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MARKERS OF INFLAMMATION AND NEURODEGENERATION IN MULTIPLE SCLEROSIS

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I have nothing to offer but blood, toil, tears and sweat

Sir Winston Churchill, speech upon becoming prime minister of the UK in 1940

To my family, past and future generations,

To my teachers,
To my patients,
To society and the research community,
To my countries

ABSTRACT

The main line of my thesis projects is searching for biomarkers and evidence of optimal correlation with clinical and para-clinical outcome measurements in multiple sclerosis (MS):
- investigating the **biochemical biomarker** in blood/plasma (cerebrosterol) and in cerebrospinal fluid (CSF) (oligoclonal bands (OCB)) and the correlation with MRI lesion load (paper 1 and paper IV);
- investigating the **genetic biomarker** (*CYP46A1*) for susceptibility and disability progression in MS (Paper III);
- comparison of the well-known positron emission tomography (PET) ligand and peripheral benzodiazepine receptor (PBR) antagonist, [^{11}C]PK11195, and a new PET-ligand [^{11}C]vinpocetine (**tissue receptor biomarker**). Both ligands denote areas of active ongoing inflammation in MS (paper II).
Paper I: How levels of cerebrosterol – a brain specific cholesterol metabolite - correlate with inflammatory and neurodegenerative markers on brain MRI.
Paper II: PET ligand and PBR agonist, [^{11}C]vinpocetine , facilitates cholesterol delivery to the mitochondrial membrane; a pilot study to investigate whether the new [^{11}C]vinpocetine is a better marker of MS inflammatory lesion areas compared to the old PET -PBR marker [^{11}C]PK11195.
Paper III: A study of how a single nucleotide polymorphism in the gene *CYP46A1*, coding for cholesterol hydroxylating enzyme 24S-hydroxylase, influences susceptibility and prognosis in MS singly and in synergism with another gene variant implicated in cholesterol turnover, *APOE ε4*.
Paper IV: Investigation of a CSF inflammation biomarker –OCB, and carriership of specific HLA gene alleles and the correlation with MRI outcome measures: total T1 and T2 lesion load, as well as T2 lesion load extension in MS specific compartments.

The **most important findings** are:
Paper I: results in this paper support the possibility that cerebrosterol in plasma is a potential marker of neurodegeneration in the relapsing remitting MS group.
Paper II: PET ligand [^{11}C]vinpocetine is a marker of activated glia in MS lesions and binds in different ways compared to [^{11}C]PK11195.
Paper III: *CYP46A1* alone or in combination with *APOE ε4* does not affect susceptibility for MS; men, as carriers of both the *CYP46A1* SNP rs754203 –AA variant and *APOE ε4* need walking assistance 17 years earlier compared with non-carrier men. However, results are lacking statistical significance, due to small sample size.
Paper IV: OCB in CSF, *HLA-DRB1*15*, and *HLA-DRB1*04* affect neither T1 lesion load on brain MRI at MS specific localizations nor total T2 lesion load. This paper supports the possibility that infratentorial T2 lesion load is associated with OCB in CSF.

LIST OF PUBLICATIONS

I. *Plasma cerebrosterol and magnetic resonance imaging measures in multiple sclerosis*
Karrenbauer VD, Leoni V, Lim ET, Giovannoni G, Ingle GT, Sastre-Garriga J, Thompson AJ, Rashid W, Davies G, Miller DH, Björkhem I, Masterman T.
Clin Neurol Neurosurg. 2006 Jul;108(5):456-60. Epub 2005 Sep 6.

II. *Functional neuroimaging in multiple sclerosis with radiolabelled glia markers: Preliminary comparative PET studies with [11C]vinpocetine and [11C]PK11195 in MS patients.*
Vas A, Shchukin Y, **Karrenbauer** VD, Cselényi Z, Kostulas K, Hillert J, Savic I, Takano A, Halldin C, Gulyás B.
J Neurol Sci. 2008 Jan 15;264(1-2):9-17. Epub 2007 Aug 28

III. Effect of *CYP46A1* genotypes on susceptibility and prognosis in multiple sclerosis.
Virginija Danylaitė Karrenbauer, Lisa Barcellos, Thomas Masterman, Kerstin, Imrell.
Manuscript

IV. Impact of cerebrospinal-fluid oligoclonal immunoglobulin bands and *HLA-DRB1* risk alleles on brain magnetic-resonance imaging lesion load in Swedish multiple sclerosis patients at age 40.
Virginija Danylaitė Karrenbauer, Robert Prejs, Thomas Masterman, Jan Hillert, Anna Glaser, Kerstin Imrell.
Manuscript submitted to Journal of Neuroimmunology

CONTENTS

LIST OF ABBREVIATIONS

AD	Alzheimer's disease
APOE	Apolipoprotein E
BBB	Blood brain barrier
BP	Binding potential
BPF	Brain parenchymal fraction
CI	Confidence interval
CIS	Clinical isolated syndrome
CSF	Cerebrospinal fluid
CNS	Central nervous system
CYP	Cytochrome P450 enzyme
DIT	Dissemination in time
DIS	Dissemination in space
EAE	Experimental autoimmune encephalomyelitis
EDSS	Expanded disability status scale
GABA	*gamma*-aminobutyric acid
Gd	Gadolinium
GWAS	Genome wide association study
IgG	Immunoglobulin G
IL7	Interleukin 7
HLA	Human leukocyte antigen
HR	Hazard ratio
MCI	Mild cognitive impairment
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
NAA	N-acetyl aspartate
NAWM	Normal appearing white matter
OCB	Oligoclonal bands
OCT	Optical coherence tomography
OR	Odds ratio
PBR	Peripheral benzodiazepine receptor
PBBS	Peripheral benzodiazepine binding site
PCR	Polymerase chain reaction
PET	Positron emission tomography
PPMS	Primary progressive multiple sclerosis
RCT	Randomized clinical trials
RERI	Relative risk due to interaction
RNFL	Retinal fiber layer thickness
RRMS	Relapsing remitting multiple sclerosis
SDMT	Symbol digit modalities test
SNP	Single nucleotide polymorphism
SCI	Slight cognitive impairment
TE	Echo time
WMLL	White matter lesion load

1 THESIS SUMMARY – MAIN SECTION

1.1 MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is a complex autoimmune CNS disease that causes inflammation, demyelination, axonal degeneration and brain and spinal cord tissue atrophy. The disease affects individuals in their third decade of life; females affected 2-3 times more often than males. One fourth of all patients never experience difficulties of daily living due to disease, but 15% of patients suffer disability in early disease stages (Compston and Coles 2002).

Main subdivision of MS clinical courses: RRMS (relapsing-remitting, 80-90%) and PPMS (primary progressive10-20%) (reviewed in (Noseworthy, Lucchinetti et al. 2000). The first decade after disease onset RRMS patients experience relapses, i.e. neurological disability that resolves with time and patients recover. With time, 10-15 years later, patients do not recover completely after bout, or handicap accumulates without bouts; this stage is called secondary progressive form. Disability progression by some researchers is considered an age dependent process, independent of relapse history (Koch, Mostert et al. 2007).

Patients might have a broad range of symptoms: motor disturbances, including walking disability, pain, coordination problems, sensory and balance disturbances as well bladder and bowel disturbances together with fatigue and cognitive impairment.

Diagnosis. There is no specific test or biomarker that singly would indicate MS. MS diagnosis is made by combining relapse history, findings on magnetic resonance imaging (MRI) and exclusion of other differential diagnosis that would mimic either clinical or MRI phenotype. Currently used are McDonald's diagnostic criteria which were modified in year 2010 (Polman, Reingold et al. 2011) from previous versions of year 2001 (McDonald, Compston et al. 2001) and year 2005 (Polman, Reingold et al. 2005).

According to the latest revision of McDonald's criteria, RRMS diagnosis requires fulfillment of at least one of the following conditions:

-at least one MS relapse and one objective lesion on brain MRI and evidence of disease dissemination in time (DIT) and dissemination in space (DIS);

-or at least one clinical attack, two MRI lesions and DIT;

-or two attacks, one MRI lesion and DIS;

-or two attacks and two MRI lesions without additional requirements.
PPMs is diagnosed when two major criteria are fulfilled:
1. One year of disease progression (retrospectively or prospectively determined)
2. and two of the three following criteria:
A. Evidence for DIS in the brain based on one T2 weighted hyperintense lesions in at least one area characteristic for MS (periventricular, juxtacortical, or infratentorial)
B. Evidence for DIS in the spinal cord based on two T2 weighted hyperintense lesions in the cord.
C. Positive CSF (isoelectric focusing evidence of OCB and/or elevated IgG index) (Polman, Reingold et al. 2011).

1.2 BIOMARKER

A biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenetic processes or pharmacological responses to a therapeutic intervention (Brex, Ciccarelli et al. 2002; Bielekova and Martin 2004). There are a few quite good biomarkers in MS (OCB or elevated IgG index are a diagnostic biomarker for inflammation in CNS; gray substance volume reduction is a marker of neurodegeneration), but we are lacking the surrogate biomarker to monitor treatment effects.

1.2.1 Characteristics of a potential biomarker for use of clinical evaluation

1.2.1.1 Biologically meaningful

The biomarker must reflect a particular pathogenetic process. As example let us use neurofilament light chain or tau proteins as markers for axonal-neuronal damage or reduction of N-acetyl aspartate (NAA) marker for loss of axonal integrity.

1.2.1.2 Clinically relevant

Biomarker level variation needs to correlate to clinical outcome endpoints. In MS patients most common used endpoints are: EDSS (Expanded disability status scale),

MSSS (Multiple sclerosis severity scale), and MSFC (Multiple sclerosis functional composite), Ambulation index and SDMT (Symbol digit modalities test).

1.2.1.3 Practical issues: easily obtainable, stable; cheap and quick sample analysis

Currently known and future identified biomarkers will be more easily accepted and used clinically if they are tested in body fluids that are easily obtainable: CSF and tissue biopsy biomarkers have a sampling disadvantage compared to biomarkers in blood, tears and urine. Quick and cheap biomarker, easy sample analysis gives an advantage as well as stable, not easily degradable compound (biomarker).

1.2.1.4 Correlates well with disease activity and accumulated disability

Disease activity might be assessed by clinical or preclinical measures. As a clinical measure might be used either number of MS relapses per patient and year or the extent of progression in one of the disability scales per time unit.
Paraclinical measures: number of Gd-enhanced lesions; accumulation of disease burden measured as WMLL (white matter lesion load), number of accumulated T1 hypointense lesions, increase in brain atrophy measures. However, disease activity does not always correlate with accumulated disability.

1.2.1.5 Correlates with treatment effect

If there is good correlation between the clinical endpoint and biomarker, treatment that predictably influences clinical endpoint, should influence levels of biomarker.

1.2.2 Criteria to evaluate clinical usefulness of biomarker

1.2.2.1 Sensitivity and specificity

Adopted from (Motulsky 1995)

Sensitivity is the fraction of all those with the disease who get a positive test result.
Sensitivity might be calculated by formula:
Sensitivity = True positives / (True positives+ False negatives)

Sensitivity measures how well the test identifies those with disease. If the test has high sensitivity, it will pick up nearly everyone with the disease.
Specificity is the fraction of individuals without disease who will get a negative test result. It might be calculated by formula:
Specificity = True negatives / (True Negatives + False positives)
Specificity measures how well the test excludes those who do not have the disease. If a test has high specificity, it will not mistakenly give a positive result to many people without disease.

1.2.2.2 Reliability

A biomarker needs to be reliable with known biological variation depending on the time point of biomarker sampling; reproducible values of measurements despite different assays (inter-and intra-assay reproducibility); known frequency of false positive and false-negative results.

1.2.2.3 Evaluation of biomarker in epidemiological studies of natural history cohorts

Currently it is unethical to conduct such studies of natural history due to early treatment initiation soon after diagnosis for both clinical isolated syndrome (CIS) and MS patients. But on the other hand studies are essential to establish the statistical relationship between the biomarker and clinical outcome measurement in treatment-naive patient.

1.2.2.4 Evaluation of biomarker in large multicenter double-blinded therapeutic trials

In such trials it is possible to evaluate the proportion of treatment effect for both clinical and biomarker endpoint and very valuable information could be collected for placebo group (untreated population).

1.2.2.5 Evaluation of a biomarker in a meta-analysis

In order to evaluate biomarker surrogacy it is important to evaluate a biomarker's relationship to clinical endpoint in meta-analysis. Meta-analysis uses results from both small and large clinical trials of biomarker relationship to the major clinical outcome.

1.3 INFLAMMATION

"Notae verae inflamationis sunt quattuor: rubor et tumor cum colore et dolore"
Cornelius Celsius

These four signs of inflammation: redness, swelling with heat and pain, - definition of inflammation by Cornelius Celsius (about 25-30 BC – 50 AD). Rudolf Ludwig Virchow (1821-1902) added the fifth sign of inflammation: *"functio laesa"* (*lat.*), reduced function. That we learnt by heart at medical school.

MS is considered an autoimmune inflammatory and neurodegenerative disease. Not all the classical signs of inflammation are present in MS-related neuroinflammation. Below are listed facts that that support the evidence of inflammation in MS pathogenesis:

- Most important loci of disease susceptibility are related to immune function as published in the International Multiple sclerosis Genetic consortium and Welcome Trust Case Control Consortium publication in Nature, 2011(Sawcer, Hellenthal et al. 2011) . GWAS, involving 9772 cases has replicated previous genes and loci of susceptibility: *HLA-DRB1*15:01* has the strongest association with MS among all classical and SNP alleles; genes that are coding for cytokine pathway (IL7R, IL7, and CXCR5), co-stimulatory (CD37, CD40, CD80) and signal transduction (TYK2, STAT3) molecules of immunological relevance.

- Genetic, cellular and tissue imaging studies give evidence for inflammation in lesions. MS lesions are characterized by infiltration of lymphocytes and antibody-producing plasma cells into the brain and spinal cord white matter. In areas of demyelination activated microglia, increased amount of astrocytes (Lassmann, Bruck et al. 2001), complement and antibody deposition are present (Frohman, Racke et al. 2006).

Mild meningeal inflammation consisting of B and T lymphocytes, plasma cells and macrophages are present.
Subpial and intracortical gray matter lesions exist; subpial lesions are extensive and cover upp to 70% of the cortical area in some individuals (Kutzelnigg and Lassmann 2005).

-Immunomodulatory treatment reduces the inflammatory burden and reduces disability progression in the early stages of disease (Giacomini, Darlington et al. 2009).

1.4 NEURODEGENERATION

Both inflammation and neurodegeneration are closely related. It is important to emphasize that there is evidence that neurodegeneration process occurs both early (Trapp and Nave 2008) and late in disease progression and in both gray (Calabrese and Gallo 2009) and white matter.
In the early disease stage both in areas of demyelination as well as in areas of normal appearing white matter (NAWM) axonal damage and transection are present (Kutzelnigg, Lucchinetti et al. 2005).
The extent of Gd-enhanced lesions correlates with subsequent brain atrophy; it means that high inflammatory burden has consequences of irreversible tissue damage, causing loss of brain parenchyma (Simon, Jacobs et al. 1999). By MR spectroscopy, loss of NAA (biomarker metabolism in neurons) is visualized early in MS disease and in patients with very few lesions (De Stefano, Narayanan et al. 2001).

In the late disease stage (approximately 10 years from disease start) immunomodulatory drugs are inefficient even if inflammatory burden has not completely disappeared. There is presence of chronic, active, slowly expanding lesions with demyelination, ongoing axonal damage and T-cell infiltration (Prineas, Kwon et al. 2001). According to a previous publication (Hochmeister, Grundtner et al. 2006) in late stages of disease inflammation may be trapped within the CNS and thus unreachable for immunomodulating treatment. Another concept of explanation for the inefficiency of immunomodulatory drugs in late stage of disease could be that neurodegeneration becomes independent from inflammation or inflammation seen in late stages of MS is consequence of neurodegeneration.
Cortical lesions and cortical atrophy (volume reduction) contribute to cognitive deficits (Calabrese, Filippi et al. 2009).
The pathological substrate of motor disability in MS, loss of corticospinal axons, correlates with clinical and pathological findings (Tallantyre, Bo et al. 2010).

2 MY PROJECTS AND PUBLICATIONS

2.1 AIMS

The overall aims in this thesis are to investigate how biochemical, genetic and tissue receptor biomarkers associate with the inflammation, neurodegeneration and correlate to clinical outcome in MS.

2.1.1 Paper I

To investigate and collect evidence as to whether variation in plasma concentration of cerebrosterol reflects ongoing inflammation or neurodegeneration in CNS expressed as volume of different brain MRI lesions.

2.1.2 Paper II

PET-pilot paper: validation of a new glia activation marker, ligand [^{11}C]vinpocetine compared to the established ligand [^{11}C]PK11195.

2.1.3 Paper III

Gene association paper: whether the *CYP46A1* SNP (rs754203) that is a suggested genetic risk factor for late onset Alzheimer's disease (AD) influences susceptibility for MS and/or disability progression both alone or in synergism with another neurodegeneration related and major gene risk allele for sporadic AD—*APOE ε4*.

2.1.4 Paper IV

To investigate the effect of OCB, *HLA-DRB1*04, HLA DRB1*15* on total T2 and T1 lesion load of T1 hypointense lesion load, and T2 lesion load in MS specific neuroanatomical compartments .

2.2 MARKERS OF INFLAMMATION AND NEURODEGENERATION

2.2.1 T2 lesion load

Definition: T_2 weighted lesions new, recurrent, newly enlarging, or persistently enlarging hyperintense lesions detected on serial T_2 MRI scans (Paty 1988).

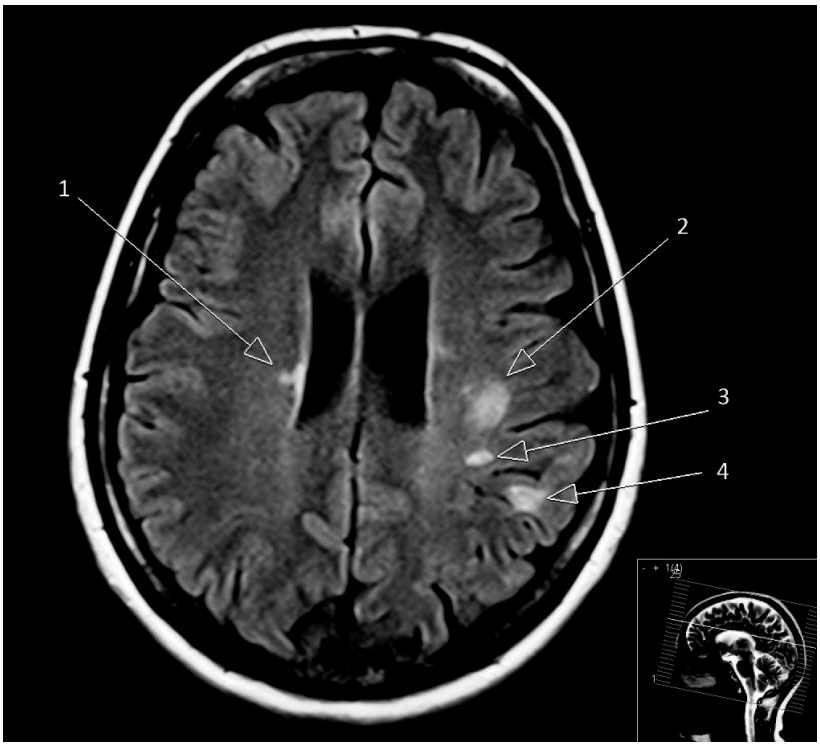


Figure1. Axial fluid-attenuated inversion recovery-weighted brain MRI with lesion localization:
1-Periventricular; 2- Deep white matter; 3-Subcortical; 4-Juxtacortical.

2.2.2 Gadolinium-enhancing lesions

Definition: Gadolinium-enhanced lesion areas of high signal intensity on T_1-weighted images that appear after intravenous injection of gadolinium. Gadolinium-enhancing lesions indicate breakdown of the blood—brain barrier and inflammation in acute MS

lesions. They tend to persist for 2 to 6 weeks, leaving behind a T_2 hyperintense lesion (Fazekas, Barkhof et al. 1999).

2.2.3 T1 hypo-intense lesions

Definition: T_1 - weighted hypointense lesions - black holes- qualitative term used to describe hypointense lesions in the white matter on T_1-weighted images (short TR/short TE spin echo image). Acute black holes represent areas of edema related to acute inflammation, whereas chronic black holes indicate persistent tissue destruction with expanded extracellular space and axonal loss (van Walderveen, Kamphorst et al. 1998).

2.2.4 Cerebrosterol (24S-hydroxycholesterol)

Projects about 24s-hydroxycholesterol (cerebrosterol) have been carried out in our clinic in close cooperation with Clinical Biochemistry Department (Professor Ingemar Björkhem) since late 1990s by Associate Professor Åke Sidén. Research on cerebrosterol was later continued by my supervisor Thomas Masterman together with a PhD student in the Department of Biochemistry, Valerio Leoni. It is great honor to be part of cerebrosterol history, carry on research on the same topic, reveal more secrets about cerebrosterol, and to try to make sense of clinical usability.

Cholesterol in CNS is found in two major pools: The myelin sheaths and plasma membranes of astrocytes and neurons (Snipes G 1998) . Approximately 70% of the brain cholesterol is associated with myelin and brain is the most cholesterol rich organ. In adult brain cholesterol synthesis rate is low and the half –life of the cholesterol in the brain is estimated to be 5 years (Bjorkhem, Lutjohann et al. 1998).

Currently two mechanisms for removal of excess of cholesterol from the brain are known: APOEE-bound cholesterol transport via CSF (1-2 mg/ day) (Pitas, Boyles et al. 1987) and excretion through BBB (blood brain barrier) of the hydroxylated form of cholesterol – cerebrosterol (6-7 mg/day) (Lutjohann, Breuer et al. 1996; Bjorkhem, Lutjohann et al. 1997; Bjorkhem, Lutjohann et al. 1998). Cholesterol is hydroxylated by a CYP46 enzyme called 24S-hydroxylase, that is found 90% in brain, and smaller amounts in testis (Lund, Guileyardo et al. 1999), eyes, bone marrow and lungs (Leoni 2009). For more about 24S-hydroxylase protein and coding gene please read the following chapter.

Both absolute plasma concentration as well as cerebrosterol/cholesterol levels are stable during the age period 5-60 years. The higher concentrations in healthy elderly individuals in the age group 60-70 yaers compared to younger ones, is an expression of brain volume and liver enzymatic capacity ratio differences between the age groups (Bretillon, Lutjohann et al. 2000).

Cerebrosterol in pathological conditions and diseases. In the EAE mouse model it was found that cerebrosterol concentration significantly increased already in day 9, before clinical symptoms appear and gradually increases up to 193% in day 17 when the animals had recovered from symptoms (Teunissen, Floris et al. 2007). It means there is no linear correlation between the neurological status severity and cerebrosterol concentration variation.

Absolute plasma levels of cerebrosterol are **elevated** in **early** AD and vascular dementia compared with healthy controls (Lutjohann, Breuer et al. 1996). An Italian group has revealed that in **early** stages of AD, levels of cerebrosterol were **higher** compared to healthy controls and vascular dementia patients (Zuliani, Donnorso et al. 2011). Compared to healthy controls, plasma concentration of cerebrosterol was **lower** in individuals with **advanced** AD, Guillain-Barré syndrome, brain tumors, ischemic stroke and brain death. Intriguingly, plasma concentration in MS was higher compared to healthy controls (Bretillon, Siden et al. 2000). In this cohort one of the MS patients had three times higher cerebrosterol concentration in plasma compared to other MS patients. Here the first hypothesis generating question has been raised: why? What was special with that particular MS patient?

We generated the hypothesis: cerebrosterol might be a marker of inflammatory relapse in MS and set out to test the hypothesis. We initiated study I "Plasma cerebrosterol and magnetic resonance imaging measures in multiple sclerosis". We did not find what we looked for, but we made other promising findings: borderline significant association of logarithmically transformed cerebrosterol/cholesterol ratio with T1 hypointense lesion volume (T1 lesions--black holes--irreversible tissue damage--sign of neurodegeneration) in the RRMS patient group.

The cerebrosterol/cholesterol ratio is reduced with advancing age in MS (Karrenbauer, Leoni et al. 2006) and AD disease progress (Papassotiropoulos, Lutjohann et al. 2000).

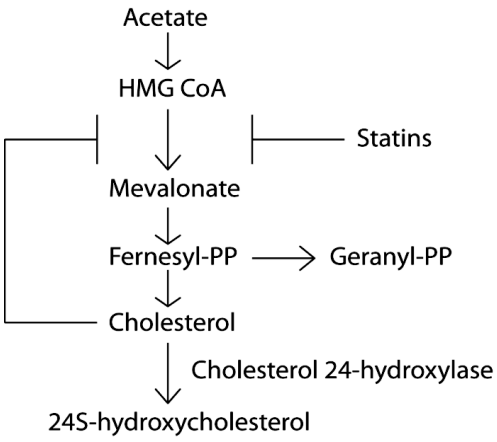


Figure 2. Cerebrosterol (24S-hydroxycholesterol) synthesis pathway in the brain.

2.2.5 CYP46A1

The gene *CYP46A1* is located on chromosome 14q32.2 and contains 15 exons and 14 introns. The enzyme CYP46 is very important for brain cholesterol metabolism and might play a role in development of AD.

In normal conditions *CYP46A1* mRNA and protein are localized in neurons that are metabolically active: pyramidal neurons in cerebral cortex, Purkinje cells of cerebellum, thalamus, dentate gyrus and hippocampus, but not in white matter or support cells in mouse brain (Lund, Guileyardo et al. 1999). In human brain tissue immunostaining of CYP46 protein was found throughout the gray matter; accentuated staining was observed in neuronal cells of pyramidal shapes and in white matter in axon-like fiber structures (Bogdanovic, Bretillon et al. 2001) . It was shown that under pathological conditions CYP46 protein can be found in other structures than neurons. In the EAE mouse model, immunostaining of Cyp46 has been shown in perivascular infiltrates (macrophages) (Teunissen, Floris et al. 2007) and Cyp46 was found to be expressed in glia cells in a rat model of brain injury (Smiljanic, Lavrnja et al. 2010). In AD, in post mortem human brain tissue, CYP46 immunostaining was localized to microglia (astrocytes) (Bogdanovic, Bretillon et al. 2001). There are no studies on *CYP46A1* expression or CYP46 protein localization in post mortem human brain tissue in MS.

In year 2003, Papassotiropoulos with colleagues investigated and found that the *CYP46A1* SNP rs754203 was associated with AD pathological features: higher beta-amyloid peptides and phosphorylated tau in CSF, and higher beta-amyloid load in brain tissue. In addition, *CYP46A1* was associated with late onset AD susceptibility both alone (OR =2.26; 95% CI 1.41-3.32) and in synergism with *APOE ε4* (OR= 9.6; 95% CI, 4.9-18.9) (Papassotiropoulos, Streffer et al. 2003).

The publication of Papassotiropoulos (Papassotiropoulos, Streffer et al. 2003) gave us inspiration to investigate whether the SNP rs754203 in intron 2 plays the role alone or in synergism with *APOE ε4* for MS susceptibility and/or whether it influences disability development in MS measured as age at EDSS 6.0.

Since 2002, when the first publication addressing the question of *CYP46A1* and AD risk was published, more than 20 publications approaching the same question with contradicting results have appeared, reviewed in (Garcia, Muniz et al. 2009). So today there is no clear consensus whether or not the SNP rs754203 is a risk factor for AD.

Back in 2003-2004, in the beginning of my PhD studies time, my supervisors and I were convinced that the *CYP46A1* gene SNP rs754203, which is of importance for AD risk as well as pathological features, might be of importance for the neurodegenerative process in MS.

2.2.1 *APOE* ε4

The *APOE* gene, situated on chromosome 19q13 is the most important established genetic risk factor for sporadic AD (Haines, Bradford et al. 2002). Exon 4 within *APOE* contains two SNP at codons 112 and 158. The gene exists in three common allelic forms: *APOE ε2, ε3, ε4* that engender six different genotypes (*ε2/ε2, ε2/ε3, ε2/ε4, ε3/ε3, ε3/ε4, ε4/ε4*) and three corresponding protein isoforms: E2, E3, E4 (Weisgraber, Rall et al. 1981). There are two minor allelic forms: *ε1* and *ε5*. Polypeptides that are 299 amino acids long are distinguished by having different combinations of the amino acids: cysteine and arginine at position 112 and 158. Due to amino acid changes the APOE isoforms displays different physical and biochemical properties.

APOE protein function. APOE functions as a major lipid and cholesterol carrier protein in the brain and nervous system (Poirier 1994). Isoforms E1 and E2 decreases

the plasma levels of cholesterol and E3 and E4 increases it. E2 and E3 clear β-amyloid plaques 20 times more effectively compared with E4. APOE exerts antioxidant activities in the following ranking: E2>E3>E4 (reviewed in (Xuan, Zhang et al. 2011)).
During the last 20 years many studies were published concerning the *APOE ε4* effect for MS susceptibility and prognosis with conflicting results. In a meta-analysis in year 2006 (22 studies, 3299 MS cases and 2532 controls), it was found that *APOE ε4* does not confer risk for MS susceptibility or affect disability progression. Results from a pooled analysis of 4048 cases indicate that *APOE ε4* status does not distinguish RR from PP disease (Burwick, Ramsay et al. 2006). A new meta-analysis published in year 2011(Xuan, Zhang et al. 2011) from 5472 MS cases and 4727 controls confirms again that there is no association between the *APOE ε4* allele and MS susceptibility.
Due to clear evidence of no effect of this gene to MS susceptibility and prognosis we did not look for an *APOE ε4* gene effect primarily but only in synergism with another gene, important for neurodegeneration-- *CYP46A1*.
The following references supports evidence that *APOE ε4* exhibits gender-depending differential effects and is the reason why we gender-stratified our cohort in paper III:

- Cognitive decline in males is associated in with *APOE ε4* (Savettieri, Messina et al. 2004).
- *APOE ε4* carriership confers 49% higher risk of mortality in males in community based cohort of healthy elderly individuals at age 75+ (Rosvall, Rizzuto et al. 2009).

2.2.2 HLA

Through the years of searching for candidate genes, one has been consistently shown to be associated with the risk of developing MS: the HLA class II gene *HLA-DRB1* . (Oksenberg, Barcellos et al. 1999; Sawcer, Hellenthal et al. 2011).
The MS-associated HLA class II haplotype consists of alleles of four adjacent genes: *DRB1*15:01*, *DRB5*01:01*, *DQA1*01:02*, *DQB1*06:02*. The carriership of this haplotype is most common in Scandinavia and more frequent in patient compare to controls; association to MS is found in almost all ethnic groups.
Linkage and association studies has established *HLA-DRB1*15:01* as a major risk factor for MS (Kantarci and Wingerchuk 2006). About 60% of Nordic MS patients carry *HLA-DRB1*15:01* and only 30% of healthy controls (Masterman, Ligers et al. 2000).

*HLA-DRB1*15:01* is associated with higher disability and severity and extent of spinal cord involvement (Qiu, Raven et al. 2011). Carriership of *HLA-DRB1*15:01* and presence of OCB lower age at attainment of EDSS 6.0. In another study it was found that *HLA-DRB1*15:01* was associated with worse performance in cognitive tests, higher T2 lesion load on MRI and reduction of NAA on spectroscopy images (Okuda, Srinivasan et al. 2009).

2.2.3 Peripheral benzodiazepine receptors, markers of activated microglia

Microglial cells represent 5% of the total cell population in the brain. Microglia represent the resident macrophages in CNS, capable of phagocytosis and antigen presentation. During physiological conditions these cells have low expression of receptors associated with macrophage function and are called "resting microglia", reviewed in (Venneti, Wiley et al. 2009). Resting microglia in healthy tissue continuously change morphology of complex processes, attempting to assess the microenvironment, to make contact with other cells and blood vessels. Hence, even in the resting state, microglia are neither dormant nor functionally at rest (Nimmerjahn, Kirchhoff et al. 2005).Previously, microglia activation was associated only with pro-inflammatory processes, but during the recent years evidence of microglia's neuroprotective and anti-inflammatory features has accumulated (Venneti, Wiley et al. 2009). The pro-inflammatory or anti-inflammatory fate of microglia is determined by specificity of the current microenvironment composition (Li, Lu et al. 2007).

It is important to distinguish the definitions of microglia and macrophages. Microglia are myeloid cells located in or derived from CNS cells present at birth and macrophages are monocytes from the bone marrow that have been trafficked to CNS after birth (spontaneously, or due to inflammation, injury, etc.) (Venneti, Lopresti et al. 2012).

Peripheral benzodiazepine receptors (PBR) are found on non-neuronal cells, activated microglia, and in case of disrupted BBB), on mononuclear-phagocytic cells invading from the blood through the disrupted BBB, reviewed in (Cagnin, Kassiou et al. 2007). PBRs are expressed in low levels on the structures without BBB (choroid plexus, ependymal cells lining ventricles). PBR are highly expressed in peripheral organs; adrenals, kidneys. (Chauveau, Boutin et al. 2008).

PBR is also called translocator protein (18 kDa). The name per se describes the function of the protein: binding and transport of cholesterol and proteins into mitochondria (Papadopoulos, Baraldi et al. 2006).
[^{11}C]PK11195 or 1-(2-chlorophenyl-) N-methyl-n-(1-metylpropil)-3-isoquinoline carboxamide is a specific ligand of PBR. In MS, [^{11}C]PK11195 binding has been identified on activated microglia- macrophages, in areas of focal pathology co-localized with T1, T2 weighted MRI lesions, NAWM, and gray matter (Banati, Newcombe et al. 2000). The biggest overlap between MRI pathology and [^{11}C]PK11195 binding is co-localized at the Gd-enhanced lesions area, where 30% of the volume showed significantly increased [^{11}C]PK11195 binding. As a tracer of activated microglia PBR, [^{11}C]PK11195 has been used in other diseases to visualize activated microglia: Rasmussen's encephalitis, idiopathic Parkinson's disease, multiple system atrophy, progressive supranuclear palsy, AD, stroke, post-herpetic encephalitis, traumatic and peripheral nerve injury (Cagnin, Kassiou et al. 2007).
Vinpocetine (ethyl-apovincaminate), structurally related to the vinca minor alkaloid-vincamine, is used as a neuroprotective drug in several European countries. Distribution of intravenously injected and orally administrated [^{11}C]vinpocetine both in monkeys(Gulyas, Halldin et al. 1999) and in humans(Gulyas, Halldin et al. 2002) is similar, with the highest uptake in basal ganglia, thalamus and visual cortex. The hypothesis that vinpocetine binds to PBR was generated from a study that showed 30% less uptake of [^{11}C]-(R)-PK11195 in monkey brains after intravenous pre-treatment with 3mg/kg of vinpocetine (Gulyas, Halldin et al. 2005). The vinpocetine molecule has highest affinity in vitro to PBR (IC_{50}=0.2 μM) and other receptors like $\alpha_2\beta$ adrenoreceptors (IC_{50}=0.9 μM) (Gulyas, Halldin et al. 2002).Despite evidence of some unspecificity of vinpocetine binding to PBR, ethical permission was obtained to conduct a pilot study in 4 MS patients to compare binding qualities to Gd-enhanced lesions and characteristics of the new ligand [^{11}C]vinpocetine compared to the established ligand [^{11}C]PK11195.

Note: IC_{50} –is the half maximal inhibitory concentration –is a measure of effectiveness of an inhibitory compound at a specific concentration to block half of the maximal biological effect. It is a measure used to evaluate potency of an antagonist drug used in pharmacological research.

2.2.4 OCB

Since the 1940s it is known that 90% of MS patients have increased intrathecal production of immunoglobulin in CSF, (reviewed in (Cross and Waubant 2011) .
OCB are present in 10% of healthy individuals and 95% of Northern European MS patients (Andersson, Alvarez-Cermeno et al. 1994), but only 50% of Japanese MS patients (Fukazawa, Kikuchi et al. 1998; Kikuchi, Fukazawa et al. 2003) harbor OCB in CSF.

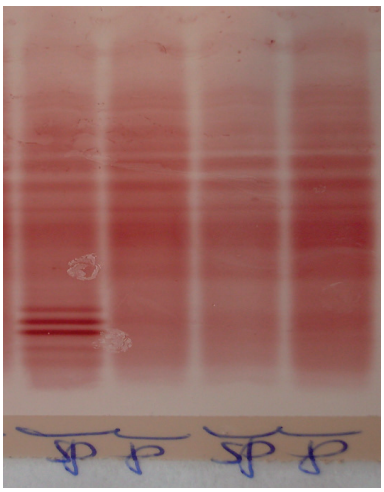


Figure 3.The readout of gel electrophoresis of CSF paired with plasma taken on the same occasion. On the left side, example with OCB in CSF (the first column) and paired plasma sample from the same patient (second column). Note the differences in disposition of lines in the first column, lower segment - these are OCB. The last two columns represent electrophoresis of CSF and plasma without OCB.

Definition: presence of one or more oligoclonal IgG bands in CSF detected by isoelectric focusing which is absent in plasma (Olsson, Kostulas et al. 1984).
OCB have a high diagnostic sensitivity (90-100%) and specificity (35%) to detect MS, but might be present in CSF due to other reasons of inflammation or due to CNS infection.
OCB are produced by clonally expanded CD138+ plasma cells and plasma blasts (von Budingen, Gulati et al. 2010). Older publications state that OCB, once present, persist

in the patient´s CSF irrespective of MS course or treatment. There have however been recent reports about quantitative (amount of bands) and qualitative (present-absent) changes during the course of disease (or treatment).
In current McDonald's criteria there are no requirement for presence of OCB as diagnostic criteria for RRMS diagnosis. For PPMS diagnosis, presence of OCB is included as one of optional requirements. In Nordic MS patients, OCB-positive MS is associated with HLA-DRB1*15:01, OCB-negative MS is associated with HLA-DRB1*04.

2.3 OUTCOME MEASURE-EDSS

Expanded disability Status Scale (EDSS) is a measurement of MS caused handicap and disease progression which is widely used in randomized clinical trials (RCT) as an endpoint measurement for clinical evaluation, despite non –linearity. The scale assesses neurological function, walking ability, and self-care functions.

EDSS shortcomings:

- based on neurological examination that is subjective,
- the scale has low reliability inter –rater and within-rater ,
- the scale is used is nonlinear-ordinal scale where the clinical importance of 1.0 change depends on starting score,
- correlates weakly with MRI measures (Sormani, Bonzano et al. 2010)
- impact of cognitive impairment for total score is low.

Despite all shortcomings and in lack of a better scale, the MS society is using EDSS both in clinical daily work and as endpoint measurement in RCT.

2.4 POPULATION

2.4.1 Paper I

This study was conducted in collaboration with the Neurology clinic at Queen square Hospital, London. We have obtained clinico-demographical data, MRI lesion volume data and plasma samples for 56 MS patients (37RR and 19PP). For 23 controls we have received demographical information and plasma samples.
RRMS was diagnosed according to McDonald's criteria (McDonald, Compston et al. 2001), and PPMS was diagnosed according to Thompson's criteria (Thompson, Montalban et al. 2000).

2.4.1 Paper II

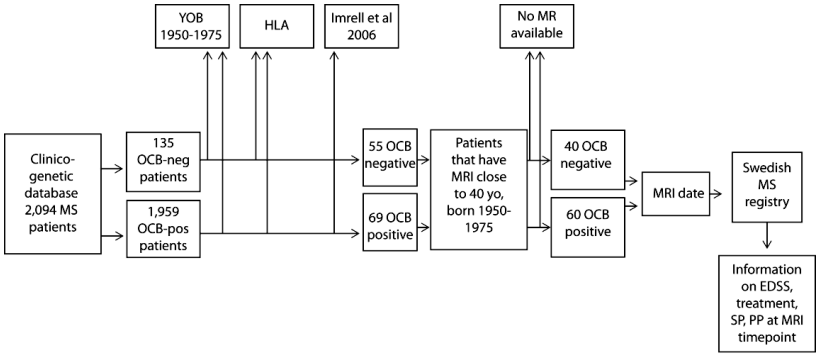
We examined 4 MS patients, who attended the MS center at Karolinska University Hospital Huddinge. Patients were examined with PET scan and MRI at the time point of relapse.

2.4.2 Paper III

We included 692 well characterized Nordic MS patients, attending the Karolinska University Hospital neurology clinic (both Huddinge and Solna sites) and 658 Nordic controls (blood donors).

2.4.3 Paper IV

Complicated study population and information gathering process I describe below as a flowchart. For detailed written description please refer to paper IV.



2.5 METHODS

2.5.1 Isotope Dilution Mass spectrometry

Detection of cerebrosterol in plasma according the method described previously (Dzeletovic, Breuer et al. 1995).

2.5.2 Brain imaging. Manual, semi-automated and automated lesion assessment.

Brain imaging. In studies I, II and IV patients were examined by 1, 5 T MRT scanner either on Siemens, Magnetom (Erlanger, Germany) or GE, Signa (General Electrics Medical Systems, Milwaukee, Wis, USA). The PET examination was made at 3D acquisition mode on a Siemens ECAT EXACT HR scanner. Patients were examined twice the same day: the first examination was performed with intravenously injected PET ligand [^{11}C] PK111195 and two hours later with [^{11}C] vinpocetine (paper II).

Lesion assessment methods. In paper I lesions were outlined by a semi-automated method (Sailer, O'Riordan et al. 1999). Lesion volumes were calculated using Dispimage software (Plummer 1992).

Lesion identification has been done manually: in paper II lesions on MRI were identified by two examiners with inter-examiner reproducibility 90% and in paper IV lesions were identified by one examiner with single rater variability 93.3%.

In paper II, on reconstructed PET-images, anatomical regions of interest were drawn using the Karolinska Institute's computerized Human Brain Atlas. PET and MRI were co-registered, regional and lesion volumes were calculated by using in-house modified version of SPM2 (Statistical Parametrical Mapping).

2.5.1 Genotyping

Paper III: Genotyping of *CYP46A1* intron 2 SNP designated rs754203 was performed by Pyrosequencing (Ronaghi 2003). The *APOE ε4* codon-112 SNP was analysed by TaqMan assay (Applied Biosystems, Foster city, California, USA).

Paper IV: Genotyping of *HLA-DRB1*15* and *HLA-DRB1*04* has been performed by sequence-specific PCR using a commercial kit (Olerup SSP AB, Saltsjöbaden, Sweden).

2.6 STATISTICS

In paper I, **multiple logistic regression analysis** was used to calculate the influence of MRI measures on cerebrosterol levels using as independent variables, the three lesion-volume parameters and the potential confounder age at sampling; and as the dependent variable, the cerebrosterol–cholesterol ratio, doubly logarithmically transformed to fulfill the assumption of normally distributed residuals. In the same paper **Scheffe's post hoc test** was used, to compare cerebrosterol–cholesterol ratio levels in RRMS patients, PPMS patients and controls.

Odds ratio (OR) is the ratio between the odds of being exposed for the variable in patients and in the healthy individuals.
OR = odds among the patients /odds among the healthy controls
odds among patients = N_{case}(gene carriers)/N_{case}(gene non-carriers)
odds among healthy = N_{contr}(gene carriers)/N_{contr}(gene non-carriers)

OR = 1 means that the odds of exposure among patients are equal to the odds of exposure among healthy individuals, i.e. that exposure has no effect on disease susceptibility. A value of more than one indicates risk variable and a value of lower than 1 indicates a protective variable.

In paper III, logistic **regression analysis** has been performed and OR estimates the influences of genotypes *CYP46 A1*AA and *APOE ε4* on risk of developing MS. In addition relative risk due to interaction (RERI) was calculated .

Cox regression analysis was performed in order to calculate hazard-ratio (HR) which estimates the influences of either *CYP46 A1*AA or *APOE ε4*or both genotypes on age at which patients reach EDSS 6.0 (need of unilateral walking assistance). HR is a measure of how often a particular event (reaching EDSS 6.0) happens in one group compared to how often it happens in another group, over time. Women and men, negative for both genotypes were defined as reference with HR estimate = 1. A HR of greater than one means that patients were at risk of reaching EDSS 6.0.

Survival analysis (Kaplan –Meier) was used in paper III to calculate cumulative proportions of patients reaching EDSS 6.0. In paper IV, associations between confounders (course, treatment and EDSS) at the time of MRI scanning and explanatory variables (OCB and HLA-allele carriage status) were investigated using **logistic regression analysis.**

Proportional ordinal logistic regression analysis was used to calculate OR reflecting influences of OCB, *HLA-DRB1*15* and *HLA-DRB1*04* for T2 lesion load extension in MS specific neuroanatomical compartments, total T1 and T2 lesion load (paper IV).

2.7 RESULTS

2.7.1 Paper I

There was statistically significant lower log transformed cerebrosterol-cholesterol concentration in PPMS (n=14; p=2.7 x 10^{-5}) and RRMS (n=20; p=0.0015) compared to healthy controls (n=6) in the age group older than 36 years; in the same age group there was no statistically significant difference between patients subgroups PPMS and RRMS (p=0.465). There was no statistically significant correlation with EDSS in neither PPMS (n=19; r= -0.06; p=0.792) nor in RRMS (n=30; r=0.14; p=0.465) [Spearman's correlation coefficient].

Multiple linear logistic analysis revealed a negative correlation between the age at sampling and log—log-transformed cerebrosterol-cholesterol ratio values with statistical significance in the PPMS group (β= -0.76; p=0.006). The T1 hypo-intense lesion volume was correlated positively with ratio values both in PPMS (β=0.064) and RRMS (β=0.59); in the latter group the correlation reached borderline statistical significance (p=0.052). For more results details please see original article enclosed in the thesis book.

2.7.2 Paper II

Volumes of gray, white matter, ventricular and subarachnoid CSF as well as total intracranial volume have been measured. Gray matter volume ranged 722-862 ml, white matter volume ranged 386-506 ml, ventricular CSF volume ranged 26-30 ml; Subarachnoidal CSF ranged from 242-278 ml and intracranial volume ranged 1486-1639 ml.

Due to differences of radioactivity per μmol of ligand at the time point of injection it was important to calculate the global uptake of radioactivity 2 min after radiolabeled ligand injection. Due to 40.4±24.3% more administered radioligand entered brain after [^{11}C]vinpocetine injection compared to after [^{11}C] PK111195 injection, 40.7±22.3% more radioligand was in the brain 2 min after [^{11}C]vinpocetine injection compared to 2 min after [^{11}C] PK111195 injection. The total brain uptake of radioactive ligand was calculated as a percentage of total injected radiolabeled ligand.

In order to show and compare the specificity of radiolabeled ligands to bind to areas adjacent to plaques, binding potential (BP) in normal brain parenchyma and BP in plaques were compared. Co-registration of MRI and PET images shows evidently the differences of binding pattern between [^{11}C]vinpocetine and [^{11}C] PK111195.

2.7.3 Paper III

***CYP46A1*AA and MS risk**

In the overall data set the carriership of *CYP46A1*AA genotype increased risk for MS (OR=1.12), but a 95% confidence interval overlapped unity (0.91-1.39).
Due to known differential effects of *CYP46A1*AA for cholesterol turnover in a knock out mouse model and *APOE ε4* differential effect for longevity in humans (Rosvall, Rizzuto et al. 2009), for further analysis we stratified the cohort by gender. Females, carriers of *CYP46A1*AA alone, carriers of *APOE ε4* alone and carriers of both genotypes were associated with reduced risk for MS, OR= 0.91; 95% confidence interval overlapped unity (0.55-1,51). Males, carriers of both genotypes were associated with increased risk for MS, OR=1.51; 95% confidence interval overlapped unity. Neither of results reache statistical significance

***CYP46A1*AA and MS prognosis**

Cox regression analysis was performed in order to calculate HR to reach EDSS 6.0 at a specific age, depending on carriership of either *CYP46A1*AA or *APOE ε4* or both. Analysis groups were stratified by gender. In females, carriership of either *CYP46 A1* AA or *APOE ε4* or both have a protective effect of reaching EDSS 6.0, but 95% HR estimate overlapped unity. In males, *CYP64A1*AA alone and in combination with *APOE ε4*, hastened attainment of EDSS 6.0. Male carriers of both genotypes attained EDSS 6.0 endpoint 17 years earlier compared to male non-carriers (HR=2.04 for carriers). Again 95% confidence interval overlapped unity.

2.7.4 Paper IV

Proportional ordinal logistic regression analysis showed no significant association of OCB status, *HLA-DRB1*15* or *HLA-DRB1*04* carriership and higher lesion load adjacent to the lateral ventricles, juxtacortical lesion load, nor with total T2-weighted lesion load or the total number of non-contrast-enhanced T1-weighted lesions in the brain. A trend was seen for a more than two-fold risk of increased lesion burden in the infratentorial compartment in OCB-positive patients; similar trends were not seen for *HLA-DRB1*15* or *HLA-DRB1*04* carriage.

2.8 DISCUSSION

2.8.1.1 Paper I

This paper was conducted in cooperation with Queen square Hospital, London .The data set with information on the T2, T1 and Gd-enhanced lesion volume and dataset with blood samples was partially matched, resulting in a total number of 37 RRMS patients and 19 PPMS patients with complete datasets.

The high value of logarithm transformed cerebrosterol - cholesterol ratio in RRMS patients in the age group younger than 36 years can be explained with current knowledge of cerebrosterol dynamics in neurodegenerative processes over time (or increasing individual age). Increased cerebrosterol in normal older individuals is a predictor of MCI eight years later.(Hughes, Kuller et al. 2012). We do not have any cognitive assessment for the current cohort but I assume that patients, younger than 36 years in the RRMS group have a slight cognitive impairment (SCI) or are cognitively normal and I could speculate that some PPMS patients older than 36 years would fulfill the criteria for MCI or dementia. This could be explained by both cholesterol and cerebrosterol in CNS being neurotoxic and causing necroptosis in vitro (Yamanaka, Saito et al. 2011)of neurons that in its turn causes neurodegeneration. PPMS patients are in another pathophysiological process with more pronounced cortical damage and atrophy and predominant neurodegeneration and here the logarithmic transformed cerebrosterol-cholesterol ratio value is low due to the already reduced amount of

metabolically active neurons and reduced bulk volume of myelin. However, the subgroup of patients is small and may not represent the general PPMS population.

EDSS does not correlate with the cerebrosterol-cholesterol ratio, and the reasons might be following:

1. EDSS is a non- linear measure of disability and handicap.
2. Processes ongoing in the spinal cord could contribute to changes of cerebrosterol concentrations in the plasma as well as to EDSS, but unfortunately this paper lacked data on MRI lesion load of the spinal cord.
3. There is no clear correlation between the disability dynamics and cerebrosterol concentration fluctuations in the EAE mouse model: cerebrosterol concentration reaches significantly higher levels already days before the development of the first EAE clinical symptoms (day 10) and reaches its peak at an approximately 200% increase when the animals have recovered and have no clinical EAE signs (day 17) (Teunissen, Floris et al. 2007).
4. EDSS does not correlate with either absolute cerebrosterol levels or cerebrosterol-cholesterol ratio in our small yet unpublished pilot study of 24 MS patients, who are assessed for EDSS and a battery of neurocognitive tests. However, we did find an inverse correlation between absolute cholesterol concentration and information processing speed.
5. Cerebrosterol is not a specific marker either for inflammation or in neurodegeneration-we do not know which ongoing process in CNS cerebrosterol fluctuation in plasma reflects.

The borderline significance of correlation ($p=0.052$, $\beta= 0.59$) between the T1 hypointense lesion volume and cerebrosterol-cholesterol ratio in the RR group is worth attention. T1 lesions (black holes,-irreversible tisseue damage and axonal loss) and could be a marker of neurodegeneration. One must be cautious interpreting statistical results with borderline significance and not to over interpret the data. However preliminary data in our pilot paper of 24 MS patients support the hypothesis of a cerebrosterol correlation with the other measures of neurodegeneration: An absolute level of cerebrosterol positively correlates with cortical volume, ventricular CSF and brain parenchymal fraction.

On a group level, the logarithm transformed cerebrosterol-cholesterol ratio concentration is reduced with increasing age in both MS groups (RR and PP) which

reflects reduction of both total brain volume and of number of metabolically active neurons as a consequence of accumulated years with tissue damaging disease.
In conclusion this paper shows that cerebrosterol is a potential biomarker of neurodegeneration in MS patients with a predominantly inflammatory process (RR group), but the study needs to be repeated in larger sample.
We wrote in the last sentence in our publication that studies with longer follow up need to be conducted and we kept our promise. We did investigate cerebrosterol levels in MS patients in a longitudinal seven year follow up paper. For more about that paper please see chapter "Current status and future prospects".
With the retrospective view of our results and publications, now is the time to sum up and see what is lacking in order for cerebrosterol to be recognized as a biomarker with potential clinical utility.
Some of the requirements are already fulfilled:

1. Biologically meaningful. The biomarker is relevant: during the pathological process with high membrane turnover the concentration of cerebrosterol both in CNS and blood is increased. Cerebrosterol is neurotoxic, causing necroptosis of neurons. The total amount of metabolically active neurons will be reduced due to both disease progress and the neurotoxic effect of cerebrosterol. The total amount of cholesterol in brain will be reduced by instant leakage of cholesterol from the brain. A combined effect of these factors will be a reduced absolute concentration of cerebrosterol in the blood.
2. Clinical relevance. It is evident that reduced amount of cortical neurons (what is found in advanced neurodegenerative processes) will lead to worse performance in neurocognitive tests. Reduced amount of neurons (reduced cortical volume) correlates with reduced total cerebral volume. In **early stages** of the neurodegenerative process cerebrosterol concentration correlates negatively with the scores of cognitive tests (high cerebrosterol, - low score). At the timepoint of **advanced** neurodegeneration low performance in neurocognitive tests correlate with low cerebrosterol concentrations in plasma (low cerebrosterol, low score).
3. Practical advantages. Cerebrosterol is a stable molecule, found in blood (plasma/serum) from easy obtainable body fluids; low amounts of plasma /serum are needed in order to analyze by isotope-dilution mass-spectrometry; the price of cerebrosterol analysis is comparable to existing routine blood sample prices.

These criteria still need to be fulfilled:

1. Confirmed correlation of cerebrosterol levels to other established biomarkers (cortical volume, cortical thickness, BPF as well as retinal cortical thickness with optical coherence tomography (OCT).
2. Confirmed correlation between cerebrosterol levels and disability (EDSS and SDMT) and prognosis.
3. Confirmed correlation of cerebrosterol levels with treatment effects.

Studies of large collections of MS patients are required in order to address these issues. For planned future studies please see chapter "Current status and future prospects".

2.8.1.2. Paper II

In our pilot study we investigated the difference of two PBR binding patterns in MS patients: well-known [^{11}C]-(R)-PK11195 and the new PBR PET marker [^{11}C]vinpocetine.
The prospective advantages of the [^{11}C]vinpocetine as PET ligand are the possibility to administer orally and better penetration through the BBB.
In the current paper [^{11}C]vinpocetine was administered intravenously.
[^{11}C]vinpocetine was binding to areas corresponding to T2 lesions on brain MRI but in a different pattern compared to [^{11}C]-(R)-PK11195. Why does [^{11}C]vinpocetine but not the [^{11}C]-(R)-PK1119 (except in one MS patient) bind to areas corresponding to T2 hyperintense lesions on MRI? Some of the possible explanations are listed below:

1. better penetrance of ^{11}C]vinpocetine through the BBB,
2. [^{11}C]vinpocetine binds to the different PBR subunit compared to [^{11}C]-(R)-PK1119. It is hypothesized that a huge molecular complex changes molecular conformation and exposes different binding sites depending on stages of glia activation.
3. Affinity of [^{11}C]vinpocetine to bind to other receptors, not only PBR: sodium and potassium channel receptors, adrenergic α receptors, peripheral $GABA_A$-benzodiazepine receptors, dopamine $D_{4.2}$ receptors (Gulyas, Halldin et al. 2002).

The fact that [^{11}C]vinpocetine uptake is increased in the brain after [^{11}C]-PK11195 pretreatment (Gulyas, Halldin et al. 2002) makes us question the study design many years later. Have we got false positive results due to microglia activation or BBB permeability alteration due to [^{11}C]-PK11195 administration prior to [^{11}C]vinpocetine injection? Would it have been better first to inject [^{11}C]vinpocetine and later [^{11}C]-PK11195 in order to find out the true [^{11}C]vinpocetine binding to MS lesion areas in PBR ligand untreated patients?
I agree with the experts in the field that [^{11}C]vinpocetine needs more research in order to map and identify better molecular, cellular and tissue structures to which [^{11}C]vinpocetine binds during MS lesion development over time.

2.8.1.2 Paper III

In this paper the weakest part is the small amount (9) of individuals of *CYP46A1* AA and *APOEε4* carriers that reach EDSS 6.0 17 years earlier compared to non-carriers. So of course the paper needs to be repeated in a bigger sample population. During my half time seminar I, together with my supervisors, have been thinking of repeating the study in a larger (hypothesis confirming) sample as a part of this paper. We have however made some changes: we chose SDMT as an outcome measure with a new paper design and different statistics methods which will result in another kind of paper and therefore we decided to make two separate projects. For project details please see chapter "Current status and future prospective".
The benign phenotype of female MS patients who are carriers of *CYP46A1* AA and *APOE*ε4 is not a big surprise. Females and males do have slightly different cholesterol metabolism and especially cerebrosterol metabolism differs between males and females. In the *Cyp46A1*-/- knockout mice, male and female phenotypes differed a lot: males were slower, had learning difficulties, and performed worse in cognitive tests (Kotti, Ramirez et al. 2006); females were active and "over performers" (personal communication Ingmar Björkhem). Animal studies concerning *Cyp46A1*-/- knockout phenotype characterization are performed only on male mice. The *APOE ε4* contribution to a female benign phenotype might be explained by *APOE ε4* gender specific effect for survival/ longevity: *APOE ε4* conferred 49% elevated risk for death for males in a community based sample of elderly individuals (Kungsholmens prospective cohort, 75+ years old, n=1094) (Rosvall, Rizzuto et al. 2009).

I admit that investigating the genes that affect neurodegeneration needs neurodeneration specific and sensitive outcome measures. Such measures are: SDMT, volumetric measures of brain tissue volumes and fractions. EDSS probably is not the optimal outcome measure for neurodegeneration.

2.8.1.3 Paper IV

The most important and critical question in this paper is whether the groups (OCB-negative and OCB-positive) in the paper are comparable due to different factors: group differences in treatment frequency and duration and a wide range of age.

	Never treated n (%)	Ever treated n (%)	Mean duration (months) of treatment per patient	
			In group	Out of all treated
OCB-negative	20 (50%)	19(47.5%) *	25 **	55.2 ***
OCB-positive	12(20%)	48(80%) *	39.4 **	49.2 ***

Two sample T- test, *p = 0.0004, **p =0.58, *** p=0.084

Table. Treatment frequency differences and mean duration in OCB-negative and OCB-positive patients.

There is a variation in the MRI protocol that depends on the time point of MRI examination: the oldest MRI examination from year 1999 and from other hospitals (Sabbatsberg) included sequences of 96 slices; in year 2001 MRI sequences contained 129 slices; the newest protocol used since year 2006 contained 169 slices. This means that sensitivity to find T2 lesions is higher on MRI examination according to the latest protocol compared to the examinations in year 1999. A higher number of slices, i.e. thinner slices, increase the sensitivity of the examination to find lesions. However, this paper might suffer from selection bias affecting the results. Blood donation for research is a voluntary act of patients in order to contribute to scientific development and research. The more often patients come to the MS center, the higher the chance to be requested to donate blood for research. These patients are less handicapped, have less fatigue, and less cognitive symptoms. In the selection of

patients, we used patients that were genotyped for HLA and within that group we searched patients that had available MRI data from close to their 40th birthday.
Now when we know the paper outcome and with the retrospective knowledge, I would rather choose diagnostic MRI made at the time point close to MS diagnosis so I could exclude the effect of covariates such as treatment. On the other hand, I still do not know at which age MS patients reach the most pronounced phenotypical diversity of T2 lesion load in total brain and in specific MS typical compartments.
This is an explorative, observational paper that did not require specific funding to conduct (except brain power work). Information has been used from the already available resources of information: genotyping database, the Swedish MS registry and clinical MRI archives. The primary endpoint of this paper is to see whether such studies are possible to conduct using retrospective data. The paper is underpowered but is still the first and largest paper to investigate the impact of OCB and *HLA-DRB1*04* and *HLA-DRB1*15* on total lesion load, T1 hypointense lesion load, contrast loading lesion load on whole brain MRI and T2 lesion load in MS specific neuroanatomical compartments. This is a pilot paper to assess how we can use our internal, already accumulated information resources, to answer questions related to clinical work, using tools that do not differ a lot from clinical situations (e.g. a clinical neuroradiologist evaluates MRI images). This is not the end of investigations of this cohort; we are planning to investigate the effect of OCB carriership and HLA genotypes for brain tissue atrophy measures using the automated volumetric method "Free surfer". For more details on that please see "Current status and future prospects".

2.8.2 Current status and future prospectives

2.8.2.1 Paper I

We have already completed the collection of data of longitudinal and cross-sectional data for 30 MS patients. Together with a neuropsychologist at our clinic, Gösta Bergendal, and a physicist, Leszek Stawiarz, we collected longitudinal and cross-sectional data for 30 MS patients; patients were examined with SDMT, at 3 time points: 1996, 2004 and 2011. Blood samples for cerbrosterol were collected in 2004 and 2011. MRI has been done once, in 2004. We did fulfill our promise to do a follow up project

on longitudinal cerebrosterol concentration changes. Advanced statistics is still remaining to be done, but preliminary observations show statistically significant correlations between cerebrosterol and MRI images in a cross-sectional sample in 2004: a positive correlation with volume of ventricular CSF and a negative correlation with cortical volume. In a prospective dataset we saw that high cerebrosterol values in 2004 are predictors of cognitive impairment in 2011. So in light of these observations, cerebrosterol is a potential marker of neurodegeneration in MS.
Another study to promote cerebrosterol as potential biomarker would be to collect evidence about treatment influence for plasma cerebrosterol levels. The first cohort I would be eager to use for such a study is the Swedish Tysabri ® cohort (IMSE project). In the frame of this project, plasma samples are already collected before Tysabri ® treatment start. Plasma is collected repeatedly yearly during Tysabri ® treatment and stored in a biobank. The MS registry will serve as a source of clinico-demographic information about the patients, included in the study. The disadvantage is that we do not have a placebo group. Deeper thoughts and detailed project design for such a study, I will develop after my thesis defence.

The next step for cerebrosterol, to qualify as a clinical biomarker, would be to involve cerebrosterol as an outcome biomarker in large multicenter placebo-controlled clinical trials. Advantages with such studies are that paper design already has been processed by authorities and other responsible persons. The difficult part is to persuade and convince primary investigators and responsible persons that cerebrosterol is a marker worthy of attention and future studies, and that multicenter trials could with certainty answer the question whether treatment affects the levels of cerebrosterol and whether there exist any differences of cerebrosterol levels between groups treated with immunomodulatory drugs and placebo groups.
And the final step, many years later, when there is a sufficient amount of positive robust studies concerning the cerebrosterol-clinical outcome relationship, will be to perform meta-analysis and to reveal mathematical relationships between cerebrosterol and clinical outcome.

2.8.2.2 Paper II

Currently, I do not plan future studies in the PET-vinpocetine area.

2.8.2.3 Paper III

From an immunochip genotyping database (approximately 2000 individuals) we will be able to get *CYP46A1* SNP rs754203 genotypes and *APOE ε4* carriership status. As an outcome measure we will choose z value of SDMT (normalized for age and absolute value of SDMT). We will perform survival analysis. As time axis we will choose age at reaching SDMT value 43, an important practical milestone indicating risk for an individual to lose his/her driving license or have impaired employment status (part-time or full time sick leave) in the next 3 years due to reduced cognitive performance.

2.8.2.4 Paper IV

Currently we are continuing the investigation of structural MRI differences between OCB-positive / OCB negative groups. By using the method FreeSurfer (automated vertex based morphometry) we will assess the differences in cortical, white matter atrophy in defined groups. With the in house developed software (validated method for volumetry of lesion), that uses a multimodal approach for lesion determination, we will assess T2, T1 and Gd-enhanced lesion volume differences in the groups. A future plan is also to conduct a prospective study on a hospital based cohort, without selection bias. For that study we will include patients prospectively. At that time we will already have power calculation for the FreeSurfer method for volumetric evaluation of gray, white substances and CSF in OCB-positive and OCB-negative groups.

2.8.1 Biomarker and outcome measure I would like to use in the future: Optical coherence tomography (OCT)

Retinal nerve fiber layer thickness (RNFL) is a non-invasive method, based on emission of low coherence infrared light through the pupil and reflection from the retina (Huang et al. 1991). The method has advantages: it is non-invasive, not painful for the patient, cost and time effective.

Retrograde trans-synaptic axonal degeneration (due to lesions along the retrochiasmatic visual pathways) is the reason for RNFL thinning. Thickness of RFNL shows significant inverse correlation with EDSS (Siger, Dziegielewski et al. 2008) and

correlation with MRI atrophy measures (Sepulcre, 2007). Measurement of RNFL by OCT still needs validation in longitudinal studies to find out whether RNFL thickness might be used as outcome measure in clinical trials of neuroprotective drugs.

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