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Multiple Sclerosis in Twins

A Finnish Twin Cohort study on
genetic and environmental factors

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CONTENTS

LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following original communications that are referred to in the text by the Roman numerals I-IV.

I Kuusisto H, Kaprio J, Kinnunen E, Luukkaala T, Koskenvuo M, Elovaara I. Concordance and heritability of multiple sclerosis in Finland: study on a nationwide series of twins. In press (Eur J Neurol)

II Kuusisto H, Hyöty H, Kares S, Kinnunen E, Saarelainen M and Elovaara I. (2005): Enteroviruses and the risk of MS in the Finnish Twin Cohort. Eur J Neurol. 12:707-9.

III Kuusisto H, Hyöty H, Kares S, Kinnunen E and Elovaara I. (2008): Human herpesvirus 6 and multiple sclerosis: a Finnish twin study. Mult Scler 14:54-8.

IV Särkijärvi S, Kuusisto H, Paalavuo R, Levula M, Airla N, Lehtimäki T, Kaprio J, Koskenvuo M, Elovaara I. (2006): Gene expression profiles in Finnish twins with multiple sclerosis. BMC Med Genet 27:7-11

ABBREVIATIONS

ab	antibody
ag	antigen
APC	antigen presenting cell
BBB	blood brain barrier
CCR	chemocine receptor
CIS	clinically isolated syndrome
CNS	central nervous system
Cp	Chlamydia pneumoniae
CSF	cerebrospinal fluid
CTLA4	cytotoxic T lymphocyte antigen
CMV	cytomegalo virus
DNA	deoxy ribo nucleic acid
DZ	dizygotic
EAE	experimental autoimmune encephalomyelitis
EBV	Ebstein barr virus
EDSS	expanded disability status scale
EV	enterovirus
Evi-5	ecotropic viral integration site 5
GA	glatiramer acetate
GIP3	Interferon-induced protein 6-16
Gd	gadolinium
HBA2	Hemoglobin alpha 2
HBB	Hemoglobin beta
HRV	human retro virus
HHV6	human herpes virus 6
HLA	human leukocyte antigen
HSV	herpes simplex virus
Ifi	interferon alpha-inducible protein
Ig	immunogloubulin
IHC	immunohistochemical
IL	interleucine
ILRA	interleucine reseptor alpha
IM	immunomodulatory
INF-β	beta interferon
IRF-5	interferon regulatory factor 5
iv	intravenous
LAPTM5	Lysosomal-associated multispanning membrane protein-5
MBP	myelin basic protein
MHC	major histocompatibility complex
MRI	magnetic resonance imaging
MS	multiple sclerosis
MX2	Myxovirus (influenza) resistance 2
MZ	monozygotic
NAB	neutaralising antibody
OCB	oligoclonal band

PBMC	peripheral blood mononuclear cell
PC	plasma cell
PCR	polymeric chain reaction
POU3F1	POU domain, class 3, transcription factor 1
PP	primary progressive
PRKCA	protein cinase C alpha
PML	progressive multifocal leucoencephalopathy
QRT	quantitative reverse transcription
RNA	ribonucleic acid
RR	relapsing remitting
RT-PCR	reverse transcription PCR
SC	spinal cord
SNPs	single nucleotide polymorphisms
SP	secondary progressive
T	tesla
TME	Theiler murine encepahlomyelitis
Tc	cytotoxic T-cell
TCR	T cell receptor
TNF	tumor necrosis factor
UV	ultra violet
VEP	visual evoked potential
VLA-4	very late antigen
VZV	varicella zoster virus

In additon, the standard one-letter abbreviations of nucleotides are used.

ABSTRACT

Multiple sclerosis (MS) is the most common autoimmune demyelinating disease of the central nervous system (CNS). Both genes and environment contribute to susceptibility to the disease, but their role is as yet poorly understood.

Ten monozygotic (MZ) and 14 dizygotic (DZ) twin pairs either dis- or concordant for MS, obtained from The Finnish Twin Cohort, participated in the present study. The aim was to evaluate the role of genetic and environmental factors in the aetiology of MS in Finland. The data was compared to the original MS Finnish Twin Cohort study performed by Kinnunen and associates in 1988. The method used to recruit the twins was the same in both studies.

The concordance of MS was assessed using probandwise concordance rates and tetrachoric correlations for MZ and DZ twin pairs. For estimating the contribution of genetic factors to susceptibility to MS, a polygenic multifactorial model was used and heritability estimated using structural equation models. In order to identify genes involved in MS pathogenesis, the gene expression profiles in peripheral blood mononuclear cells (PBMCs) obtained from MZ discordant pairs were analyzed by cDNA microarray technology detecting the expression of 8 300 genes. Expressions of the 6 most often upregulated genes were confirmed by relative quantitative reverse transcription polymeric chain reaction (QRT-PCR). Furthermore serum samples from 17 twin pairs and cerebrospinal fluid (CSF) samples from 6 twin pairs were tested by PCR specific for human herpes virus 6 (HHV6) and with reverse transcription PCR (RT-PCR) specific for enteroviruses (EVs). Immunogloubulin (Ig) G and M response against HHV6 in serum and CSF were analyzed using ELISA method.

The pairwise concordance for MZ twins was 30% and for DZ twins 14%. The corresponding probandwise concordance rates were 46% and 25%. The genetic variance (heritability) was estimated to be 15.3% (95% Cl 0.0-77.6), the common environmental variance 73.7% (95% Cl 14.1-93.9) and the unique environmental variance 11.1% (95% Cl 2.3-30.0).

We found six genes to be up-regulated by 40 % and one gene (G1P3) by 50 % in the twins with MS. The six most constantly expressed genes were G1P3, POU3F1, MX2, LAPTM5, HBA2 and HBB.

No HHV6 DNA or EV RNA was found in any serum or CSF samples. Eighty eight percent of the twins with MS and 86% of the healthy twin siblings were positive for HHV6-specific IgG in serum. One twin with MS was also postitive for HHV6 specific-IgM in serum, whereas none of the healthy twins was IgM positive. All CSF samples were negative for HHV6 specific-IgG and -IgM in both groups.

Based on these data the concordance of MS in DZ twins in Finland has increased during the past two decades, whereas in MZ twins it has remained the same. This observation, together with the estimated low heritability, would suggest that the recently reported increase in MS incidence in Finland could be mainly due to environmental factors. We were not able to demonstrate, however, that EV or HHV6 infections could have had such a causative role. The six genes that were up-regulated at least two-fold in 40% of the twins, may be related to MS pathogenesis, beta-interferon (INF-β) treatment or an unknown virus infection.

TIIVISTELMÄ

Multippeliskleroosi (MS) on parantumaton tulehduksellinen keskus-hermoston sairaus, joka aiheuttaa sekä harmaan että valkean aineen vaurioita. Se on yleisin nuorten aikuisten vakava neurologinen sairaus. MS-taudin puhkeamista ja yksilöllistä taudin kulkua on vaikea ennustaa. MS-taudin riskitekijät ovat todennäköisesti perintö- ja ympäristötekijöiden muodostamia yhdistelmiä. Toistaiseksi kuitenkin tällaisia tekijöitä tunnetaan huonosti. MS-taudin vallitsevuus lisääntyy päiväntasaajalta napoja kohti liikuttaessa ja Suomessa se on maailman korkeimpia.

Perintötekijöiden osuus MS-taudin puhkeamisessa on kiistattomasti osoitettu, joskin tauti esiintyy suvuittain vain harvoin (10 %:lla potilaista). Ensimmäisen asteen sukulaisten sairastumisriski on n. 5 %, ei-identtisten kaksosten 2-5 %, mutta identtisten kaksosten sairastumisriski jopa 30 %. Vaikuttakin siltä, että MS-tauti on polygeeninen eli osallisena on useita eri geenejä, joiden esiintyminen mahdollisesti määrittää yksilön sairastumisalttiuden. Jo pitkään on tiedetty, että human leukocyte antigen (HLA)- DRB1*15 lisää merkittävästi riskiä sairastua MS-tautiin. Tätä tyyppiä esiintyy n. 60 %:lla MS-potilaista ja 20 %:lla vertailuväestöstä.

MS taudin pahenemisvaihetta edeltää usein jokin tulehdus, ja jo kymmeniä vuosia on epäilty tulehdusten olevan yhteydessä MS taudin puhkeamiseen. Viime vuosina epäilykset ovat kohdistuneet erityisesti herpesviruksiin (EBV ja HHV6). EVs ovat puolestaan saaneet huomiota mahdollisesti laukaisevina tekijöinä autoimmuunitaudeille yleisesti, mutta niiden yhteyttä MS tautiin on tutkittu niukasti.

Tämän tutkimuksen päätavoite oli arvioida perintö- ja ympäristötekijöiden merkitystä MS-taudin etiologiassa Suomessa, joka edustaa MS-taudin suhteen korkean vallitsevuuden aluetta.

Tutkimukseemme osallistui 24 kaksosparia, joista joko molemmilla tai toisella on MS-tauti. Kaksoset kerättiin suomalaisesta kaksoskohortista. Kaikki kaksoset tutkittiin kliinisesti ja keskushermoston magneettikuvauksella. Konkordanssi ja periytyvyys, sekä yksilöllisten ja jaettujen ympäristötekijöiden osuus etiologiassa arvioitiin. MS-tautiin liittyviä perintötekijöitä tutkimme kontrolloidulla kaksostutkimus asetelmalla, johon osallistui kahdeksan diskordanttia samanmunaista (identtistä) kaksosparia, eli toinen indeksihenkilö oli terve ja toinen sairasti MS-

tautia. Sairaan kaksosen geenien ilmentymisprofiilia (8300 geeniä) verrattiin terveen kaksosen geeniprofiiliin cDNA microarray analyysia käyttämällä. Mahdollisina yksilöllisinä ympäristöriskitekijöinä määritimme EV-RNA:n seerumista ja selkäydinnesteestä. HHV6-DNA määritettiin sekä seerumista, soluttomasta selkäydinnesteestä että selkäydinnesteen valkosoluista. Lisäksi IgG ja M vaste HHV6:a vastaan määritettiin ELISA-menetelmällä sekä seerumista että selkäydinnesteestä.

Tutkimuksemme perusteella niiden erimunaisten kaksosten osuus, joissa molemmat sairastavat MS-tautia suhteessa kaikkiin pareihin, joissa esiintyi sairastapauksia, on lisääntynyt 20 vuoden aikana nollasta 14 %:iin (2/14), kun taas identtisillä kaksosilla osuus on pysynyt ennallaan (30 %). Todennäköisyys sairastua MS-tautiin, jos indeksikaksonen on sairastunut, oli 46 % identtisillä ja 25 % erimunaisilla kaksosilla. MS-taudin perinnöllisyysarvio taudin etiologiassa oli 15.3 % (95 % Cl 0.0-77.6), jaettujen ympäristötekijöiden osuus 73.7% (95 % Cl 14.1-93.9) ja yksilöllisten ympäristötekijöiden osuus 11.1% (95 % Cl 2.3-30.0). Emme voineet osoittaa EV RNA:ta tai HHV6 DNA:ta seerumista tai selkäydinnesteestä MS-tautia sairastavilla kaksosilla tai heidän terveillä kaksospareillaan. Geenien ilmentymisprofiilit erosivat identtisten MS-tautia sairastavien kaksosten ja terveiden kaksossisarusten välillä. Kuuden geenin ilmentyminen oli lisääntynyt vähintään kaksinkertaiseksi puolella kaksosista. Nämä geenit olivat: G1P3, MX2, POU3F1, LAPTM5, HBA2 ja HBB.

Johtopäätöksenä voidaan todeta, että lisääntyneen MS-taudin ilmaantuvuuden taustalla, Suomessa lienee pääasiassa muuntuneet ympäristön olosuhteet. Koska eri- ja samanmunaisten kaksosten sairastuvuus todennäköisyydessä on edelleen ero, myös perintötekijät vaikuttavat MS-taudin puhkeamisen riskiin. Yksilöllisillä ympäristötekijöillä saattaa olla niin ikään merkitystä. Emme voineet kuitenkaan osoittaa, että latentti HHV6- tai EV-tulehdus olisi yhteydessä MS-taudin riskiin. Geeniexpressiotutkimuksemme tulokset osoittavat, että ne geenit, joiden ilmentymisessä oli merkittävä ero terveen ja MS-tautia sairastavan identtisen kaksosen välillä (G1P3, MX2, POU3F1, LAPTM5, HBA2 ja HBB), osallistuvat B-solujen erilaistumisen säätelyyn, myeliinin korjaantumiseen, oxidatiiviseen stressiin ja virusperäisiin tulehduksiin. Lisääntynyt geenien ilmentyminen näyttää siis liittyvän MS-taudin tulehdukselliseen syntymekanismiin, virustulehduksiin ja mahdollisesti MS-taudin hoitoon käytettävään beetainterferonilääkitykseen.

INTRODUCTION

MS is a chronic inflammatory demyelinating disease of the CNS characterized by inflammatory lesions scattered throughout the CNS tissue. It is the most common neurological disease of young adults leading to disability (Compston, 2002).

There is a significant geographical and temporal variation in the prevalence of MS. The prevalence declines progressively in populations living near the equator and increases with latitude (Kurzke 1977). In Finland it is one of the highest in the world. Recently an increase in the incidence of MS has been observed (Sumelahti 2000). The underlying reason for this is unknown, although environmental factors have been proposed (Sumelahti et al 2001).

Despite intensive investigations, the etiopathogenesis of MS is still unknown. Interactions among possible susceptibility genes and the environment have been proposed to contribute to the development of the disease (Prat and Antel 2005). The candidate gene studies in MS have been, however, somewhat disappointing and so far only human leukocyte antigen (HLA)-DR2 has been repeatedly confirmed to be strongly associated with the disorder (Giovannoni and Ebers 2007). Viral infections in genetically susceptible individuals during childhood and early adulthood are believed to disturb the immune homeostasis and promote the development of autoreactive T cells mediating cellular damage (Hunter and Hafler 2000, Wolfson 2001). Recently EBV (Levin et al 2003, Höllsberg et al 2005, DeLorenze 2006), chlamydia pneumoniae (Cp) (Swanborg and Whittum-Hudson 2002, Swanborg et al 2003) and HHV6 (Knox et al 2000, Moore and Wolfson 2002, Swanborg and Whittum –Hudson 2002, Alvarez-Lafuente et al 2004 and 2006, Clark 2004, Höllsberg et al 2005) have been linked with MS. The findings are, however, partly controversial and no causation has been shown (Moore and Wolfson 2002, Swanborg and Whittum-Hudson 2002, Giovannoni and Ebers 2007).

A well designed and conducted twin study is a powerful means to evaluate the relative contribution of genetic and environmental factors in the aetiology of a given disease (Hawkes 1997). Therefore the access to The Finnish Twin Cohort afforded us an excellent possibility to evaluate such susceptibilities to MS in Finland. Furthermore, when evaluating the role of unique environmental factors in MS aetiology, it has been emphasised that the study population should be genetically as homogenous as possible (Moore and Wolfson 2002), thus twins can be considered optimal

subjects. For further analyses of unique environmental factors we selected HHV6 and EVs, which both can be latent in the CNS (Sawyer 2002, Alvarez-Lafuente 2004). Differences in gene expression profiles in discordant MZ twins were analysed by the cDNA microarray technique using a controlled co-twin method.

REVIEW OF THE LITERATURE

1. Clinical aspects of MS

1.1. Clinical subtypes and course of the disease

MS is a chronic autoimmune demyelinating disease of the CNS, characterised by recurrent focal blood-brain barrier (BBB) damage, perivascular lymphocyte infiltration, patchy degradation of myelin sheath and axonal loss. The symptoms and signs of MS reflect the anatomical sites of the inflammatory and degenerative CNS lesions. Any part of the CNS can be involved, most typically the cerebellum, optic nerve, brain stem and spinal cord (SC). Common symptoms are muscle weakness, sensory disturbances, vision deficits, ataxia, fatigue and cognitive impairment (Compston 2002).

The course of MS varies substantially within individuals. Typically the disease follows a fluctuating course, with relapses and remissions (relapsing-remitting MS; RRMS). In most of the patients the disease course changes into secondary progressive (SPMS) over time, the median being ten years (Confavreux et al 1980, Erikson et al 2003). About 10-15% of affected patients have chronic progressive disease from the onset (primary-progressive MS; PPMS). In RRMS the relapses are due to CNS inflammation, whereas in the SPMS and PPMS the symptoms are mainly due to irreversible axonal loss and the inflammation itself is minimal (Noseworthy et al 2000, Compston et al 2002). The course of MS is illustrated in figure 1.

Figure 1. The course of multiple sclerosis

Pre clinical phase of MS	**Relapsing-remitting (RRMS)**	**Secondary-progressive (SPMS)**

MRI activity   **Clinical course of the disease**   **Brain volume**   **Disability**
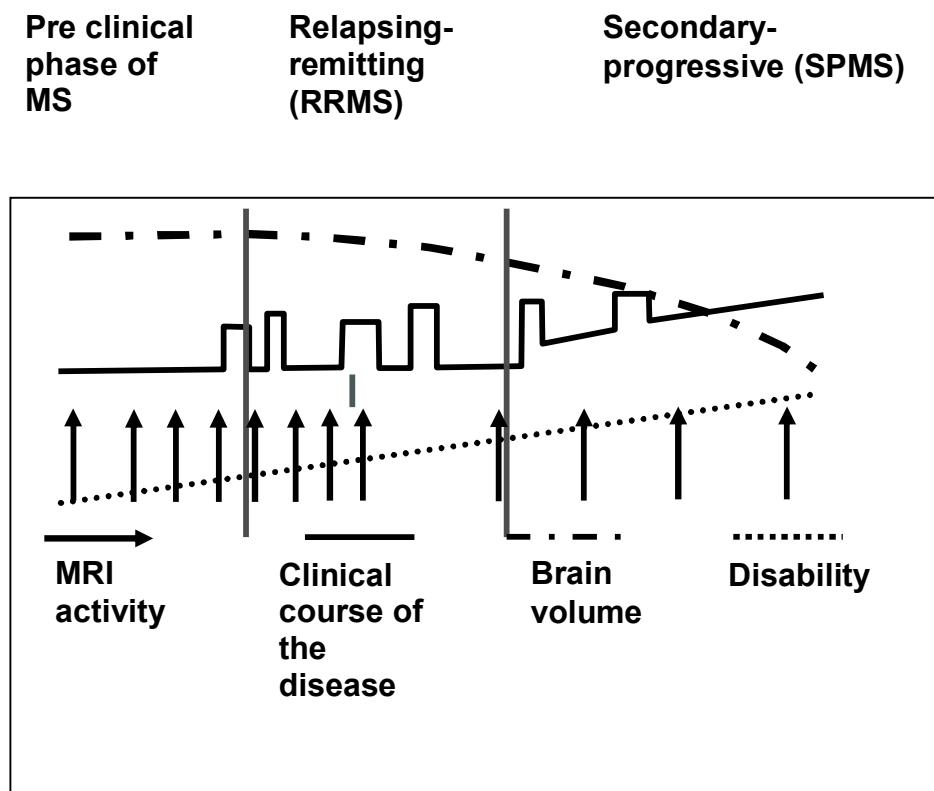
To evaluate the disability caused by the disease, the Kurtzke Expanded Disability Status Scale (EDSS) is most commonly used (Kurzke 1983), see table 1.

Table 1. The Expanded Disability Status Scale (EDSS)

Walking Ability

10.0 = Death due to MS

9.0–9.5 = Completely dependent

8.0–8.5 = Confined to bed/chair; self-care with help

Confined to a wheelchair or bed

7.0–7.5 = Confined to wheelchair

Walks with aid (<5 yards)

6.0–6.5 = Walking assistance is needed

Walks with assistance (22–220 yards or more)

5.0–5.5 = Increasing limitation in ability to walk

Walks unaided (110–220 yards or more)

4.0–4.5 = Disability is moderate

Walks unaided (330–550 yards or more)

3.0–3.5 = Disability is mild to moderate

2.0–2.5 = Disability is minimal

1.0–1.5 = No disability

Fully ambulatory

0 = Normal neurologic exam

1.2. Diagnosis of MS

Diagnosis of MS is based on the objective clinical and, if needed, paraclinical evidence of dissemination of inflammatory lesions in CNS in time and space. In addition to CNS magnetic resonance imaging (MRI) investigation, commonly used paraclinical investigations are CSF and visual evoked potential (VEP) analyses. In CSF pleocytosis, elevated IgG index and oligoclonal IgG can be seen in approximately 80% of the patients with MS (Tintore et al 2008). Over the years various diagnostic criteria for MS diagnosis have been used (Schumacher 1965, Poser 1983, Thompson et al 2000, McDonald 2001, Polman et al 2005). At first the criteria were based on clinical features alone (Schumacher 1963), but in 1983 a group of experts reached a consensus on a classification, which was widely accepted both in clinical trials and practise (Poser et al 1983). The Poser criteria were based on two or more attacks affecting at least two separate sites within the CNS, but for clinically definite disease, also allowed clinical evidence to be replaced by laboratory abnormalities at the second site. MRI, VEP and CSF examinations were used to supplement evidence for the diagnosis in situations where clinical criteria were not met (Poser et al 1983).

As a result of the demand to start disease-modifying therapy as early as possible in the disease course, when it is most likely to be useful, a need for new diagnostic criteria was recently acknowledged. The main focus was to bring forward the point at which it would be possible to make the diagnosis of MS with sufficient security. The new McDonald criteria were developed by an international panel in 2001. (McDonald et al 2001). The main change was that specific MRI features for dissemination in time and space were incorporated. Thus, if a patient has a single clinical episode characteristic of demyelination accompanied by signs only of the symptomatic lesion on examination (CIS=clinically isolated syndrome), the demonstration by MRI of dissemination in time and space allows a diagnosis of MS (McDonald et al 2001). The McDonald criteria have been revised in 2005 with minor changes mainly concerning the use of tesla (T)2-weighted lesions and SC imaging (Polman et al 2005). Recently Swanton and associates proposed a new MRI criteria for MS in which new T2 lesions can be used as a sign of dissemination in time irrespective of the timing of baseline scan in contrast to McDonald criteria, in which the baseline MRI has to be obtained at least 30 days after the initial symptom. The authors conclude that the new criteria are simpler than McDonald criteria with good specificity and accuracy (Swanton et al 2007).

1.3. MRI findings in MS

Focal white matter lesions are present in over 90% of patients with clinically definite MS (Robertson et al 1985, McDonald 1989). T1-weighted MRI is less sensitive than T2 in distinguishing MS lesions, but it reveals parenchymal destruction well (Van Waesberghe et al 1999). 20-30% of chronic T2 lesions are persistently T1 hypointense in MS, but this is very uncommon for white matter lesions associated with normal aging and vascular diseases (Uhlenbrock et al 1989).

An increase in the vascular permeability in association with inflammation can be evaluated by using gadolinium (Gd)-enhancement. Normally Gd is excluded from the parenchyma of the brain and SC by BBB. When BBB permeability is increased, Gd-enhancing lesions are detected, thus they are commonly seen during relapses. Lesions can enhance uniformly, or show a complete or partial ring-like enhancement. Enhancement is the earliest change detectable by MRI in the development of new lesions in relapsing MS. Enhancement lasts on average 4-6 weeks, and no more than 3 months. Gd-enhancing lesions are very rare in PPMS, in which only 5% of the new lesions enhance (Thompson et al 2001).

Most of white matter lesions are asymptomatic and the correlation with disability is poor (Gawne-Cain et al 1998, Nijelholt et al 1998, Charil et al 2003). Lesions seen in MS are not specific and in times difficult to distinguish from other aetiologies, especially vascular diseases. MS lesions most commonly involve deep white matter whereas peripheral white matter is affected in vascular diseases, but neither is specific. MS lesions are also typically quite large, over 3 mm in width and oval shaped. They are lined along the vessels and most often accumulate periventriculary, infratentorially, subcortically or in the corpus callosum, sites not typical for vascular lesions (Gawne-Cain et al 1998, Nijelholt et al 1998, Charil et al 2003).

The SC is commonly affected radiologically even if the patient does not have any spinal symptoms or signs. The SC lesions are normally less than one vertebral segment in length and occupy only part of the diameter of the cord. Acute lesions can display some swelling, which can lead to focal atrophy of the SC. MRI of the SC is of high value since the SC is not affected by aging (Thorpe et al 1993).

Various MRI criteria have been developed in order to predict the development of MS after CIS and to distinguish MS lesions from other aetiologies and normal aging. (Fazekas et al 1988, Paty et al 1988, Barkhof et al 1997, Tintore et al 2000). The MRI criteria of Barkhof are widely used today to demonstrate the dissemination in space (Barkhof et al 1997, McDonald et al 2001, Polman et al 2005), see table 2. The MRI criteria of Barkhof are based on a study with 74 CIS patients. Thirty three patients developed clinically definite MS in two years. The model presented in table 2 had an accuracy of 80% and performed better than the criteria of Paty and Fazekas (Barkhof et al 1997, Paty et al 1988, Fazekas et al 1988).

Table 2. MRI evidence for dissemination in space (Barkhof's criteria)
Three of the following are recuired: - One or more Gd-enhancing lesions or nine or more T2 hyperintense lesions   - One or more infratentorial lesions   - One or more juxtacortical lesions   - Three or more periventricular lesions

In addition to focal lesions brain atrophy starts to develop in the majority of the patients even from the very beginning of the disease and it seems to have at least a modest correlation with disability (Miller et al 2002, Fisher et al 2002). The decrease in brain volume in healthy subjects is approximately 0.1-0.3% yearly (Xu et al 2000, Ge et al 2002) where as in MS patients it is 0.6-1.0% (Fox et al 2000, Ge et al 2002, Kalkers et al 2002).

1.4. Treatment of inflammation in MS

Exacerbations (relapses) can be seen both in RRMS and in the relapsing form of SPMS. A relapse is commonly defined as objective evidence of a new or marked increase of old symptoms and signs lasting at least 24 hours. In 1960s Corticotropin given as pulsed intramuscular treatment was shown to shorten the duration of relapses (Miller et al 1961). For twenty years this remained the only treatment option. The introduction of intravenous (iv) high dose methylprednisolone in the 1980s, offered the benefit of far less side effects than corticotropine (Dowling et al 1980). Today the most commonly used dosage of methylprednisolone is 500-1000 mg for three consecutive days either iv or orally. The effects of methylprednisolone last only a few months and the treatment of relapses

has no impact on the course of the disease, it merely shortens the recovery period (Milligan et al 1987).

The immunomodulatory (IM) treatment of MS has developed substantially during the past decade. Large clinical trials have evaluated the efficacy of three INF-β and glatiramer acetate (GA) as disease modifying drugs in MS (PRISMS study group 1998 and 2001, The IFNB Multiple Sclerosis Study Group 1993a and b, 1995, Jacobs et al 1996, Johnson et al 1995). They all have been introduced to clinical practise and, more recently, natalizumab has been approved (Polman et al 2006, Rudick et al 2006). The efficacy of current IM treatments in different types of the disease is well known. They all reduce the relapse rate and MRI activity, but the effect on disability seems to be modest, especially at the later stages of the disease (Noseworthy et al 2000, Filippini et al 2003, Rudick et al 2005).

In addition to IM treatment, drugs that non-specifically suppress the immune response are used in selected cases. The most often used of these drugs is mitoxantrone, which has been shown to reduce relapse frequency and MRI activity in patients with very active MS (Mauch et al 1992, Edan et al 1997, Hartung et al 2002). It should be noted that mitoxantrone is a toxic agent that must be administered with care to reduce the possibility of bone marrow suppression, opportunistic infections and cardiomyoptahy.

Several novel IM approaches are being tested in ongoing clinical trials. Two of the most promising agents now in phase III trials are FTY20 and anti-CD52 (Campath-1H or alemtuzumab). FTY20 induces homing of lymphocytes to the lymphnodes and traps them at this site. As a consequence the migration of the lymphocytes to the inflamed organ compartments is prevented. The drug is orally administered. Alemtuzumab is an iv administered humanized monoclonal antibody. Phase II trial started 2004, but was temporarily discontinued due to two cases of idiopathic thrombocytopaenic purpura, one of which was fatal (Coles et al 2006).

Despite of exceptional activity in the field of clinical trials today, as for now the clinical course of the disease may be altered only by suppressing the inflammation in the CNS, which may prolong the time before entering the SP phase. Once the disease is purely progressive and there are no relapses, the therapeutical possibilities to influence the course of the disease are limited.

2. Epidemiology of MS

2.1 Incidence and prevalence of MS

Incidence measures the number of new diagnoses in a definite area over one year. The annual incidence of MS in Northern countries including Finland is approximately 3.5-6/100 000 whereas close to the equator the incidence is reported to be 1-3/100 000 (Compston 2002). Over recent decades the incidence of MS has reportedly increased both in high and low incidence areas (Compston 1997, Compston and Confavreux 2006). The underlying reason for this increase is not known. It may be in part due to the development of diagnostic tools but changes in the environmental factors have also been proposed (Sumelahti 2001).

Prevalence of MS defines the number of individuals in whom the disease has been diagnosed in a defined geographical area. MS has an uneven geographic distribution traditonally classified as low, medium and high prevalence areas, due to the differences in the genetic and ethnic backgrounds of the populations of the world (Kurzke 1977). In general the prevalence of MS gets progressively less in populations living near the equator and increases with latitude in both the northern and southern hemispheres. In addition there are some variation in MS prevalence within continents and countries themselves: In Europe the prevalence of MS is higher in southern Scandinavia, Northern Germany, parts of the United Kingdom (north east Scotland, Orkney and Shetland islands) and parts of Italy (Sicily and Sardinia) than in northern Scandinavia, France, Spain and eastern Mediterranean countries (Compston 1997). In selected parts of Finland it is as high as 200/100 000 (Sumelahti 2001). In North America prevalence is higher in the Midwest than in the south (Bulman and Ebers 1992). Among white Australians the prevalence is higher in the south than in the north (Hammond et al 1988). MS is considered to be rare in the Far East, the Arabian Peninsula, Africa, continental South America and India (see figures 2 and 3) (Compston 1997 and Compston and Confavreux 2006).

Figure 2. Distribution of multiple sclerosis in Europe. Figures are prevalence/100 000 of the population
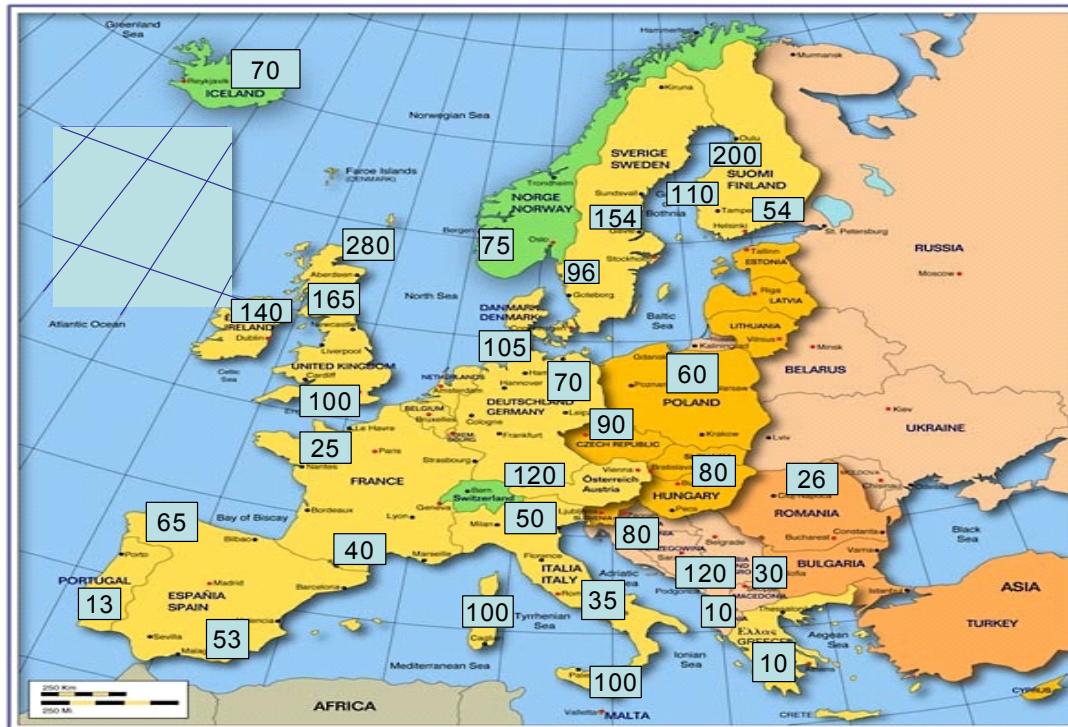

Figure 3. Distribution of multiple sclerosis in the world. Figures are prevalence/100 000 of the population
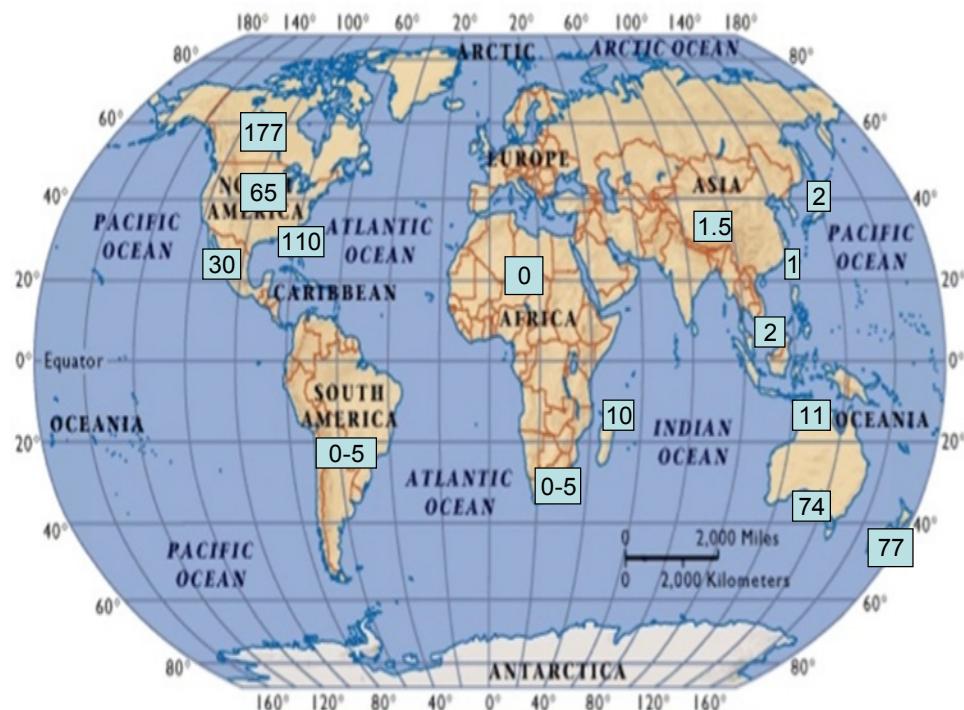
2.2 Disease concordance and heritability

All twin studies conducted in high-prevalence countries have demonstrated a higher MS concordance in MZ than in DZ twins. In MS MZ concordance in high prevalence areas is known to be approximately 25-30% with rates of 3-5% for DZ pairs (Mumford et al 1994, Sadovnick et al 1993, Willer et al 2003, Hansen et al 2005, Kinnunen et al 1988, Thorpe et al 1994). There is, however, an observation from France, a medium-prevalence country, that the concordance for MZ twins is considerably lower than in the north (French Research Group of Multiple Sclerosis 1992). This finding has recently been confirmed in two studies leading to the suggestion that frequency of MS in twins correlates with MS prevalence in the area (Ristori et al 2006, Islam et al 2006). Therefore, it has been hypothesized that genetic and environmental factors may contribute in different proportions and ways to MS risk in different populations (Ristori et al 2006). The use of the concordance rates to quantitatively examine the relative contribution of genetic and environmental factors to variation has not been performed in any of the previous twin studies in high prevalence areas. However, in a review by Hawkes, the tetracholic correlations for liability and heritabilities for MS have been calculated in the twin studies comprising more than 50 twin pairs. Heritability is the proportion of overall variance due to genetic factors. The heritability estimates range from 24% in France to 86% in Canada (Hawkes 1997). In the Italian cohort, which represents a medium prevalence area, the authors report a heritability estimate of 46% (95% CI 0.06-0.86), and an environmental contribution of 29% (95% Cl 0-0.6) for shared and 23% (95% Cl 0.12-0.39) for unique factors (Ristori et al 2006).

Kinnunen and associates studied 11 MZ and 10 DZ twins obtained from the old Finnish Twin Cohort of same sexed pairs born 1957 or earlier. The recruitment procedure for the twins was the same as used in our study. The prevalence of MS in twins was found to correspond to that of the Finnish population in 1979: in females it was approximately 60/100 000, in males 40/100 000 and in total 50/100 000. The frequency of MS was significantly higher in MZ than in DZ twins (Kinnunen et al 1987). Kinnunen and associates also report the pairwise concordance rates for MZ and DZ twins in a study published in 1988. Six DZ and seven MZ twin pairs obtained from the old Finnish Twin Cohort underwent clinical evaluation as well as brain MRI, VEP, brain stem auditory evoked potential and HLA-DR2 analyses. The pairwise MS concordance was 29% among MZ and 0% among DZ twins. Brain MRI and evoked potentials obtained from the co-twins did not show any abnormalities suggestive of MS (Kinnunen et al 1988). DR2 was present in 69% of the twins

with MS (six MZ and 3 DZ) compared to 34% of the healthy blood donors used as a control group (Kinnunen et al 1988).

2.3. Prevalence of subclinical MS in genetically susceptible and in background population

It has been hypothesized that individuals with first degree relatives with MS might have a condition called MS immunopathic trait or subclinical MS, characterized by the presence of oligoclonal bands in CSF and/or silent MS lesions in brain MRI. Haghighi and colleagues showed that 9/47 (19%) of asymptomatic siblings to MS patients had an intrathecal immunological reaction with two or more CSF-enriched oligoclonal bands (OCBs) in contrast to 2/50 (4%) unrelated healthy controls (Haghighi et al 2000 and 2003).

Numerous family and twins studies have investigated the possibility of silent MS-like brain lesions in clinically asymtomatic subjects with genetic susceptibility to MS (McFarland et al 1984, Kinnunen et al 1988, Uitdehaag et al 1989, Lynch et al 1990, Tienari et al 1992, French Research Group on Multiple Sclerosis 1992, Sadovnick et al 1993, Thorpe et al 1994, Mumford et al 1994, Fulton et al 1999, De Stefano et al 2006). Since brain white matter lesions are extremely common in the general population and their frequency increases with age, no conclusion can be drawn from the earlier studies showing frequent presentation of white matter lesions in healthy family members, but not using specific MRI criteria (McFarland et al 1984, Uitdehaag et al 1989). Only three family and three twin studies have addressed the question of silent MS in the genetically at risk population by using specific MRI criteria. From these studies it can be concluded that MS-like lesions are seen in 4-10% of asymptomatic subjects with a 1st degree relative with MS (see table 3). However, the results should be interpreted, in part, with caution, since the studies do not report chronic vascular diseases, smoking or other conditions known to increase the possibility of brain white matter lesions. Also, the majority of the asymptomatic subjects fulfilling any MRI criteria were over 45 years of age. Furthermore, it should be mentioned that Kinnunen and associates found no brain MRI abnormalities suggestive of MS in the group of 11 co-twins obtained from the Finnish Twin Cohort 20 years ago (Kinnunen et al 1988). MS-like brain lesions have been also found unexpectedly at autopsy in neurologically asymptomatic subjects with no increased risk for MS (Gilbert et al 1983, Engell 1989). The prevalence of these incidental findings equals the prevalence of clinical MS in the United States (0.1% of the population) (Brinar 2004).

Table 3. Family and twin studies on MS brain lesions using specific MRI criteria

Study	Number of subjects	Tesla	Criteria	Result
Lynch et al 1990	45 subjects with MS + 45 1st degree relatives as controls	1.5	Fazekas 1988	2 asymptomatic subjects (4.4%) over 50 fulfilled the criteria of Fazekas. Both were over 50 years of age
Tienari et al 1992	27 subjects with MS + 27 siblings as controls	1.0	Paty 1988 and Fazekas 1988	5 asymtomatic subjects (11.1%) fulfilled the criteria of Paty and one (3.7%) of those fulfilled the criteria of Fazekas. All these subjects were over 50 years of age
DeStefano et al 2006	152 asymptomatic 1st degree relatives of sporadic and 88 of familial MS + 56 healthy controls	1.0	Fazekas 1988 Barkhof 1997	7 sporadicMS relatives (4.6%), 10 familialMS (11.4%) and none of the healthy controls fulfilled both the Fazekas and Barkhof criteria
French Reseasch Group 1992	84 discordant twin pairs	not reported	Paty 1988	Nine co-twins (10.7%) fulfilled the criteria of Paty. All these subjects were over 45 years of age.
Sadovnick et al 1993	33 discordant twin pairs	not reported	Fazekas 1988	Five co-twins (15.6%) fulfilled the criteria of Fazekas. Ages not reported.
Thorpe et al 1994	48 discordant twin pairs + 37 healthy controls	0.15 0.5 1.5	Fazekas 1988	Five co-twins (10.4%) fulfilled the criteria of Fazekas. All were under 60 years of age. None of the healthy controls fulfilled the MRI criteria.

3. Pathogenesis of multiple sclerosis

Virus infections in the early life of genetically susceptible individuals seem to be able to distort immune homeostasis towards development of autoreactive T cells, which can lead, over time, to the clinical symptoms of MS. The migration of autoreactive T cells across the BBB starts the inflammatory process of MS. When encountering infectious agents, immune responses, that are cross reactive with myelin proteins, are mounted in the peripheral lymphoid system. Activated antigen specific T cells and B cells cross the BBB and target antigens expressed by oligodendrocytes and neurons. The crossing of the BBB is facilitated by adhesion molecules, chemokines and their receptors as well as cytokines. Their expression on the surface of lymphocytes and endothelial cells is regulated by different genetic and environmental factors. In the CNS, the activated T cells again interact with the antigen presenting cells (astrocyte, microglia or macrophage). The T-cell receptor (TCR) and class II major histocompatibility complex (MHC) molecules on the antigen presenting cells form a trimolecular complex, which is important in Th1 and Th2 cell stimulation. The Th1(CD4+) cells release pro-inflammatory cytokines whereas Th2(CD4+) cells release anti-inflammatory cytokines and more macrofages are stimulated. Th2 cells also activate B cells which are responsible for the antibody production. Both antibody and T-cell mediated myelin injury occur in demyelination. This process is responsible for the acute reversible symptoms of MS. Symptom recovery is due to remyelination and reversal of the conduction block. In addition to demyelination, neurodegeneration develops over time right from the onset of the disease. This, together with the altered properties of persistently demyelinated or degenerated axons is probably the reason for the stable symptoms (Prat and Antel 2005, Hemmer et al 2006). See figure 4.

Figure 4. Pathogenesis of multiple sclerosis
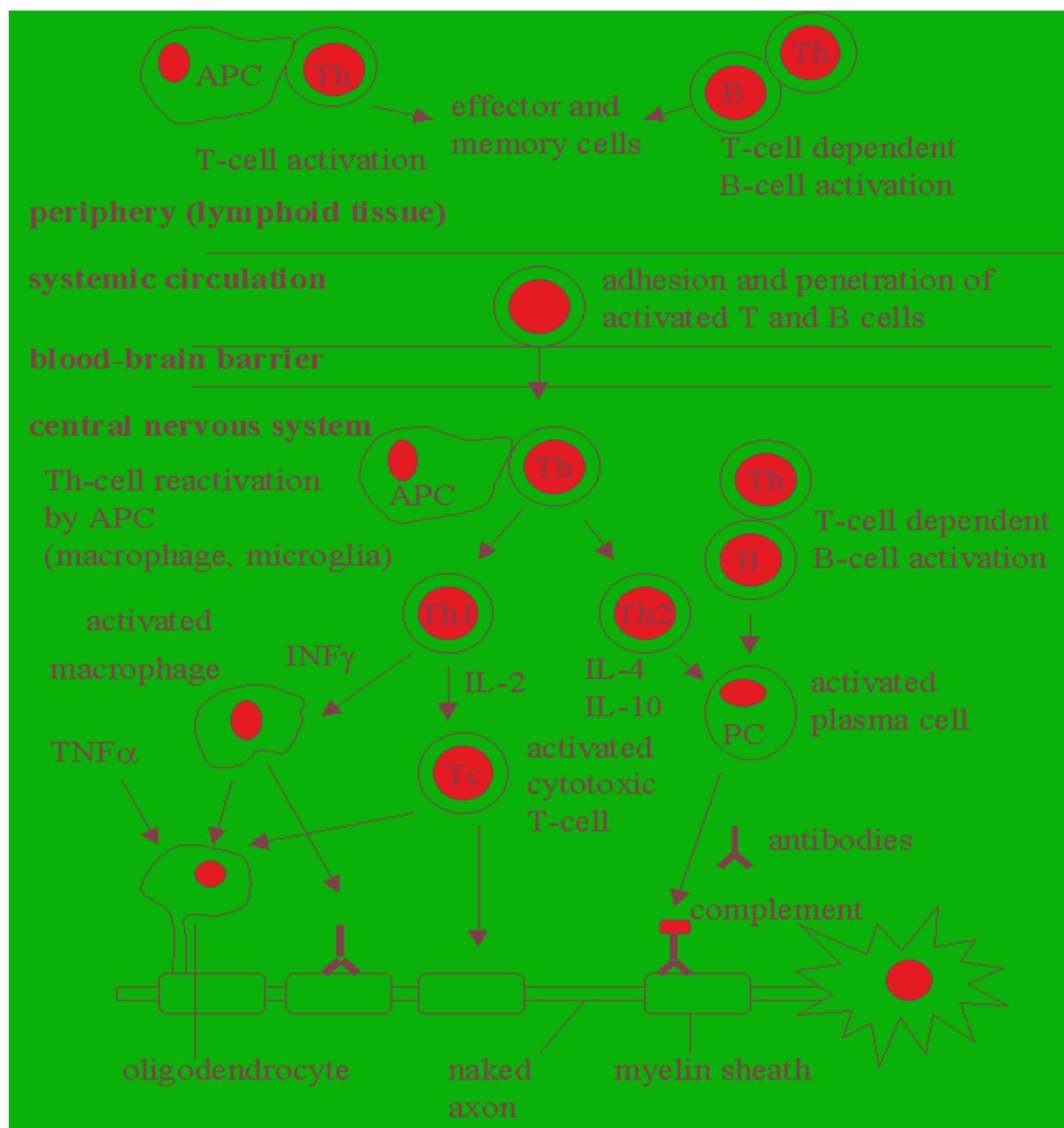
APC=antigen presenting cell, TNF=tumor necrosis factor, INF=interferon, IL=interleucine,
PC=plasma cell, Tc= cytotoxic T-cell

4. Aetiology of MS

Despite of intensive investigations of various genetic and environmental factors, the aetiology of MS remains unknown. Genetic factors are known to regulate the susceptibility to MS, but of these factors only HLA-DR2 has been confirmed to be strongly associated with the disorder. In the epidemiological surveys the term frequency is used to explain how often a particular event has occurred. The cumulative frequency, or lifetime risk, is the maximum possibility that will occur during the entire lifetime of an individual at risk. For Europeans, lifetime risk of MS is about 1:500 whereas for the MZ co-twin of an individual with MS is as high as 1:3 (Compston and Confavfreux 2006). In a child of a parent with MS the risk is less that 5%, which is nevertheless 20-40 times higher than in the general population, emphasising the role of genetic factors. On the other hand, 85% of MS patients do not have affected relatives, which suggest that environmental factors also influence the development of the disease (Ebers et al 1986, Mumford et al 1994). Infections, mainly viral, chemico-physical factors, such as sunlight exposure and vitamin D as well as population genetics have commonly been proposed as possible candidates in MS aetiology (Ebers 2008). For decades it has been recognised that there is a female preponderance in MS. Before 1930 the ratio was 1:1, but since then the ratio has been increasing and today stands at approximately 2:1 (Hader et al 1988, Duquette et al 1992) According to recent study it exceeds 3.2:1 in Canada. Since genes are unlikely to be responsible for such short term change, female-related environmental factors, such as hormonal contraceptives and increased smoking have been proposed (Orton et al 2006).

4.1. Environmental factors

Populations tend to change geographically and socially. Studies on stable isolated populations give information on genetic susceptibility, whereas studies on migrating populations are thought to reveal the acquired exogenous factors in the aetiology of the disease. One of the most important migration studies on MS demonstrating that individuals can acquire the level of MS risk of their new living surroundings if they migrate before adolescence was done by Alter and associates and updated by Kahana and associates (Alter et al 1962 and 1978, Kahana et al 1994). The original study on Israeli immigrants showed a higher prevalence among those migrating from northern Europe than from Asia and Africa, but there were very few MS cases in the cohort who migrated to

Israel before adolescence and the children born in Israel had the same prevalence of MS independent of the parents' origin (Alter et al 1962 and 1978). This implies that racially determined differences in risk for MS are modified by the environmental factors, but they must act before adulthood. However, adoption studies have shown no increased risk of adopted individuals developing MS despite of being raised from infancy with MS patients, so it appears that shared family environment alone is not enough to trigger MS (Ebers et al 1995, Sadovnick et al 1996). In the study by Ebers and associates the frequency of MS of non-biological parents (n=470), siblings (n=345) and children (n=386) of 238 index cases were compared. The observed recurrence risks (1:470, 0:345 and 0:386 respectively) equalled the lifetime risk for Europeans and Canadians (1:500) and was therefore significantly lower than that in the biological relatives of index cases (1:20 for full siblings and 1:50 for parents). Amongst the 1201 relatives of adoptees, only one had MS, so the prevalence was found to be identical to the population prevalence (Ebers et al 1995). Also the evidence from half sibling studies favours genetic factors as the basis for familial clustering (Ebers et al 2004, Sadovnick et al 1996). Sadovnick and associates studied 939 subjects with MS who had 1839 half-siblings and 1939 full siblings. The age adjusted risk for half siblings was significantly lower (1.3%) than for full siblings (3.5%). The risk for half siblings was the same for those rose together or raised apart (Sadovnick et al 1996). Therefore it is thought that common environmental risk factors for MS most likely operate on a macro-environmental, rather than a familial micro-environmental level (Giovannoni and Ebers 2007, Ebers 2008). Even though familial shared environment seems to be of little importance in regard to MS risk, the month of birth data suggest that the gestational or neonatal environment could have an effect on the risk. According to month of birth studies, significantly more MS patients are born in May and fewer in November (Willer et al 2005). The under-representation of twins in the MS population observed in a few studies (Willer et al 2003, Ristori et al 2006) and a recent finding that the rate of MS in DZ twin pairs is lower than expected from population prevalence (Hansen 2005), has raised a question about possible protective factors against MS shared by DZ twins. One of the factors could be the sharing of foetal life with a genotypically different individual, which might be beneficial for the immune system (Hansen 2005).

4.1.1. Vitamin D and sunlight exposure

The association between increasing latitude and risk of MS has been observed for decades. Even though many environmental factors change with latitude, sunlight exposure appears to be the most

likely one to be connected with MS. In Australia, the increased prevalence of MS was found to correlate with reduced sunlight exposure, ultra violet (UV) B in particular (van der Mei et al 2001). Also the impact of birth month on MS prevalence has been connected to the sunlight exposure during pregnancy. The data shows a 19% decreased risk of MS for those born in November compared to those born in May (Willer et al 2005). The reduced maternal exposure to sunlight during winter pregnancy is thought to lead to vitamin D_3 deficiency, which seems to be the mediator of the immunomodulatory effects of dermal UVB (Cantorna et al 1996, Munger et al 2006). Vitamin D may therefore protect against the development of MS and also influence the disease outcome. The effect of vitamin D could be mediated via the acquired or the innate immune system or by a combination of both (Munger et al 2006).

4.1.2. Smoking

An increase in MS symptoms after cigarette smoking (Emre and de Decker 1992) and a positive correlation between smoking before onset of MS and the risk of the disease have been detected (Ghadirian et al 2001). Most importantly a positive association has been found in each of the four prospective studies that have addressed the issue of cigarette smoking as a risk factor for MS. The relative risk for smokers compared to those who have never smoked has been found to be 1.3-1.7 (Villard-Mackintosh and Vessey 1993, Thorogood and Hannaford 1998, Hernan et al 2001, Hernan et al 2005). A positive association between smoking and MS was also found in a study of the general population in Norway. The relative risk in this study was 1.8, being in line with the prospective studies (Riise et al 2003). Furthermore a two-fold increase in the risk of paediatric MS has been reported among children exposed to parental smoking (Mikaeloff et al 2007).

4.1.3. Infectious agents

During the first decade of the 20th century there were numerous claims, which were later proved to be false, that MS could be transferred to various animals by injecting them with human CSF. It was not until In 1930s when modern virology was born, that the first report showed Theiler murine encephalomyelitis (TME) virus (a neurotropic picornavirus) to cause CNS disease. Since then TME

has served as a model to explain infectious mechanisms underlying CNS demyelination (Stüve et al 2004. In 1933 Rivers and associates established the autoimmune hypothesis for MS by inducing demyelination through immunization with CNS homogenate in the absence of any demonstrable pathogen, which led to the discovery of the experimental autoimmune encephalomyelitis (EAE) model (Rivers et al 1933). EAE acts as a model for MS and has been used to study basic mechanisms underlying CNS autoimmunity ever since. In 1930s the first organism, Spherula insularis, was linked with MS, but rapidly disappeared from interest. From that point on geographical and seasonal variations in the incidence of MS as well as migration studies further raised the enthusiasm for a possible infectious aetiology of MS (Compston et al 2006). Also, an observation that MS exacerbations are more common after infections supported this hypothesis (Sibley et al 1985), although no specific virus or bacteria has been implicated (Andersen et al 1993).

Untill 1980s there had been single, but not confirmed findings on rabies, herpes simplex virus (HSV) parainfluenza 1, measles, cytomegalo virus (CMV) and coronavirus. These earlier studies with anecdotal findings are summarised by Johson (Johnson 1994). At one point a question arose as to whether patients with MS had been exposed to human retroviruses (HRVs) (Gilden 2005), but during the last decade interest has shifted from HRVs to Cp, a gram negative bacterium. The original study showed Cp DNA and abs in the CSF of patients with MS (Shriram et al 1999). Since then many studies have explored a possible relation between Cp and MS but none have found one (Tsai and Gilden 2001).

4.1.3.1. Herpesviruses

Herpes viruses are a common neurotropic group of viruses capable of protracted latency. In the past decade two human herpesviruses have been associated with MS, namely HHV6 and EBV. They are of special interest because seroconversion to both viruses happens before adulthood, matching epidemiological evidence for the time of exposure to the disease-causing agent of MS (Gilden 2005).

EBV, the cause of infectious mononucleosis, is known to be latent in B cells. All patients with MS are seropositive, compared with 86-95% of controls (Gilden 2005). Bray and colleagues found abs to EB nuclear ag in 85% of patients with MS compared to 13% of EBV-seropositive controls (Bray et al 1992). Also a prospective study on 62439 females found a significant increase in serum titers of anti-EBV before the onset of MS (ab to EB nuclear ag in particular) (Ascherio et al 2002).

Studies on paediatric MS have shown an extremely low risk of MS in seronegative subjects (Alotaibi et al 2000, Pohl et al 2006). Compared to subjects not infected in childhood (seronegative), MS risk is approximately ten times higher among individuals who experience a subclinical EBV infection in childhood (seropositive without mononucleosis) and there is a 20-fold increase in risk among individuals with a history of clinical mononucleosis (Cohen 2000, Höllsberg et al 2005, Levin et al 2003 and 2005, DeLorenze et al 2006). In a meta-analysis of EBV and MS Ascherio reviewed eight studies that included a total of 1005 patients with MS and 1060 controls. The summary odds ratio for patients with MS compared to controls was 13.5 (95% CI 6.3-31.4) (Ascherio 2000). In the most recent meta-analysis by Thacker and colleagues the combined relative risk on MS after infectious mononucleosis from 14 studies was considerably lower 2.3 (95% CI 1.7-3.0) (Tahcker et al 2006). Today EBV is the one pathogen that is believed to stand out as a consistent and strong risk factor for MS.

4.1.3.1.1. HHV6

There are two subtypes of HHV 6, designated a and b. HHV 6b causes a common childhood infectious disease, exanthema subitum, while HHV6a is not known to cause human diseases. There is, however, a recent finding suggesting HHV6a to be pathognomic for MS (Alvarez-Lafuente et al 2004). HHV6 is prevalent worldwide. It is typically latent, but can become reactivated upon stress to the immune system (Yoshikava and Asano 2000, Berardelli 1997). HHV6 can cause inflammation of myelin forming cells and CNS complications such as febrile seizures and encephalitis/encephalopathy. Since the seropositivity for anti-HHV6 IgG ab approaches 100% in the background population (Meinl 1999), it is difficult to evaluate the possible connection with MS and HHV6 by serological surveys. Increased serum IgG ab titers to HHV6 using enzyme immunoassays or indirect immunofluorescence assays have been inconsistently reported in patients with MS (Wilborn et al 1994, Sola et al 1993, Nielsen et al 1997).

Because of the high seroprevalence it has been hypothesized that HHV6 could trigger MS only in individuals with a certain genetic profile. A study by Martinez and colleagues showed an association of the MHC2TA rs4774C with HHV6a PCR positive MS patients compared with HHV6a negative patients and healthy controls. This finding may suggest that there is a gene-environment interaction between HHV6a active replication and MHC2TA

rs4774C or maybe some other polymorphism in tight linkage disequilibrium with it
(Martinez et al 2007).

The majority of evidence supporting an association between HHV6 and MS is based on
case-control studies, which have compared the detection of HHV6 in brain tissue, CSF,
serum or PBMCs using PCR (see tables 4 and 5). Furthermore immunohistochemical (IHC)
analyses of brain biopsy specimen from patients with or without MS have been performed in
few studies. In the first study HHV6 antigens (ag) were detected using two monoclonal abs
to early [p38/p41] and late [p101] proteins. HHV6 ag was detected in the nuclei of
oligodendrocytes in 12/15(80%) of MS cases compared to none of the controls (Challoner et
al 1995). In another study IHC analyses showed only the HHV6 glycoprotein 116 ag in
some reactive astrocytes and microglia of acute lesions (Goodman et al 2003).

Table 4. Detection of HHV6 in MS and control brains by PCR

Study	Sample	Positive MS cases (%)	Positive controls (%)
Challoner et al 1995	normal white matter	25/32 (78)	40/54 (74)
Sanders et al 1996	active plaques inactive plaques normal white matter	7/22 (32) 5/29 (17) 9/37 (24)	16/37 (43)
Friedman et al 1999	active plaques normal white matter	6/16 (36) 2/12 (17)	3/22 (14)

Table 5. Detection of HHV6 in CSF, sera and PBMC from MS cases and controls by PCR

Study	CSF positive MS cases (%)	CSF positive controls (%)	sera positive MS cases	sera positive controls	PBMC positive MS cases	PBMC positive controls
Willborn et al 1994	3/21(14)	0/16	0/21	0/16		
Martin et 1997al	0/20	0/20	0/20	0/20		
Fillet et al 1998	2/32(6)	0/21	2/32(6)	1/34(3)		
Goldberg et al 1999	0/6	0/4	1/24	0/30		
Mirandola et al 1999	0/32	0/12	0/32	0/12		
Tejada-Simon et al 2002	14/30(46)	6/30(20)	22/33(66)	0/46		
Tomsone et al 2001			14/38(37)	0/43	38/56(76)	43/150(29)
Alvarez-Lafuente et al 2002			15/30(15)	0/46	55/103(53)	14/46 (30)

HHV6=human herpesvirus 6, CSF=cerebrospinal fluid, PBMC=peripheral blood mononuclear cell, MS=multiple sclerosis, PCR=

polymeric chain reaction,

From the vast variety of studies performed, it can be concluded, that even though some connections seems to exist between HHV6 and MS no causation has been shown. The main argument today for an association between HHV6 and MS is based on the presence of viral DNA in the blood from MS patients in the active phase of the disease in contrast to MS patients in the inactive phase or healthy controls (Höllsberg et al 2005, Alvarez-Lafuente et al 2006, Berti et al 2002, Chapenko et al 2003). Whether a relapse can be caused by reactivation of latent HHV6 or HHV6 is reactivated because of the relapse is not known.

4.1.3.2. Enteroviruses

Infections by EVs are common and affect people of all ages. Symptoms of EV infection vary from nonspesific fever and rash to life-threatening CNS disease. (Sawyer 2002). EVs can persist in neuronal tissue. Polioviruses are the best known EVs showing specific tropism to motoneurons in SC. Other EVs are major pathogens of the CNS causing meningitis and encephalitis (Romero 2002).

Latent EV RNA has been reported to be present in patients with varying autoimmune disorders such as diabetes mellitus (Hyöty et al 1995), chronic fatigue syndrome (Clements 1995), chronic myocarditis (Heim et al 1997) and postpolio-syndrome (Leparc-Goffart et al 1996). Thus, these observations suggest a connection between EV infection and autoimmunity. In addition to autoimmune diseases, EV RNA has been found in CSF and SC of ALS patients raising the possibility of a persisting EV infection in this neurological disorder (Berger 2000, Giraud et al 2001).

A case report study has proposed an association between acute disseminated encephalomyelitis (ADEM) and poliomyelitis vaccine (Ozawa et al 2000). Also a study performed on a Danish historical cohort of patients treated with poliomyelitis (n=6423) and the Danish MS Registry suggested that polio patients might be at an increased risk of MS (Nielsen et al 2000). On the other hand, no EV RNA was found in autopsy brain tissues from 25 MS or 33 control patients (Dessau et al 1997).

4.2. Genetic factors

The sequence of the human genome consists of over 3 billion base pairs and over 10 million genetic single nucleotide polymorphisms (SNPs) with a frequency of more than 1% are thought to exist. The majority of this data has been collected in the last few years. The growing understanding of the human genome has led to development of various tools with which to explore the genome (The international HapMap project 2003). Marked efforts to identify individual genes involved in MS aetiology have been, for the most part unsuccessful even though MS has been considered to have a strong genetic component. After a review by Dyment and associates up to 95% of initial gene association findings cannot be replicated (Dyment et al 2004). This is most likely because disease-associated genes are probably operating in different individuals or in different populations. A single gene is not likely to have a marked effect, as it is thought that the cumulative effect of several genes increases an individual's susceptibility to MS. So far only HLA DRB1*15 antigen has strongly been associated with MS. It is present in about 60% of the patients and only 20% of the general population (Rasmussen et al 2001). Recently, findings for few genes with smaller effects have been replicated in more than one population, such as protein kinase C alpha (PRKCA) (Barton et al 2004, Saarela et al 2006), interferon regulatory factor 5 (IRF-5) (Kristjansdottir et al 2008), ecotropic viral integration site 5 (Evi-5) (Hoppenbrouwers et al 2008), IL2 and 7 receptor alpha chain genes (IL2RA and IL7RA) (Gregory et al 2007, Hafler et al 2007).

4.2.1. HLA genes

MHC has been associated with MS for 30 years. The genes involved play important part in regulating the development, maturation and composition of the T cells as well as other immunological processes. The first associations reported were the HLA class I antigens A3 and B7 and HLA class II polymorphism Dw2 and DR2 (Dyment et al 2004). This has been subtyped into a strong and consistent association with the HLA DRB1*1501-DQA1*0102-DQB1*0602 extended haplotype. In northern European populations, these DRB1, DQA1 and DQB1 alleles almost always occur together. Because this allelic heterogeneity is observed, it has been suggested that there may be a hierarchy of DRB1 allelotypes that directly influence MS risk to different degrees. Also there may be another genetic variant in close proximity, which is present on all associated DRB1 haplotypes (Dyment el al 2004, Giovannoni and Ebers 2007). T cell receptor (TCR) binds directly to HLA antigens when the epitope is presented. Therefore the polymorphisms of TCR have been

considered to be potential candidates for MS susceptibility (Dyment et al 2004). Especially TCR-β genes may have an effect on MS pathogenesis, but the results have not been conclusive (Seboun et al 1989, Hockertz et al 1998). The activation of T-cells begins with the interaction of TCR and HLA-epitope complex. At this stage the expression of cytotoxic T lymphocyte antigen (CTLA4) on the surface of T cells is up-regulated. This molecule is able to bind the B7 ligand on the antigen presenting cell. CTLA4 has thus been a candidate susceptibility gene for various autoimmune diseases including MS, but the results have been inconclusive (Dyment et al 2004).

4.2.2. Linkage studies: genomic screens

Since the candidate gene approach has not been very successful, numerous genomic screen studies including one from Finland have been performed (Kuokkanen et al 1996, Ebers et al 1996, Haines et al 1996, Sawcer et al 1996). None of them have been successful in finding a candidate gene loci outside MHC at a genome wide significant level. Genomic screen studies are based on the idea that all families have their susceptibility determined by the same genes. This may not, however, be the case. Genomic screen studies have so far only provided information about the exclusion of major locus and some idea of where regions of common interest could be located. Evidence for sharing was observed in the Canadian, American, UK and Finnish studies for chromosome 6 within the MHC region (6p21) (Dyment 2004, Hafler et al 2005, Sawcer et al 2005), but other candidate gene loci have not been confirmed. A recent study on 730 families again revealed a significant linkage in the MHC, but no other regions of statistically significant linkage could be identified. The authors state that non-MHC loci of relevance in MS might escape the effective statistical power in linkage studies (Sawcer et al 2005).

4.2.3. Gene expression studies using micro array technique

Recently, new microarray technique has provided a powerful tool for investigating transcriptional changes is MS. DNA microarrays are large-scale screening techniques in which fragments of probe DNAs are chemically bound to the surface, and fluorescently labelled target cDNAs (or cRNAs) from cells or tissues are hybridized to the array. Genome-wide changes in the blood or microglia (obtained from obductions) of patients with MS have been observed by using this technique in numerous studies (Sharp et al 2006, Gebicke-Haeter 2005). The gene expression profile analysed from blood of patients with MS has been found to be different from controls and it seems to correlate with disease activity (Achiron et al 2004a, Bomprezzi et al 2003), the medications used to

treat MS (Weinstock-Guttman et al 2003) and to the treatment response (Achiron et al 2004b, Sturzebecher et al 2003). In two gene expression studies on MS brains there was very little overlap in results (Lock et al 2002, Mycko et al 2003). Only two proteins were up-regulated in both studies: Cyclin 1A, which might be involved in differentiation of the cells into phagocytes, and MEK1, which is phosphorylated through INF-β-activated intracellular signalling pathways, which leads to induction of β-chemocine mRNA and protein expression (Gebicke-Haeter 2005).

5. Twins in populations

Twins are two offsping resulting from the same pregnancy, either of the same or opposite sex. Due to the limited size of the mother's uterus, twin pregnancies are less likely to carry to full term, 37 weeks on average, and therefore the infants are subject to various health consequences (Elliot 2007, Collins 2007). There are five common variations of twinning. The most common type is DZ opposite sex twins, which constitute about 40% of all twins born. The next common types are like sexed female DZ and like sexed Male DZ twins. The other variations are like sexed female MZ twins and the least common like sexed male MZ twins. Males are more susceptible to intra uterine death, and since this is more likely for twins than in singletons, it leads to female preponderance in twins (Martin et al 1999, Collins 2007).

If both twins in a pair have the same disease they are considered concordant, while a single affected twin is one of a discordant pair. 1.1% of births in the world result from a twin pregnancy. The current rate in the United States is 3.1 twin births per 1000 women, thus 5.8% of children born in the US after 2001 are twins. It is estimated that approximately 125 million (1.9%) of the world's population are twins or triplets (Collins 2007).

5.1. Dizygotic twins

DZ, commonly known as fraternal twins, occur when two fertilized ovas are implanted in the uterine wall at the same time. DZ twins share only 50% of the genes, thus genetically they do not differ from regular siblings. The rate of DZ twinning in the early part of the 20th century was as

high as 3:1. Since the late 1950s, DZ twinning rates decreased until the 1980s, when the DZ/MZ ratio was about 2:1, but has increased since as a result of infertility treatments (Kaprio and Marttila 2004, Collins 2007, Martin et al 1999). DZ twins are more common for older mothers, with twinning rates doubling in mothers over 35 years of age (Bortolus et al 1999, Collins 2007).

5.1. Monozygotic twins

MZ, frequently referred as identical twins, occur when a single ova is fertilized to form one zygote, which then divides into two separate embryos. Even though in principle they have identical DNA (except for mitochondrial DNA), their traits and physical appearances are not exactly the same. Environmental conditions both in the uterus and through their lives influence the expression of various genes. Also there is a possibility of somatic mutations after fertilization and on rare occasion deactivation of X chromosomes in female twins. The likelihood of single fertilization resulting in MZ twins is random and rare. The estimated number of MZ twins and triplets in the world is 10 million (0.2%) (Collins 2007).

5.3. Twin models

A well-conducted twin study is considered to be an excellent means to assess the relative contribution of genetic and environmental factors to a given disease. There are three main approaches to conducting a twin study: A classical twin method, a controlled co-twin study and biometrical genetic methods.The basic aim in a classical twin method is to compare concordance rates in MZ and DZ twins with population prevalence for a given disease. When the MZ concordance is significantly higher than DZ concordance, it indicates that genetic factors are important in aetiology, whereas equal concordance rates among MZ and DZ twin pairs suggests an environmental cause.The recruitment of the twins can be based on population or volunteer ascertainment. A population based twin study has the least bias and is achieved by access to a national twin register, while a volunteer-based study is a less systematic method. In a co-twin control method only MZ twins who are discordant for a disease are studied. In this model the healthy co-twin provides a perfectly matched control to the diseased twin. Biometrical genetic

methods are used when studying the underlying liability to a disease or to disease categories assuming that twin samples are big enough. To analyse the covariance or correlations derived from the MZ and DZ pairs, genetic models may be fitted to the data. In other words this method gives an estimate of the relative contribution of genetic and environmental factors to a given disease (Hawkes 1997).

AIMS OF THE STUDY

The aims of the study were:

1. To evaluate the relative contribution of heritability and environmental factors to the aetiology of MS in Finland (Study I)

2. To investigate the possible association of EVs and MS (study II)

3. To investigate the possible association of HHV6 and MS (study III)

4. To analyse the differences in gene expression profiles in MZ twins with MS compared to their asymptomatic co-twins (Study IV)

SUBJECTS AND METHODS

1. Recruitment of the twins from The Finnish Twin Cohort

The older Finnish Twin Cohort of like-sexed twins includes all living Finnish twins born in 1957 or before (Kaprio 2006). In the 1990s the earlier cohort of only like-sexed pairs was updated to include also opposite sex-pairs in the birth years 1938 to 1949, based on a questionnaire survey and registry linkage for opposite-sex pairs born 1950-1957 (Kaprio and Koskenvuo 2002). In 2001 there were 3083 MZ and 11029 DZ same-sex and opposite-sex twin pairs in the cohort.

A computer file is maintained on patients treated in Finnish hospitals since 1972. The Finnish Twin Cohort was linked with the Hospital Discharge Register using the specific identification number assigned to each Finn. It should be noted, that MS patients that have been treated only at an out patient clinic and never hospitalized, escape the Hospital Discharge Register. The diagnosis number for multiple sclerosis only was used. The collection covered the years 1972-2001. In 2001 the total number of twin pairs born in 1957 or earlier with either one or both members recorded as having MS according to the Hospital Discharge register was 55. Of these 21 pairs were MZ and 34 DZ.

National laws and regulations prohibited us from contacting the twins directly therefore we contacted the hospitals where the twins had been treated. We then asked the heads of the departments of neurology to provide the twins with suspected MS with information concerning our study. The index twins informed further their co-twins. Subjects willing to participate were asked to contact the investigators.

Twenty eight twin pairs from the Twin Cohort contacted us. Of these eight pairs declined to participate in the study. In two twins the diagnosis of MS appeared to be false in the Hospital Discharge Register and, two had moved abroad and four were unable to travel due to severe disability. The remaining 20 pairs were invited to Tampere University hospital for two consecutive days during which the investigations were performed.

Five MZ and three DZ twin pairs had taken part in in the original Finnish Twin Cohort Study of MS 20 years ago (Kinnunen et al 1987, Kinnunen et al 1988). The recruitment method was the same in the original Finnish Twin Cohort study and the present one.

Four twin pairs outside the Twin Cohort, born after 1957 contacted us after having heard of our study. They were all included. Overall 24 pairs (10 MZ and 14 DZ) were included in the study (see figure 5).

Figure 5. Recruitment of the twins
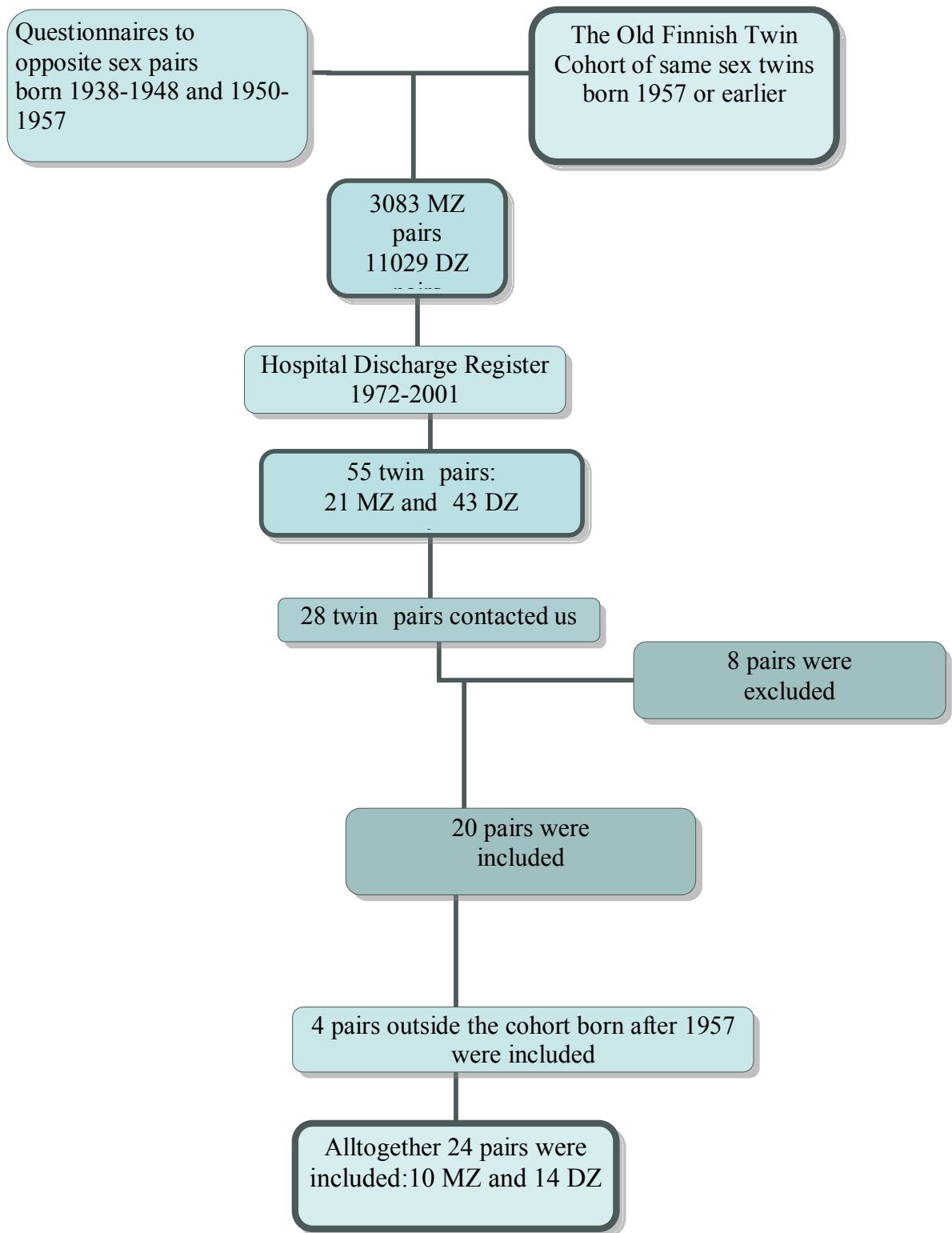
The zygosity determination of the twins in the cohort is based on self-reported similarity at school age and confusion by strangers. The accuracy of the zygosity diagnosis of twins in the cohort was checked on a subsample using 11 blood markers, showing a slight probability of misclassification (Sarna et al 1978). The zygosity of the twins in the old Finnish Twin Cohort has been reported previously by Kinnunen and associates (Kinnunen et al 1987, 1988).

2. Examination of the twins

The clinical and paraclinical assesments were performed at Tampere University Hospital during two consecutive days. The first twin pair was examined in the fall of 1999 and last at the spring of 2003. To confirm the MS diagnosis and establish the type of MS in question all subjects were examined clinically by the same neurologist (HK) and also by a neuropsychologist. MRI by using a 1.5 T MRI unit was performed on all twins. An optional CSF sample was obtained from six pairs. The CSF or MRI findings did not reveal any new MS diagnoses. The diagnosis of MS was based on Poser's criteria of a clinically definite MS (Poser 1983).The level of disability was evaluated by using the EDSS (Kurtzke 1983). For the EDSS evaluation, functional system scores (FS) were given (range 0-6) for each of eight areas of neurologic function including pyramidal, cerebellar, brain stem, mental, sensory, bowel and bladder, visual and other. The EDSS score was then based on these items. Concomitant diseases, smoking and alcohol abuse as well as family history of MS were recorded. The clinical characteristics of the twins are shown in table 6, in which the twins appear in the order of the date of the study visit.

Table 6. Clinical characteristics of the twin pairs

TWIN	AGE	SEX	ZYGOCITY	TYPE	EDSS	DISEASE DURATION (YEARS)	FAMILY	IM	CONCOMITANT DISEASES	SMOKING	ALCOHOL ABUSE
PAIRS											
1a	54	F	MZ	SP	4.0	20	neg	no	NONE	YES	NO
1b	54	F	MZ	SP	6.5	8	neg	no	NONE	NO	YES
2a	62	F	MZ	SP	6.0	28	no	no	EPILEPSY	NO	NO
2b	62	F	MZ	SP	6.5	28	no	no	HT, BREAST CANCER	NO	YES
3a	56	F	DZ	SP	2.5	20	pos	no	NONE	NO	NO
3b	56	F	DZ	NA	0	0	pos	no	NONE	NO	NO
4a	51	F	MZ	SP	1.5	0	pos	no	NONE	NO	NO
4b	51	F	MZ	NA	0	11	pos	no	SCIZOPHRENIA	NO	NO
5a	42	M	MZ	SP	6.5	22	neg	no	NONE	NO	YES
5b	42	M	MZ	NA	0	0	neg	no	NONE	YES	YES
6a	41	F	DZ	RR	1.5	25	pos	no	NONE	NO	NO

6b	41	F	DZ	NA	0	0	pos	no	PSORIASIS	NO	NO
7a	39	F	DZ	RR	1.0	22	neg	no	NONE	YES	NO
7b	39	F	DZ	NA	0	0	neg	no	ASTHMA	YES	NO
8a	30	F	MZ	RR	5.0	5	pos	INF-β	NONE	NO	NO
8b	30	F	MZ	NA	0	0	pos	no	NONE	NO	NO
9a	54	F	DZ	RR	2.5	9	neg	INF-β	NONE	NO	NO
9b	54	M	DZ	NA	0	0	neg	no	ASTHMA	YES	NO
10a	54	M	DZ	SP	6.0	13	neg	INF-β	NONE	NO	NO
10b	54	F	DZ	NA	0	0	neg	no	HT	NO	NO
11a	58	M	DZ	PP	8.0	13	pos	no	NONE	NO	NO
11b	58	F	DZ	NA	0	0	pos	no	HT	NO	NO
12a	51	F	DZ	SP	3.5	17	neg	no	NONE	NO	NO
12b	51	M	DZ	NA	0	0	neg	no	NONE	NO	NO
13a	45	F	MZ	RR	4.0	1	neg	INF-β	ASTHMA	NO	NO
13b	45	F	MZ	NA	0	0	neg	no	MIGRAINE	NO	NO
14a	53	F	DZ	SP	4.0	12	neg	no	EOS PN	NO	NO
14b	53	M	DZ	NA	0	0	neg	no	PSORIASIS	NO	NO
15a	51	F	DZ	RR	4.5	3	pos	INF-β	NONE	NO	NO
15b	51	F	DZ	NA	0	0	pos	no	NONE	NO	NO
16a	40	M	DZ	PP	6.5	4	neg	no	NONE	NO	YES
16b	40	F	DZ	NA	0	0	neg	no	NONE	YES	NO
17a	52	M	MZ	SP	6.5	23	neg	no	AF, TIA	YES	YES
17b	52	M	MZ	NA	0	0	neg	no	NONE	YES	NO
18a	49	F	DZ	RR	1.0	16	neg	no	RA	NO	NO
18b	49	F	DZ	NA	0	0	neg	no	ARTROSIS	YES	NO
19a	56	F	DZ	PP	2.5	23	pos	no	NONE	NO	NO
19b	56	F	DZ	NA	0	0	pos	no	ARTHROSIS, HYPOTHY-REOSIS	NO	NO
20a	51	F	DZ	RR	1.0	20	neg	no	ARTHROSIS	NO	NO
20b	51	F	DZ	NA	0	0	neg	no	NONE	NO	NO
21a	51	F	DZ	SP	4.5	26	pos	no	ASTHMA	NO	NO
21b	51	F	DZ	RR	0	22	pos	INFβ	NONE	YES	NO
22a	45	F	MZ	SP	6.0	6	pos	INF-β	SCIZOPHRENIA	NO	NO
22b	45	F	MZ	RR	2.5	1	pos	no	EPILEPSY, ASTHMA	NO	NO
23a	66	M	MZ	SP	6.5	11	neg	no	NONE	NO	NO
23b	66	M	MZ	NA	0	0	neg	no	CHD	NO	NO
24a	61	F	DZ	RR	2.0	17	pos	no	NONE	NO	NO
24b	61	F	DZ	SP	0.5	14	pos	no	SLE	NO	NO

F=female, M=male, MZ=monozygotic, DZ=dizygotic, RR=relapsing-remitting, SP=secondary progressive, PP=primary, progressive, NA= not applicable, pos=positive, neg=negative, family=more than two first degree family members with MS, IM=immunomodulatory treatment, CHD=coronary heart disease, HT=hypertension, RA=rheumatoid arthritis, EOS PN= eosinophilic pneumonia, AF=atrial fibrillation, TIA=transient ischemic attack

In study I all twin pairs were included (n=24) i.e. a classical twin method was used. In studies II and III only the first 17 twin pairs were included, because at that point we had to wait for approval from The National Research and Development Centre for Welfare and Health to continue the recruitment of twins from the cohort. In study IV all discordant MZ twin pairs were included (7 pairs from the actual study population and one pair from whom the blood samples were obtained by mail) i.e. a co-twin control method was used.

3. Epidemiological analyzes

MS concordance was assessed separately for MZ and DZ twin pairs using pairwise and probandwise concordance rates (Kaprio et al 1992). The tetrachoric correlations for MZ and DZ twin pairs were also computed. For estimating the contribution of genetic factors to the susceptibility of MS, a polygenic multifactorial model was used. This assumes that there is a normally distributed latent liability to disease, to which multiple genes and environmental factors contribute. When a threshold on the latent liability is exceeded, the disease becomes manifest. Because MZ pairs share all their genes, while DZ pairs share 50% of their genes identical-by-decent, a higher concordance among MZ pairs is evidence in favour of genetic factors in the aetiology of the disease (Posthuma 2003). Alternative models (say, with and without genetic effects) can be constructed to test statistically the significance of the MZ-DZ difference and provide estimates of the relative magnitude of genetic and environmental effects. Thus, structural equation models were applied for estimating variance components and to compare different genetic models (Neale and Cardon 1992). Within the structural equation model, the phenotypic variance in the trait of interest is divided into the following: 1. genetic variance 2. common environmental variance, due to the exposure shared by the twins and 3. unique environmental variance attributed to influences that are not shared by the twins. Heritability is the proportion of overall variance due to genetic factors.

The genetic analyses were performed using Mx modelling (Neale et al 2002). We assumed complete ascertainment as the cases were identified through the Hospital Discharge Register independently and then studied clinically and by MRI to ascertain that all MS diagnoses were definite. We entered the data as a contingency table and reconstructed the number of unaffected pairs based on the prevalence (100/100 000) of MS in the Finnish population. This was done because the twins were recruited from two sources and the size of actual base population was

difficult to estimate accurately. We also ran the model with other estimates of prevalence to test its sensitivity.

4. Detection of viral genome and antibodies against HHV6

HHV6 DNA and EV RNA was extracted from a 140µl volume of serum samples obtained from 17 twin pairs using the QIAamp® Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. After the extraction all samples were kept at 70°C until analysed.

CSF samples were obtained from 6 twin pairs and first used for EV RNA extraction. Then the samples were centrifuged for 15 minutes at room temperature (2000g). Supernatant and leukocytes were processed separately for HHV6 DNA extraction.

Samples from the patients and their siblings were analyzed in the same run. All analyses were made blindly from coded samples.

RT-PCR was carried out as described previously (Lönnrot et al.1999) using a primer pair from the highly conserved 5' noncoding region. PCR amplicons were hybridized using a europium-labeled enterovirus-specific probe in a liquid-phase assay on a microtiter plate (Lönnrot et al. 1999). Control samples containing a low copy number of viral RNA (70 copies per sample, which is less than usually observed during acute EV meningitis in CSF) as well as RNA negative control samples were included in all PCR runs. The samples were tested for the presence of PCR inhibitors by monitoring the amplification of internal control DNA added to the samples. Some of the samples contained PCR inhibitors, indicated by a more than 50 % reduction in amplification compared to phosphate buffered saline (the proportion of such samples was 12% for serum and 25% for CSF), while the rest of the samples were not inhibitory at all (unpublished data).

DNA amplification and detection were performed with the Argene Hybridowell™ Herpes Consensus kit (Parc Technologique Delta Sud, Varilhes, France). The primers are located in the DNA polymerase gene and they allow the simultaneous amplification of the 6 most

frequently isolated human Herpes viruses (EBV, HSV-1, HSV-2, CMV, varixella zoster (VZV) and HHV6). The amplification procedure on the GeneAmp 9600 (PE Biosystems) was the following: 6 min at 94°C then 30s at 94°C, 1min 15s at 57°C and 50s at 72 for 5 cycles, 30s at 94°C, 1min 15s at 46°C and 50s at 72 for 15 cycles and 30s at 94°C, 1min 15s at 55°C and 50s at 72 for 20 cycles.

After amplification, the amplified product was assessed by hybridization in a microtiter plate using biotinylated oligonucleotide probes specific for the virus and distributed in separate wells. All PCR runs included the HHV6-positive and -negative control samples provided by the kit manufacturer. In addition, negative and positive controls were used in nucleic acid extraction.

Six years after PCR studies, IgG and IgM class antibodies against HHV6, were measured from stored samples using a commercial enzyme-linked immunosorbent assay (ELISA) developed by PANBIO^{TM} (East Brisbane, Australia) according to the manufacturer`s protocol. Three serum samples and one CSF sample were not available for antibody analyses. Altogether 31 serum and 11 CSF samples were tested in 1:100 dilution for IgG and IgM. The results are reported as positive (presence of detectable IgM antibodies) or negative (no detectable IgM antibodies) according to the manufacturer`s guidelines.

5. Gene expression profile analysis

5.1 Isolation of total RNA for the gene analyzes

Mononuclear cells were separated from peripheral blood (PBMC) in VACUTAINER$^{®}$ CPT™ Cell Preparation Tubes (Becton Dickinson and Company, Franklin Lakes, N.J., USA) and total RNA was isolated by RNeasy$^{®}$ Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's protocols. The DNA was removed according to BD Atlas^{TM} Plastic Microarrays (BD Biosciences Clontech, Palo Alto, CA, USA) user manual for DNase treatment of total RNA for 10 µg of RNA, with the exception that RNA precipitation was carried out overnight at -20°C. The quality of total RNA was checked by gel electrophoresis and stored at -70°C until used.

5.2 cDNA microarrays

The study was performed using BD Atlas[TM] Plastic Human 8K Microarrays (BD Biosciences Clontech, Palo Alto, CA, USA), which contains duplicate DNA fragments from more than 8 300 known human genes (a list of genes is available at http://www.bdbioscience.com). 5 µg of total RNA were used for microarray analyses and samples from MZ twin pairs were analyzed at the same time. Microarrays were exposed to phosphoimaging screen and scanned by StormScan 840 Phosphoimager (Molecular Dynamics, Sunnyvale, CA, USA) after 5-7 days exposure time at a resolution of 50 µm to ImageQuant software version 5.1 (Molecular Dynamics, Sunnyvale, CA, USA).

5.3. Data analysis and normalization

Analysis was performed using the BD AtlasImage[TM] 2.7 Beta software (BD Biosciences Clontech, Palo Alto, CA, USA) and data was globally normalized by the sum method. Normalized signal intensities were compared to those of healthy siblings. Ratios of gene expression greater than two-fold were considered significant, based on a 99 % confidence interval (Chen et al. 1997; Claverie et al. 1999). The data was further analyzed and visualized with the GeneSpring software version 5.0 (Silicon Genetics, San Carlos, CA, USA).

5.4. Quantitative reverse transcription polymerase chain reaction (QRT-PCR)

The cDNA microarray data was confirmed for the interferon alpha-inducible protein gene (clone IFI-6-16) (G1P3) by relative quantitative real-time RT-PCR with LightCycler instrument (Roche Diagnostics GmbH, Mannheim, Germany). 1 µg of total RNA was converted into cDNA with Random Primer p(dN)$_6$ using 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche Diagnostics Corporation, Indianapolis, IN, USA). The primers and hybridization probes for QRT-PCR were designed and prepared by TIB MolBiol (Berlin, Germany) and the sequences are the following: forward primer: 5'-GAGTGCAGTGGCTATTCACA-3', reverse primer: 5'-GCGCATGCTTGTAATCCTAC-3', probe 5'-end labeled with acceptor dye LC Red 640: 5'-

AGCCTCAAGTGATCCTCCTGTCTCA-3' and probe 3'-end labeled with fluorecein: 5'-
CATAGTACACTGCAGCCTCCAACTCC-3'.

The PCR was performed in a 20 µl total volume for the target gene with 2 µl LightCycler FastStart DNA Master Hybridization Probes (Roche Diagnostics GmbH), 3 mM MgCl$_2$, 0.2 µM each probe, 0.5 µM each primer and 2 µl of cDNA. The PCR was performed with a denaturation at 95°C for 10 min, amplified in 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 15 s and elongation at 72°C for 12 s. The cooling was performed at 40°C for 30 s.

Glucose-6-phosphate dehydrogenase (G6PDH) was used as a reference gene. The PCR for this was performed by LightCycler – h – G6PDH Housekeeping Gene Set Kit (Roche Diagnostics GmbH) and was done at the same time and under the same PCR conditions as for the target gene. All reactions for the target and reference genes were performed in duplicates. Agarose gel electrophoresis was used to verify the PCR products. QRT-PCR results were calculated by LightCycler Relative Quantification Software with efficiency correction (Roche Diagnostics GmbH).

6. Statistical analyses

The statistical analyses were performed using SPSS for Windows, version 14.0.2 (SPSS Inc. Chicago, Illinois, USA). P-values less than 0.05 were considered statistically significant. Due to the skew distributions, continuous variables were expressed by medians and quartile range. The differences between groups were tested by the Mann-Whitney test. Categorical variables were tested by the Pearson chi-squared test or by Fisher's exact test. Associations between two continuous variables were tested by Spearmann correlation coefficients. Categorical variables were tested by the Pearson chi-squared test.

The differences in the proportions of up- and down-regulated (at least 2-fold up- or down-regulated genes) genes within MS twin groups were compared with χ-2 test using statistical software STATA version 8.0 (STATA Corporation, TX, USA). A p-value of less than 0.05 was considered significant.

7. Ethical aspects

Approval to use the Finnish Twin Cohort was obtained from the National Research and Development Centre for Welfare and Health. The study was approved by the Ethics Committee of Tampere University Hospital and all subjects gave a written, informed consent prior to study entry. The asymptomatic twins were informed, that they could receive the results of the CSF, MRI and neuropsychological investigations if they so wished.

RESULTS

1. The relative contribution of heritability and environmental factors to the aetiology of MS in Finland (Study I)

The pairwise concordance for MZ twins was 30% (3/10) and for the DZ twins 14.3% (2/14). The MZ tetrachoric correlation was 0.91 (95% Cl 0.78-1.00) and the DZ correlation was 0.80 (95% CI 0.59-1.00). The probandwise concordance rates were 46.2% for MZ and 25% for DZ twins. Based on twin modelling assuming a prevalence of 100/100 000, the genetic variance (heritability) was found to be 15.3% (95% Cl 0.0-77.6), the common environmental variance 73.7% (95% Cl 14.1-93.9) and the unique environmental variance 11.1% (95% Cl 2.3-30.0).

Five pairs were concordant for MS. One MZ pair was diagnosed within the same year, another pair five years apart and the remaining pair, 12 years apart. Concordant DZ pairs were diagnosed three and 12 years apart. Age did not associate with the concordance.

2. The association between EVs, HHV6 and MS (Studies II and III)

No EV RNA was found in any serum (n=34) or CSF (n=12) samples of the MS-patients or their twin pairs.

No HHV6 DNA was found in any serum (n=34) or CSF supernatant samples (n=12) or in CSF leukocytes (n=12) in patients with MS or their healthy twin pairs.

Of the twins with MS 88% (15/17) and of the healthy twin siblings 86% (12/14) had an IgG response against HHV6 in serum. One twin with MS and none of the healthy twins were HHV6 specific-IgM positive in serum. All CSF samples were negative for HHV6 IgG and IgM in both groups.

3. Differences in gene expression profiles between MZ twins with MS and their asymptomatic co-twins (study III)

A comparison between twin pairs showed that 305/8 300 genes were at least two-fold up- or down-regulated in at least 1/8 twins with MS. The proportion of up-regulated genes was significantly higher compared to the proportion of down-regulated genes (p=0.023 for the difference, χ-2 test). Moreover, 38/305 genes were up- or down-regulated in at least one fourth (2/8) of MS twin pairs. Of these 38 genes, 15 were down-regulated (2 to 10 fold) and 25 up-regulated (2 to 39 fold). 6/25 up-regulated genes were expressed in at least 40 % (3/8) of MS twin pairs, while none of the 15 down-regulated genes were detected with this a high frequency (p=0.01 for the difference of up- and down-regulated genes in χ-2 test). (See table 7).

Table 7. The six most constantly expressed genes

Gene	GeneBank accession no.	Description	Function	Change in expression
G1P3	X02492	Interferon-induced protein 6-16	Unknown	↑ (4 patients)
POU3F1	NM_002699	POU domain, class 3, transcription factor 1 (SCIP/Oct-6)	Serves as transcriptional transactivator in the nucleus	↑ (3 patients)
Mx2	M30818	Myxovirus (influenza) resistance 2	Possible antiviral potential	↑ (3 patients)
LAPTM5	NM_006762	Lysosomal-associated multispanning membrane protein-5	Possible involvement in B cell activation	↑ (3 patients)
HBA2	NM_000517	Hemoglobin, alpha 2	Oxygen transport	↑ (3 patients)
HBB	NM_000518	Hemoglobin, beta	Oxygen transport.	↑ (3)

Gene expressions in twin pairs are shown in Figure 6 as hierarchical clustering. The expression level of G1P3 confirmed by QRT-PCR appeared to be almost identical with the results obtained by cDNA microarray indicating that our method was working properly.

In an attempt to find correlations between clinical parameters such as type of MS, EDSS, gender, durations of the disease or age, the twins were divided into two groups. The hierarchical relationship among rations of gene expression of the 23 most significantly expressed genes between twins with MS and their co-twins were compared using cluster analysis. The results are presented in figure 6. These 23 genes were not able to separate SPMS from RRMS and no marked association between gene expressions and other clinical parameters were detected. The clinical characteristics of the twins with MS are shown in table 8 (see table 8).

Table 8. Clinical characteristics of the MZ twins with MS in study IV

twin no.	gender	age	duration of MS (years)	type of MS	EDSS	immunomodulatory treatment
1	F	54	11	SP	1.5	not treated
2	F	48	1	RR	4.0	IFN-beta 1a
3	F	55	9	SP	7.0	IFN-beta 1b
4	M	53	23	SP	6.5	not treated
5	F	33	5	RR	4.0	IFN-beta 1a
6	F	54	3	RR	4.5	IFN-beta 1b
7	M	66	11	SP	6.5	not treated
8	M	46	22	SP	6.5	not treated

F=female, M=male, SP=secondary progressive, RR=relapsing remitting, IFN=interferon

Figure 6. Hierarchical clustering showing relative gene expression comparing twins with MS to their co-twins. Genes with at least two-fold up- or down-regulation in 25% of twins with MS are presented. The colour bar on the right shows the colour representation of gene expressions. The grey colour indicates the lack of expression and "twin" represents a twin pair. The column on the left shows the similarity between the expression of different genes and the column above the similarity of the expression profiles between the twin pairs.
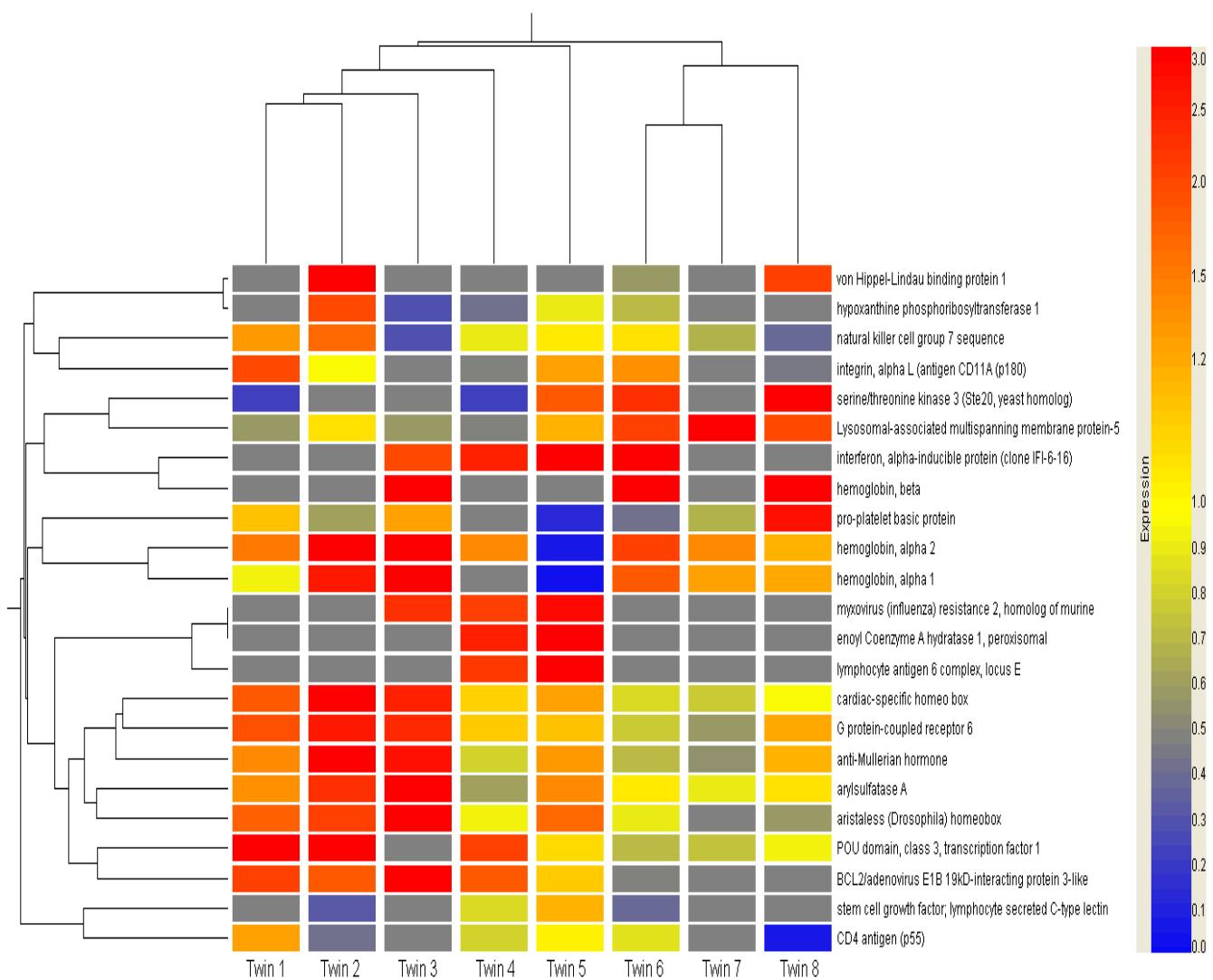
DISCUSSION

Interactions among susceptibility genes and the environment are believed to contribute to the development of MS (Poser 2006). A well-conducted twin study is an excellent means to assess the relative contribution of such genetic and environmental factors. We studied nearly half of the Finnish twin pairs born in 1957 or earlier, either dis- or concordant for MS, using a biometrical genetic method to evaluate the heritability and contribution of shared and unique environmental factors in the aetiolgy of the disease. The twins were obtained from a population based cohort, which is considered to be the most systematic approach and to have the least selection bias. The possible unique environmental factors are mainly considered to be viral infections; therefore we selected the most promising candidates that still showed inconclusive results, namely HHV6 and EVs for further analyses. When studying the role of specific environmental factors in the aetiology of MS it is important to minimize genetic heterogeneity of the patients and their controls, thus twins afforded us the best possible study population for the evaluation of HHV6 and EVs and the risk of MS. Since MZ twins share their entire genome, in discordant MZ twins the reason for MS is entirely due to environmental factors. Such factors result in differences in gene expression profiles. We addressed this question using microarray technique and a co-twin control method, in which the co-twins provided a perfect control group to the diseased subjects.

Aetiology of MS in Finland

All twin studies conducted in high-prevalence countries have demonstrated a higher MS concordance in MZ than in DZ twins, which highlights the importance of genetic factors in the aetiology of the disease (Mumford et al 1994, Sadovnick et al 1993, Willer et al 2003, Hansen et al 2005, Kinnunen et al 1988). Recent observations from medium-prevalence countries, have demonstrated, however, that the concordance for MZ twins is considerably lower than in the north (French Research Group of Multiple Sclerosis 1992, Ristori et al 2006, Islam et al 2006), suggesting that the penetrance of MS in twins correlates with MS prevalence in the area. See table 9 below for concordance rates in population based twin studies.

Table 9. Twin concordances around the world

Study	Country	MZ pairs	DZ pairs	MZ pairwise concordance	DZ pairwise concordance
Mumford et al 1994	the United Kingdom	44	61	11/44 (25%)	2/61 (3%)
Sadovnick et al 1993	Canada	26	43	8/26 (30.8%)	2/43 (4.7%)
Willer et al 2003	Canada	146	224	37/146 (25.3%)	12/224 (5.4%)
Hansen et al 2005	Denmark	32	132	5/32 (15.6%)	2/132 (1.5%)
Kinnunen et al 1988	Finland	7	6	1/7 (28.6%)	0%
French Reasearch Group of MS 1992	France	17	37	1/17 (5.9%)	1/37 (2.7%)
Ristori et al 2006	Continental Italy	51	147	4/51 (7.8%)	3/147 (2.0%)
Ristori et al 2006	Sardinia	8	10	1/8 (12.5%)	0%
Kuusisto et al in press	Finland	10	14	3/10 (30%)	2/14 (14.3%)

Pairwise concordance rates give only a little information on the aetiology of MS. The basic information is that if the MZ concordance rate is higher than that of the DZ twins, genes are important in the disease aetiology, whereas if the numbers are close to each other, environmental factors play a more significant role. The difference between MZ and DZ pairwise concordance rates in our study is clear and in that respect in line with previous reports from high prevalence areas (Mumford et al 1994, Sadovnick et al 1993, Willer et al 2003, Kinnunen et al 1988).

Further information can be acquired by using probandwise concordance rates, which in our study were estimated to be 46.2% for MZ and 25% for DZ twins. The probandwise concordance rate equals the probability of being diagnosed with MS. Hansen and associates reported propandwise concordance rate estimates of 24% for Danish MZ twins and 3% for DZ twins (Hansen et al 2005). Ristori and associates estimated probandwise concordance rates in continental Italy (a medium prevalence country) to be 14.5% for MZ and 4.0% for DZ twins and in Sardinia, which is a high prevalence area, 22.2% for MZ and 0% for DZ twins. The difference between the MZ and DZ probandwise rates in our study was smaller than in the Danish and Italian studies, which could be interpreted with extreme caution as showing that in Finland environmental factors play greater roles

in MS aetiology. It should be noted though that the small number of twins both in our and in the Sardinian study may have influenced the results obtained.

To examine the relative contribution of genetic and environmental factors to variation, we used the concordance rates quantitatively, which has not been done in any of the previous twin studies in high prevalence areas. In a review by Hawkes, the tetracholic correlations for liability and heritabilities for MS have been calculated for twin studies involving more than 50 twin pairs. The heritability estimates range from 24% in France to 86% in Canada. A prevalence of 100/100 000 for MS was assumed for Canadian and 40/100 000 for the French study (Hawkes 1997). However, the heritability estimates may change somewhat if smaller or larger population prevalence rates are used in secondary analyses. Our own analyses indicated that the modelling results are not very sensitive with respect to the prevalence estimate that is used. This is not surprising given that it largely affects the size of the unaffected twin pairs in the model. In an Italian study by Ristori and associates the heritability estimate was 48% (95% CI 6-86%), the environmental contribution 29% (95% CI 0-60%) for common and 23% (95% CI 12-39%) for unique environmental factors, when the continental Italy and Sardinia data was combined. In our study the heritability was estimated to be 15.3% (95% Cl 0.0-77.6), the common environmental variance 73.7% (95% Cl 14.1-93.9) and the unique environmental variance 11.1% (95% Cl 2.3-30.0). Since the number of concordant pairs in our study is small and the extremely wide confidence intervals overlapped with the confidence intervals of the Italian study, no definite conclusions can be drawn in regard to the low genetic variance estimate in our study.

Kinnunen et al presented the pairwise concordance rates of the Finnish twin cohort two decades ago (Kinnunen et al 1988). Our study was planned to update the original results. The twins were recruited in the same manner in both studies, so results are comparable. During recent decades the incidence of MS in Finland has increased (Sumelahti et al 2001). The fact that the pairwise concordance in DZ twin pairs has increased from 0 to 14.3% in 20 years while the pairwise concordance of MZ twins has remained the same suggests the possibility that the increase in the MS prevalence in Finland is more due to altered environmental than genetic factors even though there is a difference between the MZ and the DZ concordance. A longer follow-up also permits more DZ twins to become concordant if there is a shorter delay in onset in the MZ co-twins of MS cases than among DZ pairs. Also, it is unlikely that genes could be responsible for the increase in MS incidence observed over just a few decades.

Kinnuen and associates found MS prevalence to be higher among MZ than DZ twins (Kinnunen et al 1987). Furthermore the under-representation of twins in the MS population observed in a few studies (Willer et al 2003, Ristori et al 2006) and a recent finding that the rate of MS in DZ twin pairs is lower than expected from population prevalence (Hansen et al 2005), has raised the question of possible protective factors against MS shared by the twins. The DZ/MZ ratio in our study was only 1.4:1 compared to the estimated 2:1 in the general population. Our results thus may support a recent hypothesis suggesting that being a DZ twin may protect against MS (Hansen et al 2005). On the other hand, being a MZ twin may increase the risk.

The age of onset of MS may provide a clue for assessment of the impact of genetic and environmental factors influencing the development of the disorder. Bulman and colleagues found that concordant siblings received the diagnosis of MS within a span of a few years, emphasising the importance of genes in MS susceptibility (Bulman et al 2001). In accordance with these observations, three of the five concordant twin pairs in our series were diagnosed within five years, one, in fact, within the same year.

There is a recent report from Canada suggesting that the sex ratio in MS has changed over the past 100 years with with the rate of MS in females has increased rapidly while in males it has remained the same. The ratio prior to 1930 was reported to be 1:1 and currently exceeds 3.2:1 (Orton et al 2006). In our study the female/male ratio of twins with MS was 3.8:1, which is in line with the Canadian observation, but it may also be influenced by the greater likelihood of women to participate in research studies than men. Since genes are unlikely to be responsible for change over such a short time, environmental factors are thought to be responsible. Several female related factors have been suspected as being associated with MS, such as contraceptive pills, but no single factor has been confirmed to have such an association (Orton et al 2006).

Viruses and MS

Viral infections, vaccinations, nutrition and other factors that vary within individuals represent so-called unique environmental factors in the aetiology of MS. We found the contribution of these factors to the aetiology of MS in Finland to be only 11.7% (95% Cl 2.5-30.7). Therefore, for further analyses we selected one of most promising DNA viruses, HHV6, which has been linked to MS in numerous studies but the results have remained inconclusive. In addition to HHV6 we

selected one hypothetically interesting RNA virus group, namely EVs, which have been connected with varying autoimmune disorders other than MS, but scarcely studied in MS (Hyöty et al 1995, Clements 1995, Heim et al 1997, Leparc-Goffart et al 1996).

There are two major hypotheses about viruses and their association with MS. One is that persistent or reactivated latent virus infection may cause an autoimmune response leading to injury within the CNS, and the other, that MS is an acquired infectious disease caused by so far an unknown virus infection (Gilden 2005). It is known that demyelination can be initiated by infection with variety of DNA and RNA viruses. The possible mechanisms leading to virus induced demyelination appear to be diverse. The rationale for an infectious cause of MS is based on the hypothesis that demyelination can result from a direct viral infection of oligodendroglia, as happens in progressive multifocal leucoencephalopathy (PML), or virus infection can initiate immunopathology leading to demyelination as happens in animals infected with strains of TME virus, coronaviruses and lentiviruses. Bases for the autoimmune theory lie in the observation that some viruses can cross-react with MBP and activate an immune response against myelin components, which may lead to demyelination and possibly to the development of MS. This so-called molecular mimicry has been observed for example in HHV6 (Fotheringham and Jacobson 2005, Tejada-Simon et al 2003, Stüve at al 2004). Also it is thought that a virus-induced unspecific inflammatory response, in which the various immune mediators damage the oligodendroglia or the myelin sheath, could be the mechanism leading to development of MS (Stohlman and Hinton 2001).

In the present study we did not find HHV6 DNA in serum, acellular CSF or leukocytes separated from the CSF in any of the MS patients or their healthy siblings, which is in line with the absence of HHV6 specific antibodies in CSF in our study. The rate of seropositivity for serum HHV6 IgG was nearly 90% in both our groups, which is in line with previous reports.

Studies measuring HHV6-spesific IgG and IgM antibodies in the CSF have suggested that there may be a relationship between HHV6 infection and MS though studies on serum have failed to confirm such an association (Ongradi et al 1999,Ablashi et al 1998, Nielsen et al 1997). One reason to this may be the fact that the seropositivity for anti-HHV6 IgG antibodies approaches 100% in the background population (Meinl 1999), which makes it difficult to evaluate this question by serological surveys. It has been suggested that HHV6

might be more likely to cause MS only in selected subjects despite the high seroprevalence in the general population. On the other hand it might merely be an innocent bystander (Enbom et al 1999) or even a symptom of MS rather than a cause, since due to the breakdown of the BBB, HHV6 could simply migrate into the CNS (Berardelli 1997). The presence of HHV6 DNA in serum of healthy individuals appears to be low. Secchiero and associates found that 86% of exanthema subitum pediatric patients were positive for HHV6 DNA in serum, whereas only 3% of chronic fatigue syndrome patients and none of the healthy adult controls displayed such positivity (Secchiero et al 1995). Recent meta-analyzes on HHV6 and MS found PCR studies to be inconclusive (Moore and Wolfson 2002, Swanborg and Whittum-Hudson 2002 Swanborg et al 2003). Is seems, however, that there is a connection between HHV6 and disease activity. Today, the main argument for an association between HHV6 and MS is based on the presence of viral DNA in the blood from MS patients in active phase of the disease in contrast to MS patients in the inactive phase or healthy controls (Höllsberg et al 2005, Alvarez-Lafuente et al 2006, Berti et al 2002, Chapenko et al 2003). Whether HHV6 is able to cause a relapse upon reactivation or become reactivated because of the relapse is not known. Our samples were collected during clinical and radiological remission of the disease, and therefore this study neither supports nor refutes the observations showing a correlation between the presence of HHV6 DNA in plasma and disease activity (Höllsberg et al 2005, Alvarez-Lafuente et al 2006, Berti et al 2002, Chapenko et al 2003). Since the twins were from all over Finland and the travel distances were very long, regrettably, it would have been impossible to obtain samples during an acute exacerbation.

Also the EV RNA in serum or CSF from MS patients, or their healthy twin siblings, using a high sensitive RT-PCR assay was negative in our study. The absence of EV RNA in serum and CSF indicates that there is no persisting or latent enteroviral infection in patients with MS. Our results support the earlier finding of absence of enteroviral RNA in autopsy brain tissues from MS patients or control patients (Dessau et al 1997).

When studying the role of viruses in the aetiology of MS, many methodological challenges need to be taken in consideration. One major issue is the selection of subjects and controls. The subjects with MS should be uniform and clearly defined as having definite MS and the controls should be examined and other neurological or autoimmune diseases ruled out. The two groups should be as similar as possible; otherwise differences found may be due to

differences between cases and controls other than MS. For example some viruses may be more prevalent in different populations or at different ages. Also in the majority of the studies using CSF, the control samples are collected from patients with other neurological diseases than MS, and, for ethical reasons, not from healthy volunteers. It is also important to have patients with a recent onset of the disease. For example in studies that have used specimen collected post mortem, many years after the initial diagnosis of MS, it is impossible to find evidence for a positive finding that a specific virus actually has caused MS decades ago. The methods used in viral studies should be well controlled. Especially studies using PCR, which is very prone to contamination, should always include negative and positive controls. All analyses should be done blind from coded samples (Moore and Wolfson 2002, Gilbert 2005).

In the EV and the HHV6 studies our purpose was to minimize genetic heterogeneity of the patients and their controls. The use of the FinnishTwin Cohort allowed us to avoid the selection bias and optimized the control selection. The presence of definite MS was confirmed by careful neurological, neuropsychological and radiological examination of both affected and healthy twins. Other neurological or immunological diseases both in patients and controls were recorded. All analyses were done blindly from coded samples. We used a well established commercial kit with high sensitivity. In the EV study, we tested the samples, for the presence of PCR inhibitors by monitoring the amplification of internal control DNA added to these samples. The majority of the samples were not inhibitory.

The notion that MS might be infectious has mainly relied on assumed epidemics. The most famous MS epidemic study suggested a point-source epidemic on the Faroe Islands acquired over a wide age range (Kutrzke 2001). There has been, however, criticism of the assumed epidemics, pointing out that the increase in incidence in the area was most likely attributable to increased facilities to diagnose the disease. Also, the fact that majority of the patients with MS have high concentration of IgG, manifested as oligoclonal bands, in the brain and CSF, and that many chronic inflammatory CNS disorders (subacute/chronic sclerosisng panencephalitis caused by measles and rubella viruses, mumps meningitis or PML caused by human papova virus), are infectious, is thought to support the hypothesis that MS is caused by a virus. On the other hand, although there have been vigorous attempts to show that IgG in patients with MS is directed against a certain virus, the results remain negative (Gilden 2005). Furthermore the general rules for micro-organisms to cause a disease do not

support the hypothesis that MS is acquired. The rules are that the organism must be present in every case of the disease, it must be isolated from the host with the disease and grow in pure culture, it must be reproduced when a pure culture of the microbe is transferred in to a healthy susceptible host and the pathogen must be recoverable from the experimentally infected host (Stüve 2004). Finally it should be briefly mentioned that there is a paper by Hawkes claiming that MS is a sexually transmitted disease (Hawkes 2002). At the time the article attracted a lot of general attention, especially because it suggested that paediatric MS is caused by child abuse. Understandably, patients with MS were upset and the hypothesis was quickly discredited.

In end it is very difficult to interpret the results from virus studies on MS, even the positive ones since it may be that the immune system activation and BBB breakdown in patients with MS make signs of prior virus infection more easily detectable.

Gene expressions are influenced by the environment

Transcriptional changes impact the expression of the gene product. Therefore, studying gene expression profiles can help in gaining better understanding of molecular disturbances in MS. We found six genes that were over two-fold up-regulated in nearly half of the MZ twins with MS, compared to their healthy co-twins. These genes (G1P3, MX2, POU3F1, LAPTM5, HBA2 and HBB) seem to be related with MS pathogenesis, virus infections or treatment of MS.

G1P3 is transcriptionally induced by IFN-α and -β (Friedman et al. 1984, Hibbert and Foster 1999, Kelly et al. 1986), virus infections (Clauss et al. 1990, Wathelet et al. 1989, Zhu et al. 2003) and tumour necrosis factor (Chernajovsky and Reid 1990), but its function is not known. Since three of the four twins, who presented significant up-regulation of GIP3 were treated with IFN-β, the up-regulation of G1P3 in their PBMC is most likely explained by the IFN-β treatment. There was, however, one twin who had been treated with IFN-β, but did not express GIP3. One reason could be a possibility of a development of neutralizing antibodies (NABs) against INF-β treatment. At the time of the study knowledge of NABs was limited and their measurement was not routine in general practise. The lack of expression may also be due to possible suboptimal clinical efficacy of INF-β treatment. GIP3 expression may also be related to some unknown virus infection. Mx proteins are

induced specifically by IFN-α and -β (Simon et al. 1991, Staehili 1990) and also have antiviral activities (Haller et al. 1998). The antiviral potential or other functions of the MxB protein encoded by MX2, one of the up-regulated genes in our study, are not fully understood, while human MxA protein has a wide antiviral spectrum (Haller and Kochs 2002) and high levels of its mRNA have been detected after treatment with IFN-β (Bertolotto et al. 2001). Since, up-regulated MX2 was observed in two twins treated with IFN-β, its up-regulation is most likely due to this treatment, especially since in these two twins GIP3 was also up-regulated. An unknown virus infection is also a possibility for the up-regulation. Up-regulated HBA2 and HBB genes detected in three twins with MS translate proteins that are part of a hemoglobin molecule. Heme units contain iron, which is involved in myelin production by oligodendrocytes and participates in the initiation of oxidative stress-induced injury in the CNS. This process plays a role also in the pathogenesis of MS (Connor and Menzies 1996). All the twins that had up-regulated HBA2 and HBB were female INF-β users. The protein (SCIP/Oct-6) translated by POU3F1 has been associated with Schwann cells in the process of remyelination (Sim et al. 2002). In oligodendrocytes, the SCIP/Oct-6 can stimulate the expression of the papovaviral JC regulatory genes in PML (Wegner et al. 1993), which has recently been associated with natalizumab tratment (Kleinschmidt-DeMasters and Tyler 2005, Langer-Gould et al 2005). Two of the three twins that presented significant up-regulation of POU3F1 had SPMS and had never been treated with IFN-β. The up-regulated LAPTM5 gene detected in three twins with MS is conserved across evolution but it encodes protein which has no homology to any of the other lysosomal proteins (Adra et al. 1996). LAPTM5, also known as Clast6, has been found to be highly expressed in progenitor and precursor B cells (Seimiya et al. 2003). The protein may function during B cell activation or it could have a role in the antigen processing in lysosomes (Seimiya et al. 2003), which may be of relevance in MS. Two of the three twins who expressed LAPTM5 were male with SPMS. They had severe disability and had never been treated with IFN-β.

MS in MZ twins discordant for the disorder, is entirely due to environmental factors. Since transcriptional changes impact the expression of the gene product, the differences in the gene expression profiles that we detected can help in gaining a better understanding of molecular disturbances in MS. We do recognize that the differences in gene expression profiles in our study may also be due to various exogenous factors other than MS, such as infections or the treatment used for the disease. When trying to repeat earlier gene findings in MS, it should be noted that there is virtually no overlap between genes identified in different studies. The challenges in microarray studies are that there is no stereotypical inflammatory reaction for MS and the onset and

progression of inflammatory events can last for either short or long period of time. Furthermore gene expression patterns anywhere in the organism are subject to circadian rhythms. Therefore, comparable results can be obtained only from subjects with comparable histories (Gebicke-Haerter 2005).

CONCLUSIONS

The following conclusions are drawn from this study:

1. The pairwise concordance for MZ twins in Finland is 30% (3/10) and for the DZ twins 14.3% (2/14). The probandwise concordance rates are 46.2% for MZ and 25% for DZ twins. Based on twin modelling assuming a prevalence of 100/100 000, the genetic variance (heritability) is 15.3% (95% Cl 0.0-77.6), the common environmental variance 73.7% (95% Cl 14.1-93.9) and the unique environmental variance 11.1% (95% Cl 2.3-30.0).

Compared to the pairwise concordance rate reported in Finland 20 years ago it has increased in DZ twins from 0/6 to 2/14 twin pairs, but in MZ twins remained the same. Though the numbers are small and the confidence intervals of the heritability estimate wide, we make a caucious conclusion that the known increase in MS incidence in Finland is due mainly to common (shared) environmental factors. The difference in concordance between MZ and DZ twins, however, still remains, suggesting that genes also have a significant role in the development of MS.

2. Our results do not support the concept of an aetiological role for persistent EV infection in MS.

3. During a clinical remission of MS the detection of HHV6 specific antibodies in CSF and HHV6 DNA in serum, CSF supernatant or CSF leukocytes is unlikely. However, the results do not exclude a possibility of HHV6 reactivation during MS exacerbation or acute HHV6 infection being one of the triggering agents in the development of MS long before its clinical manifestation.

4. There is a difference in the gene expression profiles of MZ twins with MS and their co-twins. The detected up-regulated expression of G1P3-, MX2-, POU3F1-, LAPTM5-, HBA2- and HBB- genes in nearly half of MZ twins with MS is related either to the pathogenesis of MS or various exogenous factors such as the INF-β treatment or viral infections.

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Research article

Gene expression profiles in Finnish twins with multiple sclerosis

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Abstract

Background: Since genetic alterations influencing susceptibility to multiple sclerosis (MS), the most common autoimmune demyelinating disease of the central nervous system (CNS), are as yet poorly understood, the purpose of this study was to identify genes responsible for MS by studying monozygotic (MZ) twin pairs discordant for MS.

Methods: In order to identify genes involved in MS development, the gene expression profiles in blood mononuclear cells obtained from eight MZ twin pairs discordant for MS were analyzed by cDNA microarray technology detecting the expression of 8 300 genes. The twins were collected from the Finnish Twin Cohort Study and both affected subjects and their healthy siblings underwent neurological evaluation and cerebral and spinal magnetic resonance imaging. Gene expressions were confirmed by relative quantitative reverse transcription PCR.

Results: It appeared that 25 genes were at least two-fold up-regulated and 15 genes down-regulated in 25% (2/8) of twins with MS when compared to their healthy siblings. Moreover, 6/25 genes were up-regulated in 40% of MS twins and one gene, interferon alpha-inducible protein (clone IFI-6-16) (G1P3), in 50% of them. The six most constantly expressed genes are (1) G1P3, (2) POU domain, class 3, transcription factor 1, (3) myxovirus resistance 2, (4) lysosomal-associated multispanning membrane protein-5, (5) hemoglobin alpha 2 and (6) hemoglobin beta.

Conclusion: Over two-fold up-regulation of these six genes in almost half of MZ twins with MS suggests their role in MS pathogenesis. Studies using MZ MS twins obtained from genetically homogeneous population offer a unique opportunity to explore the genetic nature of MS.

Background

Multiple sclerosis (MS) is the most common autoimmune demyelinating disease of the central nervous system (CNS) characterised by inflammatory lesions scattered

throughout the brain tissue. According to recent data the prevalence of MS in Finland is the highest in the world being 100/100 000, although the figures vary in different regions of the country [1]. The reasons for the increase in the disease prevalence are not known, although viral infections and other environmental factors have been suggested [1].

Family and twin studies have shown that the concordance rate of MS for monozygotic (MZ) twins is about 30% and 2 – 5% for dizygotic (DZ) twins and siblings [2-4]. In a Finnish twin cohort of 15 815 pairs, the concordance rate of MS was 29% in MZ twins and the concordance rate was 0% in DZ twins [5].

It is well known that genetic factors regulate susceptibility to MS, but of these factors only HLA-DR2 has been confirmed to be associated with the disorder [6]. In our previous studies several susceptibility genes and their associations have been reported [7-15]. These are the protective effect of HLA-DR1 and HLA-DR53 combination against MS [9], decreased risk of severe MS of IL-10-1082 AG genotype carriers [12] and high chemokine receptor 5 (CCR5) RNA expression in peripheral blood in primary progressive MS [8]. Increased risk of MS in women has been detected with interleukin-1 receptor antagonist (IL-1RA) allele 2 [11], 5G5G genotype of plasminogen activator inhibitor 1 (PAI-1) gene [10] and interaction between estrogen receptor 1 (ESR1) and HLA-DR2 [13]. Other associations with MS in Finnish population are myelin basic protein (MBP) short tandem repeat [15], intercellular adhesion molecule-1 (ICAM-1) AA genotype (Lys[469]/Lys[469]) [14] and preliminary evidence of two distinct MS susceptibility genes, proximal rs3977 and distal D2S1271-associated genes, on 2q33 outside of cytotoxic T-lymphocyte-associated 4 (CTLA4) gene [7]. Taken together, these observations suggest that experimental approach using MZ twin pairs discordant for MS obtained from Finnish genetically relatively homogeneous population may provide a unique opportunity to explore the genetic nature of MS. Interestingly, one study performed on monozygotic twins with MS reported deficient expression of the inhibitory transcription factor Sp3 in mononuclear blood cells [16].

Since genetic factors influencing MS susceptibility and progression are as yet poorly understood, the purpose of this study was to identify genes responsible for MS development by studying MZ twin pairs discordant for MS identified from the Finnish Twin Cohort Study and using cDNA array technology involving the expression profiles of 8 300 known genes.

Methods
Study subjects
We studied eight MZ twin pairs discordant for MS obtained from the Finnish Twin Cohort Study. Patients of MS were identified by linkage to the national hospital discharge registry, which covers all hospitalization in Finland since 1972. All the twin pairs, both affected subjects and their healthy twin siblings, underwent neurological evaluation and magnetic resonance imaging (MRI) of the CNS using 1,5 Tesla MRI unit during a clinical remission of the disease. From the MRI protocol, axial 3 dimensional (3D) T2 fast spin echo (FSE), T1 3D axial spoiled gradient echo (SPGR) and FLAIR sequences were used. T2 hyperintense plaques were analyzed from 3D T2 FSE images, T1 hypointense plaques from 3D T1 SPGR images and FLAIR lesions from FLAIR images. All MS patients showed T1- and T2-lesions characteristic to MS, but Gadolinium-enhanced focal lesions were not detected. The diagnosis of MS was based on Poser's criteria and all diagnoses were definite [17]. The affected twins had no other diseases and their twin siblings were all healthy. The mean age of twin pairs was 51.1 ± 9.1 (SD) years. The neurological disability evaluated by the Expanded Disability Status Scale (EDSS) score was 5.1 ± 1.9 (mean ± SD). The clinical characteristics of twin pairs are shown in Table 1. Four out of 8 twins with MS were treated with interferon beta (IFN-β). One of them had received this treatment for 1 year (patient No2, table 1) and the remaining three patients for two to three years. (patients No3, 5 and 6). In addition most of MS patients had symptomatic medication. The peripheral blood samples were collected during a clinical remission of the disease and the analyses were performed blind to disease status.

Isolation of total RNA
Mononuclear cells were separated from peripheral blood (PBMC) in VACUTAINER® CPT™ Cell Preparation Tubes (Becton Dickinson and Company, Franklin Lakes, N.J., USA) and total RNA were isolated by RNeasy® Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's protocols. The DNA was removed according to BD Atlas™ Plastic Microarrays (BD Biosciences Clontech, Palo Alto, CA, USA) user manual for DNase treatment of total RNA for 10 μg of RNA, with the exception that RNA precipitation was carried out overnight at -20°C. The quality of total RNA was checked by gel electrophoresis and stored at -70°C until used.

cDNA microarrays
The study was performed using BD Atlas™ Plastic Human 8 K Microarrays (BD Biosciences Clontech, Palo Alto, CA, USA), which contains duplicate DNA fragments from more than 8 300 known human genes (a list of genes is available at http://www.clontech.com/clontech/atlas/genelists/). 5 μg of total RNA were used for microarray

Table 1: Clinical characteristics of monozygotic twin pairs.

Twin pair no	Gender	Age	Duration of MS (years)	Type of MS	EDSS	Immunomodulatory treatment
1	F	54	11	SP	1.5	n.t.
	F	54	Healthy/0	Healthy		
2	F	48	1	RR	4.0	IFN-β-1a
	F	48	Healthy/0	Healthy		
3	F	55	9	SP	7.0	IFN-β-1b
	F	55	Healthy/0	Healthy		
4	M	53	23	SP	6.5	n.t.
	M	53	Healthy/0	Healthy		
5	F	33	5	RR	4.0	IFN-β-1a
	F	33	Healthy/0	Healthy		
6	F	54	3	RR	4.5	IFN-β-1b
	F	54	Healthy/0	Healthy		
7	M	66	11	SP	6.5	n.t.
	M	66	Healthy/0	Healthy		
8	M	46	22	SP	6.5	n.t.
	M	46	Healthy/0	Healthy		

F female; *M* male; *n.t.* no treatment; *EDSS* Expanded Disability Status Scale; *IFN-*β beta-interferon

analyses and samples from MZ twin pairs were analyzed at the same time. Microarrays were exposed to phosphoimaging screen and scanned by StormScan 840 Phosphoimager (Molecular Dynamics, Sunnyvale, CA, USA) after 5–7 days exposure time at a resolution of 50 µm to ImageQuant software version 5.1 (Molecular Dynamics, Sunnyvale, CA, USA). The comparison was done for all 8 discordant monozygotic twin pairs and all 8300 genes by using widely used cDNA subtraction procedure according to manufacturer's instructions (Clontech, Palo Alto, CA, USA). In brief, in our array, RNA sample obtained from healthy MZ twin (tissue No1) was compared to corresponding RNA obtained from MZ twin with MS (tissue No2) by using cDNA subtraction method.

Data analysis and normalization

Analysis was performed using the BD AtlasImage™ 2.7 Beta software (BD Biosciences Clontech, Palo Alto, CA, USA) and data was globally normalized by the sum method. Normalized signal intensities were compared to those of healthy siblings. Ratios of gene expression greater than two-fold were considered significant, based on a 99% confidence interval [18,19]. The data was further analyzed and visualized with the GeneSpring software version 5.0 (Silicon Genetics, San Carlos, CA, USA) and the detailed principles of the cluster analysis and dendrograms can be found from the GeneSpring GX animated tutorial from the internet http://www.chem.agilent.com/scripts/pds.asp?lpage=27881.

Quantitative reverse transcription polymerase chain reaction (QRT-PCR)

The cDNA microarray data was confirmed for interferon alpha-inducible protein gene (clone IFI-6-16) (G1P3) by relative quantitative real-time RT-PCR with LightCycler instrument (Roche Diagnostics GmbH, Mannheim, Germany). 1 µg of total RNA was converted into cDNA with Random Primer p(dN)$_6$ using 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche Diagnostics Corporation, Indianapolis, IN, USA). The primers and hybridization probes for QRT-PCR were designed and prepared by TIB MolBiol (Berlin, Germany) and the sequences are the following: forward primer: 5'-GAGTGCAGTGGCTATTCACA-3', reverse primer: 5'-GCGCATGCTTGTAATCCTAC-3', probe 5'-end labeled with acceptor dye LC Red 640: 5'-AGCCTCAAGTGATCCTCCTGTCTCA-3' and probe 3'-end labeled with fluorecein: 5'-CATAGTACACTGCAGCCTCCAACTCC-3'.

The PCR was performed in a 20 µl total volume for target gene with 2 µl LightCycler FastStart DNA Master Hybridization Probes (Roche Diagnostics GmbH), 3 mM MgCl$_2$, 0.2 µM each probe, 0.5 µM each primer and 2 µl of cDNA. The PCR was performed with a denaturation at 95°C for 10 min, amplified in 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 15 s and elongation at 72°C for 12 s. The cooling was performed at 40°C for 30 s.

Glucose-6-phosphate dehydrogenase (G6PDH) was used as a reference gene. The PCR for this was performed by LightCycler – h – G6PDH Housekeeping Gene Set Kit (Roche Diagnostics GmbH) and was done at the same time and under the same PCR conditions as for the target gene. All reactions for target and reference gene were performed in duplicates. Agarose gel electrophoresis was used to verify the PCR products. QRT-PCR results were calculated by LightCycler Relative Quantification Software with efficiency correction (Roche Diagnostics GmbH).

Statistical analyses

The significantly expressed genes in our array experiments were defined according to the instructions given by the cDNA array manufacturer (Clontech, Palo Alto, CA, USA). cDNA subtraction ratios of gene expression greater than two-fold were considered significant, based on a 99% confidence interval. The differences of at least 2-fold up- or down-regulated genes within MS twin groups were compared with McNemar test and χ-2 test using statistical software STATA version 8.0 (STATA Corporation, TX, USA) A p-value of less than 0.05 was considered significant.

Results

Gene expressions in MZ twin pairs discordant for MS

The numbers of significantly up- or down-regulated genes (at least two-fold difference in expression between twin pairs) in the PBMC of MZ pairs discordant for MS detected by cDNA microarray are shown in Table 2. Comparison between twin pairs showed that 305/8 300 genes were at least two-fold up- or down-regulated in at least 1/8 twins with MS. The proportion of up-regulated genes was significantly higher compared to the proportion of down-regulated genes (p = 0.023 for the difference, χ-2 test). Moreover, 38/305 genes were up- or down-regulated in at least one fourth (2/8) of MS twin pairs (Table 2 and 3). Of these 38 genes, 15 were down-regulated (2 to 10 fold) and 25 up-regulated (2 to 39 fold). 6/25 up-regulated genes were expressed in at least 40% (3/8) of MS twin pairs (Table 2), while none of the 15 down-regulated genes were detected with this high frequency (p = 0.01 for the difference of up- and down-regulated genes in χ-2 test). One gene, interferon alpha-inducible protein (clone IFI-6-16) (G1P3), appeared to be up-regulated in 50% (4/8) of the MZ MS twin pairs.

The hierarchical relationship among ratios of gene expression of 23 most significantly expressed genes between healthy sibling and otherwise identical sibling with MS were compared with cluster analysis. These results are displayed as a dendrogram in Figure 1. As shown by the figure the twin pair samples are roughly divided into two main branches, left branch including twin pairs 1–5 and right branch including twin pairs 6–7. In order to find clinical differences between the branches we characterized these two groups according to the clinical criteria shown in Table 1. From the left branch pairs 1–5 three had SP type (3/5, 60%) and two RR type of MS and from the right branch two of the twins had SP (2/3, 67%) and one RR type of MS (Table 1). Thus these 23 genes were insufficient to separate between relapsing-remitting and secondary progressive MS. From the left branch pairs three (3/5, 60%) of the diseased siblings got IFN-β-1b treatment and from the right branch pair one of the three siblings (1/3, 33%) received this treatment. Also the average EDSS,

Table 2: Number of up- or down-regulated genes in the discordant twins with MS compared to their healthy siblings.

	Number of twin pairs		
	1/8*	2/8**	3/8
Up-regulated	194	25	6
Down-regulated	111	15	0
Total no of genes with change	305	38	6

Genes with at least two-fold change are included.
The proportion of up-regulated genes was significantly higher compared to the proportion of down-regulated genes within twin pair groups *p = 0.023; **p = 0.01 in X-2 test.

duration of the MS and mean age of the sibling pairs tended to be lower in the left branch subjects (mean EDSS = 4.6, and mean duration 9.8 years, mean age 48.6 years, respectively) than in the three subjects involved in the right branch (mean EDSS 5.8, mean duration 12.0 years, and mean age 55.3 years, respectively). Furthermore, four of the total five siblings (4/5, 80%) in the left branch were women and in the right branch two of the total tree pairs were men (2/3, 67%).

The six most constantly expressed genes are shown in the Table 4. They are the following: (1) G1P3, (2) POU domain, class 3, transcription factor 1 (POU3F1), (3) myxovirus resistance 2 (MX2), (4) lysosomal-associated multispanning membrane protein-5 (LAPTM5), (5) hemoglobin alpha 2 (HBA2) and (6) hemoglobin beta (HBB). Over two-fold up-regulation of these six genes in 40% of MZ MS twins suggests their role in MS pathogenesis. However, no marked associations between gene expressions and neurological or MRI findings were detected. The expression level of G1P3 confirmed by QRT-PCR appeared to be almost identical with the results obtained by cDNA microarray indicating that our method was working properly (Figure 2).

Discussion

The present study applying modern technique and experimental approach where gene expression profiles of MZ twin pairs discordant for MS were compared to each other revealed differential expression of 305 genes out of 8300 genes studied. Among the differentially expressed genes the proportion of up-regulated genes was significantly higher than that of down-regulated ones (64% vs. 36%). This observation may reflect the balance between immunoactivating and immunoinhibitory factors during the complex inflammatory cascade in MS.

It is noteworthy that up-regulated expression of six genes was found in half of twins with MS. This observation together with the fact that the MS twins were obtained from genetically homogeneous Finnish population sug-

Table 3: The most constantly expressed genes (n = 38) having at least two-fold change, up- (n = 25) or down- (n = 15) regulation, simultaneously in 2 of 8 discordant identical twins with MS when compared to their healthy siblings.

Gene group/symbol	GenBank accession no.	Description	Up (↑) or down (↓) – regulation of genes
Basic transcription factors			
POU3F1	NM_002699	POU domain, class 3, transcription factor 1	↑
NKX2-5	NM_004387	Cardiac-specific homeobox	↑
PHOX2A	NM_005169	Aristaless (drosophila) homeobox	↑
Cell surface antigens			
MX2	M30818	Myxovirus (influenza) resistance 2	↑
LY6E	NM_002346	Lymphocyte antigen 6 complex, locus E	↑
NKG7	NM_005601	Natural killer cell group 7 sequence	↓
ITGAL	NM_002209	Integrin, alpha L, lymphocyte function-associated antigen 1 (CD11A)	↓
CD4	M12807	T cell surface glycoprotein CD4 antigen (p55)	↓
Growth factors, cytokines and chemokines			
PPBP	M54995	Pro-platelet basic protein	↓
SCGF	D86586	Stem cell growth factor	↓
Intracellular transducers/effectors/modulators			
VBP1	NM_003372	Von Hippel-Lindau binding protein 1	↑
STK3	NM_006281	Serine/threonine kinase 3 (STE20 homolog, yeast)	↑ and ↓
Metabolism			
HBA1	NM_000558	Hemoglobin, alpha 1	↑
HBA2	NM_000517	Hemoglobin, alpha 2	↑
HBB	NM_000518	Hemoglobin, beta	↑
ECH1	NM_001398	Enoyl Coentzyme A hydratase 1, peroxisomal	↑
ARSA	NM_000487	Arylsulfatase A	↑
HPRT1	V00530	Hypoxanthine phosphoribosyltransferase1	↓
GAPD	X01677	Glyceraldehyde-3-phosphate dehydrogenase	↓
Ribosomal proteins			
RPS9	U14971	Ribosomal protein S9	↑
RPS12	NM_001016	Ribosomal protein S12	↑
RPS20	NM_001023	Ribosomal protein S20	↑
RPS29	NM_001032	Ribosomal protein S29	↑
RPL3	NM_000967	Ribosomal protein L3	↑
RPL9	NM_000661	Ribosomal protein L9	↑
RPL39	NM_001000	Ribosomal protein L39	↑
LAMR1	U43901	Laminin receptor 1	↑ and ↓
RPS5	NM_001009	Ribosomal protein S5	↓
RPL27A	NM_000990	Ribosomal protein L27a	↓
RPL30	NM_000989	Ribosomal protein L30	↓
RPL38	NM_000999	Ribosomal protein L38	↓
Others			
G1P3	X02492	Interferon-induced protein (6–16, IFI6-16)	↑
LAPTM5	NM_006762	Lysosomal-associated multispanning membrane protein-5	↑
AMH	NM_000479	Anti-Mullerian hormone	↑
GPR6	NM_005284	G protein-coupled receptor 6	↑
BNIP3L	NM_004331	BCL2/adenovirus E1B 19 kD-interacting protein 3-like	↑
ACTB	X00351	Actin, beta	↓

gests the importance of these genes in MS. To the best of our knowledge none of the six genes has previously been reported to be associated with MS. The sample was relatively small, but not unexpected given that MS is relatively rare, as is MZ twinning (about 0.4% of births are MZ twin births), The Finnish Twin Cohort covers virtually all twins alive in 1975 and born before 1958 [20], while the hospital discharge registry identified nearly all MS cases in Finland.

Expression of G1P3 was up-regulated in half of our MS twins. It is known that this gene is transcriptionally induced by IFN-α and -β [21-23], virus infections [24-26] and tumour necrosis factor [27], but its function remains unknown. It is interesting that Ifi-6-16 peptide translated by G1P3 has been identified as an abundant self-peptide induced following measles virus (MV) infection [28], which has previously been associated with MS [29-31]. Since most of our MS twins were treated with IFN-β, the
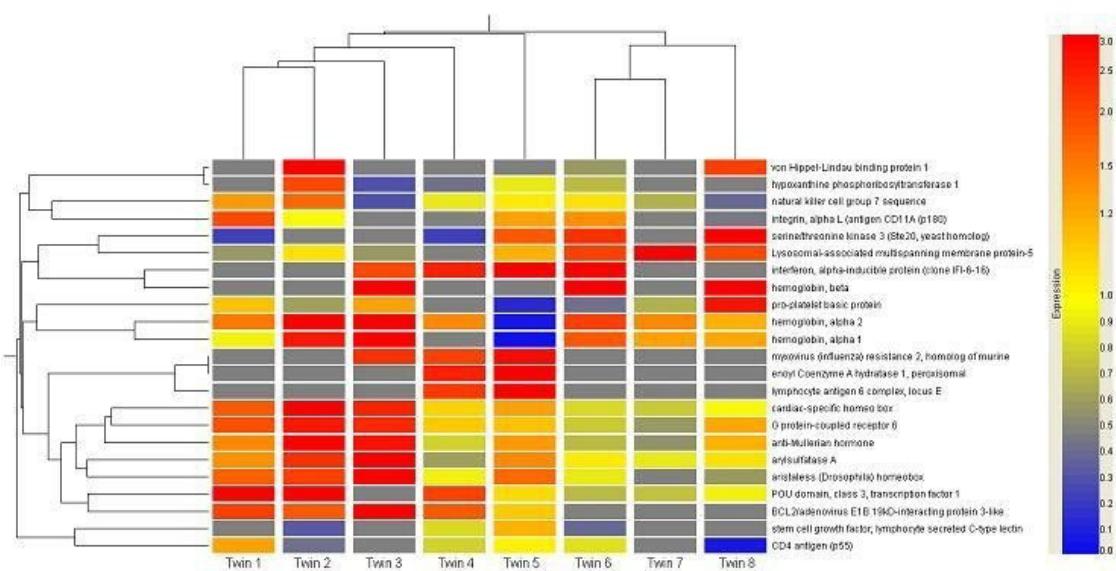
Figure 1
Hierarchical clustering showing relative gene expressions comparing patients with MS to their healthy siblings. Genes with at least two-fold up- or down-regulation in 25% of MS twins are presented. Housekeeping genes and ribosomal protein genes are not included. The colorbar on the right shows the color representation of gene expressions. Grey color indicates the lack of expression in cDNA microarray and twin represents twin pair. The column dendrogram on the left shows the similarity between the expression of different genes and column dendrogram above the similarity in the gene expression profiles of twins. The data was visualized with the GeneSpring software version 5.0 (Silicon Genetics, San Carlos, CA, USA) and the detailed principles of the cluster analysis and dendrograms can be found from the GeneSpring GX animated tutorial from the internet http://www.chem.agilent.com/scripts/pds.asp?lpage=27881. See also text for the interpretation of the figure.

up-regulation of G1P3 in their PBMC can most likely be explained by the IFN-β treatment or some unknown virus infection.

Twins with MS had up-regulated expression of POU3F1. The protein (SCIP/Oct-6) translated by this gene has mostly been studied in the nervous system, where it is associated with Schwann cells in the process of remyelination [32]. In oligodendrocytes, cells producing myelin in the CNS, the SCIP/Oct-6 can stimulate the expression of the papovaviral JC regulatory genes in progressive multifocal leukoencephalopathy (PML) [33], a demyelinating disease of the CNS. However, immune response to the JC virus has not so far been detected in MS.

Table 4: The six most constantly expressed genes detected by cDNA microarray.

Gene symbol	GeneBank accession no.	Description	Function	No of twin pairs with up-regulated gene expression
G1P3	X02492	Interferon-induced protein 6–16	Unknown	4
POU3F1	NM_002699	POU domain, class 3, transcription factor 1 (SCIP/Oct-6)	Serves as transcriptional transactivator in the nucleus	3
MX2	M30818	Myxovirus (influenza) resistance 2	Possible antiviral potential	3
LAPTM5	NM_006762	Lysosomal-associated multispanning membrane protein-5	Possible involvement in B cell activation	3
HBA2	NM_000517	Hemoglobin, alpha 2	Oxygen transport	3
HBB	NM_000518	Hemoglobin, beta	Oxygen transport	3

Särkijärvi

Figure 2

a

Twin 3

Twin 4

Twin 5

Twin 6

b

Twin	cDNA microarray result	QRT-PCR result	IFN-β treatment
Twin 3	2.0	2.8	Yes
Twin 4	2.5	1.5	No
Twin 5	8.2	6.8	Yes
Twin 6	3.2	3.1	Yes
Mean	4.0	3.6	
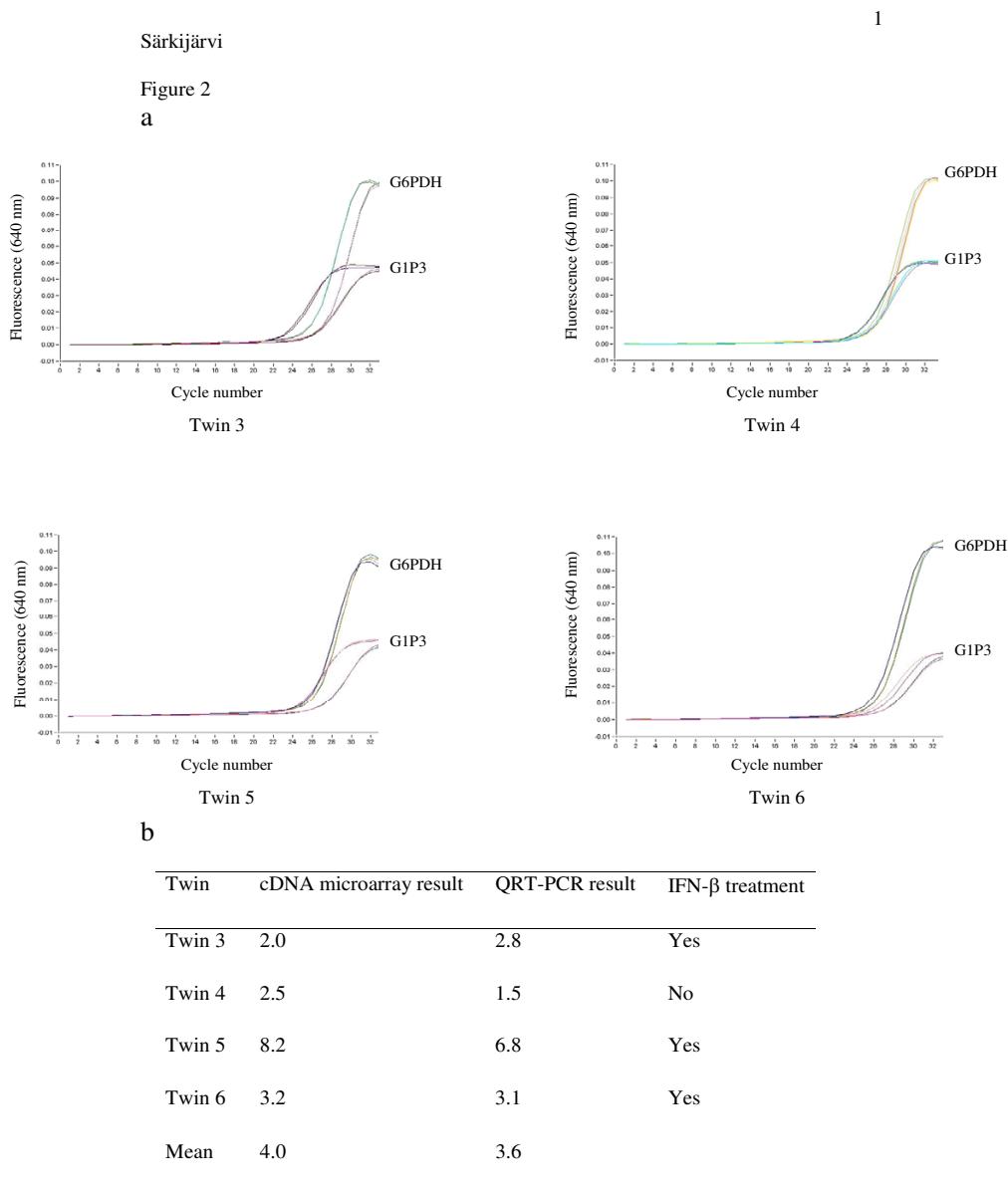
Figure 2
G1P3 gene analyzed by QRT-PCR. Glucose-6-phosphate dehydrogenase (G6PDH) was used as a reference gene in relative quantification. The expression of G1P3 was on average 3.6 times higher in MS twins compared to their healthy siblings, and the results concurred with those obtained from cDNA microarray. Panel **a** shows the real-time QRT-PCR amplifications duplicate in each twin pair (3 – 6) and the table (panel **b**) shows the comparison between the cDNA microarray results and the QRT-PCR results. The y axis indicates fluorescence intensity and x axis PCR cycle numbers. G1P3 gene amplification begins earlier in MS patients than in their healthy siblings, indicating higher gene expression in MS twins.

The MX2 gene was also up-regulated in twins with MS. Mx proteins are induced specifically by IFN-α and -β [34,35] and have antiviral activities [36]. The antiviral potential or other functions of the MxB protein encoded by MX2 gene are not fully understood, while human MxA protein has a wide antiviral spectrum [37] and relatively high levels of its mRNA have been detected after treatment with IFN-β [38]. Since up-regulated MX2 gene was observed in MS twins treated with IFN-β, its up-regulation may be explained by this treatment or alternatively by unknown virus infection.

The up-regulated LAPTM5 gene detected in twins with MS is conserved across evolution but it encodes protein which has no homology to any of the other lysosomal proteins [39].[37] LAPTM5 is also known as Clast6 and has been found to be highly expressed in progenitor and precursor B cells [40]. The protein may function during B cell activation or it could have a role in the antigen processing in lysosomes [40], which may be of relevance in MS.

Up-regulated HBA2 and HBB genes detected in MZ MS twins translate proteins that are part of a hemoglobin molecule. Heme units contain iron, which is involved in myelin production by oligodendrocytes and participates in the initiation of oxidative stress-induced injury in the CNS [41]. It is noteworthy that this process plays a role also in the pathogenesis of MS [41].

Conclusion
Taken together, in this study comparison of gene expression profiles in MZ MS twins to the corresponding profiles of their healthy siblings showed over two-fold up-regulation of six genes in almost half of twins with MS. This observation is of importance taking into account the restricted availability of MZ twins discordant for MS. However, given the sample size, clinical variation among subjects and limitations of the cross-sectional design, our results should be regarded as descriptive and hypothesis generating. To confirm the data MZ pairs discordant for MS need to be studied in other populations. Currently we are in a process of confirming our data with higher number of patients with MS.

Abbreviations
CCR5 = chemokine receptor 5

CNS = central nervous system

CTLA4 = cytotoxic T-lymphocyte-associated 4 gene

DZ = dizygotic

EDSS = Expanded Disability Status Scale

ESR1 = estrogen receptor 1

G1P3 = Interferon-induced protein

HBA2 = hemoglobin alpha 2

HBB = hemoglobin beta

ICAM-1 = intercellular adhesion molecule-1

IL-1RA = interleukin-1 receptor antagonist

LAPTM5 = Lysosomal-associated multispanning membrane protein-5

MBP = myelin basic protein

MRI = magnetic resonance imaging

MS = multiple sclerosis

MV = measles virus

MX2 = Myxovirus (influenza) resistance 2

MZ = monozygotic

PAI-1 = plasminogen activator inhibitor 1

POU3F1 = POU domain, class 3, transcription factor 1

QRT-PCR = quantitative reverse transcription polymerase chain reaction

Competing interests
The author(s) declare that they have no competing interests.

Authors' contributions
SS carried out the experimental work and helped drafting of the manuscript. HK carried out the neurological examination of the patients and participated in drafting of the manuscript. RP, MK and NA participated in the experimental work. TL, JK and MK helped to plan the study and to draft the manuscript. IE was the responsible investigator of the study and participated in planning of the study and drafting of the manuscript.

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