow rate; ii) massive release from damaged neuronal cells and/or myelin; and iii) increased permeability of the BBB as consequence of inflammation or direct barrier damage.

Another possible explanation to our findings is that an increase in the concentrations of plasma-derived lipoproteins, as a result of a decreased CSF flow rate, results in a more efficient extraction of 24S-hydroxycholesterol from the brain. In view of the relation between molecular size and flux over the blood-brain and blood-CSF barriers, and given the size of a typical lipoprotein particle, this is likely to occur only in situations with a massive loss of integrity and functionality of blood-brain and blood-CSF barriers.

Finally the possibility must be considered that there is a "replacement" of some of the 24S-hydroxycholesterol in the brain with the 27-hydroxycholesterol fluxing in from the circulation.

Recently, consistently lower levels of 24S-hydroxycholesterol were found in all areas of the brain of AD patients compared to controls (71). In the same samples the 27-hydroxycholesterol levels were significantly increased with a general increase of the

27-hydroxycholesterol:24S-hydroxycholesterol ratio. The plant sterols were also found to be higher (71).

Attempts to define CSF 24S- and 27-hydroxycholesterol levels in a normal population (Paper III and IV)

A general problem with studies on CSF is to obtain suitable control material. CSF is almost exclusively collected from subjects with some type of symptoms. The general strategy is to utilize CSF samples from subjects who have been subjected to a number of clinical and biochemical investigations without any positive findings. According to Reiber (98,99) normal control patients can be defined clinically (absence of brain-organic diseases according to clinical and imaging criteria) and via their CSF and blood analysis.

In order to define a control group to evaluate the CSF levels of both 24- and 27-hydroxycholesterol, in paper III we used the following criteria for inactive MS-disease: *i*) "normal" $Albumin_{CSF}$:$Albumin_{plasma}$ ratio ($< 8 \times 10^{-3}$); *ii*) normal levels of 24S-hydroxycholesterol in the plasma ($\leq$ 120 ng/mL) and CSF ($\leq$ 3.5 ng/mL), as defined by Ref. 15; *iii*) age $\leq$ 60 years; *iv*) absence of gadolinium-enhancing lesions on the MRI investigation immediately preceding or following plasma and CSF collection. In this group of patients (analysed in a previous study (cfr Paper I) (n = 49, mean age, 41 years, female:male, 33:15) the CSF levels of 24 and 27-hydroxycholesterol were 1.96 ± 0.1 □g/L and 0.76 □g/L, respectively. There was a low but statistically significant correlation between CSF and plasma levels of both 24S-hydroxycholesterol ($P < 0.05$) and 27-hydroxycholesterol ($P < 0.01$).

The control group used in Paper III was not regarded to be optimal for a broader category of patients. In the expanded investigation (see Paper IV), a selected group of

headache patients with normal biochemical parameters were used as controls. The levels of 24S-hydroxycholesterol were 1.38 ± 0.07 μg/L and the levels of 27-hydroxycholesterol 0.51 ± 0.02 μg/L. The 95% confidence interval was 0.4 – 2.5 μg/L and 0.2 - 1 μg/L, for 24S-hydroxycholesterol and 27-hydroxycholesterol, respectively.

No statistically significant differences with respect to age and sex were found. There was however a statistically significant correlation between CSF-levels of 24S-hydroxycholesterol and 27-hydroxycholesterol ($r = 0.58$, $P < 0.001$). As expected there was no significant correlation between 24- or 27-hydroxycholesterol levels and $Albumin_{CSF}$:$Albumin_{plasma}$ quotient or between the 24S-hydroxycholesterol/27-hydroxycholesterol ratio in CSF and $Albumin_{CSF}$:$Albumin_{plasma}$ quotient. A significant but low correlation was found between the plasma levels of 24S- and 27-hydroxycholesterol ($r = 0.38$, $P<0.001$) . A significant correlation was found also between plasma and CSF levels of 27-hydroxycholesterol ($r = 0.3$, $P < 0.05$). No correlation was found between the plasma and CSF levels of 24S-hydroxycholesterol.

It should be pointed out that the above control group may not be optimal when evaluating older patients with multiple sclerosis and Alzheimer's disease.

Table 4. $Albumin_{CSF}$:$Albumin_{plasma}$ ratio (QAlb), absolute plasma and CSF levels of 24S-hydroxycholesterol (24OHC) and 27-hydroxycholesterol (27OHC) in the different diagnostic groups.

	Size	Qalb[a]	24OHC CSF[a]	27OHC CSF[a]	24OHC plasma[a]	27OH plasma[b]
	n	x 10^{-3}	μg/L	μg/L	μg/L	μg/L
Controls	58	5.9 ± 0.2	1.38 ± 0.07	0.51 ± 0.02	73.1 ± 3.42	122 (109 – 142)
MS-Gdnegative	56	6.1 ± 0.15	1.74 ± 0.08	0.71 ± 0.04	72.4 ± 2.02	106 (91 – 125)
MS-Gdpositive	32	8.8 ± 1.1	2.62 ± 0.13[d]	1.06 ± 0.10[c]	78.8 ± 3.93	112 (92 – 141)
Alzheimer	54	9.7 ± 1.0	2.47 ± 0.12[d]	1.51 ± 0.12[d]	70.5 ± 2.68	138 (98 – 153)
Demyelinating	21	32.3 ± 4.9	6.41 ± 0.58[d]	4.02 ± 0.69[d]	69.2 ± 3.29	127 (106 – 141)
SAH	8	17.9 ± 3.7	1.81 ± 0.18	2.34 ± 0.25[d]	68.5 ± 4.50	143 (90 – 152)
Vmeningitis	12	13.1 ± 2.1	3.46 ± 0.4[d]	2.43 ± 0.26[d]	65.4 ± 6.39	131 (96 - 170)
Borrelia	14	14.0 ± 1.2	2.51 ± 0.23	2.52 ± 0.38[d]	76.2 ± 7.26	154 (120 – 187)

[a]data are expressed as mean ± SEM. [b]Data are expressed as median and interquartiles range. [c]P <0.05 compared with cont rols (ANOVA). [d]P<0.001 compared with controls (ANOVA). SAH, subarachnoid haemorrhage; Demyelinating: Guillain -Barrè-syndrome and chronic inflammatory demyelinating polyradiculoeuropathy

Diagnostic use of 24S- and 27-hydroxycholesterol determination in CSF (Paper III and IV)

Levels of 24S- and 27-hydroxycholesterol in CSF and plasma in the different groups of patients (Paper III and IV)

The CSF and plasma levels of 24S- and 27-hydroxycholesterol in the different diagnostic group are reported in Table 4 and Figure 2. These represent a summary of the data presented and discussed in the two different papers.

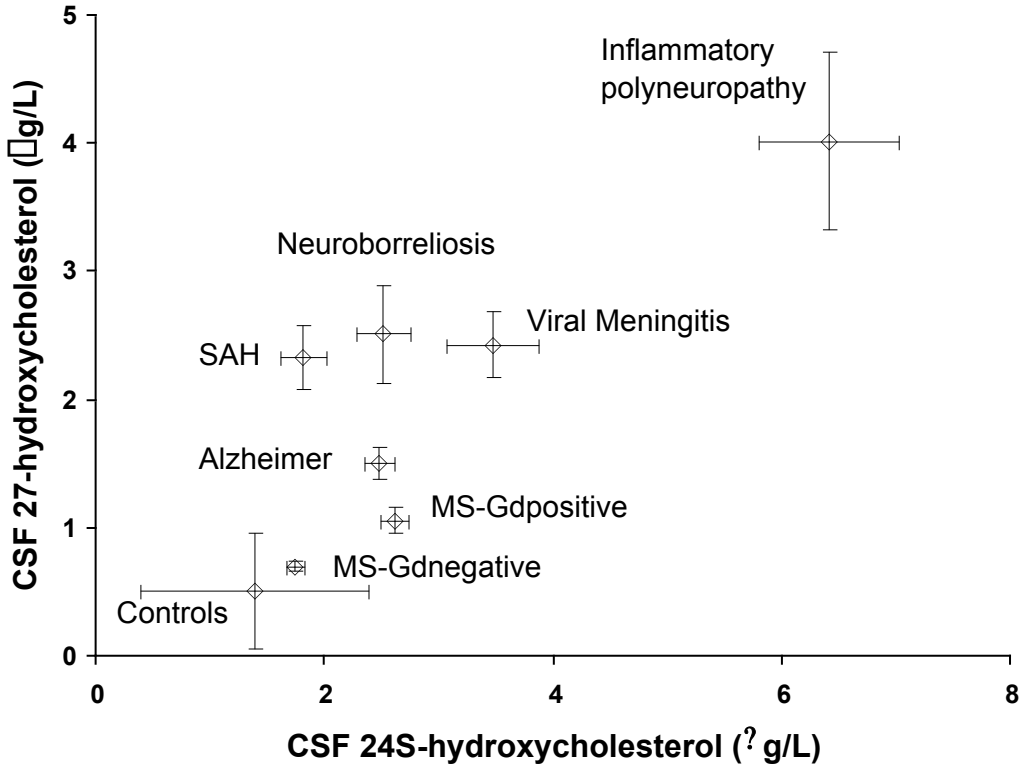

Figure 2. CSF 24S- and 27-hydroxycholesterol levels in the different diagnostic groups (results presented as mean ± SEM). The control group is expressed as mean and 95% confidence interval for both 24S- and 27-hydroxycholesterol.

No statistically significant differences in plasma levels of 24S-hydroxycholesterol were found (one-way ANOVA). In contrast to the slight changes of the plasma levels of the two oxysterols, marked changes of the CSF-levels were found in the different groups. The CSF levels of 24S-hydroxycholesterol were significantly increased in case of the Demyelinating ($P<0.001$), Vmeningitis ($P<0.001$), MS-Gdpositive ($P<0.001$), and

Alzheimer (P<0.001) groups when compared to those of the controls. No statistically significant differences were found between the MS-Gdnegative, SAH and Control groups. The CSF 24S-hydroxycholesterol levels of the patients with viral meningitis were significantly higher than those of the SAH (P<0.01) and Alzheimer groups (P<0.05) but not different from those of the MS-Gdpositive and Borrelia- groups (see also Figure 2).

The CSF 27-hydroxycholesterol levels of the Demyelinating (P<0.001), SAH (P<0.001), Vmeningitis (P<0.001), Borrelia (P<0.001), the Alzheimer (P<0.001), MS-Gdpositive (P<0.001), and MS-Gdnegative (P<0.05) groups were all found to be significantly increased as compared to the Controls. A statistically significant correlation was found between 24S- and 27-hydroxycholesterol CSF levels in all the different diagnostic groups. Low but significant correlations were also found between the CSF and the plasma levels of 27-hydroxycholesterol in the case of SAH ($r = 0.7$, P<0.01) and MS-Gdpositive ($r = 0.55$, P<0.001), but not in the other pathological groups.

24S-hydroxycholestero:27-hydroxycholesterol ratio in CSF in the different groups of patients (Paper IV)

The 24S-hydroxycholesterol:27-hydroxycholesterol ratio was calculated for each of the different groups. After application of ANOVA, the levels of the SAH, Vmeningitis, Borrelia, Demyelinating and Alzheimer-groups were found to be significantly lower than those of the Controls (P<0.05). No significant differences were found between the two different MS-groups and the Controls.

Fraction of patients with elevated values in the different diagnostic group(Paper IV)

From the normal distribution of the 24S- and 27-hydroxycholesterol levels in the CSF of the Control population, a 95% confidence interval was calculated for both of the oxysterols. The upper cut-off value was calculated to be 2.5 μg/L and 1.0 μg/L for 24S- and 27-hydroxycholesterol, respectively.

The individual values of each patient in each group were compared with the two cut-off values. The absolute and relative (expressed as percentage) values are given in Table 5 and Figure 3.

In case of diseases with a predominant BBB-dysfunction, increased levels of 27-hydroxycholesterol were frequently found (Borrelia, Vmeningitis and SAH-groups). However in the case of a disease with predominant demyelination the increase of 24S-hydroxycholesterol was higher (56% vs 34 % for 24S-hydroxycholsterol and 27-hydroxycholesterol, respectively, in MS-Gdpositive group).

Table 5. Patients (absolute number and percentage) with CSF of either 24 - (24OHC) or 27-hydroxycholesterol (27OHC) or both oxysterols increased above the 95th percentile of the confidence interval.

	Total	Patients with increased CSF 24OHC	Patients with increased CSF 27OHC	Patients with at least one oxysterol increased
	n	n (%)	n (%)	n (%)
MS-Gdnegative	56	5 (9)	8 (14)	13 (23)
MS-Gdpositive	32	18 (56)	11 (34)	22 (69)
Alzheimer	54	26 (48)	33 (61)	37 (69)
Borrelia	14	6 (43)	11 (79)	12 (86)
Vmeningitis	12	7 (58)	12 (100)	12 (100)
SAH	8	1 (13)	7 (88)	7 (88)
Demyelinating	21	17 (81)	18 (86)	19 (91)

The combined data suggests that different neurological diseases affect the levels of the two oxysterols in CSF to a much higher extent than the corresponding levels in the circulation. It should be emphasized that less than 1% of the total flux of 24S-hydroxycholesterol from the brain corresponds to a primary secretion of the oxysterol

into the CSF. This means that changes in plasma levels of 24S-hydroxycholesterol are likely to reflect changes in the flux of 24S-hydroxycholesterol from the brain whereas changes in CSF-levels reflect changes of a very small part only of the total flux. Why the latter flux is more sensitive to intracerebral changes in the over-all cholesterol homeostasis is not clear. According to a previous pilot study (42) serious neurological damage seems to be required to significantly affect the plasma levels of 24S-hydroxycholesterol.

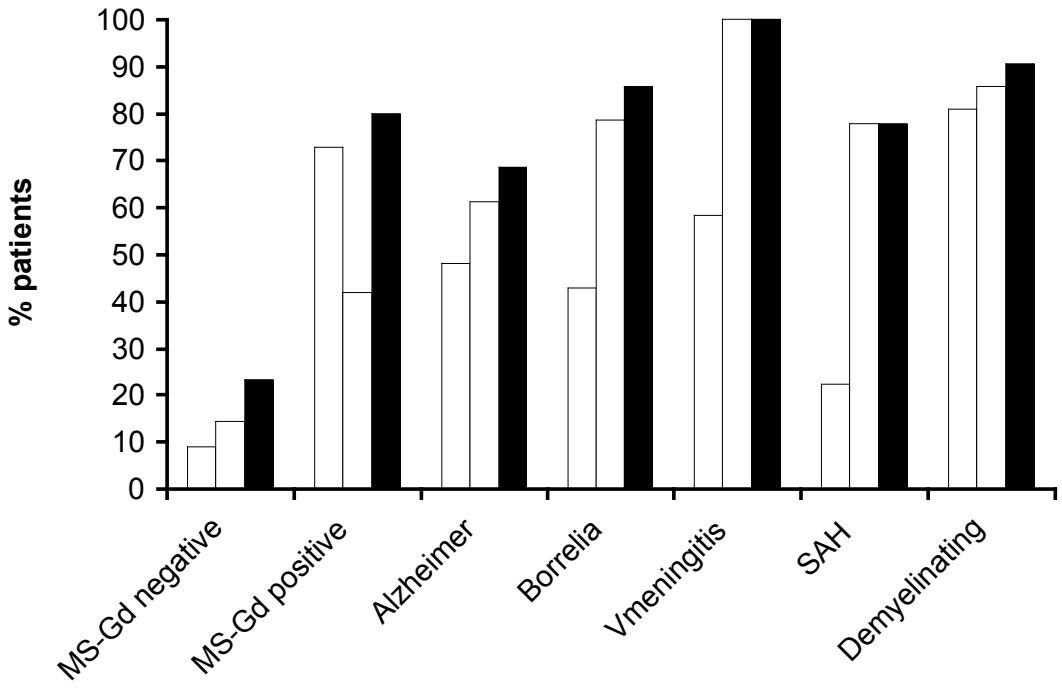

Figure 3. Fraction of patients in the different diagnostic groups with CSF-levels of one or both of the two oxysterols increased above the upper reference value (95th percentile). The proportion was calculated as number of patients with increased 24S-hydroxycholesterol (grey bar) or 27-hydroxycholesterol (white bar) or both oxysterols (black bar) divided by number of patients with specific disease and expressed as %.

The hypothesis tested here is that neuronal damage and/or demyelination causes increased CSF levels of 24S-hydroxycholesterol, and that a compromised BBB causes increased levels of 27-hydroxycholesterol.

Theoretically a demyelination and/or a neuronal degeneration would not be expected to affect the levels of 27-hydroxycholesterol and a defect in BBB functionality would be

expected to only slightly affect the levels of 24S-hydroxycholesterol. In patients with active MS and AD, the CSF levels of both oxysterols were increased. The levels of 24S-hydroxycholesterol were however increased considerably more than those of 27-hydroxycholesterol. The increased levels of 27-hydroxycholesterol may be due to some dysfunction in BBB, and it has been reported that patients with AD as well as those with MS may have an increased $Albumin_{CSF}$:$Albumin_{plasma}$ ratio (152). Using the age-related $Albumin_{CSF}$:$Albumin_{plasma}$ ratio upper-reference limits (98,99), the present AD patients were divided in two subgroups. The 27-hydroxycholesterol levels were significantly higher in the patients with increased $Albumin_{CSF}$:$Albumin_{plasma}$ ratio than in those with normal $Albumin_{CSF}$:$Albumin_{plasma}$ ratio. Similar results were also observed in the MS-Gdpositive patients (data not shown). As expected, the levels of 27-hydroxycholesterol were increased more than the levels of 24S-hydroxycholesterol in patients with viral and Borrelia meningitis. Part of the reason for the simultaneous increase in the levels of 24S-hydroxycholesterol in these patients could be that these conditions often are associated with encephalitis with some demyelination and/or a direct involvement of the neurons. Another explanation could be that an increased flux of lipoproteins into CSF may increase the extraction of 24S-hydroxycholesterol from the brain. In the case of SAH there is a direct flux of plasma into the subarachnoidal space and thus a direct contamination of the CSF. As expected, the CSF-levels of 27-hydroxycholesterol were found to be markedly increased in these patients without a significant increase in 24S-hydroxycholesterol.

In view of the low fraction of patients in the different diagnostic groups without increased levels of at least one of the two oxysterols (cf. table 5), it seems likely that normal levels of both oxysterols in CSF may have a high negative predictive value.

Levels of 7-oxocholesterol in CSF in patients with multiple sclerosis are more than one thousand lower than reported (Paper V)

Levels of 7-oxygenated steroids in CSF from controls and MS patients (Paper V)

The median CSF concentrations, ranges [minimum – maximum] and Inter quartile (25%-75%) confidence intervals of cholesterol, oxysterols and the ratios of the oxysterols to cholesterol concentrations from control and MS patients are shown in table 6.

Table 6. Absolute cholesterol and oxysterol levels and ratios of oxysterols to cholesterol in CSF from control (Control) and MS patients.

	Controls	MS
	n = 24	n = 29
Cholesterol (mg/L)	4.50 [2.23 – 19.3] (3.86 - 5.93)[a]	4.20 [1.93 - 21.7] (3.04 - 5.14)
7□-OHC (□g/L)[a]	1.5 [0.67 - 4.43] (1.12 - 2.33)	2.01 [0.65 – 6.68] (1.20 - 2.81)
7□-OHC (□g/L)	1.11 [0.88 – 3.75] (0.79 - 1.24)	1.43 [0.52 – 4.70] (0.98 - 2.26)
7-oxo (□g/L)	0.86 [0.30 – 2.34] (0.69 – 1.05)	1.24 [b] [0.44 – 4.60] (0.97 – 1.72)
24OHC (□g/L)	1.54 [0.88 – 3.12] (1.20 - 2.29)	1.52 [0.45 – 7.16] (1.06- 2.09)
27OHC (□g/L)	0.80 [0.51 – 3.75] (0.65 - 0.95)	0.97 [0.41 – 6.70] (0.71 - 1.35)
7□-OHC/cholesterol (□g/mg)	0.29 [0.12 - 0.88] (0.21-0.52)	0.51 [0.06 - 1.10] (0.21 - 0.82)
7□-OHC/cholesterol (□g/mg)	0.19 [0.07 - 0.84] (0.17 - 0.28)	0.39 [0.05 - 1.10] (0.21 - 0.54)
7-oxo/cholesterol (□g/mg)	0.20 [0.06 - 0.45] (0.16 - 0.25)	0.32 [c] [0.04 - 1.47] (0.19 - 0.52)
24OHC/cholesterol (□g/mg)	0.35 [0.15 - 0.66] (0.25 - 0.51)	0.33 [0.09 - 1.93] (0.26 - 0.52)
27OHC/cholesterol (□g/mg)	0.20 [0.02 - 0.39] (0.14 - 0.25)	0.23 [0.05 - 0.64] (0.15 - 0.32)

[a]Abbreviations: 7□-OHC:7□-hydroxycholesterol; 7□-OHC: 7□-hydroxycholesterol;
7oxo: 7-oxocholesterol; 24OHC: 24S-hydroxycholesterol; 27OHC: 27-hydroxycholesterol;
[b] Significant difference from Controls; p = 0.002; [c] significant difference from Controls: p = 0.018

The levels of 24S-hydroxycholesterol (24OHC) and 27-hydroxycholesterol (27OHC) were similar to those reported in Paper III and IV and the levels of 27-hydroxycholesterol were slightly higher in the MS patients than in the controls. The levels of the 7-oxygenated oxysterols were in the same order of magnitude as those of 24S-hydroxychoelsterol and 27-hydroxycholesterol. In the case of 7-oxocholesterol the

levels were slightly higher in the MS patients than in the controls (p=0.002). No significant difference was found for 7α- and 7β-hydroxycholesterol between MS patients and controls (p = 0.23 and p = 0.097, respectively).

The ratio of 7-oxocholesterol to cholesterol was significantly higher in MS patients compared with Controls (p = 0.018).

Increase in levels of 7-oxygenated steroids after oxidation of cholesterol in CSF (Paper V)

Uncontrolled lipid peroxidation by incubation of a pool of CSF at room temperature for 24 hours was found to increase the levels of 7β-hydroxycholesterol by 36% (p = 0.002), 7α-hydroxycholesterol by 102% (p = 0.002), and 7-oxocholesterol by 44% (p = 0.033). No significant changes in the concentrations of cholesterol, 24OHC and 27OHC could be found.

Finally, in order to evaluate the effect of in vitro induced lipid peroxidation, diluted (1:1 v/v) CSF was incubated with Cu^{2+} (as $CuSO_4$) or with an hydroxyl-radical generator, ABAP as lipid peroxidation triggers.

In the first experiment the formation of oxysterols in CSF with copper as a triggering agent was studied (Figure 4A). The metal-ion dependent mechanism represents a "classic" way to induce lipid peroxidation (153,154).

After a 24-hour incubation period there was a significant increase (2.6 times) in levels of 7-oxocholesterol. 7α- and 7β-hydroxycholesterol were also significantly increased, (4.7 and 3.1 times, respectively). There was a slight decrease in the levels of 27-hydroxycholesterol, while there was a more marked decrease in the levels of 24S-hydroxycholesterol. A similar effect on 24S- and 27-hydroxycholesterol has been previously reported in connection with lipid peroxidation of LDL from plasma (77,78).

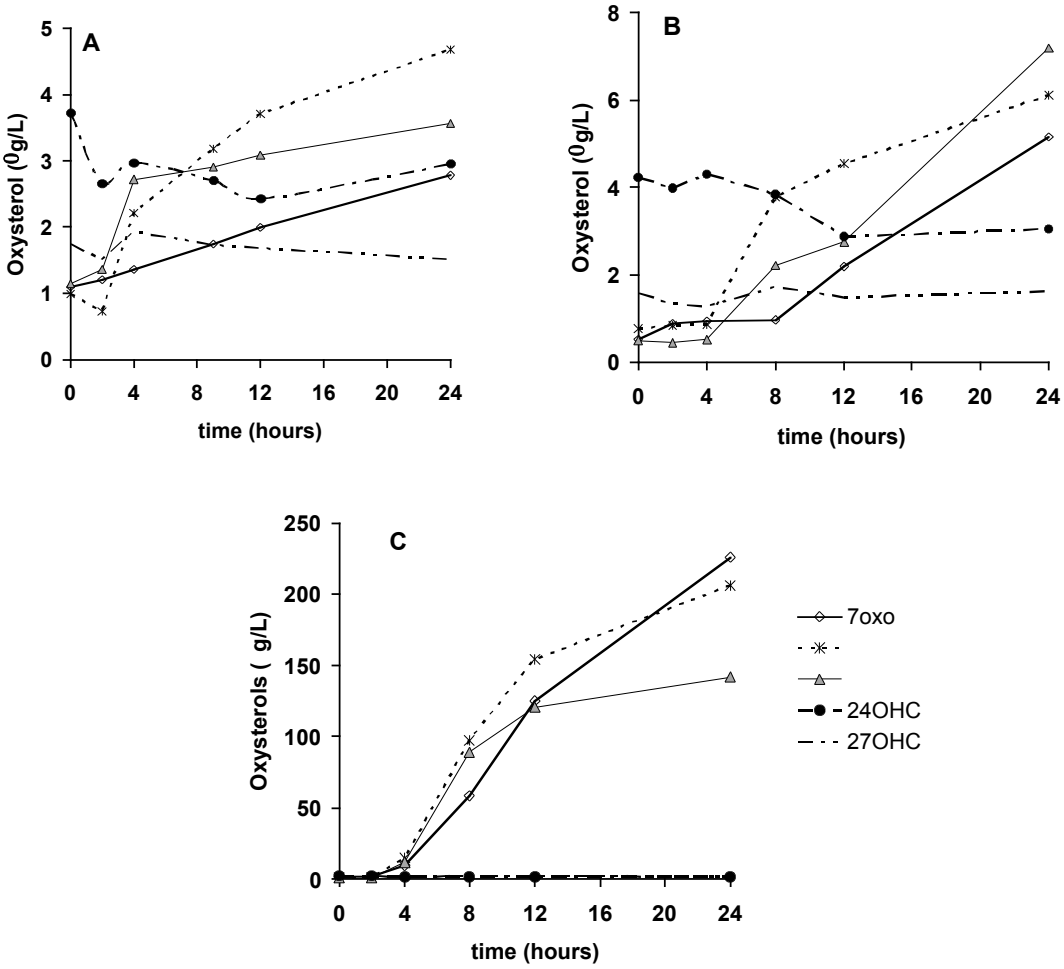

Figure 4. The 7-oxygenated sterols are increased by in-vitro induced lipid peroxidation. The CSF was diluted 1:1 with NaCl 0.9%. At time 0 the lipid peroxidation was induced adding Cu^{2+} (as $CuSO_4$) (Panel A), ABAP 2mM (Panel B) or 5mM (Panel C). The reaction was stopped by the addition of 50 □L of a solution of BHT (5 mg/L) and 50□L of EDTA (1 g/L). The samples were capped, vortexed and placed in the -20°C freezer until the oxysterols levels could be analysed as described in Material and Methods.

Figure 4B summarises the data on oxysterol formation during lipid peroxidation induced by using 2 mM of ABAP. Such conditions represent a non-metal dependant model of lipid peroxidation with a low intensity of the flux of the initiator peroxy radicals (155). In similarity with the above experiment there was a significant increase in all the 7-oxygenated products (between 8 to 14 times). The levels of 27-hydroxycholesterol remained stable throughout the 24-hour period, while the levels of 24S-hydroxycholesterol decreased slightly.

In Figure 3C results are shown of an experiment using a higher concentration of ABAP (5 mM). Under these conditions the increase in the levels of 7α-hydroxy-, 7β-hydroxy- and 7oxo-cholesterol became more pronounced (up to about 300-times). Also in this experiment the levels of 24S- and 27- hydroxycholesterol were slightly decreased.

In our study the CSF levels of 7-oxocholesterol measured in CSF appeared to be more than one thousand times lower than those reported by Diestel et al (75). In vitro induced lipid peroxidation of CSF increased the levels more than 100-fold, but still considerably lower than the levels reported. The much higher levels reported previously (75) cannot be solely interpreted on the basis of an uncontrolled lipid peroxidation or cholesterol autoxidation of the samples during the sample handling. In our hands incubation at room temperature in air was found to be responsible for an increase of the 7-oxygenated oxysterols lower than twice. In accordance with the findings by Diestel et al. (75) the absolute and the cholesterol corrected concentrations of CSF 7-oxocholesterol were significantly lower in the control population than in the patients with MS. This difference was relatively small, however. A similar difference was observed in the case of 7β-hydroxycholesterol, an enzymatic as well as autoxidative metabolite of cholesterol and 7α-hydroxycholesterol.

The slight difference in 7-oxocholesterol concentrations between the Swedish MS-patients and controls may be due to increased oxidative stress in the MS-patients as a consequence of the demyelination. However, definitive evidence for this is lacking.

We can only speculate about the reason for the very marked difference between our results and those of Diestel et al (75). Cholesterol is present in great excess in all biological fluids from mammals, and there is always a risk that auto-oxidation and uncontrolled lipid peroxidation may result in falsely high levels of 7-oxygenated products.

The plasma levels of 7-oxygenated steroids reported in plasma have decreased markedly during the last two decades in parallel with the development of better methodology (156). The levels of the 7-oxocholesterol in human plasma have been reported to vary with a factor of almost 200 in some previous work (156). In a recent interlaboratory comparison study in which our laboratory participated together with 7 others (157), the levels of 7-oxocholesterol varied however with a factor of less than 3. In view of the present findings the hypothesis of Diestel et al that 7-oxocholesterol may be a link between demyelination and progressive damage should be reconsidered.

In view of the growing evidence for the importance of oxidative stress in the pathogenesis of neuronal damage such as in MS and AD, it appears reasonable that oxidative stress and lipid peroxidation may result in increased auto-oxidation of cholesterol and a subsequent increase in CSF levels of 7-oxocholesterol and the other 7-oxygenated cholesterol derivates.

Measurement of the 7-oxysterols in addition to 24- and 27-hydroxycholesterol may be a have the potential to be uses to evaluate the oxidative stress in case of CNS diseases. Further work on this is in progression.

GENERAL SUMMARY

1) Plasma levels of 24S-hydroxycholesterol are significantly decreased in MS patients with advanced disease, and are likely to reflect the degree of brain atrophy.

2) Neurological diseases were found to affect the levels of 24S-hydroxycholesterol and 27-hydroxycholesterol to a much higher degree in CSF than in plasma. The 95% confidence interval for CSF levels in a normal population was found to be 0.4 - 2.5 and 0.2 - 1.0 μg/L for 24- and 27-hydroxycholesterol, respectively.

3) Most of the 27-hydroxycholesterol in CSF originates from the circulation and a compromised BBB is associated with increased CSF levels of this oxysterol.

4) Neuronal damage or demyelination are associated with increased flux of 24S-hydroxycholesterol from brain into CSF.

5) In case of CNS disease both oxysterols may be increased in CSF, depending upon the relative importance of the above two processes.

6) The high proportion of the neurological patients with increased CSF levels of one or both of the two side-chain oxidised oxysterols is consistent with the contention that a normal level of both these oxysterols is likely to have a high negative predictive value.

7) Patients with MS were found to have slightly increased levels of 7-oxocholesterol in CSF, possibly reflecting increased lipid peroxidation. The CSF levels were found to be three order of magnitude lower than previously reported.

8) Determination of both plasma and CSF oxysterols levels may offer important information about the different processes in CNS diseases.

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