The role of past sun exposure in Multiple Sclerosis

by

Ingrid A.F. van der Mei

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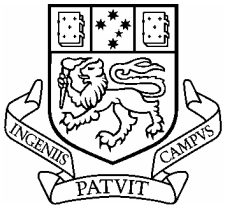

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ABSTRACT

This epidemiological thesis firstly reviews the disease Multiple Sclerosis (MS): its history, pathology, clinical expression, and the current views on immunopathogenesis, aetiology and treatment. A separate review on ultraviolet radiation (UVR) and MS indicates that recent work in photoimmunology provides evidence that UVR can attenuate T helper 1 cell mediated processes through several mechanisms, and that epidemiological features of MS, such as the striking latitudinal gradient and seasonal variation in month of birth, MS onset and disease activity, are at least in part consistent with the hypothesis that UVR exposure may reduce the risk of MS. An ecological analysis was conducted as part of the PhD to demonstrate that regional variation in MS prevalence in the continent of Australia could be closely predicted by regional UVR levels, but analytical epidemiological studies are required to further investigate the UVR hypothesis.

The project central to this thesis was a population based case-control study on Multiple Sclerosis, conducted in Tasmania, Australia. It examined: (i) whether high past sun exposure was associated with a reduced risk of MS, (ii) whether sibship structure and past infections influenced the risk of MS and (iii) whether having had children and differences in prevalence and strength of MS risk factors between men and women could explain the sex difference in MS. Interviews were conducted with 136 cases with MS and 272 controls randomly drawn from the community and matched on sex and birth year. In one of the methodology chapters, a measure-retest and method comparison was conducted to examine aspects of reliability of the sun exposure measures used in the case-control study. A separate study on 104 healthy volunteers was carried out to examine the effect of seasonal variation and body hair on melanin density estimates based on skin reflectance.

The case-control study showed that higher past sun exposure, particularly during childhood and early adolescence, was associated with a reduced risk of MS, which is compatible with UVR having a protective role against MS. Having younger siblings, but not older siblings, was also associated with a reduced risk of MS, while having had glandular fever or having high antibody titers against the Epstein-Barr virus was associated with an increased risk of MS. Among women, a negative association was found between having had children and MS.

The finding of an inverse association between sun exposure during childhood and early adolescence and MS, if confirmed in future work, will have important public health implications.

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In 1998, I joined the Menzies Centre for Population Health Research on a one year Australian-European Program Award to conduct epidemiological research and to bring back my newly acquired knowledge to the Netherlands. Rather than returning to the Netherlands, Professor Dwyer (director of the Menzies Centre) gave me the opportunity to conduct research on Multiple Sclerosis. There are a number of people I would like to acknowledge for their contributions they have made to the research I have conducted at the Menzies Centre.

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TABLE OF CONTENTS

PUBLICATIONS AND PRESENTATIONS AT SCIENTIFIC MEETINGS

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van der Mei IAF, Ponsonby A-L, Blizzard L, Dwyer T. Regional variation in Multiple Sclerosis prevalence in Australia and its association with ambient Ultraviolet radiation. Scientific meeting on 'Progress in Multiple Sclerosis Research', Melbourne, November 2000 (Oral presentation)

HONOURS RECEIVED IN THE COURSE OF THIS WORK

Travel bursary for Australasian Epidemiological Association Annual Meeting, Perth, September 2003 for oral presentation: Past sun exposure, skin phenotype and risk of multiple sclerosis: a case-control study.

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ABBREVIATIONS

AF	Attributable fraction
CGRP	calcitonin gene related peptide
CI	confidence interval
CMV	cytomegalovirus
CNS	central nervous system
CSF	cerebrospinal fluid
DSS	Disability Status Scale
EAE	experimental allergic encephalomyelitis
EA-R	early antigen complex (restricted), viral protein of Epstein-Barr virus
EA-D	early antigen complex (diffuse), viral protein of Epstein-Barr virus
EBNA	Epstein-Barr nuclear antigen, viral protein of Epstein-Barr virus
EBV	Epstein-Barr virus
EDSS	Expanded Disability Status Scale
HHV	human herpes virus
HLA	human leukocyte antigen
HSV	herpes simplex virus
ICC	intraclass correlation coefficient
IDDM	type 1 diabetes mellitus
Ig	immunoglobulin
IL	interleukin
IM	infectious mononucleosis
INF	interferon
κ	kappa statistic
MBP	myelin basic protein
MED	mean erythemal dose
MHC	major histocompatibility complex
MOG	myelin oligodendrocyte glycoprotein
MRI	magnetic resonance imaging
MS	Multiple Sclerosis
ON	optic neuritis
OR	odds ratio
PLP	proteolipid protein
RR	rate ratio
SD	standard deviation
SE	standard error
Th	T helper
TNF	tumour necrosis factor
TGF	transforming growth factor
UVR	ultraviolet radiation
VCA	viral capsid antigen, viral protein of Epstein-Barr virus
VZV	varicella zoster virus

Chapter 1

Introduction

1.1 THE CURRENT STATE OF MULTIPLE SCLEROSIS RESEARCH

Multiple Sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system affecting over 15,000 Australians. Although essential features of the disease pathology have been known for many years, recent achievements in basic neurobiology and immunology have led to an increased understanding of the mechanisms responsible for the pathology.[1] A large amount of descriptive data has demonstrated that there is considerable regional variation in MS prevalence and that there has been an increase in MS prevalence over time, which is not solely due to increased case-ascertainment. Both genetic and environmental factors seem to interact to cause the disease. Case-control studies and other epidemiological studies have identified some risk factors for MS. A region on the genome, the human leukocyte antigen (HLA) complex, and an environmental exposure, the Epstein-Barr virus, have consistently been found to be positively associated with MS. Recent positive developments in areas such as diagnostic precision (e.g. the use of magnetic resonance imaging), genetic information, computer technology and improved measures for epidemiological research (e.g. the use of biomarkers) should assist analytical studies that aim to identify risk factors for MS.

1.2 TASMANIA AS A LOCATION TO CONDUCT EPIDEMIOLOGICAL RESEARCH ON MS

The most recent Australian prevalence survey, conducted in 1981, showed a striking latitudinal gradient of MS with the highest prevalence recorded in Hobart, the capital of the southern-most state of Australia, Tasmania. The age-standardised prevalence of 75.6 per 100,000 in Hobart was more than six times higher than the age-standardised prevalence of 11.8 per 100,000 in tropical Queensland.[2]

Tasmania comprises about 0.9% of the total area of Australia and lies between 40°38'S and 43°39'S, placing it on similar latitudes to the northern part of the south island of New Zealand. The mean maximum temperature of Hobart is 21.5°C in January and 11.6°C in July and the mean effective ultraviolet radiation level is relatively high in summer (20.7 mean erythemal doses (MEDs) in January), but low in winter (1.7 MEDs in July).[3] Tasmania has approximately 2.5% of the Australian population.[4] At the 2000 census, Tasmania had a population of 470,336 persons of whom 75% were located in urban centres of 1000 or more persons.[4] The Tasmanian population is relatively stable. In 1978, the population was 413,538, and between 1978 and 2000, the rate of population growth was less than 1% per year.[4, 5] From an ethnic point of view, Tasmania is relatively homogenous, with more than 90% of Tasmanians being able to trace their ancestry back to the British Isles. Due to the isolated geographical position of Tasmania and limited migration in the twentieth century, 60-65% of the current population descend from the original 10,000 settling families, indicating that a large founder effect exists.[6]

Tasmania has become increasingly recognised as an excellent location to conduct epidemiological and genetic research because of its compact physical size, good medical infrastructure, relatively stable population, high cooperation of the Tasmanian population, large genetic founder effect, and relatively high genealogical knowledge of the population.

These features together with the relatively high prevalence of MS makes Tasmania suitable to conduct research examining environmental and genetic risk factors for MS.

1.3 THE PROJECT CENTRAL TO THIS THESIS

From 1999 to 2001, we conducted a population-based epidemiological case-control study to examine whether environmental factors might influence the risk of MS. This "Tasmanian MS case-control study" was part of a larger program "The Tasmanian MS Research Program" which started in 1998 and included a genetic project that aimed to identify MS susceptibility genes in the Tasmanian population. The program was a collaboration between the Australian National Register of MS families (Canberra), The Walter and Eliza Hall Institute (Melbourne), Royal Melbourne Hospital, the Murdoch Institute (Melbourne), Tasmanian neurologists and the Menzies Centre for Population Health Research. The Tasmanian MS case-control study was largely funded by the National Health and Medical Research Council, the federal government funding body for medical research. The set-up phase was funded by MS Australia and the Australian Rotary Health Research Fund. The genetic study was funded by the National MS Society of the United States and the Cooperative Research Centre for Discovery of Genes for Common Human Diseases (Australia).

1.4 MAIN OBJECTIVES OF THE PROJECT CENTRAL TO THIS THESIS

At the commencement of the study in 1999, the main objectives of the Tasmanian MS case-control study were:

1. to examine if past high ultraviolet radiation exposure (determined by a biological measure of photoageing, previously validated questionnaire measures and a calendar) during childhood and adolescence is associated with a reduced risk of MS after controlling for confounding factors such as childhood infections and melanin density of the skin.

2 A) to examine the relationship between past infections and MS by testing the hypothesis that major infection, particularly during childhood and adolescence, is independently associated with increased risk of MS using subject recall and serological evidence of past infection.

B) to perform a detailed assessment of the influence of sibship structure (birth order, sibling number, number of younger and older siblings, and intersibling interval) on MS risk as an indirect measure of the timing of infection load.

3. to examine the association between other environmental exposures (parity, immunisations, diet, exposure to chemicals, animal exposure, concussion, tobacco smoke) and MS after adjustment for confounding factors.

1.5 OUTLINE OF THE THESIS

This thesis focuses in particular on the "UVR hypothesis", the first objective of the project as outlined above. Different methodological and statistical techniques have been applied in this thesis. The principal statistical components are: a case-control analysis, an ecological analysis and an examination of different aspects of reliability of important measures used in the case-control study.

In chapter 2, an overview is given on the history of MS, clinical aspects, current insights on the immunopathogenesis, epidemiological features and current treatments. In chapter 3, recent immunological findings and epidemiological features are discussed which are consistent or not consistent with the primary hypothesis of this thesis that past UVR exposure is associated with a reduced risk of MS. In Chapter 4, an ecological analysis is conducted which examines to what extent regional UVR levels might explain the regional variation of MS prevalence in Australia. Both chapter 3 and 4 provide evidence that analytical epidemiological studies are warranted to examine the UVR hypothesis. Chapter 5 outlines the methods of the analytical case-control study that was conducted to examine this hypothesis. Chapter 6 and 7 are methodological chapters that assess aspects of reliability of past sun exposure, skin type and other measures used in this thesis. Chapter 8 shows the results of the UVR hypothesis. Chapter 9 shows the results of the associations between sibship structure, past infections and MS, the second objective of the Tasmanian MS case-control study. Chapter 10 discusses the female excess observed in MS by examining the female to male ratio by age, the relationship between puberty development and MS, the relationship between having children among women and MS onset and sex differences in prevalence and strength of particular risk factors of MS. The final chapter will discuss the main conclusions of this thesis, future research that might assist in the causal inference of the finding regarding UVR and MS and public health implications that this finding might have in the future.

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Chapter 2

A review of Multiple Sclerosis: History, diagnosis, clinical expression, pathogenesis, aetiology and treatment.

2.1 PREFACE

This chapter gives an overview of the disease Multiple Sclerosis (MS): its origin, pathology, clinical expression, and the current views on immunopathogenesis, aetiology and treatment. It provides information relevant for the understanding of the thesis.

2.2 INTRODUCTION

Multiple Sclerosis (MS) is a chronic demyelinating disease of the central nervous system (CNS). The CNS consists of a large, complex brain and an elongated spinal cord. MS is characterised by focal areas of destruction of the myelin sheath in the brain and spinal cord. A myelin sheath surrounds axons and permits rapid conduction of nerve impulses. MS is the most common neurological disease of young adult Caucasians of North European ancestry (prevalence United Kingdom 74-193 per 100,000 population),[1] with about 75% having a disease onset prior to the age of 50 years.[2, 3] The disease is more common in females than in males, usually in a ratio of about two to one.[4] The mortality rate due to MS is low (1-4% per year), leading to a large burden of chronic disease morbidity.

2.3 HISTORY

Pathological descriptions of MS cases appeared for the first time around 1838.[5] Carswel (1938) and Cruvelhier (1941) both provided pathological anatomical illustrations of the human body, which were later interpreted as representing the macroscopical appearances of the plaques seen in MS.[5] J-M Charcot is seen as the first to recognise that MS was a distinct entity. His publications in the late 1860s emphasised the frequency of the disease and included clinico-pathological correlations and speculations on the pathophysiology.[6] The following excerpt from a diary, written between 1822 and 1846 by Augustus d'Este, an illegitimate grandson of King George III, was published by Douglas Frith in 1940.[7] It seems to represent the first recorded case fulfilling the diagnostic criteria for clinically definite MS.

> In the month of December 1822 I travelled from Ramsgate to the Highlands of Scotland for the purpose of passing some days with a relation for whom I had the affection of a son. On my arrival I found him dead. – I attended his funeral: – Shortly after the funeral I was obliged to have my letters read to me, and their answers written for me, as my eyes were so attacked that when fixed upon *minute objects indistinctness of vision was the consequence: …Soon after I* went to Ireland, and without anything having been done to my eyes, they completely recovered their strength and distinctness of vision. – Now (December 1827) a new disease began to show itself: every day I found gradually (by slow degrees) my strength leaving me: I could clearly *perceive each succeeding day that I went up and down the staircase with greater difficult…..I remained in this extreme state of weakness for about 21 days…* (then) my strength gradually returned. I never was able to run so fast as formerly, nor could I venture to dance.

Charcot referred to the disease as la sclerose en plaques disseminées, la sclerose multiloculaire or la sclerose generalisée.[6] These names were translated in the New Sydenham Society edition of his lectures as disseminated (cerebrospinal) sclerosis. Many name variations were used (insular sclerosis, lobular and diffuse sclerosis, multiple Sklerose, multiple inselformige Sklerose, multiple Sklerose des Nervensystems) and it was not until the 1950s that consistency of nomenclature was achieved.[5] The process of renaming the disease gathered momentum with the formation of patient support societies and the publication of the monograph Multiple Sclerosis written by Douglas McAlpine, Nigel Compston and Charles Lumsden.

The work of the 19th century investigators focussed on pathological observations within the brain and a description in the variations in clinical presentation. A review from 1849 to early 1900s,[5] showed that many pathological and clinical features currently well recognised, were already described in those early days. In 1849 for example, autopsies revealed plaques, seen as locations with abnormal firmness or leathery consistency, were observed in irregularly circumscribed parts of the white matter, but rarely in the grey matter of the brain. In the 1860s, plaques were found to be orientated around a central blood vessel, which was interpreted by Rindfleisch as evidence for a chronic inflammatory process. In addition, the loss of myelin sheaths in lesions was described as the associated increased density of connective tissue in the form of scar tissue. In the early 1900s, Marburg showed that the disease process was not confined to the brain and spinal cord, but that demyelination also existed in the peripheral nervous system in some cases. Around 1900, MS was seen as a primary inflammatory demyelinating disease possibly mediated by a soluble myelinotoxic factor and with relative sparing of axons. Clinically, Charcot played a significant role in correlating clinical symptoms, such as amblyopia, nystagmus, dysarthria and ataxia with the anatomical locations in the brain involved (cerebrum, cerebellum, brainstem). He also outlined the cognitive manifestations of the disease, while Marie, one of Charcot's pupils, reported disordered bladder, bowel and sexual functions in some cases. Importantly, Marie also recognised the variable symptoms at onset and the subsequent clinical course, which could be progressive from onset or progressive at a later stage of disease. In a group that was progressive from onset, he noted a later age of onset, a worse prognosis, a relative absence of histological involvement of the cerebrum and a more frequent axon degeneration.

The work of the 19th century investigators highlighted the need for an epidemiological approach to the disease. The period 1900-1950 saw a gradual evolution of the conduct and statistics of population-based studies. The initial studies provided snapshots in frequency and indicated the need for improved methods of case ascertainment. In 1921, Davenport[8] noted that MS was more common among drafted men in the northern states of the United States compared to the southern states. He also pointed out that a large component of variation between studies could be related to selection of the denominator rather than variations in numerator, arguing for carefully designed regional studies.[8]

Although the quality of the studies increased, a material increase in information on the epidemiology of MS did not occur until the 1950s. A milestone was achieved in 1950 with a publication from the Association for Research in Nervous and Mental Diseases showing that mortality rates were greater in temperate zones than in the tropics or sub-tropics and figures were higher in northern parts of the United States and Italy than in southern regions.[9] A more extensive survey in 31 countries between 1951 and 1958 showed similar regional variations although overall mortality had decreased, reflecting the impact of improved health care following the introduction of antibiotics and other treatments of complications of MS.[10] Scientists were not only interested in case numbers, but aetiological hypotheses relating to the impact of environmental factors such as domicile, climate and soil conditions were also

explored.[11-14] Latitude seemed more important than altitude[12] and both lower temperature[12, 13] and diminished solar radiation[14] correlated with the MS distribution. The inverse association between solar radiation and MS prevalence even persisted after adjustment for latitude.[14]

2.4 PATHOLOGY

Individual nerve cells (neurons) contain a cell body, dendrites and an axon (a structure specialised to conduct electric al signals). Communication between nerve cells usually occurs from the terminal of the transmitting neuron via a synapse. Synaptic junctions are often located between an axon and a dendrite, but can also be located between an axon and a nerve cell body, between two axons, and between two dendrites. Impulse transmission at most synaptic sites involves the release of a chemical transmitter substance. Many axons are covered by multiple concentric layers of myelin, a lipid-rich insulating material produced by Schwann cells in the peripheral nervous system and by oligodendrocytes (specialised glial cells that produce myelin in the brain, optic nerves and spinal cord) in the central nervous system. The myelin sheath permits rapid conductions of nerve impulses. The smallest axons are unmyelinated and are not very prominent in histologic preparations, even though they are far more numerous than the larger, myelinated axons.

MS is currently described as a chronic inflammatory demyelinating disease of the CNS. The disease process is characterised by focal areas of destruction of the myelin sheath in the brain and spinal cord. These areas of destruction are known as plaques or lesions. By gross inspection of the unfixed brain, inactive plaques appear as grey discoloured areas with firm tissue texture.[15] Microscopically, myelin sheaths are completely lost in inactive plaques, axons are spared and embedded in dense astroglial scar tissue.[15] Active plaques, in contrast, macroscopically have a pink discolouration and their tissue texture is soft, while microscopically they reveal demyelination with little astroglial scar formation and the lesions are infiltrated by numerous inflammatory cells (such as macrophages) that contain myelin and tissue debris in their cytoplasm.[15] All lesions appear to result from a selective and localised attack on the myelin of the nerves, or on the cells responsible for making myelin, the oligodendrocytes.[16]

Chronic persistent inflammation in the CNS is another characteristic pathological feature. This inflammation is not restricted to the areas of demyelination, but also affects large parts of the so-called normal white and grey matter. However, the density of inflammatory infiltrates is in general higher within demyelinated plaques compared to the surrounding white matter.[17]

Although the essential features of MS lesions have been known for many years, understanding of the mechanisms has lead to the notion that MS is a much more complex disease than originally thought.[18] For example, different mechanisms of demyelination operate in different subgroups of MS patients and different patterns of oligodendrocyte pathology can be found in MS lesions.[18, 19] However, early symptoms are widely believed to result from axonal demyelination,[18] while the regression of symptoms has been attributed to the resolution of the inflammatory response and to partial remyelination.[16] Repeated episodes of disease activity may result in, for example, irreversible axonal injury and exhaustion of the oligodendrocyte progenitor pool which will lead to progressive loss of neurologic function.[16]

2.5 DIAGNOSTIC CRITERIA

With the increase of epidemiological studies in the 1950s, the need for standard diagnostic criteria became critical. Allison and Millar[20] (1954) classified cases as early (few physical signs

but a recent history of remitting symptoms), probable (soon changed to early probable or latent: no reasonable doubt about the diagnosis), possible (findings suggesting the diagnosis and no other cause found but the history static or progressive and with insufficient evidence for scattered lesions) and the unusual term of discarded disseminated sclerosis. Until the mid-1980s, most surveys of MS used the Allison and Millar criteria with some modifications within categories, including the introduction of the term (clinical) definite in 1965.[21] Schumacher et al.[22] (1965) categorised definite cases as those showing objective evidence for disease affecting two or more white matter parts of the CNS, occurring in episodes separated by more than 24 hours or with progression over six months, in a person aged between 10 and 50 years at onset and in whom a competent observer can find no better explanation. Further modifications adapted by Rose et al.[23] (1976) were definitions for probable MS (two episodes but signs at a single site or a single episode with signs of widespread disease) and possible disease (two episodes with no or few signs). The McDonald and Halliday criteria[24] (1977) added a definition for proven MS (evidence from autopsy or biopsy), refined the early probable or latent cases (two episodes and a single affected site or a single episode and two affected sites), and dealt with the difficult issues of progressive probable (progressive history with multiple sites affected), progressive possible disease (progressive history affecting a single site), and suspected MS (one episode at a single site unless the optic nerves are affected).

More recently, the Poser committee criteria[25] (1983) incorporated information available from laboratory investigations within the categories of clinically definite and probable MS. The Poser criteria have gained widespread acceptance. Changing from the Allison and Millar classification to the Poser criteria does not materially affect estimates for the total number of identified cases but differences do arise when surveys are restricted to the categories of definite and probable (Poser), and probable and early (Allison and Millar) cases, since the proportion in the suspected and possible categories needing exclusion differs significantly between the two classifications.[5]

The Poser criteria[25] have been the basis for the research conducted for this thesis and will be outlined in more detail. The criteria include laboratory-based evidence such as examination of cerebrospinal fluid (CSF) obtained at lumbar puncture and the so-called paraclinical evidence such as evoked potentials. The examination of the CSF for oligoclonal immunoglobulin (Ig) G can provide evidence of the immune and inflammatory nature of lesions(s), while the presence of delayed evoked potentials can contribute to determine whether there is a pathological process of demyelination present. An attack is defined as a symptom or symptoms of neurological dysfunction with or without objective confirmation, lasting more than 24 hours, and separate lesions are signs or symptoms that are involved in different parts of the CNS.

The Poser criteria classify MS into two major groups, (1) definite and (2) probable MS, with each having two subgroups, (a) clinical and (b) laboratory supported. (1a) Clinically definite MS: the patient should have had two attacks with clinical evidence of two separate lesions, or, the patient should have had two attacks with clinical evidence of one lesion and paraclinical (e.g. visual evoked potentials) evidence of another separate lesion. The two attacks should involve different parts of the CNS and must be separated by a period of at least one month. (1b) Laboratory-supported definite MS: the laboratory support is required from CSF, where an increase has to be demonstrated in CNS synthesis of IgG or where IgG oligoclonal bands have to be demonstrated. There are three situations for this type of MS: (i) two attacks, either clinical or paraclinical evidence of one lesion and laboratory support in CSF; (ii) one attack, clinical evidence of two separate lesions and laboratory support in CSF; and (iii) one attack, clinical evidence of one lesion, paraclinical evidence of another separate lesion and laboratory support in CSF. (2a) Clinically probable MS: (i) two attacks and clinical evidence of one lesion; (ii) one attack and clinical evidence of two separate lesions; and (iii) one attack, clinical

evidence of one lesion and paraclinical evidence of another, separate lesion. (2b) Laboratory probably MS is used in the situation where there were two attacks and laboratory support in CSF.

Since 1981, magnetic resonance imaging (MRI) of the brain has developed as one of the more sensitive diagnostic tools. MRI scans show MS lesions which have been demonstrated to correspond with demyelinating plaques at autopsy.[26] Abnormalities on MRI scans are observed in about 95% of patients with clinically definite MS.[27] A detailed description of this technique is beyond this chapter, but magnetic resonance images depend on the relative amounts and physico-chemical environment of water protons in each area of the brain and the signal depends on a complex set of (tissue) parameters including hydrogen density and two separate relaxation times (T_1 and T_2) which characterise a tissue by the way the nuclei relax. These relaxation times can be measured and provide T_1-weighted and T_2-weighted images. In addition, administration of intravenous gadolinium (gadopentetate dimeglumine) five to ten minutes prior to the scan increases the signal intensity on T_1-weighted magnetic resonance images. Gadolinium enhanced lesions seem to depict the early inflammatory phase of MS lesions, while non-enhanced lesions seem to represent older and inactive lesions. Gadolinium enhancement has been shown to increase the specificity of the diagnosis of MS compared to the use of T_2-weighted lesions.[28]

Since comparable MRI abnormalities may be found in a variety of other diseases and health volunteers, criteria have been developed by which MRI scans can be classified as suggestive of MS or not. Paty et al.[27] developed the following criteria for the use of MRI scans in 1988, which have been used as additional diagnostic criteria for our research work. According to those criteria,[27] an MRI scan: (1) is strongly suggestive of MS if it presents with four lesions, or with three lesions of which one is in a periventricular location; (2) is suggestive of MS if it presents with three lesions or two lesions of which one periventricular; (3) shows possible MS if two lesions are present or one lesion in a periventricular location; (4) falls in category four (unnamed category) if it shows one lesion not in a periventricular location; and (5) does not show MS if the MRI is normal or shows another diagnosis. Lesions are considered as typical of MS if they are of high intensity on T_2-weighted images, measured greater than 3 mm in diameter, and were located predominantly in the white matter.[27]

New MRI criteria have been proposed by Barkhof et al.[29] in 1997, which rely on T_2-weighted imaging as well as gadolinium enhanced imaging. The model includes four dichotomised criteria: the presence of (a) one gadolinium enhanced lesion, (b) one juxtacortical lesion, (c) three periventricular lesions, and (d) one infratentorial lesion. This final model does not include the total number of lesions, but rather shows the importance of the type and location of the lesions. Barkhof et al.[29] compared their criteria with the criteria from Paty et al.[27] and Fazekas et al.[30] MRI scans were taken from patients who were diagnosed with clinically isolated neurological symptoms suggestive of MS. The gold standard was the diagnosis of clinically definite MS, allowing four years to convert from suspected MS to clinically definite MS using the Poser criteria. They calculated the sensitivity (percentage of people with definite MS where the test correctly identifies them as having an abnormal MRI), specificity (percentage of people without a final diagnosis of definite MS where the test correctly identifies them as having a normal MRI), accuracy (percentage of people where the test correctly identifies them as having an abnormal or normal MRI), positive predictive value (percentage of people with an abnormal MRI that indeed had definite MS), and negative predictive value (percentage people with a normal MRI that indeed did not have MS). The criteria proposed by Paty et al. and by Fazekas et al. showed identical results: sensitivity, 88%; specificity, 54%; accuracy, 69%; positive predictive value, 60%; and negative predictive value, 85%. The criteria proposed by Barkhof et al. showed the following: sensitivity, 82%; specificity, 78%; accuracy, 80%; positive

predictive value, 75%; and negative predictive value, 84%. This shows that the four dichotomised MRI parameters proposed by Barkhof et al. are more specific and accurate than the criteria proposed by Paty et al. or Fazekas et al. for predicting conversion to clinically definite MS. Tintore et al.[31] conducted a similar study and reached the same conclusion.

New diagnostic criteria have been published by the International Panel on the Diagnosis of MS in 2001.[32] The focus has remained on the objective demonstration of dissemination of lesions in both time and place. The MRI criteria from Barkhof et al.[29] have been integrated with clinical and other paraclinical diagnostic methods (CSF and visual evoked potentials). The revised criteria facilitate the diagnosis of MS in patients with a variety of presentations, including "monosymptomatic" diseases suggestive of MS, disease with a typical relapsing-remitting course, and disease with insidious progression, without clear attacks and remissions. Previously used terms such as "clinically definite" and "probable MS" are no longer recommended. The outcome of a diagnostic evaluation is either "MS", "possible MS" (for those at risk for MS, but for whom diagnostic evaluation is equivocal), or "not MS".

2.6 CLINICAL FEATURES

The symptoms and signs experienced by people with MS are variable but in general reflect the involvement of those parts of the CNS that are most heavily demyelinated. The prevalence of the symptoms reported in different studies varies greatly, caused by the different types of samples (hospital, community, armed forces, autopsies), the diagnostic criteria used in each study and the time and method of assessment of the symptoms (retrospective recall, records).

Table 1. Frequency of symptoms in 301 prevalent patients in South Glamorgan who were interviewed (%).

Symptom	Ever?	At onset?	At prevalence?
Weakness	268 (89)	66 (22)	241 (80)
Sensory symptoms	263 (87)	103 (34)	219 (73)
Ataxia	248 (82)	32 (11)	218 (72)
Bladder symptoms	213 (71)	3 (1.0)	188 (62)
Fatigue	171 (57)	5 (2)	144 (48)
Cramps	156 (52)	2 (0.6)	133 (44)
Diplopia (double vision)	155 (51)	25 (8)	77 (26)
Visual symptoms	148 (49)	38 (13)	98 (33)
Bowel symptoms	126 (44)	0 (0)	112 (37)
Dysarthria	110 (37)	2 (0.6)	74 (25)
Vertigo	107 (36)	13 (4.3)	57 (19)
Facial pain	106 (35)	5 (2)	42 (14)
Poor memory	96 (32)	1 (0.3)	81 (27)
Headache	90 (30)	6 (2)	51 (17)
Mental symptoms	68 (23)	1 (0.3)	49 (16)
Deafness	51 (17)	2 (0.6)	38 (13)
Facial weakness	48 (16)	4 (1)	15 (5)
Dysphagia	40 (13)	1 (0.3)	29 (10)

Data from Matthews (1998)

Table 1 provides an overview of the prevalence of symptoms at onset and during the course of the disease of a large population-based series of cases from southeast Wales in the United Kingdom.[33] The most common initial symptoms recorded include weakness in one or more

limbs, sensory disturbances, impaired vision due to optic neuritis, ataxia (loss of coordinated muscular contractions required for the production of smooth movements), diplopia (double vision) and vertigo (illusory feeling of giddiness with disorientation in space).[33] Common additional symptoms that develop during the course of the disease were bladder symptoms, fatigue, cramps, bowel symptoms, dysarthria (slurred speech), facial pain, poor memory and headache. It has been recognised that during the course of the disease some MS patients experience considerable cognitive impairment, difficulties with memory, concentration and other mental skills.[34]

MS is generally categorised as being either relapsing-remitting or primary progressive in onset. The relapsing-remitting form is characterised by a series of attacks that result in varying degrees of disability from which patients recover partly or completely. It is usually followed by a remission period of variable duration before the next exacerbation. At onset, about 85% have this type of MS, while about 15% have primary progressive MS. The progressive form of the disease lacks the acute relapses and instead typically involves a gradual clinical decline. During the course of the disease, about one-third of the relapsing-remitting patients enter a progressive form of MS known as secondary-progressive MS.

2.7 CLINICAL FACTORS INFLUENCING PROGNOSIS

In the past, the most widely used measures of progression have been the Disability Status Scale (DSS) and the Expanded Disability Status Scale (EDSS).[35] The DSS scale discriminates the level of neurological impairment of people with MS in 10 grades beyond normal (grade 0) with grade 10 being death due to MS. The DSS scale was adapted in 1983 into the EDSS scale and includes half step increments. Although there has been criticism on the scales, mainly because of its lack of sensitivity and the influence of the walking ability of the patient in determining the score at the higher end of the scale, it is still widely used as a clinical measure of progression. As described above, MRI measures are currently also available as alternative measures of progression. A new inexpensive and precise method that seems promising as a predictive method in patients with a first demyelinating event to conversion to clinically definite MS is the presence of serum antibodies against myelin oligodendrocyte glycoprotein (MOG) and myelin basic protein (MBP).[36] People with those antibodies had more often relapses (95% of people with antibodies against both MOG and MBP, 23% in antibody-negative people) and the mean time to relapse was shorter (7.5 (SD 4.4) months for people with antibodies against both MOG and MBP, 45.1 (SD 13.7) months for antibody-negative people).[36]

Natural history studies prior to the use of current immunomodulatory treatment showed that, patients deteriorated to an EDSS score of 6 (requires unilateral assistance with ambulation) at a median of 15 years, following diagnosis.[37] Furthermore, at 15 years following diagnosis, about 10-15% required the use of a wheelchair, while 20-25% remained unrestricted in their ambulation (EDSS $\leq$ 3), demonstrating the large amount of variation between patients.[38] Although it might be difficult to estimate prognosis in individual patients at the time of diagnosis, there are some clinical features at onset and after the first two to five years that influence long-term progression.

Weinschenker et al.[39] followed 1099 MS patients referred to the MS Clinic at the University Hospital in London, Canada, between 1972 and 1984. The time to reach DSS level 6 was used as the endpoint variable. Multivariate analysis showed that at onset, male sex, an older age at onset, and cerebellar involvement or insidious onset of a motor deficit as first symptom, were all significantly associated with an adverse outcome ($p<0.005$). Factors ascertained later which were associated significantly with a worse outcome, even after controlling for those

previously mentioned, included persisting deficits in brainstem (p=0.01), cerebellar (p<0.001) or cerebral systems (p=0.01), a higher frequency of attacks in the first two years after onset of disease (p<0.001), a short first interattack interval (p<0.001) and higher disability (DSS) at two and five years from onset (p<0.001). A progressive course from onset was also associated with a worse outcome, but not independent of the insidious presentation of a motor deficit and pyramidal tract as first symptoms. Similar results were obtained in an Australian study[40] which compared patients with moderate or severe disability (DSS 4-9) with patients with mild disability (DSS 0-3) and found that worse prognosis was associated with older age of onset, progressive disease and onset symptoms that are multiple, pyramidal or cerebellar. The relationship between a worse prognosis and male sex was not independent of a progressive course of MS. In a study following 71 patients for a mean of 14.1 years (range 12.5-16.8) using serial MRI data showed that the EDSS score at 14 years correlated with the T_2 lesion volume at five years (r=0.60) and with the increase in T_2 lesion volume over the first five years (r=0.61), indicating a worse prognosis if the lesion volume in the first five years is high.[41]

2.8 IMMUNOPATHOGENESIS

The immune system plays a central role in MS. Before discussing the immunopathogenesis of MS, an overview of the immune system will be given, discussing the immunological aspects that are of importance for MS.

The term immunity refers to all mechanisms used by the body as protection against environmental agents that are foreign to the body. Immunity may be innate or acquired. Innate immunity is conferred by all those elements with which an individual is born and which are always present, including physiological and chemical barriers, cells that kill micro-organisms, inflammation, fever and biologically active substances that are harmful to micro-organisms. Acquired immunity is more specialised and is acquired by contact with the invader and is specific to that invader only. The initial contact with the foreign agent (immunisation) triggers a chain of events that leads to the activation of certain cells (including lymphocytes) and the synthesis of proteins, some of which exhibit specific reactivity against the foreign agents. By this process, the individual acquires the immunity to withstand and resist a subsequent attack by, or exposure to, the same offending agent.

There are three major cell types involved in acquired immunity. Two of these cell types come from a common lymphoid precursor cell but differentiate along different developmental lines. One line matures in the thymus and is referred to as T cell; the other matures in the bone marrow and is referred to as B cell. Cells of the B- and T-lymphocyte series differ in many functional aspects but share one of the important properties of the immune response, namely, they exhibit specificity towards an antigen (foreign material that is specifically bound by antibody or lymphocytes) or an epitope (a particular portion of the antigen). Antigen-presenting cells such as macrophages constitute the third cell type that participate in the acquired immune response. Although these cells do not have antigen-specific receptors, their function is to “process” the antigen and “present” it to the specific receptors on T lymphocytes. The antigen-presenting cells have on their surface two types of special molecules that function in antigen presentation. These molecules are called major histocompatibility complex (MHC) class I and MHC class II molecules in vertebrate species. Processed antigenic peptides compete for binding to the cleft of the MHC molecule. In humans, the gene region on chromosome 6 that encodes the MHC complex is called the human leukocyte antigen (HLA) region. In addition to activation by MHC-peptide binding, T cell receptors can be stimulated by superantigens. These are microbial products which bind to the outside of MHC molecules and the T cell receptor, rather than to its groove. Activation of T cells by microbial superantigens

may contribute to the pathogenic activation of potentially self-reactive T cell clones, which persist in the healthy immune repertoire in a dormant state.

There are two branches of acquired immunity – humoral and cell-mediated immunity – that interact with each other. Humoral immunity is mediated by serum antibodies, which are proteins (globulins) secreted by B cells in response to immunisation. All serum globulins with antibody activity are referred to as immunoglobulins (Ig). There are five major classes – IgG, IgM, IgA, IgE, IgD – each of which has several unique biological properties. Cell-mediated immunity is mediated by T cells. There are several subpopulations of T cells, each of which may have the same specificity for an antigenic determinant (i.e epitope), although each subpopulation may perform different functions. T helper (Th) cells cooperate with B cells to enhance the production of antibodies. They function by releasing cytokines (soluble substances secreted by cells, which have a variety of effects on other cells) that provide various activation signals for the B cells. T cytotoxic cells are able to kill their target cells, while T suppressor cells are able to suppress the immune response leading to a downward modulation or a shutoff in reactivity of other effector cells.

T cells carry a receptor composed either of α-β chains or γ-δ chains. α-β T cells generally dominate in the mature immune system and characterise the vast majority of T lymphocytes in blood, lymph nodes and spleen. These cells are mainly responsible for specific immune functions involved in the elimination of pathogens. The γ-δ T cells are mostly found in the lymphatic system of the gut and other mucosal tissues, and are involved in early defence against bacteria and other cellular pathogens.

T cells have surface molecules all of which have different functions. For example, the surface molecules CD4 and CD8 act as adhesion molecules that help to tighten the binding of T cells to antigen-presenting cells. Studies in mice indicate that the Th cells that cooperate with B cells, $CD4^+$ T cells, can be divided into subsets based on the different cytokines they produce. Activated Th_1 cells secrete for example large amounts of the cytokines interleukin(IL)-2, IL-12, tumour necrosis factor(TNF)-α, and interferon(IFN)-γ, while Th_2 cells produces IL-4, IL-5, IL-6 and IL-10. Cytokines produced by one subset can inhibit the function of the other subset, reinforcing the functional differentiation of Th_1 and Th_2 subsets. In humans, however, the cytokine profile is generally more diverse, and forms a continuous spectrum in which Th_1 and Th_2 cells may be only be two of the possible phenotypes.[42] It seems that naïve T cells have the option to differentiate either in the Th_1 or Th_2 direction and the cytokines present during initial and subsequent antigen presentation appear to be crucial factors dictating the prospective cytokine pattern of a responsive T cell. This initial cytokine profile might be influenced by factors including particular antigen-presenting cells, co-stimulatory factors and the nature of the peptides presented.

Under normal circumstances, the immune system reliably attacks foreign invaders and ignores self-structures. T cell clones with receptors recognising self-antigens such as myelin specific antigens (myelin reactive T cells) are eliminated in the thymus,[43] kept in a state of anergy or non-reactivity, or down regulated.[44] Thus, the existence of regulated self-reactive T cell clones is part of the healthy immune repertoire. If, however, these T cells escape, they can attack normal tissues and cause tissue damage and autoimmune disease.

MS is thought to be an example of an autoimmune disease where Th_1 lymphocytes play a major role. There are several lines of evidence for this. Firstly, MS has many pathological similarities with experimental allergic encephalomyelitis (EAE), the animal model of MS. EAE is an autoimmune disease that can be induced in a variety of animal species by injections of myelin antigens. The disease is characterised by focal demyelination and Th_1 cell infiltration.[45] Secondly, the active lesions in MS are almost indistinguishable from areas of inflammation

seen in EAE. Thirdly, in MS plaques the majority of infiltrating lymphocytes are T cells [46-48] and cytokines TNF-α and IFN-γ, often produced by Th_1 cells, have been observed in plaques of people with MS.[49-51]. Fourthly, there is currently good evidence that the genetic HLA class II region is associated with MS (see section genetic factors). Peptides bound to class II antigen-presenting cells are known to be presented to CD4+ T cells, while class I-embedded peptides, which are relatively short peptides, are presented to CD8+ T cells.

Hellings et al.[52] has provided a schematic overview of the current hypothesis on the immunopathogenesis of MS (Figure 1). The first hypothetical event is the activation of myelin reactive T cells in the blood in the periphery, outside the CNS. This initial activation could be triggered by "molecular mimicry", bacterial or viral superantigens or non-specific mechanisms. According to the hypothesis of molecular mimicry, some foreign agents (including infectious and dietary agents) are comprised of peptides that mimic autoantigenic epitopes.[53] Upon infection for example, presentation of those peptides in the periphery by infected antigen presenting cells may cross-activate autoreactive T cells. Bacterial or viral superantigens may also be able to activate autoimmune T cells, because they can activate T cells irrespective of their antigen specificity by crosslinking MHC class II molecules to a specific T cell receptor segment.[54] Lastly, non-specific mechanisms, such as the exposure to high local concentrations of cytokines, secreted as a result of immune inflammatory responses to foreign invaders, could activate autoimmune T cells.[55] As mentioned above, activation of myelin reactive T cells in the periphery is actually part of the normal T cell repertoire. They are found in similar frequencies in people with MS as well as in healthy controls.[56, 57] There is, however, evidence that the frequency of activated myelin reactive T cells is significantly higher in peripheral blood from people with MS compared to controls with other neurological diseases after stimulation with for example myelin basic protein or myelin proteolipid protein,[58-60] indicating that myelin reactive T cells of MS patients exist in an in vivo activated state.

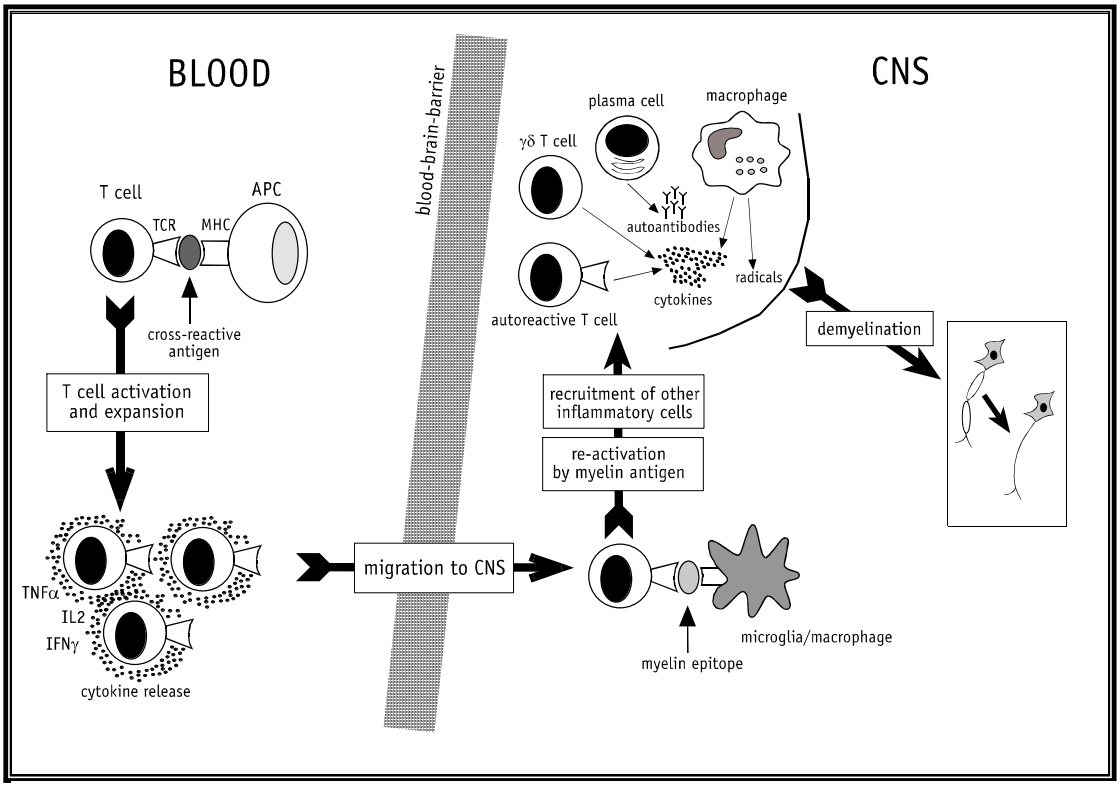

Figure 1. Current concepts of the multiple sclerosis pathogenesis.
TCR: T cell receptor; APC: antigen presenting cell; MHC: Major histocompatibility complex; CNS: central nervous system; TNF: Tumor necrosis factor; IL: Interleukin; IFN: Interferon.
(Figure reproduced from Hellings et al. (2002) with approval from the authors)

Once these myelin reactive T cells are activated, they expand and traffic to the CNS by crossing the endothelial blood brain barrier.[61] The blood brain barrier includes vascular endothelial barriers that separate blood within the brain from blood outside the brain and separates blood from cerebrospinal fluid. It provides a blocking function of substances that the brain does not require. Myelin reactive T cells are found in the cerebrospinal fluid of people with MS but not in patients with other neurological diseases.[58] Within the CNS, they are thought to become reactivated once they encounter their specific myelin epitope presented by resident antigen presenting cells: microglia cells or perivascular macrophages.[62]

The reactivated T cells will locally produce proinflammatory cytokines such as TNF and IFN-γ leading to the up-regulation of MHC class II molecules on astrocytes and microglia and adhesion molecules on the blood brain barrier endothelium. This will facilitate the further influx of T cells, B cells and macrophages, thus contributing to the amplification of the immune inflammatory response. Although T cells are though to be key players in the immunopathogenesis of MS, autoantibodies produced by B cells such as plasma cells may also be involved. Demyelination will be the ultimate result of this vicious circle of events. Most probably, the myelin breakdown is brought about by the combined effects of cytotoxic cells (macrophages and γ-δ T cells), demyelinating autoantibodies, and cytokine-induced toxicity.[49, 63-65] Although the vast majority of T cells in MS plaques carry the α-β receptor (most $CD4^+$ and $CD8^+$ T cells), a variable number of γ-δ T cells (1-10%) can also be found, [15] but their role in MS remains enigmatic.[66] The degradation of the myelin sheath is the main event in MS and proteins within the CNS myelin are considered to contain candidate antigens eliciting the pathogenic autoimmune responses. Possible candidate antigens are MOG, MBP, and proteolipid protein (PLP).

The fact that functional rather than quantitative differences exist between autoreactive T cell populations of MS patients and control subjects in the periphery may indicate that regulatory mechanisms which are responsible for controlling autoreactive T cells in normal conditions are impaired in MS. There is evidence of the existence of a peripheral regulatory T cell network that prevents uncontrolled expansion of potentially pathogenic T cells. The knowledge about the immunoregulatory mechanisms are partially based on T cell vaccination and T cell receptor vaccination studies in both animal models and MS. This experimental therapy is aimed at enhancing the regulatory networks to specifically suppress the circulating autoreactive T cells. These studies showed evidence for the involvement of several immune cell types in the immunoregulatory T-T cell interactions: (a) anti-idiotypic T cells (cells that recognise T cell receptor related structures), (b) anti-ergotypic T cells (cells that recognise unknown markers commonly expressed by activate T cells), (c) γ-δ T cells, (d) natural killer cells, and (e) $CD8^+$ suppressor cells.[52] Thus, although the active role of regulatory T cells in the pathogenesis of MS remains to be proven,[66] it is possible that an imbalanced regulatory network could lead to the suboptimal suppression of activated pathogenic T cells, which may finally result in autoimmunity.[52]

2.9 MS MORBIDITY IN TIME, PLACE AND PERSON

The complex interplay of genetic and environmental factors is reflected in the distribution of many diseases. Morbidity studies examining prevalence and incidence rates can contribute to assess the importance of genetic and environmental influences on the risk of the disease. Observations that point to a role for environmental influences are: (1) a change in morbidity rates over time, (2) a difference in morbidity rates in different geographical areas, (3) a difference in morbidity rates in groups that migrated to another location compared to the population of origin, and (4) clustering within a small geographical area. Observations that

point to a role for genetic influences are: (1) a difference in morbidity rates in different racial groups and (2) the extent of familial clustering.

As mentioned before, Davenport[8] noted in 1922 that MS was more common among drafted men in the northern states of the United States compared to the southern states, but inaccurate denominators could have influenced the estimates. The next report in 1950 reported that mortality rates were greater in temperate zones than in the tropics or sub-tropics and that the rates were higher in northern parts of the United States and Italy than in southern regions.[9] Other carefully designed studies confirmed this pattern.[67] In addition, MS prevalence among United States veterans correlated well with latitude (r=0.76).[14] In 1975, Kurtzke suggested that the distribution of MS prevalence could broadly be classified into bands of low, medium, and high prevalence.[68, 69] High risk (>30 per 100,000) was found throughout northern Europe, the northern United States, Canada, southern Australia, and New Zealand; medium risk (5-30 per 100,000) was found in southern Europe, the southern United States and northern Australia; and low risk (<5 per 100,000) areas included Asia, South America, and many uncharted regions.[68, 69] Thus, regional differences were thought to be related to latitude and studies were conducted to search for environmental factors that correlated with latitude.

Currently, over 400 publications are published on the prevalence of MS.[1] Although comparison of the surveys is often difficult due to differences in diagnostic criteria, case ascertainment, and variability of the surveyed populations in terms of size, age structure, ethnic origin and composition, a number of observations can be made: (1) the increase of morbidity rates over time, (2) an increased awareness of the importance of genetic factors in the development of MS, and (3) the remaining importance of environmental factors in the development of MS. These observations will now be further discussed.

Systematic updating of prevalence and incidence rates showed that the absolute number of cases identified in different parts of the world has risen steadily since the 1950s.[70] The question is whether this was partly due to a real increase, or that improved diagnosis and increased awareness fully accounted for the difference.[70] A study in a province of Italy indicated that the increase in this location was partly due to a real increase and that a better diagnosis could not fully explain the difference.[71] In northern Sardinia (Sassari) a steady increase in incidence rate was found from 2.0 per 100,000 (1968-1972) to 6.8 per 100,000 (1993-1997).[71] Over time, improvement in diagnosis would decrease the lag time between symptomatic onset and diagnosis. When they compared the trend in lag time between symptomatic onset and diagnosis with another region in Italy (Ferrara), no differences were detected, but a clear difference in the incidence trend was observed between the two areas, with Ferrara having a much lower increase in incidence (1.4 fold) than Sassari (3.4 fold).[71, 72] Therefore, although it is often difficult to separate true increases from increases due to diagnosis and awareness, there are locations such as northern Sardinia where the increase is thought to be partly real. Another study conducted in Newcastle, Australia, found a steady and significant rise in MS prevalence and incidence from 1961 to 1996.[73] Although there is the possibility that a better case ascertainment contributed to the increase, the authors concluded that the homogeneity of the population studied on each occasion and the application of identical study methods suggests a true increase in prevalence, and that the increase in prevalence was attributed to an increased incidence in females and an increased survival in the MS population.[73]

While the regional differences of early prevalence studies were mostly explained in terms of latitude and environmental factors underlying those geographical differences, more recent studies also highlight the importance of genetic background. In many locations, it is difficult to distinguish to what extent the geographical patterns reflect genetic and environmental differences. For example, the north-south gradient that was observed in the United States in

the early seventies could also be partly explained by the distribution of ancestral background.[74] Bulman and Ebers found a high correlation ($r=0.73$, $p<0.01$) between the rank order of case control ratios among veterans in each state and the percentage of people with a Scandinavian origin in those states.[74]

The influence of genetic factors using prevalence data might be best assessed by comparing populations with different ethnic background within the same geographical location, limiting environmental confounding. For example, indigenous populations might be compared with groups that colonised the area. One limitation is that in many circumstances the way of life of the indigenous population is also substantially different from the population that colonised the region, so they differ not only genetically but also in their exposure to many environmental agents.[75] In addition, a uniform case ascertainment for both populations should improve the interpretation of the results, but it is known that well-designed surveys are not always conducted in different sub-populations.[75] Lastly, a prevalence is a cross product of the incidence rate and duration of disease, and thus, differences in prevalence between regions and/or populations could also be attributed to differences in survival.

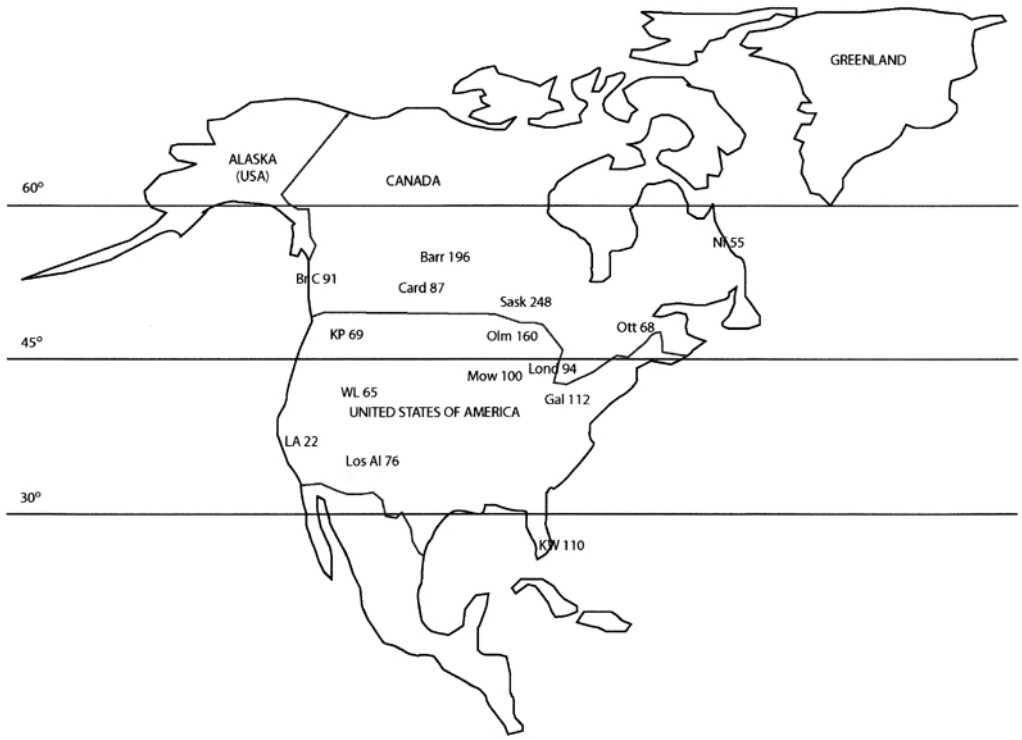

Figure 2. Distribution of MS (prevalence per 100,000) in Canada and the United States of America. (Data from Rosati (2001))

Region: study area, state (prevalence year)

Canada: NF, Newfoundland (1984); Ott, Ottawa, Ontario (1975); Lond, London and Middlesex County, Ontario (1984); Sask, Saskatoon, Saskatchewan (1999); Card, Cardston Region, southern Alberta (1988); Barr, Barrhead, Alberta (1990); Br C, British Columbia (1982).

United States of America: KP, King-Pierce Counties, Washington (1970); Olm, Olmsted County, Minnesota (1985); Mow, Mower County, Minnesota (1978); Gal, Gallion, Ohio (1987); WL, Weld-Larimer Counties, Colorado (1982); LA, Los Angeles County, California (1970); Los Al, Los Alamos County, New Mexico (1979); KW, Key West Island, Florida (1983).

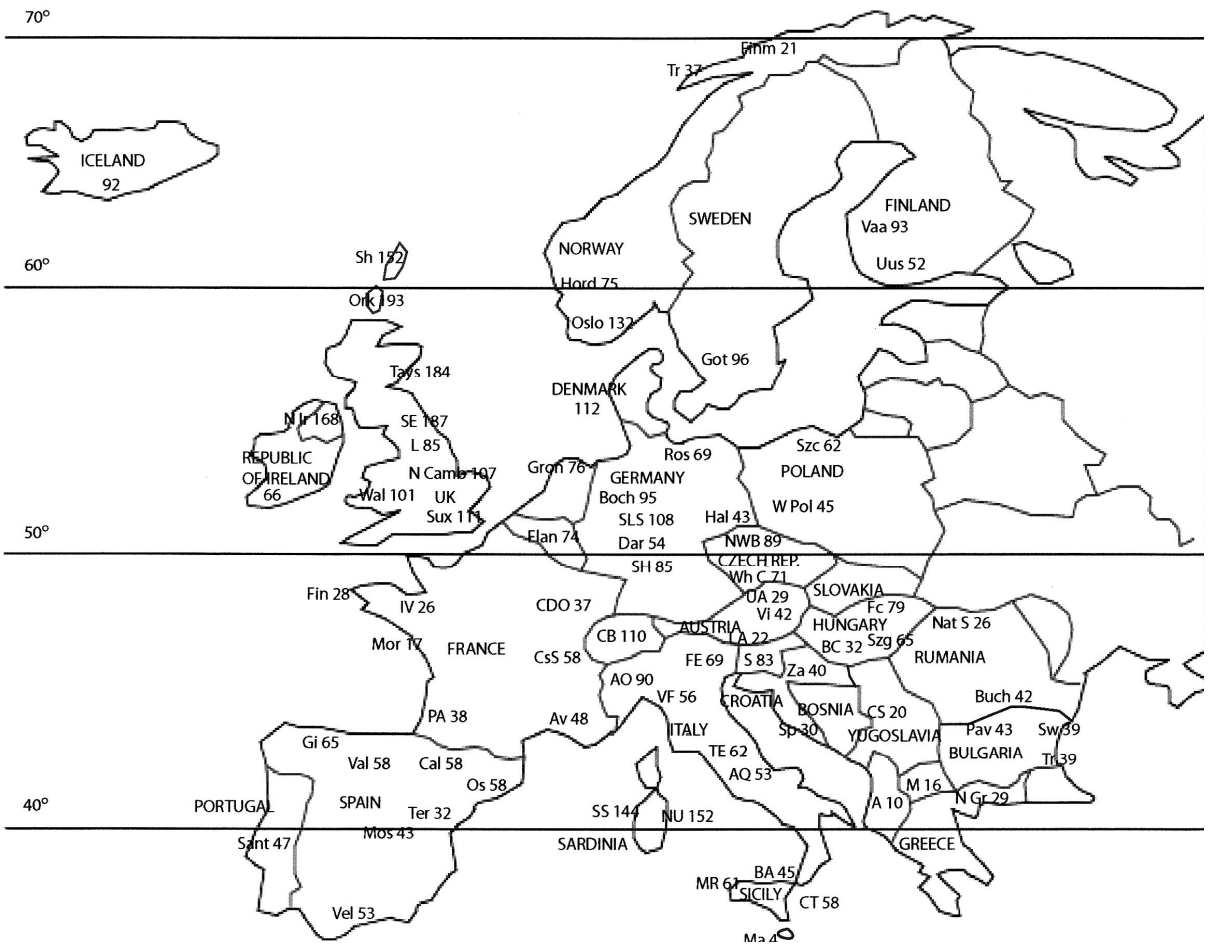

Figure 3. Distribution of MS (prevalence per 100,000) in Europe (excluding the countries of the former USSR). (Data from Rosati (2001))

Region: study area (prevalence year)

Scandinavia: Finm, Finmark (1983); Tr, Tröms (1983); Hord, Hordaland (1983); Oslo, Oslo (1995); Got, Gotenburg (1988); Vaa, Vaasa (1979); Uus, Uusimaa (1979); Iceland (1989); Denmark (1990).

United Kingdom, Republic of Ireland: Sh, Shetland Islands (1974); Ork, Orkney Islands (1983); Tays, Tayside (1996); SE, South East Scotland (1995); L, Leeds Health Authority (1996); N Camb, North Cambridgeshire (1993); Sux, Sussex (1990); Wal, South East Wales (1985); N Ir, Northern Ireland (1996); Republic of Ireland (1971).

Netherlands, Belgium, France: Gron, Groningen, The Netherlands (1992); Flan, Flanders, Belgium (1992); Fin, Finistére (1978); IV, Ille et Vilaine (1978), Mor, Morbihan (1978), CDO, Côte D'Or (1983); CsS, Chalon sur Saône (1984); Av, Avignon (1984); PA, Pyrérées Atlantiques (1988).

Spain, Portugal: Gi, Gijon (1994); Val, Valladolid (1997); Cal, Calatayud (1995); Os, Osona (1993); Ter, Teruel (1996); Mos Mòstoles (1998); Vel, Vélez-Malaga (1991); Sant, Santarem (1998).

Germany, Switserland, Austria: Ros, Rostock (1983); Boch, Bochum (1990); SLS, South Lower Saxony (1992); CB, Canton of Berne, Switzerland (1986); UA, Upper Austria (1981); LA, Lower Austria (1981); Vi, Vienna (1981).

Poland, Czech Republic, Hungary: Szc, Szczecin Region (1992); W Pol, Western Poland (1981); NWB, North West Bohemia (1992); Wh C, Whole Country (1984), FC, Feyer County (1992); Szg, Szeged City (1996); BC, Baranya County (1993).

Italy: AO, Valle d'Aosta (1995); FE, Ferrara province (1993); VF, Valdarno-Firenze district (1991); TE, Terni province (1994); AQ, L'Aquila province (1996); SS, Sassari province (1996); NU, Nuoro province (1994); MR, Monreale (1991), BA, Bagheria (1994); CT, Catania province (1995), Ma, Malta (1978).

Slovenia, Croatia, Yugoslavia, Rumania: S, Slovenia (1992), Za, Zagreb (1979); Sp, Split (1981); CS, Central Serbia (1981); Nat S, National Survey 34 counties (1984); Buch, Bucharest (1977).

Albania, Macedonia, Greece, Bulgaria: A, Albania (1988); M, Macedonia Republic (1991); N Gr, Northern Greece (1984); Sw, Swoge (1995); Tr, Trojan (1995); Pav, Pavlikeny (1998).

Rosati has provided a detailed analysis on the prevalence of MS in 2001.[1] Figure 2 and 3 provide the regional prevalences of MS in the United States of America and Canada, and Europe. As can be seen from those figures, MS prevalence seems to be higher in people with a northern European ancestry (England prevalence 74-112 per 100,000 (1985-1993), Scotland 145-193 per 100,000 (1983-1996), Norway 75-132 per 100,000 (1983, 1995), Gothenburg (Sweden) 96 per 100,000 (1988), Denmark 112 per 100,000 (1990)). The highest prevalences in the world seem to be in Scotland, although the highest rate reported ever was in an area in Canada in Saskatoon (248 per 100,000 (1999), probably due to an exceptionally high case ascertainment. The rates in Spain, Portugal and the mainland of Italy have been reported to be around 50 per 100,000, but a striking exception is the Italian island Sardinia, which has reported prevalences around 144 to 152 per 100,000 (1994, 1996). This is thought to reflect their different genetic structure compared to other Italians. In contrast, Malta reported a prevalence of 4 per 100,000, which could also be related to their genetic background, but major problems in case ascertainment cannot be excluded. The Gypsy population has a much lower prevalence than the surrounding populations in some regions (Baranya county in Hungary: prevalence 5 per 100,000 among Gypsies versus 32 per 100,000 in the white population; Sofia in Bulgaria: prevalence 19 per 100,000 among Gypsies versus 45 per 100,000 in the white population), but not in all (Feyer county in Hungary: prevalence 98 per 100,000 among Gypsies versus 79 per 100,000 in the white population). In the most northern counties of Norway, the prevalence is lower (21-37 per 100,000 (1983)), than the rest of Norway (75-132 per 100,000 (1983, 1995)). This might be in accordance with the Scandinavian neurologists' clinical experience that MS very rarely affects the Lapps (who are genetically an admixture of Mongoloid and Caucasoid people), but also demonstrates the importance of environmental factors as the Lapps only represent about 10% of the most northern counties of Norway. In Siberia, prevalences range from 12 to 41 per 100,000 (1980s), and until 1972, no MS case had been observed among native Siberian people. North American Indians living in Canada, and Canadian Hutterites, a socially isolated ethnic group originally immigrated from southern Germany to Canada, both have lower prevalences than other Canadians. Although the number of studies conducted in China is low (three), and some doubts have been raised about the validity of diagnosis in both Japan and China, it seems that MS is relatively rare in those countries (China 1-2 per 100,000 (1980s), Japan 1-4 per 100,000 (1975-1983)). In South Africa, MS is more common among with English speaking South Africans (13 per 100,000, 1960) than among Afrikaners (4 per 100,000, 1960) and hardly at all reported among black Africans. Similarly, MS was reported to be rare among Maoris, the indigenous population of New Zealand. Thus, this prevalence data provides evidence that genetic differences appear to be important in the development of MS.

Regional differences in ethnically homogeneous populations might reflect the influence of environmental factors. For example, the lower MS prevalence at high altitude areas of Switzerland compared to low altitude areas has been attributed to a higher ultraviolet radiation intensity, and the lower MS prevalence in coastal areas of Norway compared to inland areas has been attributed to the higher consumption of fish, a dietary source rich in vitamin D_3.[76]

If latitude-related factors are important in the development of MS that show a latitudinal gradient, then a latitudinal gradient might be expected in the MS prevalences. Although the original evidence in the 1970s seemed convincing, the current regional differences seem much more complicated. The influence of a latitudinal gradient using prevalence data might be best assessed in regions with a substantial latitudinal spread and an ethnically homogenous population, limiting genetic confounding. When the latitudinal spread is relatively small, variation in prevalence due to differences in study methods or chance might limit the detection of an effect due to environmental factors. In addition, uniform case ascertainment should improve the interpretation of the results.

There are not many regions that have the features previously mentioned. The former Soviet Union, China and Brazil all have a large latitudinal spread, but the first has large genetic differences, while the number of prevalence studies conducted in the other two areas is not sufficient to examine whether there is a gradient. In Canada, a number of studies have been conducted with very different levels of case ascertainment. In addition, there is a slightly higher density of French-speaking population in the eastern seaboard and Newfoundland, as compared to western Canada, where people of British and North European ancestry are more represented. The prevalence studies conducted since the 1970s in the United States do not support the same north-south gradient as was seen prior to that time, but the low level of comparability of the studies makes the interpretation difficult. However, two cohort studies of American women, the Nurses' Health Study (NHS and NHS II), currently follow up two groups of female nurses: those born between 1920 and 1946 (NHS) and those born between 1947 and 1964 (NHS II). The incidence rate of MS among NHS participants (181 definite/probable patients) increased significantly with latitude (p=0.03, trend).[77] Adjusted rate ratios {95% confidence intervals} were 3.5 {1.1—11.3} for the north and 2.7 {0.8—8.9} for the middle tiers relative to the southern tier. Among NHS II women (131 definite/probable patients), no association between latitude and MS was found (p=0.89, trend). Adjusted rate ratios were 0.8 {0.4—1.6} for the northern areas and 0.9 {0.4—1.8} for the middle areas, relative to the southern areas.[77] Thus, they confirmed the association between latitude and risk of MS in the United States in NHS, while they showed an attenuation of the north-south gradient over time by comparing NHS II to NHS. Australia, another country with a large latitudinal spread, has ethnically a relatively homogeneous population and prevalence surveys were conducted in six

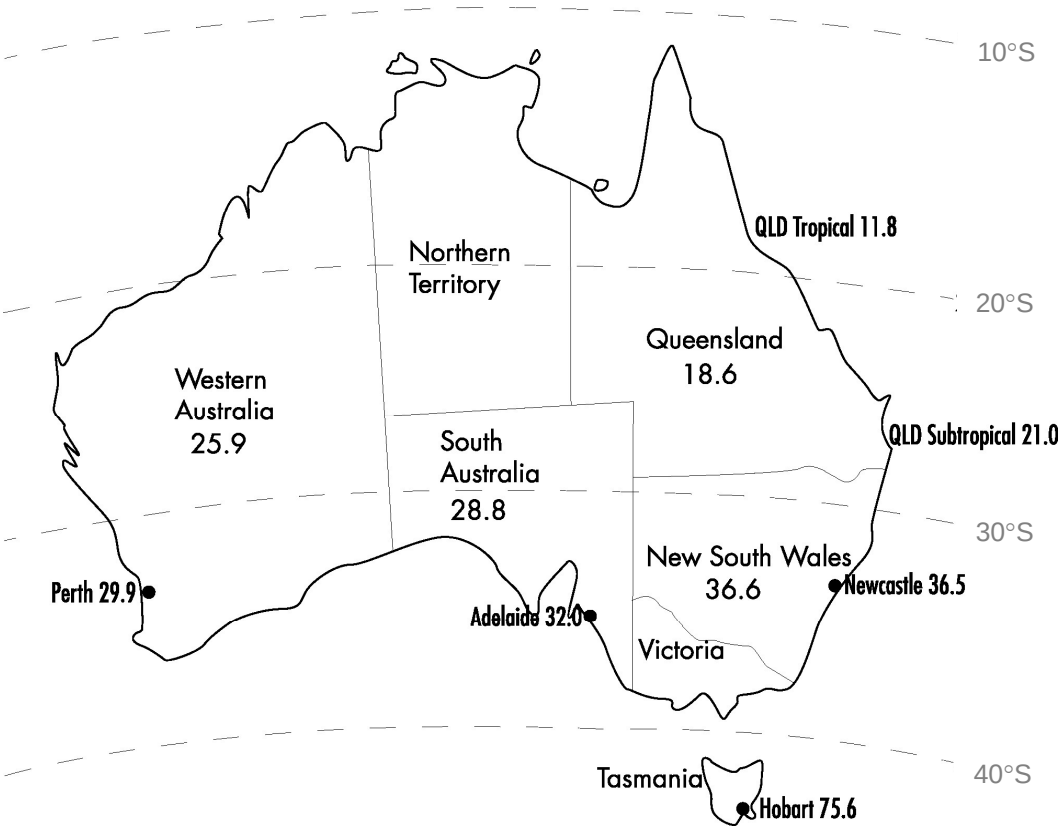

Figure 4. Distribution of multiple sclerosis (age-standardised prevalence per 100,000 population) in Australia in 1981.

(Data from McLeod et al. (1994))

regions in 1981 using similar case ascertainment methods. Cases were ascertained from hospital records, treating health notifications were also used in the Hobart region.[78] All patients were interviewed and examined, in regions other than New South Wales, where only 57% of the patients were interviewed and examined due to the large number of patients notified.[79] However, almost all of the remaining 43% of patients in New South Wales had been examined previously by a neurologist.[79] Patients were classified according to the clinical criteria of Rose et al.[23] Figure 4 shows the age-standardised prevalences in those regions of Australia. As can be seen, a more than six-fold increase in age-standardised MS prevalence from tropical Queensland (Brisbane, 11.8 per 100,000, latitude 19°S) to Tasmania (Hobart, 75.6 per 100,000, latitude 43°S).[79] This gradient was also evident among UK and Irish immigrants to Australia ($p<0.001$, trend), a population subgroup that is predominantly Caucasian.[80]

Thus, based on the criteria of a substantial latitudinal spread, an ethnically homogeneous population, sufficient prevalence studies conducted in the area using similar methodologies, it seems that Australia was particular suitable for a latitudinal analysis of prevalence data, and this region clearly shows an increasing prevalence with increasing latitude. This suggests the importance of exposure of one or more environmental factors that differ by latitude. In addition, the cohort studies conducted in the United States not only showed a latitudinal gradient with MS incidence, but also showed that the latitudinal gradient diminished over time, which could suggest that the latitudinal gradient in exposure of populations to the environmental factor might also be diminishing.

There are other lines of evidence that support the contribution of both genetic and environmental factors in the development of MS. Familial clustering has been used to indicate the influence of genetic factors on the risk of MS. Monozygotic twins have a higher concordance rate (approximately 25%) than dizygotic pairs (3%).[81, 82] Meta-analysis of recurrence risk among relatives showed that the age-adjusted risk is highest for siblings (3%), than parents (2%) and children (2%), with lower rates in second- and third-degree relatives.[83] The risk of adopted children is more or less identical to the population risk[84] and the risk for half siblings is significantly lower than for full siblings.[85]

If MS was, however, purely genetic, a concordance rate close to 100% would theoretically be expected among monozygotic twins. A concordance rate of approximately 25% suggests that environmental factors also influence disease development. Other evidence often used to illustrate the importance of environmental factors is the change in disease occurrence after migration of populations. The overall consensus is that migration alters the prevalence of MS, whether people move from a high risk country to a low risk country or vice versa.[86] Some studies also show that the risk of MS only changed when people migrated prior to the age of 15 years,[87, 88] indicating the importance of an environmental factor in childhood and/or early adulthood, but other studies do not confirm that,[80] maybe indicating that the risk from environmental factors operates over a period of many years and not only in childhood and early adult life. Clusters of people with MS in a particular area could also be used as evidence that an environmental factor is critical in the development of MS. Kurtzke provided some evidence of increasing MS rates in Iceland and the Faroe Islands after the occupation by British troops in 1945.[89, 90] In Iceland, the average annual incidence rate was higher during 1945 to 1954 (3.2 per 100,000), compared to the period prior to an during the war (1923 to 1944, 1.6 per 100,000) or after 1954 (1955 to 1974, 1.9 per 100,000). On the Faroe Islands, no MS patients among the native population were reported prior to 1943 and an epidemic was observed in the following years. He suggested the transmission of a virus by the British troops to the local population, however the increases may have been related to improved recognition and diagnostic procedures rather than a true increase.[91]

In conclusion, different lines of evidence — trends over time, complex geographical patterns of MS prevalence within continents, countries and small regions, familial clustering and migration studies — represent the importance of both genetic and environmental factors in this disease.

2.10 GENES OR GENE REGIONS INFLUENCING THE DEVELOPMENT OF MS

The association between MS and human leucocyte antigen (HLA) class II polymorphisms on chromosome 6p21 is the only consistently replicated genetic result in MS research.[92] This region has been refined to the DR15 and DQ6 subtypes of DR2 and DQw6 respectively, which correspond to phenotypic expression of the DRB1*1501-DRB5*0101 (DR15) and DQA1*0102-DQB1*0602 (DQ6) genotypes. More recently, the class I HLA region has been re-identified as harbouring genes that independently modulate susceptibility to MS.[93] Yet, given the relatively weak results of linkage analysis for both HLA class I and II regions, they can account for but a small portion of overall susceptibility.[92]

Many studies have been performed on candidate genes, where genes are selected for analysis based on functional knowledge. A review by Dyment[92] outlines that the use of the transmission disequilibrium test and affected family-based controls have made it easier to discard potential candidate loci such as T cell receptor α, interleukin-1 receptor agonist, interferon α, β and γ and a variety of complement and cytokine and enzyme loci. There are other candidate loci such as myelin basic protein, T cell receptor β locus and immunoglobulin variable gene loci, where positive results have been found by some groups, but the results have not been consistent.[92] An association of MS with the tumour necrosis factor locus has also been found, but not independent of the HLA associations.[92]

Because of the lack of success of the candidate gene approach, a number of groups have turned to full genome searches using a large number of microsatellite markers. Linkage with the HLA region was found by all four groups that used this approach, but no other regions of the genome appeared linked in all the studies.[94-97] The relative disappointing results may have been caused by a number of factors such as power limitations, incomplete penetrance, interactions with environmental factors or other genes, the use of incorrect model assumptions and the incorporation of different clinical subtypes of MS.[98] Some interactions with environmental factors will be discussed further in chapter 3.

2.11 ENVIRONMENTAL FACTORS INFLUENCING THE DEVELOPMENT OF MS

Many studies have been conducted to assess the relationship between environmental factors and the development of MS. Most studies were case-control studies, a design often used when a disease is relatively rare and large cohorts are required for prospective cohort studies. A number of prospective cohort studies on MS have been conducted in the United States and the United Kingdom. In contrast to case-control studies, prospective cohort studies assess exposure prior to disease onset. This provides certainty that exposure preceded disease and avoids recall bias, which is a differential recall between cases and controls. A short overview is provided on the environmental factors most commonly studied. The evidence of the possible influence of ultraviolet radiation on the risk of MS will be discussed in chapter 3, and the evidence of the possible influence of female hormones and pregnancies will be discussed in chapter 10.

Infections have are often considered to be involved in the development of an autoimmune disease, but the only virus with consistent proof that it might be involved in the development of MS is the Epstein-Barr virus (EBV).[99, 100] In a recent systematic review, Ascherio and Munch[99] combined the results of eight sero-epidemiological case-control studies and reported an estimated odds ratio of 13.5 {6.3—31.4} for seropositivity against EBV indicating a strong association between markers of past infection with EBV and the risk of MS. A prospective cohort study and nested case-control study with samples taken prior to MS onset, also support a role of EBV in the aetiology of MS.[101, 102] For example, the nested case-control study found a strong association with MS for antibodies to the Epstein-Barr nuclear antigen type 2 (relative risk 3.9 {1.1—13.7} for a four-fold difference in titers).[101] More information on causality of EBV will be provided in chapter 9.

Infections might also play a protective role. Bach demonstrated that the increased incidence over time of autoimmune and allergic diseases such as MS, type 1 diabetes, Crohn's disease and asthma is associated with a decreased incidence of measles, mumps, rheumatic fever, hepatitis A and tuberculosis.[103] These observations might be related to infection load during early childhood. In atopic disease, there is evidence for the so-called "Hygiene Hypothesis", where an increased hygiene and smaller family size over the last decades is thought to be associated with a reduced opportunity of infection in early childhood and an increased risk of atopic disease. A similar situation might be occurring in MS. Chapter 9 will discuss in detail the effect of family structure on MS and will discuss the effects of specific viruses such as EBV, herpes simplex viruses, cytomegalovirus, measles, mumps and rubella.

Infections could also be transmitted via animals. Hodge and Wolfson[104] conducted a review of the epidemiological evidence in regard to dog ownership and canine distemper, an infection of dog like animals (dog, fox, coyote, wolf) that has similarities with the human measles virus. Ecological studies provide no clear evidence of an association between canine distemper prevalence among dogs and MS prevalence. Studies describing a temporal association between canine distemper rates and MS incidence are inconsistent, often involve small numbers of cases, do not have supportive evidence from accurate time series, and have no consistent pattern in the latency interval between canine distemper epidemics and the reported increased incidence of MS (ranging from 3 to 15 years). Some studies found higher serum antibody titers in cases compared to controls.[105, 106] Antibodies to canine distemper virus can, however, cross-react with antibodies to the measles virus, and there is no evidence of the infectivity of canine distemper virus in human populations. A number of case-control studies report significant positive associations between dog or cat contact or ownership and MS, but the case-control studies that are methodologically more rigorous do not provide a consistent pattern. One of those studies, however, found that occupational exposure to dogs, but also other animals such as cats, poultry, horses and swine, were reported to occur more frequently among men with MS than among the controls,[107] suggesting that occupational exposure to animals broadly could be important rather than exclusively or even predominantly to those known to harbour canine distemper virus.[104] The number of subjects exposed were, however, low and the associations were not seen in women.

If infections could influence the development of MS, then immunisations could also possibly influence MS.[108] Compston et al.[109] conducted a case-control study where controls were matched on HLA-DR2 genotype and did not find a difference between cases and controls for typhoid and tetanus toxoid immunisation. Two other case-control studies found no differences between cases and controls in the frequency of immunisations against poliomyelitis,[110] tuberculosis,[110] smallpox,[111] diphtheria,[111] and thypoid.[111] A nested case-control study (relative risk 0.9 {0.5—1.6} for any hepatitis B immunisation, 0.7 {0.3—1.8} for immunisation within two years before onset) and a retrospective cohort study on immunisation against hepatitis B did

not show an increased risk of MS.[112, 113] Both used vaccination records and did not rely on self-report. The nested case-control study showed that a slight increased risk was found if they had relied on self-reported immunisations against hepatitis B (relative risk 1.2 {0.8—1.7} for any immunisation, 1.9 {1.1—3.3} for immunisation within two years before onset). This may have been the reason why another case-control study found a slight, but non-significant increased risk.[114]

In relation to dietary intake, positive correlations have been found in ecological studies between MS rates and intake of total energy, total fat, animal fat, butter fat, meat fat and milk, while inverse correlations have been found with intake of fish, vegetable fat, fruit and vegetables.[115] However, results of case-control studies have been inconsistent,[110, 116-119] but most of them reporting a null association with intakes of fruit,[110, 120, 121] vegetables,[110, 120, 121] fat,[110] meat[110] and dairy products.[110, 111, 116] Although some case-control studies found an increased risk for higher intakes of animal-derived fats,[118, 119] pooled results from two large cohort studies (92,422 nurses followed for 14 years and 95,389 nurses followed for four years) did not find evidence to support the hypotheses that higher total fat or saturated fat intake increased the risk of MS, or that higher intake of vegetable fat, monounsaturated fat, polyunsaturated fat, cholesterol or omega-3 fatty acids from fish, carotenoids, vitamin C, and vitamin E decreased the risk of MS.[122, 123]

Exposure to organic solvents might also play a role, because organic solvents have been demonstrated to alter immune function, change the permeability of the blood brain barrier, induce alterations in the peripheral and central nervous system such as axonal swellings, and organic solvents have been associated with other diseases such as peripheral neuropathy, leukaemia, Hodgkin's disease and liver damage.[124] A meta-analysis conducted on 13 published studies showed a relative risk point estimate that ranged from 1.7—2.6.[125] In addition, a recent record linkage study in Norway (1970-1986) found nine people with a disability pension for MS among a cohort of 11,542 painters (cohort exposed to organic solvents), compared to 18 among a cohort of 36,899 construction workers and 9,313 food-processing workers (unexposed cohort) (relative risk 2.0 {0.9—4.5}).[126] In contrast, a record linkage study using the Danish MS Register (3,241 men) and the National Bureau of Statistics in Denmark, which has data on occupational status, showed no increased risk of MS among housepainters, carpenters/cabinet makers, and typographers/printers (exposed cohort) compared to skilled electricians, bricklayers, and butchers (unexposed cohort) (standardised incidence ratio 0.9 {0.7—1.1}).[127] Ionising radiation in the form of radiological work and X-ray examination has also been associated with MS.[128] Axelson et al.[128] combined two previously published case-control studies and obtained odds ratios of 4.4 {1.6—11.6} for radiological work and 1.8 {1.2—2.6} for X-ray examinations. Ionising radiation may be involved in the pathogenesis of MS via free radical formation and oxidative damage.

Several research groups examined the association between smoking and MS. The results of case-control studies have been conflicting,[121, 129-131] but prospective cohort studies found a modest increased risk with relative risks ranging from 1.4 to 1.8 for the top category (more than 15 cigarettes per day or more than 25 pack-years) compared to non-smokers with also evidence of dose-response ($p<0.05$, test for trend).[132-134]

It has been suggested that mild, concussional trauma to the central nervous system could produce alteration of the blood brain barrier and therefore act as a trigger or facilitator in the development of MS lesions.[135] However, a review of the relevant published data by the Therapeutics and Technology Assessment Subcommittee of the American Academy of Neurology concluded that there is no association between physical trauma and MS onset.[136]

Results on breastfeeding history and MS have been inconsistent with most studies finding no effect,[110, 116, 137] but two case-control studies finding that cases were less likely to have been breastfed for a prolonged period compared to controls.[138, 139] Cow's milk contains lower amounts of unsaturated fatty acids, and a different composition of cortex grey matter has been described in bottle fed infants. This could be associated by means of the formation of defective membranes with easier entry of an infective agent across the blood-brain barrier or with accelerated degradation of myelin itself.[138] Breastfeeding could also change the pattern of childhood infections.

Although a number of ecological studies have been conducted, only one case-control study has been carried out on the influence of dental amalgam and this study did not find an association between the number of dental amalgams or the duration of exposure to mercury amalgams and MS.[140]

In conclusion, there seems sufficient evidence only for the EBV to conclude that this factor might be causally related to MS. In addition, MS is a multifactorial disease where genetic and environmental factors interact with each other. Environmental factors may be critical at different stages in life. It is possible that the immune susceptibility is initially determined by a genetic component and early life exposures.[141] This susceptible system may then be primed, probably via the phenomenon of molecular mimicry, by EBV and/or other viruses.[141] Finally there may be a proximal trigger – an event that again distorts normal immune response – perhaps a stressful life event or an exposure that was also important in early life.[141]

2.12 IMMUNOMODULATORY TREATMENTS

It has been difficult to develop the first effective therapeutic agents for MS, because: (i) the disease is complex and the pathogenesis is not adequately understood, (ii) different therapies seem to be useful for different sub-types of MS, (iii) the animal model of MS (EAE) is an imperfect approximation of MS and a high number of drugs effective against EAE have been shown to be inefficacious in the context of MS, and (iv) the definition of reliable endpoints has been problematic in clinical trials.[142]

There are now a number of immunomodulating agents available that modestly reduce the accumulation of disability, decrease the frequency of new enhancing lesions, decrease relapse rates, decrease the appearance of fixed lesions on T_2 weighted MRI scans, and decrease the rate of cerebral atrophy.[143-147] Therapies approved for people with relapsing remitting MS are interferon β-1b (Betaseron®), interferon β-1a (Avonex®, Rebif®) and glatiramer acetate (Copaxone®). In phase III clinical trials, all three treatments showed approximately a 30% lower annual relapse rate in the treatment group compared to the randomised placebo group.[144-146] Also, a reduced number of active lesions was found in the treatment group compared to the placebo group for interferon β-1b (65% reduction within six months; 78% reduction after 19-24 months), interferon β-1a (78% reduction after 24 months), and glatiramer acetate (29% reduction after 9 months).[144, 145, 147] The β-interferons are naturally occurring cytokines. Although their predominant mechanism of action is unknown, they have been found to suppress T cell proliferation, reduce T cell migration from the systemic circulation into the central nervous system, and alter the T cell cytokine secretion repertoire from relatively proinflammatory Th_1 to relatively anti-inflammatory Th_2 response.[148] Glatiramer acetate is a random polymer of four amino acids (L-glutamic acid, L-lysine, L-alanine, and L-tyrosine) that compose myelin basic protein and evidence points to induction of tolerance to myelin basic protein specific T cells as well as alteration of the immune response from Th_1 to Th_2 predominance.[149]

In a subgroup of patients with a very high relapse rate, the intravenously administered immunosuppressive agent mitoxantrone reduces relapse rate by up to 66% over two years, and this has established mitoxantrone as a second-line therapy for aggressive MS not controlled with beta-interferon or glatiramer acetate.[150-152]

A recent review[153] indicates that a number of studies showed the benefit of early initiation of treatment, even as early as the diagnosis of the first demyelinating event, indicating that a delay of treatment may result in the accumulation of permanent disability, however long term data are not yet available. A number of other drugs that target aspects of immune function are also under development, the most advanced of which is the monoclonal antibody natalizumab. This agent binds to and blocks the α4 chain of integrin adhesion molecules, which are required for lymphocytes to enter into the CNS across endothelial membranes. A randomised, placebo-controlled, multi-centre, six-month study of monthly natalizumab showed a relapse rate reduction of 50%, and a greater than 90% reduction in new MRI lesion formation.[154] A much larger, two-year trial is in progress.

A recent review on treatments[155] indicates that for people with secondary progressive MS, interferon-β-1b showed modest effects in EDSS, wheelchair status, time to a change in EDSS score of 2.0 and T_2 lesion load. Despite these modest beneficial effects, patients continue to deteriorate, and advancing disability seems likely in most treated patients. In addition, the effect on T_2 lesion load seemed to wane in year three, and it does not prevent atrophy progression. Interferon-β-1a has not shown consistent results in regard to EDSS change when different doses and protocols are used.[155] Currently, mitoxantrone is emerging as a front-line option, as results of three recent, randomised, placebo-controlled trials indicate a significant reduction of disability-progression and its toxicity profile is relatively limited.[150-152] In the largest of these three trials, mitoxantrone therapy, tested only in a subgroup of patients with very active MS, reduces disability progression by 19% at three years compared with placebo.[152] However, the major limitation of mitoxantrone treatment in MS is the risk of dose-dependent cardiac toxicity, and therefore only one course of treatment, not exceeding a cumulative dose of 96 mg/m^2, should ever be given to any individual patient. At a cumulative dose of >100 mg/m^2, asymptomatic reduced left ventricular function develops in approximately 5% of MS patients, and clinically symptomatic heart failure occurred in two of 779 patients.[156] There are currently no convincing studies to suggest that it is possible to impact on continued disability progression in patients with primary progressive MS.[155]

2.13 RECENT DEVELOPMENTS AND OPPORTUNITIES IN EPIDEMIOLOGICAL RESEARCH ON RISK FACTORS OF MS

The use of magnetic resonance imaging has improved the precision in diagnosing people with MS. Recent validation studies have demonstrated which MRI variables best predict the outcome of having MS or not. A decreased misclassification in disease outcome increases the chance of finding genetic and environmental factors that are associated with risk of MS. In addition, measurement of exposure variables has improved over the last decade. New measures have been developed such as the use of silicon casts on the dorsum of the hand to measure actinic damage which is a proxy for cumulative sun exposure, the use of spectrophotometry to measure skin phenotype, and the use of sibling structure as a proxy measure of infection load in early childhood. Well-validated questionnaires have become available to measure, for example, dietary factors (Food Frequency Questionnaire of the Nurses' Health Studies in the United States) and physical activity (International Physical Activity Questionnaires), and different cognitive interviewing techniques have been used to produce more accurate long-term recall. Improvements in communication technology has

enhanced opportunities to conduct large multi-centre studies. The sequencing of the human genome, the development of better and cheaper genetic markers and the rapid expansion in computer technology will assist the identification of susceptibility genes and will also give rise to opportunities to identify interactions between environmental and genetic factors. Both research into environmental factors and genetic factors will benefit from an increased knowledge of immunological and pathological aspects of MS. The combination of factors mentioned above will make it worthwhile to conduct analytical studies that aim to identify risk factors for MS. The case-control study central to this thesis has utilised a number of those new developments.

2.14 SUMMARY

MS is a chronic inflammatory demyelinating disease of the central nervous system. The demyelinating event is the result of an inflammatory process, which is thought to involve a helper T type 1 cell mediated attack on myelin proteins. The clinical expression of symptoms depend on the location of the areas of destruction in the brain and spinal cord. Different types of MS have been identified: primary progressive, relapsing remitting and secondary progressive MS. The disease is multifactorial in that genetic and environmental factors interact with each other to cause the disease. The human leukocyte antigen complex is the only region of the genome so far that has consistently been found to be associated with MS, while the Epstein-Barr virus is, at present, the only environmental factor consistently found to be associated with MS. There is presently no cure, but immunomodulating agents are available that modestly reduce the progression of the disease. Recent developments in areas such as diagnostic precision, exposure measurement and genetic technology will make it worthwhile to continue to conduct analytical studies that aim to find risk factors of MS.

2.15 POSTSCRIPT

This chapter has provided some key information about different aspects of MS. This information should assist in the understanding of the rest of the thesis. The chapter has discussed the increasing prevalence with increasing latitude. Ultraviolet radiation is one climatic variable that changes with latitude. The evidence on the possible influence of ultraviolet radiation on the risk of MS will be discussed in the next chapter.

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Chapter 3

Ultraviolet radiation and Multiple Sclerosis: Insights from epidemiological research.

3.1 PREFACE

The previous chapter provided a general background into the disease Multiple Sclerosis (MS). In this chapter we will assess which epidemiological features are consistent or inconsistent with the primary hypothesis of this thesis, that the amount of ultraviolet radiation (UVR) exposure may reduce the risk of MS. Where applicable, information about type 1 diabetes mellitus (IDDM) is included. It is recognised that MS and IDDM are different diseases and probably differ in aetiology, but both are thought to be Th_1-mediated autoimmune diseases and the effects of UVR may be similar, making a comparative assessment of these diseases warranted. Parts of this chapter have been published in Toxicology 2002;181-182:71-78.

3.2 INTRODUCTION

Evolution has selected for darker skin pigmentation at low latitudes with high sun exposure, whereas lighter pigmentation may have been favoured at higher latitudes to enhance cutaneous synthesis of vitamin D.[1] However, many human groups have defied this evolutionary selection and migrated rapidly into new areas — too recently to have adapted naturally to the environment. For example, skin cancer rates are high in Australia among Caucasians that have migrated from the British Isles, and vitamin D deficiency disorders such as tuberculosis is common among Asian populations that migrated to the United Kingdom, because their increased skin pigmentation reduces the production of vitamin D. Thus, we now see high rates of skin cancer in lightly pigmented populations displaced to areas of high ambient UVR and vitamin D deficiency disorders in deeply pigmented people who have moved to places of high latitude.

Accumulating evidence that excessive exposure to solar UVR can increase the risk of skin cancer has led to health promotion activities aimed at reducing human UVR exposure.[2] For example, it has been recommended by the American Academy of Pediatrics (1999) that infants less than six months should not be exposed to direct sunlight and that paediatricians should incorporate sun protection advice into their health supervision practices.[3] However, it is inappropriate to eschew all exposure to UVR. There are diseases related to insufficient as well as excessive UVR exposure, and the burden of disease related to UVR is likely to be a U-shaped, not linear, relation (Figure 1).[4] Thus, the possible benefits of UVR exposure on human health should therefore be assessed alongside the adverse effects.[2] This review will focus on the epidemiological evidence of a possible beneficial effect of UVR exposure on the development of MS.

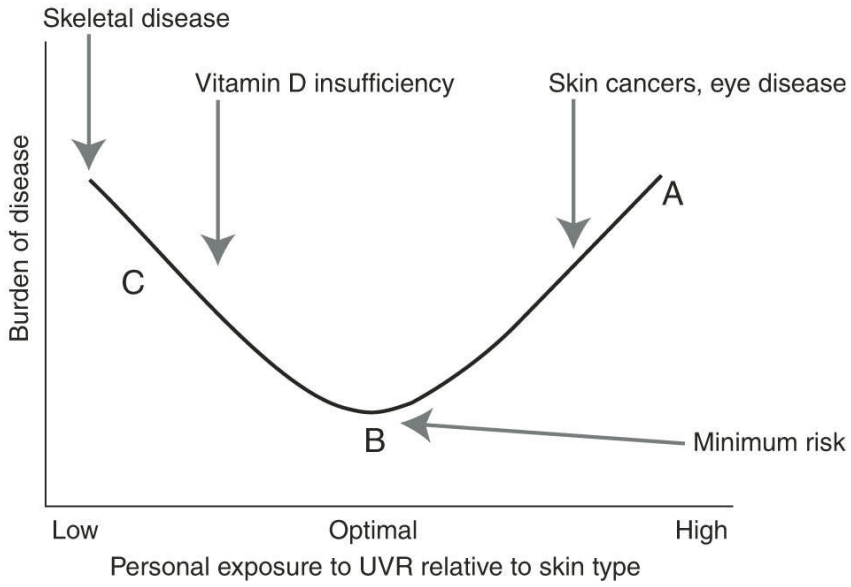

Figure 1. Schematic diagram of the relation between ultraviolet radiation (UVR) exposure and the burden of disease.
Points A and C represent inappropriate UVR exposure. Europeans in Australia with high outdoor UVR exposure typify point A. Point C represents people with insufficient UVR exposure, whose dietary vitamin D intake will also be important in determining their vitamin D status. Point B represents optimal UVR exposure: a person with careful titration of correct UVR dose for skin type. (Figure reproduced from Lucas & Ponsonby (2002) with approval from the authors)

3.3 EFFECTS OF UVR ON THE IMMUNE SYSTEM

As discussed in the immunopathogenesis section of chapter 2, the demyelinating event in MS is the result of an inflammatory process, which is thought to involve myelin reactive T-cells in the periphery that become activated after which they traffic to the central nervous system.[5] In the central nervous system they become activated once more when they encounter their specific myelin epitope.[5] In experimental allergic encephalomyelitis (EAE), the animal model of MS, the infiltrating T-cells are members of the T helper(h) type 1 family and they secrete a specific repertoire of cytokines which can modulate other immune responses.[5] Recent work suggests that UVR exposure may be one factor that can attenuate Th_1-mediated immune responses through several mechanisms, which will be particularly important as MS is thought to be a Th_1-mediated immune disease.

UVR can cause local as well as systemic immunosuppression.[6-8] It has demonstrated effects such as a decreased number and function of Langerhans cells, decreased natural killer cell activity, and isomerisation of trans-urocanic acid to cis-urocanic acid favouring Th_1 cell suppression.[7, 8] UVR also increases the cytokines interleukin (IL)-1, IL-6, IL-10 and tumour necrosis factor-alpha (TNF-α).[7] IL-10 plays an important antagonistic role towards interferon-gamma (IFN-γ), inducing down-regulation of MHC class II expression, and therefore antigen presentation, on macrophages.[7] In 1999, a review concluded there is evidence to suggest that the change in antigen presentation by UVR-induced IL-10 preferentially activates Th_2 cells and suppresses Th_1 cell mediated immune responses.[7] It has been demonstrated that certain neuropeptides, such as calcitonin gene related peptides (CGRPs) but not tachykinins,[9] which are released by sensory nerves, are involved in cutaneous inflammation after UVB irradiation.[6] CGRPs have several immunomodulatory effects and the increase in TNF-α, found after

irrradiation of UVR, might have been caused by these peptides, because it has been shown that these peptides impair contact sensitivity through a mast cell release of TNF-α,[10] while an CGRP antagonist abrogate the immunosuppression.[9] Increased production of TNF-α, IL-6 and IL-10 have also been shown to result in UVR-induced DNA damaged skin cells and those soluble mediators migrate from the skin to other places in the body.[6] Research on EAE showed that UVR can prevent or delay the clinical symptoms of EAE in mice.[11] UVR can be divided into two major regions, UVB (290-320 nm) which comprises less than 5% of the UVR that reaches the biosphere and UVA (320-400 nm) which comprises at least 95% of the remaining UVR. Although most research has focused on UVB, because of its role in skin cancer, recent research shows that UVA may also play a significant role in immune suppression, especially in regard to systemic immune suppression.[12, 13]

UVR could also act via alterations in vitamin D_3.[14] The active form of vitamin D_3 (1,25-$(OH)_2$ D_3) can be synthesised from dietary vitamin D or, alternatively, dermal stores of 7-dehydrocholesterol can be converted into previtamin D_3 under the influence of UVB and further conversions result into the active form of vitamin D_3.[15] Ninety to 100% of most human being's vitamin D requirement comes from exposure to sunlight.[16] The active form of vitamin D_3 has been shown to display immunomodulatory properties in vivo as well as in vitro.[17] Its actions on the immune system are exerted predominantly by intracellular vitamin D receptors, present in several immunological cells such as monocytes, macrophages and activated lymphocytes.[18] In vitro, 1,25-$(OH)_2D_3$ selectively inhibits T-cell activation and secretion of several cytokines including IL-12, TNF-α and IFN-γ partly by direct action on T cells itself and partly by monocytic effects.[19, 20] Moreover, 1,25-$(OH_2)D_3$ might create a shift of CD4+ Th differentiation toward Th_2 by decreasing the macrophage secretion of IL-12.[21] Direct inhibition of IFN-γ synthesis however could explain by itself this differentiating selectivity, since IFN-γ favours Th_1 formation.[22] Research on EAE has shown that administration of 1,25-$(OH)_2D_3$ at the time of immunisation has protective effects, such as delayed onset, decreased severity of symptoms and prolonged survival,[23] while vitamin D deficiency accelerates EAE onset.[24] The protective effects of 1,25-$(OH)_2D_3$ on EAE appear largest if vitamin D_3 is provided in combination with a high calcium diet.[15] In addition, vitamin D supplementation increases IL-4 and transforming growth factor(TGF)-β1 transcripts, whereas there is a reduction, if anything, in IFN-γ and TNF-α gene expression.[25] These results do not differentiate between the direct effects of vitamin D on cytokine gene expression and the indirect effects of vitamin D as regulators of other cells and genes that result in a net change in cytokine expression.[15] However, EAE was more severe in IL-4 knock-out mice, which were also resistant to treatment with vitamin D_3,[15] suggesting a role for IL-4 in the mechanism whereby vitamin D can act as a selective immunosuppressant.

Light-induced melatonin suppression has also been suggested to attenuate the autoimmune process underlying MS.[26, 27] An increased amount of light decreases the melatonin production by the pineal gland.[28] Melatonin or constant darkness, which stimulates melatonin secretion, have been shown to enhance Th_1-cell-mediated autoimmunity.[29-31] In contrast, pinealectomy or continuous light exposure procedures, which suppress melatonin production, have been demonstrated to have an inhibitory effect on autoimmunity in animal models.[29] Blocking the interaction between melatonin and its receptor by luzindole prevented the development of EAE in 22 of 23 mice.[29] In addition, activation of melatonin receptors on T helper cells appears to enhance T lymphocyte priming and the release of Th_1 type cytokines such as IFN-γ.[32, 33] A role for UVR in promoting the secretion of melanocyte stimulating hormone, which may suppress Th_1 cell activity, has also been proposed[34]. Overall, these findings indicate that UVR can suppress Th_1-mediated immune activity and that more than one mechanism might be involved to induce this suppression.

3.4 STUDIES ON LATITUDE, SEASONAL VARIATION AND MS

3.4.1 Latitude

One of the most striking epidemiological features of MS is a gradient of increasing prevalence with latitude. This is consistent with the hypothesis of a protective effect for UVR-induced immunosuppression on MS,[35] because annual averaged UVR levels decrease with increasing latitude. For Australia, the decrease is 1kJm^{-2} per 10° latitudinal increase.[36] An increase in MS is generally found with increasing latitude in Europe and the USA, with some exceptions.[37] In the US, differences in ethnic ancestry by latitude may also contribute to the latitude gradient.[38] However, in the first Nurses' Health Survey, a gradient of increasing MS incidence with latitude was observed after adjustment for confounders, including ancestry.[39] Also, an earlier report showed that the association between MS prevalence and latitude at birth did not persist after adjustment for winter solar radiation.[40]

In Australia, a six-fold increase in MS prevalence from North Queensland (latitude 19°S) to Hobart, Tasmania (43°S) exists.[41] The gradient persists even among immigrants from the United Kingdom and Ireland, a subgroup of similar ancestry.[42] The amplitude of the change in MS prevalence by latitude (a nearly four-fold increase between Brisbane (28°S) and Hobart (43°S))[41] is more consistent with the UVR difference between Hobart and Brisbane mid-winter (a 4.9-fold difference in daily total effective UVR in mean erythemal doses (MEDs))[43] than mid-summer (a 1.2-fold difference in daily total effective UVR (in MEDs)).[43] A latitudinal gradient has also been reported for childhood IDDM. An examination of childhood IDDM incidence across 15 countries reported that a model based on temperature and latitude appeared to explain 40% of the variation in IDDM risk.[44] In Europe, an approximately three-fold incidence increase has been observed with increasing latitude[45] and a gradient of increasing incidence with latitude has also been reported with China.[46] A recent review has noted that RA has also been reported to be more common at higher latitudes.[47]

3.4.2 Seasonal variation

The seasonal variation of UVR, with a winter nadir, increases with increasing latitude. For example, within Australia, the mid-summer to mid-winter ratio of daily effective UVR (in MEDs), in Darwin (12°S) is 1.1 while the ratio is 3.0 for Brisbane (28°S) and 12.8 in Hobart (43°S).[43] If UVR indeed played a role in the development and/or progression of MS, several seasonal effects could potentially be observed. Firstly, the prevalence of MS might show a seasonal pattern with the month of birth, if very early life exposures were important. Secondly, there might be a seasonal variation in the onset of MS, and thirdly, disease activity of MS might vary by season.

Month of birth

Variations in MS prevalence by month of birth are thought to be related to exposures around the birth, either in utero or in the first year of life. Templer et al.[48] demonstrated in 1992 that the monthly distribution of birth of a series of Danish MS cases (n=6,276) differed significantly from the general population of Denmark (around 56ºN). There were on average 9.3% more births than expected in the months of March to June and fewer than expected in other months, particularly in October and November (9.4% less). Sadovnick and Lee[49] found a similar pattern in British Columbia (Canada, around 50ºN) (n=2,229) and James[50] concluded that the seasonal pattern was significant. In addition, the month of birth for MS patients in Denmark and British Columbia correlated well (Spearman's rho=0.664, $p<0.01$, one way) and the seasonalities were concurrent with May being the peak month in both.[50] In contrast, Salemi et

al.[51] reported an excess of births in MS patients (n=965) from Sicily (Italy, around 37.5ºN) in June to October, with a peak in August. A small group of 42 MS patients from Budapest were assessed for their month of birth, but the pattern of births in MS patients was not compared to the pattern of births of the general population.[52] An excess was observed in April and October.[52]

To see whether the patterns could have been related to UVR, we conducted an additional analysis examining the association between month of birth of MS patients and the monthly UVR levels. This was conducted for the three studies that compared the pattern of MS patients to that of the normal population.[48, 49, 51] The results show that the pattern of month of birth of MS patients in two different areas in the northern hemisphere at a latitude of 50-56ºN (Denmark and British Columbia) was closely correlated with the inverse of the monthly pattern of UVR when the UVR pattern was lagged four months (Figure 2). This means that the lowest level of UVR (December, January and February) is four months prior to the peak in month of birth (April, May and June) of MS cases, or alternatively, eight months after the peak in month of birth. In Sicily, which is located at a lower latitude (around 37.5ºN), there was a close correlation when the UVR pattern was lagged eight months (Figure 2). This means that the lowest level of UVR (December, January and February) is eight months prior to the peak in month of birth (August, September, October), or alternatively, four months after the peak in month of birth.

A lag time of four months, as seen in the data from Denmark and British Columbia, could point to a low UVR exposure of the mother around the fifth month of pregnancy, while a lag time of eight months, as seen in the data from Sicily, could point to a low UVR exposure in the first month of pregnancy. If vitamin D deficiency or insufficiency of the mother were to be important in the development of MS in the offspring, then we could expect the lag time to be different at different latitudes. It is thought that at latitudes greater than 40º, ambient UVR levels in winter are insufficient to produce vitamin D.[53] The duration of this vitamin D 'blackout window' increases with higher latitudes.[53] The duration of being vitamin D insufficient not only depends on the duration of this blackout window but also on the amount of UVR available prior to and after this vitamin D blackout window.[54] In our situation, pregnant women in Denmark and British Columbia, might become vitamin D insufficient at an earlier point in time in winter compared to Sicily, which could explain part of the difference in lag time that we observed.

In addition, it might be expected that the magnitude of becoming vitamin D insufficient would be less in Sicily compared to Denmark and British Columbia, which could have resulted in a weaker pattern in month of birth. When we compared the magnitude of the month of birth effects by dividing the difference of the observed and expected rates by the total sample of observed MS patients in each study, no difference could be observed between the studies (data not shown). Sicily has higher ambient UVR levels in each month of the year compared to Denmark and British Columbia, but the difference is much smaller in winter compared to summer.

Although this month of birth data provides some support for the possible importance of UVR and/or vitamin D in the development of MS, the analyses need to be interpreted with caution because of possible ecologic biases. A (large) study measuring vitamin D levels during pregnancy could overcome some of the limitations. We could not conduct a month of birth analysis in the project central to this thesis, because the number of participants was not sufficient to conduct this type of analysis.

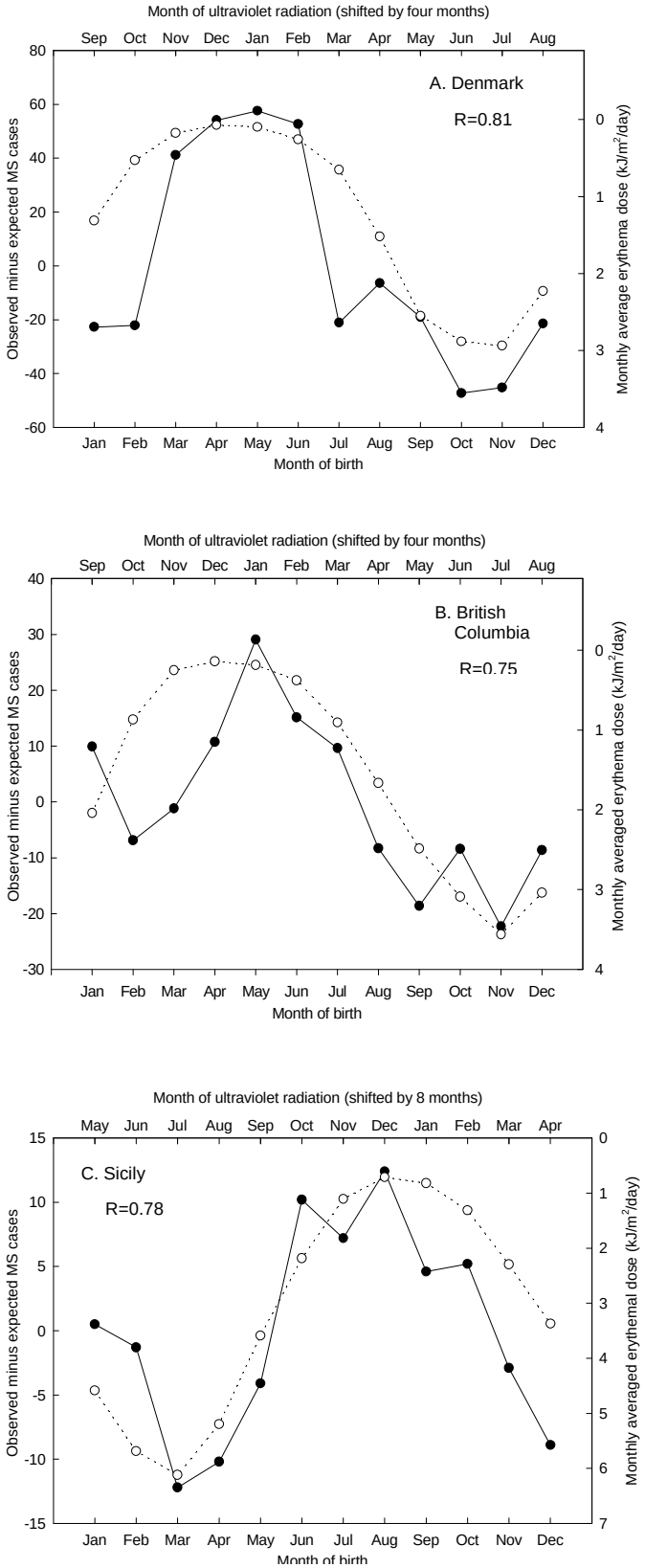

Figure 2. Observed minus expected number of births of Multiple Sclerosis cases by month (solid line) and ultraviolet radiation (dotted line) in Denmark (panel A), British Columbia (panel B) and Sicily (panel C).

A consideration of seasonal variation in UVR and month of birth variation in MS – Methods of analysis

We compared those monthly distributions of the surplus of births of MS patients with the monthly distribution of UVR in the three regions. Average monthly UVR data of Denmark, British Columbia and Sicily were obtained from satellite-based observations of atmospheric ozone and cloud reflectivity over the period 1979-1992. The UVR of the closest latitudes and longitudes to the cities Aarhus, Vancouver and halfway between Palermo and Catania were used as estimates of UVR for Denmark, British Columbia and Sicily respectively. These data were published on the website of the National Centre for Atmospheric Research (Germany). The correlation between the monthly distribution of birth and the inverse of the monthly distribution of UVR was calculated after alignment of the two patterns. The probability value for the correlation coefficient was calculated for n–2=10 degrees of freedom (that is, a penalty of 1 degree of freedom was imposed because the lag was selected to maximize the correlation).

As an underlying mechanism of seasonal variation in month of birth data, some groups have suggested that infections may play a role.[48, 55] McGrath recently proposed that low pre- and perinatal vitamin D 'imprint' on a range of tissues, leaving the affected individual at increased risk of developing, for example, MS. This is in accordance with the hypothesis from Barker et al.,[56] who suggested that environmental factors may 'program' the foetus during a critical periods in early life, such as pregnancy, and contribute to adult health. An analogous situation might occur for diseases such as IDDM, schizophrenia, osteoporosis, prostate cancer, breast cancer and colorectal cancer.[57] All these diseases have shown similarities with MS in some epidemiological features, such as latitudinal gradient, effects of vitamin D on animal models and in vitro studies, evidence of an association with UVR or intake of vitamin D, and season of birth pattern.[57] For example, a latitudinal gradient of increasing prostate cancer mortality has been observed[58] and a case-control study demonstrated an increased risk of prostate cancer for low levels of sun exposure.[59] Schizophrenia has shown seasonality in birth with an excess of births in winter and spring[60] and long-term perinatal sunshine duration was associated with rising schizophrenia birth rates for males and an earlier age of first registration for both males and females.[54] The early life exposure could contribute to risk status in addition to other factors such as genetic susceptibility and adult exposures.[57] In rats, vitamin D has been shown to have a role in neural[61, 62] and immunological[63] development. Offspring of vitamin D_3 depleted mother rats also had different shaped brains (longer but not wider) with larger lateral ventricles.[64] In addition, central immunological tolerance, resulting in the elimination of self-reactive lymphocytes during lymphopoiesis, develops primarily in foetal life.[65]

MS onset and disease activity

Seasonality in disease onset is a well described feature of childhood IDDM with most[66-68], not all,[69] studies reporting seasonality in IDDM incidence. The onset of MS, however, may be more insidious than IDDM, thus temporal onset patterns are more difficult to examine. Optic neuritis (ON) is a common presentation of MS characterised by acute disease onset with a short latent time to diagnosis.[70] Jin et al.[71] conducted a meta-analysis on the seasonal variation in ON and MS onset. The pooled weighted patterns of nine reports on ON and six reports on MS onset, which fulfilled specific criteria for report quality and data homogeneity, suggested a similar pattern for ON and MS with highest frequencies in spring and lowest frequencies in winter. One study reported a positive correlation ($r=0.67$, $p=0.02$) between the seasonal monthly incidence of ON and the average number of sunny hours. This correlation, at first examination, may appear inconsistent with previous reports of an inverse association between UVR and MS. However, the presentation of ON and MS may well reflect an underlying pathological process that may have commenced some months prior and, if this were the case, disease initiation, rather than presentation, may still be inversely related to UVR.

Jin et al.[71] also conducted a meta-analysis on the seasonal variation in MS exacerbations in people with relapsing-remitting MS. Similarly to the onset of ON and MS, the number of exacerbations was highest in spring and lowest in winter.[71] An examination of 202 MRI scans of 53 German patients with untreated relapsing remitting and secondary progressive MS showed a clear biphasic seasonal fluctuation of mean number of active lesions (n_{actles}), which was highest in spring and early summer (April n_{actles} 4.1) and lowest in autumn (October n_{actles} 0.77).[72] In addition, the mean number of active lesions were significantly higher in the first compared with the second half of the years ($p<0.006$).[72] Embry et al. correlated this pattern with published data on monthly 25(OH)D levels in 415 people (aged 50-80 years) from southern Germany, and found a striking inverse correlation ($r=-0.85$) when the 25(OH)D data were lagged by two months behind the MRI data.[73] Another study[74] found the highest mean number of active lesions in March (n_{actles} 5.7, SD 9.5) and the lowest in December (n_{actles} 3.3,

SD 5.0) when they conducted 11 consecutive scans obtained every four weeks on 120 relapsing remitting patients who were part of the untreated arm of a European/Canadian multi-centre, randomised, double-blind, placebo-controlled trial,[75] but the seasonal pattern over the four seasons was not significant. The fact that the results were not as strong as the German study could have been due to the selection of patients.[76] They were from different geographical locations and were in a very active phase of disease due to the strict inclusion criteria that were applied in the clinical trial.[76] A third study with serial MRI scans on a small sample (n=28) of Dutch MS patients also found a non-significant seasonal variation over the four seasons, with the highest mean number of active lesions in summer (n_{actles} 1.79, SD 3.60) and the lowest in winter (n_{actles} 1.18, SD 2.23).[77]

There is also some evidence of a seasonal change in immunoregulation in MS.[77, 78] In a cross-sectional study, the production of IFN-γ and IL-12 have been found to be highest in autumn and winter and lowest in spring and summer in 60 patients with progressive MS, compared to 53 healthy controls.[78] Even though the Dutch study described above did not find large differences in the mean number of active lesions, they did observe significant seasonal variation in IFN-γ and TNF-α, with maximum values found in autumn.[77] These patterns would be in line with the expected effects of UVR or vitamin D_3 on cytokine profile, because in vitro, UVR and/or 1,25-$(OH)_2D_3$ has been shown to selectively decrease the secretion of several cytokines including IL-12, TNF-α and IFN-γ.[7, 19, 20] Thus, low levels of those cytokines could be expected when UVR and/or vitamin D_3 levels are high, such as in summer, while high levels of these cytokines could be expected when UVR and/or vitamin D_3 levels are low, such as in winter.

3.4.3 Conclusions from studies on latitude, seasonal variation and MS

Overall, these studies have some epidemiological features that are consistent with the hypothesis that UVR-induced immune suppression is beneficial for MS. However, the evidence is far from conclusive. These ecological studies lack individual exposure level data and within populations there is a wide log normal distribution of personal sun exposure.[79] Because levels of 25(OH)D and UVR are correlated, ecological studies cannot identify whether UVR mediates its effect through the vitamin D pathway or whether it has other UVR-induced processes that independently suppress disease activation. Furthermore, these studies cannot control for the confounding effect of other possible causal factors in the aetiology of these diseases that may also vary by latitude or season, such as infection or diet. The lack of data on joint exposures at the individual level makes it also impossible to study interactions between environmental exposures. Low dietary omega 3 fatty acids have been implicated in MS.[26] and have also been shown to increase UVB-erythemal sensitivity.[80] Infections may play a role in autoimmune disease development but UVR exposure may act as a modifier, down-regulating infection-induced T helper cell overactivity.[35]

3.5 ANALYTICAL STUDIES ON UVR AND MS

There has been a lack of observational analytical epidemiological studies on the association between UVR exposure and MS. Potential cohort studies to examine relationships between past UVR exposure and MS are hampered by difficulties in serial exposure measurement during childhood, a long latent period and the low incidence at the population level. Cohort studies to examine the effect of personal UVR exposure on disease progression are more feasible. Case-control studies may be hampered by poor exposure measurement of past sun exposure and recall bias. The measurement of lifetime sun exposure has been problematic in skin cancer epidemiology over time.[81] The rarity of MS has generally limited to prevalent

rather than incident cases. However, since sun exposure and dietary vitamin D intake may be affected by the presence, severity or duration of disease, the contribution of disease to the measurement of these exposures must be carefully considered.

Recent improvements in communication technology now enhances the opportunity to conduct multicentre incident case-control studies, which include the additional benefit of providing large variation in field exposures such as regional UVR. Study measurements to assess past sun exposure are also developing. These include measurements of age-adjusted actinic damage on the dorsum of the hand,[82] the possible use of a ratio of spectrophotometric melanin density in exposed to unexposed sites to indicate cumulative lifetime sun exposure[83] and the possible use of mitochondria DNA deletions in the epidermis or CGRPs as a candidate biomarker for past UVR exposure.[84] The use of dietary-adjusted serum 25(OH)D levels as a biomarker for UVR exposure over the past 1-2 months also requires consideration. Furthermore, the classification of skin phenotype, a possible confounder or effect modifier of UVR-induced suppression, has improved with the use of spectrophotometric skin measures at 400-420nm which correlate (r=0.68) with melanin density of non-sun-exposed skin histologically.[85] These recent advances, together with our growing understanding of photoimmunology, indicate that future analytical observational studies on this issue should prove worthwhile.

A recent analytical case-control study on MS mortality reported that among outdoor workers, the adjusted odds ratios {95% confidence interval} for low, medium and high regional sunlight were 0.89 {0.64—1.22}, 0.52 {0.38—0.71} and 0.24 {0.15—0.38} for MS compared to indoor workers with low ambient sunlight.[86] A small study of vitamin D and mineral intervention in MS patients showed that, after a period of one to two years, less than half the number of exacerbations were observed compared to the expected number based on patient case histories.[87] Recently, it was demonstrated that vitamin D supplementation also influenced the cytokine profile of MS patients with low levels of vitamin D (25(OH)D < 20 ng/ml).[88] Compared to the untreated MS patients, the patients who were treated for six months with 1000 IU vitamin D_3 and 800 mg calcium had increased levels of TGF-β1, while the results for IL-2, TNF-α, IFN-γ and IL-13 were variable and inconclusive.[88] Vitamin D treatment of mice with EAE has also been shown to induce TGF-β1.[25] A cohort study conducted in northern Finland on IDMM, demonstrated that regular vitamin D supplementation in the first year of life was associated with a reduced risk of subsequent disease (rate ratio 0.12 {0.03—0.51}).[89]

3.6 INFLUENCE OF GENETIC FACTORS

Genetic variations could influence the relationship between UVR and MS. For example, genes regulating skin type and skin type associated differences in UVR-induced immunosuppression and/or outdoor behaviour could potentially confound or modify the relationship between UVR and disease.

As was discussed in chapter 2, the prevalence of MS differs by race, and thus skin colour, with higher rates among Caucasians compared to darker-skinned populations such as Amerindians, Chinese, Japanese, African blacks and New Zealand Maoris.[90] Skin type among Caucasians might also influence risk as was observed in a study on IDDM, where German cases tended to be fairer than controls.[91] Although the prevalence of MS is higher in fairer-skinned populations, fairer-skinned people absorb more UVR for a given level of ambient UVR compared to darker-skinned people,[1] leaving them less prone to vitamin D deficiency.[16] In addition fairer-skinned people require a lower dose to induce the same immunosuppressive effect of UVR.[92, 93] These observations seem not consistent with the UVR hypothesis. This apparent anomaly could reflect one or more of the following four explanations: (a) MS

prevalence is under-reported in vitamin D deficient populations; (b) vitamin D deficiency during early life may be more important (in the United Kingdom, whereas MS was uncommon among adult immigrants from India, Asia and Africa, the children of these immigrants had a higher MS prevalence similar to the general English population[94]); (c) people with darker skin may have other immunological changes related to skin pigmentation, not mediated by vitamin D, that can counter any effect of vitamin D deficiency on autoimmune up-regulation[95]; (d) protective factors operating outside the pathway of UVR-induced suppression are more common in dark-skinned populations (e.g. earlier age of childhood infections with Epstein-Barr virus).

In prostate cancer, another disease associated with sun exposure, an interaction has been demonstrated between sun exposure and a genetic marker of skin type TYR (tyrosinase).[96] Some mutations in TYR have been shown to cause albinism. TYR A2/A2 homozygotes were at reduced risk of prostate cancer (OR=0.48, $p<0.01$).[96] Stratification of cases and controls by quartiles of sun exposure showed that the protective effect of TYR A2/A2 was particularly strong in subjects who had received the greatest amount of sun exposure (OR=0.06, $p<0.01$).[96]

There might be other genetic factors that could influence the relationship between UVR and MS. There is currently good evidence that the human leukocyte antigen (HLA) region is involved in the development of MS. HLA molecules are involved in the presentation of short pathogen-derived peptides to T cells, a process that initiates the adaptive immune response.[97] The HLA class I molecules influence a T cell to differentiate into a CD8+ cytotoxic killer T cell, while HLA class II molecules influence a T cell to differentiate into A CD4+ helper T cell.[97] In MS, the association with a class II haplotype (DRB1*1501-DQB1*0602) has been the most consisted finding.[98] This haplotype encodes the β chain of the class II molecule.[97] However, a genetic study on MS conducted in Tasmania demonstrated an independent effect of a haplotype in the class I region.[98] The nature of the association with the HLA haplotypes has, however, not been fully elucidated. Somehow autoreactive T cells, that have escaped negative selection, can be activated by complexes of certain HLA molecules with particular self peptides such as myelin basic protein.[99] The fact that self-peptides, like all other peptides, are bound and presented by some HLA molecules but not by others presumably provides the basis for the association of MS with specific alleles.[99] If the HLA haplotypes influence the relationship between sun exposure and MS, then there must be some overlap in biological pathway. UVR has been shown to increase IL-10 which down-regulates HLA class II expression on antigen presenting cells such as macrophages through the down-regulation of IFN-γ.[7] It seems plausible that the effect of the UVR-induced increase of IL-10 is different depending on the genotype of a critical gene in the HLA class II region, and thus, gene-environment interaction might be possible based on this biological knowledge.

It has also been shown that people with relapsing-remitting MS and progressive MS have more often an innate production of low IL-10 and high TNF compared to people in a control population.[100] As a proxy measure for the innate production of cytokines for cases and controls, the mean production of five healthy first-degree relatives was calculated.[100] This pro-inflammatory cytokine profile of low IL-10 and high TNF was also associated with an increased risk of MS and seemed to act independently of the HLA class II association.[100] A study in a Japanese population found that individuals with vitamin D receptor gene allelic variants were at increased risk of MS,[101, 102] but no evidence for linkage or association of vitamin D receptor gene for MS was found in a Canadian population.[103] Variation in vitamin D receptor gene status has also been associated with IDDM[104, 105] and tuberculosis,[106] and an interaction between vitamin D deficiency and the vitamin D receptor gene was demonstrated for tuberculosis in an Asian population that migrated to the United Kingdom.[107] In this study, there was no evidence for an increased risk of a particular genotype (Fok1 or Taq1, and

Bms1), but for the Fok1 genotype, the combination of genotype ff and vitamin D deficiency was associated with tuberculosis (OR 2.8 {1.2—6.5}) and the presence of this genotype ff and undetectable levels of vitamin D was even more strongly associated with disease (OR 5.1 {1.4—18.4}).[107] Thus, genetic markers involving IL-10, TNF and the vitamin D receptor might also potentially influence the relationship between UVR and MS.

The identification of genes that increase or decrease MS risk would enhance the identification of interactions with sun exposure and might be useful for explaining the mechanisms by which UVR works. However, gene-environment interactions have been identified without the observation of a genetic risk association with disease, and the use of environmental subgroups might even assist in the identification of genes through an increase in power.

3.7 CONCLUSION

In conclusion, the epidemiological features of MS are, in part, consistent with recent photoimmunological work showing UVR-mediated immune suppression through several mechanisms. Some studies suggest that higher vitamin D levels may mediate any beneficial effect of UVR. However, the data are not conclusive and further analytical epidemiological and biomolecular work is required to assess the health risks and benefits and hence, the correct titration of this important natural exposure for optimal human health.

3.8 SUMMARY

This review examined the epidemiological evidence that suggests that UVR may play a protective role in MS. Recent work in photoimmunology has shown that UVR can specifically attenuate Th_1 cell mediated processes through several mechanisms. To date, most of the information has accumulated from population studies that have studied the relationship between geography or climate and disease prevalence. An interesting gradient of increasing prevalence with increasing latitude has been observed. Seasonal influences on both disease incidence and clinical course and, more recently, analytical studies at the individual level have provided further support for a possible protective role for UVR in MS but the data are not conclusive. Future analytical observational studies on this issue should prove worthwhile.

3.9 POSTSCRIPT

This chapter demonstrated that the epidemiological features of MS are, at least in part, consistent with the hypothesis that UVR exposure may reduce the risk of MS. However, further analytical studies are required to test this specific hypothesis. Before we do that we will investigate to what extent UVR levels might explain the regional variation of MS in Australia.

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Chapter 4

Regional variation in Multiple Sclerosis prevalence in Australia and its association with ambient ultraviolet radiation.

4.1 PREFACE

One of the most striking epidemiological features of Multiple Sclerosis (MS) is a gradient of increasing prevalence with latitude. A prevalence survey conducted in Australia in 1981 revealed a sixfold increase when moving from northern Queensland to Tasmania. In this chapter we assess to what extent ultraviolet radiation levels might explain the regional variation of MS in Australia. Most of this chapter has been published in Neuroepidemiology 2001;20:168-174.

4.2 INTRODUCTION

MS is a chronic demyelinating disease of the central nervous system (CNS). It is the most common neurological condition occurring during young adulthood and, despite extensive research, the aetiology of this serious disease remains unclear. To date, the key pathology of MS appears to involve a defect in immunological self-tolerance, possibly in conjunction with an infectious agent, resulting in a subsequent attack on myelin proteins mediated by T helper cells.[1] It is thought that the type 1 T helper cells (Th1) mediate this attack and that experimental allergic encephalomyelitis (EAE), the animal model of MS, is initiated by Th1 cells.[2] There is accumulating evidence that one or more infectious agents trigger the disease in a genetically susceptible host but the diverse nature of viral peptides that are capable of stimulating myelin basic protein specific T cells indicate that a number of common pathogens may initiate the disease.[1] Migration studies emphasise the timing of environmental agents and/or immunological development, with country-of-origin risk persisting if migration occurs after adolescence.[3] If one or more infectious agents trigger the disease, the status of the Th1 cell activity at the time of infection or during the period of host response could be of critical importance.

One of the most striking epidemiological features of MS is a gradient of increasing prevalence with increasing latitude.[4] In Australia, a more than sixfold increase in age-standardised MS prevalence has been demonstrated from tropical Queensland to Tasmania.[5-8] Within Europe and the United States, there is also at least a two- to threefold gradient of increasing MS prevalence with increasing latitude.[4] These geographical differences were initially interpreted to represent environmental influences which varied by latitude, such as climatic factors,[9] dietary characteristics[10, 11] and infectious agents.[12] More recent analyses indicate that geographical MS variation, at least in North America, may result from a complex interplay of genes and environment.[13] The marked Australian latitudinal gradient found in the national prevalence survey of 1981[5-8] is unlikely to be explained by genetic factors only, because the gradient is evident even among those of UK and Irish descent, a population subgroup that is predominantly Caucasian.[14] These findings together with the large latitudinal spread across the continent, stretching from 10 to 44° South in latitude, and a uniform health care system

suggest Australia provides a good opportunity to examine the relationship between latitude related factors and MS.

Several early studies reported MS prevalence to be inversely associated with levels of solar radiation,[9, 11, 15], especially winter solar radiation,[9] but others did not.[16, 17] In the study by Acheson et al.,[9] a strong inverse association was found between solar radiation at birthplace and MS prevalence among US veterans (r=-0.73 for average annual hours of sunshine; r=-0.80 for average December daily solar radiation). The correlations with latitude and a measure of the severity of winter (average annual degree days) were 0.76 and 0.67, respectively. Including all three terms (a solar radiation term, latitude and annual degree days) in a linear regression model left the contribution of solar radiation, but not latitude and annual degree days, significant. The possible protective effect of solar radiation on MS was not intensively investigated, partly because research evidence to support UVR-mediated changes in immune function was not then available. However, new insights into photoimmunology have provided support for the possibility that solar radiation exposure may have a beneficial effect on the autoimmune processes that underlie MS via an immunosuppressive effect on Th1 cell activity.[18-21] Possible pathways for this immunosuppressive effect include the synthesis of the active form of vitamin D_3, the suppression of melatonin secretion or a modification of local epidermal intracellular and intercellular signalling mechanisms, resulting in antigenic-specific systemic immunosuppression.[22-24] Thus, recent immunological evidence indicates that a re-examination of the relationship between UVR, latitude and MS is required.[18]

The aim of this chapter was to conduct an ecological analysis of the extent to which UVR levels might explain the regional variation of MS in Australia. We contrasted the relationship between UVR and MS prevalence with that of UVR and melanoma incidence, because the latter association has previously been demonstrated to be causal.[25]

4.3 METHODS

4.3.1 Sources of data

Australian MS data (crude prevalence, age-standardised prevalence, number of MS patients, total populations) for tropical Queensland, subtropical Queensland, Western Australia, New South Wales, South Australia and Hobart (Tasmania) were obtained from MS prevalence surveys carried out in 1981.[5-8] The state of Queensland is divided by the Tropic of Capricorn (at ca. 23.5°S latitude) into a tropical and a subtropical zone. The crude prevalence was defined as the ratio of persons with an acceptable diagnosis of MS living in the defined area on June 30th, 1981 (National Census Day) to the total population of the same area on the same day, and was expressed per 100,000 of population.[5-8] Age-standardised prevalences were calculated using the Australian population on June 30th, 1981 as the reference population.[5-8] Cases were ascertained from hospital records, treating doctors, MS Societies, Department of Veteran Affairs records and the Australian Bureau of Statistics.[5] The State Chronic Care Hospital Register and Commonwealth Department of Health notifications were also used in the Hobart region.[7] All patients were interviewed and examined, in regions other than New South Wales, where only 57% of the patients were interviewed and examined due to the large number of patients notified.[5] However, almost all of the remaining 43% of patients in New South Wales had been examined previously by a neurologist.[5] All patients in whom the diagnosis of MS was considered to be correct were classified, according to the diagnostic criteria of Rose et al.,[26] into clinically Definite, Probable or Possible groups. A 10% sample of examined MS patients were also reviewed by an independent assessor to assess the correctness of the diagnosis and of the diagnostic classification of each patient.[5-8] The veracity of the basic diagnosis was not disputed in any case in Queensland, Western Australia and Hobart, but the category of subtype disease was reclassified in 17, 30 and 20% of cases, respectively.[6-8] This information was not provided for New South Wales and South Australia.[5]

Monthly climate data — bright sunshine hours per day, maximum temperature (°C), minimum temperature (°C) and rainfall (mm) — were obtained from the Bureau of Meteorology of the Commonwealth of Australia for the largest city in each region (Townsville, Brisbane, Perth, Sydney, Adelaide and Hobart) for the 10-year period 1971-1980. Erythemal (skin-reddening) estimates for the nearest latitude and longitude to those cities were taken from data published on the website of the National Centre for Atmospheric Research (Germany). Those measurements were derived from satellite-based observations of atmospheric ozone and cloud reflectivity over the period 1979-1992. Spectral irradiance was calculated every 30 minutes at 1-nm steps from 280 to 400nm (covering the UVA and UVB spectrum) using a geographical resolution of 1.25° longitude by 1.00° latitude.

A latitudinal gradient of opposite direction compared to MS has been demonstrated for cutaneous malignant melanoma. Because UVR is an important environmental causal factor for cutaneous malignant melanoma,[25] melanoma incidence data were compared with MS prevalence data. Melanoma incidence data were used for the regions Western Australia, New South Wales, South Australia and Tasmania for the period 1978–1982.[27] For Queensland, data for the entire state in 1982 were used. Separate data for tropical and subtropical Queensland and for the other years were not available. The incidence densities were the average the annual world age-standardised rates per 100,000 population by sex and were provided by the State Cancer Registries.

4.3.2 Data analysis

Average annual means of climatic variables were calculated for the largest city of each region. The latitudes used for each region were the latitudes of those cities. Poisson regression under standard assumptions (log-linear relationship, Poisson errors, logarithm of population as an offset, maximum likelihood fitting) was used to calculate the crude prevalence for each region relative to Tasmania using binary (0, 1) terms for regions other than Tasmania. The 95% confidence intervals were calculated from the standard errors. To estimate the prevalence in each region that was predicted by its climate, a linear predictor taking the values for each city of one of the climate variables was used in the Poisson regression model in place of binary (0, 1) predictors. In a separate regression for each region the predictor variable was centred to have the zero value for that region. The predicted prevalence of that region was then calculated by exponentiating the model intercept. To estimate the prevalence of a region relative to the predicted value for Tasmania, the logarithm of the predicted value for Tasmania was subtracted from the offset term. The prevalence ratio was then obtained by exponentiating the intercept. The 95% confidence intervals were calculated from the standard error of the intercept. Pearson correlation coefficients were calculated for associations of climatic variables and latitude with age-standardised MS prevalence. For the correlations with the melanoma incidence, the age-standardised MS prevalence in total Queensland was used (18.3 per 100,000), but data for Brisbane (its capital city) were used for the climatic variables. Correlation coefficients of climatic variables with MS prevalence and melanoma incidence were compared using Fisher's transform. The relationships between UVR, sunshine, latitude, melanoma incidence and age-standardised MS prevalence were fitted with polynomial curves which included the predictor and its square. Comparing statewide prevalence data with climate or latitude of the largest city in the region would be subject to error if the climate or latitude of the city was not representative of the entire region. However, the representative latitudes, temperatures or rainfalls calculated for the populations of the six largest cities of South Australia, New South Wales and Western Australia in every case differed by less than 3% from the value of that variable for the capital city. For Hobart, the MS prevalence and climate data were obtained from the same area. To check whether the annual UVR levels changed substantially from 1979 to 1992, the linear association between annual UVR levels and year was estimated in a linear regression model that allowed the intercept but not the slope to differ between states (there was no evidence that the slope differed between cities). The annual increase in UVR levels was small (β=0.003, p=0.41).

4.4 RESULTS

4.4.1 Relationship between climatic variables and MS prevalence

In Australia, average annual UVR levels increased by a factor of 2.1, from 2.4 kJ/m^2/day in Tasmania to 5.1 kJ/m^2/day in tropical Queensland. The age-standardised MS prevalences were 75.6, 36.6, 28.8, 25.9, 21.0 and 11.8 for Hobart, New South Wales, South Australia, Western Australia, subtropical Queensland and tropical Queensland, respectively. Correlation coefficients for the association of the different climatic variables and latitude with the age-standardised MS prevalences are shown in Table 1. UVR levels and maximum temperature were most strongly correlated with observed MS prevalence, providing correlations of -0.91 (p=0.01) and -0.93 (p=0.01) respectively.

Table 1. Pearson correlations between climatic variables, latitude and age-standardised MS prevalence in Australia

	Correlation	p-value
Mean annual maximum temperature (°C)	–0.93	0.01
Mean annual UVR level (kJ/m^2/day)	–0.91	0.01
Latitude	0.89	0.02
Mean annual bright sunshine (hrs)	–0.87	0.03
Mean annual minimum temperature (°C)	–0.84	0.04
Mean annual rainfall (mm)	–0.54	0.26

Although the correlation for hours of bright sunshine in summer was much lower than the correlation in autumn, winter and spring (Table 2), this pattern was not observed with UVR and maximum temperature.

Table 2. Pearson correlations between sunshine, UVR and maximum temperature and age-standardised MS prevalence per season.

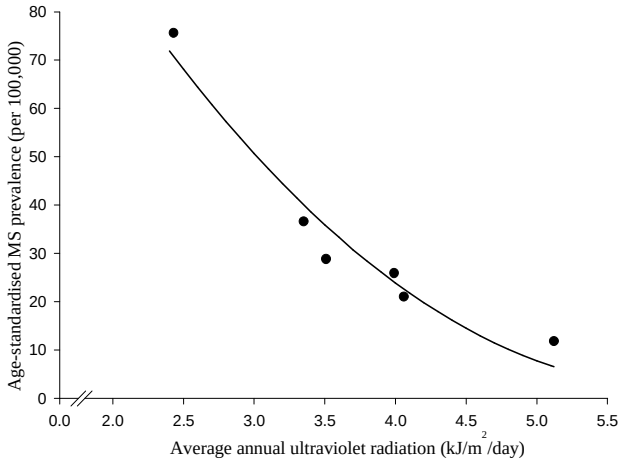
	Summer	Autumn	Winter	Spring
Mean sunshine (hrs)	-0.14	-0.84	-0.78	-0.90
Mean UVR (kJ/m^2/day)	-0.90	-0.89	-0.80	-0.89
Mean T_{max} (°C)	-0.97	-0.94	-0.86	-0.87

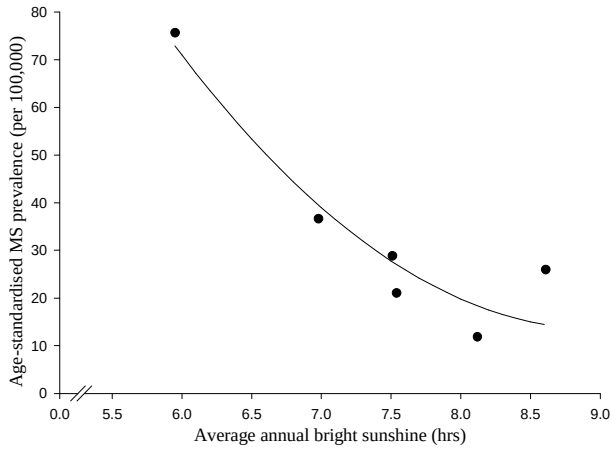
Table 3 shows that the UVR-predicted MS prevalences closely matched the actual crude MS prevalences observed in each region. Similarly, the UVR-predicted prevalence ratios for each of the five regions compared to Tasmania were very close to the actual prevalence ratios, with the UVR-predicted prevalence ratios within the 95% confidence interval of the observed prevalence ratios for each region. Prevalence ratios estimated from mean annual maximum temperature were also close to those actually observed, but prevalence ratios estimated from annual minimum temperature were not (data not shown). Figure 1 shows scatter diagrams with polynomial curves fitted to show the relationships between UVR, hours of bright sunshine and latitude and age-standardised MS prevalence. The UVR level was highly correlated with maximum temperature (r=0.94, p=0.05) and bright sunshine hours (r=0.82, p=0.05).

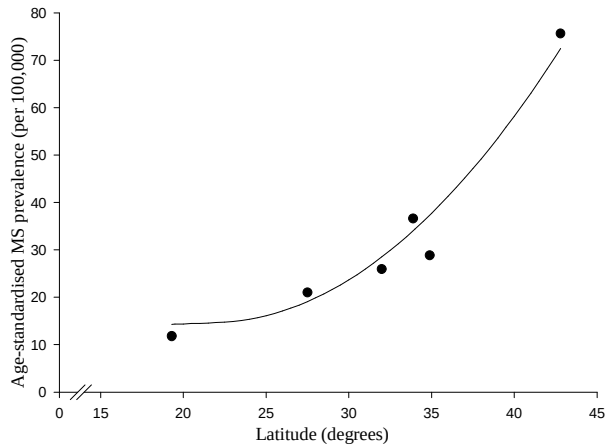
Table 3. Observed MS prevalences in Australia and theoretical UVR-predicted MS prevalences with prevalence ratios (PR) relative to Tasmania and 95% confidence intervals (CI).

	Crude Observed			Predicted from UVR levels		
Region	Prev	PR	{95% CI}	Prev	PR	{95% CI}
Hobart (Tasmania)	74.2	1.00		71.7	1.00	
New South Wales	37.2	0.50	{0.42—0.60}	36.7	0.51	{0.49—0.53}
South Australia	29.4	0.40	{0.32—0.49}	32.7	0.46	{0.44—0.47}
Western Australia	25.0	0.34	{0.27—0.41}	23.0	0.32	{0.30—0.34}
Subtropical Queensland	20.9	0.28	{0.23—0.35}	22.0	0.31	{0.29—0.32}
Tropical Queensland	11.1	0.15	{0.11—0.21}	10.1	0.14	{0.12—0.16}

Figure 1. The relationships of ultraviolet radiation, bright sunshine and latitude with Australian age-standardised MS prevalence with fitted polynomial curves.



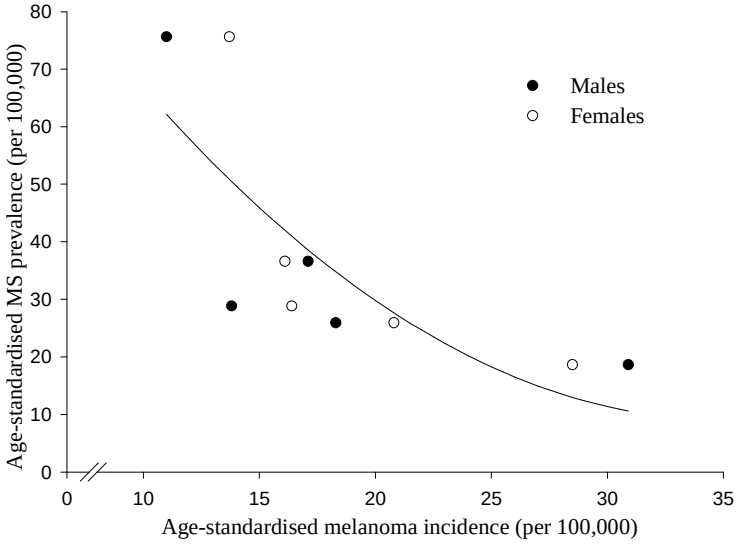




4.4.2 Comparison of melanoma and MS prevalence with regard to UV radiation.

The correlation of melanoma incidence with latitude (r=-0.88, p=0.05 for both males and females) was of similar magnitude but in opposite direction to the correlation of MS prevalence with latitude, while the correlations with UVR (r=0.75, p=0.15 for males and r=0.80, p=0.10 for females) and bright sunshine (r=0.42, p=0.49 for males and r=0.54, p=0.35 for females) were both non-significantly lower than for MS. The correlations between melanoma incidence and MS prevalence were -0.69 (p=0.19) for males and -0.72 (p=0.17) for females. The scatter diagram with fitted polynomial curves shows the inverse relationship between melanoma incidence and age-standardised MS prevalence (Figure 2). Thus, an increasing MS prevalence is associated with a decreasing melanoma incidence.

Figure 2. The relationship of melanoma incidence with Australian age-standardised MS prevalence with a fitted polynomial curve.



4.5 DISCUSSION

We examined associations of regional MS prevalence within Australia and UVR levels experienced by a significant proportion of the population in several regions. We found a close association between theoretical prevalence predicted from the UVR levels and the actual MS prevalence by region.

The development of MS appears to involve a complex interaction between genetic and environmental factors.[13] This study was conducted on a relatively homogeneous population, which has advantages when examining environmental exposures as conducted in this study. Furthermore, the population covers a large geographic area with marked climatic variation, and the data of the prevalence studies used had been obtained by a nearly identical method of estimating prevalence in the different regions which makes the occurrence of selection bias unlikely.

These ecological data support the previously proposed inverse relationship between UVR and MS, but they need to be interpreted with caution because of possible ecologic biases. Exposure misclassification may have occurred as UVR doses have not been measured at an individual level. For most individuals, personal UVR exposure varies between 5 and 15% of daily total ambient UVR.[28] The dose an individual receives will depend on ambient UVR, the time spent outside and the amount of clothing/sunscreen worn. We were unable to take those behavioural factors into account, although it seems likely that if we could control for them the observed inverse pattern between UVR and MS would be strengthened. In a colder climate, behavioural changes (less outdoor activity and more outdoor clothing) will further decrease personal UV exposure. Here, the finding that maximum, but not minimum, temperature correlated highly with the age-standardised MS prevalence is consistent with maximum temperature increasing individual sun exposure behaviour as well as with the correlation between ambient UVR and maximum temperature. Bright sunshine in summer seemed less important than in the other seasons, but this pattern was not seen for ambient UVR and maximum temperature. We cannot exclude the contribution of selective migration after onset of MS as we examined prevalence, not incidence, rates. We were unable to control for latitude when examining the relationship between UVR and MS due to collinearity and the low number of data points. In addition, other potential ecological confounders or effect modifiers, such as gender, age or skin type, were also unable to be assessed in this ecological analysis. In particular, the transmission of infectious disease is influenced by climate, and infectious agents have been implicated in the pathogenesis of MS.[12] The observation that MS is generally more common in females[29] is consistent with our findings, as it has been previously reported that females generally have a lower UVR exposure than males.[28] It will be important to examine whether control for personal UVR exposure reduces the previously established[29] positive association between female gender and MS prevalence.

Chapter 3 discussed in detail that the new insights into photoimmunology have provided support for the possibility that UVR could attenuate the autoimmune process that underlies MS.[18] Different mechanisms could be involved. In short, cytokine signalling alterations can induce soluble mediators which can exert systemic immunosuppression.[30] An effect could be mediated via the active form of vitamin D_3 (1,25-$(OH)_2$ D_3),[19] a hormone which is synthesised under the influence of UVR, and melatonin, which is suppressed through sunlight, can influence Th1-cell-mediated autoimmunity.[31]

These ecological data are unable to provide information on timing of sun exposure effects. UVR could play a critical role in the acquisition, clinical manifestation and progression of the disease. Migration studies imply that an environmental agent could be operating prior to puberty in predisposed individuals and that there is on average a 10-year latency time before

clinical manifestation of the disease.[3] This is in line with the observation from Acheson et al.[9] who found that the correlation between average December solar radiation and MS prevalence was higher for solar radiation at birthplace (r=-0.80) than for solar radiation at residence (r=-0.59). Animal models have demonstrated that UVR, vitamin D injections, continuous light exposure or melatonin blocking could prevent autoimmunity, delay the onset of EAE, decrease the severity of symptoms and/or prolong survival.[24, 32, 33] In EAE, mice who were radiated with UV 30 minutes daily for seven consecutive days before immunisation with mouse spinal cord homogenate did not show any clinical signs, while neuropathological signs were absent in 75% of the mice.[32] When the UV treatment was administered for the first time at the onset of clinical signs of EAE, it did not abort any ongoing EAE nor prevented relapses after the re-immunisation.[32] Mice injected with vitamin D_3 (1 or 3 days before immunisation) did not show any clinical EAE signs either.[33, 34] However, in contrast to UVR, vitamin D_3 administration at the onset of clinical signs halted the EAE progression.[34] Furthermore, vitamin D deficient mice had earlier EAE onset by one day.[34] So it seems that the immunological status may be altered by UVR or vitamin D_3 and that this status at the time of immunisation is important in order to prevent the clinical signs of EAE. In humans, it is known that UVR can suppress Th_1 cell activity for several weeks.[35] The status of the Th_1 cell activity, influenced by the amount of sunlight, could potentially play a critical role in the pathogenesis of MS, either at the time of initial infection or during the period of host response to that infection. A viral candidate for infection could be the Epstein-Barr virus (EBV). A meta-analysis reviewing case-control studies that assess the relationship between anti-EBV seropositivity and MS estimated a summary odds ratio {95% confidence interval} of 13.5 {6.4—31.4},[36] and a prospective cohort study and case-control study, with samples taken prior to MS onset, also support a role of EBV in the aetiology of MS.[37, 38] For example, the cohort study found a strong association {95% confidence interval} with MS for antibodies to the Epstein-Barr nuclear antigen type 2 (relative risk 3.9 {1.1—13.7}).[37]

The inverse associations between MS prevalence and melanoma incidence in this analysis were also consistent with a postulated protective effect of UVR on MS. UVR is a well-established environmental causal factor for melanoma, although the associations are complex in regard to timing, sun exposure intensity and duration and skin type.[39] A systematic review of MS case-control study reported that intermittent sun exposure (odds ratio 1.71) and sunburn at any age (odds ratio 1.91) related more strongly to malignant melanoma than total sun exposure (odds ratio 1.18).[40] The association of latitude with melanoma incidence was of a magnitude similar to that with MS prevalence, but the correlation between UVR and malignant melanoma was weaker than for that between UVR and MS prevalence. Freedman et al.[40] contrasted the relative strength of associations between solar radiation and MS and non-melanoma skin cancer. Using a case-control approach, the negative association between the combined effect of the highest level of residential and occupational exposure to sunlight and MS mortality was high (odds ratio 0.24 {0.15—0.38}) compared with the positive association for non-melanoma skin cancer (odds ratio 1.38 {1.12—1.69}). Both comparative analyses indicate that further assessment of UVR as a causal protective factor in the aetiology of MS is warranted.

Other autoimmune diseases may also be inversely related to UVR exposure.[18] For type 1 diabetes, for example, a gradient of increasing incidence with latitude in Europe and North America has been observed,[41, 42] the incidence seems lower (odds ratio 0.67 {0.53—0.86}) among children who were supplemented with vitamin D in infancy,[43] and the onset shows seasonal variation with a winter peak.[44] In addition, a recent cohort study in northern Finland demonstrated that regular vitamin D supplementation in the first year of life was associated with a strong reduced risk of subsequent type 1 diabetes (relative risk 0.12 {0.03—0.51}).[45]

In conclusion, this ecological analysis demonstrates that the regional variation of MS prevalence in the continent of Australia can be closely predicted by regional ambient UVR levels. This is consistent with recent immunological evidence suggesting that personal exposure to ultraviolet radiation may reduce the risk of acquiring MS due to UVR-induced immunosuppression. Analytical epidemiology studies investigating this specific hypothesis are now required.

4.6 SUMMARY

The aim of this chapter was to conduct an ecological analysis of the extent to which UVR levels might explain the regional variation of MS in Australia. MS prevalence data for six Australian regions were compared with UVR levels of the largest city in each region, with some other climatic variables and with the melanoma incidence in the same regions. A close association was found between the theoretical MS prevalence predicted from UVR levels and the actual prevalence. Furthermore, the negative correlation between UVR and MS prevalence ($r=-0.91$, $p=0.01$) was higher than the positive correlation observed for UVR and malignant melanoma incidence ($r=0.75$, $p=0.15$ for males and $r=0.80$, $p=0.10$ for females). This chapter demonstrated that the regional variation in MS prevalence in the continent of Australia could be closely predicted by regional UVR levels. It is consistent with the hypothesis that UVR exposure may reduce the risk of MS possibly via T lymphocyte-mediated immunosuppression. Analytical epidemiology studies are required to investigate this specific hypothesis.

4.7 POSTSCRIPT

This chapter and chapter 3 have provided evidence as to why an analytical epidemiology study is required that measures UVR at an individual level to investigate the hypothesis that UVR exposure may reduce the risk of MS. We have conducted a case-control study that examined this particular hypothesis. The methodology of this study will be outlined in the next chapter; the results of the case-control study in regard to this hypothesis are discussed in chapter 8.

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Chapter 5

Methodology of the Tasmanian Multiple Sclerosis case-control study.

5.1 PREFACE

An analytical study was conducted that measures environmental factors at an individual level to investigate the hypothesis that ultraviolet radiation (UVR) exposure may reduce the risk of Multiple Sclerosis (MS), and to examine whether other environmental factors might influence the development of MS. The following chapter outlines the methodology of this Tasmanian MS case-control study.

5.2 STUDY DESIGN

We conducted an epidemiological case-control study, using prevalent cases and controls matched to cases on sex and birth year. This design was most feasible and efficient for achieving the aims of this analytical work.

A prospective cohort study would have been the preferred option, because it assesses exposure prior to disease onset. Therefore, there would be certainty that exposure preceded disease. In addition, recall bias, which is a differential recall between cases and controls, is not a major issue. This design was not possible due to the rarity of MS and the expected latency between the time of exposure and disease onset. A prospective study would have required a sample size of 2,085,714 person years to detect a relative risk of two (assuming a type I error of 0.05, power of 0.80, probability of exposure of 50%, probability of disease 3.5 per 100,000 person years). The probability of disease is based on the incidence rate data available when starting this study (3.5 per 100,000 person years, Hobart, Tasmania, 1971—1981).[1] If the study would run for five years, 458,857 people would need to enter the study (assuming a loss of follow up and disease occurrence midway through the study in 18% of the subjects). This is slightly more than the total Tasmanian population. During the study it became obvious that the incidence rate of 1981 is an underestimate of the current incident rate. In Southern Tasmania, which has 49% of the Tasmanian population in June 1999,[2] there were on average 20.5 incident MS cases per year in 1999—2002 (data from Dr B Taylor, review records of the Royal Hobart Hospital and private rooms of the two neurologists in southern Tasmania). This provides an incidence rate of 8.5 per 100,000 person years for Tasmania, but even with this higher incidence rate a cohort study is not a very feasible option.

An alternative design would have been an incident case-control study, using cases that were newly diagnosed with MS. This design limits recall bias and disease related changes in exposure variables. Using the incidence rate available when starting this study (3.5 per 100,000 person years), an average of 16 new cases would have been diagnosed per year in the Tasmanian population (n=470,803)[2]. With an identification rate of 90% and a response rate of 90%, this would have provided us with 13 study participants a year. Over seven years would have been required to recruit 100 cases. An additional criterion for our study that subjects had to have at least one grandparent who was born in Tasmania was essential for the genetic study that ran concurrent to our study. Conducting the two studies in this fashion provided an opportunity to examine gene-environment interactions and resulted in

considerable savings compared to the cost of conducting two separate studies. However, this additional criterion even further decreased the feasibility of conducting an incident case-control study. Given the new estimates we have available now, conducting an incident case-control study without the additional criterion of a grandparent born in Tasmania could become feasible for a future study. Power could be further increased if this study was conducted in more than one geographical location in Australia.

5.3 TIMELINE

The recruitment of people with MS started in August 1998 due to the start of the concurrent genetic project on MS. The interviews with cases and controls commenced in March 1999 and were completed in June 2001. The project received ethics approval from the Human Research Ethics Committee of the Royal Hobart Hospital, and written consent was obtained from all participants.

5.4 CONCURRENT GENETIC STUDY

In collaboration with the Walter and Eliza Hall Institute in Melbourne, a genetic study on MS was conducted. The fieldwork, coordinated by the author, was carried out between August 1998 and March 2001. A haplotype-based case-control strategy was developed to search genome-wide for shared MS susceptibility genes in people of Tasmanian ancestry. The recruitment strategy focussed on cases and controls who had at least one grandparent who was born in Tasmania, because these participants had a high likelihood of being descendants of the initial settlers, from the early to mid 1800s. It is estimated that 65% of Tasmania's current population of 470,000 are descendants of those initial settlers who were mainly of English, Scottish and Irish ancestry.[3] Also, rates of immigration to Tasmania have lagged far behind the mainland since the 19th century, which has resulted in a more genetically homogeneous population than mainland Australia. These factors together make Tasmania an attractive place to study the genetics of complex disease. For each case and control, genotype information on a number of close relatives were used to reconstruct the haplotypes probabilistically. Recruitment of relatives was prioritised as (in order of preference): parents, grandparents, siblings, and offspring and spouses. Two hundred and twenty-two people with MS were assessed, including 28 people recruited in Victoria by the Walter and Eliza Hall Institute. Of those, 181 were admitted to the study utilising the diagnostic criteria that were also applied to the Tasmanian MS case-control study (see below). Eight hundred and forty-three close relatives (4.7 per case on average) were recruited.

Controls without a history of MS and at least one grandparent born in Tasmania were nominated by the cases. Controls of this genetic study are referred to as "genetic controls". With this approach, genetic controls would be more likely to match closely on age, geographic origin, socio-economic background and ancestral background to the case. Matching on ancestral background should limit population stratification, which is the observation of a difference in allele frequencies between cases and controls that is attributable to diversity in background population rather than disease status.[4] In addition, this method increased the chances of recruiting close relatives of the genetic controls, which was deemed more likely to succeed if the control had a relationship (non-genetic) to the case. Where MS patients were unable to nominate a person to act as a genetic control, controls from the Tasmanian MS case-control study were recruited to also participate in this genetic study. Non-biological relatives (spouses, 44% (46/105), in-laws, 5% (5/105)) accounted for approximately half the genetic control cohort with friends (18% (19/105)) and controls from the Tasmanian MS case-

control study (33% (35/105)) representing the remainder. In total, samples for 105 genetic controls and 400 of their close relatives were collected.

A genome-wide search has been performed at a resolution of 5 cM to detect shared chromosomal regions inherited identical-by-descent in distantly related people with MS using 811 genetic markers from the ABI PRISM™ HD-5 set.[5] In 2002, a publication was published in the American Journal of Human Genetics where the haplotype approach was successfully applied at the Human Leukocyte Antigen region.[5] We replicated the association with the DRB1*1501-DQB1*0602 haplotype and determined that the class I and/or extended class I region, defined by a genomic segment of ~350 kb between MOGCA to D6S265, contained susceptibility and protective haplotypes that independently influenced risk of MS.[5] The analysis of the full genome search is being conducted in 2003 and 2004.

5.5 SOURCE POPULATION AND STUDY SAMPLE

5.5.1 Source population

The source population for the Tasmanian MS case-control study was the cohort of persons under the age of sixty years, resident on the mainland of Tasmania, with at least one grandparent born in Tasmania. The two sparsely populated islands on the north-east and north-west coast of Tasmania were excluded for logistical reasons.

5.5.2 Recruitment of cases

Cases were members of the source population who were diagnosed with MS. They were volunteers who responded to requests for participants, conveyed by several means. The study was advertised on radio stations, in local newspapers and MS Society magazines. Information evenings were held at the local MS Societies and information kits were sent out to neurologists, general physicians, general practitioners and pharmacists, who were encouraged to publicise the posters and to inform people with MS about the program. All neurologists in the south of the state searched their records, sent letters to patients inviting them to participate if they were eligible, and encouraged newly diagnosed MS patients to participate in the program.

The source population of the concurrent genetic project was broader. There were no age restrictions and locality boundaries. In total, 194 cases were recruited by the Menzies Centre to participate. Twenty-five could only participate in the genetic project, because they fell outside the source population of the Tasmanian MS case-control study. Of those, eighteen people were sixty years or older and seven lived on the mainland of Australia. Of the remaining 169 people, another 30 (17.8%) did not meet the criteria set in regard to the diagnosis of MS. For one person we were unable to conduct a neurological assessment to confirm the MS diagnosis, one person died before we could conduct the interview and one person deteriorated to the extent that the disease became too severe to participate. In total, 136 cases participated in the study.

The exact case response rate is unknown, because: 1) cases volunteered to participate, 2) there is no disease register available in Tasmania and 3) our source population consisted of people under the age of sixty years, resident on the mainland of Tasmania, with at least one grandparent born in Tasmania. Our best estimate, based on available prevalence data,[1] is that there were 183 people with definite MS in this source population eligible to participate in the study (see the text in the block below for calculations of this estimate). Firstly, this number has to be interpreted with caution because of the number of estimates, extrapolations and

assumptions that had to be applied in order to calculated this number. Secondly, our criteria set for this research work were more stringent (see case ascertainment) than the criteria of Rose et al.[6] for clinical definite MS used for the prevalence surveys. Our estimate could, however, suggest that between 76% (139/183) and 92% (169/183) of the eligible people with MS were recruited which means the case response is at least as high as that from the controls, and it might have led to some selection bias.

1. Estimate of the prevalence for definite MS in Hobart in 1981 for people under the age of 60 years.
   The most recent prevalence survey in Hobart in 1981 provides us with an unadjusted prevalence for definite and probable MS for the total population of 74.2 per 100,000 (125 cases, 168,363 population at risk). With the data available in the publication we could calculate that the prevalence was 69.1 per 100,000 for those under the age of 60 years (100 cases, 144,750 population at risk). Also provided is a prevalence of 54.0 per 100,000 for definite MS for the total population (91 cases, 168,363 population at risk). No age groups were provided to calculate the exact prevalence for definite MS for people under the age of 60 years, but the best estimate was to take the fraction of people with definite MS over those with definite and probable MS (91/125=0.73) and to multiply that with the total number of cases with definite and probable MS under the age of 60 years. This provides us with a prevalence of 50.3 per 100,000 for definite MS under the age of 60 years (cases 0.73*100=73, 144,750 population at risk).

2. Extrapolation to a prevalence for definite MS in Hobart in 1999 for people under the age of 60 years.
   The same prevalence survey in 1981 was also conducted in Newcastle (New South Wales, Australia).[1] An age-standardised prevalence for definite and probable MS for the total population of 37.1 per 100,000 was observed. A more recent prevalence survey conducted in 1996 in the same region using the same methodology showed an age-standardised prevalence of 59.1, indicating a 1.57 fold increase compared to 1981. If we apply this 1.57 fold increase to the data in Hobart, then we expect a prevalence of 79.0 per 100,000 for definite MS for people under the age of 60 years.

3. Estimate of the population at risk
   Our population at risk consists of people under the age of 60 years who had a grandparent born in Tasmania. In 1999, Tasmania had a total population of 470,803 with 388,070 (82.4%) under the age of 60 years. Our best estimate of the percentage of people with a grandparent born in Tasmania comes from our controls from the electoral roll. Figure 1 shows that 163 people informed us that they did not have a grandparent born in Tasmania, while 359 people were eligible to participate and thus had a grandparent born in Tasmania. Of the 43 not resident in Tasmania and the control with MS we do not know whether or not they had a grandparent born in Tasmania and were therefore not included in the calculation. The percentage born in Tasmania is then estimated to be 68.8% (359/(359+163)). This gives us a total population at risk of 232,592 (0.688*338,070).

4. Estimate of total number of cases in Tasmania with definite MS under the age of 60 years with a grandparent born in Tasmania
   We assume that the estimate of the prevalence for definite MS under the age of 60 years in Hobart is similar for that in total Tasmania and similar for those with or without a grandparent born in Tasmania. Using that assumption, we multiply the prevalence of 79.0 per 100,000 with the population at risk estimated above (n=232,592) and we find a total number of cases of 184.

5.5.3 Recruitment of controls

Eligible controls were all members of the source population not diagnosed with MS. They were selected from the roll of registered electors, a comprehensive computerised listing of the population, maintained by the State Electoral Office of Tasmania. For each case, two control subjects were randomly selected. They were matched to the index case on gender and birth year.

A letter of invitation and an information booklet was sent out to each potential control who was drawn. This was followed up with a phone call from one of the two research assistants approximately a week later. The research assistant ascertained the eligibility and willingness of the potential control. In the situation that no phone number was available, the potential control was requested to contact the Menzies Centre on a toll free number. When a potential control was not eligible, was not able or unwilling to participate, the process was repeated until an eligible control was found that was able and willing to participate. Because this was a time consuming process, especially when people were difficult to trace, the average time between the interview of the cases and controls was 5.2 ± 3.7 months. Figure 1 shows the details of eligibility and participation. For the 136 cases included in the study, 566 potential controls were approached, 359 were eligible as controls (no MS, living in Tasmania and at least one grandparent born in Tasmania) and 272 of the eligible controls participated (response rate 76%). A response rate of 76% is high, reducing non-response bias, but it is conceivable that some selection bias may have occurred.

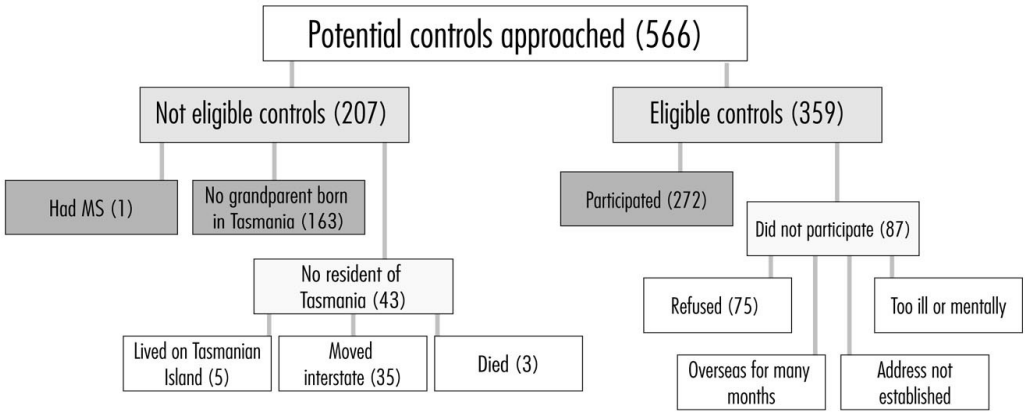

Figure 1. Overview of the eligibility and participation of controls.

5.5.4 Case ascertainment

All respondents were interviewed and examined by one of the six participating neurologists. In addition, available or newly performed magnetic resonance images (MRI) were assessed (n=134) or, at a minimum, MRI reports (n=2) were obtained. The criteria from Poser et al.[7] (discussed in chapter 2) of clinically definite or laboratory-supported definite MS were set as the minimum standard by which patients were included in the study. In addition, patients were required to have cerebral MRI abnormalities consistent with MS, as defined by the criteria of Paty et al.[8] (see chapter 2). All patients with a classification of primary progressive MS had to exhibit progressive neurological disability for at least one year, to have no other better explanation for the clinical features and to have not only relevant spinal cord abnormalities but also changes on cerebral MRI consistent with demyelination.

Of the 169 people under the age of sixty and living on the mainland of Tasmania, 139 were admitted to the study utilising the above criteria, with the final decision concerning inclusion based on the consensus opinions of two neurologists (T Kilpatrick and H Butzkueven) after all relevant data had been compiled and assessed. Thirty patients were excluded from the study: 17 had a normal, non-diagnostic or atypical MRI; 5 had monophasic disease (3 optic neuritis, 1 transverse myelitis, 1 brainstem demyelination); 5 had other neurological disorders (spinocerebellar ataxia, syringomyelia, radiculopathy, devic's disease and multiple lacunal strokes); 2 had other non-neurologic disease (sjoegren's syndrome, fibromyalgia syndrome); and 1 had MS as well as demyelinating peripheral neuropathy neuritis.

5.6 DATA COLLECTION AND STUDY MEASUREMENTS

5.6.1 Main interviews

Two research staff experienced in interviewing conducted all interviews and measurements. One interviewer was responsible for conducting interviews with cases and controls who lived in the north of Tasmania. The northern region was defined as the area served by the 63 and 64 telephone codes. The second interviewer was responsible for interviewing cases and controls in the south of Tasmania (the 62 telephone district). The average interview time (SD) of the main interviews was 54.1 (17.7) minutes (cases 61.9 (19.1) minutes, controls 50.1 (15.6) minutes).

5.6.2 Proxy interviews

At the end of the interview, subjects were asked to nominate a proxy, someone who had close contact with the subject during the subject's childhood and adolescence. This proxy was then approached to participate in a telephone interview. To overcome the limitations of a telephone interview only and to provide sufficient time to think about the questions, a questionnaire was sent out to the proxies before the telephone interview took place.

Of all the subjects, 84.1% (343/408) had a proxy available who was willing and able to participate (cases 84.6%, controls 83.8%). The reasons for not having a proxy were: no suitable proxy available (41), unable to help because the nominated proxy did not know enough about the subject (21), the proxy refused to participate (1) and the proxy that was nominated already contributed to a large extent in the case interview due to memory problems of the case (1).

The participating proxies were mostly mothers (70.9%), while fathers (5.0%), older brother or sisters (16.9%) and younger brothers or sisters (4.4%) participated less frequently.

The proxy questionnaire contained those questions of the main questionnaire that referred to the subject's childhood and adolescence (Appendix E). The study factors assessed were sun exposure, chemical exposure, infections, immunisations, dietary intake, breastfeeding and age of menarche (for women only). The average interview time (SD) of the proxy interview was 13.7 (5.9) minutes (cases 15.5 (6.7) minutes, controls 12.8 (15.5) minutes).

5.6.3 Repeat interviews

To assess the reliability of the measurements of the main study factors, repeat interviews were conducted by the project coordinator (the author) on cases and controls living in the south of the state. It was aimed to conduct the analysis on about 50 cases and 50 controls. The first 58 cases for which interviews were conducted were recontacted after approximately two months to request their participation in a repeat interview. Of those, 55 (94.8%) were willing to conduct the repeat interview. Three had to be excluded after the repeat interview had taken place, because they showed to have a negative MS diagnosis. For controls, the first 52 subjects for which an interview was conducted were recontacted for a repeat interview and all of them (100%) were willing to participate in a repeat interview. The final analysis was conducted on 52 cases and 52 controls.

The repeat questionnaire contained those questions of the main questionnaire that referred to the main study factors of the study (Appendix F). The factors assessed were sun exposure, sunburn and skin sensitivity, exposure to chemicals, infections, immunisations, dietary intake

of vitamin D, breastfeeding and age of menarche (for women only). The average interview time (SD) of the repeat interviews was 31.2 (8.6) minutes (cases 31.9 (8.8) minutes and controls 31.9 (8.8) minutes). The average time (SD) between the main interview and the repeat interview was 11 weeks (77.6 days (20.3), 75.1 days (16.8) for cases and 80.1 days (23.1) for controls).

5.6.4 Evaluation forms

After each interview (main, proxy or repeat interview), the interviewer completed an evaluation form rating the subject's recall ("very well", "fairly well, some difficulty recalling", "unable to recall" or "declined questions") and the difficulties perceived (Appendix G). Separate assessments were made for sun exposure, medical history, and other factors such dietary intake, chemicals, smoking, alcohol consumption, medication and family information, depending on the questionnaire. In addition, the person's English communication, cooperation and ability to recall were rated as "good", "fair" or "poor" and it was noted whether other people participated in the interview and what their contribution was to the different sections ("none", "little", "some", "a lot").

5.6.5 Personal residence and work calendars

Lifetime calendars were used as an additional method to obtain information, because different cognitive interviewing technique have been shown to produce more accurate long-term recall in some circumstances.[9] Prior to the main interview, subjects were sent a personal residence and work calendar (Appendix D) and asked to fill out, for each year of their life, information regarding residence, schools and occupations and exposure to pets and farm animals. During the interview, the interviewer checked the calendar and asked two sun exposure questions (see below) for each year of their lives, but the information already filled out on the calendar was utilised to identify blocks of years where the sun exposure lifestyle was constant or not. Where participants indicated that they had owned dogs, more detailed exposure data was obtained for those particular periods.

5.6.6 Information bias

Information bias is a systematic error which can arise when information is collected about or from subjects that is erroneous. This can lead to misclassification if a variable is measured on a categorical scale. A common type of information bias is recall bias, which can occur in case-control studies where subjects are interviewed to obtain information after disease has occurred. Cases might report exposures differently from controls because of their knowledge and feelings about the disease, or due to a cognitive decline associated with the disease. To obtain insights whether recall bias might have occurred, our subjects were asked to indicate, as the first question of the interview, what they thought was the importance of the following factors as a possible cause of MS: smoking, infections, genetic influence, high body weight, allergy, stress, concussion, exposure to chemicals, lack of exercise, climatic temperature, other climatic factors (e.g. amount of sunlight exposure) or any other factors. The factors could be rated as 1 = "possible cause, very important", 2 = "possible cause, moderately important". 3 = "possible cause, not very important", 4 = "not a possible cause", 5 = "don't know".

Another type of information bias is interview bias, where interviewers influence the subjects in their answers by the way they are behaving or administering the questionnaire. To limit interview bias, a standardised questionnaire and interview protocol was employed. The wording to be used by the interviewer was agreed in advance and typed on the questionnaire. Interviewers were not permitted to re-word the questions, or to ask them in any different

sequence to that on the questionnaire. Every six months, the interviewers were observed while conducting interviews and meetings were held to ensure standardisation over time and to discuss the coding of the questions.

5.6.7 Study measurements

A variety of instruments were used to obtain the best information possible on the study factors investigated while limiting the burden on the subjects. Many questionnaire-based indices were applied, but where possible, objective measures were used. The same information was obtained from cases and controls.

Migration studies have suggested that MS might be acquired during childhood or adolescence, indicating that the timing of exposure might be important. We obtained data on the timing of exposures using five-year age categories (0—5 years, 6—10 years, 11—15 years, 16—20 years, etc.) as recommended by the guidelines reported in Neurology 1997.[10] Because it was our aim to identify factors that might influence the development of MS, for many factors we obtained information until the age of diagnosis of the cases. For controls we used the age of diagnosis of the case they were matched too. For some factors we also obtained data on recent exposure. Appendix A, B and C show the questions that were used and the measurements conducted during the main interview. All factors examined will be described in detail below.

Past sun exposure. For the amount of ultraviolet radiation exposure in the age periods 6-10 years, 11-15 years, 16-20 years and the last three years, subjects were asked about the amount of time they would normally have spent in the sun during weekends and holidays ("< 1 hr a day", "1 to 2 hrs per day", "2 to 3 hrs per day", "3 to 4 hrs per day" and "≥ 4 hrs a day") and about how much their activities (such as playing, day sports, spectator sports, gardening, walking and working activities) took them outside ("not that often", "a moderate amount", "quite a lot", "virtually all the time"). Both questions were asked for summer as well as for winter. The "Time in the sun" question in summer (but not winter) was also used in the personal residence and work calendar and this information was therefore available for each year of the subjects' life. Both questions have been previously validated in Tasmania using polysulphone badge readings as the comparison method and showed to be reliable and valid measure of habitual sun exposure in teenagers (see chapter 6 for more detail).[11] They also have predictive validity for UVR related diseases in adults based on results from other studies recently conducted at the Menzies Centre (melanoma skin cancer, non-melanoma skin cancer, prostate cancer).[12, 13]

Occupational exposure was assessed by the question as to whether their jobs were overall mainly indoors, both indoors and outdoors or mainly outdoors. In addition, similarly to the "Time in the sun" question, the lifetime calendar had a question on how much time their occupation took them normally outside during working hours each year. Participants could answer in hours and/or minutes.

Lifetime sun exposure was objectively assessed by measuring actinic damage on the hand and by counting the number of naevi greater than 5mm on the left arm. Actinic damage was measured by making silicon skin surface moulds. The research assistant mixed silicone liquid with catalyst and applied that on the dorsum of left hand of the subject (Figure 2). After seven minutes, the cast was removed. The method is simple and safe and has no side effects. The fine lines on the underside of the cast were examined under a low-power dissecting microscope and graded by a single observer according to the method of Beagley and Gibson.[14] This provides grades ranging from one (undamaged skin) to six (severe deterioration). The method has a high observer repeatability (intra class correlation coefficient for inter-observer reliability = 0.89)[15] and actinic damage has been found to be associated with

age,[16] lifetime sun exposure[17], outdoor occupations and leisure activities,[15] solar keratosis,[15, 16] and basal and squamous cell cancer.[15]

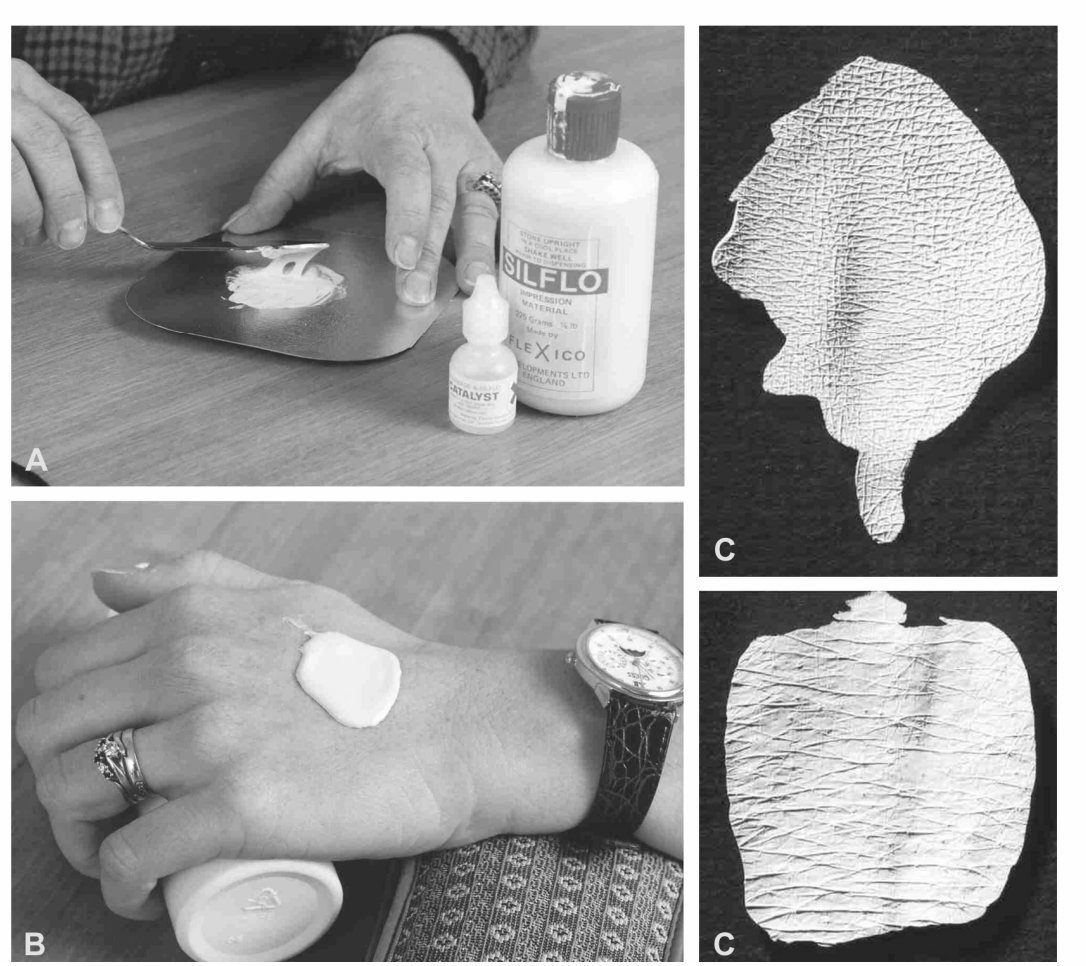

Figure 2. Making a silicon skin surface cast. The research assistant mixes silicon with catalyst (A) and applies it on the left hand of the participant (B). After 7 minutes the cast is removed and the fine lines on the underside of the cast (C) is graded using a low-power dissecting microscope.

Vitamin D. The amount of dietary vitamin D was assessed over the age period 10-15 years and in the last three years by recording the consumption of fish, eggs, milk and vitamin D supplements (see also dietary intake). The amount of 25(OH)D, a precursor of vitamin D which is produced under the influence of UVR, was measured in serum samples taken at the time of interview. 25(OH)D can act as a measure of recent sun exposure. Levels of 25(OH) in cases are influenced by disease duration and disability and can therefore not be used as a marker of sun exposure prior to disease onset in order to obtain a risk estimate of disease. It was, however, used to: (1) compare 25(OH)D status between cases and controls; (2) describe characteristics associated with low 25(OH)D status within each group; and (3) quantify what proportion of the case-control difference in 25(OH)D status could be attributed to differences in these characteristics. This analysis was not part of this thesis.

Skin phenotype, sun sensitivity, lifetime sunburn and sun avoidance behaviour. Skin phenotype was assessed objectively at the upper inner arm and the buttock, body sites usually not exposed to the sun, with a spectrophotometer and chroma-meter. We have previously shown that skin phenotype based on melanin density can be estimated objectively and relatively accurately by spectrophotometry (see chapter 7 for more detail).[18] At the start of

the project, the spectrophotometer was not available for all subjects. For that reason a chroma-meter was also used. A chroma-meter measures colour and provides estimates of lightness (L*), yellowness (a*) and redness (b*) of the skin.[19] Although these measures have been used in a considerate number of studies as a measure of skin phenotype and the lightness estimate has been shown to correlate with melanin density in skin biopsies(r=-0.46),[18] the measure obtained with the spectrophotometer correlated far better with melanin density in skin biopsies (r=0.68).[18]

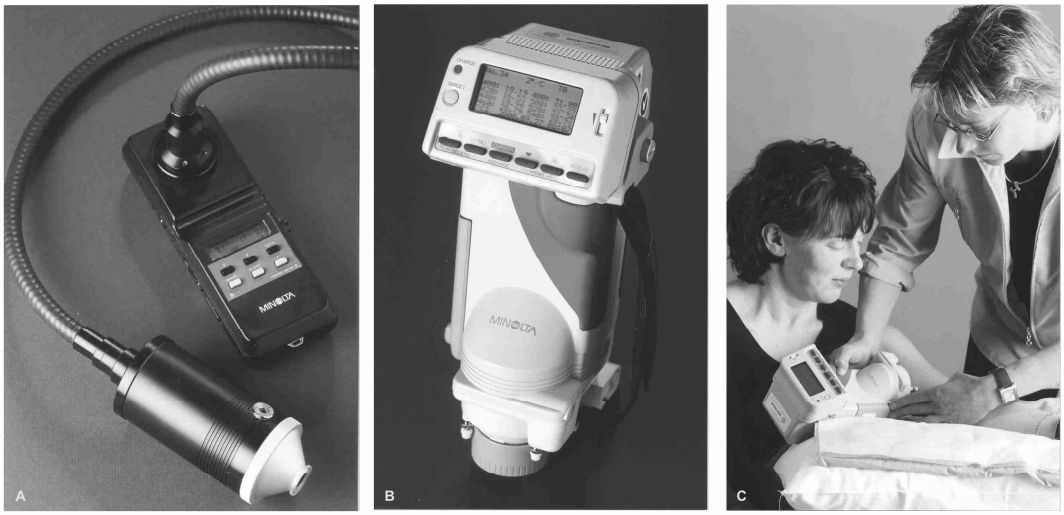

Figure 3. A chroma-meter (A) and spectrophotometer (B), used to estimate melanin density of the skin at a body site usually not exposed to the sun by measuring skin reflectance (C).

The research assistant rated the subjects' eye colour and skin colour at the upper inner arm. The natural hair colour as a teenager (aged 14-19 years) was assessed together with the subject using badges of imitation hair. In the questionnaire, subjects were asked to assess their propensity to burn when in the sun in the middle of the day for the first time in summer without sunscreen, and what kind of tan they would have at the end of summer or after a two week holiday in the sun. An estimate was obtained of the number lifetime burns, where the pain lasted two or more days. This measure partly reflects skin phenotype as well as sun exposure behaviour. Sun avoidance behaviour was examined by asking how often they used a sunscreen or made sure they were 'covered up' in different age periods.

Infections by questionnaire and calendar. Subjects were asked whether and in which five-year age period they had had any of the following infections: chicken pox, measles, rubella (German measles), mumps, pertussis (whooping cough), herpes labialis (cold sores) herpes genitalis, infectious mononucleosis (glandular fever), impetigo (school sores) and any other infectious illnesses serious enough to lead to an absence from school or work for two or more weeks. In relation to canine distemper virus, a virus that could possibly be transmitted to humans, the calendar identified dog ownership and disease at any stage in life.

Infections by serology. IgG serum antibody titers were assessed using enzyme immunoassays of the following viruses: Epstein-Barr virus (nuclear antigen (EBNA) and viral capsid antigen (VCA)), rubella, human simplex virus 1, human simplex virus 2 and cytomegalovirus (see chapter 9 for more detail).

Sibship structure. Sibship structure can be used as a proxy for the frequency and timing of infections.[20] We collected information on the date of birth of the parents and the birth order among their natural brothers and sisters. Each sibling (full or half) was asked whether they

were a full or half sibling, the date of birth of the sibling and whether the subject lived with the sibling when the subject was 0-5 years, 6-10 years, 11-15 years and 16-20 years. With this information, calculations could be made of the total number of siblings, the number of younger and older siblings and the interbirth intervals to the nearest siblings.

Female reproduction. Women were asked about the age menarche. In June 2000 the questionnaire was supplemented with questions on the number of children women had, the date of birth of the children, whether the children were breastfed and the number of completed months of breastfeeding for each child.

Chemicals. Questions on exposure to chemicals were modified from questionnaires previously used to examine the relationship between chemicals and radiological aspects and MS.[21] Exposure to chemicals was assessed under the age of 17 years, and between the age of 17 years and the age of onset. Under the age of 17 years, exposure during their own hobbies, chores or jobs was recorded, as well as exposure through the work or hobby of other family members. The groups of chemicals assessed were: “paint or varnish”, “glues or adhesives”, “metals (including copper, lead, zinc, mercury)”, “petroleum products (such as exhaust gases, LP gas, petrol, kerosene and motor oil)”, “acids, alkalis or ammonium products”, “smoke fumes (such as fumes from industry, machines and incinerators)”, “fibre glass or resin”, “wood dust or sawn wood”, “pesticides (such as insecticides, herbicides and fungicides)”. Occupational exposure to anaesthetic vapours, radioactive radiation or x-ray and animals was additionally assessed in the age group of 17 years and over. When subjects answered that they had been exposed to any of the chemicals listed above, additional information was obtained on the hobby or work, the exact substances, total time of exposure and age period. Separate questions were used to assess whether subjects rode on a trail bike at age 12—17 years and whether they lived within an area of 10 square kilometres where farmers or primary producers sprayed with pesticides.

Dietary intake. For dietary intake it was decided after piloting not to include a Full Frequency Questionnaire in order to limit the burden and total time involvement of the participants. The questions were developed in assistance with a nutritional epidemiologist and were based on standard questions used in Food Frequency Questionnaires.[22] Childhood (age 10—15 years) and recent (in the last 12 months) intake of certain foods was assessed. Subjects could indicate how often on average (times a day, per week, per month or never/rarely) they ate the following types of foods: “meat (pork, lamb, beef)”, “fish of all types (including prawns, scallops, crayfish and canned fish)”, “brains of any type”, “other offal foods of any type (e.g. kidney, liver or tripe)” and “eggs (including boiled and fried eggs, omelettes, quiche and all other forms of egg)”. The intake of milk was recorded (“less than 150 ml (or 5 ozs) per day”, “150—300 ml (300 ml is a small carton)”, “301—600 ml (600 ml is an old ‘pint’) or “more than 600 ml”) and the use of vitamin D supplements (yes/no, name or type, dose and frequency of use).

Pets and farm animals. The calendar identified whether and when subjects had farm animals or pets in their life, the type of farm animals or pets people had and the number of pets. During the interview, additional exposure information was sought for dogs: where the dog spent most of its time (mostly outdoors, more outdoors than indoors, more indoors than outdoors, or mostly indoors), how often on average they would cuddle, pat, nurse or stroke the dog (less than 3 times a day, 3-6 times a day or more than 6 times a day) and whether the dog had a disease at a certain stage in life.

Immunisations. Information was collected on whether subjects were immunised against diphtheria, pertussis, tetanus and polio, the childhood immunisations that were recommended before or on starting school. For immunisations against other (childhood) illnesses given

before the age of 16 years, the five-year age period that they were immunised was also requested. These were immunisations against measles, mumps, rubella (german measles), smallpox, tuberculosis and immunisations required for travelling. All immunisations that the subjects could remember after the age of 16 years and before the age of onset of the case were noted, including the five-year age group.

Medical history. Subjects were asked about the following medical conditions: asthma diagnosed by a doctor, hay fever, allergic reaction to food which included a skin rash, swelling of the mouth or throat or difficulty in breathing, rheumatoid arthritis diagnosed by a rheumatologist, insulin dependent diabetes mellitus, systemic lupus erythematosus diagnosed by a specialist and any other specific medical condition. Additional information was obtained about the age of onset of the condition and whether the disease had been present in the last 12 months (where applicable). In regard to skin cancer, information was collected on whether a doctor had ever told them that they had melanoma skin cancer and whether they had a non-melanoma skin cancer removed. Further details on the type and site of cancer, the diagnosis, form of treatment and the name of the doctor were obtained, so the information could be checked with the records of the Cancer Registry of Tasmania (a registry maintained by the Menzies Centre).

Concussion. Data was collected on whether subjects ever had concussion resulting from a head injury, which led to some confusion, some loss of memory or loss of consciousness, the number of times that had happened and grading of the severity (1= “confusion without amnesia and no loss of consciousness”, 2= “confusion with amnesia, but without loss of consciousness”, and 3= “loss of consciousness”).[23]

Place of living, occupation and socio-economic status. The calendar identified for each year of their life in which place they lived, where they went to school or what their occupation was. The questionnaire included previously used questions on the highest level of completed education or training, the occupation they had for the longest time in their life and their current employment status.

Smoking. Questions were asked about their smoking history (whether ever smoked, age of commencement, total period of quitting, amount they smoked per day, the brand and type they smoked) and current smoking status (whether smoking, the amount they were smoking per day, the brand and type they were smoking). These questions have been used previously by the Menzies Centre in a study on lung cancer[24] and are similar to questions used by other groups.[25] Current self-reported smoking has also been shown to have a high validity when compared to serum cotinine levels.[26]

Alcohol intake. Information was obtained on the frequency of drinking alcohol and the amount and type of alcohol they usually drank. This was done for the age periods 18—20 years, 21—30 years or 21—age of diagnosis if the age of diagnosis was under the age 30 years, 31—age of diagnosis and in the last six months. The questions included were previously used in studies of the Menzies Centre.[27]

Remaining factors. The height and weight of the subject was measured by the research nurse. Detailed information was obtained on any medication taken. All participants were asked whether they were breastfed as a baby and for how long. The proxy questionnaire contained one additional question on puberty development: whether the subject was an early, average or late developer compared with others in his or her school class.

5.7 DATA ANALYSIS

The main type of analysis used to analyse the case-control study was conditional logistic regression. Logistic regression is the preferred method to use in case-control studies, where the outcome factor is binary.[28]

We matched our controls on sex and birth year to the cases. As Rothman points out,[29] without matching there might be some strata with many cases but few controls, and others with few cases and many controls. Matching on birth year and sex will provide a constant ratio of cases to controls over age and sex strata and will minimise this loss of efficiency. The ratio of two controls for each case rather than one control for each case was chosen to increase the power to detect differences between cases and controls.

5.7.1 Unmatched analysis

Before outlining the use of conditional logistic regression in a statistical package, the concepts that underlie this type of regression analysis will be discussed. Table 1 shows the two by two table of the presence or absence of the disease (outcome variable) by the presence or absence of the exposure of interest in a situation where controls are not matched to cases. In this table, a denotes the number of exposed cases, b the number of exposed controls, c the number of unexposed cases and d the number of unexposed controls. The estimation of the odds ratio is the key parameter in the analysis of case-control studies, because it approximates the incidence rate ratio that would have been found in a cohort study of a rare disease (the cohort in question being the entire population or a subset of it). The incidence rate ratio is the incidence of disease among exposed subjects divided by the incidence of disease among unexposed subjects.

Table 1. Frequency of exposure in an unmatched sample n_1 cases and n_2 controls.

Exposure	Cases	Controls	Total
+	a	b	m_1
–	c	d	m_2
Total	n_1	n_2	n

Notes
1. Exposed (+), unexposed (-)

The maximum likelihood estimate of the odds ratio (ψ) can be calculated by:

$$\hat{\psi} = \frac{a/c}{b/d} = \frac{a \times d}{b \times c}$$

This ratio is the odds of exposure for cases divided by the odds of exposure for controls.

5.7.2 Matched analysis

Conditional logistic regression is used in a situation where the case is personally matched to one or more controls. In the situation where one control is matched to one case, the observations may be represented as matched case-control pairs (Table 2).

Table 2. Frequency of exposure among N case-control pairs.

		Control		
		+	–	Total
Case	+	A	B	A+B
	–	C	D	C+D
Total		A+C	B+D	N

Notes

1. Exposed (+), unexposed (-)

There are four possible outcomes for each pair when the exposure is dichotomous: case and control both exposed (+ +), case exposed but control unexposed (+ –), case unexposed but control exposed (– +) and case and control both unexposed (– –). In Table 2, the number of pairs (+ +) are denoted A, the number of pairs (+ –) are B, the number of pairs (– +) C, and the number of pairs (+ +) D. The term N denotes the total number of pairs, so that the total number of cases and controls is 2N.

In a comparison of the proportion of exposed cases (A+B)/N) versus the proportion of exposed controls (A+C)/N, the case-control difference is (B-C)/N, and information regarding differential exposure to a study factor is revealed by the number of discordant pairs B and C. The maximum likelihood estimate of the odds ratio is now:[30]

$$\hat{\psi} = \frac{B}{C}$$

In our study we matched two controls to one case. The observations may now be represented as matched case-control triplets (case, control 1, control 2). There are eight possible outcomes for a triplet when the exposure is dichotomous (Table 3). The term n_1 for example, denotes the total number of sample triplets in which the case and the first control are exposed and the second control is not. The ordering of the two controls does not influence the results.

Table 3. Sample frequencies of eight possible outcomes[1] for matched triplets (case, control 1, control 2).

Outcome	Frequency	Outcome	Frequency
+ + +	n_0	– + +	n_4
+ + –	n_1	– + –	n_5
+ – +	n_2	– – +	n_6
+ – –	n_3	– – –	n_7

Notes

1. Exposed (+), unexposed (-)

Table 4 shows all possible two by two tables for a case-control triplet, ignoring the ordering of the two matched controls. By regarding each triplet as a separate subgroup, the estimate of the odds ratio may be written as:[31]

$$\hat{\psi}_{mh} = \frac{(n_1 + n_2 + 2n_3)}{(2n_4 + n_5 + n_6)}$$

Table 4. Possible 2 x 2 tables for case-control triplet, ignoring the ordering of the two matched controls.

	1		2		3	
	Case	Control	Case	Control	Case	Control
Exposure						
+	1	2	1	1	1	0
–	0	0	0	1	0	2
a_i d_i	0		1		2	
b_i c_i	0		0		0	
Frequency	n_0		$n_1 + n_2$		n_3	

	4		5		6	
	Case	Control	Case	Control	Case	Control
Exposure						
+	0	2	0	1	0	0
–	1	0	1	1	1	2
a_i d_i	0		0		0	
b_i c_i	2		1		0	
Frequency	n_4		$n_5 + n_6$		n_0	

Notes

1. Frequency refers to the frequency with which the 2x2 table occurs in the sample (from Table 3)

To analyse the data in our study with conditional logistic regression, we used the statistical package STATA 7.0. This package obtains the maximum likelihood estimator (β_1) of the exposure of interest from a likelihood function, which expresses the probability of the observed data as a function of the unknown parameter (the effect of exposure on disease). The maximum likelihood estimator of this parameter is chosen to be that value that maximises this function. Each case-control triplet is treated as a stratum and the contribution to the likelihood of each individual stratum is assessed. The estimate of the odds ratio is then obtained by taking the anti-logarithm of β_1. The 95% confidence interval is calculated by using the model-based estimator of the standard error (SE) of β_1 and assuming a normal distribution for the distance of the upper and lower limit to the maximum likelihood estimator (β_1):

$$\hat{\beta}_1 \pm z_{1-\alpha/2}\hat{SE}(\hat{\beta}_1)$$

Hosmer and Lemeshow[28] provide the formulas used for calculating the estimators of the standard errors.

5.7.3 Assessment of confounding

Adjustment for confounders could have been achieved by calculating an odds ratio for exposure and disease in each stratum (at each level) of the potential confounder, and calculating an "average" odds ratio from the stratum-specific odds ratios. To avoid giving small strata undue influence on the overall results, the stratum-specific odds ratios would be weighted by a function of the number of case-control pairs when calculating this average. However, in logistic regression adjustment for confounders can be achieved through inclusion of those exposures in the model.[28] To decide whether an exposure was a confounder of the association of interest, the criteria described by Rothman and Greenland[32] were applied. Firstly, a confounder must be associated with the disease (either as a cause or as a proxy for a cause but not as an effect of the disease). Secondly, a confounder must be associated with

the exposure in the source population. In a case-control study, an estimate of this association can be obtained from the control group. Thirdly, a confounding factor must not be affected by the exposure or the disease. In particular, it cannot be an intermediate step in the causal path between the exposure and the disease. This can be assessed by examining the association between the confounder and disease among those that are not exposed to the factor of interest. In addition to evaluating these criteria in the analysis, we considered whether there was a plausible mechanism for the potential confounder to confound the association of interest. Also, the amount of confounding was evaluated and a stratified approach was used to check the assumption of homogeneity across strata.

5.7.4 Assessment of interaction

If the odds ratios were not homogenous across strata, interaction was assumed to be present. With interaction, the association between the exposure of interest and outcome variable differs or depends in some way on the level of the covariate. If so, the covariate modifies the effect of the exposure of interest and can be termed an effect measure modifier. Statistical interaction between two variables is used to refer to departure from the underlying form of a statistical model, while biologic interaction refers to the coparticipitation in a causal mechanism of two or more component causes.[33]

A statistical test for interaction was conducted by comparing a model that includes the exposure of interest and the covariate that might interact (naïve model) with a model that includes the exposure of interest, the covariate plus a product term of the two variables (saturated model). The likelihood-ratio test now uses a statistic G that follows a chi-square distribution with 1 degree of freedom, and examines whether there is any advantage of including the protect term in the model. When interaction was assessed between the exposure of interest and a matching variable, a model including the exposure of interest (naïve model) was compared with a model including the exposure of interest and the product term of the exposure of interest and the matching variable. Thus, the matching variable was not included in the model, because it was identical for all strata. Hosmer and Lemeshow[28] provide additional details on the use, the interpretation and the statistical concepts underlying the likelihood-ratio test.

Interaction can be assessed on a multiplicative and additive scale. Examining interaction with the likelihood-ratio test will assess interaction on a multiplicative scale. Interaction between the exposure of interest (exposure A) and a covariate (exposure B) can also be assessed by creating a variable that includes four categories: (1) people with exposure A and B absent (reference category); (2) those with exposure A present while B absent; (3) those with exposure B present while A absent, and; (4) those with exposure A and B both present (Table 5). This will partition the effect measure for those with joint exposure to A and B into four parts: the background effect, the effect relating to each of the two exposures in the absence of the other, and the effect attributable to biologic interaction. In the example provided in Table 5, there would be interaction on an additive scale, because an odds ratio of 13.6 is larger than an odds ratio of 9.0 (1.0 + (3.1 – 1.0) + (6.9 – 1.0), the sum of the background effect and the effects relating to each of the two exposures in the absence of the other. The odds ratio of 9 is what would have been expected on an additive scale. The proportion attributable to the interaction of these two exposures would be 34% ([13.6 – 9.0] / 13.6). In contrast, there would not be interaction on a multiplicative scale, because 13.6 is smaller than 21.4 (3.1 * 6.9), the value that would have been expected on a multiplicative scale. In fact, evaluation of statistical interaction based on a multiplicative model (likelihood-ratio test) would indicate that those with joint exposure have a smaller effect than that predicted from the separate effects of the two exposures.

Table 5. Example of interaction.

	Odds ratio
Exposure A and B absent	1.0 (reference)
Exposure A present while B absent	3.1
Exposure B present while A absent	6.9
Exposure A and B present	13.6

5.7.5 Odds ratios and test for trend

For categorical variables having k categories, k-1 odds ratio estimates were obtained when the variable was replaced by k-1 binary (0, 1) predictors that used the first category as a reference (odds ratio set to one). A test for trend was then undertaken by replacing the binary variables with the single predictor taking, for example, rank scores for the categories (1st category=1, 2nd category=2, etc). The significance of the test for trend was evaluated by examining the p-value of the Wald test.

Continuous variables can be analysed as continuous predictors provided they are approximately "linear in the logit". To assess this modelling assumption, we categorised the continuous variables into five levels and plotted the logit against the category number or its midpoint. Alternatively, these variables can be divided into categories and analysed as categorical variables.

5.8 SUMMARY

A population based case-control study was conducted in Tasmania, to investigate whether high past ultraviolet radiation exposure may reduce the risk of MS, and to examine whether other environmental factors might influence the development of MS. Interviews were conducted with 136 cases with MS and 272 community controls. Both cases and controls were drawn from the cohort of persons under the age of sixty years, resident on the mainland of Tasmanian, with at least one grandparent born in Tasmania. Cases were people with MS, defined by both clinical and magnetic resonance imaging criteria. Controls were randomly drawn from the community and matched on sex and birth year. Subjects were interviewed using an extensive questionnaire and were asked to complete a personal residence and work calendar. Phone interviews were held with proxies (someone who had close contact with the subject during the subject's childhood and adolescence) regarding exposures that occurred during childhood and adolescence. Repeat interviews were conducted with 52 cases and 52 controls, about 11 weeks after the main interview took place, to examine the reliability of measurements of the main study factors. Measurements were included on past sun exposure, vitamin D, skin type and sun avoidance behaviour, history of infections, immunisations, concussion, smoking and specific diseases, sibship structure, puberty development and female reproduction, exposure to chemicals, pets and farm animals, dietary intake (including alcohol), place of living, occupation and socio-economic status. Where possible objective measures included, such as the measurement of lifetime actinic damage using silicon casts of the hand, assessment of skin type by spectrophotometry and serum analysis of 25(OH)D and IgG levels of specific infections. Conditional logistic regression was used as the main type of analysis of the data.

5.9 POSTSCRIPT

We have now established the methodological aspects of the Tasmanian MS case-control study, the study that is central to this thesis. A number of chapters will refer back to this chapter, in order to limit duplication of information. Other aspects, however, will be discussed in more detail in other chapters when required. The next two chapters assess the reliability of measurements of the Tasmanian MS case-control study.

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Chapter 6

Reliability of measurements of sun exposure in a case-control study of Multiple Sclerosis.

6.1 PREFACE

The previous chapter outlined the methods of the Tasmanian MS case-control study. The main objective of this study was to assess whether past sun exposure influenced the risk of Multiple Sclerosis (MS). This chapter and the next chapter of the thesis deal with the reliability of measurements of the Tasmanian MS case-control study. This chapter examines the reliability of measurements of past sun exposure and will compare this to the reliability of measures of other environmental factors, while the next chapter will examine aspects of the reliability of the spectrophotometric assessment of skin type.

6.2 INTRODUCTION

Measurement error of exposure variables is one of the major sources of bias in case-control studies. Measurement error is defined as the difference between the measured exposure and the true value of the exposure of interest.[1] Examples of sources of measurement error are: errors in the design of the instrument, e.g. lack of coverage of all sources of the exposure on a questionnaire; errors, omissions or poor execution of the study protocols, e.g. failure to specify the protocol in sufficient detail to data collectors, failure of the data collectors to follow the protocol in the same manner for all subjects; limitations of subjects, e.g. poor recall of exposures, influence of recent exposures on memory of past exposures, tendency of subjects to over-report socially desirable behaviours and under-report socially undesirable behaviours; and errors during data entry and analysis. Measurement error can become differential if the error differs according to disease status or another outcome being studies.[1] One example of differential measurement error is recall bias, where cases report exposures differently from controls because of their knowledge and feelings about the disease, or due to a cognitive decline associated with the disease.

Reduction of measurement error is of critical importance because of its influence on the odds ratio observed in a case-control study. The true odds ratio is the odds ratio that would have been found if the exposure were measured without any error. When exposure measurement is the only source of error and the exposure is measured on a dichotomous scale, non-differential measurement error leads to attenuation toward the null value of no association in the odds ratio.[1] Similarly, when the exposure is measured on an ordered or continuous scale, non-differential measurement error also leads to attenuation toward the null value, but only when a number of assumptions apply such as the size of the error is independent of the magnitude of the exposure.[1] The effect of differential measurement error is less predictable. Compared to the true odds ratio, the observed odds ratio can diminish, increase or even change direction.[1]

Validity refers to the capacity to measure the true exposure in a population of interest.[1] Validity includes a systematic error component (bias) and a random error component (precision). The

validity is usually difficult to ascertain, because a perfect exposure measure is generally not available. In such situations the reliability can reveal something about the validity of a measurement. Reliability refers to the reproducibility of a measure, that is, how consistently a measure can be repeated in the same subjects.[1] It can be assessed in a number of ways, of which two are covered this chapter. Intramethod reliability is a measure of reproducibility of an instrument, either applied in the same manner to the same subjects at two or more points in time (measure-retest reliability) or applied by two or more data collectors to the same subjects (inter-rater reliability). Intermethod reliability is a measure of the ability of two different instruments that measure the same underlying exposure to yield similar results on the same subjects.

In this study, a measure-retest comparison was conducted on a sub-sample of cases and control who participated in the MS case-control study. They were re-interviewed a few months after the main interview took place. A method comparison was applied to one of the main validated questions of sun exposure. This particular question was part of the questionnaire but also incorporated in a personal residence and work calendar. Different cognitive interviewing techniques have been shown to produce more accurate long-term recall in some circumstances.[2] A life events calendar, using memorable events as guideposts, has shown to be an effective strategy to record exposure histories accurately.[3-5] A life events calendar for sun exposure has successfully been used previously in the Geraldton Skin Cancer Prevention Survey.[6, 7]

It is known that MS can affect the cognitive function of the brain.[8-10]. This might affect the recall of the cases and could lead to an increased measurement error in cases compared to controls, especially when prevalent cases are used.

The first objective of this chapter was to assess the reliability of past sun exposure for the entire sample and to compare this to the reliability of exposure to other environmental factors, such as the exposure to childhood infections, immunisations and chemicals. The second objective was to assess whether the reliability of past sun exposure and other variables differed by disease status. The third objective was to assess whether the reliability of self-reported sun exposure differed across the range of other variables, such as age, gender, education, melanin density at the upper inner arm, and ability to recall sun exposure.

6.3 METHODS

6.3.1 Subjects

The subjects in this study were a subset of the subjects described in chapter 5. See chapter 5 for a detailed description of the recruitment and case ascertainment.

6.3.2 Measurements

As described in chapter 5, interviewing was conducted between March 1999 and June 2001, with one interviewer responsible for conducting interviews with subjects who lived in the south of Tasmania (62 telephone district) and the second interviewer responsible for interviewing subjects in the north of Tasmania (the 63 and 64 telephone district).

The main questionnaire (Appendix A) collected information on a large range of environmental and lifestyle factors. For the amount of sun exposure in four age periods (6-10 years, 11-15 years, 16-20 years and the last three years), subjects were asked about the amount of time they would normally have spent in the sun during weekends and holidays ("< 1 hr a day", "1 to 2 hrs per day", "2 to 3 hrs per day", "3 to 4 hrs per day" and "≥ 4 hrs a day") and about how much their activities (such as playing, day sports, spectator sports, gardening, walking and working activities) took them outside ("not that often", "a moderate amount", "quite a lot", "virtually all the time"). The first question will be referred to as the "Time in the sun" question and the second question as the "Activities outside" question. Both these questions were asked for summer as well as for winter. The "Time in the sun" question was also used in the personal residence and work calendar for each year of their life (Appendix D).

Both questions have been previously validated in Tasmania using polysulphone badge readings as the comparison method and showed to be reliable and valid measure of habitual sun exposure in teenagers.[11] Polysulphone badges are personal UV dosimeters that are worn on the body (eg dorsum of the hand). The polysulphone film measures ultraviolet radiation (UVR) by relating the change in the optical absorption of the film, determined before and after exposure to sunlight, to biologically effective solar UVR. In November (spring) 1992, a group of 125 school students aged 14-15 years were interviewed about their habitual and recent sun exposure.[11] Exactly one year later, these children were requested to wear polysulphone badges on four weekend days. The "Time in the sun" and "Activities outside" questions showed to be highest correlated to the amount of UVR assessed by polysulphone badges (r=0.38, r=0.32, respectively).[11] As a comparison, the number of days at the beach in the previous year, the typical number of weeks of holidays in the sun, and the frequency of sun bathing had correlations of 0.13, 0.10 and 0.10, respectively.[11] In addition to this validation study, the "Time in the sun" question in winter has also been shown to be a predictor of 25(OH)D in eight year old Tasmanian children (r=0.20, p<0.01 for exposure during winter school holidays; r=0.16, p=0.02 for exposure during winter weekends).[12] 25(OH)D is a precursor of vitamin D which is produced under the influence of UVR. Another validation study conducted on 44 healthy adults in Denmark showed that total hours outdoors estimated in 30-min intervals in a diary, using a very similar question, correlated highly (especially during the holiday period) with the UVR measured by a personal UVR dosimeter worn on the wrist.[13] The correlation was 0.86 for the holiday period and 0.52 for the working period, and adjusted for total skin area exposure the correlations were 0.82 and 0.63, respectively.[13]

Occupational exposure was assessed by the question whether their jobs were overall mainly indoors, both indoors and outdoors or mainly outdoors. Silicon skin surface casts on the hand, measuring actinic damage, were used as an objective marker of cumulative lifetime sun exposure. Actinic damage has been found to be associated with age, [14] outdoor occupations[15], outdoor leisure activities[15], solar keratosis[14 15], skin cancer[15] and tendency to sunburn.[15] To create the casts, the research assistant mixed silicone liquid with catalyst and applied some of the mixture on the left hand of the subject. The silicone set in approximately seven minutes after which the cast was removed gently. The fine lines on the underside of the cast were then examined under a low-power dissecting microscope and graded by a single observer according to the method of Beagley and Gibson[16] which classifies severity of skin surface changes on a six-point scale. Grade 1 indicates undamaged skin while grade 6 indicates most severe deterioration.

The standardised questionnaire also included questions on sun sensitivity, sun avoidance behaviour, infection and immunisation history, exposure to chemicals, dietary intake (fish, milk, eggs), intake of vitamin D supplements, whether breastfed or not, age of menarche (women only) and socio-economic status. After the interview was conducted, the interviewer completed an evaluation form (Appendix G) rating the subject's recall for different sections of the questionnaire ("very well", "fairly well, some difficulty recalling", "unable to recall" or "declined questions"). It included a separate assessment for the ability to recall sun exposure.

6.3.3 Reliability method 1: Measure-retest comparisons

Repeat interviews were conducted by the project coordinator (the author) on cases and controls living in the south of the state, while the main interviews with those participants were all conducted by the research assistant that conducted interviews in the south of the state. The first 58 cases with whom repeat interviews were conducted, were recontacted after approximately two months to request their participation in a repeat interview. Two months was chosen because it was expected to limit respondents remembering their responses from the initial interview, while controlling genuine change in exposure. Of the 58 cases recontacted, 55 (94.8%) were willing to participate in the repeat interview. Three had to be excluded after the repeat interview had taken place because they had been shown to have a negative MS diagnosis. The first 52 controls for which an interview was conducted were recontacted for a repeat interview and all (100%) were willing to participate in a repeat interview. Thus, the analysis of measure-retest comparisons was conducted on 52 cases and 52 controls. The average time (SD) between the main interview and the repeat interview was 11.1 (2.9) weeks (10.7 (2.4) weeks for cases and 11.4 (3.3) weeks for controls).

The repeat questionnaire (Appendix F) contained questions regarding sun exposure, skin type, chemical exposure, infections, immunisations, dietary intake of vitamin D, breastfeeding and age of menarche (for women only). In addition, new silicon skin surface casts on the hand were made and graded by the same person that conducted the grading of the first casts. The grader was unaware of the grading of the first casts.

6.3.4 Reliability method 2: Method comparison

One of the main validated questions assessing past UVR exposure, the "Time in the sun" question, was used in two different ways in the total sample of 136 cases and 272 controls of the Tasmanian MS case-control study. In the main questionnaire, this question was asked for four different age periods, age 6-10 years, 11-15 years, 16-20 years and the last three years. In the second method, a personal residence and work calendar was used, where participants answered the "Time in the sun" question for each year in their life. Prior to the interview, subjects were sent the lifetime calendars and asked to fill out the calendar for each year of their life (residence, school, occupation, and exposure to pets and farm animals). An example is given in Figure 1. During the interview, subjects were asked to answer the "Time in the sun" question for each year of their life, but the information already filled out was utilised to identify blocks of years where the "Time in the sun" lifestyle was constant and where changes could have occurred. In figure 1, the column with "S" (summer sun exposure) was used for this purpose.

6.3.5 Data analysis

For the measure-retest comparison, the Cohen's kappa statistic[17] and proportion in exact agreement were calculated for categorical measurements. The kappa calculates agreement over and above what might be expected by chance alone and it is influenced by random error as well as systematic bias. The latter is preferred, because in a measure-retest comparison, systematic bias influences the precision. The proportion in exact agreement was calculated by dividing the number of response pairs in exact agreement by the overall number of response pairs. The weighted kappa[18] based on table row and column scores, with the weights of Cicchetti and Allison,[19] was used to measure agreement for ordered categorical variables. To assist the interpretation of the results, the suggestions of Landis and Koch[20] were followed. They characterised the levels of agreement for different values of kappa as follows: agreement was "almost perfect" for kappa values in the range 0.81—1.00, "substantial" for 0.61—0.80, "moderate" for 0.41—0.60, "fair" for 0.21—0.40, "slight" for 0.01—0.20, and "poor" for 0.00.[20] To assess systematic under or over reporting, the misclassification matrices were assessed and Bowker's test of symmetry was applied.

For ordered categorical variables where three or less participants responded with "Don't know", the "Don't know" respondents were not included to preserve the ordinal groupings. If more than three responded with "Don't know", an unweighted kappa statistic was used. Agreement was not calculated for the questions that were answered with "Yes", "No" or "Don't know" if the proportion that answered "Yes" at the main interview was less than 5.0%, because in those situations the kappa statistic is overly sensitive for small departures of agreement. Agreement of the age period of an exposure was not calculated if the prevalence of the actual exposure was less than 15.0%.

For actinic damage, analysis was performed on 323 high quality casts, because 39 casts were difficult to grade and 46 were unable to be graded. We examined the influence of excluding the casts that were difficult to grade by comparing the results of the sample that included those casts with the results of the sample that excluded those casts. Exposure to chemicals in adulthood was assessed up to the age of diagnosis of MS. Controls were given the same age of diagnosis as the case they were matched to. For a small number of participants, the data on exposure to chemicals was missing. Firstly, the first two repeat interviews were conducted

Surname: Smith Given Names: Jane ID number 0 1 0 - 0 1

① Age	② Year	③ Where living *Town and state/country*	④ Farm *Yes or No*	⑤ Type of animals *Cattle, sheep, horses, pigs, poultry, other or None*	⑥ Type and number of pets *Dogs, cats, rabbits, mice, guinea pigs, birds, other (excluding fish)*	*Office Use*	⑦ School or type of job	⑧ Days worked per week in paid Employ-ment	*Office Use* S	W
Born	19 __	Hobart TAS	No	None	None					
1	19 __	↓	↓	↓	↓		Not applicable	not applicable		
2	19 __									
3	19 __				↓					
4	19 __				1 dog, 1 cat					
5	19 __				↓					
6	19 __	↓	↓	↓	↓		Lindisfarne Primary			
7	19 __	Hagley TAS	Yes	Cattle/poultry	2 dogs, 1 cat		Hagley Primary			
8	19 __	↓	↓	↓	↓		↓			
9	19 __									
10	19 __									

Figure 1. An example of the personal residence and work calendar.

without the questions on chemicals included in the questionnaire. Secondly, there was one person whose age of diagnosis was at age 17 years and, therefore, the section on adult exposure between the age of 17 years and the age of diagnosis was not required to be completed.

The continuous variables age of menarche and dietary intake of fish and eggs were categorised so comparisons could be made with the other variables. For those continuous variables we also calculated an intraclass correlation coefficient. An intraclass correlation coefficient was preferred over the Pearson correlation coefficient because the latter does not take systematic errors into account. Even though different interviewers were used for the main interview and the repeat interview, the main interest was the difference in answers that the subjects gave the two consecutive times. For that reason, a one-way random effects model was used for the calculation of intraclass correlation using formula ICC(1,1) of Shrout and Fleiss:[21]

$$\text{ICC} = \frac{BMS - WMS}{BMS + (k-1)WMS}$$

with BMS being the between-subjects mean square, WMS being the within-subjects mean square and k the number of ratings, which is two in our situation.

For the method comparison, the “Time in the sun” question (5 levels) in the questionnaire was asked for the time periods age 6—10 years, 11—15 years, 16—20 years and the last three years. In the calendar, this question was answered for each year in their lives. To compare these two methods, rounded off mean values of the calendar data were calculated for the age groups 6—10 years, 11—15 years, 16—20 years and the last three years. A Pearson or Spearman correlation is normally the preferred option for a method comparison, because in contrast to the measure-retest comparison, systematic bias due to the a different scale of the two measurements used in the two methods should not affect the precision. However, in this study the scale of questionnaire-based measure and calendar measure was identical, so the weighted kappa coefficient can be used, which will also allow us to make comparisons with the intramethod reliablility.

Differences in kappa statistic between cases and controls or other groups were tested by assuming that the difference between the kappa statistics divided by an estimate of the standard error of the difference was normally distributed, i.e. that the test-statistic:

$$z = \frac{K_1 - K_2}{SE(K_1 - K_2)} = \frac{K_1 - K_2}{\sqrt{[SE(K_1)]^2 + [SE(K_2)]^2}}$$

with K_1 being the kappa statistic of cases, K_2 the kappa statistic of controls and SE the standard error.[22] To estimate SE(K_1-K_2), we used the property that the variance of the difference between independent variables is equal to the sum of the variances.

In addition, both the agreement (overall proportion in exact agreement and kappa statistic) on retest as the method comparison for the “Time in the sun” question was assessed within subgroups. For the analysis on retest for “Time in the sun” during childhood and early adolescence, the answers for summer and winter and for the age groups 6—10 years, 11—15 years and 16—20 years were combined, because the agreement was similar for those groups. The subgroups analysed were age, sex, education, melanin density at the upper inner arm, ability to recall sun exposure, time interval between interviews, and sun exposure in the last three years. For the analysis on retest for “Time in the sun” in the last three years, the

answers for summer and winter were combined and the subgroups analysed were the same as for sun exposure during childhood and early adolescence except for the subgroup sun exposure in the last three years, which could not be performed. For the method comparison of the “Time in the sun” question in summer during childhood and early adolescence, again, the age groups 6—10 years, 11—15 years and 16—20 years were combined, while the “Time in the sun” in the last three years was separately analysed by subgroups. The subgroups analysed were age, sex, education, melanin density at the upper inner arm, ability to recall sun exposure and interviewer.

6.4 RESULTS

6.4.1 Characteristics of the samples

Measure-retest comparisons were made on 52 cases and 52 controls. The sample of subjects with who repeat interviews were conducted was similar in structure as the total sample of subjects (Table 1).

Table 1. Characteristics of the cases and controls of the total sample and the repeat sample.

	Total sample		Repeat sample	
	Cases n=136	Controls n=272	Cases n=52	Controls n=52
Characteristics for both cases and controls				
Female/Male ratio	2.1	2.1	1.7	2.2
Mean age, years (SD)	44.0 (9.2)	44.4 (9.2)	43.6 (10.6)	44.7 (10.0)
Height, cm (SD)	166.9 (9.0)	166.1 (8.9)	167.0 (8.5)	165.6 (7.7)
Weight, kg (SD)	72.9 (14.4)	76.2 (16.1)	73.2 (14.4)	71.7 (12.5)
Disease specific characteristics of cases				
Mean age at diagnosis, years (SD)	34.8 (9.1)	-	35.5 (10.3)	-
Mean age at first symptoms, years (SD)	32.2 (9.1)	-	33.3 (10.3)	-
Mean duration of MS since diagnosis, years (SD)	9.4 (7.5)	-	8.7 (6.3)	-
Mean duration of MS since first symptoms, years (SD)	12.0 (8.0)	-	10.9 (6.3)	-
Mean EDSS score (SD)	3.6 (2.2)	-	3.4 (2.4)	-
Type of MS				
Relapsing remitting MS, %	65.4	-	64.7	-
Secondary progressive MS, %	26.5	-	23.5	-
Primary progressive MS, %	8.1	-	11.8	-

6.4.2 Agreement of measurements of sun exposure

Reliability method 1: Measure-retest comparisons

Total sample. Table 2 summarises the agreement on retest for measurements of sun exposure. The column headed “a/N” shows the number (a) of subjects whose reports were identical on each occasion, and the total number (N) of subjects for comparison. The weighted kappa statistic (κ) is a chance-corrected index of agreement. Overall, the agreement of self-reported sun exposure was moderate. The kappa statistic for the “Time in the sun” question ranged from 0.37 to 0.56, and the “Activities outside” question ranged from 0.46 to 0.53. The mean kappa statistic for the “Time in the sun” question and “Activities outside” question in summer (mean 0.47, SD 0.05) was similar to that in winter (mean 0.48, SD 0.06). The kappa statistic for the “Time in the sun” question was highest for the most recent time period, the last three years, but this pattern was not observed for the “Activities outside” question. The agreement was similar when the variables were assessed as dichotomised variables. This was done for sun exposure during childhood and early adolescence (age 6—10 years, 11—15 years and 16—20 years) for summer and winter. The kappa statistic ranged from 0.30 and 0.62, the mean kappa statistic was 0.45 (SD 0.08) and the mean percentage in exact agreement was 78.3% (SD 7.4). Occupational exposure was assessed by the question

whether their jobs were overall “mainly indoors”, “both indoors and outdoors” or “mainly outdoors”. The level of agreement of this question was substantial (Table 2).

Table 2. Agreement on retest of measurements of sun exposure.

	Total sample		Cases		Controls	
Measurement	a/N	κ {95%CI}	a/N	κ {95%CI}	a/N	κ {95%CI}
Time in the sun in summer (5 level)						
When 6-10 yrs	57/104	0.49 {0.35—0.63}	26/52	0.49 {0.29 – 0.68}	31/52	0.48 {0.27 – 0.69}
When 11-15 yrs	55/104	0.44 {0.30—0.57}	25/52	0.43 {0.26 – 0.61}	30/52	0.45 {0.25 – 0.65}
When 16-20 yrs	41/104	0.38 {0.25—0.51}	17/52	0.38 {0.20 – 0.56}	24/52	0.39 {0.20 – 0.58}
Last 3 yrs	55/103	0.56 {0.42—0.69}	31/52	0.62 {0.42 – 0.81}	22/51	0.45 {0.27 – 0.64}
Time in the sun in winter (5 level)						
When 6-10 yrs	50/104	0.50 {0.36—0.63}	26/52	0.49 {0.31 – 0.68}	24/52	0.50 {0.30 – 0.69}
When 11-15 yrs	44/104	0.37 {0.25—0.50}	20/52	0.38 {0.20 – 0.56}	24/52	0.36 {0.18 – 0.55}
When 16-20 yrs	45/104	0.46 {0.33—0.59}	23/52	0.50 {0.32 – 0.69}	22/52	0.42 {0.24 – 0.60}
Last 3 yrs	58/104	0.57 {0.44—0.70}	34/52	0.65 {0.46 – 0.85}	24/52	0.48 {0.29 – 0.66}
Activities outside in summer (4 level)						
When 6-10 yrs	59/104	0.46 {0.32—0.59}	26/52	0.43 {0.24 – 0.62}	33/52	0.47 {0.27 – 0.67}
When 11-15 yrs	58/104	0.46 {0.32—0.59}	25/52	0.40 {0.22 – 0.58}	33/52	0.51 {0.30 – 0.72}
When 16-20 yrs	58/104	0.49 {0.36—0.62}	28/52	0.49 {0.31 – 0.68}	30/52	0.48 {0.28 – 0.68}
Last 3 yrs	56/104	0.48 {0.34—0.61}	30/52	0.49 {0.29 – 0.68}	26/52	0.41 {0.22 – 0.59}
Activities outside in winter (4 level)						
When 6-10 yrs	52/104	0.46 {0.33—0.59}	24/52	0.41 {0.23 – 0.59}	28/52	0.50 {0.31 – 0.69}
When 11-15 yrs	52/104	0.48 {0.35—0.61}	24/52	0.41 {0.22 – 0.59}	28/52	0.54 {0.36 – 0.73}
When 16-20 yrs	62/104	0.53 {0.40—0.67}	31/52	0.56 {0.37 – 0.74}	31/52	0.51 {0.33 – 0.69}
Last 3 yrs	58/104	0.47 {0.34—0.61}	37/52	0.57 {0.37 – 0.78}	21/51	0.35 {0.17 – 0.54}
Occupational exposure	89/104	0.80 {0.64—0.96}	44/50	0.81 {0.58 – 1.00}	45/52	0.79 {0.57 – 1.00}
Actinic damage	52/78	0.68 {0.53—0.83}	18/35	0.52 {0.30—0.75}	34/43	0.79 {0.59—0.99}

Notes

1. a/N =number of subjects with exact agreement on both reports / total number of subjects; κ {95%CI}=Kappa statistic {95% confidence interval}, weighted if more than 2 ordered categories.

Actinic damage on the dorsum of the hand is an objective measure that is thought to reflect cumulative lifetime sun exposure. The agreement for actinic damage was substantial (Table 2) and higher than the self-reported assessment of sun exposure. Including the casts that were difficult to grade did not influence the results (a/N=62/93, κ=0.69, 95% confidence interval 0.56—0.83). The level of agreement of actinic damage on retest includes an error component for the creation of the two casts and an error component for the grading. To assess the reliability of the grading separately, 31 casts were graded twice by the same grader. Of those, 19 (61.3%) had perfect agreement in grading and the weighted kappa statistic {95% confidence interval} was 0.66 {0.42—0.90}. So, the level of agreement of actinic damage of two different casts on the same subject was similar to the agreement of the same cast graded twice, suggesting that a large component of the error was caused by the grading.

The misclassification matrices were examined to see whether subjects systematically over or under-reported at the second interview. No evidence was found that this was the case for any of the above measurements (data not shown).

By disease status. When the level of agreement for cases and controls were compared, differences were especially small for self-reported sun exposure during childhood and early adolescence (at age 6—10 years, 11—15 years and 16—20 years) and occupational sun exposure (mean difference in kappa statistic –0.01, SD.06). For sun exposure in the last three years, a higher agreement for cases was observed (mean kappa statistic 0.16, SD.06), but the differences could have occurred by chance (Time in the sun in summer, p=0.20; and winter, p=0.19; Activities outside in summer, p=0.55; and winter p=0.09). There was also some difference in kappa statistic for actinic damage (-0.27), with cases having a lower kappa statistic than controls, but again, this difference could have occurred by chance (p=0.08). We examined whether the agreement was dependent on the level of actinic damage, but this was not the case (grade 3-4, κ=0.37 {0.14—0.60}; grade 5-6, κ=0.46 {0.22—0.70}.

Reliability method 2: Method comparison

Total sample. The inter-method agreement was assessed for one of the main validated questions of sun exposure, the "Time in the sun" question. This question was used in a questionnaire, but also in a personal residence and work calendar. In the questionnaire, the "Time in the sun" question was asked for four different time periods (age 6-10 years, 11-15 years, 16-20 years and the last three years). The calendar had this information available for each year of their lives, and to make comparisons, the average value was calculated for each of those four age periods. The agreement is shown in Table 3.

Table 3. Agreement between questionnaire and calendar measurements of the time in the sun in summer during weekends and holidays.

	Total sample		Cases		Controls	
Measurement	a/N	κ {95%CI}	a/N	κ {95%CI}	a/N	κ {95%CI}
Time in the sun in summer (5 level)						
When 6-10 yrs	271/404	0.54 {0.47—0.61}	90/136	0.60 {0.47 – 0.73}	181/268	0.50 {0.41 – 0.58}
When 11-15 yrs	250/408	0.51 {0.44—0.58}	81/136	0.54 {0.41 – 0.66}	169/272	0.50 {0.41 – 0.58}
When 16-20 yrs	182/407	0.44 {0.37—0.50}	55/136	0.41 {0.29 – 0.53}	127/271	0.45 {0.36 – 0.53}
Last 3 yrs	153/406	0.42 {0.35—0.48}	49/136	0.39 {0.28—0.50}	104/270	0.41 {0.33—0.49}

Notes

1. a/N =number of subjects with exact agreement on both reports / total number of subjects; κ {95%CI}=Kappa statistic {95% confidence interval}, weighted if more than 2 ordered categories.

The kappa statistic for the total sample ranged from 0.42 to 0.54, representing moderate agreement, which was similar to the agreement on retest. The agreement decreased with age, with a significant difference between the kappa statistic for sun exposure at age 6—10 years and sun exposure in the last three years (p=0.01).

When the misclassification matrices were examined, it became evident that participants reported on average higher levels of sun exposure with the calendar approach (Table 4). This higher reporting at the calendar was especially strong for sun exposure in the last three years, but was also significant for age groups 6—10 years and 11—15 years (Table 4).

It was possible that the over reporting at the calendar for sun exposure in the last three years occurred with the extraction of the data from the calendar, because the last calendar year was an incomplete year, so the mean over the last three years was in actual fact a mean value over anything between two or three years. To see whether this could have partly accounted for the observed effect, an additional year was taken (sun exposure in the last four years) to calculate the average level of sun exposure. The high proportion reporting higher levels of sun

exposure with the calendar approach remained, and was in fact slightly higher (52.0% vs 48.8% in the previous occasion). This means that the computation of sun exposure in the last three years using an incomplete year did not account for higher levels of sun exposure found using the calendar approach.

Table 4. Comparison of proportions of subjects reporting hours in the sun in summer via the questionnaire and calendar.

	Reported lower at calendar % (n)	Reported the same at questionnaire and calendar % (n)	Reported higher at calendar % (n)	p-value **Bowker's test** of symmetry
6—10 years	11.6 (43)	67.1 (271)	22.3 (90)	<0.01
11—15 years	15.9 (65)	61.3 (250)	22.8 (93)	0.02
16—20 years	26.8 (109)	44.7 (182)	28.5 (116)	0.26
Last 3 years	13.5 (55)	37.7 (153)	48.8 (198)	<0.01

Obtaining data via a life events calendar requires a certain degree of interaction between the interviewer and the subject and could therefore lead to differences between interviewers. Stratification of the misclassification matrices by interviewer resulted in the same pattern of reporting on average higher levels of sun exposure at the calendar for both interviewers for three of the four age categories. For sun exposure at age 16—20, participants from interviewer two reported slightly lower at the calendar. Even though generally a similar pattern was observed, the results were somewhat stronger for interviewer one. The difference between the proportion of people reporting higher at the calendar and the proportion of people reporting lower at the calendar, and the p-value of Bowker's test of symmetry for interviewer one were: age 6—10, 17.8%, p<0.01; age 11—15, 10.7%, p=0.07; age 16—20, 10.3%, p=0.13; the last three years, 44.8%, p<0.01. For interviewer two these were: age 6—10, 4.8%, p=0.14; age 11—15, 2.6%, p=0.45; age 16—20, -7.8%, p=0.40; the last three years, 25.0%, p<0.01. Thus, although some differences were observed by interviewer, the pattern that subjects reported on average higher levels of sun exposure at the calendar compared to the questionnaire was observed for both interviewers.

By disease status. When the level of agreement for self-reported "Time in the sun" in summer for cases and controls were compared, no systematic differences could be observed and the mean difference in kappa statistic between cases and controls was 0.02 (SD 0.06). The same pattern of over reporting at the calendar was observed for both cases and controls (data not shown).

6.4.3 Agreement of measurement of other factors

Sun sensitivity, lifetime sunburns and sunscreen use

Total sample. Table 5 shows the agreement for measurements of sun sensitivity, lifetime sunburns and sunscreen use. Substantial agreement was observed for ability to tan, while time until burning, tendency to burn and the number of lifetime burns had moderate agreement. Although people with a high sun sensitivity are likely to get more readily sunburnt, the number of lifetime burns is also influenced by the number of episodes of intense sun. The use of sunscreen also had moderate agreement and ranged from 0.43 to 0.58 for the different age groups.

The misclassification matrices were examined to see whether subjects systematically over or under-reported at the second interview. No evidence was found that this was the case for the assessment of sun sensitivity, lifetime sunburns and sunscreen use (data not shown).

By disease status. When comparing the level of agreement of Table 5 for cases and controls, none of the differences were significant and there was no consistent pattern. Cases had a slightly lower agreement for the tendency to burn and the use of sunscreen in three of the four age groups, but the agreement was similar for time until burning and ability to tan and slightly higher for the number of lifetime burns.

Table 5. Agreement on retest of categorical measurements of sun sensitivity and sunscreen use.

	Total sample		Cases		Controls	
Measurement	a/N	κ {95%CI}	a/N	κ {95%CI}	a/N	κ {95%CI}
Time until burning (5 level)	46/104	0.45 {0.33—0.58}	23/52	0.45 {0.27 – 0.63}	23/52	0.46 {0.28 – 0.64}
Tendency to burn (3 level)	71/104	0.54 {0.39—0.70}	30/52	0.42 {0.21 – 0.63}	41/52	0.69 {0.47 – 0.91}
Ability to tan (4 level)	75/104	0.67 {0.54—0.80}	37/52	0.66 {0.47 – 0.84}	38/52	0.68 {0.50 – 0.86}
Lifetime burns (5 level)	56/103	0.55 {0.43—0.68}	28/51	0.58 {0.40 – 0.76}	28/52	0.52 {0.35 – 0.69}
Use of sunscreen (4 level)						
When 6-10 yrs	73/101	0.58 {0.43—0.74}	33/49	0.33 {0.12 – 0.55}	41/52	0.73 {0.51 – 0.95}
When 11-15 yrs	66/103	0.50 {0.35—0.65}	28/51	0.25 {0.04 – 0.45}	38/52	0.66 {0.44 – 0.88}
When 16-20 yrs	57/103	0.43 {0.29—0.56}	34/51	0.50 {0.30 – 0.70}	23/52	0.35 {0.16 – 0.55}
Last 3 yrs	52/103	0.45 {0.31—0.59}	22/52	0.39 {0.19 – 0.59}	30/51	0.49 {0.30 – 0.69}

Notes

1. a/N =number of subjects with exact agreement on both reports / total number of subjects; κ {95%CI}=Kappa statistic {95% confidence interval}, weighted if more than 2 ordered categories.

Infections

Total sample. Table 6 lists the proportion of answers in each of the categories ('Yes", "No" "Don't know") for the infections included in the main interview. The proportion of people that reported that they had particular infections was extremely variable, ranging from 3.9% to 84.5%. Table 6 also shows that about 7—8% of the subjects did not know whether they had had the infections chickenpox, measles, German measles and mumps, while for the other infections this percentage was below 2%.

Table 6. Proportions of responses at the main interview for infections.

	% Yes	% No	**% Don't know**
Chicken pox	76.9	16.4	6.7
Measles	84.5	8.7	6.8
German measles	20.2	73.1	6.7
Mumps	51.5	39.8	8.7
Cold sores	48.1	51.9	0.0
Glandular fever	25.0	75.0	0.0
Whooping cough	11.5	87.5	0.96
School sores	4.8	93.3	1.9
Herpes genitalis	3.9	96.2	0.0
Any other infectious illnesses	24.0	76.0	0.0

The agreement on retest for whether subjects had particular infections ranged from moderate to nearly perfect, but was generally substantial (Table 7). The agreement for glandular fever (κ=0.87) and cold sores (κ=0.89) was almost perfect. For measles and German measles, the kappa statistic was around 0.50, which might have been caused by a certain amount of confusion between the two infections. The research assistants reported at the regular meetings that subjects had difficulty recalling whether they had the 'normal' measles or German measles.

Table 7. Agreement on retest of categorical measurements of infections.

	Total sample		Cases		Controls	
Measurement	a/N	κ {95%CI}	a/N	κ {95%CI}	a/N	κ {95%CI}
Infections (Yes/No/Don't know)						
Chicken pox	92/104	0.68 {0.52—0.84}	45/52	0.64 {0.43—0.86}	47/52	0.71 {0.46—0.96}
Measles	86/103	0.48{0.34—0.63}	41/52	0.43 {0.22—0.63}	44/51	0.55 {0.32—0.77}
German measles	81/104	0.52 {0.37—0.67}	40/52	0.52 {0.28—0.69}	41/52	0.54 {0.33—0.76}
Mumps	86/103	0.71 {0.55—0.87}	44/51	0.78 {0.57—0.98}	42/52	0.63 {0.39—0.87}
Cold sores	98/104	0.89 {0.70—1.00}	49/52	0.88 {0.62—1.00}	49/52	0.88 {0.62—1.00}
Glandular fever	99/104	0.87 {0.68—1.00}	48/52	0.81 {0.55 – 1.00}	51/52	0.94 {0.67—1.00}
Whooping cough	94/104	0.60 {0.43—0.76}	46/52	0.52 {0.30—0.74}	48/52	0.67 {0.42—0.93}
Any other infectious illnesses	91/104	0.65 {0.46—0.85}	43/52	0.55 {0.28 – 0.82}	48/52	0.77 {0.50—1.00}
Age of infection (5 level)						
Chickenpox	57/72	0.81 {0.66—0.96}	29/35	0.83 {0.61 – 1.00}	28/37	0.79 {0.57 – 1.00}
Measles	61/70	0.48 {0.30—0.65}	29/32	0.68 {0.43 – 0.93}	27/38	0.34 {0.10 – 0.58}
Rubella	10/14	0.67 {0.12—1.00}	5/5	1.00 {0.12—1.00}	5/9	0.50 {0.06—0.94}
Mumps	35/46	0.75 {0.54—0.96}	14/19	0.71 {0.39 – 1.00}	21/27	0.72 {0.47 – 0.97}
First cold sore	27/45	0.67 {0.47—0.87}	13/21	0.68 {0.41- 0.96}	14/24	0.65 {0.37– 0.94}
Glandular fever	15/20	0.75 {0.42—1.00}	7/11	0.58 {0.17 – 1.00}	8/9	0.89 {0.37 – 1.00}

Notes

1. a/N =number of subjects with exact agreement on both reports / total number of subjects; κ {95%CI}=Kappa statistic {95% confidence interval}, weighted if more than 2 ordered categories.

The agreement for the age period that subjects had the infections was generally substantial. Even though the kappa statistic was lower for the age of the measles infections, the exact agreement was high (87.1%) and the lower kappa statistic was caused by a high proportion (77.1% at the main interview) of subjects reporting their measles infection at age 6—10.

The misclassification matrices were examined to see whether subjects systematically over or under-reported at the second interview. No evidence was found that this was the case for the assessment of infections (data not shown).

By disease status. Comparing the level of agreement of reporting infections for cases and controls, we observed that cases had more often (six out of eight) a lower agreement than controls. The mean difference in kappa statistic was -0.07 (SD 0.11). None of the differences were statistically significant. For the age period of infections, cases had on average a higher agreement (mean difference 0.11, SD 0.29), but the relatively low numbers for some infections makes the interpretation difficult.

Immunisations

Total sample. Table 8 lists the proportion of answers in each of the categories ('Yes", "No" "Don't know") for the immunisations of the main interview.

Table 8. Proportions of responses at the main interview for immunisations prior to the age of 16 years.

	% Yes	% No	% Don't know
Diphtheria	90.4	5.8	3.9
Whooping cough	88.5	8.7	2.9
Tetanus	78.9	17.3	3.9
Polio	75.0	22.1	2.9
German measles	36.5	63.5	0.0
Measles	29.8	58.7	11.5
Tuberculosis	13.5	76.0	10.6
Smallpox	8.7	75.7	15.5
Mumps	6.7	85.6	7.7
Travel immunisations	2.9	95.2	1.9
Any other immunisations	28.9	71.2	0.0

The proportion of people that reported that they had particular immunisations was extremely variable, ranging from 2.9% to 90.4%. Subjects had generally some difficulty reporting whether or not they were immunised against particular infections, with percentages that did not know whether they were immunised against measles, tuberculosis and smallpox exceeding 10%. No participants reported that they did not know whether they were immunised against German measles, probably because participants remembered the mass immunisations in their teenage years.

Table 9. Agreement on retest of categorical measurements of immunisations prior to age 16 years.

	Total sample		Cases		Controls	
Measurement	a/N	κ {95%CI}	a/N	κ {95%CI}	a/N	κ {95%CI}
Immunisations prior to age 16 (Yes/No/Don't know)						
Diphtheria	84/104	0.28 {0.15—0.42}	40/52	0.28 {0.09 – 0.47}	44/52	0.28 {0.08 – 0.47}
Whooping cough	82/104	0.36 {0.23—0.49}	39/52	0.29 {0.11 – 0.47}	43/52	0.43 {0.24 – 0.63}
Tetanus	84/104	0.51 {0.36—0.66}	38/52	0.44 {0.24 – 0.63}	46/52	0.61 {0.36 – 0.85}
Polio	80/104	0.52 {0.38—0.67}	35/52	0.40 {0.21 – 0.59}	45/52	0.68 {0.44 – 0.92}
German measles	90/104	0.73 {0.55—0.91}	37/52	0.78 {0.53 – 1.00}	43/52	0.67 {0.42 – 0.92}
Measles	77/104	0.54 {0.40—0.69}	40/52	0.59 {0.38 – 0.80}	37/52	0.49 {0.30 – 0.69}
Tuberculosis	71/103	0.33 {0.19—0.47}	34/52	0.33 {0.15 – 0.52}	37/51	0.33 {0.13 – 0.54}
Smallpox	76/101	0.37 {0.22—0.52}	38/52	0.43 {0.23 – 0.64}	38/52	0.29 {0.07 – 0.51}
Mumps	78/104	0.34 {0.20—0.47}	39/52	0.36 {0.17 – 0.55}	41/52	0.30 {0.10 – 0.51}
Any other immunisations	78/102	0.46 {0.27—0.65}	37/49	0.44 {0.17 – 0.70}	40/52	0.48 {0.21 – 0.75}
Age of immunisation prior age 16 (3 level)						
German measles	23/30	-0.11 {0 — 0.19}	8/12	-0.11 {0 — 0.25}	15/17	-0.08 {0 — 0.35}
Measles	10/10	1.00 {0.50—1.00}	5/5	1.00 {0.26—1.00}	5/5	1.00 {0.12—1.00}

Notes

1. a/N =number of subjects with exact agreement on both reports / total number of subjects; κ {95%CI}=Kappa statistic {95% confidence interval}, weighted if more than 2 ordered categories.

The agreement for immunisations was generally lower than for infections with the exception of the immunisations against German measles which had a substantial agreement (Table 9). Immunisations against diphtheria and whooping cough had a rather low kappa statistic, even

though the percentage of exact agreement was rather high. This was because nearly all subjects were immunised against diphtheria (90.4%) and whooping cough (88.5%).

No age period was requested for immunisations against diphtheria, whooping cough, tetanus and polio, because it was recommended to parents to give these immunisations prior to school, at age 0—5 years. Only for immunisations against measles and German measles, the prevalence was high enough to assess agreement for the age of the immunisations. The agreement for the age of the immunisation against measles was perfect (Table 9). The exact agreement for the age of the German measles immunisation was 76.7%, but a negative kappa statistic was found partly because the highly skewed matrix and some zero counts on the diagonal axis (Table 10).

Table 10. Misclassification matrix for the age of the German measles immunisations

Age period reported	Age period reported at the repeat interview		
at the main interview	**0—5 years**	**6—10 years**	**11—15 years**
0—5 years	0	0	0
6—10 years	0	0	3
11—15 years	1	3	23

The misclassification matrices were examined to see whether subjects systematically over or under-reported at the second interview. No evidence was found that this was the case for the assessment of infections (data not shown).

By disease status. Comparing the level of agreement for cases and controls showed some differences, but these were not significant and did not reveal a systematic pattern. For example, the kappa statistic was slightly lower for cases for the immunisation against polio (difference –0.28, p=0.07) and tetanus (difference –0.17, p=0.28), but higher for smallpox (difference 0.14, p=0.36). Overall, the mean difference in kappa statistic was –0.02 (SD 0.14).

Exposure to chemicals

Table 11. Proportion of subjects reporting exposure to different chemicals at the main interview.

	% reporting "Yes"		
	Up to age 17		Exposure from age 17
Chemicals	Own exposure	Family exposure	to age of diagnosis
Petroleum products	28.7	18.8	30.3
Wood dust or sawn wood	26.7	19.8	25.0
Glues or adhesives	24.8	8.9	19.0
Paint or varnish	23.8	21.8	28.0
Metals	17.8	5.9	15.0
Smoke fumes	13.9	11.9	19.0
Acids, alkalis or ammonium products	9.9	3.9	22.0
Pesticides	9.9	16.8	13.0
Fibre glass or resin	5.0	3.0	6.0
Animals*	-	-	11.0
Radioactive radiation or x-ray *	-	-	4.0
Anaesthetic vapours*	-	-	0.0

Notes

1. * denotes to occupational exposure only

Total sample. We obtained information on a range of chemicals during early life (up to age 17) and from age 17 to the age of diagnosis. The latter will be referred to as adult exposure. For the controls, the same age of diagnosis was used as the case they were matched to. For early life exposure, it was not only assessed whether participants were exposed to chemicals through their own hobbies, chores or jobs, but also through the work or hobbies of other family members. Table 11 lists the proportion of participants that reported that they were exposed to each of the chemicals. The exposure ranged from 0% to 30.3%. Exposure to petroleum products, wood dust or sawn wood, glues or adhesives and paint or varnish was rather common, but exposure to fibre glass or resin or occupational exposure to anaesthetic vapours and radioactive radiation or X-ray was rather uncommon.

Table 12. Agreement on retest of categorical measurements of exposure to chemicals

	Total sample		Cases		Controls	
Measurement	a/N	κ {95%CI}	a/N	κ {95%CI}	a/N	κ {95%CI}
Exposure up to age 17 from hobbies, chores or jobs						
Paint or varnish	83/97	0.62 {0.43—0.82}	38/48	0.45 {0.17 – 0.73}	45/49	0.79 {0.51 – 1.00}
Glues or adhesives	86/97	0.72 {0.52—0.91}	40/48	0.59 {0.32 – 0.86}	46/49	0.85 {0.57 – 1.00}
Metals	83/97	0.48 {0.28—0.67}	39/48	0.45 {0.18 – 0.73}	44/49	0.49 {0.21 – 0.77}
Petroleum products	83/97	0.64 {0.44—0.84}	40/48	0.60 {0.31 – 0.88}	43/49	0.69 {0.41 – 0.97}
Acids, alkalis or ammonium products	86/97	0.46 {0.26—0.66}	43/48	0.49 {0.21 – 0.77}	43/49	0.43 {0.16 – 0.71}
Smoke fumes	84/97	0.53 {0.34—0.72}	43/48	0.67 {0.39 – 0.95}	41/49	0.35 {0.11 – 0.60}
Wood dust / sawn wood	86/97	0.71 {0.51—0.91}	42/48	0.65 {0.37 – 0.93}	44/49	0.76 {0.48 – 1.00}
Pesticides	91/97	0.59 {0.39—0.79}	45/48	0.54 {0.26 – 0.82}	46/49	0.63 {0.36 – 0.91}
Exposure up to age 17 through family members						
Paint or varnish	78/97	0.50 {0.31—0.69}	40/48	0.55 {0.31 – 0.79}	38/49	0.46 {0.18 – 0.74}
Glues or adhesives	82/97	0.32 {0.13—0.51}	40/48	0.34 {0.07 – 0.61}	42/49	0.29 {0.02 – 0.56}
Metals	83/97	0.69 {0.50—0.89}	46/48	0.78 {0.50 – 1.00}	47/49	0.48 {0.20 – 0.76}
Petroleum products	71/97	0.34 {0.16—0.52}	37/48	0.46 {0.20 – 0.71}	34/49	0.22 {0.00 – 0.48}
Smoke fumes	80/97	0.39 {0.20—0.58}	41/48	0.50 {0.22 – 0.78}	39/49	0.29 {0.06 – 0.52}
Wood dust / sawn wood	80/97	0.53 {0.33—0.72}	41/48	0.64 {0.36 – 0.92}	39/49	0.39 {0.13 – 0.65}
Pesticides	73/97	0.48 {0.29—0.67}	44/48	0.67 {0.40 – 0.95}	36/49	0.32 {0.06 – 0.58}
Exposure from age 17 to age of onset						
Paint or varnish	80/96	0.60 {0.41—0.80}	39/47	0.59 {0.31 – 0.88}	41/49	0.62 {0.34 – 0.90}
Glues or adhesives	73/96	0.49 {0.29—0.69}	39/47	0.46 {0.18 – 0.73}	40/49	0.52 {0.24 – 0.80}
Metals	89/96	0.73 {0.53—0.92}	43/47	0.78 {0.49 – 1.00}	46/49	0.54 {0.26 – 0.82}
Petroleum products	81/96	0.65 {0.45—0.85}	43/47	0.80 {0.52 – 1.00}	38/49	0.50 {0.23 – 0.78}
Acids, alkalis or ammonium products	79/96	0.51 {0.31—0.71}	39/47	0.58 {0.30 – 0.86}	40/49	0.41 {0.13 – 0.69}
Smoke fumes	78/96	0.45 {0.25—0.65}	40/47	0.62 {0.35 – 0.90}	38/49	0.22 {0.00 – 0.50}
Fibreglass / resin	93/96	0.75 {0.55—0.95}	45/47	0.78 {0.49 – 1.00}	48/49	0.66 {0.39 – 0.92}
Wood dust / sawn wood	87/96	0.75 {0.55—0.95}	44/47	0.84 {0.56 – 1.00}	43/49	0.67 {0.39 – 0.95}
Pesticides	86/96	0.52 {0.32—0.72}	42/47	0.55 {0.27 – 0.84}	44/49	0.49 {0.21 – 0.77}
Animals (occupational)	90/96	0.73 {0.53—0.93}	45/47	0.64 {0.36 – 0.93}	46/49	0.76 {0.48 – 1.00}

Notes

1. a/N =number of subjects with exact agreement on both reports / total number of subjects; κ {95%CI}=Kappa statistic {95% confidence interval}.

Table 12 shows the agreement for exposure to chemicals. For early life exposure from hobbies, chores or jobs, the agreement was moderate to substantial. The kappa statistic ranged from 0.48 to 0.72. The agreement was generally similar for adult exposure, but lower for early life exposure through other family members, where the kappa statistic ranged from 0.32 to 0.69. Assessing early life exposure from hobbies, chores or jobs and adult exposure, we observe the best agreement for exposure to "sawn wood and wood dust" (mean kappa above 0.70) and "petroleum products" or "paint and varnish" (mean kappa between 0.60 and 0.70). "Pesticides" had a mean kappa statistic between 0.50 and 0.60 and "smoke fumes" and "acids, alkalis or ammonium products" had a mean kappa statistic between 0.40 and 0.50. For "metals" and "glues or adhesives", the difference in kappa statistic was larger than 0.20 and a mean kappa value was not calculated. For "fibreglass or resin" and "occupational animal exposure", the agreement was only assessed for adult exposure and the kappa statistic was in both instances above 0.70.

The misclassification matrices were examined to see whether subjects systematically over or under-reported at the second interview. For chemical exposure during childhood and early adolescence from hobbies, chores or jobs the misclassification matrices were symmetrical for all exposures except smoke fumes (Bowker's test of symmetry, $p=0.05$) where slightly more subjects reported that they were exposed at the second interview. For exposure to chemicals during childhood and early adolescence through family members, more subjects reported at the second interview that they were exposed to petroleum products ($p<0.01$), smoke fumes ($p=0.03$) and pesticides ($p=0.02$). The misclassification matrices were symmetrical for adult exposure to chemicals.

By disease status. Comparing the agreement for cases and controls shows that cases had on average a slightly lower agreement for early life exposure from hobbies, chores or jobs (mean difference in kappa statistic –0.07, SD 0.20) but a slightly higher agreement for early life exposure by other family members (mean difference in kappa statistic 0.21, SD 0.11) and adult exposure (mean difference in kappa statistic 0.14, SD 0.15). However, only the difference between cases and controls for exposure to smoke fumes during adult life was significantly different (difference in kappa statistic 0.40, $p=0.04$).

Breastfed, menarche, dietary intake of vitamin D containing foods or supplements

Total sample. Table 13 shows the agreement for measurements on whether subjects were breastfed, the time of being breastfed, age of menarche and dietary intake of vitamin D containing foods and supplements. The level of agreement was substantial for being breastfed and the age of menarche, while the agreement was moderate for the time they were breastfed and for vitamin D containing foods and supplements. The agreement for vitamin D containing foods and supplements in the last 12 months was only slightly higher (mean $\kappa=0.55$, SD 0.09) compared to when aged 10 to 15 years (mean $\kappa=0.50$, SD 0.11).

The number of times per month subjects ate fish and eggs and the age of menarche were the only variables that could be assessed as continuous variables. In Table 13 we treated them as categorical variables and calculated a kappa statistic in order to compare them with the other variables. However, we have also treated them as continuous variables and calculated an intraclass correlation coefficient. The intraclass correlation coefficients {95% confidence interval} were for age of menarche 0.89 {0.84—0.94}, fish intake in the last 12 months 0.61 {0.49—0.73}, egg intake in the last 12 months 0.68 {0.56—0.78}, fish intake at age 10—15

years 0.39 {0.22—0.55}; and egg intake at age 10—15 years 0.44 {0.28—0.60}. Thus, the intraclass correlations were slightly higher than the kappa statistics.

Table 13. Agreement on retest of categorical measurements of breastfeeding, menarche and dietary intake of vitamin D containing foods/supplements.

	Total sample		Cases		Controls	
Measurement	a/N	κ {95%CI}	a/N	κ {95%CI}	a/N	κ {95%CI}
Ever breastfed (Y/N/DK)	79/104	0.63 {0.49—0.78}	41/52	0.58 {0.37 – 0.78}	44/52	0.69 {0.49 – 0.90}
Time breastfed (5 level)	34/61	0.43 {0.29—0.56}	13/30	0.25 {0.06 – 0.44}	21/31	0.59 {0.41 – 0.77}
Age menarche (9 level)	48/69	0.76 {0.62—0.91}	23/33	0.73 {0.51—0.91}	25/36	0.79 {0.59—0.98}
Dietary intake in last 12 months						
Fish (≤3x vs >3x)	82/104	0.58 {0.38—0.77}	42/52	0.61 {0.34 – 0.88}	40/52	0.51 {0.24 – 0.78}
Eggs (≤10x vs >10x)	86/104	0.62 {0.43—0.80}	46/52	0.65 {0.38 – 0.93}	40/52	0.55 {0.29 – 0.81}
Milk (4 level)	65/104	0.57 {0.43—0.71}	33/52	0.52 {0.32 – 0.72}	32/52	0.59 {0.41 – 0.78}
Vit D suppl. (Y/N)	87/104	0.42 {0.22—0.61}	42/52	0.46 {0.19 – 0.73}	45/52	0.29 {0.02 – 0.56}
Dietary intake at age 10—15 years						
Fish (≤3x vs >3x)	79/104	0.52 {0.33—0.71}	29/52	0.11 {0 – 0.38}	36/52	0.38 {0.13 – 0.64}
Eggs (≤10x vs >10x)	69/104	0.34 {0.15—0.52}	38/52	0.43 {0.16 – 0.70}	31/52	0.25 {0.02 – 0.48}
Milk (4 level)	57/103	0.57 {0.43—0.70}	26/52	0.53 {0.35 – 0.71}	31/52	0.61 {0.43 – 0.80}
Vit D suppl. (Y/N)	90/104	0.57 {0.38—0.76}	43/52	0.38 {0.12 – 0.63}	47/52	0.72 {0.45 – 0.99}

Notes

1. a/N =number of subjects with exact agreement on both reports / total number of subjects; κ {95%CI}=Kappa statistic {95% confidence interval}, weighted if more than 2 ordered categories; Egg and fish intake is quantified in times per month.

The misclassification matrices were examined to see whether subjects systematically over or under-reported at the second interview. Only for the intake of eggs at age 6—10 years, subjects reported less at the second interview compared to the first interview (Bowker's test of symmetry, p=0.01). For the other variables there was no difference.

By disease status. Comparing the agreement for cases and controls showed that cases had a slightly lower agreement for being breastfed and the age of menarche (mean difference in kappa statistic –0.17, SD 0.15). The differences were not significant. No pattern was observed for the dietary intake of vitamin D containing foods and supplements (mean difference in kappa statistic –0.03, SD 0.20).

6.4.4 Agreement of measurements of self-reported sun exposure by subgroups

Reliability method 1: Measure-retest comparisons

We assessed whether the agreement on retest for measurements of the time in the sun during weekends and holidays differed by subgroups. For the analysis of sun exposure during childhood and early adolescence, we combined the answers for summer and winter and for the age groups 6—10 years, 11—15 years and 16—20 years, so the total number of subjects was six times the sample size of 104 subjects. For the analysis of sun exposure in the last three years, we combined the answers for summer and winter and the total number of subjects was two times the sample size of 104 subjects.

Sun exposure during childhood and early adolescence—total sample. The agreement for sun exposure during childhood and early adolescence for the total sample did not differ by the

level of education, melanin density at the upper inner arm, the ability to recall sun exposure (assessed by research assistant), the time between the main interview and the repeat interview, and recent sun exposure (Table 13). The kappa statistic was somewhat higher for younger (difference 0.11, p=0.05) and female subjects (difference 0.10, p=0.09).

Sun exposure during childhood and early adolescence—*by disease status.* When cases and controls were analysed separately (Table 14), no significant differences were observed.

Table 14. Agreement on retest of the time in the sun during weekends and holidays during childhood and early adolescence by subgroups.

	Total sample		Cases		Controls	
Subgroup variable	a/N	κ {95%CI}	a/N	κ {95%CI}	a/N	κ {95%CI}
Age						
≤ 44 years	155/318	0.54 {0.46—0.62}	69/156	0.55 {0.44 – 0.65}	86/162	0.53 {0.42 – 0.64}
> 44 years	137/306	0.43 {0.35—0.51}	69/156	0.44 {0.33 – 0.55}	69/150	0.43 {0.32 – 0.54}
Difference		0.11		0.10		0.10
Sex						
Men	101/210	0.40 {0.30—0.49}	49/114	0.38 {0.25 – 0.51}	52/96	0.39 {0.25 – 0.53}
Women	191/414	0.50 {0.43—0.57}	88/198	0.53 {0.44 – 0.63}	103/216	0.47 {0.38 – 0.56}
Difference		-0.10		-0.14		-0.08
Education						
Year 10 or below	169/354	0.50 {0.43—0.57}	72/156	0.54 {0.42 – 0.65}	97/198	0.47 {0.37 – 0.57}
Year 11 or higher	121/264	0.47 {0.39—0.56}	65/156	0.45 {0.34 – 0.55}	56/108	0.50 {0.37 – 0.63}
Difference		0.03		0.09		-0.03
Melanin density at the upper inner arm						
≥ 2%	133/252	0.53 {0.44—0.62}	52/108	0.54 {0.42 – 0.67}	81/144	0.51 {0.39 – 0.63}
< 2%	69/252	0.52 {0.44—0.61}	71/162	0.52 {0.41 – 0.62}	48/90	0.53 {0.39 – 0.68}
Difference		0.01		0.02		-0.02
Ability to recall sun exposure						
Very well	228/462	0.50 {0.44—0.57}	91/186	0.54 {0.44 – 0.64}	137/276	0.47 {0.39 – 0.55}
Some difficulty	64/162	0.45 {0.34 – 0.55}	46/126	0.43 {0.31 – 0.56}	18/36	0.51 {0.31 – 0.71}
Difference		0.05		0.11		-0.04
Time between interviews						
≤ 71 days	138/324	0.48 {0.40—0.55}	86/192	0.50 {0.39 – 0.59}	62/132	0.45 {0.34 – 0.57}
> 71 days	144/300	0.50 {0.41—0.58}	51/120	0.50 {0.37 – 0.62}	93/180	0.48 {0.38 – 0.59}
Difference		-0.02		0.00		-0.03
Sun exposure in the last 3 years						
≤ 1—2 hrs a day	143/342	0.45 {0.37—0.52}	77/198	0.45 {0.36 – 0.55}	66/144	0.44 {0.33 – 0.55}
≥ 2—3 hrs a day	146/276	0.49 {0.41—0.58}	60/114	0.54 {0.42 – 0.67}	86/162	0.44 {0.33 – 0.56}
Difference		-0.04		-0.09		0.00

Notes

1. The data for questions for summer and winter and age 6—10, 11—15 and 16—20 were combined, so the total number of subjects was 6x sample size of 104 subjects.
2. a/N =number of subjects with exact agreement on both reports / total number of subjects; κ {95%CI}=Kappa statistic {95% confidence interval}, weighted if more than 2 ordered categories.

Sun exposure during childhood and early adolescence—by disease related factors. Among cases, there was no difference between the agreement for sun exposure during childhood and early adolescence for subjects with an EDSS score lower than or equal to three (κ=0.51 {0.41—0.61}) compared to subjects with an EDSS score higher than three (κ=0.51

{0.39—0.63}). Similarly, no differences were seen for subjects with a duration shorter than 11 years (κ=0.51 {0.41—0.62}) compared to subjects with a duration equal to or longer than 11 years (κ=0.47 {0.35—0.58}), or for subjects with the relapsing remitting form of MS (κ=0.51 {0.42—0.61}) compared to subjects with the progressive form of MS (κ=0.50 {0.36—0.63}).

Sun exposure in the last three years—total sample. The agreement for sun exposure in the last three years for the total sample did not differ by age, sex, level of education, and melanin density at the upper inner arm (Table 15). After each interview, the interviewer rated, as part of the evaluation form, the subject's ability to recall the sun exposure questions. There was some difference in agreement for sun exposure by the subject's ability to recall sun exposure rated in the above way, but this difference was not significant (p=0.29). There was a significant difference by the time between the main interview and the repeat interview (difference 0.20, p=0.04), but the direction of the difference was against the expected direction. We expected that a shorter time between the two interviews would increase the observed agreement. In stead, we found that the agreement was lower for subjects with a shorter duration between the interviews (<71 days) compared to a longer duration (≥71 days).

Table 15. Agreement on retest of the time in the sun during weekends and holidays in the last three years by subgroups.

	Total sample		Cases		Controls	
Subgroup variable	a/N	κ {95%CI}	a/N	κ {95%CI}	a/N	κ {95%CI}
Age						
≤ 44 years	58/104	0.59 {0.46—0.72}	33/52	0.68 {0.49 – 0.87}	25/52	0.47 {0.29 – 0.64}
> 44 years	53/102	0.55 {0.41—0.68}	32/52	0.58 {0.38 – 0.77}	21/50	0.48 {0.29 – 0.67}
Difference		0.04		0.10		-0.01
Sex						
Men	36/70	0.58 {0.41—0.74}	22/38	0.63 {0.41 – 0.85}	14/32	0.38 {0.15 – 0.62}
Women	75/136	0.53 {0.42—0.64}	43/66	0.64 {0.47 – 0.81}	32/70	0.41 {0.26 – 0.56}
Difference		0.05		-0.01		-0.03
Education						
Year 10 or below	64/112	0.57 {0.44—0.70}	32/52	0.54 {0.35 – 0.73}	32/66	0.56 {0.39 – 0.73}
Year 11 or higher	46/86	0.56 {0.41—0.70}	34/52	0.72 {0.53 – 0.92}	13/34	0.30 {0.10 – 0.51}
Difference		0.01		-0.18		0.26
Melanin density at the upper inner arm						
≥ 2%	40/82	0.51 {0.35—0.66}	21/36	0.56 {0.32 – 0.80}	18/46	0.41 {0.20 – 0.62}
< 2%	34/84	0.54 {0.39—0.69}	35/54	0.67 {0.48 – 0.86}	12/30	0.34 {0.11 – 0.56}
Difference		-0.03		-0.11		0.07
Ability to recall sun exposure						
Very well	71/152	0.52 {0.41—0.63}	32/62	0.59 {0.43 – 0.76}	39/90	0.47 {0.33 – 0.61}
Some difficulty	40/54	0.64 {0.45—0.84}	33/42	0.66 {0.41 – 0.90}	7/12	0.48 {0.16 – 0.80}
Difference		-0.12		-0.07		-0.01
Time between interviews						
≤ 71 days	55/108	0.46 {0.33—0.60}	40/64	0.53 {0.36 – 0.71}	15/44	0.34 {0.15 – 0.54}
> 71 days	56/98	0.66 {0.52—0.79}	25/40	0.75 {0.53 – 0.98}	31/58	0.58 {0.40 – 0.75}
Difference		-0.20		-0.22		-0.24

Notes

1. The data for questions for summer and winter were combined, so the total number of subjects is 2 x sample size of 104 subjects.
2. a/N =number of subjects with exact agreement on both reports / total number of subjects; κ {95%CI}=Kappa statistic {95% confidence interval}, weighted if more than 2 ordered categories.

Sun exposure in the last three yea*rs—by disease status.* When cases and controls were analysed separately, no significant differences could be observed. However, the difference in agreement by the time between the two interviews was of similar magnitude and in the same direction for both cases (difference 0.22, p=0.13)) and controls (difference 0.24, p=0.08) compared to the total sample.

Sun exposure in the last three years—by disease related factors. Among cases, there was no significant difference between the agreement for sun exposure in the last three years for subjects with an EDSS score lower than or equal to three (κ=0.59 {0.42—0.77}) compared to subjects with an EDSS score higher than three (κ=0.62 {0.40—0.83}). Similarly, no differences were seen for subjects with a duration shorter than 11 years (κ=0.60 {0.42—0.77}) compared to subjects with a duration equal to or longer than 11 years (κ=0.68 {0.47—0.89}), or for subjects with the relapsing remitting form of MS (κ=0.63 {0.47—0.80}) compared to subjects with the progressive form of MS (κ=0.51 {0.27—0.74}).

Reliability method 2: Method comparison

We assessed whether the agreement between questionnaire and calendar measurements of the time in the sun in summer during weekends and holidays differed by subgroups. For the analysis for sun exposure in summer during childhood and early adolescence, we combined the answers for the age groups 6—10 years, 11—15 years and 16—20 years, so the total number of subjects was 3x sample size of 408 subjects.

Sun exposure during childhood and early adole*scence—total sample.* The agreement for sun exposure during childhood and early adolescence for the total sample did not differ significantly by age, sex, the ability to recall sun exposure (assessed by research assistant), and interviewer (Table 16). The kappa statistic was higher for subjects with a higher education (difference 0.10, p=0.02) and subjects with low levels of melanin (difference 0.10, p=0.02). Note that due to combining the data of the three age groups, the samples sizes have become sufficiently large to detect significant differences even though the magnitude of the difference was modest. As a comparison, a difference of 0.10 was not significant in the measure-retest analysis by subgroups.

Sun exposure during childhood and early adolescence—*by disease status.* When cases and controls were analysed separately, there was no significant difference by sex, level of education and interviewer. However, the difference in agreement by education was of similar magnitude and in the same direction for both cases (difference 0.10, p=0.17)) and controls (difference 0.09, p=0.08) compared to the total sample. Similarly to the total sample, cases with less melanin density had a higher kappa statistic (difference 0.16, p=0.03) than cases with more melanin density. For controls, this difference was smaller (difference 0.06, p=0.28). We did not find a difference for the total sample by ability to recall sun exposure rated by the research assistants, but controls who recalled their sun exposure well had a higher kappa statistic (difference 0.24, p<0.01) than controls who had some difficulty recalling their sun exposure. Among cases there was no difference (difference –0.08, p=0.21). Similarly, we did not find a difference for the total sample by age and interviewer, but younger cases (difference 0.14, p=0.05) and cases interviewed by interviewer 1 (difference 0.18, p<0.01) had a higher kappa statistic compared to older cases and cases interviewed by interviewer 2. In controls, there was no difference.

Sun exposure *during childhood and early adolescence—by disease related factors.* Among cases, there was no difference between the agreement for sun exposure during childhood and

early adolescence for subjects with an EDSS score lower than or equal to three (κ=0.46 {0.36—0.55}) and compared to subjects with an EDSS score higher than three (κ=0.60 {0.49—0.71}). Similarly, no differences were seen for subjects with a duration shorter than 11 years (κ=0.47 {0.37—0.58}) compared to subjects with a duration equal to or longer than 11 years (κ=0.55 {0.45—0.65}), or for subjects with the relapsing remitting form of MS (κ=0.48 {0.39—0.57}) compared to subjects with the progressive form of MS (κ=0.59 {0.46—0.71}).

Table 16. Agreement between questionnaire and calendar measurements of the time in the sun in summer during weekends and holidays during childhood and early adolescence by subgroups.

	Total sample		Cases		Controls	
Subgroup variable	a/N	κ {95%CI}	a/N	κ {95%CI}	a/N	κ {95%CI}
Age						
≤ 44 years	368/638	0.52 {0.46—0.57}	125/213	0.58 {0.49 – 0.68}	181/268	0.50 {0.41 – 0.58}
> 44 years	335/581	0.50 {0.44—0.56}	101/195	0.44 {0.34 – 0.55}	234/386	0.53 {0.45 – 0.60}
Difference		0.02		0.14		-0.03
Sex						
Men	259/393	0.44 {0.37—0.51}	82/132	0.50 {0.37 – 0.63}	175/261	0.40 {0.31 – 0.48}
Women	444/826	0.51 {0.46—0.55}	142/276	0.51 {0.42 – 0.59}	302/550	0.50 {0.44 – 0.57}
Difference		-0.07		-0.01		-0.10
Education						
Year 10 or below	375/661	0.46 {0.40—0.52}	109/198	0.45 {0.34 – 0.55}	266/463	0.47 {0.40 – 0.53}
Year 11 or higher	321/546	0.56 {0.50—0.62}	114/207	0.56 {0.46 – 0.66}	207/339	0.56 {0.48 – 0.64}
Difference		-0.10		-0.10		-0.09
Melanin density at the upper inner arm						
≥ 2%	312/568	0.46 {0.40—0.52}	83/174	0.42 {0.32 – 0.53}	229/394	0.48 {0.41 – 0.55}
< 2%	308/517	0.56 {0.50—0.62}	125/207	0.58 {0.48 – 0.68}	183/310	0.54 {0.46 – 0.62}
Difference		-0.10		-0.16		-0.06
Ability to recall sun exposure						
Very well	585/989	0.52 {0.47—0.56}	151/279	0.49 {0.40 – 0.57}	434/710	0.53 {0.48 – 0.58}
Some difficulty	106/209	0.47 {0.37 – 0.56}	77/126	0.57 {0.45 – 0.69}	35/83	0.29 {0.15 – 0.44}
Difference		0.05		-0.08		0.24
Interviewer						
Interviewer 1	391/811	0.54 {0.49—0.60}	150/240	0.59 {0.50 – 0.68}	241/402	0.51 {0.44 – 0.58}
Interviewer 2	312/577	0.47 {0.41—0.53}	76/168	0.41 {0.30 – 0.52}	236/409	0.49 {0.42 – 0.56}
Difference		0.07		0.18		0.02

Notes

1. The data "Time in the sun" question at age 6—10, 11—15 and 16—20 years were combined, so the total number of subjects was 3 x sample size of 408 subjects.
2. a/N =number of subjects with exact agreement on both reports / total number of subjects; κ {95%CI}=Kappa statistic {95% confidence interval}, weighted if more than 2 ordered categories.

Sun e*xposure in the last three years—total sample.* The agreement for sun exposure in the last three years for the total sample did not differ by any of the subgroups.

Sun exposure in the last three years—by disease status. Also, when cases and controls were analysed separately, no significant differences were observed by any of the subgroups.

Sun exposure in the last three years—by disease related factors. Among cases, there was no significant difference between the agreement for sun exposure in the last three years for subjects with an EDSS score lower than or equal to three (κ=0.32 {0.18—0.46}) compared to

subjects with an EDSS score higher than three (κ=0.41 {0.25−0.57}). Similarly, no differences were seen for subjects with a duration shorter than 11 years (κ=0.40 {0.24−0.55}) compared to subjects with a duration equal to or longer than 11 years (κ=0.35 {0.20−0.50}), or for subjects with the relapsing remitting form of MS (κ=0.34 {0.21−0.47}) compared to subjects with the progressive form of MS (κ=0.37 {0.19−0.56}).

Table 17. Agreement between questionnaire and calendar measurements of the time in the sun in summer during weekends and holidays in the last three years by subgroups.

	Total sample		Cases		Controls	
Subgroup variable	A/N	κ {95%CI}	a/N	κ {95%CI}	a/N	κ {95%CI}
Age						
≤ 44 years	80/211	0.39 {0.30−0.48}	24/71	0.36 {0.21 – 0.50}	56/140	0.40 {0.30 – 0.51}
> 44 years	73/195	0.43 {0.34−0.52}	25/65	0.39 {0.23 – 0.54}	48/130	0.41 {0.29 – 0.53}
Difference		-0.04		-0.03		-0.01
Sex						
Men	53/132	0.42 {0.30−0.53}	15/44	0.41 {0.23 – 0.58}	38/88	0.34 {0.20 – 0.49}
Women	100/274	0.37 {0.30−0.45}	34/92	0.38 {0.25 – 0.51}	66/182	0.36 {0.27 – 0.45}
Difference		0.05		0.03		-0.02
Education						
Year 10 or below	88/221	0.43 {0.34−0.52}	27/66	0.44 {0.28 – 0.60}	61/155	0.41 {0.30 – 0.51}
Year 11 or higher	63/181	0.40 {0.30−0.49}	22/69	0.35 {0.20 – 0.49}	41/112	0.41 {0.28 – 0.53}
Difference		0.03		0.09		0.00
Melanin density at the upper inner arm						
≥ 2%	74/188	0.43 {0.33−0.52}	22/58	0.40 {0.23 – 0.57}	52/130	0.42 {0.30 – 0.54}
< 2%	60/173	0.39 {0.30−0.49}	22/69	0.36 {0.22 – 0.50}	38/104	0.39 {0.26 – 0.51}
Difference		0.04		0.04		0.03
Ability to recall sun exposure						
Very well	124/329	0.42 {0.35−0.49}	29/93	0.34 {0.21 – 0.47}	96/236	0.44 {0.35 – 0.52}
Some difficulty	28/70	0.37 {0.21−0.53}	20/42	0.45 {0.25 – 0.64}	8/28	0.26 {0.00 – 0.45}
Difference		0.05		-0.11		0.18
Interviewer						
Interviewer 1	79/212	0.40 {0.32−0.49}	32/80	0.41 {0.28 – 0.55}	47/132	0.38 {0.27 – 0.48}
Interviewer 2	74/194	0.43 {0.33−0.53}	17/56	0.36 {0.19 – 0.53}	57/138	0.44 {0.32 – 0.55}
Difference		-0.03		0.05		-0.06

Notes

1. a/N =number of subjects with exact agreement on both reports / total number of subjects; κ {95%CI}=Kappa statistic {95% confidence interval}, weighted if more than 2 ordered categories.

Conclusions subgroup analysis

By reviewing the results of agreement of self-reported sun exposure on retest and the agreement between questionnaire and calendar measurements by subgroups, we did not find many substantial differences. For self-reported sun exposure in the last three years, we observed a substantial difference in the measure-retest analysis by the time between the main interview and the repeat interview. Against the expectation, a lower agreement was observed for subjects with a shorter time between the two interviews. This pattern was exhibited for both cases and controls, but was not observed for sun exposure during childhood and early adolescence. There were other significant differences in the method comparison analysis that only occurred in cases or in controls, such as a higher agreement in younger cases, a higher

agreement among controls who, according to the perception of the research assistants, recalled sun exposure well, and a higher agreement by cases interviewed by interviewer 1. Assessment of all tables lead to the conclusion that there was some consistency in a slightly higher agreement in younger subjects for sun exposure during childhood and adolescence, but there was no consistent pattern by perceived ability to recall sun exposure or by the interviewer. Reassuringly, among cases, there were no differences by subgroups of disease related factors such as disability (EDSS score), disease duration or type of MS.

6.5 DISCUSSION

This chapter presents the results of a study of reliability of measurements of sun exposure. Measure-retest comparisons for a range of other factors were used for comparison. A method comparison was conducted comparing questionnaire indices to a lifetime calendar approach.

6.5.1 Measure-retest reliability of the total sample

In general, the agreement for self-reported levels of sun exposure was moderate with the kappa statistic ranging from 0.37 to 0.56. There was no difference in the level of agreement between the "Time in the sun" question and the "Activities outside" question and no difference was observed between the agreement for sun exposure in summer compared to that in winter. The agreement for occupational sun exposure, which was assessed in broad categories (jobs overall mostly indoors, both indoors and outdoors, or mainly outdoors), was substantial (κ=0.80). The agreement for actinic damage on the dorsum of the hand, an objective measure which is thought to reflect cumulative lifetime sun exposure,[14, 15, 23] was also substantial (κ=0.68).

Only a few studies have been conducted on the reproducibility of measurements of sun exposure.[23, 24] English et al.[23] carried out a retest on 164 subjects with basal cell and squamous cell carcinoma and 102 population controls after five years of conducting a case-control study on non-melanoma skin cancer. Rosso et al.[24] conducted a reliability study on 115 cases with basal cell and squamous cell carcinoma and 119 population controls, 18-26 months apart, in four centres of Italy, Spain and France. A few studies have been conducted on the reliability of measures of sunburn and sun sensitivity but these are not discussed in detail here.[25-28]

English et al. observed a good level of agreement (ICC 0.77 {0.71—0.83} using a one-way random effects model) for lifetime sun exposure measured on a continuous scale using a lifetime calendar. Even though the agreement appeared good, they stated that they observed differences for a small percentage of people (<3%) between the two different interviews as much as 30,000 hours over a lifetime which was large in relation to the range of sun exposure (mean 48,788, SD 22,868 for the total sample). In a situation where the variance between subjects is much larger than the variance within subjects, it is possible to obtain a relatively high intraclass correlation coefficient even though the variance within subjects is considered to be high. An intraclass correlation coefficient can only be compared with a weighted kappa if the weights are used that were proposed by Fleiss and Cohen.[29] We used the weights proposed by Cichetti and Allison, but using the weights from Fleiss and Cohen increased the mean (SD) weighted kappa for the "Time in the sun" question at age 6-10 years, 11-15 years, 16-20 years and the last three years for summer and winter from 0.47 (0.07) to 0.60 (0.07), which is much closer to the agreement observed by English et al. Rosso et al. also found a good agreement for a weighted index of sun exposure hours in a lifetime (ICC 0.79 {0.72—0.85} during holidays, ICC 0.68 {0.58—0.77} for outdoor work). As expected, single items of the indices exhibited a lower reliability, because it is known that a compounded index has a higher reliability than its single component, provided it is internally consistent.[30] This might also partially explain the higher agreement in these studies compared to our study.

Interestingly, both studies found that exposure in the recent past was not much more reproducible than exposure in the distant past (English et al.: ICC=0.55 between age 8-14

years, ICC=0.77 for age 15-19, ICC=0.73 for age 20-24 years; Rosso et al.: ICC=0.65 for outdoor hours at the beach in childhood). Our study observed a similar pattern for the "Activities outside questions', but the agreement for "Time in the sun" question was higher for sun exposure in the last three years compared to childhood sun exposure. In addition, younger people recalled their sun exposure during childhood and adolescence slightly better than older people, while there was no difference for sun exposure in the last three years. Although there was more variation for childhood sun exposure on retest, the agreement between the questionnaire and calendar measurements showed a slightly higher agreement for childhood sun exposure than for sun exposure in the last three years, suggesting that if we had done a measure-retest comparison using the calendar method, we would have found a slightly higher agreement for childhood sun exposure than for sun exposure in the last three years. The order of asking first about sun exposure during childhood and adolescence and then during adult life was similar in both situations.

The agreement for measurement of other factors ranged from fair to nearly perfect. The agreement for measures of sun sensitivity, lifetime burns and use of sunscreen was similar to the agreement for self-reported sun exposure. However, the agreement for infections were generally higher, especially for glandular fever (κ=0.87) and cold sores (κ=0.89), which both had a nearly perfect agreement and was even higher than the agreement for occupational sun exposure. For infections, the agreement was lowest for measles (κ=0.48) and German measles (κ=0.52), probably because subjects had difficulty discriminating between the two. Agreement for the immunisation against German measles was substantial (κ=0.73), but agreement for other immunisations were generally lower, with the kappa statistic ranging of 0.28 to 0.54. The agreement for exposure to chemicals from hobbies, chores or jobs and adult exposure up to the age of diagnosis ranged from moderate to substantial, depending on the type of chemical assessed. The agreement was best (κ>0.60) for "sawn wood and wood dust", "petroleum products" and "paint and varnish". The agreement was lower when the subject was exposed through other family members during childhood or adolescence years and did not actually perform the task of exposure him or herself. The agreement for whether subjects were breastfed and the age of menarche was substantial, but the agreement for more detailed information on the time of being breastfed was only moderate. The kappa statistic for vitamin D containing foods and supplements ranged from 0.34 to 0.62 and was only slightly higher (mean κ=0.54, SD 0.09) for intake in the last 12 months compared to when aged 10 to 15 years (mean κ=0.50, SD 0.11).

Other studies have found that the reliability of measurements depended on a number of factors, such as the nature of the factor, the amount of detail sought and the amount of complexity.[31] The nature of the exposure includes aspects such as variability over time, severity of the exposure and how common the exposure is.[31] Indeed, we observed that agreement was higher when an objective measure was used (i.e. actinic damage), when the exposure was more dramatic (i.e. glandular fever), when the exposure reoccurred throughout life (i.e. cold sores), when the information sought was not complex (i.e. occupational sun exposure) or when the subject conducted the actions him- or herself (i.e. exposure to chemicals during childhood and early adolescence from hobbies, chores or jobs). In contrast, agreement was lower when the information sought was more complex (i.e. self-reported sun exposure and sun sensitivity), when more detail was requested (i.e. time of being breastfed), or when subjects were exposed through others (exposure of chemicals during childhood and early adolescence though family members).

Of the sun exposure indices, the reliability was highest for the question on occupational sun exposure and the assessment of cumulative sun exposure via actinic damage. In a case-control study, the high reliability would result in low measurement error and would provide us with an observed odds ratio close to the true odds ratio, which is the odds ratio that would have been found if the measurement of the exposure of interest was perfect. However, if sun exposure in childhood or adolescence years were a more important risk factor of disease than occupational sun exposure or lifetime sun exposure, then we need to rely on the use of self-reported sun exposure in the distant past.

The question is how much the observed odds ratio in a case-control study would be attenuated compared to the true odds ratio. The results showed that sun exposure during childhood and early adolescence assessed as a dichotomised variable had on average 78.3% exact agreement and thus 21.7% of the subjects were misclassified. We will use our MS case-control study with 136 cases and 272 controls to assess the effect of 21.7% misclassification on the observed odds ratio. We assume that the misclassification is non-differential between cases and controls and that 50% of the controls had high sun exposure. If high sun exposure had a protective effect on the risk of MS and the true odds ratio would have been 0.25 (which equals to 27 cases low sun exposure and 109 high sun exposure), then the observed odds ratio would have only been 0.49 (45 cases low and 91 cases high sun exposure). Similarly, a true odds ratio of 0.10 (12 cases low and 124 cases high sun exposure) would attenuate to 0.36 (36 cases low and 100 cases high sun exposure). Thus, this substantial misclassification could hamper finding an association between sun exposure and MS. Statements of these kind have also been made for melanoma and non-melanoma skin cancer,[32] but there is currently substantial evidence to suggest that sun exposure is an independent risk factor of melanoma and non-melanoma skin cancer. Moreover, the ecological analysis conducted in chapter 4 showed that the correlation between regional UVR levels and MS prevalence was stronger (r=-0.91) than the correlation between UVR and malignant melanoma incidence (r=0.75, p=0.15 for males and r=0.80, p=0.10 for females), which could suggest that sun exposure might be a stronger risk factor in MS compared to malignant melanoma. In addition, important associations have been found between disease and exposure factors that have a similar reliability as sun exposure. Physical activity, for example, has a reliability of similar magnitude,[4] and analytical studies have shown that an increased amount of physical activity decreases the risk of non-insulin-dependent diabetes mellitus.[33]

6.5.2 Method comparison of self-reported sun exposure of the total sample

One of the main validated sun exposure questions, the “Time in the sun” question, was obtained via two different methods. In the first approach, the question was incorporated in the main interview and asked for four different time periods, age 6—10 years, 11—15 years, 16—20 years and in the last three years. In the second approach, the question was part of a life events calendar. This approach has been previously used in epidemiology.[3-7] The life events calendar makes use of a cognitive interviewing technique that includes memory probes and has a specific recall strategy.[34] Prior to the interview, subjects were sent the calendars and asked to fill out the part of the calendar regarding residence, schools and occupations and exposure to pets and farm animals. This provided life events references, which were then used by the interviewer and participant to identify blocks of years where the “Time in the sun” lifestyle was fairly constant and where changes could have occurred. This part of the interview was less standardised and there was some interaction between interviewer and participant about the start and finish of certain blocks, but the research assistants were trained to guide

the subject through the calendar and directed that it should always be the participant reporting how many hours per day they would spend in the sun during weekends and holidays. The use of this method could improve the reliability and validity of the data obtained.[2]

We observed moderate agreement between the two methods, with the kappa statistic ranging from 0.42 to 0.54. The agreement decreased with age with sun exposure at age 6—10 years having the highest and sun exposure in the last three years having the lowest agreement. Interestingly, participants reported on average higher levels of sun exposure using the calendar approach compared the questionnaire indices for the same age groupings. This was especially pronounced for sun exposure in the last three years. We did not conduct a measure-retest comparison using the calendar and therefore cannot assess the random error of sun exposure using this method. Also, this study does not give us information on which method is more valid. If the calendar would be the better method to use, we could potentially expect odds ratios of a higher magnitude in our case-control study.

The pattern of over reporting at the lifetime calendar was observed for the participants of both interviewers, but there were some differences by interviewer, which demonstrates the importance of standardisation. At the start of the study, training sessions were held discussing the interviewing techniques and how to avoid differences between interviewers. To maintain a high standard during the study period, meetings were held and both interviewers were observed while conducting a number of interviews every six to eight months.

6.5.3 Agreement of self-reported sun exposure by disease status

It is known that MS can affect the cognitive function of the brain.[8-10] We limited our sample to subjects under the age of sixty. By doing this, we anticipated to decrease the number cases with cognitive dysfunction. Estimates of cognitive dysfunction in MS patients have been high. In clinic populations estimates ranged from 54% to 65%, while a case-control study, comparing a general MS population to a healthy control sample found impairment on four or more of the 31 cognitive test indices in 48% of the MS patients and 5% of the controls, giving an overall frequency rate of 43% for the MS group.[8] There are, however, many aspects of cognitive function, such as immediate, recent and remote memory, sustained attention, verbal fluency, conceptual reasoning, visuospatial perception and language,[9] and we suspected that only some of those aspects, such as recent and remote memory would influence the reliability of the measures in our case-control study. Measures of recent memory have been shown to be influenced in MS patients but not remote memory.[8] A review by Rao[9] indicated that some studies found that patients recalled poorly on tasks which require spontaneous and free recall of information. Therefore, the well-structured questionnaire might limit potential recall problems.

If cognitive dysfunction in cases would decrease the recall of exposure measures in our case-control study, it would lead to an increased measurement error in cases compared to controls which in turn could reduce the observed odds ratio. We did not assess cognitive function in our participants, but one way to assess whether the recall in cases could be a concern in our case-control study is to compare the level of agreement on retest of cases to that of controls. Participants having difficulties in their recall were likely to provide more variable answers in the two consecutive times of measurement resulting in a lower level of agreement.

For measurements of self-reported sun exposure and occupational sun exposure, we found no evidence that agreement was lower for cases than for controls. In fact, the agreement for

self-reported sun exposure in the last three years was marginally higher for cases. The agreement for actinic damage, an objective measure not dependent on recall, was slightly lower for cases. The reason for this difference is unclear, but the agreement was not related to the degree of actinic damage that subjects had.

For other factors, the agreement for cases and controls was similar for sun sensitivity, lifetime sunburns, the use of sunscreen, having had immunisations, and intake of vitamin D containing foods and supplements. A slightly lower agreement for cases was observed for most infections and exposure to some chemicals during childhood and early adolescence. However, a slightly higher agreement for cases was observed for most ages of infections, early life exposure to some chemicals through other family members, adult exposure to some chemicals, whether breastfed and age of menarche.

Among cases, cognitive function has been found to be positively associated with higher disability (measured by EDSS score), the progressive form of MS and higher age.[10] We found no evidence that among cases the agreement differed by disability (EDSS score) type of MS and duration of MS. We found some evidence that higher age was related to a lower agreement for sun exposure during childhood and early adolescence, but this effect was weak.

Overall, we found little evidence that the reliability of measures of exposure from cases was lower than that of controls. This suggests that even if there was some degree of cognitive dysfunction among cases, it will not likely affect the results of the MS case-control study.

6.5.4 Agreement of self-reported sun exposure by other subgroups

We did not observe any substantial and consistent differences in the agreement of self-reported sun exposure by sex, education, melanin density at the upper inner arm, ability to recall sun exposure or interviewer. The implication for the analysis of past sun exposure in the case-control study is that we are not required to undertake stratified analyses for these variables.

As mentioned before, older subjects had a slightly lower agreement compared to younger subjects for sun exposure during childhood and adolescence, while the agreement was similar for sun exposure in the last three years. This effect is in the direction we expected. The effect was not strong, but it is possible that in the control study we might observe an odds ratio closer to one for older people, which could have been caused by increased measurement error in the older group compared to the younger group. It might therefore be useful to limit the sample to younger subjects for some analyses.

The agreement for self-reported sun exposure in the last three years varied by the time between the main interview and the repeat interview, with a lower agreement for subjects with a shorter time between the two interviews. We expected that people with a shorter time between the two interviews might have a higher agreement, because they had a potentially higher chance of remembering answers and a lesser chance that a change in season influenced their answers. The reason for this observation is unknown.

6.5.5 Limitations of this study

In this study we were able to assess the reproducibility of our measures but not the validity. However, another study validated measures of self-reported sun exposure in teenagers by

using polysulphone badge readings as the comparison method.[11] It showed that the "Time in the sun" question and the "Activities outside" question were valid measures of habitual sun exposure in this setting[11]. We assessed the precision of our measures by estimating a random measurement error component using agreement, but were unable to identify whether the measurement error was different for cases compared to controls. Comparing agreement for cases and controls does not reveal differential measurement error, because if cases, for example, had a tendency to overestimate a particular exposure factor during the main interview, then they were likely to do the same during the repeat interview. Objective measures, such as actinic damage, are generally less prone to differential measurement error because they do not rely on the subject's perception.

Although the main interview and repeat interview were conducted by different interviewers, the standardised questionnaire and interview protocol that was employed limited interviewer-administration effects. Also, the wording that was used by the interviewers was agreed in advance and typed on the questionnaire, and interviewers were not permitted to re-word the questions or to ask them in any different sequence to that on the questionnaire.

6.5.6 Conclusions

In conclusion, we found that agreement for self-reported sun exposure was moderate and could lead to a substantial attenuation of the observed odds ratio in a case-control study. Agreement of an objective measure of cumulative lifetime sun exposure, actinic damage, was substantial. There was no evidence that the reliability for different exposure measures was lower in cases compared to controls, and reassuringly, among cases, there were no differences in reliability by subgroups of disease related factors such as disability (EDSS score), disease duration or type of MS. This provides confidence that even if there was some degree of cognitive dysfunction among cases, it is not likely to affect the results of the MS case-control study. No differences were observed in the agreement of self-reported sun exposure by sex, education, melanin density at the upper inner arm, ability to recall sun exposure or interviewer, but the agreement differed slightly by age.

6.6 SUMMARY

Measurement error of exposure variables is one of the major sources of bias in case-control studies. It was our objective in this chapter to assess the reliability of measures of past sun exposure and to examine whether self-reported sun exposure differed by disease status or other variables, such as age, gender, education, melanin density at the upper inner arm, and ability to recall sun exposure. A measure-retest comparison was conducted on 52 prevalent cases and 52 community controls, who were re-interviewed after 11 weeks on average. In addition, questionnaire administration of 136 cases and 272 controls of a validated sun exposure question for different age periods was compared to the use of this question in a life events calendar, where memorable events were used as guideposts. We found that agreement for self-reported sun exposure was moderate and similar for the two comparison methods. Agreement of an objective measure of cumulative lifetime sun exposure – actinic damage – was substantial. There was no evidence that the reliability for different exposure measures was lower in cases compared to controls, and reassuringly, among cases there were no differences in reliability by subgroups of disease related factors such as disability (EDSS score), disease duration or type of MS. Also, no differences were observed in the

agreement of self-reported sun exposure by sex, education, melanin density at the upper inner arm, ability to recall sun exposure or interviewer, but the agreement differed slightly by age. In conclusion, the moderate agreement for self-reported sun exposure found in this study could lead to a substantial attenuation of the observed odds ratio in a case-control study, while the similar reliability between cases and controls suggests that, even if there was some degree of cognitive dysfunction among cases, it is unlikely to affect the results of the Tasmanian MS case-control study.

6.7 POSTSCRIPT

In this chapter we discussed the reliability of one of our main exposures of interest – sun exposure. We found that self-reported sun exposure is recalled only with moderate agreement, which could lead to a substantial attenuation of the observed odds ratio in our Tasmanian MS case-control study, and could limit us in finding an association. The reliability of actinic damage, an objective measure of cumulative lifetime sun exposure, was however, substantial. The results of the case-control study in regard to the UVR hypothesis (whether high levels of past UVR exposure is related to a decreased risk of MS) will be discussed in chapter 8. The next chapter will focus on the reliability of the spectrophotometric assessment of skin type.

6.8 REFERENCES

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Chapter 7

Misclassification due to body hair and seasonal variation on melanin density estimates for skin type using spectrophotometry.

7.1 PREFACE

The previous chapter discussed the reliability of measures of past sun exposure, because this relationship between sun exposure and risk of Multiple Sclerosis (MS) is the primary hypothesis of this thesis. Skin phenotype is another factor that might be related to the risk of MS. Alternatively, it could modify or confound the relationship between sun exposure and MS. To measure skin phenotype, it is possible to rely on self-reported indices, but the previous chapter showed only a moderate agreement for measures of self-reported sun sensitivity. Reflectometers such as spectrophotometers have been shown to produce results that are more reproducible, but the choice of where on the body to take the measurements has implications on the reliability of the measurements. A separate study on healthy volunteers was conducted to examine this topic. Most of this chapter has been published in the Journal of Photochemistry and Photobiology 2002;68:45-52.

7.2 INTRODUCTION

Melanoma is more common among light-skinned than dark-skinned populations living at the same latitudes,[1] and research on the aetiology of melanoma[2] and non-melanoma skin cancer[3] indicates that light skin colour is associated with risk of skin lesions. In MS, the prevalence is also higher among Caucasians compared to darker-skinned populations such as Amerindians, Chinese, Japanese, African blacks and New Zealand Maoris.[4] In type 1 diabetes, also thought to be a Th_1-mediated autoimmune disease, a case-control study with subjects of German origin showed that cases tended to be fairer than controls (odds ratio 3.7, $p<0.01$).[5] Therefore, it seems possible that risk of MS differs by skin phenotype, even within a Caucasian population.

The commonly used markers of skin phenotype have been eye, hair and skin colour. The various measures of skin colour included self-report, interviewer appraisal using skin tone charts or prosthesis[6-8] or by personal assessment,[9] and skin reflectance of light at the infrared end of the visible light spectrum.[10, 11] These phenotype properties have been selected for research because they appear to be markers for the biological differences that distinguish northern Europeans from lower risk populations. While the biological basis for these measures has not usually been made explicit, it is likely that they have been used as proxies for the density or type of melanin in the skin.[12]

There have been attempts to estimate melanin density in the skin using reflectometers. As early as 1939, Edwards and Duntley[13] demonstrated that the reflectance of light in the visible spectrum (400-700 nm) is influenced by the presence of melanin, as well as by haemoglobin and carotene. Melanin was found to absorb more light at shorter wavelengths and to exhibit steadily decreasing absorption up to 700 nm. Absorptions by haemoglobin and by carotene

were more intermittent, with peaks at 418-429 nm and 542-576 nm (haemoglobin) and at 482 nm (carotene). The subsequent development of non-invasive skin reflectance measurements of melanin density focused on reflectance at wavelengths of 650-700 nm in the belief[14] that although absorption by melanin at those wavelengths was relatively low, the total absorption of light could be attributed solely to the presence of melanin because there was no contribution by haemoglobin or carotene.

To find the best reflectance estimate of melanin density, we recently compared epidermal concentrations of melanin, analysed histologically in skin biopsy specimens with reflectance measures using a spectrophotometer at the upper inner arm, an area usually not exposed to the sun.[15] The spectrophotometer output also included the L* value (the measurement of lightness of colour across the visible light spectrum) used with the Commission International d'Eclairage system of colour assessment.[16] The study, conducted on 82 volunteers, showed that cutaneous melanin in Caucasians can be estimated best by the difference between two measurements of reflectance: those at wavelengths 400 and 420 nm.[15] This measure explained 46.5% of the variance (r=0.68), while L* explained 21.3% of the variance (r=-0.46) and reflectance at 680nm explained 10.7% of the variance (r=-0.33).[15] The measure of Kollias and Baqer[17] was not included in this validation study, but when we reanalysed the data using that method we found that it explained 29.1% of the variance (r=-0.54). According to their method,[17] we used the average of three reflectance readings from the white surface in the cap of the spectrophotometer as a reference. Then, the ratio of the reference reflectance at each wavelength (20nm intervals over the range 620-700nm) to the skin reflectance at the upper inner arm at that wavelength was taken and the measure of Kollias and Baqer was calculated by taking the slope of the regression of the log of the ratios at the each wavelength. Absorption at 400 and 420 nm is principally due to the presence of cutaneous melanin and haemoglobin, while there is a lesser contribution by carotene. It is assumed that by subtracting the spectrophotometric estimate of reflectance at 400 nm from that at 420 nm, the contribution by haemoglobin is removed, because it is similar at both wavelengths.[15] Cutaneous melanin density at the upper inner arm estimated in this way has been shown to predict the occurrence of common melanocytic naevi,[18] the presence of large numbers of which is a very strong risk factor for cutaneous malignant melanoma.[8] A recent publication from Menzies Centre colleagues demonstrated that this measure, particularly for men, is a strong predictor of cutaneous malignant melanoma, basal carcinoma and squamous cell carcinoma of the skin.[12]

The upper inner arm was selected as the site for reflectance measurements on the grounds that this skin is not often exposed to the sun. It is rarely completely unexposed to ultraviolet radiation however, and seasonal variation in skin reflectance has been observed.[19, 20] A significant darkening of the skin was found to occur in spring and summer.[19, 20] Because seasonal variation was less pronounced for buttock skin,[20] Lock-Andersen and Wulf[20] have suggested that the buttock rather than the upper inner arm should be the body site used for reflectance measurements of constitutive pigmentation.

The ideal site for measuring constitutive pigmentation would provide skin type estimates that were not unduly influenced by other factors such as body hair or temporary alterations related to seasonal ultraviolet radiation exposure. If the site is influenced by other factors, then this could lead to skin type misclassification.

The first aim of this chapter was to assess the effect of body hair on melanin density estimates based on skin reflectance at the buttock and the upper inner arm. Because the seasonal variation previously reported at the upper inner arm[19, 20] was in reflectance of light near 660 nm, and seasonal variation in a new method of estimating melanin based on skin reflectances

at 400 nm and 420 nm[15] had not been investigated, a second aim was to assess whether the estimates at the buttock and the upper inner arm made with the new method were also subject to seasonal influence. Thirdly, we sought to assess the consequences of constitutive skin type misclassification due to body hair and seasonal variation, by determining the impact that it would have on the results of analytical case-control studies.

7.3 METHODS

7.3.1 Subjects

To recruit participants, leaflets were placed at the main teaching hospital and the associated university department in Hobart, Tasmania, Australia. The participants were visitors, staff and their friends, not patients of the hospital. The sample was limited to healthy Caucasian volunteers between the age of 20 and 59 years who had not been on a holiday outside the island of Tasmania in the two months prior August 1998. Subjects were measured five times: end of winter 1998 (last week of September and the first week of October), end of spring 1998 (last week of November and the first week of December), end of summer 1999 (last week of February and first week of March), end of autumn 1999 (last week of May and first week of June) and end of winter 1999 (last week of September and first week of October). The project was approved by the Ethics Committee of the University of Tasmania and all participants provided informed written consent prior the study. Of the 122 subjects, 104 (85.2%) completed all five sessions.

7.3.2 Spectrophotometric measurements and body hair assessment

A hand-held Minolta 508 spectrophotometer was used to measure skin reflectance on two usually unexposed body sites, the upper inner arm and buttock. The exact position at the upper inner arm was halfway along the line between the mid point of the axilla and the medial epicondyle of the humerus. The buttock measurements were taken at the medial and upper quadrant of the buttock. Both measurements were taken on the left side of the body. For the upper inner arm measurements, the subject was seated in a chair with the arm rested at heart height to reduce variation in measurement due to blood flow. For the buttock measurements, the subject was in the standing position.

The measurements were taken firstly with body hair and secondly after gentle shaving to remove body hair. In order to minimize the possible effect of increased blood flow due to shaving, the second measurement was delayed for three minutes after the shaving took place.

During each measurements session, four characteristics of the hair at the measurement sites were recorded: amount of hair (‘a lot’, ‘a fair bit’, ‘not that much’, ‘little’, ‘no hair’), colour of hair (‘dark’, ‘medium’, ‘light’), length of hair (‘long’, ‘medium’, ‘short’) and type of hair (‘coarse’, ‘medium’, ‘fine’). The assessments were conducted by a single observer (the author). The pair wise weighted kappa coefficients of agreement for the ratings of the amount of hair at the buttock in the five sessions were in the range of 0.66 - 0.77.

7.3.3 Estimation of melanin density

The spectrophotometer measured reflectance at 20 nm intervals in the wavelength range 400-700 nm. The equation for the estimation of melanin density was based on previous results:[15]

$$MD_{400} = 100 \times [0.035307 + 0.009974 \times (R_{420} - R_{400})]$$

where MD_{400} is an estimate of the percentage of the epidermis of the skin at the upper inner arm that contains melanin, and R_{400} and R_{420} denote respectively the averages of three measurements of reflectance at 400 nm and 420 nm made at that site. Melanin estimated in this way was previously found to be closely correlated (r=0.68) with histopathological

measurements of the density of cutaneous melanin in 3 mm punch biopsies taken at the upper inner arm after the reflectance measurements were taken.[15] As evidence of the high reproducibility of measurements made by the single observer, the percentage of total variation in MD_{400} at the upper inner arm in session one attributed to within-person variation in the three measurements was 0.3 percent. The intraclass correlation was 0.99, calculated from a one-way random effects model using ICC(1,1) from Shrout and Fleiss:[21]

$$\text{ICC} = \frac{BMS - WMS}{BMS + (k-1)WMS}$$

with BMS being the between-subjects mean square, WMS being the within-subjects mean square and k the number of ratings, which is three in our situation.

7.3.4 Data analysis

For each subject, mean melanin density was calculated by averaging the measurements made at the end of spring, summer, autumn and winter. The average of the two end of winter readings was used as the winter measurement. These means were then averaged to provide the group means presented in Table 1. For analyses where melanin density estimates at the buttock were involved, three male and four female subjects were excluded because they had a recent history of nude sunbathing or had used a solarium without wearing underwear. One person, with readings more than four standard deviations higher than the mean of the sample, was excluded from calculation of means and standard deviations.

Effect of body hair on the melanin density estimates.

A paired t-test was used to test whether removal of hair produced a significant effect on the melanin density estimates and a t-test was used to compare the means of men and women. An intraclass correlation coefficient was calculated between the unshaved and shaved measurements from a two-way mixed effects model using formula ICC [3,1] from Shrout and Fleiss:[21]

$$\text{ICC} = \frac{BMS - EMS}{BMS + (k-1)EMS}$$

with BMS being the between-subjects mean square, EMS being the residual sum of squares and k the number of ratings, which is two in our situation. This model was applied because the unshaved and shaved measurements can be seen as two fixed judges.

The relationship between the effect of hair and the four hair characteristics was further investigated using analysis of variance. The amount of hair was dichotomised as little hair (combining categories of 'no hair' and 'little') and more than a little (combining categories of 'not that much', 'a fair bit' and 'a lot').

Seasonal variation in the melanin density estimates.

To depict the seasonal variation in melanin estimates at the buttock and upper inner arm, a sinusoidal model was fitted by linear regression with a period of 12 months:

$$MD_{400\,i} = \beta_0 + \beta_1 \sin\frac{2\pi t_i}{12} + \beta_2 \cos\frac{2\pi t_i}{12}$$

where t_i is the month of measurement (0-12) of the i^{th} MD_{400} reading. The ANOVA F-test was used to decide whether there was significant seasonal variation and the amplitude was calculated using the formula $\sqrt{\beta_1^2 + \beta_2^2}$.

Effect of body hair on skin type classification.

The percentage of subjects misclassified when melanin at the buttock was measured in the presence of body hair was calculated. Measurements after shaving are the best estimates of the true values, and the measurements with hair are the observed values that would be seen during fieldwork if subjects were not shaved. The melanin estimates were dichotomised at the median. Subjects with melanin estimates lower than the median are referred to as having low melanin and subjects with melanin estimates higher than the median are referred to as having high melanin.

We next assessed to what extent any non-differential measurement error due to body hair would influence the results of a hypothetical unmatched case-control study with 150 cases and 300 population-representative controls. With melanin dichotomised at the median, 150 would have lower-than-median melanin. A ratio of 2:1 comparing the odds of low melanin among cases with the odds of low melanin among controls is suggested by the results of our recent case-control study.[12] This would require 100 cases to have low melanin. This categorisation is referred to as the true classification. To calculate the observed classification based on melanin estimated without shaving, we applied the misclassification percentages estimated as outlined in the paragraph above. The odds ratio was then re-calculated based on the categorisation with misclassification.

Measurement error is differential when the misclassification is different for cases compared to controls. This could occur when hair characteristics at the buttock such as amount, colour, type and length of hair are different for cases compared to controls, so that the effect of shaving of the hair at the buttock is different for cases and controls. As far as we are aware, no studies have investigated this possibility. There is evidence that both melanoma and non-melanoma cases have on average 15% (women) to 30% (men) less cutaneous melanin at the buttock than controls.[12] As a test of whether there could be differential measurement error, we calculated the Spearman correlation between the effect of shaving (measured by the difference between the melanin estimates at the unshaved and the shaved buttock) and the amount of melanin at the buttock. To assess the extent to which differential measurement error due body hair would influence the results of the hypothetical unmatched case-control study, we created a hypothetical group of cases (those of our subjects with the lowest melanin estimates) and a hypothetical group of controls (all of our subjects). Sufficient subjects were chosen as cases to make the average melanin at the shaved buttock 30% lower for the cases than for controls. The melanin estimates of both groups were dichotomised at the median of the controls. The misclassification due to body hair for the group of cases and controls was then calculated using the buttock melanin estimates with and without shaving.

To calculate the effect on the odds ratio, we calculated first the observed classification by applying the misclassification percentages in our hypothetical example. The odds ratio was then re-calculated based on the observed categorisation with differential misclassification.

Effect of seasonal variation on skin type classification.

The percentage of subjects misclassified when measurements were made at different times of the year was calculated. The true value for each person was taken to be the winter measurement and the observed value was one of those four seasonal measurements chosen at random. The percentage of subjects misclassified when melanin was measured randomly in any of the four seasons was then calculated. Because the misclassification that resulted depended upon which value happened to be chosen for each individual (one of the four

seasonal values), we repeated this 10,000 times, and used the mean of the 10,000 misclassification percentages as the estimated misclassification.

To assess to what extent any non-differential measurement error due to seasonal variation would influence the results of a hypothetical unmatched case-control study, we used the same hypothetical case-control data as outlined above. To calculate the observed classification based on melanin estimates randomised according to their time of measurement, we used the misclassification percentages estimated. The odds ratio was then re-calculated base on the categorisation with misclassification.

Measurement error could be differential when different proportions of cases and controls are measured in any period of the year. The extreme possibilities are that all cases were measured in summer with all controls measured in winter, and that all cases were measured in winter with all controls measured in summer. The true values (the winter measurements) were dichotomised at the median. The observed values were dichotomised at the median of values for the controls (measured in winter on one occasion, and in summer on the other). The misclassification percentages were then calculated.

To calculate the observed classification, we applied the relevant misclassification percentages to the case and control groups separately. The odds ratio was re-calculated based on this classification.

7.4 RESULTS

The phenotypic characteristics of the study subjects who completed all five sessions are shown in Table 1. More women than men happened to have brown, hazel or green eyes ($p<0.03$) and women also had higher average melanin estimates at the upper inner arm ($p<0.01$).

Table 1. Demographic details of study subjects stratified by gender.

Study factor	Men (n=49)	Women (n=55)
Age distribution		
20-29 years	24.5% (12)	23.6% (13)
30-39 years	26.5% (13)	27.3% (15)
40-49 years	30.6% (15)	29.1% (16)
50-59 years	18.4% (9)	20.0% (11)
Eye colour		
Brown	14.3% (7)	25.5% (14)
Hazel or green	28.6% (14)	38.2% (21)
Blue or grey	57.1% (28)	36.4% (20)
Hair colour		
Black or dark brown	24.5% (12)	32.7% (18)
Mid or light brown	46.9% (23)	40.0% (22)
Mousy or light blond	22.4% (11)	25.5% (14)
Red	6.1% (3)	1.8% (1)
Mean melanin density % [SD]		
Upper inner arm	1.66 [0.96]	2.17 [0.85]
Shaved buttock	0.61 [0.67]	0.65 [0.61]
Unshaved buttock	0.87 [0.62]	0.66 [0.59]

Notes

2. For the mean melanin density, the average was taken of the spring value, summer value, autumn value and average two winter values.
3. For the melanin density results of the buttock, three men and four women were excluded due to nude sunbathing and/or solarium use.

7.4.1 Effect of body hair on the melanin density estimates

None of our subjects had hair covering the upper inner arm, and the melanin density estimates were not changed by shaving ($p=0.55$).

The mean estimates of melanin density at the buttock before and after shaving are shown in Table 1. The effect of removing body hair by shaving at the buttock reduced the melanin estimates in men ($p<0.01$), but not in women ($p=0.40$). The intraclass correlation coefficient between shaved and unshaved readings was 0.93. Prior to shaving, the mean buttock readings were higher for men than for women ($p=0.05$), but there was no difference in mean melanin estimates by gender after shaving ($p=0.77$).

The effect of shaving at the buttock increased with the amount of body hair ($p<0.01$)(Figure 1). Of the people who had any hair at the buttock, colour of hair on its own did not have significant effect ($p=0.89$) and length and type of hair were not independent of amount of hair.

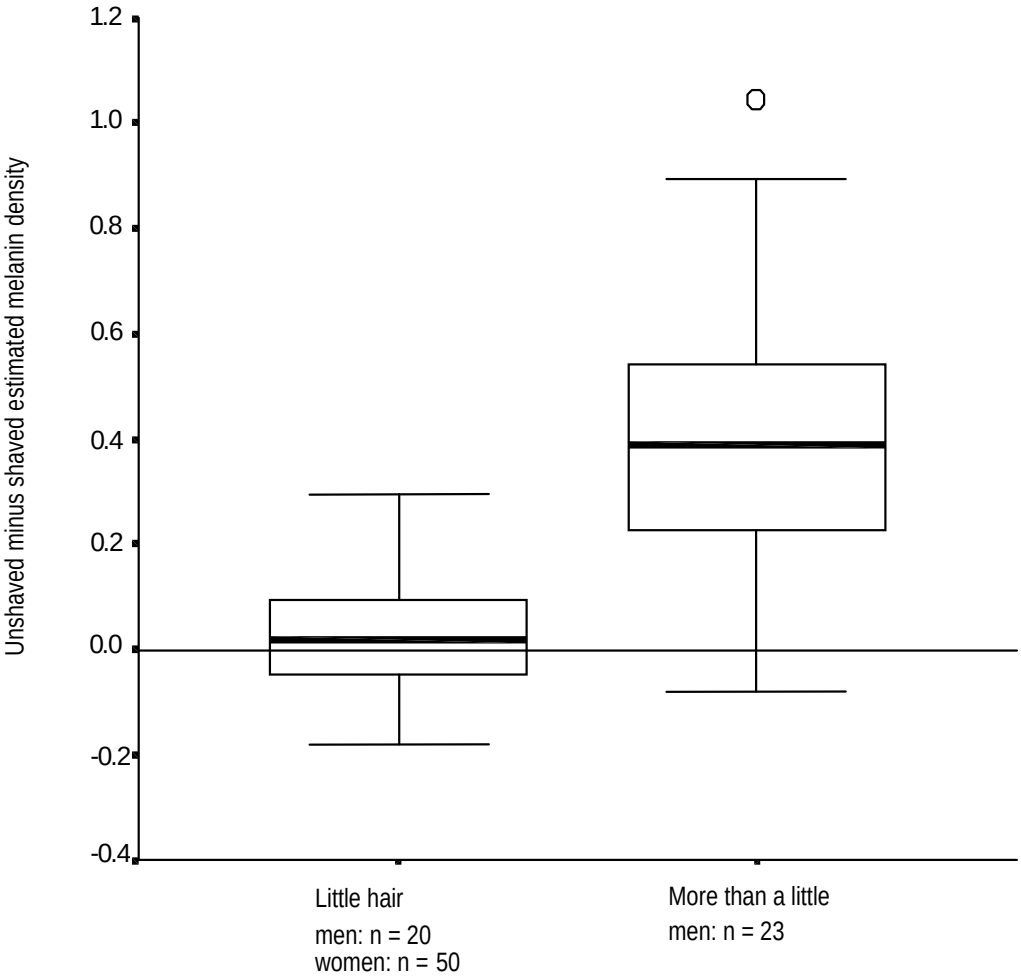

Figure 1. Box and whisker plot of the effect of shaving at the buttock on the estimates of melanin density by the amount of hair. The top and bottom of the box represent the 75th and 25th percentiles, respectively, and the whiskers extend to the 90th and 10th percentiles. An individual point above the 90th percentile is plotted as an open circle.

7.4.2 Seasonal variation in the melanin density estimates

To analyse the effect of season, a sinusoidal model was fitted to the quarterly estimates of melanin density at the upper inner arm and the buttock. There was statistically significant seasonal variation at the upper inner arm (p=0.02), but not at the buttock (shaved p=0.28, unshaved p=0.37) (Figure 2). At the upper inner arm, 1.6% of the variance could be explained by seasonal variation. The amplitude (half the difference between the maximum and minimum values) of the seasonal variation in melanin density was 0.17%. The maximum value of 2.08% was reached in early April, just after summer. For the buttock only 0.50% of the variance was explained by seasonal variation with an amplitude of 0.07% of melanin.

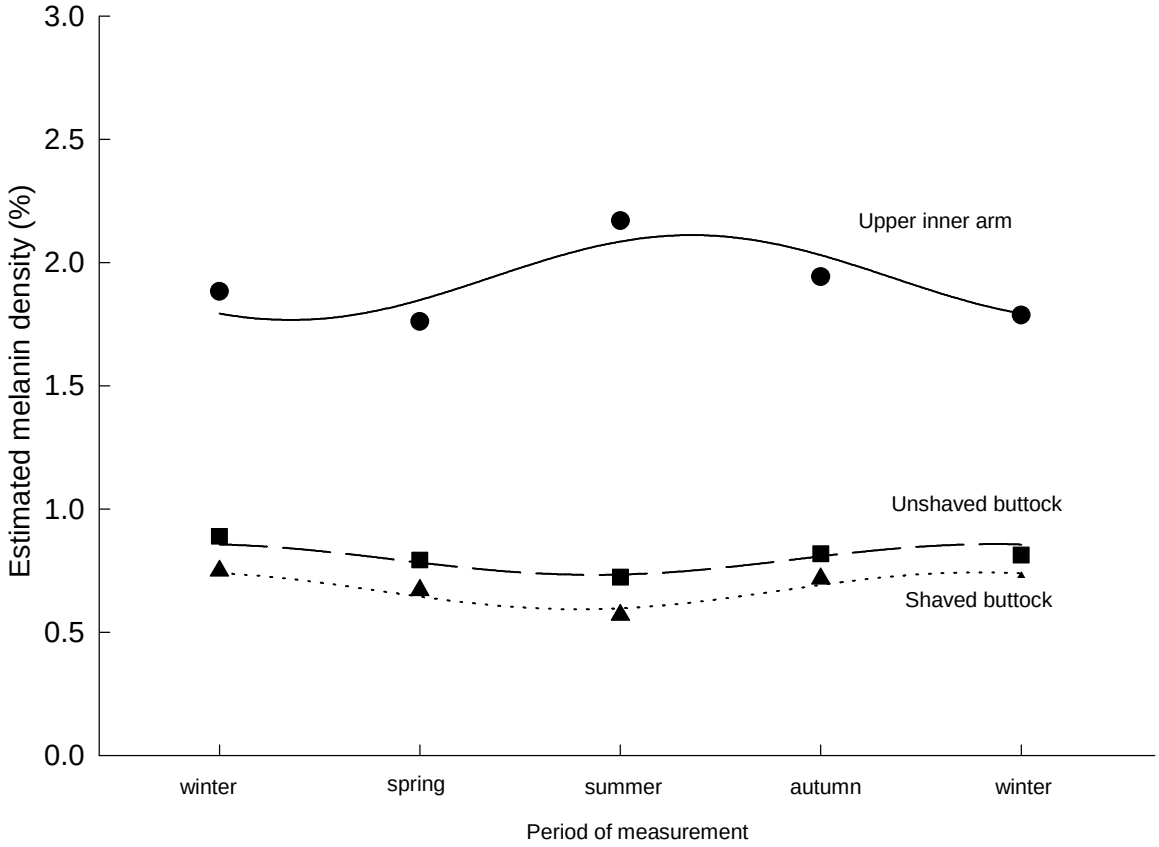

Figure 2. Seasonal variation in melanin density at the upper inner arm, unshaved and shaved buttock.

7.4.3 Effect of body hair on skin type classification

Table 2 (panel A) shows the misclassification that is possible when the melanin estimates are dichotomised. Twelve subjects out of 96 (12.5%) were misclassified.

We assessed to what extent this misclassification due to body hair, if non-differential, would influence the results of an unmatched case-control study with 150 cases and 300 controls and an odds ratio of 2. Table 2 (panel B) demonstrates that non-differential misclassification due to body hair decreased the true odds ratio of 2 to an observed odds ratio of 1.68, using the percentage misclassification shown in panel A.

To test whether differential misclassification due to body hair could occur, we sought to determine whether the effect of shaving might differ between cases and controls. If cases have more or less hair at the buttock than controls, the effect of shaving would be differential. We found that there was an association ($r=-0.28$, $p<0.01$) between the effect of shaving (measured by the difference between the melanin estimates at the unshaved and the shaved buttock) and the estimates of melanin density at the shaved buttock. Melanoma and non-melanoma cases on average have less melanin than controls, and the negative correlation

Table 2. Misclassification matrix due to body hair for melanin density at the buttock *(A)*, and the effect of this (non-differential) misclassification on the odds ratio (OR) *(B)*.

(A)

	True melanin (without body hair)	
Observed melanin (with body hair)	Low melanin	High melanin
Low melanin	87.5% (42)	12.5% % (6)
High melanin	12.5% (6)	87.5% (42)
	100% (48)	100% (48)

(B)

True classification			Observed classification		
	Cases	Controls		Cases	Controls
Low melanin	100	150	Low melanin	94	150
High melanin	50	150	High melanin	56	150
	OR = 2			OR = 1.68	

Notes

1. The observed classification was calculated by applying the misclassification matrix from panel A to the true classification. See equations on the right.

$94 \approx 93.75 = (100 \times 0.875) + (50 \times 0.125)$
$56 \approx 56.25 = (50 \times 0.875) + (100 \times 0.125)$
$150 = (150 \times 0.875) + (150 \times 0.125)$

Table 3. Misclassification matrices due to body hair for melanin density at the buttock for a hypothetical group of cases (those with the lowest melanin estimates) and a hypothetical group of controls (all our subjects) *(A)*, and the effect of this (differential) misclassification on the odds ratio (OR) *(B)*.

(A)

Observed melanin (with body hair)	Hypothetical group of cases		Hypothetical group of controls	
	True melanin (without body hair)		True melanin (without body hair)	
	Low melanin	High melanin	Low melanin	High melanin
Low melanin	87.5% (42)	16.2% % (6)	87.5% (42)	12.5% % (6)
High melanin	12.5% (6)	83.8% (31)	12.5% (6)	87.5% (42)
	100% (48)	100% (37)	100% (48)	100% (48)

(B)

True classification			Observed classification		
	Cases	Controls		Cases	Controls
Low melanin	100	150	Low melanin	96	150
High melanin	50	150	High melanin	54	150
	OR = 2			OR = 1.78	

Notes

1. For the hypothetical group of cases, sufficient subjects were chosen as cases to make the average melanin density 30% lower for the cases than for the controls.
2. The observed classification was calculated by applying the misclassification matrices from panel A to the true classification. See equations on the right.

$96 \approx 95.6 = (100 \times 0.875) + (50 \times 0.162)$
$54 \approx 54.4 = (50 \times 0.838) + (100 \times 0.125)$
$150 = (150 \times 0.875) + (150 \times 0.125)$

suggests that they would have a greater reduction in buttock melanin as the result of shaving. If measurements were made without shaving, relatively more cases than controls would tend to be misclassified into the high melanin category. Table 3 (panel A) shows the misclassification percentages when a hypothetical group of cases was created with an average melanin density at the shaved buttock that was 30% lower than that of controls. Table 3 (panel B) demonstrates that this differential misclassification decreased the true odds ratio of 2 to an observed odds ratio of 1.78.

Effect of seasonal variation on skin type classification

Table 4 (panel A) shows the misclassification matrix when the winter measurements were used as the true values and one of the four seasonal measurements was chosen at random for each person as the observed value. Ten subjects (9.6%) were on average misclassified.

Table 4. Average misclassification matrix due to seasonal variation for melanin density at the upper inner arm *(A)*, and the effect of this (non-differential) misclassification on the odds ratio (OR) *(B)*.

(A)

Observed melanin (one of four seasonal values)	True melanin (winter value)	
	Low melanin	High melanin
Low melanin	90.4% (47)	9.6% (5)
High melanin	9.6% (5)	90.4 (47)
	100% (52)	100% (52)

(B)

	True classification			Observed classification	
	Cases	Controls		Cases	Controls
Low melanin	100	150	Low melanin	95	150
High melanin	50	150	High melanin	55	150
	OR = 2			OR = 1.73	

Notes

1. The observed classification was calculated by applying the misclassification matrix from panel A to the true classification. See equations on the right.

$95 \approx 95.2 = (100 \times 0.904) + (50 \times 0.096)$
$55 \approx 54.8 = (50 \times 0.904) + (100 \times 0.096)$
$150 = (150 \times 0.904) + (150 \times 0.096)$

The effect of this measurement error due to seasonal variation on the observed odds ratio of our hypothetical example is shown in Table 4 (panel B). A misclassification of 9.6% decreased the true odds ratio of 2 to an observed odds ratio of 1.73.

Table 5 demonstrates how differential misclassification due to seasonal variation affects the odds ratio when all cases were measured in summer and all controls were measured in winter. The observed odds ratio decreased to 0.97. When the same was done for the situation that all cases were measured in winter and all controls were measured in summer, the observed odds ratio increased to 3.05 (data not shown). Thus, in two extreme situations of differential misclassification, a true odds ratio of 2 could be measured to be as low as 0.97 or as high as 3.05.

Table 5. Misclassification matrix due to seasonal variation for melanin density at the upper inner arm when all cases are measured in summer and all controls in winter *(A)* and the effect of this (differential) misclassification on the odds ratio (OR) *(B)*.

(A)

Observed melanin (value of the season when measured)	Cases measured in summer		Controls measured in winter	
	True melanin (winter value)		True melanin (winter value)	
	Low melanin	High melanin	Low melanin	High melanin
Low melanin	73.1% (38)	1.9% (1)	100% (52)	0% (0)
High melanin	26.9% (14)	98.1% (38)	0% (0)	100% (52)
	100% (52)	100% (52	100% (52)	100% (52)

(B)

	True classification			Observed classification		
	Cases	Controls			Cases	Controls
Low melanin	100	150		Low melanin	74	150
High melanin	50	150		High melanin	76	150
	OR = 2				OR = 0.97	

Notes

1. Observed melanin density is dichotomised at the median of the winter values of the controls.
2. The observed classification was calculated by applying the misclassification matrices from panel A to the true classification. See equations on the right.

$74 \approx 74.05 = (100 \times 0.731) + (50 \times 0.019)$
$76 \approx 75.95 = (50 \times 0.981) + (100 \times 0.269)$
$150 = (150 \times 1) + (150 \times 0)$

7.5 DISCUSSION

In this chapter our purpose was to assess the effect of body hair and seasonal variation at the upper inner arm and the buttock on measurements of constitutive skin type based on estimates of melanin density. Melanin density was estimated from skin reflectance of wavebands of light centred at the two wavelengths of 400 nm and 420 nm using a method we have recently developed.[15] This measure has been shown to be a stronger predictor of melanin in skin biopsy samples than other commonly used measures of skin phenotype,[15] or the measure suggested by Kollias and Baqer.[17] It has also been demonstrated to predict risk of cutaneous malignant melanoma, basal carcinoma and squamous cell carcinoma of the skin, particularly for men.[12]

We found no effect of body hair at the upper inner arm of either men or women, but the melanin estimates at the buttock of men were substantially reduced by removing the body hair at that site. This suggests that if the aim is to accurately measure constitutive melanin and the site for the measurement is to be the buttock, it will be necessary to remove the body hair by shaving prior to taking measurements of skin reflectance. We found shaving to be well-tolerated by our volunteer subjects, but there may be more reluctance among less motivated subjects. It might be possible to create an increased local blood flow at the measurement site due to shaving. We delayed the measurements for three minutes after the shaving took place.

A significant seasonal variation in the melanin estimates at the upper inner arm was observed that was of modest magnitude (on average a 0.34 percentage point difference between summer and winter), but more marked than the variation in measurements made at the buttock. The melanin estimates for the same subjects at the upper inner arm were highest when made in summer-autumn than when made in winter-spring. Lasker[19] and Lock-Anderson and Wulf[20] have previously found seasonal variation in other skin reflectance measures that parallel these results. The upper inner arm has been selected as the site for skin reflectance measurements in epidemiological studies[10, 11, 15, 22, 23] because the skin at this site is generally not exposed to the sun. Our results suggest that there must nevertheless be some exposure in summer, when lightweight clothing or clothing without long sleeves is worn.

These findings – of differences in melanin estimates at the buttock due to the presence of hair and at the upper inner arm due to seasonal effects – raise the possibility of misclassification of subjects when constitutive skin type is being assessed. We therefore attempted to evaluate whether this misclassification was substantial.

In the simplest scenario in which misclassification of subjects due to body hair at the buttock and seasonal variation at the upper inner arm is non-differential, we found that the extent of misclassification was similar for each reason and considerable, because misclassification reduced an odds ratio from 2 to around 1.7 (a reduction in excess risk of approximately 30%).

If misclassification is differential, however, its effects are less predictable. In the case of the effect of body hair on melanin estimated at the buttock, differential misclassification could occur when hair characteristics at the buttocks such as amount, colour, type and length of hair are different for cases compared to controls, so that the effect of shaving of the hair at the buttock would be different for cases and controls. We inferred this could be the case because those with least melanin in our sample tended to have a larger reduction in buttock melanin estimated after shaving, and male cases have been found to have on average 30% less melanin than male controls.[12] However, we found that the reduction in the odds ratio would be of the same order as that produced by non-differential misclassification. We did not have actual measurements on cases and controls. Therefore, our inference is speculative and needs to be tested.

Misclassification due to seasonal variation could be differential when more cases are measured in one period of the year and more controls in another period. We demonstrated that the effect of differential misclassification on the odds ratio of a case-control study in two extreme situations would be more severe than the effect of non-differential misclassification. However, this differential misclassification can be prevented by taking the simple precaution of measuring similar proportions of cases and controls in each season, or by making all measurements at the same time of the year.

In summary, our results suggest that misclassification of constitutive skin type based on melanin density measured at the buttock due to body hair can be avoided by the removal of body hair at this site. Differential and non-differential misclassification at the upper inner arm due to seasonal variation can also be avoided, but the latter only if all measurements are made at the same time of the year. We cannot yet recommend the buttock as the site of measurements of constitutive skin type, because the buttock melanin density is a different measure than upper inner arm melanin density and the predictive value of the buttock measurements has not been demonstrated. Low levels of melanin density at the upper inner arm have been shown to predict the occurrence of melanoma, basal cell carcinoma and squamous cell carcinoma[12] but there is no evidence yet of the value of buttock melanin density as a predictor of risk. For analytical studies assessing the relationship between upper inner arm melanin density and skin cancer risk, seasonal variation in constitutive skin type should be considered as a confounder and considered in the design and analysis.

7.6 SUMMARY

Recent advances have enabled quite accurate estimation of cutaneous melanin density by spectrophotometry using reflectance of light at wavelengths 400 and 420 nm. The purpose of this chapter was to assess the effect of body hair and seasonal variation at the upper inner arm and buttock on measurements of melanin density. We estimated melanin density of 104 volunteers at three-monthly intervals over 12 months both before and after shaving. Removing body hair at the upper inner arm had no effect, but substantially reduced melanin estimates at the buttock in men. Significant seasonal variation was only observed at the upper inner arm, with highest readings in summer-autumn. In case-control studies, misclassification due to body hair at the buttock and seasonal variation at the upper inner arm could affect the observed odds ratio substantially. However, both sources of error can be reduced by careful attention to key aspects of study design.

7.7 POSTSCRIPT

This chapter and the previous chapter discussed the reliability of measures of past sun exposure and skin phenotype. In the spectrophotometric assessment of skin phenotype, we found significant seasonal variation when measurements were taken at the upper inner arm. In our matched case-control study, seasonal variation could be avoided when the cases and their matched controls are measured within the same season. In addition, we described in this chapter a significant effect of body hair in men when measurements were taken at the buttock. The effect of body hair on the spectrophotometric assessment of skin type at the buttock in a case-control study can be avoided by removal of the body hair. In our case-control study, the magnitude of this effect is probably small, because two thirds of our study participants are female and we found no effect of body hair in women. The next chapter will discuss the results of the MS case-control study in regard to past sun exposure and skin phenotype, the measures central to the main hypothesis of this thesis.

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Chapter 8

Past sun exposure, skin phenotype and risk of Multiple Sclerosis: a case-control study.

8.1 PREFACE

The review in chapter 3 demonstrated that the descriptive epidemiology of Multiple Sclerosis (MS) was, at least in part, consistent with the hypothesis that ultraviolet radiation (UVR) exposure may reduce the risk of MS. In chapter 4 we demonstrated, using an ecological analysis, that the regional variation in MS prevalence in the continent of Australia could be closely predicted by regional UVR levels. In both chapters, the importance of conducting analytical studies, such as case-control studies, was discussed. In addition, past UVR exposure may be examined more accurately if assessed at an individual level rather than taking, for example, ambient UVR levels of locations that participants lived at particular ages. We have conducted a case-control study where we assess past UVR exposure at an individual level. In this chapter we will examine the results of the main hypothesis of this thesis: whether high past UVR exposure is associated with a reduced risk of MS. Most of this chapter has been published as a short version in the British Medical Journal 327(7410): 316, and as a long version on the British Medical Journal website (http://bmj.com).

8.2 INTRODUCTION

One of the most striking epidemiological features of MS is a gradient of increasing prevalence with latitude.[1] Four decades ago, an inverse association between solar radiation and MS prevalence was first observed but the significance of this finding was unclear.[2]

Recent photoimmunological work has rekindled scientific interest in this observation because solar radiation has important immunosuppressive properties.[3] UVR attenuates Th_1-mediated immune responses through several mechanisms. UVR can exert local as well as systemic immunosuppression through cytokine signalling.[4] In addition, UVR promotes the production of the active form of vitamin D_3 (1,25-$(OH)_2D_3$), a hormone with immunomodulatory properties.[5] Studies in mice on experimental allergic encephalomyelitis (EAE), an animal model of MS, have shown that administration of either UVR or 1,25-$(OH)_2D_3$ has protective effects, such as reduced risk or delayed onset, symptom severity reduction and prolonged survival.[6, 7]

In human populations, UVR or vitamin D may also be protective for MS. In chapter 4, we reported a strong ecologic association between regional UVR levels and MS prevalence in the continent of Australia ($r=-0.91$, $p=0.01$). A strong inverse correlation ($r=-0.79$, $p<0.01$) between population levels of serum 25(OH)D, a vitamin D precursor,[8] and mean lesional activity among people with MS has also been reported,[9] suggesting that UVR could also play a role in the disease progression.

However, analytical studies are now required to evaluate the significance of these ecological findings. A death certificate based case-control study in the United States has reported that high residential or occupational sunlight exposure was more strongly associated with reduced

MS mortality than deaths from other disease.[10] Among outdoor workers, the adjusted odds ratio {95% confidence interval} for low, medium and high regional sunlight were 0.89 {0.64—1.22}, 0.52 {0.38—0.71} and 0.24 {0.15—0.38} for MS compared to indoor workers with low ambient sunlight.[10] Data on the relationship between past UVR exposure at the individual level and the development of MS have not been available. Early life exposure to UVR may alter immune development during a critical development window but the finding of a strong latitudinal gradient of MS prevalence in Australia even among immigrants from the UK and Ireland (70% who migrated after age 15)[11] suggests that cumulative or later life UVR exposure might also be important. Furthermore, UVR-related immunomodulation may be of importance at the time of host exposure to dietary or infectious peptides that could induce autoimmunity through molecular mimicry or other processes.

Tasmania, the island state of Australia, is located at latitudes 41-43°S and has a high estimated prevalence of MS at 75.6 per 100,000 population.[12] We conducted a matched case-control study in Tasmania of 136 cases and 272 community controls to examine whether high past UVR exposure was associated with a reduced risk of MS.

8.3 METHODS

8.3.1 Subjects

As was outlined in chapter 5, the source population for case and control selection consisted of subjects under the age of 60 years who were residents of Tasmania, Australia, and had at least one grandparent who was born in Tasmania. Cases were members of the source population who were diagnosed with MS, and controls were selected from the same source population that gave rise to the cases using the roll of registered electors, a comprehensive listing of the population maintained by the State Electoral Office of Tasmania. For each verified case, two control subjects were randomly selected and matched to the index case on sex and birth year. For the 136 cases included in the study (response rate estimated to be between 76% and 92%), 566 potential controls were approached, 359 were eligible as controls (no MS, living in Tasmania and at least one grandparent born in Tasmania) and 272 of the eligible controls participated (response rate 76%). The average time between the interview of the cases and controls was 5.2 ± 3.7 months, but 44.1% of the controls were interviewed within three months of the interview of the case.

8.3.2 Measurements

Reported sun exposure by questionnaire. Subjects were asked about the amount of time they would normally have spent in the sun during weekends and holidays ("< 1 hr a day", "1 to 2 hrs per day", "2 to 3 hrs per day", "3 to 4 hrs per day" and "≥ 4 hrs a day") and about how much their activities (such as playing, day sports, spectator sports, gardening, walking and working activities) took them outside ("not that often", "a moderate amount", "quite a lot", "virtually all the time") (Appendix A). The first question will be referred to as the "Time in the sun" question and the second question as the "Activities outside" question. Both questions were asked for summer and winter during three periods of life: 6 – 10 years of age, 11 – 15 years of age and 16 – 20 years of age. Occupational exposure was assessed by the question whether the participants' jobs were overall mainly indoors, both indoors and outdoors or mainly outdoors. As discussed in chapter 6, the weighted kappa statistic {95% confidence interval} of the "Time in the sun" question on retest for the age spans 6-10 years, 11-15 years and 16-20 years in summer was 0.49 {0.35—0.63}, 0.44 {0.30—0.57} and 0.38 {0.42—0.69}, respectively. There was no difference in agreement between cases and controls. Proxies also answered the "Time in the sun" and "Activities outside" question for summer and winter for the three different age periods.

Reported sun exposure by lifetime calendar. The same "Time in the sun" question was also incorporated in a separate personal residence and work calendar (Appendix D). In addition, exact time outside on a working day during working hours was estimated for each year of occupation. A lifetime calendar has been shown to produce more accurate long-term recall in some circumstances because of the different cognitive interviewing technique that is used.[13] Prior to the interview, subjects were sent a lifetime calendar and asked to fill this out for each year of their life (residence, school, occupation, exposure to pets and farm animals). During the interview, subjects answered the "Time in the sun" question for summer (but not winter) and occupational sun exposure for each year of their life, and the information already filled out on the calendar was utilised to identify blocks of years where the sun exposure lifestyle was constant or not. The weighted kappa statistic between the questionnaire-based "Time in the sun" measure and the calendar measure (using the mean value) for the age spans 6-10 years,

11-15 years and 16-20 years, was 0.54 {0.47—0.61}, 0.51 {0.44—0.58} and 0.44 {0.37—0.50}, respectively. Again, there was no difference between cases and controls.

Assessment of actinic damage by silicon cast. Silicon skin surface casts of the hand, measuring actinic damage, were used as an objective marker of cumulative lifetime sun exposure. This measure has been previously found to be associated with residence in a high UVR location,[14] lifetime sun exposure,[15] outdoor occupations and leisure activities,[16] solar keratosis,[16, 17] and basal and squamous cell cancer.[16] Silicone liquid was mixed with catalyst and applied to the dorsum of the left hand of the subject. After seven minutes, the cast was removed. The fine lines on the underside of the cast were examined under a low-power dissecting microscope and graded by a single observer according to the method of Beagley and Gibson.[18] This provides grades ranging from one (undamaged skin) to six (severe deterioration). A previous study conducted in four different Australian cities has indicated that by the age of 14 years up to 70% of those teenagers have detectable sun damage of their skin.[14] Another study found more advanced deterioration of the skin among 72% of men and 47% of women living in Nambour (latitude 27°S) by the third decade of life.[16] In addition, age and sex have been shown to strong predictors of the amount of actinic damage,[14, 16, 17] and these two factors were controlled for through our matched design. The weighted kappa statistic on a sub-sample for which the casts were repeated was 0.68 {0.53—0.83}, substantially higher than the repeatability for the questionnaire-based sun exposure measures.

Skin phenotype. Skin phenotype was assessed objectively from skin reflectance using a spectrophotometer and a chroma-meter at the upper inner arm and the buttock, body sites usually not exposed to the sun. Cutaneous melanin density (MD_{400}) was estimated from spectrophotometric measurements of skin reflectance of wavebands of light centred at the two wavelengths of 400 nm and 420 nm[19]. The chroma-meter includes a L* value, the measurement of lightness of colour across the visible light spectrum used with the Commission International d'Eclairage system of colour assessment.[20] Skin colour at the upper inner arm was also assessed visually by the research assistant. The questionnaire allowed several other less objective assessments of skin phenotype from questions of skin reaction to unaccustomed sun, tendency to burn when in the sun, and end-of-summer tan. Two indicators that partially reflect sun exposure and skin phenotype were also included. The number of naevi greater than 5 mm on the left arm was assessed by the research assistant and a question was asked about the number of lifetime burns, where the pain lasted two or more days ("Never", "Once". "2-5 time". "6-10 times" or "More than ten times").

Other study factors. Other study factors are discussed in detail in chapter 5. In short, research assistants assessed the hair and eye colour and measured the height and weight of each subject. The standardised questionnaire included questions on sun avoidance behaviour, medical history including reported infections and immunisations, residential history, exposure to chemicals, exposure to pets and farm animals, dietary intake at age 10-15 years (meat, fish, offal, milk, eggs, vitamin D containing supplements), occupation, education, smoking, alcohol intake, whether the subject was breastfed, and reproductive history for women (age of menarche, number of children and breastfeeding). For the timing of exposures, we obtained, in accordance to the recommendations of Boiko [21], either the exact age or the five-year age group of the exposure.

8.3.3 Data analysis

Pearson correlations were calculated as measures of linear association. Odds ratios and 95% confidence intervals were estimated by conditional logistic regression (STATA 7.0).[22] Tests for

trend of categorical variables were undertaken by replacing the binary predictors with a single predictor taking category rank scores for the categories. For the analyses in Table 2, the scaled variables melanin and naevi were dichotomised at previously-used cut-points.[23]

Spectrophotometric assessment of skin phenotype was performed on 358 of the 408 subjects. There was no difference between the group with and the 50 subjects without spectrophotometric measurements in distribution of the L* reading of the chroma-meter or skin type assessed by the research assistant. As evidence of the high reproducibility of measurements made, the percentage of total variation in MD_{400} at the upper inner arm attributed to within-person variation in the three measurements was 0.3 percent (intraclass correlation=0.99, calculated from a one-way random effects model using ICC(1,1) from Shrout and Fleiss.[24]

For actinic damage, analysis was performed on 323 high quality casts, because 39 casts were difficult to grade and 46 were unable to be graded. We assessed the influence of including the casts that were difficult to grade.

The recording of year-by-year sun exposure by the lifetime calendar allowed us to estimate average exposure at any age. For the "Time in the sun" question, we did this for the age spans 6-10 years, 11-15 years and 16-20 years (Table 3), and also for age spans 6-10 years, 6-15 years, 6-20 years and so on (Table 7, Figure 1). To aggregate and then average annual exposure, the categories were assigned rank scores one to five. For example, for average sun exposure at age span 6-15 years, the rank scores of the "Time in the sun" answers were added up for age 6 through to age 15 years and divided by 10 years. For Figure 1, the average sun exposure for each age span was dichotomised at 2-3 hours per day. For Table 7 and Figure 1, the sample was limited to cases (and their matched controls) who had not experienced any MS symptoms prior or during the age span. For occupational sun exposure (Table 5), weekly exposure was aggregated for each year that the subjects worked before the age of onset (cumulative occupational sun exposure). Weekly exposure was calculated by multiplying the estimated average time outside per working day by the number of days worked per week. Mean occupational sun exposure was then calculated by dividing cumulative occupational sun exposure by the number of years that the subjects worked before the age of onset.

To take account of duration of disease, we stratified by time elapsed since the first MS symptom: 0–5, 6–10, 11–15, 16–20, >20 years. To see whether the odds ratios for sun exposure across the strata of disease duration changed in magnitude, a likelihood-ratio test of interaction was conducted using a product term of sun exposure and disease duration. In analysis, controls were given the years of duration or the age at onset (age of first MS symptom) of their case pair. Proportional hazard regression was used among cases to assess the effect of sun exposure and skin phenotype on the age at disease onset (time elapsed from birth to onset of first symptoms). Linear regression was used to regress vitamin D levels among controls on predictor variables such as melanin density.

Population attributable fraction is the proportion by which the incidence rate of the outcome in the entire population would be reduced if exposure were eliminated, assuming that the exposure is causally related to the outcome and that causes other than the one under investigation have had equal effects on the exposed and unexposed groups.[25] For adjusted (and unadjusted) risk estimates, it can be estimated by the formula:[25]

$$AF_p = \frac{P(RR-1)}{RR}$$

where RR is the estimate of the rate ratio (odds ratio in a case-control study) and P is the proportion of all cases that are exposed. The population attributable fraction was calculated for two measures of past sun exposure.

8.4 RESULTS

8.4.1 Characteristics of the sample

Table 1 shows characteristics of the sample. There were twice as many females as males and 66.4% of the cases had the relapsing remitting type of MS. A very high percentage of participants were born and living in Tasmanian at the age of 10 (Table 1).

Table 1. Characteristics of the cases and controls

	Cases	Controls
Personal characteristics of cases and controls		
Female/Male ratio (females/males) (matching factor)	2.1/1 (92/44)	2.1/1 (184/88)
Mean age, years (SD) (matching factor)	43.5 (9.3)	43.6 (9.2)
% born in Tasmania (n/N)	96.3 (131/136)	95.2 (258/271)
% living in Tasmania at the age of 10 (n/N)	96.3 (131/136)	97.4 (265/272)
Height, cm (SD)	166.9 (9.0)	166.1 (8.9)
Weight, kg (SD)	72.9 (14.4)	76.2 (16.1)
Disease specific characteristics of cases		
Mean age at diagnosis, years (SD)	34.6 (9.1)	-
Mean age at first symptoms, years (SD)	31.0 (9.1)	-
Mean duration of MS since diagnosis, years (SD)	9.4 (7.5)	-
Mean duration of MS since first symptoms, years (SD)	12.1 (8.0)	-
Mean EDSS score (SD)	3.5 (2.2)	-
DRB1*1501—DQB1*0602 haplotype frequency, % (n/N)	53.7 (66/123)	-
Type of MS		
Relapsing remitting, % (n)	65.4 (89)	-
Secondary progressive, % (n)	26.5 (35)	-
Primary progressive, % (n)	7.5 (10)	-

8.4.2 Risk for measures of skin phenotype

We examined how measures of skin phenotype were associated with MS (Table 2). The odds of having fair skin type at the upper inner arm assessed by the spectrophotometer (melanin<2%) and chroma-meter (L* > 0.68) was nearly 1.6 times higher for cases than for controls. Skin colour at the upper inner arm assessed by the research assistant also showed a relation (p=0.03), but skin type measures at the buttock using a spectrophotometer or chroma-meter, reported tendency to burn, or tanning ability were not associated with MS (Table 2). As discussed in chapter 7, seasonal variation at the upper inner arm could have influenced the upper inner arm results of melanin density and L*, because we were not always able to examine cases and their matched controls within a three month period. However, when we limited to cases and their controls who were examined within three months from each other, no substantial differences were observed in the odds ratios {95% confidence intervals} compared to the total sample (melanin density upper inner arm 1.45 {0.75—2.83}, L* upper inner arm 1.67 {0.93—3.00}). Similarly, body hair at the buttock in men could have biased the buttock results, but limiting to women – who did not show an effect of body hair on spectrophotometer results (see chapter 7) – did not change the odds ratios substantially either (melanin density buttock 0.94 {0.54—1.62}, L* buttock 0.98 {0.58—1.66}).

Table 2. Unadjusted odds ratios for Multiple Sclerosis and measures of skin phenotype, sunburn history and skin nevi.

Factor	Cases exposed n/N (%)	Controls exposed n/N (%)	Unadjusted OR {95% CI}
Melanin density at the upper inner arm (< 2% vs ≥ 2%)	69/127 (54.3)	104/236 (44.1)	1.59 {0.99—2.55}
L* at the upper inner arm (> 68 vs ≤ 68)	78/130 (60.0)	134/271 (49.5)	1.58 {1.02—2.44}
Skin colour (fair or medium/fair vs olive/medium or olive)	87/132 (65.9)	142/264 (53.8)	1.62 {1.05—2.51}
Melanin density at the buttock (< 0.8% vs ≥ 0.8%)	66/119 (55.5)	131/233 (56.2)	0.97 {0.62—1.54}
L* at the buttock (> 68 vs ≤ 68)	63/120 (52.5)	131/269 (48.7)	1.09 {0.70—1.68}
Tendency to burn (burn within 1 hr vs burn after > 1 hr)	81/136 (59.6)	151/272 (55.5)	1.19 {0.77—1.83}
Ability to tan (light or no tan vs dark or medium tan)	48/136 (35.3)	91/272 (33.5)	1.11 {0.68—1.80}
Lifetime sunburns (any vs none)	104/135 (77.0)	232/272 (85.3)	0.55 {0.32—0.96}
Number of naevi >5mm on left arm (≥ 3 vs < 3)	25/129 (19.4)	51/263 (19.4)	0.94 {0.54—1.63}

Notes
1. OR = odds ratio; {95% CI} = {95% confidence interval}
2. Skin colour is assessed at the upper inner arm by the research assistant

We then examined among controls, using linear regression, whether vitamin D levels were associated with amount of melanin at the upper inner arm. People with more melanin tended to have higher vitamin D levels, even after adjusting for time in the sun in the last three years, use of sunscreen and amount of clothing to cover up, and month the serum sample was taken (coefficient 4.53, p<0.01). The association between melanin and vitamin D was stronger for people older than 44 years (coefficient 8.98, p<0.01) compared to younger people (coefficient 2.20, p=0.19).

8.4.3 Risk for measures of sun exposure in childhood and adolescence

People with less melanin reported more severe sunburn episodes in their lifetime (controls: r=-0.25, p<0.01; cases: r=-0.22, p=0.01). However, although people with MS had fairer skin, they were less likely (p=0.04) to report severe sunburn episodes (Table 2), which could suggest that people with MS had fewer episodes of intense sun exposure. Indeed, a strong inverse association between sun exposure during weekends and holidays in childhood and adolescent years and MS was observed (Table 3). For example, cases were less likely than controls to report higher levels (more than one hour a day) of sun exposure during winter at age 6-10 years (OR 0.47 {0.26—0.84}). There was consistency between the questionnaire data and the calendar data in that the age periods 6-10 and 11-15 seemed more important than age period 16-20. Results for the "Activity outside" question showed similar patterns but the results were not as consistent (data not shown).

The inverse association was observed for both winter and summer sun exposure (Table 3). Compared to bivariate analysis, including both summer and winter questionnaire-based sun exposure measures as dichotomised terms in the model left the estimated effect of winter sun exposure almost unchanged (adjusted odds ratio 0.52 {0.28—0.95} at age 6-10 years), but greatly reduced the effect of summer sun exposure (0.63 {0.30—1.35} at age 6-10 years). This was found irrespective of the ages at which exposure occurred.

Table 3. Unadjusted odds ratios for Multiple Sclerosis and reported measures of sun exposure in childhood and adolescence.

	Age 6—10			Age 11—15			Age 16—20		
	Cases n (%)	Controls n (%)	Unadjusted OR {95% CI}	Cases n (%)	Controls n (%)	Unadjusted OR {95% CI}	Cases n (%)	Controls n (%)	Unadjusted OR {95% CI}
Time in the sun in winter, by questionnaire									
Less than 1 hr a day	26 (19.1)	27 (10.0)	1	22 (16.3)	28 (10.3)	1	29 (21.3)	37 (13.6)	1
1—2 hrs a day	29 (21.3)	61 (22.7)	0.50 {0.24—1.00}	29 (21.5)	69 (25.4)	0.55 {0.27—1.10}	37 (27.2)	84 (30.9)	0.59 {0.32—1.07}
2—3 hrs a day	26 (19.1)	64 (23.8)	0.43 {0.21—0.87]	30 (22.2)	65 (23.9)	0.61 {0.30—1.22}	26 (19.1)	53 (19.5)	0.63 {0.32—1.24}
3—4 hrs a day	14 (10.3)	34 (12.6)	0.44 {0.19—1.01}	22 (16.3)	37 (13.6)	0.76 {0.36—1.61}	17 (12.5)	39 (14.3)	0.56 {0.26—1.22}
More than 4 hrs a day	41 (30.2)	83 (30.9)	0.50 {0.26—0.98}	32 (23.7)	73 (26.8)	0.57 {0.28—1.14}	27 (19.9)	59 (21.7)	0.60 {0.30—1.17}
Test for trend			p=0.18			p=0.45			p=0.22
Dichotomised (≥ 1—2 a day vs < 1 hr a day)			0.47 {0.26—0.84}			0.60 {0.33—1.09}			0.59 {0.35—1.01}
Time in the sun in summer, by questionnaire									
Less than 1 hr a day	6 (4.4)	8 (3.0)		3 (2.2)	2 (0.7)		11 (8.1)	13 (4.8)	
1—2 hrs a day	15 (11.0)	15 (5.6)	1	13 (9.6)	26 (9.6)	1	19 (14.0)	37 (13.6)	1
2—3 hrs a day	20 (14.7)	39 (14.4)	0.59 {0.27—1.28}	20 (14.7)	37 (13.6)	0.94 {0.40—2.17}	22 (16.0)	64 (23.6)	0.55 {0.28—1.10}
3—4 hrs a day	17 (12.5)	48 (17.8)	0.39 {0.17—0.88}	27 (19.9)	61 (22.4)	0.77 {0.35—1.68}	26 (19.1)	61 (22.5)	0.71 {0.38—1.35}
More than 4 hrs a day	78 (57.4)	160 (59.3)	0.55 {0.30—1.03]	73 (53.7)	146 (53.7)	0.88 {0.44—1.74}	58 (42.7)	96 (35.4)	1.00 {0.57—1.78}
Test for trend			p=0.15			p=0.72			p =0.56
Dichotomised (≥2—3 hrs a day vs ≤1—2 hrs a day)			0.50 {0.24—1.02]			0.86 {0.44—1.66}			0.79 {0.47—1.33}
Time in the sun in summer, by calendar									
Less than 1 hr a day	2 (1.5)	2 (0.7)		4 (2.9)	2 (0.7)		6 (4.4)	8 (2.9)	
1—2 hrs a day	17 (12.5)	14 (5.2)	1	11 (8.1)	12 (4.4)	1	20 (14.7)	47 (17.3)	1
2—3 hrs a day	18 (13.2)	23 (8.5)	0.63 {0.23—1.73}	27 (19.9)	40 (14.7)	0.65 {0.26—1.61}	27 (19.9)	58 (21.3)	0.99 {0.50—1.95}
3—4 hrs a day	19 (14.0)	44 (16.3)	0.35 {0.14—0.88}	22 (16.2)	53 (19.5)	0.39 {0.16—0.95}	30 (22.1)	55 (20.2)	1.16 {0.59—2.26}
More than 4 hrs a day	80 (58.8)	187 (69.3)	0.34 {0.16—0.74}	72 (52.9)	165 (60.7)	0.40 {0.18—0.89}	53 (39.0)	104 (38.2)	1.09 {0.60—1.97}
Test for trend			p<0.01			p=0.01			p=0.70
Dichotomised (≥2—3 hrs a day vs ≤1—2 hrs a day)			0.36 {0.17—0.76}			0.44 {0.20—0.94}			1.07 {0.63—1.85}

Notes

1. OR = odds ratio; {95% CI} = {95% confidence interval}

We also assessed the association between sun exposure in childhood and adolescence and MS using proxy recall. The associations were weak and in some occasions in opposite direction (winter sun exposure at age 6-10 years: OR 1.31 {0.66—2.61}; winter sun exposure at age 11-16 years: OR 1.29 {0.59—2.83}), but when we limited to proxies of whom the research assistants recorded that they had a "good" recall of the subject's sun exposure, the associations improved and were inversely associated with MS (winter sun exposure age 6-10 years: OR 0.72 {0.30—1.72}; winter sun exposure age 6-11 years: OR 0.69 {0.26—1.83}). The weaker results might be partly explained by the low agreement that was found between the recall of sun exposure for these ages of subjects and proxies. The average weighted kappa coefficient (SD) for winter and summer sun exposure for the three age groups was 0.24 (0.03).

8.4.4 Risk for measures of lifetime sun exposure

We then examined the effect of sun exposure throughout life. The recording of year-by-year sun exposure by the lifetime calendar allowed us to estimate average exposure to any age. We did so for sun exposure from age 6 to ages 10, 15, 20, 25, 30 and 35. The risk estimates using these measures are shown in Figure 1.

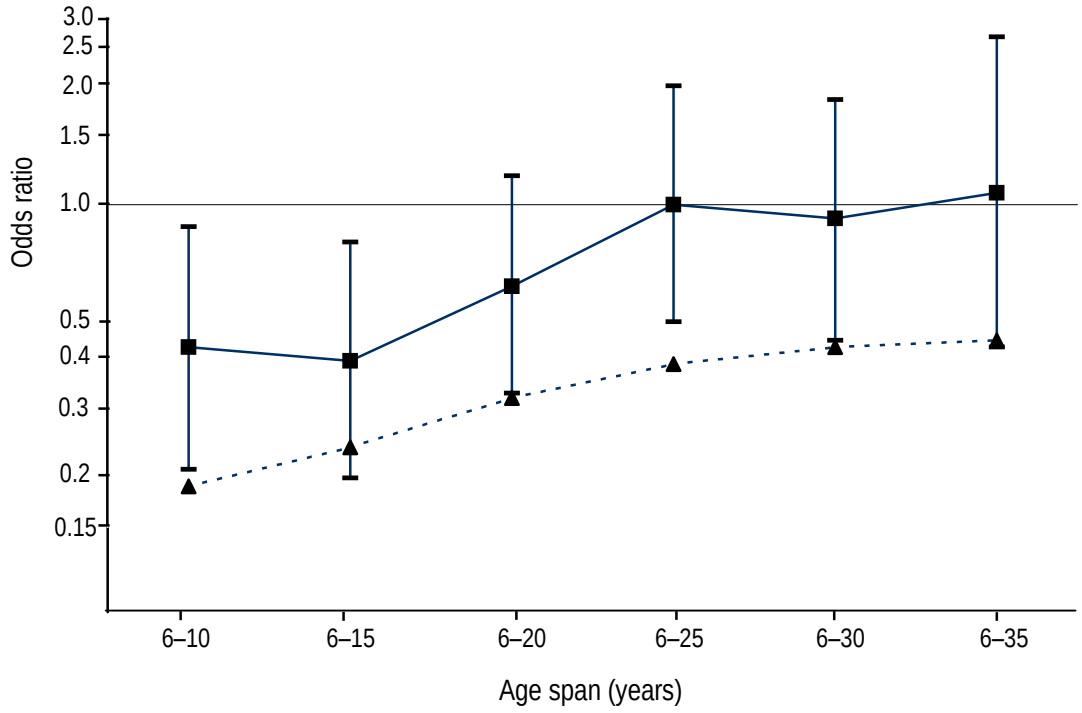

Figure 1. The association between sun exposure and Multiple Sclerosis for different age spans. Odds ratios and 95% confidence intervals for higher (≥2-3 hrs per day on average) sun exposure in summer on weekends and holidays for the total sample (solid line) and for the sub-sample of subjects who did not believe that sun exposure was an important cause of MS (broken line).

To calculate these odds ratio estimates, sun exposure during each year of age in the age span considered was averaged as explained in the methods, and the resultant estimates were dichotomised at 2-3 hours per day. For each analysis, the sample was limited to cases (and their matched controls) who had not experienced any MS symptoms prior or during the age span. The odds ratio estimates of the apparent protective effect of high levels of sun exposure was greatest prior to the age of 15 years (OR 0.43 {0.21—0.88} for 6-10 years; OR 0.40 {0.20—0.80} for 6-15 years). Inclusion of later years into the cumulative lifespan measure diluted the effect. To assess the possible contribution of reporting bias, we repeated the analysis on a subgroup of subjects who had indicated in the pre-interview checklist that they

did not believe that climatic factors such as sun exposure were an important cause of MS. Again, the protective effect of sun exposure appeared to be greatest at younger ages, but for this group the protective effect was even stronger than for the total group (OR 0.19 {0.05—0.67} for 6-10 years; OR 0.24 {0.09—0.69} for 6-15 years; OR 0.32 {0.12—0.85} for 6-20 years; OR 0.39 {0.14—1.07} for 6-25 years; OR 0.43 {0.17—1.13} for 6-30 years; OR 0.45 {0.16—1.26} for 6-35 years) (Figure 1).

Higher levels of actinic damage were also associated with a reduced risk of MS (OR 0.39 {0.17—0.90} for grade 4-6 versus grade 3) with evidence of dose response (test for trend, p<0.01) (Table 4). Including the casts that were difficult to grade only marginally affected the results (OR 0.44 {0.20—0.95} for grade 4-6).

Table 4. Unadjusted odds ratios for Multiple Sclerosis and a range of levels of actinic damage on the dorsum of the hand.

	Cases n (%)	Controls n (%)	Unadjusted OR {95% CI}
Grade 3	18 (17.1)	21 (9.6)	1
Grade 4	34 (32.4)	59 (27.1)	0.47 {0.19—1.13}
Grade 5	41 (39.0)	78 (35.8)	0.37 {0.15—0.90}
Grade 6	12 (11.4)	60 (27.5)	0.14 {0.05—0.42}}
Test for trend			p<0.01
Dichotomised (grade 4-6 vs grade 3)			0.39 {0.17—0.90}

Notes
1. OR = odds ratio; {95% CI} = {95% confidence interval}

8.4.5 Risk for measures of sun exposure immediately prior to MS onset

To assess the effect of sun exposure at a specific time period immediately prior to disease onset, we created variables of the subjects' sun exposure at particular years prior to MS onset using the calendar (10 years, 5 years and 1 year). For the controls, the age of onset of the index case was used. The odds ratios for 2-3 hrs or more sun exposure in summer during weekends and holidays and MS were 0.95 {0.55—1.64}, 0.92 {0.55—1.54} and 1.06 {0.65—1.7}, respectively, for 10 years, 5 years and 1 year prior MS onset. Thus, in contrast to the inverse association between sun exposure in early life or actinic damage and MS, there was no evidence that sun exposure at these particular years in the decade prior to disease onset was important.

8.4.6 Risk for measures of occupational sun exposure

Whether jobs overall were indoor and/or outdoor jobs was not associated with MS, although there was some protective effect seen for people who had mainly outdoor jobs compared to those with indoor jobs (Table 5). The year-by-year calendar data allowed us to estimate cumulative and average amount of time that participants spent outside during their occupations. There was no association between the cumulative or average time participants spent outside prior to the age of onset (Table 5). Occupational sun exposure occurs during late adolescence and adulthood. In our sample, 95% of the participants started their first job when they were between 15 and 22 years (mean 17.3 years, SD 2.3). The finding of no association between occupational sun exposure is therefore in accordance with the finding of a diluted effect or no association for sun exposure during weekends and holidays after the age of 16 years.

Table 5. Unadjusted odds ratios for Multiple Sclerosis and occupational sun exposure

	Cases n (%)	Controls n (%)	Unadjusted OR {95% CI}
Type of job			
Mainly indoors	95 (71.4)	185 (68.3)	1
Both indoors and outdoors	25 (18.8)	52 (19.2)	1.00 {0.57—1.78}
Mainly outdoors	13 (9.8)	34 (12.5)	0.72 {0.33—1.56}
Test for trend			p=0.49
Cumulative occupational sun exposure before the age of onset			
≤ 5 hrs	33 (24.3)	76 (27.9)	1
5.1—30 hrs	46 (33.8)	64 (23.5)	1.73 {0.96—3.14}
30.1—90 hrs	27 (19.9)	66 (24.3)	0.93 {0.50—1.74}
>90 hrs	30 (22.1)	66 (24.3)	0.98 {0.48—2.01}
Test for trend			p=0.60
Mean occupational sun exposure before the age of onset			
0—1.0 hrs a week	31 (24.4)	59 (23.1)	1
1.1—3.0 hrs a week	33 (26.0)	69 (27.1)	0.88 {0.49—1.56}
3.1—8.0 hrs a week	35 (27.6)	61 (23.9)	1.07 {0.58—1.96}
>8.0 hrs a week	26 (22.1)	66 (25.9)	0.81 {0.41—1.61}
Test for trend			p=0.74

Notes
1. OR = odds ratio; {95% CI} = {95% confidence interval}

8.4.7 Other factors associated with MS

A number of other factors in this study were found to be associated with MS (Table 6). Factors positively associated with MS were having smoked tobacco prior to the age of diagnosis, a high level of education, exposure to fibre glass or resin prior to age 17, exposure to smoke fumes prior to age 17, exposure to smoke fumes between age 17 and the age of onset, and owning a cat prior to MS onset (p=0.02). Having had an immunisation against rubella was negatively associated with MS. A history of infectious mononucleosis (p<0.01), being seropositive for the Epstein-Barr virus nuclear antigen (p<0.01) and viral capsid antigen (p<0.01), and having high IgG levels against the Epstein-Barr virus nuclear antigen (>2.50 units) (p<0.01) and viral capsid antigen (>2.00 units) (p=0.01) were also positively associated with MS risk, but these factors will be discussed in the next chapter. Intake of vitamin D containing supplements at age 10-15 years was not associated with MS (OR 0.89 {0.45—1.75}).

Table 6. Unadjusted odds ratios for Multiple Sclerosis and smoking history, education level, immunisation against rubella, exposure to fibre glass and resin, smoke fumes, and cats.

	Cases n (%)	Controls n (%)	Unadjusted OR {95% CI}
Ever smoker prior to the age of diagnosis			
No	51 (37.5)	129 (47.4)	1
Yes	85 (62.5)	143 (52.5)	1.53 {0.99—2.36}

Table 6 continued at next page

Table 6 continued

	Cases n (%)	Controls n (%)	Unadjusted OR {95% CI}
Education			
No formal education, primary school	17 (12.6)	51 (19.0)	1
Secondary school until year 10	49 (36.3)	104 (38.7)	1.47 {0.74—2.91}
Higher secondary school, Tafe, trade and apprenticeship	44 (32.6)	73 (27.1)	1.91 {0.94—3.85}
University	25 (18.5)	41 (15.2)	1.93 {0.88—4.20}
Test for trend			p=0.07
Rubella immunisation			
No	83 (61.5)	125 (46.0)	1
Yes	44 (32.6)	107 (39.3)	0.45 {0.24—0.85}
Don't know	8 (5.9)	40 (14.7)	0.28 {0.12—0.64}
Exposure to fibre glass or resin prior to age 17 years			
No	125 (91.9)	261 (96.0)	1
Yes	11 (8.1)	11 (4.0)	2.62 {0.93—7.33}
Exposure to smoke fumes prior to age 17 years			
No	116 (85.3)	246 (90.8)	1
Yes	20 (14.7)	25 (9.2)	1.80 {0.93—3.50}
Exposure to smoke fumes between age 17 and the age of diagnosis			
No	102 (76.1)	226 (84.3)	1
Yes	32 (23.9)	15 (15.7)	1.90 {1.06—3.40}
Owning a cat prior to onset using calendar data			
No	22 (16.2)	71 (26.1)	1
Yes	114 (83.8)	201(73.9)	1.87 {1.09—3.24}

Notes
1. OR = odds ratio; {95% CI} = {95% confidence interval}

8.4.8 Effect of confounders and disease duration on the relationship between sun exposure and MS

Sun exposure in childhood and early adolescence

We estimated the effect of average sun exposure at age 6 to 15 years from the year-by-year calendar on MS (Table 7) taking into account other factors that related to MS. After examining the effects of the possible confounders, using the strategy discussed in chapter 5, melanin density at the upper inner arm and whether ever smoked prior to the age of onset were considered to confound the association between sun exposure at age 6 to 15 years and MS. Adjusting for melanin density at the upper inner arm marginally increased the magnitude of the odds ratios (OR 0.36 {0.19—0.68} for 2-3 hours of sun a day or more) and adjusting for smoking further increased the magnitude of the protective effect (OR 0.31 {0.16—0.59} for 2-3 hours of sun a day or more). Additional adjustment for the other factors listed in the previous section made no material difference to the results shown Table 7.

Table 7. Unadjusted and adjusted odds ratios for Multiple Sclerosis and average time in the sun in summer on weekends and holidays at age 6 – 15 years using the calendar measure.

	Cases n (%)	Controls n (%)	Unadjusted OR {95% CI}	Adjusted OR {95% CI}
Less than 1 hr a day	4 (3.0)	4 (1.5)		
1—2 hrs a day	16 (11.9)	14 (5.2)	1	1
2—3 hrs a day	22 (16.3)	39 (14.4)	0.51 {0.22—1.17}	0.42 {0.16—1.09}
3—4 hrs a day	30 (22.0)	65 (24.1)	0.40 {0.18—0.89}	0.32 {0.13—0.80}
More than 4 hrs a day	63 (46.7)	148 (54.8)	0.37 {0.18—0.76}	0.26 {0.11—0.60}
Test for trend			p=0.01	p<0.01
Dichotomised (≥2-3 hrs vs <2-3 hrs per day)			0.39 {0.22—0.70}	0.31 {0.16—0.59}

Notes
1. OR = odds ratio; {95% CI} = {95% confidence interval}
2. Adjusted for melanin density at the upper inner arm and whether ever smoked prior to the age of diagnosis.

There was no evidence that these results differed by the duration of disease (time elapsed since the first MS symptom) of the cases. Disease duration was not strongly associated with sun exposure at age 6 to 15 years (r=-0.08). Moreover, the relative risk estimates did not differ by duration of disease (test for interaction: p=0.49), and the protective effects were also observed among recent onset cases (≤5 years) (adjusted odds ratio 0.58 for ≥2-3 hrs of sun exposure per day), although this estimate was more imprecise.

Actinic damage

We adjusted the estimate of actinic damage for melanin density at the upper inner arm, because it was associated with less actinic damage (p<0.01), and doing so increased the magnitude of the odds ratios (OR 0.33 {0.12—0.89} for grade 4-6). Adjusting for smoking further increased the magnitude of the protective effect (OR 0.31 {0.11—0.83}. We also adjusted for total sun exposure after MS onset to remove the contribution of this factor to the observed associations shown in Table 8.

Table 8. Unadjusted and adjusted odds ratios for Multiple Sclerosis and a range of levels of actinic damage on the dorsum of the hand.

	Cases n (%)	Controls n (%)	Unadjusted OR {95% CI}	Adjusted OR {95% CI}
Grade 3	18 (17.1)	21 (9.6)	1	1
Grade 4	34 (32.4)	59 (27.1)	0.47 {0.19—1.13}	0.32 {0.10—0.98}
Grade 5	41 (39.0)	78 (35.8)	0.37 {0.15—0.90}	0.33 {0.12—0.96}
Grade 6	12 (11.4)	60 (27.5)	0.14 {0.05—0.42}	0.17 {0.05—0.60}
Test for trend			p<0.01	p<0.01
Dichotomised (grade 4-6 vs grade 3)			0.39 {0.17—0.90}	0.32 {0.11—0.88}

Notes
1. OR = odds ratio; {95% CI} = {95% confidence interval}
2. Adjusted odds ratios are adjusted for melanin density at the upper inner arm, whether ever smoked prior to the age of diagnosis and amount of sun after disease onset.

Similarly to sun exposure prior to age 15, the results for actinic damage did not differ by the duration of disease of the cases. Disease duration was not associated with actinic damage (r=-0.02 after adjustment for age), the relative risk estimates for actinic damage did not differ by duration of disease (test for interaction: p=0.62), and the protective effects were also

observed among recent onset cases (≤5 years) (adjusted odds ratio 0.50 for actinic damage grade 4-6), although the estimate was more imprecise.

8.4.9 Relative importance of sun exposure in childhood and early adolescence, and actinic damage

We assessed whether higher sun exposure prior to age 15 and higher actinic damage were each important in predicting risk of MS. Compared to univariate analysis, including both in the same model as linear terms left the estimated effects of both factors almost unchanged, suggesting that not only sun exposure during childhood and early adolescence reduces the risk of MS, but that cumulative sun exposure might also be important as an independent factor of reducing MS risk.

8.4.10 Effect of sun exposure on age at onset among cases

To assess whether sun exposure among cases was associated with age at onset of disease, we modelled time elapsed from birth to onset of first symptoms using proportional hazard regression. There was no evidence that higher (≥ 2-3 hours a day on average) sun exposure in summer on weekends and holidays over the age span of 6-15 years (p=0.87 for linear term) or lifetime actinic damage (p=0.71 for linear term adjusted for current age) were associated with earlier onset of disease. However, skin phenotype did relate to age of onset. Fair skin assessed by the research assistant and low melanin density at the buttock, another body site used to measure skin phenotype, were associated with earlier onset of disease (p=0.04 and p=0.02, respectively).

8.4.11 Population attributable fraction

To conclude, we calculated the proportion by which the incidence rate of MS would be reduced if we were able to eliminate (1) low (1-2 hrs a day or less) sun exposure in summer during weekends and holidays at age 6 to 15 years, and (2) low (grade 3) actinic damage. In our cases, 15.6% had low sun exposure in summer during weekends and holidays at age 6-15 years, and 17.1% had low actinic damage. The adjusted odds ratios were 3.23 (1/0.31) and 3.13 (1/0.32), respectively. By using the formula provided in the methods, we can calculate that the incidence rate of MS would be reduced by 11% if we were able to eliminate low sun exposure in summer during weekends and holidays at age 6-15 years and the incidence rate of MS would be reduced by 12% if we were able to eliminate low actinic damage.

8.5 DISCUSSION

This is the first analytical study to measure relative risk of MS for past sun exposure in individuals. Higher sun exposure during childhood and early adolescence was associated with a decreased risk of MS. Higher actinic damage was also associated with a decreased MS risk. Both exhibited a dose-response relationship with MS. The striking inverse association between past UVR exposure and MS was consistently found regardless of whether past sun exposure was measured by questionnaire, calendar or the biomarker - actinic damage.

These epidemiological findings are consistent with other work that indicates that UVR may be beneficial for MS. The only analytical study to date on sun exposure and MS is a death certificate case-control study.[10] A strong negative association was found in this study where among outdoor workers, the adjusted odds ratios for low, medium and high regional sunlight were 0.89 {0.64 to 1.22}, 0.52 {0.38 to 0.71} and 0.24 {0.15 to 0.38} for MS compared to indoor workers with low ambient sunlight.[10] As discussed in detail in chapter 3, both UVR and vitamin D_3 have been found to suppress T helper 1 cell immune responses through cytokine signalling and other mechanisms.[4, 5, 26] Clinical symptoms of experimental allergic encephalomyelitis, an animal model of MS, can be prevented or delayed through administration of UVR or 1,25-$(OH)_2D_3$ (the active form of vitamin D_3) at the time of immunisation.[5, 7, 27] A strong inverse correlation ($r=-0.79$, $p<0.01$) between population levels of serum 25(OH)D and mean lesional activity among people with MS has also been reported.[9] Vitamin D deficiency has been noted among people with MS [28] and a small vitamin D and mineral intervention study in MS patients showed that less than half the number of exacerbations were observed after one to two years compared to the expected number based on patient case histories.[29] UVR or vitamin D may also relate to other T helper 1 related autoimmune diseases such as type 1 diabetes. In a Finnish birth cohort, regular vitamin D supplementation in the first year of life was associated with a reduced risk of subsequent disease (rate ratio 0.12 {0.03 to 0.51}).[30]

The case sample appeared similar to other populations with MS of North European ancestry with regard to disease-related features such as the type of MS, age at diagnosis and sex ratio.[12, 31, 32] Also, the phenotypic frequency of the human leukocyte antigen haplotype DRB1*1501-DQB1*0602[33, 34] was similar. Tasmania provides a good setting for this type of study. Unlike Northern Australia, the region has relatively low winter ambient UVR levels, and winter sun exposure is a major determinant of serum 25(OH)D levels in this location.[35] Participation rates for cases and controls were high, reducing non-response bias, but it is conceivable that some selection bias may have occurred. The use of past “Time in the sun” measures could have lead to substantial misclassification of the measurement of past sun exposure if participants had resided in locations with varying ambient UVR levels, but a very high proportion of participants lived in Tasmania for most of their life and their estimated UVR exposure would not be confounded by their residential history. A possible weakness of the study was that prevalent, not incident cases were studied. It is unlikely that recall bias fully explains the observed strong reported associations. The inverse association between estimated average sun exposure in early life and MS did not seem to be caused by subject knowledge of the hypothesis. In fact, the odds ratios for sun exposure were more protective for the group that prior to the interview indicated they did not believe climatic factors such as sun exposure were an important cause of MS. Also, if the results were caused by recall bias, we would expect this to affect the results of sun exposure after the age of 20 or sun exposure immediately prior to the age of onset in a similar manner, but this was not the case. In addition, actinic damage, an objective marker of past sun exposure, also demonstrated an inverse association with MS and this objective marker is free of recall bias. Disease-related

changes in behaviour also did not explain the findings because (i) the protective effect of high actinic damage or child sun exposure was evident even among the recent onset MS group, (ii) the strong inverse association between actinic damage and MS persisted after adjustment for differences in sun exposure that occurred after MS onset and (iii) the association did not differ by disease duration. Chapter 6 indicated that reliability of the "Time in the sun" question was only moderate, but not different for cases compared to controls. Therefore, the odds ratios for the questionnaire and calendar-based measures observed in this chapter are maybe an underestimate of the true effect.

In evolutionary terms, the levels of skin pigmentation in indigenous populations have evolved to optimise the amount of UVR absorbed by the skin in terms of the balance of biological benefits and risks.[36] It would be expected that if host response to UVR were part of the causal pathway for MS, risk would be likely to vary by skin pigmentation levels. Here, within a Caucasian population, fair skin phenotype was associated with an increased risk of MS. A similar association has been described in a German population for type 1 diabetes (OR 3.7, $p<0.01$).[37] In accordance with this, controls (in particular older people) with less melanin were more often vitamin D insufficient, even after adjustment of recent sun exposure. This finding is, however, in contrast with the belief that fairer-skinned people absorb more UVR for a given level of ambient UVR compared to darker-skinned people,[36] leaving them less prone to vitamin D deficiency.[38] Also, a study among eight-year-old Tasmanian children found a non-significant negative correlation ($r=-0.09$, $p=0.19$) between melanin and vitamin D levels,[35] indicating that vitamin D levels were not much affected by skin type among Caucasian children. Behavioural sun avoidance when having a fair skin type may partly counter the increased vitamin D synthesis.

The apparent protective effect appeared to be greatest for sun exposure during childhood and early adolescence. This is consistent with the hypothesis that early life exposure, including UVR, may be important in priming immune system susceptibility.[39] In this model of causation, other early life events including childhood infections may act in combination with UVR to produce MS in genetically susceptible individuals.[39] The finding of no association between occupational sun exposure and MS in our sample is in accordance with that observation, but not in line with the results of a death certificate case-control study conducted in the United States that found a moderate inverse association between occupational sun exposure and MS (compared to indoor occupations, odds ratio for indoor and outdoor occupations 0.89 {0.78—1.02}, odds ratio for outdoor occupations 0.74 {0.61—0.89}).[10] However, their outcome measure is mortality due to MS, while our outcome is MS morbidity. We can only address the timing issue through self-reported data, because the objective biomarker – actinic damage – measures cumulative damage but cannot provide additional data on timing of sun exposure. The finding of no association between sun exposure in the decade prior to disease onset and MS may indicate that the timing of low sun exposure may relate more to age-related immunological development than disease onset. Subsequent work by others will be important to further assess the relevant timing of sun exposure.

When we assessed whether sun exposure in summer and winter were each important in predicting risk of MS, we found that higher levels of winter sun exposure were particularly important in reducing risk. This is not surprising, because in our setting (Tasmania, 41-43°S) the ambient UVR levels in Hobart are more than ten-fold lower in mid-winter than in mid-summer[40] and therefore very low levels of UVR exposure might be expected only at this time. Table 3 indicates that the proportion of personal UVR dose received is likely to be even lower in winter because at all ages, the proportion of children spending less than an hour in the sun on weekends or holidays was substantially greater in winter compared to summer. It is also important to note that the threshold for MS risk reduction with winter sun exposure appeared to occur at more than one hour a day compared to less. Thus, while our results are consistent

with a public health message against total sun avoidance, the findings do not need to jeopardise health promotion activities to reduce excessive exposure to UVR aiming to reduce skin cancer rates, particularly in Australia. However, any general public health message of using sun protection all year round might require some modification to account for regional variation, seasonal variation and skin phenotype in order to prevent negative health outcomes due to insufficient UVR exposure, particularly if vitamin D intake is low.[41, 42] In conclusion, the findings from this case-control study indicate that higher sun exposure during childhood and early adolescence appears to be associated with a reduced risk of MS. This is consistent with insufficient UVR influencing the development of MS. UVR is a natural exposure that is likely to require titration, not avoidance, for optimal health.

8.6 SUMMARY

The striking latitudinal gradient of MS prevalence may reflect variation in UVR. A Th_1 cell mediated attack on myelin proteins appears to be important in the pathogenesis of MS and recent immunological work shows that UVR tends to suppress the Th_1 cell mediated activity. In this chapter, we used the data of the Tasmanian MS case-control study, the study central to thesis, to examine whether high past sun exposure was associated with a reduced risk of MS. Interviews were conducted with 136 cases with MS and 272 controls randomly drawn from the community and matched on sex and birth year. Measures included reported sun exposure, dermal actinic damage (a marker of cumulative lifetime sun exposure), and skin spectrophotometry. MS cases had a fairer skin phenotype, but were less likely to report sunburn (odds ratio 0.55 {0.32—0.96}. In addition, higher ($\geq$ 2-3hours a day on average) sun exposure in summer on weekends and holidays over the age span of 6-15 years was associated with a decreased risk of MS (adjusted odds ratio 0.31 {0.16—0.59}. Higher (grade 4-6) actinic damage was also independently associated with a decreased MS risk (adjusted odds ratio 0.32 {0.11—0.88}. A dose response relationship was observed in both sun exposure over the age span of 6-15 years ($p<0.01$) and actinic damage ($p<0.01$). In conclusion, higher sun exposure during childhood and early adolescence appears to be associated with a reduced risk of MS, which is compatible with UVR having a protective role against MS.

8.7 POSTSCRIPT

This chapter showed that higher sun exposure was associated with a reduced risk of MS. MS is a complex disease where a number of environmental factors are likely to influence its development. The next chapter will examine another environmental factor – infections. In line with the second hypothesis of the Tasmanian MS case-control study, we will examine whether sibship structure and past infections are associated with the risk of MS.

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Chapter 9

Sibship structure, past infections and risk of Multiple Sclerosis.

9.1 PREFACE

In this chapter we will examine another environmental factor that might be involved in the development of Multiple Sclerosis (MS) – infections. We will not only focus on specific past infections, using subject and proxy recall, and serological evidence of past infections, but we will also apply an indirect measure of timing of infection load through a detailed assessment of the influence of sibship structure (birth order, sibling number, number of younger and older siblings, and intersibling interval) on MS risk. The use of sibship structure as an indirect measure of timing of infection load has been successfully used in epidemiological work on atopic disease.

9.2 INTRODUCTION

Viruses have long been suspected as aetiological agents that trigger an autoimmune response, with Pierre Marie postulating in 1884 that an infectious agent might be involved in the development of MS.[1] Viruses that have been implicated in the aetiology of MS are measles, rubella, mumps, canine distemper, retrovirus, and herpes viruses.[2] Infections might induce an autoimmune response via molecular mimicry.[3] Some peptides of the virus or bacteria can mimic autoantigens and presentation of those peptides in the periphery by infected antigen presenting cells may cross-activate autoreactive T cells.[3] Also, viruses might disrupt the regulated network of the immune system, leading to an imbalanced response possibly giving rise to autoimmunity.[1] For example, an increased expression of human leukocyte antigen molecules through the induction of cytokines may raise presentation levels of autoantigens to such a level that autoreactive T cells become activated. Another possibility envisaged is that viruses incorporate host antigens, which then might act, for example, as antigen-presenting entities.[1] More direct mechanisms include the virus being cytopathic to myelin-producing oligodendrocytes, toxic effects of soluble factors that are part of the immune response to the virus invasion, or an increased permeability of the blood brain barrier.[1]

Even though viruses have been thought to trigger autoimmune diseases, the incidence of many infectious diseases have decreased as a result of improved hygiene, better socio-economic conditions, antibiotics and vaccination, while the incidence of many autoimmune diseases (MS, type 1 diabetes mellitus, Crohn's disease) have increased over the past three decades in developed countries, suggesting that infections might also play a protective role.[4] The timing of the infections might be critical in determining whether they act as a protective factor or increase risk.[4] This hypothesis, known as the "Hygiene Hypothesis", proposes that having infections early in life might down regulate host susceptibility to allergic and autoimmune disorders, while having infections later in life might increase the risk.[4]

9.2.1 Family size and sibship structure

The Hygiene Hypothesis was first proposed in 1989 to explain the inverse relation between large sibship size and prevalence of allergic rhinitis at age 11 and 23 years.[5] Children of large

families had an increased risk of early respiratory infections[6] and sibship size could be therefore be seen as a marker of early life infections. Since then, inverse associations between family size and atopic disease and skin prick positivity have been consistently found.[7] A recent meta-analysis reported weighted average odds ratios for having three or more siblings of 0.66 for eczema, 0.72 for asthma or wheezing, and 0.44 for hay fever.[8] Family size and sibship structure has been examined to a greater extent by not only using sibling number and birth order, but also the number of older siblings, the number of younger siblings and interbirth interval to the next sibling. In asthma for example, having older siblings seems more important than having younger siblings, while in hay fever having both older and younger siblings seems important.[9, 10] It suggests that the apparent protective effect of large family size for asthma appears to operate during infancy, while for hay fever it seems to operate at the childhood as well as infant stage.[9, 10] Hay fever has been closely linked to ryegrass or pollen sensitisation, while asthma has been shown to be more strongly linked to house dust mite.[11] The weaker associations with family size that are generally observed in asthma, might be related to the type of allergen that children are sensitised to, because family size was inversely related to asthma among children who had a sensitisation to rye grass but not to house dust mite, while this association was absent among children who had a sensitisation to house dust mite but not to rye grass.[11]

Although family size and sibship markers were thought of as surrogate markers for timing and frequency of infections after birth, Karmaus et al.[12] recently demonstrated that IgE levels of the umbilical cord also decreased with birth order, suggesting that birth order might also reflect foetal-maternal interactions during the prenatal period. Although it is conceivable that birth order, sibling number and the number of older siblings have their origin to some extent in utero, the number of younger children would be free of this effect and can only reflect events after the birth of the index case.

To prove that family size and sibship structure are surrogate markers of the timing and/or frequency of infections, cross-sectional and cohort studies have been conducted to directly link specific infections or the total burden of infectious illness in early life to allergic disease or birth order and family size. However, the results have been disappointing in that no substantial inverse relationships have emerged.[7] This may be because the wrong infections have been studied, or because the timing, rather than the nature, of the infection is particularly important.[7] Alternatively, it might be that infections are not the influential exposure, but another developmental, lifestyle, or environmental influence that varies strongly with birth order and family size.[7]

In MS, migration studies have suggested that the timing of an environmental risk factor might be important. People migrating prior to age 15 years seem to obtain the risk of the host country, while people migrating after the age of 15 years retain the risk of the country of origin.[13-15] In fact, Leibowitz et al.[16] proposed in 1966 (similarly to the Hygiene Hypothesis) that: “In environments with a high sanitary level, infection may be postponed until an age when the central nervous system is more susceptible to the process which provokes demyelination”. In line with this hypothesis it was observed that the frequency of MS was low in regions where childhood diseases were acquired early in life, while MS frequency was high in regions where childhood diseases tended to occur nearer adolescence.[17] Also, MS is less frequent among people belonging to low socioeconomic classes or living in countries where the general level of sanitation is low.[18] Animal studies on autoimmune diseases such as type 1 diabetes mellitus and collagen-induced arthritis showed that mice or rats develop disease earlier and at a higher rate among animals bred in a specific pathogen-free environment than among animals bred in a conventional environment.[19, 20] In addition, treatment with mycobacteria protects against experimental allergic encephalomyelitis.[21] Importantly, a recent case-control

study on childhood diabetes showed that increased social mixing through attendance at daycare in early infancy appeared to confer protection.[22]

In contrast to the research on allergic disease and sibship structure, in MS the attention has been mainly focused on birth order, which reflects older sibling number, but not younger sibling number or total number of siblings. Younger sibling number, however, might also be important particularly when the putative protective infection is common in infancy and/or re-exposure to this infant infection is important. Re-exposure to active viral infection is known to cause immune boosting (as assessed by rising immunoglobulin (Ig) G titres) in seropositive individuals,[23] and repeated viral antigen challenge may lead to immune refinement.[24]

If the hypothesis were true that having childhood infections later in life might increase the risk of MS, then one would expect that earlier born members (low birth order) have a higher risk compared to later born members (high birth order). Of the studies that compared the mean birth order of a sample of cases with the expected birth order that would have occurred by chance, most found no effect,[25-28] but some found, against the expectation, that the mean birth order was higher than expected.[29, 30] However, as outlined by James, this method is dependent on the assumption that family size is stable over time, which is not the case.[31-33] A case-control study would therefore be the preferred study design. Two case-control studies found no effect in birth order.[34, 35] Isager et al. and Visscher et al. found a reduction in risk for having a birth order higher than three, but the confidence interval was wide in the study from Visscher et al. (Isager et al.[36]: OR 0.29 {0.10—0.79}; Visscher et al.[25]: OR 0.52 {0.23—1.23}). A recent case-control study nested in a large cohort of nurses conducted a more detailed analysis of sibship structure. They found little effect for being first born or being an only child, a weak negative association with the number of older siblings, and there appeared to be an increased risk for first-born children in families with four or more children (OR 2.1 {1.2—3.5}, which was absent in families with two or three children (OR 0.8 {0.5—1.2}).[37]

9.2.2 Specific infections/viruses

As was briefly discussed in chapter 2, the only specific virus with consistent evidence that it might be involved in the development of MS is the Epstein-Barr virus (EBV), one of the herpes viruses.[38, 39] In young children, infection with EBV is mild and generally asymptomatic. In older children, adolescents, and adults, EBV presents as infectious mononucleosis (IM) in 50% of those who become EBV positive. After primary infection, EBV persists for life in a latent state in peripheral B-cells. The infection is associated with the appearance and persistence of specific antibodies to EBV. IgG antibodies to EBV viral capsid antigen (VCA) and anti-early antigen complex (diffuse) (EA-D) emerge during the late incubation period or in the course of the acute phase of IM, whereas IgG antibodies to the Epstein Barr nuclear antigen (EBNA) family and anti-early antigen complex (restricted) (EA-R) arise only weeks or months after onset of the disease.[40] Antibodies to EBNA-2 arise before those to EBNA-1 and usually decline over several months, whereas antibodies to EBNA-1 persist indefinitely. Persistently high levels of antibodies to VCA and EBNA-2 with low EBNA-1 titers have been associated with chronic IM.[40, 41]

Similarities in the epidemiology of MS and IM led to the proposition in 1981 that MS could be caused by infection with EBV during or after adolescence in genetically susceptible individuals.[42] This hypothesis is supported by observations suggesting an increased risk of MS for those with a history of IM,[34, 37, 43, 44] which includes a prospective study.[44] The age of IM might also be important. Martyn et al. found a higher risk in a case-control study for those who had IM prior to age 17 years (the median age of IM) and were seropositive against VCA (OR 7.0 {1.7—37.0}), compared to the total group that had IM and was seropositive against VCA (OR 2.9 {1.1—7.2}).[43] A nested case-control study by Hernan et al. found no increased risk

when IM was diagnosed prior to age 10 years, but increased risk estimates for a diagnosis after the age 10 years (OR 1.9 {0.8—4.2} for IM at age 11-15 years; OR 2.2 {1.5—3.2} for IM after age 15 years).[37]

MS is rare among individuals without serum anti-EBV antibodies.[38, 39] Seroepidemiologic studies are consistent in reporting an increased prevalence of antibodies to EBV in MS cases (as high as 100% prevalence) as compared with healthy controls (85-95%).[38] In a recent systematic review, Ascherio and Munch combined the results of eight seroepidemiological studies and reported an estimated odds ratio of 13.5 {6.3—31.4} for seropositivity against EBV antibodies.[38] Six of the eight studies used seropositivity against VCA as their marker of past infection with EBV.[38]

However, it is important to know whether the EBV infection precedes the disease or whether altered EBV IgG levels reflect a disease-related immune disturbance. Some indirect evidence, by analysing different types of EBV antibodies, has suggested a lack of primary infections (positivity of anti-EA-IgM and/or anti-EA-IgG in the absence of anti-EBNA-1 antibodies) among people with MS compared to controls.[39, 45] This indicates that the EBV infection already occurred at some earlier point in time which strengthens the concept that this occurred prior to MS onset. More direct evidence has recently become available from a prospective cohort study and nested case-control study, both with samples taken prior to MS onset.[46, 47] The prospective cohort study found that risk of MS increased monotonically with increasing serum levels of antibodies to VCA (test for trend, $p<0.01$) and EBNA complex (test for trend, $p<0.01$).[47] The relative risk (RR) in persons in the highest category of VCA ($\geq$2560) compared with those in the lowest ($\leq$160) was 19.7 {2.2—174}, and in persons in the highest category of EBNA complex ($\geq$1280) compared with those in the lowest ($\leq$40), the RR was 33.9 {4.1—283}.[47] Similarly strong positive associations between EBV antibodies and risk of MS were present in samples collected five or more years before MS, suggesting the results were not due to an early manifestation of the preclinical phase of the disease. In fact, repeated serological determinations in the same cases revealed that all antibodies titers were similar in the earliest available sample (mean, 3.6 years before onset) and the sample collected after MS onset (mean, 1.0 years after MS onset).[47] The nested case-control study of 144 cases and 288 age-matched controls included 18 cases who had their blood sample taken prior to the age of onset.[46] They found significant associations in both the group with samples taken prior to MS onset and the group with samples taken after MS onset, but the associations were stronger for the group with samples taken prior to MS onset. The strongest association with MS was seen for antibodies to the EBNA-2 (RR 3.9 {1.1—13.7} for a four-fold difference in titers), and they found an RR of 1.6 {0.7—3.7} for VCA, 2.5 {1.0—6.3} for EBNA complex and 1.8 {1.0—3.1} for EA-D for a four-fold difference in titers.[46] Both studies observed a simultaneous elevation of titers to VCA and EBNA, which seems to suggest a more severe or more recent primary infection among subjects who developed MS than in subjects who remained healthy.[46, 47] Studies on MS plaques using in situ hybridisation or polymerase chain reaction have not shown any EBV DNA, suggesting that the central nervous system is not directly infected, but rather that the T-cell response to EBV infection could include clones that are potentially cross-reactive with self-antigens.[48] Prior to this T cell cross-reactivity, Pender suggests a role for autoreactive B cells infected by EBV that proliferate and become latently infected memory B cells, which are resistant to apoptosis that occurs during normal B-cell homeostasis.[49] Genetically susceptible people might have an increased number of those latently infected autoreactive memory B cells.[49] These B cells might act as antigen-presenting cells in organs where the target antigen is expressed and present the cognate antigen or other physically lined self-antigen to activated autoreactive T cells that are trafficking through the target organ.[49]

Other herpes viruses (cytomegalovirus (CMV), human herpes virus 6 (HHV-6) and 8 (HHV-8), herpes simplex virus 1 (HSV-1) and 2 (HSV-2), varicella zoster virus (VZV)) are also attractive candidates for a role in the development of MS. The majority of the herpes viruses are neurotropic, establish latency, are periodically reactivated and have the capacity to induce demyelination.[50] However, no consistent pattern has emerged for any of those viruses. There is currently interest in the relationship between HHV-6 and MS. A recent subjective but systematic review of the literature indicated that there is some pathological evidence for a relationship between HHV-6 and MS.[50] Even though these pathological studies found positive staining for HHV-6 around plaques and in oligodendrocytes in MS patients but not controls, the studies rated low on methodological rigor, in particularly in regard to the selection of cases and controls. The data do not conclusively show that theses findings could not have been attributed to selection bias.[50] In addition, other experimental methods (including serum IgG and IgM antibody studies and polymerase chain reaction studies for HHV-6 DNA using serum, cerebrospinal fluid, peripheral blood mononuclear cells or brain tissue) failed to support a relationship between HHV-6 and MS.[50] Despite these results, further studies on the relationship between HHV-6 and MS using better designs or different methods (for example by examining the geographic variation in HHV-6 prevalence or similarities between HHV-6 proteins and human myelin proteins) seem worthwhile.[50] In addition, herpes viruses have the ability to interact with each other and those interactions may play a role in the pathogenesis of MS.[51] For example, it has been reported that increased replication from the latent EBV genome was induced by HHV-6 variant A infection.[51]

The results of a meta-analysis of 26 case-control studies of a number of other common childhood infectious diseases and MS showed no significant differences in frequency between cases and controls (measles, OR 1.06 {0.92—1.22}; rubella, OR 0.97 {0.83—1.13}; mumps, OR 0.91 {0.79—1.06}; whooping cough, OR 0.98 {0.92—1.3}; chickenpox, 0.98 {0.85—1.12}).[52] However, 13 of 15 studies with information on the timing of infections found that people with MS tended to have their childhood infections at a later age.[52] Some studies found this for measles, others for rubella, mumps, varicella zoster, IM or a group of childhood diseases. A recent well-conducted nested case-control study found increased risks for measles (OR 2.8 {0.8—9.1}) and mumps (OR 2.3 {1.2—4.3}) if they occurred after the age of 15 years.[37] Serologic studies have found elevated measles antibody levels in people with MS compared to controls,[53, 54] but the results regarding mumps antibodies have been conflicting.[55] The nested case-control study also found a moderately increased risk of MS among dog owners (OR 1.5 {0.9—2.3}), but the 95% confidence interval was wide. Canine distemper virus is a measles-like virus that can cause demyelination in the central nervous system of dogs and other mammals. A review of 17 case-control studies concluded that the overall evidence of a role of the canine distemper virus in the causation of MS remains at best equivocal, and that it is unlikely that this virus plays a major role in the aetiology of MS in the majority of patients.[56]

In conclusion, review of the available literature on sibship structure and MS shows that a number of studies have assessed the effect of birth order and MS, but that there is a lack of studies that assessed other important indicators of sibship structure. Epidemiological studies on sibship structure and atopic disease have demonstrated that it is worthwhile to examine those additional indicators. Review of the literature on specific infections and MS shows that the only specific virus with consistent proof that it might be involved in the development of MS is EBV. Even though no clear patterns have emerged from other specific viruses, a delay in infections might be an important risk factor, because people with MS tended to have their childhood infections at a later age. The use of sibship structure might assist regarding this issue as it seems a proxy for the timing of non-specific infections.

In this chapter, we firstly assess the relationship between a number of indicators of sibship structure and MS – birth order, sibling number, number of younger and older siblings, and intersibling interval. Secondly, we investigate the association between the history of specific infections and MS using subject recall, proxy recall and serum antibody analysis.

9.3 METHODS

9.3.1 Subjects

Chapter 5 outlined in detail the multiple strategies that were used to recruit cases, the approach to obtain community controls matched age and birth year to the case and the clinical[57] and MRI[58] criteria used for diagnosis. The final total sample consisted of 136 cases (response rate estimated to be between 76% and 92%) and 272 controls (response rate 76%). A blood sample for serum analysis was taken from all cases and 262 controls (98%). The research assistant was not successful in obtaining blood samples from four controls, six controls declined to have a sample taken.

9.3.2 Measurements

Sibship structure

We collected information on the date of birth of the parents and the birth order among their natural brothers and sisters (Appendix A). For each sibling it was asked whether: they were a full or half sibling, the date of birth of the sibling and whether the subject lived with the sibling when the subject was 0-5 years, 6-10 years, 11-15 years and 16-20 years. The questionnaire allowed for six siblings and research assistants were instructed to write any additional siblings on the next page. With this information, calculations could be made of the total number of siblings, the number of younger and older siblings and the interbirth interval to the nearest siblings.

Infections

Self-reported infections. Subjects and proxies were asked whether and in which five-year age period the subject had any of the following infections: chicken pox, measles, rubella (German measles), mumps, pertussis (whooping cough), herpes labialis (cold sores) herpes genitalis, infectious mononucleosis (glandular fever), and impetigo (school sores) (Appendix A).

IgG serum antibodies. Blood samples of 10 ml were taken. The blood was centrifuged and the removed serum frozen at -80°C. The frozen samples were sent to the Westmead hospital, stored in liquid nitrogen, where the antibody analyses were performed. IgG antibodies to EBV nuclear antigen (EBNA) and EBV capsid antigen (VCA) were determined by enzyme linked immunosorbent assay (ELISA) (PANBIO, Brisbane, QLD, Australia); herpes simplex virus 1 (HSV-1) and herpes simplex virus 2 (HSV-2) by enzyme linked immunosorbent assay (ELISA) (Focus Technologies, California, USA); cytomegalovirus (CMV) and rubella by a microparticle enzyme immunoassay (Axsym system, Abbott Laboratories, Illinois). All assays included negative and positive controls.

9.3.3 Data analysis

Pearson correlations were calculated as measures of linear association. Odds ratios and 95% confidence intervals were estimated by conditional logistic regression (STATA 7.0). Tests for trend of categorical variables were undertaken by replacing the binary predictors with a single predictor taking category rank scores for the categories. The Cohen's kappa statistic[59] was used to calculate agreement between proxies and subjects in recall of self-reported infections. Cut-points for negative, equivocal, and positive serum antibody results were taken from the instructions of the manufacturer of the antibody test.

Birth order was assessed among natural live born brothers and sisters. For other analyses on sibship structure, we included full and half siblings. We excluded 100 siblings that never lived with the subject under the age of 20 years. The interbirth interval to the nearest sibling was not calculated for three subjects for whom we did not have the date of birth of each sibling available. The category of subjects that did not have older siblings included subjects without any siblings and subjects with only younger siblings. Similarly, the category of subjects that did not have younger siblings included subjects without any siblings and subjects with only older siblings. The number of younger siblings and interbirth interval between the subject and each sibling was combined by adding up the number of days that the subject shared his/her life with each sibling by the time that the subject was six years of age. This was done using the dates of birth of the subject and each of the siblings. This cumulative number of sibling days at the age of six years was then converted into years by dividing it by 365.25. The procedure was repeated to calculate cumulative sibling infant years by age six, but rather than counting total shared days with each sibling, shared days were only counted when the sibling was an infant younger than two years of age.

Similarly to chapter 8, population attributable fraction (the proportion by which the incidence rate of the outcome in the entire population would be reduced if exposure were eliminated, assuming that the exposure is causally related to the outcome and that causes other than the one under investigation have had equal effects on the exposed and unexposed groups) was estimated by the formula:[60]

$$AF_p = \frac{P(RR-1)}{RR}$$

where RR is the estimate of the rate ratio (odds ratio in a case-control study) and P the proportion of all cases that are exposed. The population attributable fraction was calculated for risk estimates of (1) younger sibling number, (2) teenage IM, (3) high IgG levels against EBNA (>2.50 units), and (4) high IgG levels against VCA (>2.00 units).

9.4 RESULTS

9.4.1 Risk for measures of sibship structure

Of all 408 subjects, 392 subjects (132 cases and 260 controls) had one or more siblings, while 16 subjects did not have any siblings. Those 392 subjects had 1226 siblings (1170 (95.4%) full siblings, 49 (4.0%) half siblings and 7 (0.6%) missing data for type of sibling).

The study sample of cases and controls in this chapter was identical to the study sample described in chapter 8. See Table 1 of chapter 8 for the description of the characteristics of the cases and controls.

We found no association between birth order among the natural live born siblings and MS (Table 1). The age of the parents when the subject was born correlated highly (mothers, r=0.60; fathers, r=0.50) with birth order. The association between the age of the mother when the subject was born and MS was slightly stronger compared to that of birth order, but there was no dose-response relationship (Table 1). There was, however, a positive association between the age of the father when the subject was born and MS, with evidence of a dose-response trend. Further analysis revealed that this association was caused by its correlation with the number of younger siblings (r=-0.33). Adjustment for younger sibling number removed the effect between age of the father when the subject was born and MS. The adjusted odds ratios {95% confidence interval} compared to age ≤25 years were: OR 1.78 {0.94—3.36} for 26-30 years; OR 1.08 {0.55—2.13} for 31-35 years; 1.51 {0.71—3.21}; test for trend, p=0.56).

Table 1. Unadjusted odds ratios for Multiple Sclerosis and birth order among natural live born siblings.

	Cases n (%)	Controls n (%)	Unadjusted OR {95% CI}
Birth order			
First born	43 (31.6)	94 (34.6)	1
Birth order = 2	34 (25.0)	75 (27.6)	0.99 {0.58—1.70}
Birth order = 3	22 (16.2)	46 (16.9)	1.06 {0.58—1.94}
Birth order = 4 or more	37 (27.2)	57 (21.0)	1.42 {0.82—2.47}
Test for trend			p=0.23
Age mother when subject born			
≤20 years	17 (12.7)	41 (15.4)	1
21—25 years	36 (26.9)	93 (35.0)	0.92 {0.45—1.87}
26—30 years	43 (32.1)	64 (24.1)	1.59 {0.79—3.18}
>30 years	38 (28.4)	68 (25.6)	1.32 {0.66—2.65}
Test for trend			p=0.13
Age father when subject born			
≤25 years	22 (16.5)	71 (27.1)	1
26—30 years	45 (33.8)	80 (30.5)	1.77 {0.96—3.27}
31—35 years	30 (22.6)	62 (23.7)	1.52 {0.80—2.88}
>35 years	36 (27.1)	49 (18.7)	2.60 {1.31—5.16}
Test for trend			p=0.02

Notes
1. OR = odds ratio; {95% CI} = {95% confidence interval}

Controls tended to have more full and half siblings compared to cases, resulting in a negative association between the number of siblings and MS (Table 2). When we assessed whether it

was of importance whether subjects had older or younger siblings, we observed that having younger siblings was strongly associated with a reduced risk of MS, and the magnitude of the effect increased with an increased number of younger siblings. No association was observed for having older siblings (Table 2). The magnitude of the effect of the number of younger siblings remained similar after adjustment for the number of older siblings and a negative association was also observed for the subgroup of subjects who where the eldest in the sibship or were only children, but the results were less precise due to lower numbers (Table 2).

Table 2. Unadjusted odds ratios for Multiple Sclerosis and family structure indicators.

	Cases n (%)	Controls n (%)	OR {95% CI}
Family size			
Increase in sibling number			
No siblings	10 (7.5)	15 (5.5)	1
One sibling	39 (29.1)	47 (17.3)	1.28 {0.49—3.41}
Two siblings	25 (18.7)	62 (22.9)	0.57 {0.22—1.48}
Three siblings	16 (11.9)	55 (20.3)	0.40 {0.14—1.15}
Four or more siblings	44 (32.8)	92 (33.9)	0.68 {0.27—1.74}
Test for trend			p=0.04
Increase in sibling number (per sibling)			0.90 {0.81—1.00}
Older or younger siblings			
No siblings	10 (7.5)	15 (5.5)	1
Only older siblings	48 (35.8)	59 (21.8)	1.23 {0.49—3.11}
Only younger siblings	38 (28.4)	82 (30.3)	0.69 {0.27—1.75}
Both younger and older siblings	38 (28.4)	115 (42.4)	0.44 {0.17—1.23}
Younger siblings (total sample)			
Having younger siblings			
No	58 (43.3)	74 (27.3)	1
Yes	76 (56.7)	197 (72.7)	0.47 {0.30—0.74}
Increase in younger sibling number			
No younger siblings	58 (43.3)	74 (27.3)	1
One younger sibling	38 (28.4)	69 (25.5)	0.68 {0.39—1.18}
Two younger siblings	20 (14.9)	64 (23.6)	0.37 (0.20—0.70}
Three or more younger siblings	18 (13.4)	64 (23.6)	0.35 {0.18—0.66}
Test for trend			p<0.01
Increase in younger sibling number (per sibling)			0.77 {0.67—0.90}
Increase in younger sibling number (per sibling)*			0.78 {0.66—0.91}
Younger siblings (for oldest or only children)			
Having younger siblings			
No	10 (20.8)	15 (15.5)	1
Yes	38 (79.2)	82 (84.5)	0.80 {0.21—2.98}

Table 2 continued at next page

Table 2 continued

	Cases n (%)	Controls n (%)	OR {95% CI}
Increase in younger sibling number			
No younger siblings	10 (20.8)	15 (15.5)	1
One younger sibling	18 (37.5)	22 (22.7)	2.70 {0.45—16.35}
Two younger siblings	10 (20.8)	30 (30.9)	0.54 (0.13—2.29}
Three or more younger siblings	10 (20.8)	30 (30.9)	0.75 {0.13—4.15}
Test for trend			p=0.15
Increase in younger sibling number (per sibling)			0.84 {0.60—1.18}
Interbirth interval total sample			
Interval to nearest younger sibling			
No younger siblings	58 (43.3)	74 (27.3)	1
Nearest younger sib >6.00 years younger	11 (8.2)	12 (4.4)	0.71 {0.46—2.89}
Nearest younger sib 4.01—6.00 years younger	12 (9.0)	31 (11.4)	0.50 (0.23—1.08}
Nearest younger sib 2.01—4.00 years younger	28 (20.9)	60 (22.1)	0.56 (0.30—1.01}
Nearest younger sib 0—2.00 years younger	25 (18.7)	94 (34.7)	0.33 {0.19—0.59}
Test for trend			p<0.01
Interval to second younger sibling			
No younger siblings	58 (60.4)	74 (26.5)	1
2nd younger sib >6.00 years younger	10 (10.4)	43 (21.2)	0.31 (0.13—0.74}
2nd younger sib 4.01 —6.00 years younger	18 (18.8)	29 (14.3)	0.82 (0.35—1.92}
2nd younger sib 0—4.00 years younger	10 (10.4)	57 (28.1)	0.21 {0.08—0.52}
Test for trend			p<0.01
Older siblings (total sample)			
Having older siblings			
No	48 (35.8)	97 (35.8)	1
Yes	86 (64.2)	174 (64.2)	0.99 {0.65—1.52}
Increase in older sibling number			
No older siblings	48 (35.8)	97 (35.8)	1
One older sibling	36 (26.9)	77 (28.4)	0.96 {0.56—1.63}
Two older siblings	19 (14.2)	44 (16.2)	0.86 (0.47—1.61}
Three or more older siblings	31 (23.1)	53 (19.6)	1.18 {0.67—2.07}
Test for trend			p=0.69
Increase in older sibling number (per sibling)			1.06 {0.94—1.19}
Increase in older sibling number (per sibling)**			1.01 {0.88—1.14}

Notes
1. OR = odds ratio; {95% CI} = {95% confidence interval}
2. * refers to an odds ratio that is adjusted for the number of older siblings
3. ** refers to an odds ratio that is adjusted for the number of younger siblings

To assess the time frame over which the apparent protective effect of having younger siblings might operate, we examined the interval between the subject and the nearest younger sibling. The results indicate a strong inverse association with MS when the interval between the subject and the nearest younger sibling was less than two years, a moderate association when the interval was between two and six years, and only a weak association when the interval between the subject and the nearest younger subject was more than six years (Table 2). The pattern for the interval to the second younger sibling was less obvious among subjects

that had two or more siblings, probably due to the influence of the first younger sibling (Table 2). Although theoretically close siblings could mediate their protective effect at any age, it seems more plausible that they mediate most of their protective effect in the first years of life rather than later.

To gain further insight into which age period the apparent protective effect might be operating, we reanalysed the effects of having younger siblings at different ages of the subject (age two, four, six, and eight years). Although a strong protective effect for having younger siblings was observed when the subject was only two years of age (OR 0.48 {0.29—0.78}), a similar effect was seen when the subject was, for example, eight years of age (OR 0.49 {0.31—0.76}) and adjusting for each other was not possible due to the high correlations between the variables. Also, the inclusion of the number of siblings and interbirth interval did not contribute to a better understanding of the age period that the apparent protective effect might be operating (data not shown).

The fact that younger but not older siblings mediate a protective effect might indicate that: (1) primary infections of the subjects in the first year of life are not of critical importance, because those infections are likely to be transmitted by older siblings, (2) but rather that repeated stimulation of the immune system through early life infections of the younger siblings is important.

Table 3. Unadjusted odds ratios for Multiple Sclerosis and younger sibling years by age six.

	Cases n (%)	Controls n (%)	OR {95% CI}
Cumulative sibling years by age six			
Zero	66 (49.3)	84 (31.0)	1
Less than three years	21 (15.7)	47 (17.3)	0.56 {0.30—1.05}
Three to six years	32 (23.9)	76 (28.0)	0.53 {0.31—0.92}
More than or equal to six years	15 (11.2)	64 (23.6)	0.28 {0.15—0.56}
Test for trend			p<0.01
Cumulative sibling years by age six (per sibling year, range 0-16 years)			0.86 {0.80—0.93}
Cumulative sibling infant years by age six			
Zero	66 (49.3)	84 (31.0)	1
Less than two years	12 (9.0)	30 (11.1)	0.50 {0.23—1.07}
Two to 3.5 years	41 (30.6)	91 (33.6)	0.58 {0.34—0.97}
More than or equal to 3.5 years	15 (11.2)	66 (24.4)	0.28 {0.14—0.54}
Test for trend			p<0.01
Cumulative sibling infant years by age six (per sibling year, range 0-12 years)			0.80 {0.70—0.90}

Notes

1. OR = odds ratio; {95% CI} = {95% confidence interval}
2. Cumulative sibling years by age six takes into account the number of siblings and the timing of their births by adding up the number of days that the subject shared with each sibling by the age of six years. Cumulative sibling infant years takes only into account the shared days where the sibling was younger than the age of two years.

We next combined the number of younger siblings and the interbirth interval of each sibling by counting the number of days that the subject shared his or her life with each sibling by the age of six years using the dates of births of the subject and the siblings. This was then converted into years by dividing the number of cumulative sibling days by age six by 365.25. The aim was to see whether this combination of number of younger siblings and interbirth interval predicted risk better than just the number of younger siblings. In addition, we repeated the procedure, but rather than counting total shared days with each sibling, we only counted

shared days where the sibling was an infant younger than two years of age (cumulative sibling infant years by age six). The aim here was to see whether shared infant days up to age two were more important than total shared days.

Both cumulative sibling years by age six and cumulative sibling infant years by age six were associated with a strong reduced risk of MS in a dose-response manner (Table 3). Firstly, compared to the risk estimates for the number of younger siblings provided in Table 2, cumulative sibling years by age six did not seem to be a better predictor of MS. Among controls, the correlation between the two variables was 0.73. Secondly, the risk estimates for cumulative sibling years were nearly identical to the risk estimates for cumulative sibling infant years. This was not surprising when we examined the correlation between the two variables (r=0.97). The extremely high correlation made it impossible to draw conclusions regarding the more or lesser importance of shared infant days compared to total shared days.

9.4.2 Risk for specific viral infections

Epstein-Barr virus

History of infectious mononucleosis. More cases than controls had a history of IM (Table 4). When we assessed the age at which subjects had the infectious disease, we observed a positive association between IM and MS for subjects who had IM between age 11 and 20 years, but no association when the disease occurred at age 6 to 10 years or after the age of 20 years (Table 4), indicating that having IM was especially important when it occurred during adolescence years. We will refer to IM that occurred between 11 and 20 years as "teenage IM".

As shown in chapter 6, the agreement on retest for self-reported history of IM was very high (κ=0.87) and was not significantly different for cases (κ=0.81) compared to controls (κ=0.94). We assumed that people who reported that they had IM but who were found to be seronegative to EBNA and VCA were incorrect in their report on IM. Excluding those people (n=4) and their matched pair from the analysis made no substantial difference to the association between IM and MS (OR 2.29 {1.32—3.95}). The odds ratios for MS for infection at different ages slightly increased for age 6-10 years (OR 1.35 {0.30—6.13}), age 11-15 years (OR 5.53 {1.76—17.38}) and age 16-20 (OR 3.54 {1.27—9.86}) and reduced to below one for age >20 years (OR 0.88 {0.31—2.57}).

Table 4. Unadjusted odds ratios for Multiple Sclerosis and history of infectious mononucleosis and serum IgG antibodies to Epstein-Barr virus.

	Cases n (%)	Controls n (%)	Unadjusted OR {95% CI}
Self-reported history of IM			
No	99 (72.8)	232 (85.3)	1
Yes	35 (25.7)	39 (14.3)	2.14 {1.26—3.62}
Don't know	2 (1.5)	1 (0.4)	4.68 {0.42—52.48}
Self-reported age of IM			
Did not have IM	99 (74.4)	232 (86.2)	1
At age 6—10 years	3 (2.0)	5 (1.9)	1.20 {0.29—5.02}
At age 11—15 years	13 (9.8)	8 (3.0)	4.74 {1.11—17.8}
At age 16—20 years	12 (9.0)	10 (3.7)	3.09 {1.18—8.13}
Above age 20 years	6 (4.5)	14 (5.2)	1.04 {0.39—2.74}
History of teenage IM (IM at age 11—20 years vs no IM or no IM at age 11—20 years			3.88 {1.88—7.99}

Table 4 continued at next page

Table 4 continued

	Cases n (%)	Controls n (%)	Unadjusted OR {95% CI}
Serum IgG antibody analysis			
Antibodies to EBNA			
Negative (<0.9 units)	0 (0)	39 (14.9)	
Equivocal (0.9—1.1 units)	1 (0.7)	8 (3.1)	1
Positive (>1.1 units)	135 (99.3)	214 (82.0)	28.00 (3.81—205.7)
Antibody titers to EBNA			
0—2.50 units	26 (19.1)	150 (57.5)	1
2.51—3.00 units	31 (22.8)	52 (19.9)	3.49 {1.82—6.70}
3.01—4.00 units	79 (58.1)	59 (22.6)	7.47 {4.16—13.41}
Test for trend			p<0.01
Antibody titers to EBNA (>2.50 vs ≤2.50 units)			5.69 {3.33—9.71}
History of teenage IM and antibody titers to EBNA			
No teenage IM + EBNA titers ≤ 2.50	19 (15.3)	133 (55.6)	1
Teenage IM + EBNA titers ≤ 2.50	6 (4.8)	5 (2.1)	10.01 {2.45—40.98}
No teenage IM + EBNA titers > 2.50	80 (64.5)	89 (37.2)	6.40 {3.32—12.32}
Teenage IM + EBNA titers > 2.50	19 (15.3)	12 (5.0)	20.37 {6.35—65.39}
Test for trend			p<0.01
Antibodies to VCA			
Negative (<0.9 units)	3 (2.2)	37 (14.2)	
Equivocal (0.9—1.1 units)	1 (0.7)	4 (1.5)	1
Positive (>1.1 units)	132 (97.1)	220 (84.3)	5.64 {2.00—15.94}
Antibody titers to VCA			
0—2.00 units	21 (15.4)	70 (26.8)	1
2.01—2.50 units	23 (16.9)	41 (15.7)	1.79 {0.91—3.54}
2.51—3.00 units	38 (27.9)	78 (29.9)	1.63 {0.87—3.08}
3.01—4.00 units	54 (39.7)	72 (27.6)	2.58 {1.37—4.85}
Test for trend			p<0.01
Antibody titers to VCA (>2.00 vs ≤2.00)			1.97 {1.15—3.40}
Antibody titers to VCA (>2.50 vs ≤2.50)			1.57 {0.99—2.47}
History of teenage IM and antibody titers to VCA			
No teenage IM + VCA titers ≤ 2.50	37 (29.8)	98 (41.0)	1
Teenage IM + VCA titers ≤ 2.50	4 (3.2)	7 (2.9)	1.82 {0.44—7.45}
No teenage IM + VCA titers > 2.50	62 (50.0)	124 (51.9)	1.21 {0.72—2.05}
Teenage IM + VCA titers > 2.50	21 (16.9)	10 (4.2)	8.97 {2.94—27.40}
Test for trend			p<0.01

Notes
1. OR = odds ratio; {95% CI} = {95% confidence interval}
2. IM = infectious mononucleosis; EBNA = Epstein Barr virus Nuclear Antigen; VCA = Viral Capsid Antigen

Serum IgG antibodies to EBV. Being seropositive to EBNA and VCA was strongly associated with MS (Table 4). There was evidence of a dose response relationship with increased antibody titers (p<0.01). A strong correlation was seen among controls between EBNA and VCA antibody titers (r=0.46, p<0.01), which was not so evident among cases (r=0.14, p=0.10). The effect on MS was stronger for EBNA compared to VCA.

History of teenage IM and serum IgG antibodies to EBV. There was a moderate correlation between having had a history of teenage IM and the level of EBNA antibody titers (controls

r=0.12, p=0.06; cases r=0.07, p=0.45), and between having had a history of teenage IM and VCA antibody titers (controls r=0.07, p=0.26; cases r=0.17, p=0.05).

We assessed the combined effect of a history of teenage IM and high EBNA antibody titers by creating one variable with rank scores for four categories: "no history of teenage IM and low EBNA titers (≤2.5 units)" (reference category), "history of teenage IM and low EBNA titers", "no history of teenage IM and high EBNA titers", and "history of teenage IM and high EBNA titers" (Table 4). Having had teenage IM showed a strong positive association with MS when EBNA titers were low, and having high EBNA titers showed a strong positive associated with MS even in the absence of teenage IM (Table 4). From a biological point of view, an additional risk increase was seen for those who both had teenage IM and high EBNA antibody titers (24.3% of the estimate of the risk ratio was attributed to the interaction ([20.37–1.00–9.01–5.40]/20.37)),[61] although from a statistical point of view, the additional risk was far less than would be expected on a multiplicative scale. Indeed, using a model with teenage IM, EBNA titers (high versus low) and an interaction term for teenage IM and EBNA titers, the combined effect of a history of teenage IM and high EBNA was lower than we would have expected on a multiplicative scale (p=0.06).

We used a similar approach to examine the combined effect of teenage IM and high VCA antibody titers. Table 4 shows that compared to those without a history of teenage IM and low levels of VCA, there was no significant independent effect for those with a history of teenage IM when VCA levels were low, or for those with high VCA levels and no history of teenage IM. From a biological point of view, a large risk increase was seen for those who both had teenage IM and high VCA antibody titers (77.4% of the estimate of the risk ratio was attributed to the interaction ([8.97–1.00–0.82–0.21]/8.97)). Indeed, the combined effect of a history of teenage IM and high VCA was higher than we would have expected on a multiplicative scale (p=0.05) when using a model with teenage IM, VCA titers (high versus low) and an interaction term for teenage IM and VCA titers.

Thus, EBNA and VCA are two different antibodies to EBV. They both were positively associated with MS, but the effect of increasing EBNA titers appeared to be stronger than the effect of increasing VCA titers. In addition, the combined effects of having high antibody titers and a history of teenage IM were different. For VCA, high IgG levels against VCA or having had teenage IM did not appear to be associated with MS in absence of the other, but having both was positively associated with MS. In contrast, for EBNA, strong associations were found for high IgG levels against EBNA or having had teenage IM in absence of the other, while the combined effect did not appear to result in a high additional risk.

Rubella

History of rubella. Self-reported history of rubella was not associated with MS, and although there was some evidence that having had rubella under the age of 5 years was positively associated with MS (OR 2.01 {0.80—5.02}), the confidence intervals were wide (Table 5). Interestingly, having had an immunisation against rubella was negatively associated with MS (OR 0.45 {0.24—0.85}).

Serum IgG antibodies to rubella. A very high percentage of cases and controls was seropositive to rubella and being seropositive to IgG rubella was not associated with MS. Assessment of the IgG antibody titers showed a dose-response relationship between IgG antibody levels and MS, with a significant positive association for people with titers above 200 IU/ml (OR 2.43 {1.24—4.76}) (Table 5).

History of rubella and serum IgG antibodies to rubella. There was no correlation between history of rubella and serum IgG antibody titers against rubella (controls r=-0.03, p=0.59; cases r=0.14, p=0.12). Assessing the combined effect of a history of rubella and antibody

titers against rubella (>100.0 vs ≤100.0 IU/ml) did not show any significant associations (Table 5) and including an interaction term of self-reported history of rubella and serum IgG antibody levels against rubella in the model did not show to be significant (p=0.27).

Table 5 Unadjusted odds ratios for Multiple Sclerosis and history of rubella and serum IgG antibodies to rubella,

	Cases n (%)	Controls n (%)	Unadjusted OR {95% CI}
Self-reported history of rubella			
No	88 (64.7)	184 (67.6)	1
Yes	32 (23.5)	55 (20.2)	1.23 {0.73—2.06}
Don't know	16 (11.8)	33 (12.1)	1.02 {0.54—1.92}
Self-reported age of rubella			
Did not have rubella	88 (73.3)	184 (78.0)	1
At age 0—5 years	10 (8.3)	9 (3.8)	2.01 {0.80—5.02}
At age 6—10 years	13 (10.8)	25 (10.6)	1.10 {0.53—2.31}
Above age 10 years	9 (7.5)	18 (7.6)	0.85 {0.35—2.08}
Serum IgG antibody analysis			
Antibodies to rubella			
Negative (<5.0 IU/ml)	4 (2.9)	5 (1.9)	
Equivocal (5.0—9.9 IU/ml)	1 (0.7)	6 (2.3)	1
Positive (≥10.0 IU/ml)	131 (96.3)	250 (95.8)	1.11 {0.36—3.44}
Antibody titers to rubella			
0—50.0 IU/ml	20 (14.7)	51 (19.5)	1
50.1—100.0 IU/ml	24 (17.6)	64 (24.5)	0.91 {0.44—1.90}
100.1—200.0 IU/ml	38 (27.9)	87 (33.3)	1.20 {0.60—2.38}
>200.0 IU/ml	54 (39.7)	59 (22.6)	2.43 {1.24—4.76}
Test for trend			p<0.01
Antibody titers to rubella (>100.0 vs ≤100.0 IU/ml)			1.76 (1.11—2.79}
Rubella infection and antibody titers to rubella			
No rubella + titers ≤ 100.0 IU/ml	29 (24.2)	77 (33.5)	1
Rubella + titers ≤ 100.0 IU/ml	6 (5.0)	22 (9.6)	0.58 {0.18—1.91}
No rubella + titers >100.0 IU/ml	59 (49.2)	101 (43.9)	1.56 {0.88—2.79}
Rubella + titers > 100.0 IU/ml	26 (21.7)	30 (13.0)	1.88 {0.93—3.83}
Test for trend			p=0.03

Notes
1. OR = odds ratio; {95% CI} = {95% confidence interval}
2. IU/ml = international units per ml

Herpes simplex-1

History of cold sores. Self-reported history of cold sores was not associated with MS.

Serum IgG antibodies to HSV-1. Being seropositive to HSV-1 IgG was negatively associated with MS (OR 0.60 {0.37—0.99}) (Table 6). There was no strong evidence for a dose-response relationship between the IgG antibody titers and MS (p=0.10)

History of cold sores and serum IgG antibodies to HSV-1. There was a strong correlation between having had cold sores and high HSV-1 titers (r=0.49, p<0.01 in controls; r=0.43, p<0.01 in cases). Assessing the combined association of a history of cold sores and antibody titers against HSV-1 (≥4.50 vs <4.50 units) did not show any significant associations (Table 6) and including an interaction term of self-reported history of cold sores and serum IgG antibody levels against HSV-1 in the model did not show to be significant (p=0.83).

Table 6. Unadjusted odds ratios for Multiple Sclerosis and history of cold sores and serum IgG antibodies to herpes simplex virus 1 (HSV-1)

	Cases n (%)	Controls n (%)	Unadjusted OR {95% CI}
Self-reported history of cold sores			
No	73 (53.7)	131 (48.2)	1
Yes	63 (46.3)	141 (51.8)	0.80 {0.53—1.21}
Self-reported age of cold sores			
Did not have cold sores	73 (54.1)	131 (48.2)	1
At age 0—10 years	17 (12.6)	37 (13.6)	0.83 {0.44—1.56}
At age 11-15 years	16 (11.0)	48 (17.6)	0.61{0.32—1.15}
At age 16-20 years	17 (12.6)	21 (7.7)	1.45 {0.71—2.99}
Above age 20 years	12 (8.8)	35 (12.9)	0.61 {0.30—1.24}
Test for trend			p=0.34
Serum IgG antibody analysis			
Antibodies to HSV-1			
Negative (<0.9 units)	37 (27.2)	48 (18.4)	
Equivocal (0.9—1.1 units)	3 (2.2)	3 (1.1)	1
Positive (>1.1 units)	96 (70.6)	210 (80.5)	0.59 {0.37—0.96}
Antibody titers to HSV-1			
0—1.49 units	41 (30.2)	58 (22.2)	1
1.50—4.49 units	13 (9.6)	26 (10.0)	0.72 {0.32—1.62}
≥4.50 units	82 (60.3)	177 (67.8)	0.67 {0.42—1.07}
Test for trend			p=0.10
Antibody titers to HSV-1 (≥4.50 vs <4.50 units)			0.72 {0.47—1.11}
Cold sores and antibody titers to HSV-1			
No cold sores + titers < 4.50 units	44 (32.4)	68 (26.1)	1
Cold sores + titers < 4.50 units	10 (7.4)	16 (6.1)	0.93 {0.37—2.32}
No cold sores + titers ≥ 4.50 units	29 (21.3)	56 (21.5)	0.81 {0.46—1.43}
Cold sores + titers ≥ 4.50 units	53 (39.0)	121 (46.4)	0.67 {0.41—1.11}
Test for trend			p=0.11

Notes
1. OR = odds ratio; {95% CI} = {95% confidence interval}

Herpes Simplex 2

Neither self-reported history of herpes genitalis, nor being seropositive to HSV-2 was associated with MS (Table 7).

Table 7. Unadjusted odds ratios for Multiple Sclerosis and history of herpes genitalis and serum IgG antibodies to herpes simplex virus 2 (HSV-2)

	Cases n (%)	Controls n (%)	Unadjusted OR {95% CI}
Self-reported history of herpes genitalis			
No	132 (97.1)	267 (98.2)	1
Yes	4 (2.9)	5 (1.8)	1.60 {0.43—5.96}
Serum IgG antibody analysis			
Antibodies to HSV-2			
Negative (<0.9 units)	120 (88.2)	234 (89.7)	
Equivocal (0.9—1.1 units)	0 (0)	1 (0.4)	1
Positive (>1.1 units)	16 (11.8)	26 (10.0)	1.11 {0.55—2.24}
Antibody titers to HSV-2			
0—0.05 units	26 (19.1)	63 (24.1)	1
0.06—0.15 units	43 (31.6)	73 (28.0)	1.47 {0.81—2.68}
0.16—0.25 units	29 (21.3)	56 (21.5)	1.24 {0.66—2.32}
≥0.25 units	38 (27.9)	69 (26.4)	1.27 {0.71—2.28}
Test for trend			p=0.59

Notes
1. OR = odds ratio; {95% CI} = {95% confidence interval}

Cytomegalovirus

A weak negative association was observed between being seropositive to the cytomegalovirus and MS (Table 8). There was a dose-response relationship with increasing antibody titers, and people with the highest level of antibodies had a significantly reduced risk.

Table 8. Unadjusted odds ratios for Multiple Sclerosis and serum IgG antibodies to cytomegalovirus.

	Cases n (%)	Controls n (%)	Unadjusted OR {95% CI}*
Antibodies to cytomegalovirus			
Negative (<15.0 AU/ml*)	55 (40.4)	92 (35.3)	1
Positive (≥15.0 units)	81 (59.6)	169 (64.8)	0.79 {0.50—1.23}
Antibody titers to cytomegalovirus			
0—3.0 AU/ml	44 (32.4)	61 (23.4)	1
3.00—75.0 AU/ml	23 (16.9)	48 (18.4)	0.62 {0.32—1.21}
75.1—150.0 AU/ml	25 (18.4)	46 (17.6)	0.72 {0.38—1.39}
≥150.0 AU/ml	44 (32.4)	106 (40.6)	0.53 {0.30—0.92}
Test for trend			p=0.04

Notes
1. OR = odds ratio; {95% CI} = {95% confidence interval}
2. AU/ml = Antibody Units per ml

Other infections

There was no association between self-reported chickenpox, measles, mumps, pertussis or impetigo and MS (Table 9).

Table 9. Unadjusted odds ratios for Multiple Sclerosis and self-reported history of chickenpox, measles, mumps, pertussis and impetigo.

	Cases n (%)	Controls n (%)	Unadjusted OR {95% CI}
Chickenpox			
No	18 (13.2)	49 (18.0)	1
Yes	108 (79.4)	202 (74.3)	1.43 {0.81—2.55}
Don't know	10 (7.4)	21 (7.7)	1.27 {0.51—3.15}
Age of chickenpox			
Did not have chickenpox	18 (14.5)	49 (19.8)	1
At age 0—5 years	19 (15.3)	43 (17.4)	1.07 {0.49—2.34}
At age 6—10 years	67 (54.0)	113 (45.7)	1.63 {0.87—3.07}
At age >10 years	20 (16.1)	42 (17.0)	1.17 {0.54—2.53}
Test for trend			p=0.31
Measles			
No	15 (11.0)	37 (13.7)	1
Yes	109 (80.2)	210 (77.5)	1.28 {0.68—2.42}
Don't know	12 (8.8)	24 (8.9)	1.24 {0.48—3.18}
Age of measles			
Did not have measles	15 (12.2)	37 (15.5)	1
At age 0—5 years	29 (23.6)	48 (20.1)	1.44 {0.68—3.05}
At age 6—10 years	72 (58.5)	135 (56.5)	1.35 {0.70—2.62}
At age >10 years	7 (5.7)	19 (7.9)	0.87 {0.30—2.57}
Test for trend			p=0.82
Mumps			
No	55 (40.7)	115 (42.3)	1
Yes	67 (49.6)	143 (52.6)	1.00 {0.64—1.57}
Don't know	13 (9.6)	14 (5.2)	1.97 {0.85—4.57}
Age of mumps			
Did not have mumps	55 (46.6)	115 (45.1)	1
At age 0—5 years	4 (6.4)	13 (5.1)	0.66 {0.21—2.12}
At age 6—10 years	39 (33.1)	86 (33.7)	1.01 {0.61—1.68}
At age 11—15 years	14 (11.9)	27 (10.6)	0.96 {0.47—1.95}
At age >15 years	6 (5.1)	14 (5.5)	1.00 {0.36—2.79}
Test for trend			p=0.99
Pertussis			
No	118 (86.8)	244 (89.7	1
Yes	13 (9.6)	20 (7.4)	1.32 {0.66—2.67}
Don't know	5 (6.7)	8 (2.9)	1.31 {0.42—4.03}
Age of pertussis			
Did not have pertussis	118 (90.1)	244 (92.4)	1
At age 0—5 years	7 (5.3)	12 (4.6)	1.17 {0.46—2.96}
At age >6 years	6 (4.6)	8 (3.0)	1.79 {0.57—5.59}
Test for trend			p=0.31

Table 9 continued at next page

Table 9 continued

	Cases n (%)	Controls n (%)	Unadjusted OR {95% CI}
Impetigo			
No	126 (92.6)	251 (92.6)	1
Yes	6 (4.4)	18 (6.6)	0.66 {0.26—1.68}
Don't know	4 (2.9)	2 (0.7)	6.61 {0.72—60.86}
Age of impetigo			
Did not have impetigo	126 (95.5)	37 (93.3)	1
At age 0—5 years	1 (0.8)	1 (0.4)	2.00 {0.13—32.00}
At age 6—10 years	2 (1.5)	12 (1.5)	0.31 {0.07—1.44}
At age >10 years	3 (2.3)	5 (1.9)	1.20 {0.29—5.02}

Notes
1. OR = odds ratio; {95% CI} = {95% confidence interval}

Infections by proxy recall

There was moderate agreement in recall between proxies and subjects for IM (κ=0.57) and the agreement was substantial when proxies and subjects that answered with "Don't know" were excluded (κ=0.65). As we saw before, IM recalled by subjects was positively associated with MS (OR 2.14 {1.26—3.62}). Using proxy recall, we also observed a significant positive association between IM and MS (Table 10). In fact, the association was even stronger. In addition, with subject recall, IM seemed only important when the subject had IM between the ages 11 and 20 years. With proxy recall, a high risk estimate was seen for all age groups although the number of subjects were insufficient to detect significant associations in three of the four age groups.

The agreement in recall between proxies and subjects of the other infections was only slight to fair. The kappa statistics for the different infections were: mumps, κ=0.40; cold sores, κ=0.37; chickenpox, κ=0.36; pertussis, κ=0.24; measles, κ=0.19; rubella, κ=0.17; impetigo, κ=0.14. As we saw before, with subject recall, we did not observe any significant associations between those self-reported infections and MS. The same was observed using proxy recall (Table 10).

Table 10. Unadjusted odds ratios for Multiple Sclerosis and proxy-reported history of infectious mononucleosis, rubella, cold sores, herpes genitalis, chickenpox, measles, mumps, mumps, pertussis and impetigo.

	Cases n (%)	Controls n (%)	Unadjusted OR {95% CI}
Infectious mononucleosis (IM)			
No	89 (78.1)	201 (88.2)	1
Yes	25 (21.9)	17 (7.5)	3.90 {1.79—8.51}
Don't know	0 (0)	10 (4.4)	
Age of infectious mononucleosis			
Did not have IM	89 (78.1)	201 (92.2)	1
At age 6—10 years	5 (4.4)	2 (0.9)	4.86 {0.90—26.31}
At age 11—15 years	5 (4.4)	2 (0.9)	4.86 {0.90—26.31
At age 16—20 years	11 (9.7)	8 (3.7)	3.74 {1.34—10.40}
Above age 20 years	4 (3.5)	5 (2.3)	2.61 {0.57—11.87}
Test for trend			p<0.01

Table 10 continued at next page

Table 10 continued

	Cases n (%)	Controls n (%)	Unadjusted OR {95% CI}
Rubella			
No	64 (56.1)	119 (52.4)	1
Yes	29 (25.4)	59 (26.0)	0.92 {0.51—1.66}
Don't know	21 (18.4)	49 (21.6)	0.70 {0.36—1.37}
Age of rubella			
Did not have rubella	64 (69.6)	119 (67.2)	1
At age 0—5 years	8 (8.7)	20 (11.3)	0.78 {0.28—2.20}
At age 6—10 years	17 (18.5)	28 (15.8)	1.11 {0.49—2.53}
Above age 10 years	3 (3.3)	10 (5.7)	0.55 {0.14—2.20}
Test for trend			p=0.64
Cold sores			
No	78 (68.4)	144 (63.2)	1
Yes	31 (27.2)	61 (26.8)	0.93 {0.54—1.61}
Don't know	5 (4.4)	23 (10.1)	0.34 {0.11—1.04}
Age of cold sores			
Did not have cold sores	78 (71.6)	144 (72.0)	1
At age 0—10 years	11 (10.1)	22 (11.0)	0.88 {0.39—1.98}
At age 11-15 years	11 (10.1)	18 (9.0)	1.22{0.53—2.80}
At age 16-20 years	6 (5.5)	10 (5.0)	0.97 {0.32—2.93}
Above age 20 years	3 (2.8)	6 (3.0)	1.50 {0.25—8.98}
Test for trend			p=0.69
Herpes genitalis			
No	107 (93.9)	208 (91.2)	1
Yes	1 (0.9)	2 (0.9)	0.78 {0.07—8.88}
Don't know	6 (5.3)	18 (7.9)	0.76 {0.24—1.81}
Chickenpox			
No	14 (12.3)	44 (19.3)	1
Yes	88 (77.2)	168 (73.7)	1.55 {0.80—3.00}
Don't know	12 (10.5)	16 (7.0)	2.95 {1.03—8.44}
Age of chickenpox			
Did not have chickenpox	14 (13.9)	44 (21.2)	1
At age 0—5 years	24 (23.8)	40 (19.2)	1.62 {0.72—3.63}
At age 6—10 years	50 (49.5)	101 (48.6)	1.45 {0.71—2.95}
At age >10 years	13 (12.9)	23 (11.1)	1.99 {0.74—5.38}
Test for trend			p=0.24
Measles			
No	15 (13.2)	25 (11.0)	1
Yes	87 (76.3)	183 (80.2)	0.81 {0.41—1.58}
Don't know	12 (10.5)	24 (8.8)	1.10 {0.41—2.95}
Age of measles			
Did not have measles	15 (14.9)	25 (12.5)	1
At age 0—5 years	38 (37.6)	54 (27.0)	1.24 {0.56—2.71}
At age 6—10 years	46 (45.5)	113 (56.5)	0.70 {0.33—1.49}
At age >10 years	2 (2.0)	8 (4.0)	0.43 {0.07—2.51}
Test for trend			p=0.13

Table 10 continued

	Cases n (%)	Controls n (%)	Unadjusted OR {95% CI}
Mumps			
No	44 (38.6)	88 (38.6)	1
Yes	61 (53.5)	113 (49.6)	0.98 {0.58—1.64}
Don't know	9 (7.9)	27 (11.8)	0.62 {0.25—1.52}
Age of mumps			
Did not have mumps	44 (44.0)	88 (44.7)	1
At age 0—5 years	10 (10.0)	15 (7.6)	1.34 {0.53—3.34}
At age 6—10 years	33 (33.0)	71 (36.0)	0.81 {0.43—1.55}
At age 11—15 years	10 (10.0)	17 (8.6)	1.21 {0.47—3.10}
At age >15 years	3 (3.0)	6 (3.1)	1.45 {0.20—10.69}
Test for trend			p=0.99
Pertussis			
No	97 (86.4)	197 (86.4)	1
Yes	13 (11.4)	20 (8.8)	1.50 {0.62—3.64}
Don't know	4 (3.5)	11 (4.8)	0.99 {0.28—3.52}
Age of pertussis			
Did not have pertussis	97 (88.2)	197 (91.6)	1
At age 0—5 years	7 (6.4)	9 (4.2)	1.49 {0.49—4.55}
At age >6 years	6 (5.5)	9 (4.2)	1.57 {0.48—5.17}
Test for trend			p=0.38
Impetigo			
No	102 (89.5)	193 (84.7)	1
Yes	9 (7.9)	17 (7.5)	0.92 {0.38—2.24}
Don't know	3 (2.6)	18 (7.9)	0.33 {0.09—1.13}
Age of impetigo			
Did not have impetigo	102 (91.9)	193 (91.9)	1
At age 0—5 years	2 (1.8)	1 (0.5)	4.0 {0.36—44.1}
At age 6—10 years	7 (6.3)	14 (6.7)	0.70 {0.26—1.87}
At age >10 years	0 (0)	2 (1.0)	
Test for trend			p=0.64

Notes
1. OR = odds ratio; {95% CI} = {95% confidence interval}

Conclusions regarding specific viral infections

We observed a strong positive association between a history of IM between 11 and 20 years and MS. In addition, serum IgG levels against EBNA and VCA both appeared to be positively associated with MS in a dose-response manner, with EBNA showing a stronger association than VCA. The combined effect of having high antibody titers and a history of teenage IM was different for VCA and EBNA. For VCA, high IgG levels against VCA or having had teenage IM did not appear to be associated with MS in absence of the other, but having both was positively associated with MS. In contrast, for EBNA, strong associations were found for high IgG levels against EBNA or having had teenage IM in absence of the other, while the combined effect did not appear to result in a high additional risk.

For rubella we found some evidence of an increasing positive association with rising serum IgG levels, but being seropositive for rubella and self-reported history of rubella were not

associated with MS. For HSV-1, being seropositive for HSV-1 was negatively associated with MS, but there was no dose-response relationship and a history of cold sores was not associated with MS. For cytomegalovirus, the evidence was more consistent with observing a mild negative association for seropositivity, a dose-response relationship with increasing antibody titers, and people with the highest level of antibodies having a significantly reduced risk. No association was found between serum IgG for HSV-2 or self-reported herpes genitalis and MS. In addition, no association was seen between history of chickenpox, measles, mumps, pertussis or impetigo and MS, neither by self-report nor proxy-report.

We will now restrict our attention to the relationship between younger sibling number and MS, and between EBV and MS. For EBV, we will focus on the history of teenage IM, IgG antibody titers to EBNA, and IgG antibody titers to VCA. A full multivariate analysis will not be conducted for rubella, HSV-1 and cytomegalovirus.

9.4 3 Effect of confounders and disease-related factors on the relationship between younger sibling number and MS, and Epstein-Barr virus and MS

As discussed in chapter 8, other factors positively associated with MS were low melanin density at the upper inner arm (p=0.06), smoking (p=0.05), high education (p=0.07), exposure to fibre glass and resin prior to age 17 (p=0.02), exposure to smoke fumes prior to age 17 (p=0.05), exposure to smoke fumes between age 17 and the age of diagnosis (p=0.03), and owning a cat prior to MS onset (p=0.02). Factors negatively associated with MS were higher levels of summer sun exposure during weekends and holidays at age 6—15 years (p<0.01), higher levels of actinic damage (p<0.01), and having had an immunisation for rubella in early life (p<0.01).

Younger sibling number and MS

Firstly, we assessed, among controls, whether the number of younger siblings was associated with serum IgG antibody titers to specific viruses and self-reported history of infections. The number of younger siblings was not associated with IgG antibody titers to EBNA, VCA, rubella, CMV and HSV-2 but was associated with increased levels of antibodies to HSV-1 (r=0.23, p<0.01). The number of younger siblings was also associated with self-reported cold sores (r=0.18, p<0.01), pertussis (r=0.17, p<0.01) and mumps (r=0.16, p=0.01). Some mild associations were seen for self-reported rubella (r=0.11, p=0.07), teenage IM (r=-0.10, p=0.12), and herpes genitalis (r=-0.09, p=0.14), but no associations were seen for self-reported chickenpox and measles.

Although there was a strong correlation among controls between younger sibling number and IgG antibody titers against HSV-1, adjusting for IgG antibody titers against HSV-1 did not alter the risk estimates of younger sibling number. Also, adjustment for IgG antibody titers to rubella, CMV and HSV-2 did not alter the results, as well as adjusting for self-reported history of the infections mentioned above. Adjusting for IgG antibody titers to EBNA and VCA did alter the results, even though we did not observe an association among controls between those titers and younger sibling number. After further analysis we decided not to treat EBV antibody titers as a routine confounder, and thus not to adjust for it, because it potentially mediates part of the effect between younger sibling number and MS and also seemed to modify the relationship between younger sibling number and MS.

Other factors associated with MS mentioned previously such as smoking, sun exposure and education level did also not alter the association between younger sibling number and MS. Thus, the effect of younger sibling number did not seem to be affected by confounding factors.

Epstein-Barr virus and MS

We estimated the effect of a history of teenage IM, IgG antibody titers to EBNA, and IgG antibody titers to VCA, taking into account other factors that related to MS, including other specific infections. After examining the effects of all possible confounders, using the strategy discussed in chapter 5, a number of factors were considered to confound at least one of the three associations between EBV and MS: summer sun exposure during weekends and holidays at age 6-15 years, melanin density at the upper inner arm, whether ever smoked prior to the age of diagnosis, exposure to smoke fumes and IgG antibody titers to rubella. Adjustment for those factors decreased the odds ratio for teenage IM, but the association remained significant. Also, the association between IgG antibody titers to VCA decreased somewhat, while the association between IgG antibody titers to EBNA and MS became stronger.

Table 11. Unadjusted and adjusted odds ratios for Multiple Sclerosis and history of teenage infectious mononucleosis and serum IgG antibodies to Epstein-Barr virus.

	Cases n (%)	Controls n (%)	Unadjusted OR {95% CI}	Adjusted OR {95% CI}
History of teenage IM				
No	108 (81.2)	251 (93.3)	1	1
Yes	25 (18.8)	18 (6.7)	3.88 {1.88−7.99}	2.49 {1.04−5.98}
Serum IgG antibody titers to EBNA				
0−2.50 units	26 (19.1)	150 (57.5)	1	1
2.51−3.00 units	31 (22.8)	52 (19.9)	3.49 {1.82−6.70}	4.35 {1.93−9.78}
3.01−4.00 units	79 (58.1)	59 (22.6)	7.47 {4.16−13.41}	8.85 {4.26−18.41}
Test for trend			p<0.01	p<0.01
Antibody titers to EBNA (>2.50 vs ≤2.50 units)			5.69 {3.33−9.71}	6.92 {3.52−13.60}
Serum IgG antibody titers to VCA				
0−2.00 units	21 (15.4)	70 (26.8)	1	1
2.01−2.50 units	23 (16.9)	41 (15.7)	1.79 {0.91−3.54}	1.33 {0.61−2.88}
2.51−3.00 units	38 (27.9)	78 (29.9)	1.63 {0.87−3.08}	1.55 {0.71−3.43}
3.01−4.00 units	54 (39.7)	72 (27.6)	2.58 {1.37−4.85}	2.51 {1.18−5.35}
Test for trend			p<0.01	p=0.02
Antibody titers to VCA (>2.00 vs ≤2.00 units)			1.97 {1.15−3.40}	1.76 {0.93−3.30}

Notes
1. OR = odds ratio; {95% CI} = {95% confidence interval}
2. IM = infectious mononucleosis; EBNA = Epstein Barr virus Nuclear Antigen; VCA = Viral Capsid Antigen
3. Adjusted for sun exposure at age 6-15 years, melanin density at the upper inner arm, whether ever smoked prior to the age of diagnosis, exposure to smoke fumes and IgG antibody titers to rubella.

Serum samples of cases were taken after disease onset. Immunomodulatory treatment could have potentially affected the IgG titers against EBV and affected the risk estimates. However, among cases, there was no correlation between using immunomodulatory treatment and IgG levels against EBNA (r=0.05, p=0.59) or VCA (r=0.05, p=0.55) and mean (SD) IgG antibody titers were similar for cases that were treated (EBNA, 3.01 (0.56); VCA, 2.79 (0.59)) and untreated (EBNA, 2.95 (0.64); VCA, 2.63 (0.76)). In addition, the relative risk estimates for IgG titers against EBNA and VCA and MS did not differ by treatment status (test for interaction: EBNA, p=0.90; VCA, p=0.85). Thus, the results for IgG antibody titers against EBNA and VCA did not differ by immunomodulatory treatment status of the cases.

Similarly, duration of disease was not correlated with IgG levels against EBNA (r=-0.04 after adjustment for age) and VCA (r=0.04 after adjustment for age) among cases and the risk

estimates for EBNA and VCA did not differ by duration of disease (test for interaction: EBNA, p=0.26; VCA, p=0.31).

9.4.4 Do the effects of younger sibling number and Epstein-Barr virus operate independently from the effect of sun exposure?

Younger sibling number

We assessed whether the effect of younger sibling number operated independently from the effect of sun exposure (summer sun exposure during weekends and holidays at age 6-15 years, actinic damage). Compared to univariate analysis, including both younger sibling number and sun exposure at age 6-15 years, or younger sibling number and actinic damage in the same model as linear terms left the estimated effects of all factors almost unchanged, suggesting that the protective effect of younger sibling number and sun exposure are not confounded by each other. Although the effect of having younger siblings appeared slightly stronger among those who had higher (≥ 2-3 hours a day on average) levels of sun exposure in summer on weekends and holidays over the age span of 6-15 years (OR 0.31 {0.15—0.65}) compared to those who had lower levels of sun exposure (OR 0.56 {0.18—1.72}), this difference was not significant (test for interaction: p=0.52). There was also no difference in the effect of having younger siblings for different levels of actinic damage. Thus, the protective effect of younger sibling number seems to operate independently from the protective effect of sun exposure.

Epstein-Barr virus

Similarly, we assessed whether the effect of EBV (self-reported history of teenage IM, IgG antibody titers to EBNA, IgG antibody titers to VCA) operated independently from the effect of sun exposure (summer sun exposure during weekends and holidays at age 6-15 years, actinic damage). Again, for all three EBV measures, the estimated effects were left mostly unchanged when including an EBV measure and sun exposure measure compared to univariate analysis. Where the change in coefficient was more than 10% (for example for summer sun exposure during weekends and holidays at age 6-15 years and IgG antibody titers to VCA), the magnitude of the effect became stronger rather than weaker. In addition, no significant interactions were found between EBV measures and sun exposure measures. This suggests that the effects of EBV operated independently from the protective effect of sun exposure.

9.4.5 Effect of younger sibling number and Epstein-Barr virus on age at onset among cases

Younger sibling number

We assessed whether the number of younger siblings influenced the age of onset among cases by modelling time elapsed from birth to onset of first symptoms using proportional hazard regression. Compared to not having any younger siblings, having one, two, or three or more younger siblings was associated with a hazard ratio of 0.97 {0.64—1.47}, 0.68 {0.40—1.15} and 0.61 {0.36—1.05}, respectively (test for trend, p=0.04). Although none of the categories were significantly associated, probably due to the limited number of cases, it seems that having an increasing number of younger siblings delays the onset of MS in a dose-response manner.

Epstein-Barr virus

There was no evidence that teenage IM (p=0.63) or increasing IgG titers against EBNA (p=0.84 for linear term) were associated with earlier onset of disease, but there was some association between increasing IgG titers against VCA and a later onset of MS (p=0.07 for the linear term), although none of the categories were significantly associated (compared to having VCA IgG levels of 0-2 units, having 2.01-2.50 units, 2.51-3.00 units, or 3.01-4.00 units was associated with a hazard ratio of 1.03 {0.57—1.87}, 1.23 {0.72—2.12}, 1.51 {0.90—2.53}, respectively).

9.4.6 Population attributable fraction

Having younger siblings

We calculated the proportion by which the incidence rate of MS would be reduced if we were able to eliminate not having younger siblings. In our cases, 43.3% did not have a younger sibling and the unadjusted odds ratio for not having a younger sibling is 2.13 (1/0.47). By using the formula provided in the methods, we can calculate that the incidence rate of MS would be reduced by 23% if the whole population had younger siblings.

Epstein-Barr virus

We calculated the proportion by which the incidence rate of MS would be reduced if we were able to eliminate (1) teenage IM, (2) high IgG levels against EBNA (>2.50 units), and (3) high IgG levels against VCA (>2.00 units). In our cases, 18.8% had teenage IM, 80.9% had high IgG levels against EBNA, and 84.6% had high IgG levels against VCA. The adjusted odds ratios were 2.49, 6.92 and 1.76, respectively. The incidence rate of MS would be reduced by: 11% if we were able to eliminate teenage IM, 69% if we were able to eliminate high IgG levels against EBNA and 37% if we were able to eliminate high IgG levels against VCA.

9.5 DISCUSSION

9.5.1 Sibship structure

Our results on sibship structure showed that having younger siblings was strongly associated with a reduced risk of MS. The magnitude of the protective effect increased with increasing sibling number, and was stronger when the interval between the subject and the sibling was smaller. For example, a strong inverse association with MS was observed when the interval between the subject and the nearest younger sibling was less than two years, while the association disappeared when the interval was longer than six years. This seems to suggest that most of sibling effect is mediated in the first years of life.

As discussed in chapter 8, the case sample appeared similar to other populations with MS of North European ancestry with regard to disease-related features such as the type of MS, age at diagnosis and sex ratio.[62-64] Also, participation rates for cases and controls were high, reducing non-response bias, but it is conceivable that some selection bias may have occurred. Measurement error in sibling information including the number of siblings and their date of birth was thought to be low and this information seems not prone to recall bias. The results of the effect of younger siblings did not appear to be influenced by confounding factors and the effect of younger siblings operated independently of the effect of sun exposure that we discussed in the previous chapter. Also, among cases, an increasing number of younger siblings seemed to delay the onset of MS in cases, strengthening the case that the association between younger sibling number and MS might be causal.

Our results are in accordance with another case-control study which showed that the degree of social mixing was associated with a reduced risk of childhood type 1 diabetes. Attendance to daycare centres for infants below the age of one year was associated with an odds ratio of 0.71 {0.51—1.00}, and an infectious exposure index (a composite of attendance at daycare under one year of age, any reported infection before one year of age and the number of other children in the house at birth) was negatively associated in a dose-response manner (index of none as reference: OR 0.75 {0.35—1.61} for low index; OR 0.55 {0.26—1.19} for medium index; OR 0.54 {0.23—1.23} for high index; test for trend, p=0.05).[22] They state that the use of daycare attendance is a good proxy for infectious exposure in this group, particularly as it may 'mark' the presence of asymptomatic infection. In our sample of adults, where daycare facilities were not likely to be used as frequently as nowadays, siblings seem a good proxy for infectious exposure.

Birth order did not seem to be an important factor in our study, which is in accordance with the results of two case-control studies.[34, 35] Two other case-control studies[25, 36] found a reduction in risk for having a birth order higher than three (equivalent to three or more older siblings) although the confidence interval was wide in the study from Visscher et al.[25] As far as we are aware, only one study to date has been conducted where authors performed a more detailed analysis on sibship structure and MS.[37] This nested case-control study (301 cases, 1416 controls) showed no association for first-born or only children (OR 1.2 {0.9—1.6}). First-born or only children are children who do not have any older siblings. Their variable is the inverse of our variable of having older children where we also did not find an association. In contrast to our data, they found a weak inverse trend between number of older siblings and risk of MS (OR 0.9 {0.7—1.1} for one or two older siblings; OR 0.9 {0.6—1.4} for three or four older siblings; OR 0.6 {0.3—1.4} for five or more older siblings, compared with first-born and only children). The authors did not report directly on younger siblings, but it was observed that among families with four or more children, being the first born (equivalent to having three or more younger siblings, but no older siblings) was associated with an increased risk (OR 2.1

{1.2—3.5}), while this was not the case in families with two or three children (equivalent to having one or two younger siblings, but no older siblings) (OR=0.8 {0.5—1.2}). This suggests an increased risk for having more than three younger siblings compared to having one or two younger siblings, which is opposite to the results that we found. Half-siblings did not seem to be included in their analysis, but the effect of including half-siblings was probably small, because in our sample only 4% were half-siblings. The case sample included people with definite MS (262/301) and probable MS (84/301), which could have introduced some bias. On the other hand, their study was a nested case-control study, a design less prone to selection bias, because both cases and controls arose from a cohort. In contrast to our analysis, they did not examine interbirth interval.

The fact that younger but not older siblings mediated a protective effect in our study and that the effect was strongest if the interval to the next sibling was small might indicate that not primary infections in the first year of life of the subject are of critical importance but rather that repeated stimulation of the immune system through early life infections of the younger siblings are important. Re-exposure to infections can lead to immune boosting in seropositive individuals.[23, 65] A recent case-control study found that contact with children protected latently infected adults against herpes zoster, a clinical syndrome caused by varicella zoster virus reactivation after cell-mediated immunity has waned.[66] Immune boosting after re-exposure via contact with children is thought to underlie this protective effect.[66] Perhaps more importantly, repeated viral antigen challenge leads to immune refinement. For example, recurrent infection induces genetic mutational change in B cell germ centres, leading to higher affinity B cells that are more specific for the viral antigen.[24]

A number of alternative explanations have been suggested in the literature for the protective effect of infections in autoimmune diseases, but the importance of the timing of those infections has not been incorporated in those mechanisms. Infections could stimulate the production of regulatory cells and induce a systemic suppression. The production of IL-10 has been shown to be increased in a number of infectious diseases.[67] Interleukin-10, which is produced by Th_2 cells, monocytes, and macrophages, slows the progression of EAE[67] and in adults, it has been observed that members of families with low IL-10 and high TNF production had a fourfold increased risk of developing typical MS compared with members of families with high IL-10 and low TNF production.[68] Infections could also be involved in antigenic competition, where the immune response to an antigen is decreased by a concomitant immune response against an unrelated antigen.[69, 70] The transfer of maternal antiviral antibodies of newborns could also play a role. Zinkernagel suggested that decreased exposure of women to particular viruses before pregnancy may subsequently reduce the degree of protection against these viruses afforded to their newborns.[71] When exposed to these viruses, an immune response could be provoked that could ultimately lead to an autoimmune disease. Toll-like receptors (TLRs), which are receptors for bacterial components, could be involved, because binding can stimulate mononuclear cells to produce cytokines, some of which in turn could down-regulate autoimmune responses.[72] Lastly, a large variety of parasitic infections have been associated with generalised immunosuppression, an effect that is not directly related to the anti-parasitic immune response.[73]

If it were true that early life infections of the younger siblings are important, many viruses could potentially contribute to this effect. For example, infants attract a large number of respiratory infections (pre-school children on average 6-8 per years) and are often infected by enteroviruses.[74] Another group of viruses, the human herpes viruses might potentially be more important because of their ability to interact with each other, where HHV-6 variant A, for example, can increase the replication of EBV.[51] HHV-6 infection is generally acquired at a very early age.[75] More than 70% of babies in the United Kingdom are infected by the age of two years, a rate that is similar to the rate in adults.[75] VZV and EBV are acquired throughout

childhood and adolescence, with about 60-70% infected by the age of 10 years.[75] HSV-1 and CMV are also common infections during childhood,[75] with CMV commonly transmitted before the age of one year by mothers via breast milk.[76]

9.5.2 Epstein-Barr virus

We showed that both history of IM and high IgG antibody titers to EBNA and VCA were related to an increased risk of MS. The association was stronger for antibodies to EBNA compared to VCA, something which has been found in other studies.[46, 47] IgG antibodies to VCA emerge during the late incubation period or in the course of the acute phase of IM, while IgG antibodies to EBNA emerge weeks or months after onset. Both persist indefinitely. The simultaneous elevation of titers to VCA and EBNA has been suggested to indicate a more severe or more recent primary infection among people who developed MS compared to people who remained healthy.[46, 47]

The associations between IM and MS were similar regardless of whether recalled by the subject or the proxy. In addition, the test-retest reliability of self-reported IM was high and not different for cases and controls. Among cases, IgG antibody titres to EBV were not affected by disease duration or the use of immunomodulatory treatment, which is in line with results from a prospective study that showed that IgG antibody titers to EBV in the same cases were similar for samples collected prior to MS onset and after MS onset.[47] Adjustment for confounders did not eliminate the effects, and the effects appeared to operate independent of the effect of sun exposure. Among cases, IgG antibody titers to VCA were associated with a slightly later onset of MS, but a similar effect was not observed for teenage IM and IgG antibody titers to EBNA.

We found that having had IM was especially important when people had the infection between age 11 and 20 years. This is in accordance with a nested case-control study that found no increased risk for those who had IM prior to age 10 years, but an increased risk for those with IM after the age 10 years (OR 1.9 {0.8—4.2} for IM at age 11-15 years; OR 2.2 {1.5—3.2} for IM after age 15 years).[37] Also, Martyn et al. found a higher risk in a case-control study for those who had IM prior to age 17 years (the median age of IM) and were seropositive against VCA (OR 7.0 {1.7—37.0}), compared to the total group that had IM and was seropositive against VCA (OR 2.9 {1.1—7.2}).[43] For some other infections, timing has also been shown to be important. A meta-analysis showed that people with MS tended to have different childhood infections at a later age.[52] A recent well-conducted nested case-control study found, for example, an increased risk for measles (OR 2.8 {0.8—9.1}) and mumps (OR 2.3 {1.2—4.3}) if the infections occurred after the age of 15 years.[37] We did not observe such an effect for any of the other self-reported infections.

We found a protective effect for younger sibling number and an increased risk for EBV. The question is whether those two factors could be related to each other. If there is a link, then the biological plausibility of the protective effect for younger sibling number would increase. Controls with more younger siblings had slightly less often teenage IM. No correlation was found between younger sibling number and IgG antibody titers to EBNA or VCA. However, adjusting younger sibling number for IgG antibody titers to EBNA changed the risk estimates, indicating some link between younger sibling number and EBV antibodies. An in-depth analysis of the relationship between younger sibling number and EBV was beyond the scope of this thesis, but it is conceivable that having younger siblings reduces the risk estimates for IM and high antibody titers to EBV. In addition, we did not perform a full multivariate analysis of cytomegalovirus, HSV-1 and rubella, to see whether the strength of the mild associations that we observed with MS increased or decreased after adjustment for other factors.

9.5.3 Conclusions

We provided novel results regarding the relationship between sibship structure and MS. Having younger siblings, but not older, was strongly associated with a reduced risk of MS in a dose response manner, and the effect was strongest when the interval to the next sibling was small. This might indicate that not primary infections in the first year of life of the subject are of critical importance, but rather that repeated stimulation of the immune system through early life infections of the younger siblings may be important. The positive associations between IM, high IgG antibody titers to EBNA and high IgG antibody titers to VCA and MS, that have been observed in other studies, were also seen in our study. IM seemed especially important when subjects had the infection between the age of 11 and 20 years. Further work might reveal whether there is a link between the negative association between having younger siblings and MS and the positive association between EBV and MS.

9.6 SUMMARY

A delay in infections might be an important risk factor for MS, because people with MS tended to have a number of childhood infections at a later age compared to controls. A detailed assessment of sibship structure as a marker of timing of infections has been successfully used in epidemiological work on atopic disease, but in MS the attention has been mostly limited to the assessment of birth order, a measure that only takes number of older siblings into account. The only specific virus that has been consistently associated with MS is the Epstein-Barr virus. In this chapter we used the data of the Tasmanian MS case-control study to conduct a detailed examination of sibship structure and MS and to investigate the association between history of specific infections and MS using subject recall, proxy recall and serum antibody analysis. The results show that having younger siblings, but not older, was strongly associated with a reduced risk of MS (odds ratios 0.68 {0.39—1.18}, 0.37 {0.20—0.70}, 0.35 {0.18—0.66} for one younger sibling, two younger siblings, and three or more younger siblings, respectively, compared to no younger siblings) in a dose response manner (test for trend, $p<0.01$), and the effect was strongest when the interval to the next sibling was small (odds ratio 0.33 {0.19—0.59} for an interval between zero and two years). This might indicate that not primary infections in the first year of life of the subject are of critical importance, but rather that repeated stimulation of the immune system through early life infections of the younger siblings are important. Among cases, a temporal pattern was observed in that younger sibling number was associated with a later onset of MS. We found positive associations between infectious mononucleosis (unadjusted odds ratio 2.14 {1.26—3.62}), high (>2.50 units) IgG antibody titers to Epstein-Barr nuclear antigen (adjusted odds ratio 6.92 {3.52—13.60} and high (>2.00 units) IgG antibody titers to Epstein-Barr virus capsid antigen (adjusted odds ratio 1.76 {0.93—3.30} and MS, associations that have also been observed in other studies. Infectious mononucleosis seemed especially important when the subjects had the infection between the age of 11 and 20 years (adjusted odds ratio 2.49 {1.04—5.98}). Further work is required to confirm the apparent protective effect of younger sibling number and the underlying biological mechanism.

9.7 POSTSCRIPT

The more detailed analysis of sibship structure in relation to MS has provided some novel results. In the last chapter we will examine why there is an excess of women that have MS. We will not only focus on female specific factors, but unlike other studies also make comparisons between females and males in the prevalence and strength of MS risk factors or protective factors that were observed in this thesis.

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Chapter 10

The female excess in Multiple Sclerosis:

(i) A comparison of prevalence and strength of MS risk factors between females and males; (ii) female specific factors.

10.1 PREFACE

Chapter 8 and 9 investigated the first two objectives of the Tasmanian MS case-control study: to examine whether (1) high past sun exposure was related to a decreased risk of MS, and (2) sibship structure and past infections were related to MS.

The third objective was to examine whether other factors were associated with MS. Chapter 8 briefly examined other factors that were associated with MS such as tobacco smoking prior to the age at onset, education level, immunisation against rubella, exposure to fibre glass or resin prior to age 17, exposure to smoke fumes prior to age 17, and exposure to smoke fumes between age 17 and the age at onset and having cats prior to the age at onset. One factor we did not report there, as it was only conducted on a subgroup of the total sample, was whether having had children prior to MS onset was associated with MS among women.

The sex imbalance in MS onset is a striking epidemiological feature of MS, which we will further explore in this chapter. Although we can only show new results of our case-control study on risk of onset of MS in this chapter, for completeness we will also discuss the literature on progression of MS in relation to this topic.

10.2 INTRODUCTION

10.2.1 Sex difference in MS

Many autoimmune diseases, but not all,[1] have been shown to occur more often in women than in men, with female to male ratios in the order of 2:1 for MS, 3:1 for rheumatoid arthritis and even higher ratios for diseases such as Hashimoto's thyroiditis, Grave's disease and Sjögren's syndrome.[2] In addition, the sex ratio in MS among incident cases differs by age. It is highest during the reproductive years and decreases thereafter.[3, 4] For example, among 637 incident MS cases diagnosed between 1968 and 1997 in Sassari, Sardinia, the female to male ratio was 3.8:1 for cases diagnosed before age 20 years, 2.5:1 for cases diagnosed between 20 and 29 years, 2.2:1 for cases diagnosed between 30 and 39 years and 1.8:1 for cases diagnosed after age 39 years.[4] Also, a significantly higher female to male ratio of 3:1 has been reported in Canada among children and adolescents with an MS onset prior to age 16 years compared to a ratio of 2:1 for an onset after the age of 16 years ($p<0.01$).[5] A slightly higher female to male ratio was also observed in an Italian population among children and adolescents with an MS onset prior to age 16 years (ratio 2.2:1) compared to people with an onset after the age of 16 years (ratio 1.6:1) ($p=0.12$).[6] The question is what might be causing this difference in onset between females and males. Epidemiological studies have been conducted, predominantly in women, to examine the effects of different stages in reproductive life on disease risk and progression and to examine the effects of medications such as oral

contraceptives and hormone replacement therapy on MS risk and progression. We will review the available literature both regarding MS risk and MS progression.

10.2.2 Effect of puberty development on MS risk

The Sardinian study mentioned previously also showed that among children with an onset prior to age 12 years, the female to male ratio was 0.8:1, while the ratio increased to 3.0:1 for children with an onset between age 12 and 16 years.[6] This might suggest that hormonal changes related to puberty play a role in triggering MS onset, because puberty is associated with an increase in sex hormones including testosterone in males and estrogen in females. Only two studies have examined the effect of puberty development on MS risk. A case-control study conducted in the United States found that female cases had a lower mean age of menarche compared to female controls (12.3 versus 12.7, $p<0.01$)[7] but no difference was found in three other case-control studies.[8-10]

10.2.3 Effect of parity on MS risk

A number of studies have assessed the effect of parity (number of pregnancies beyond a certain point of gestation) or having had children prior to the age at onset on MS risk.[7, 10-14] Runmarker and Anderson[14] used an incident cohort of MS patients (1950-1964) and found that women with MS were more often childless prior to MS onset (74 childless women out of 153 women with a diagnosis of definite or probable MS) compared to what was expected (50.9/153) based on the general population ($p<0.01$). Two case-control studies conducted in 1961 and 1983 showed no difference between cases and controls for having a child[7] or having been pregnant.[10] Three prospective cohort studies have now been conducted that examined the effect of parity. Two found no effect of parity.[11, 12] The study reported by Hernan et al.[11] included 238,371 women at baseline of whom 315 developed MS during follow-up. Compared to a parity status of zero, they found rate ratios {95% confidence interval} of 0.8 {0.5—1.3}, 0.8 {0.6—1.2}, 0.9 {0.6—1.4}, 1.1 {0.6—1.7} for a parity of one, two, three, or four or more, respectively.[11] The study from Thorogood and Hannaford[12] included 46,000 women of whom 114 developed MS. They found rate ratios of 1.2 {0.5—2.9}, 1.1 {0.5—2.4} for a parity of one, and two or more, respectively.[12] One prospective study (17,032 women, 63 developed MS) found a protective effect for having had three or more pregnancies compared to none (rate ratio 0.4 {0.2—1.4}), but the confidence interval was wide due to the low numbers and no dose-response trend was observed.[13] Having children is not only accompanied by endocrine effects, but also by lifestyle changes during and after the pregnancy. None of the described studies have assessed these effects.

10.2.4 Effect of the use of oral contraceptives on MS risk

Three cohort studies examined the effect of use of oral contraceptives on the incidence of MS in women, but they found little effect.[11-13] The studies by Hernan et al.,[11] Thorogood and Hannaford,[12] and Villard-Mackintosh and Vessey,[13] found a rate ratio of 1.1 {0.9—1.5}, 1.3 {0.9—2.0} and 0.8 {0.5—1.4}, respectively, for ever users of oral contraceptives. The study of Villard-Mckintosh and Vessey used women with other types of contraceptives (diaphragm, intrauterine devices) as their reference group, which might have influenced their results.[13] The other two studies[11, 12] used women that never took oral contraceptives as their reference group. These two studies also found no effect for current users of oral contraceptives (Hernan et al.: 1.0 {0.6—1.6}; Thorogood and Hannaford: 1.2 {0.7—2.0}).

10.2.5 Effect of pregnancy on MS progression

Although there is not much evidence that pregnancies or having had children influenced the risk of MS onset, there is good evidence that being pregnant while having MS influences the disease activity. The relapse rate has been shown to be lowest in the third trimester of pregnancy in women that have relapsing remitting MS,[15-17] while the rate is higher during the early postpartum period.[15-21] In a well designed prospective study conducted by Confavreux et al.,[15] 254 women were followed during their pregnancies and for up to 12 months after delivery. They reported a mean relapse rate $\pm$ SD of: (a) 0.7 ± 0.9 per women per year in the year before pregnancy, (b) 0.5 ± 1.3 ($p=0.03$ for the comparison with the rate before pregnancy), 0.6 ± 1.6 ($p=0.17$) and 0.2 ± 1.0 ($p<0.001$) during the first, second and third trimester, respectively, and (c) 1.2 ± 2.0 ($p<0.001$) during the first three months postpartum. The rate returned to the pre-pregnancy rate after three month postpartum.[15]

Several studies have evaluated the progression before and after pregnancies using outcome measures such as the total number of relapses[15-18] and the change in Expanded Disability Status Scale (EDSS),[15, 18] but found no overall effect. As discussed in chapter 2, the EDSS has been criticised for its lack of sensitivity, which could explain the null results in studies where the EDSS was used as an outcome factor. More recently developed MRI measures might improve the measurement of MS progression. Others compared the progression of MS in women who had pregnancies after onset of MS with those with pregnancies before onset of MS and/or with no pregnancies at all.[20-25] Some found no difference,[20-22, 24, 25] but Verdru et al.[23] reported, in a cohort of women with MS, a 50% longer duration to become wheelchair dependent for those who had a pregnancy after MS onset compared to those without a pregnancy after MS onset. Schapira et al.[26] reported a lower disability status in patients whose first pregnancy occurred after onset of MS, but this could have been attributed to the younger mean age at onset of this group, compared to patients whose first pregnancy occurred prior to MS onset, because they did not adjust for age at onset. A long term follow-up study of 25 years by Runmarker and Andersen[14] compared women with a pregnancy after MS onset with women without a pregnancy after MS onset, matching on neurological deficit, disease duration and age. They showed that pregnancy after MS onset was associated with a subsequent lower risk ($p=0.02$) of conversion from a relapsing-remitting course to a chronic progressive disease course. For each year of observation the risk of entering a progressive course was 3.2 {1.1—10.3} times higher in the non-pregnant state compared with that after pregnancy.[14] Another study,[18] following 125 MS patients for 10 years, reported that patients with pregnancies after onset of MS had lower relapse rates ($p=0.07$) compared to patients without pregnancies after onset of MS, after adjusting for age at onset and duration of MS. Although they adjusted for age at onset and duration of MS, they did not adjust for pre-cohort relapse rates, so it is possible that the relapse rate influenced the decision to have a child.

Taken together, the relapse rate has been shown to be lower in the third trimester of pregnancy, higher during the first three months post-partum and there is some evidence for favourable long-term effects on MS progression when having pregnancies after MS onset.

10.2.6 Effect of breastfeeding on MS progression

No subsequent beneficial effect of breastfeeding was found by Confavreux et al.[15] as compared to not breastfeeding (OR 0.8 {0.4—1.6}), although over the total period of the study (33 months), women who breastfed their children had a significantly lower relapse rate ($p=0.02$), but not disability ($p=0.27$), than women who did not breastfed their children.[15] Another study using a cohort of people with MS (n=435), conducted in the 1980s, found that: (a) the relapse rate in women who breastfed their child (37.5%) was not significantly higher

than the relapse rate among those who did not breastfed their child (30.5%); (b) breastfeeding did not delay the mean time to relapse (3.0 vs 3.1 months); and (c) 69% of relapses in the breastfeeding group occurred before the cessation of breastfeeding.[19] Thus, although the number of studies conducted is low, there is not sufficient evidence to suggest that breastfeeding is beneficial for the progression of MS.

10.2.7 Effect of menstrual cycle on MS progression

Zorgdrager and de Keyser[27] showed that over a period of two years, 42% (22/53) of women with relapsing remitting MS had at least one relapse starting in the premenstrual phase. They indicated that for the group that had relapses both during the premenstrual period and the remaining period of the menstrual cycle (n=12), the proportion of premenstrual relapses was greater than could be expected by chance (p=0.006). However, they did not provide the number of women in each of the groups that would have occurred by chance (number of women with only relapses during the premenstrual phase, number of women with relapses both during the premenstrual phase and the remaining phase, and number of women with relapses only during the remaining phase). This makes the interpretation of these results difficult, because the results could have been due to chance. Serial magnetic resonance imaging (MRI) examinations over four menstrual cycles in eight women with relapsing remitting MS showed that the number (r=0.6, p=0.03) and volume (r=0.7, p=0.009) of gadolinium enhanced lesions was correlated with the ratio of progesterone and 17-β-estradiol during the luteal phase (day 21-28 if day 1 is the first day of the menstrual cycle), while the individual hormones were not related to MRI activity neither during the luteal phase nor the follicular phase (day 3-9).[28] This suggests that the balance of progesterone and 17-β-estradiol may be important for the development of a relapse.

10.2.8 Effect of the use of oral contraceptives, hormone replacement therapy and menopause on MS progression

A retrospective study of 179 women with MS found that the 21% that used oral contraceptives at the time of interview had a similar mean disability (DSS) as the group that did not use oral contraceptives, indicating a possible lack of effect of oral contraceptives on disease progression.[22] A small study (n=19) using a retrospective questionnaire enquiring about perceived changes in severity of symptoms of MS with menopause and use of hormone replacement therapy showed that 54% (10/19) of postmenopausal women reported a worsening of symptoms with the menopause, and 75% (6/8) of those who had tried hormone replacement therapy reported an improvement.[29] No clinical trials have yet been conducted to examine the effect of hormone replacement therapy on MS progression.

10.2.9 Possible mechanisms

The epidemiological evidence above shows that a higher proportion of females has MS compared to males and that the ratio of females to males is highest during the early reproductive period starting during puberty. One mechanism responsible for this effect seems to be the involvement of sex-related hormones such as testosterone, estrogen and/or progesterone, which will now be discussed.

Testosterone in males may have a protective effect. A review by Voskuhl and Palaszynski[30] shows that experimental allergic encephalomyelitis (EAE) was more severe in male mice that were castrated. Female mice with EAE treated with high doses of dihydrotestosterone experienced a reduced disease severity, while in males, only a low-dose (physiologic) treatment was required to reduce disease severity.[30] In vivo and in vitro studies have

demonstrated that dihydrotestosterone can directly bind to androgen receptors on CD4+ T cells to induce an increase in the production of interleukin(IL)-10, which has a beneficial effect on EAE. The higher female to male ratio starting during puberty might be partly explained by the protective effect of testosterone in young males. Serum testosterone increases during puberty and begins to decline in healthy men around the age of 30 to 40 years. Androgen levels and metabolism vary widely among healthy men, but despite this variability in the normal range, it has been reported that 24% of males with MS had significantly lower testosterone levels as compared with age-matched normal men.[31]

In female mice, administration of estradiol in mice can also reduce the onset and severity of EAE and produce a shift from T helper(h)$_1$ cell cytokines to Th_2 cell cytokines, because decreased levels of tumour necrosis factor(TNF)-α, interferon(IFN)-γ and IL-12 and increased levels of IL-4 and IL-10 have been observed.[32, 33] However, the dose required in animals is above levels that occur naturally during the menstrual cycle.[30] The lack of effect of oral contraceptives on disease progression of people with MS, and the lack of effect of past and current use of oral contraceptives in healthy women on MS risk could possibly be explained by the fact that the dose is not sufficient to mediate a protective effect. Ovariectomy, which leads to decreased levels of endogenous estrogen, led to an earlier onset and enhanced severity of EAE in one study,[34] but this was not confirmed in another study.[30] Administration of mice with progesterone did not show any delay in onset or decrease in severity compared to placebo mice.[32]

Estriol, seems to be more effective than estradiol in suppressing EAE. This hormone, an estrogen made by the foetal placental unit that is not present in appreciable amounts in non-pregnant states but increases progressively with time during pregnancy, is thought to be, at least in part, responsible for the decrease in relapse rate found in people with MS during the third trimester of the pregnancy. In EAE, estriol doses that induce serum estriol levels that approximated estriol levels during late pregnancy were capable of ameliorating disease.[30] Pathological studies of EAE mice treated with estriol revealed decreased inflammation and decreased demyelination in spinal cord sections as compared with placebo-treated mice.[32] In a small cross-over study (n=10) of non-pregnant female MS patients, administration of estriol for six months resulted in women with relapsing remitting (n=6) into a decreased number (82% reduction) and volume (82% reduction) of gadolinium enhanced lesions, decreased IFN-γ levels in peripheral blood mononuclear cells and decreased delayed type hypersensitivity responses to tetanus.[35] Subsequent withholding the administration for six months increased the number and volume of enhancing lesions to pre-treatment levels, while reinstitution for four months, decreased the number (48%) and volume (88%) of enhancing lesions again as compared with the original baseline.[35] No significant effects of estriol were found in women with secondary progressive MS, but the sample was small (n=4).

During pregnancy there is an immune deviation characterised by a decrease in Th_1 responses and an increase in Th_2 responses that is evolutionarily advantageous because it promotes foetal survival by decreasing Th_1 responses involved in rejection of the foetus as an allograft.[36, 37] The influence of Th_2 cells during the last trimester of pregnancy may lead to suppression of disease, which then escapes control when the shift from Th_2 to Th_1 cells occurs during the postpartum period.[15] Apart from estriol, other hormones such as estradiol, cortisol, norepinephrine and 1,25-dihydroxyvitamin D_3 could contribute to these changes.[38] In healthy pregnant women, the production of monocytic IL-12 in the third trimester was about three-fold lower and TNF-α production was approximately 40% lower than postpartum values, while at the same time serum cortisol and norepinephrine excretion and serum levels of 1,25-dihydroxyvitamin D_3 were 2 to 3-fold higher than postpartum values.[38] In vitro, these hormones have been shown to directly suppress IL-12 and TNF-α. An increase of estradiol and

progesterone may also facilitate a Th_2 shift during pregnancy because they can directly up-regulate the production of IL-4 and IL-10.[39, 40]

Although the protective effect of testosterone in young men might partly explain the preponderance of females in MS, differences in levels of exposure between men and women to, for example, sun exposure or particular infections, such as the Epstein-Barr virus (EBV), could provide an additional explanation. Also, differences between men and women in immunological responsiveness following an infection may be important. Infections of animals to viruses have shown sex differences in disease pathogenesis. For example, compared to male mice, female mice show a heightened immune response in almost every aspect of innate and acquired immunity involved in viral clearance of the vesicular stomatitis virus (VSV), a virus that leads to an acute infection of the central nervous system.[41] Such an elevated immune responsiveness may be beneficial to the host response against the viral infections, yet may be detrimental in autoimmune disease. Hormones such as estradiol might underlie these differences. Estradiol has been shown to act positively in the regulation of the IFN-γ promoter and female patients with MS have been shown to have stronger IFN-γ responses than males.[42] Although estrogen seems to play a role in the susceptibility to those infections, other immune response mechanisms might also underlie those differences. As we discussed in the previous chapter, timing of common or specific infections or other exposures might be of importance in the immune development and the effect might be different for men and women due to behavioural differences. In addition, differences in genotypes between men and women are likely to influence the immune system, especially if linked to the X chromosome.

In this chapter we examine: (1) the female to male ratio by age; (2) whether early or late puberty was associated with risk of MS; (3) whether among women, having had children prior to MS onset was associated with a decreased risk of MS; and (4) whether differences in exposure levels and risk estimates for summer sun exposure during weekends and holidays at age 6-15 years, actinic damage, younger sibling number and EBV antibody titers between females and males could explain the female to male ratio.

10.3 METHODS

10.3.1 Subjects

Chapter 5 outlined in detail the multiple strategies that were used to recruit cases, the approach to obtain community controls matched age and birth year to the case, and the clinical[43] and MRI[44] criteria used for diagnosis. The final total sample consisted of 136 cases (response rate estimated to be between 76% and 92%) and 272 controls (response rate 76%). Interviews were conducted between March 1999 and June 2001. Midway through the study (June 2000) it was decided to supplement the questionnaire for women with questions on childbirth and breastfeeding history.

10.3.2 Measurements

As part of the verbal questionnaire, information was obtained from women on the age of menarche (Appendix A). Puberty development was assessed by proxy recall by asking whether with regard to puberty, the subject was an early, average of late developer compared with others in the school class (Appendix E). The supplementary questions for women on childbirth and breastfeeding were on the number of children the female subjects had had, the date of birth of the children, whether the children were breastfed and the number of completed months of breastfeeding for each child (Appendix B). Measurements regarding sun exposure are outlined in chapter 8 and measurements regarding sibship structure and infections are discussed in chapter 9.

10.3.3 Data analysis

Pearson correlations were calculated as measures of linear association. Odds ratios and 95% confidence intervals were estimated by conditional logistic regression (STATA 7.0). Tests for trend of categorical variables were undertaken by replacing the binary predictors with a single predictor taking category rank scores and using the p-value of the Wald test.

Analysis of the female to male ratio by age and age at onset was conducted on the total sample of 136 cases, while the analysis on the age of menarche included all women of the study (92 cases and 184 controls). Age of menarche was also assessed by proxy recall and puberty development for women was only assessed by proxy recall (74 case proxies, 149 control proxies), with 158 of the 223 (70.9%) being mothers of the subjects (55 mother case proxies, 103 mother control proxies). The intraclass correlation {95% confidence interval} between the subject and proxy recall of age of menarche was 0.60 {0.51—0.69}. The supplementary questions on childbirth and breastfeeding were answered by 39 cases and 122 controls. Of the 39 cases, 38 had two matched controls with this information while one case had one matched control with this information, providing us with 77 case-control pairs to use for conditional logistic regression. Children born or breastfed after the age at onset could not have influenced the development of MS and were therefore not included in the analysis. Controls were given the same age at onset as their case pair.

To see whether the odds ratios for having a child prior to the age at onset across the strata of age at onset changed in magnitude, a likelihood-ratio test of interaction was conducted, by comparing a model with having a child prior to MS onset (naïve model) with a model that also includes the product term of having a child prior to MS onset and age at onset (saturated model). Age at onset can be seen as a matching variable and was therefore not included in the models (see also chapter 5, assessment of interaction). Controls were given the age at

onset (age of first MS symptom) of their case pair. We tested whether there were differences in particular exposures between females and males by modelling sex as a function of the exposure of interest using unconditional logistic regression and examining the p-value of the Wald test. We adjusted the models for age. To evaluate differences in odds ratios of particular exposures for females and males, we conducted a stratified analysis by sex and tested the difference using a likelihood–ratio test of interaction by comparing a model with the exposure of interest (naïve model) to a model with the exposure of interest and the product term of the exposure of interest and sex (saturated model). Sex was not included separately in the models, because it was a matching variable.

Similarly to chapter 8 and 9, population attributable fraction (the proportion by which the incidence rate of the outcome in the entire population would be reduced if exposure were eliminated, assuming that the exposure is causally related to the outcome and that causes other than the one under investigation have had equal effects on the exposed and unexposed groups) was estimated for adjusted risk estimates by the formula: [45]

$$AF_p = \frac{P(RR-1)}{RR}$$

where RR is the estimate of the rate ratio (odds ratio in a case-control study) and P is the proportion of all cases that are exposed. The population attributable fraction was calculated for females and males for past summer sun exposure during weekends and holidays at age 6-15 years.

10.4 RESULTS

10.4.1 Female to male ratio

For the total sample of cases, the female to male ratio was 2.1. The ratio was higher for younger subjects and decreased with older age (test for trend, p<0.01) (Table 1). A similar pattern was observed for age at onset, but not as strong (test for trend, p=0.12).

Table 1. Female to male ratio among cases by age and age at onset.

Age	Males n (%)	Females n (%)	F/M	Age at onset	Males n (%)	Females n (%)	F/M
≤ 30 years	1 (2.3)	10 (10.9)	10.0	≤ 20 years	3 (6.8)	11 (12.2)	3.7
31—40 years	11 ((25.0)	25 (27.2)	2.3	21—30 years	17 (38.6)	32 (35.6)	1.9
41—50 years	18 (40.1)	34 (37.0)	1.9	31—40 years	13 (29.5)	31 (34.4)	2.4
51—60 years	14 (31.8)	23 (25.0)	1.6	41—60 years	11 (25.0)	16 (17.8)	1.5
Total sample	44	92	2.1	Total sample	44	90	2.0

Notes
1. F/M, female to male ratio; n, number of cases
2. Information on age at onset was not available for two women.

10.4.2 Puberty development and risk of MS

For the total sample of women, compared to controls, cases reported more often an age of menarche of 12 years than an earlier age of menarche (Table 2), but there was no dose-response relationship for those who reported an age of menarche above 12 years. Recall by proxies gave similar results. The mean age of menarche was similar for cases (12.5 years) and controls (12.7 years) (p=0.44). Mothers of all cases (men and women) reported more often that their child was an average or late developer compared to an early developer, but the confidence interval was wide (Table 2). Including proxies that were not mothers showed no effect (average developer, OR 1.14 {0.54—2.19}, late developer, OR 0.91 {0.30—2.71}, early developer as reference category). The positive association between being an average or late developer and MS was stronger in males compared to females (test for difference, p=0.04), suggesting possibly a stronger protective effect of the increase of testosterone during early puberty in males.

Overall, there is some evidence that having a late puberty development tends to be positively associated with MS. The tendency seems stronger in males than in females.

Table 2. Unadjusted odds ratios for Multiple Sclerosis and age of menarche among women and puberty development among men and women.

	Cases n (%)	Controls n (%)	Unadjusted OR {95% CI}
Age of menarche (subject recall)			
8—11 years	14 (15.4)	45 (24.7)	1
12 years	35 (38.5)	47 (25.8)	2.28 {1.06—4.89}
13 years	22 (24.2)	46 (25.3)	1.45 {0.65—3.26}
14—17 years	20 (22.0)	44 (24.2)	1.40 {0.62—3.21}
Test for trend			p=0.92

Table 2 continued at next page

Table 2 continued

	Cases **n (%)**	Controls n (%)	Unadjusted OR {95% CI}
Age of menarche (proxy recall)			
8—11 years	7 (11.1)	26 (20.6)	1
12 years	29 (46.0)	42 (33.3)	2.32 {0.83—6.49}
13 years	16 (25.4)	39 (31.0)	1.47 {0.49—4.39}
14—17 years	11 (17.5)	19 (15.1)	1.65 {0.50—5.46}
Test for trend			p=0.83
Puberty development (proxy recall of mothers)			
Early developer	14 (16.9)	31 (20.3)	1
Average developer	64 (77.1)	115 (75.2)	1.99 {0.83—4.78}
Late developer	5 (6.0)	7 (4.6)	1.75 {0.42—7.40}
Test for trend			p=0.24
Puberty development females only (proxy recall of mothers)			
Early developer	11 (20.0)	25 (22.7)	1
Average or late developer	44 (80.0)	85 (77.3)	1.69 {0.65—4.38}
Puberty development males only (proxy recall of mothers)			
Early developer	3 (10.7)	6 (14.0)	1
Average or late developer	25 (89.3)	37 (86.0)	3.68 {0.42—32.04}

Notes
1. OR = odds ratio; {95% CI} = {95% confidence interval}

10.4.3 Childbirth and breastfeeding history and risk of MS

Female sample characteristics

Table 3. Characteristics of the cases and controls of women of the total sample and the sample of women with a history on childbirth and breastfeeding.

	Total sample (Females)		Sample of females with childbirth and breastfeeding history		
	Cases n=92	Controls n=184	Cases n=39	Controls n=122	Controls* n=77
Characteristics for both cases and controls					
Mean age, years (SD)	43.0 (9.7)	43.0 (9.7)	44.7 (9.9)	43.8 (9.4)	44.5 (9.8)
Mean height, cm (SD)	162.8 (6.3)	161.8 (5.8)	161.6 (6.4)	161.8 (6.1)	162.2 (5.7)
Mean weight, kg (SD)	69.2 (14.5)	71.6 (14.6)	72.0 (17.4)	72.8 (15.7)	73.2 (16.9)
Average time in the sun in summer on weekends and holidays at age 6 – 15 years, hrs (SD)	3.39 (1.52)	3.70 (1.33)	3.15 (1.48)	3.74 (1.39)	3.77 (1.31)
Mean actinic damage, grade (SD)	4.35 (0.92)	4.73 (0.98)	4.16 (0.95)	4.80 (0.97)	4.77 (1.06)
Disease specific characteristics of cases					
Mean age at diagnosis, years (SD)	34.4 (9.2)	-	34.8 (9.6)	-	-
Mean age at first symptoms, years (SD)	31.5 (8.9)	-	32.1 (9.4)	-	-
Mean duration of MS since diagnosis, years (SD)	8.6 (9.1)	-	9.3 (9.1)	-	-
Mean duration of MS since first symptoms, yrs (SD)	11.6 (7.8)	-	12.0 (9.7)	-	-
Mean EDSS score (SD)	3.3 (2.2)	-	3.5 (2.4)	-	-
Type of MS					
Relapsing remitting MS, % (n)	71.1 (64)	-	76.3 (29)	-	-
Secondary progressive MS, % (n)	24.4 (22)	-	18.4 (7)	-	-
Primary progressive MS, % (n)	4.1 (4)	-	5.3 (2)	-	-

Notes
1. * denotes to controls that had a matching case available.

The sample of women with information on their childbirth and breastfeeding history was similar in structure as the total sample of women of the study for most characteristics (Table 3). However, female cases with information on childbirth and breastfeeding history tended to have a slightly higher weight and lower average levels of summer sun exposure during weekends and holidays at age 6-15 years and actinic damage compared to the total sample of female cases, while there was no difference between the female controls with information on their childbirth and breastfeeding history and the total sample of female controls.

Childbirth and breastfeeding history

Having had any children prior to the age at onset was associated with a strong decreased risk of MS (Table 4). There was no substantial additional risk reduction in having more than one child. Although cases less often had children prior to the age at onset than controls, among those who had more than one child prior to age at onset, stronger protective risk estimates were seen for a longer duration between the pregnancies (Table 4), which could indicate a beneficial effect of having a child around while not pregnant. The age of the mother when the first child was born did not seem to be associated with risk of MS onset.

Table 4. Unadjusted odds ratios for Multiple Sclerosis and childbirth history prior to the age at onset of MS.

	Cases n (%)	Controls n (%)	Unadjusted OR {95% CI}
Having had children			
No	18 (46.2)	21 (27.3)	1
Yes	21 (53.8)	56 (72.7)	0.21 {0.06−0.76}
Number of children			
No children	18 (46.2)	21 (27.3)	1
One child	3 (7.7)	10 (13.0)	0.23 {0.04−1.20}
Two children	7 (17.9)	19 (24.7)	0.19 {0.04−0.93}
More than two children	11 (28.2)	27 (35.1)	0.21 {0.05−0.89}
Test for trend			p=0.05
Interval between 1st and 2nd child			
0−2.50 years	10 (55.6)	11 (23.9)	1
2.51−3.50 years	4 (22.2)	10 (21.7)	0.41 {0.06−2.68}
>3.50 years	4 (22.2)	25 (54.3)	0.24 {0.05−1.02}
Test for trend			p=0.05
Interval between 2nd and 3rd child			
≤4.5 years	7 (63.6)	12 (44.4)	1
>4.5 years	4 (26.4)	15 (55.6)	0.47 {0.04−5.68}
Age mother when 1st child born			
<20 years	6 (28.6)	16 (28.6)	1
20-22 years	5 (23.8)	17 (30.4)	0.92 {0.19−4.36}
23-25 years	6 (28.6)	11 (19.6)	1.62 {0.32−8.21}
>25 years	4 (19.0)	12 (21.4)	066 {0.12−3.57}
Test for trend			p=0.54
≤22 years	11 (52.4)	33 (58.9)	1
>22 years	10 (47.6)	23 (41.1)	1.10 {0.39−3.22}

Notes
1. OR = odds ratio; {95% CI} = {95% confidence interval}

We examined whether the effect of having children could have been (partly) mediated through breastfeeding and whether breastfeeding modified the relationship between having had children prior to the age at onset and MS. Among women who never breastfed prior to the age at onset, there were women who had never had a child prior to MS onset and women who had given birth to a child prior to MS onset but did not breastfed that child. We examined the effect of breastfeeding and having children using different reference groups (Table 5). Firstly, compared to women who never had a child or breastfed a child (Table 5 "Children and breastfeeding (1)"), breastfeeding a child prior to the age at onset was associated with a reduced risk of MS (OR 0.57 {0.23—1.41}), but the strength of the association was less than half compared to the strength of the association between having children and MS that we observed in Table 4 (OR 0.21 {0.06—0.76}). This suggests that having a child is more important than breastfeeding a child. Secondly, compared to women that never had a child (Table 5 "Children and breastfeeding (2)"), having a child but not breastfeeding them was also negatively associated with MS (OR 0.07 {0.005—0.75}) with a magnitude that was not weaker than the magnitude of the association for all children that we observed in Table 4 (OR 0.21 {0.06—0.76}). Although the number of women who had children but did not breastfeed them was low, it seems to suggest that the effect of having had children was not mediated through breastfeeding. Thirdly, again compared to women that never had a child (Table 5 "Children and breastfeeding (2)"), having a child and breastfeeding them was negatively associated with MS with an odds ratio of 0.22 {0.06—0.82}. The strength of this association was not stronger than the association between having children and not breastfeeding them and MS (OR 0.07 {0.005—0.75}), indicating that breastfeeding did not modify the relationship between having had children prior to MS onset and MS.

In addition, we conducted multivariate analysis and included two variables - "having had children prior to onset" and "having ever breastfed prior to onset" (Table 5 "Children and breastfeeding (1)") - in the model. Although the correlation between variables was high (r=0.83), compared to univariate analysis the magnitude of the effect and standard error (SE) did not change substantially for "having had children prior to MS onset" (univariate model: OR 0.21, SE 0.14; multivariate model: OR 0.17, SE 0.16), but both the magnitude of the effect and standard error largely increased for "having ever breastfed prior to onset" (univariate model: OR 0.57, SE 0.26; multivariate model: OR 1.05, SE 0.61). This indicates that having had children prior to MS onset seems more important than having ever breastfed prior to MS onset. It also suggests that the effect of having had children prior to MS onset was not mediated through breastfeeding, because there was no residual effect of breastfeeding after adjustment for having children, but rather that the apparent inverse association between breastfeeding and MS was a result of the association between having had children and breastfeeding and the association between having had children and MS.

Assessment of the number of children that were breastfed did not show an additional risk reduction for more than one child compared to one child (test for trend, p>0.20) (Table 5). There was some evidence of a dose-response for increased duration (>3 months) of breastfeeding of the first child when the reference category included women who did not have children (test for trend, p=0.03), but this was not evident when the reference category also included women that had children but not breastfed them (test for trend, p=0.64).

On the whole, although the overall sample size is limited and there is a high correlation between having had children and breastfeeding, these results do not provide evidence that the effect of having had children is (partly) mediated through breastfeeding or that breastfeeding modifies the effect of having children.

Table 5. Unadjusted odds ratios for Multiple Sclerosis and childbirth and breastfeeding history prior to the age at onset of MS.

	Cases n (%)	Controls n (%)	Unadjusted OR (95% CI)
Children and breastfeeding (1)			
No child or ≥1 child but never breastfed child	19 (48.7)	29 (37.7)	1
≥1 child and breastfed ≥1 child	20 (51.3)	48 (62.3)	0.57 {0.23−1.41}
Children and breastfeeding (2)			
No child	18 (46.2)	21 (27.3)	1
≥1 child but never breastfed	1 (2.6)	8 (10.4)	0.07 {0.005−0.75}
≥1 child and breastfed ≥1 child	20 (51.3)	48 (62.3)	0.22 {0.06−0.82}
Test for trend			p=0.06
Number of children breastfed (1)			
No child or ≥1 child but never breastfed child	19 (48.7)	29 (37.7)	1
Breastfed 1 child	5 (12.8)	22 (28.6)	0.32 {0.09−1.09}
Breastfed more than 1 child	15 (38.5)	26 (33.8)	0.83 {0.30−2.30}
Test for trend			p=0.72
Number of children breastfed (2)			
No child	18 (47.4)	21 (30.4)	1
Breastfed 1 child	5 (13.2)	22 (31.9)	0.08 {0.01−0.51}
Breastfed more than 1 child	15 (39.5)	26 (37.8)	0.31 {0.08−1.25}
Test for trend			p=0.31
Duration of breastfeeding of first child (1)			
No child or did not feed 1st child	21 (53.8)	39 (50.6)	1
Breastfed 0-3 months	8 (20.5)	14 (18.2)	1.07 {0.36−3.20}
More than 3 months	10 (25.6)	24 (31.2)	0.80 {0.32−2.03}
Test for trend			p=0.64
Duration of breastfeeding of first child (2)			
No child	18 (50.0)	21 (27.3)	1
Breastfed 0-3 months	8 (22.2)	14 (18.2)	0.32 {0.07−1.38}
Breastfed more than 3 months	10 (27.8)	24 (31.2)	0.17 {0.04−0.793}
Test for trend			p=0.03

Notes
1. OR = odds ratio; {95% CI} = {95% confidence interval}

Assessment of confounders

We estimated the effect of having had children prior to MS onset and the number of children prior to MS onset by taking into account other factors related to MS. Even though education level (r=-0.24) and occupational level (r=-0.28) were strongly associated with the number of children prior to the age at onset among controls, both did not alter the association between having had children prior to the age at onset and MS. Having high IgG antibody titers against cytomegalovirus (CMV) was also positively associated with the number of children prior to the age at onset among controls (r=0.21, p=0.03). The increase in antibody titers might reflect a reactivation pattern of the mother when their children obtain the primary infection of CMV early in life. However, adjusting for IgG antibody levels to CMV did not affect the risk estimate of having had children prior to the age at onset.

As discussed in chapter 8, other factors positively associated with MS were low melanin density at the upper inner arm (p=0.06), smoking (p=0.05), having had glandular fever (p<0.01), being seropositive for the EBV nuclear antigen (p<0.01) and viral capsid antigen

(p<0.01), and having high IgG levels against the EBV nuclear antigen (>2.50 units) (p<0.01) and viral capsid antigen (>2.00 units) (p=0.01), exposure to fibre glass and resin prior to age 17 (p=0.02), exposure to smoke fumes prior to age 17 (p=0.05), exposure to smoke fumes between age 17 and the age of diagnosis (p=0.03), and owning a cat prior to MS onset (p=0.02). Factors negatively associated with MS were higher levels of summer sun exposure during weekends and holidays at age 6—15 years (p<0.01), higher levels of actinic damage (p<0.01), having younger siblings (p<0.01) and having had an immunisation for rubella in early life (p<0.01).

Adjusting for summer sun exposure during weekends and holidays at age 6—15 years marginally increased the magnitude of the odds ratio for having had children prior to MS onset (0.18 {0.05—0.73}) (Table 6). Actinic damage was strongly associated with having had children prior to MS onset (r=0.29, p=0.01) or the number of children prior to MS onset among controls (r=0.36, p=0.01) and adjusting for actinic damage increased the magnitude of the odds ratio for having had children prior to MS onset substantially (0.06 {0.008—0.52}). We decided not to adjust for actinic damage, because the risk estimates were not homogeneous across the strata of actinic damage, indicating interaction between having had children and actinic damage. We used unconditional regression (with an age adjustment) because of the limited number of subjects. There was no effect of having had children among those who had low levels of actinic damage (OR 1.14 {0.33—3.93}), but a strong protective effect among those who had high (grade 5-6) levels of actinic damage (OR 0.13 {0.04—0.50}) (test for interaction: p=0.04). The risk estimates might have been influenced by some sampling error, because as was shown in Table 3, the cases in the sample of women with childbirth and breastfeeding history had a lower mean actinic damage compared to the cases of the total sample of women, while the controls in both groups had a similar mean actinic damage.

Table 6. Unadjusted and adjusted odds ratios for Multiple Sclerosis and having had children prior to the age at onset.

	Cases n (%)	Controls n (%)	Unadjusted OR {95% CI}	Adjusted OR {95% CI}
Having had children				
No	18 (46.2)	21 (27.3)	1	1
Yes	21 (53.8)	56 (72.7)	0.21 {0.06—0.76}	0.18 {0.05—0.73}
Number of children				
No children	18 (46.2)	21 (27.3)	1	
One child	3 (7.7)	10 (13.0)	0.23 {0.04—1.20}	0.27 {0.05—1.49}
Two children	7 (17.9)	19 (24.7)	0.19 {0.04—0.93}	0.17 {0.03—0.90}
More than two children	11 (28.2)	27 (35.1)	0.21 {0.05—0.89}	0.14 {0.03—0.70}
Test for trend			p=0.05	p=0.02

Notes
1. OR = odds ratio; {95% CI} = {95% confidence interval}
2. Adjusted for summer sun exposure during weekends and holidays at age 6—15 years.

Additional adjustment for the other factors listed above made no material difference to the odds ratio. Thus, the negative association between having had children prior to MS onset and MS did not disappear after adjustment for confounding factors.

<u>Does the effect of having children operate independently from the effects of sun exposure, younger sibling number and Epstein-Barr virus?</u>

We assessed whether the effect of having had children prior to MS onset operated independently from the effect of sun exposure (summer sun exposure during weekends and holidays at age 6-15 years, actinic damage), younger sibling number and EBV (self-reported

history of teenage IM, IgG antibody titers to EBNA, IgG antibody titers to VCA). As discussed in the previous paragraph, the effect of having had children prior to MS onset appeared to interact with actinic damage. This interaction needs to be interpreted with some caution because of the limited number of subjects and the possible sampling error that might have occurred. There was no interaction between having had children and summer sun exposure during weekends and holidays at age 6-15 years and adjusting for each other slightly increased the magnitude of the effects. There also was no interaction between having had children prior to MS onset and younger sibling number or EBV, and compared to univariate analysis of the effect of having had children and each of those factors, including both in the same model as linear terms did not affect the magnitude of the estimated effect of having had children prior to MS onset or any of the factors.

Effect on the age at onset among cases

Similar to the analysis conducted in chapter 8, we aimed to assess whether having had children prior to the age at onset among cases was associated with age at onset of disease by modelling time elapsed from birth to onset of first symptoms using proportional hazard regression. We could, however, not use this approach, because the age at onset was related to the opportunity that women had to have children at that point in time. Alternatively, we examined whether the effect of having had children prior to the age at onset was different for people who had an early onset of disease (<32 years) compared to people who had a later onset of disease (≥32 years) (Table 7). The effect of having had children prior to the age at onset seems stronger in people with an earlier onset of disease, but given the limited number of people in each stratum and the uneven distribution of having had children in each stratum, the test for interaction was not significant (p=0.22).

Table 7. Unadjusted odds ratios for Multiple Sclerosis and childbirth history prior to the age at onset of MS stratified by age at onset

	Cases n (%)	Controls n (%)	Unadjusted OR {95% CI}
Cases (and matched controls) with age at onset <32 years			
Having had children			
No	15 (83.3)	17 (48.6)	1
Yes	3 (16.7)	18 (51.4)	0.09 {0.01−0.76}
Cases (and matched controls) with age at onset ≥32 years			
Having had children			
No	3 (14.3)	4 (9.5)	1
Yes	18 (85.7)	38 (90.5)	0.55 {0.08−3.60}

Notes
1. OR = odds ratio; {95% CI} = {95% confidence interval}

10.4.4 Sex differences in prevalence and strength of MS risk factors

We then examined for some factors that were associated with MS whether, among controls, females had different levels of exposure compared to males and whether the risk estimates were different for females compared to males. We included measures that showed a significant association in chapter 8 and chapter 9: summer sun exposure during weekends and holidays at age 6 to 15 years, actinic damage, number of younger siblings, self-reported history of teenage infectious mononucleosis (IM), and antibodies against Epstein-Barr virus nuclear antigen (EBNA) and viral capsid antigen (VCA). To test whether there were differences in exposure between females and males, we used unconditional logistic regression to model sex as a function of the exposure of interest. We adjusted the model for age, because among controls there was some association between each of the exposures of

interest and age (summer sun exposure during weekends and holidays at age 6-15 years, r=0.14, p=0.02; actinic damage, r=0.43, p<0.01; younger sibling number, r=0.13, p=0.04; teenage IM, r=-0.16, p<0.01; EBNA antibody titers, r=-0.10, p=0.10; VCA antibody titers, r=0.06, p=0.34), and as we discussed under the heading of female to male ratio, we observed differences in the age structure in our sample of females cases and male cases, which will also be seen in the age matched controls.

Table 8. Differences among controls between females and males in summer sun exposure during weekends and holidays at age 6-15 years, actinic damage, number of younger siblings, self-reported history of infectious mononucleosis (IM), IgG antibody titers to Epstein-Barr Virus nuclear antigen (EBNA) and IgG antibody titers to Epstein-Barr virus viral capsid antigen (VCA).

	Female controls n (%)		Male controls n (%)
Measures of sun exposure			
Summer sun exposure during weekends and holidays at age 6-15 years			
Less than 1 hr a day	4 (2.2)		0 (0)
1—2 hrs a day	12 (6.5)		2 (2.3)
2—3 hrs a day	36 (19.6)		3 (3.5)
3—4 hrs a day	54 (29.4)		11 (12.8)
More than 4 hrs a day	78 (42.4)		70 (81.4)
Test for trend		p<0.01	
Actinic damage			
Grade 3	20 (12.4)		1 (1.8)
Grade 4	45 (27.8)		14 (25.0)
Grade 5	55 (34.0)		23 (41.1)
Grade 6	42 (25.9)		18 (32.1)
Test for trend		p=0.13	
Sibship structure			
Number of younger siblings			
No younger siblings	50 (27.3)		24 (27.3)
1 younger sibling	47 (25.7)		22 (25.0)
2 younger siblings	46 (25.1)		18 (20.5)
≥3 younger siblings	40 (21.9)		24 (27.3)
Test for trend		p=0.87	
IM and Epstein-Barr virus			
Self-reported history of teenage IM			
No	196 (91.9)		85 (96.6)
Yes	15 (8.2)		3 (3.4)
Test for difference		p=0.25	
IgG antibody titers to EBNA			
≤2.5 units	99 (56.9)		51 (58.6)
>2.5 units	75 (43.1)		36 (41.4)
Test for difference		p=0.84	
IgG antibody titers to VCA			
≤2.0 units	49 (28.2)		21 (24.2)
>2.0 units	125 (71.8)		66 (75.9)
Test for difference		p=0.55	

Notes

1. The test for trend or difference, using a logistic regression model of sex and the exposures of interest, was adjusted for age.

Among controls, females had less sun exposure at age 6 to 15 years compared to males (Table 8). The age adjustment only had a small influence on the risk estimate (4.6% change in the coefficient). With low sun exposure at age 6 to 15 years being a risk factor of MS, the higher percentage of females with low sun exposure could partly explain the female to male ratio. However, when we examined the risk estimates for females and males, we observed that the protective effect between higher sun exposure at age 6 to 15 years and MS was significantly stronger for males compared to females (test for interaction, p=0.03) (Table 9). Adjusting for melanin density at the upper inner arm and smoking prior to MS onset did not affect this difference substantially (females: OR 0.65 {0.37—1.17}; males: OR 0.12 {0.02—0.63}). Thus, even though females had low levels of sun exposure more often, the effect of having low levels of sun exposure on MS was weaker than the effect of sun exposure on MS for males. We examined whether these estimates could have been influenced by recall bias by limiting to subjects who did not think that sun exposure was an important cause of MS. The difference between the risk estimates for higher sun exposure and MS between males (OR 0.15 {0.04—0.52}) and females (OR 0.26 {0.10—0.67}) decreased but we did not have a sufficient number of subjects to determine whether this difference was significant or not.

Table 9. Unadjusted odds ratios for Multiple Sclerosis and sun exposure at age 6-15 years, actinic damage, number of younger siblings, self-reported history of infectious mononucleosis (IM), IgG antibody titers to Epstein-Barr Virus nuclear antigen (EBNA) and IgG antibody titers to Epstein-Barr Virus capsid antigen (VCA) by sex.

	Females			Males		
	Cases n	Controls n	Unadjusted OR {95% CI}	Cases n	Controls n	Unadjusted OR {95% CI}
Measures of sun exposure						
Sun exposure at age 6-15 years						
≤ 2-3 hrs a day	31	52	1	11	5	1
≥ 3-4 hrs a day	61	132	0.77 {0.45—1.33}	32	81	0.16 {0.04—0.57}
Test for interaction			p=0.02			
Actinic damage						
Grade 3-4	41	65	1	11	15	1
Grade 5-6	37	97	0.51 {0.26—0.98}	16	41	0.55 {0.20—1.53}
Test for interaction			p=0.90			
Sibship structure						
Having younger siblings						
No	44	50	1	14	24	1
Yes	48	133	0.41 {0.24—0.70}	28	64	0.70 {0.29—1.73}
Test for interaction			p=0.90			
Teenage infectious mononucleosis (IM) and Epstein-Barr virus						
Self-reported history of IM						
No	74	168	1	35	85	1
Yes	16	15	2.91 {1.26—6.73}	9	3	8.29 {1.78—38.69}
Test for interaction			p=0.22			
IgG antibody titers to EBNA						
≤2.5 units	16	99	1	10	51	1
>2.5 units	76	75	6.52 {3.27—12.97}	34	36	4.50 {1.92—10.58}
Test for interaction			p=0.51			
IgG antibody titers to VCA						
≤2.0 units	13	49	1	8	21	1
>2.0 units	79	125	2.33 {1.18—4.59}	36	66	1.44 {0.58—3.53}
Test for interaction			p=0.41			

1. OR = odds ratio; {95% CI} = {95% confidence interval}

We examined the combined effect of a higher prevalence and a lower risk estimate of low (2-3 hrs a day or less) summer sun exposure during weekends and holidays at age 6-15 years in women compared to men by calculating the population attributable fraction for females and males. For females, 33.7% of the cases had low sun exposure and the adjusted risk estimate was 1.54 (1/0.65), giving us a population attributable fraction of 12%. For males, 25.6% of the cases had low sun exposure and the adjusted risk estimate was 8.33 (1/0.12), giving us a population attributable fraction of 23%. The attributable fraction does not take into account the difference in age structure between females and males, but, as discussed, this influence was only small. Thus, it seems that the incidence rate of MS would be reduced to a lesser extent if we were able to eliminate low sun exposure in summer during weekends and holidays at age 6-15 years in females compared to males, and thus, sun exposure at age 6-15 years cannot explain part of the female excess.

For actinic damage, female controls had lower levels than male controls, but the difference was not significant after adjusting for age (Table 8). The age adjustment had a large influence on the risk estimate (20.0% change in the coefficient), because the correlation between actinic damage and age was high ($r=0.42$, $p<0.01$). There was also no difference in the risk estimates for actinic damage between females and males (test for interaction, $p=0.90$)(Table 9). Adjusting for melanin density at the upper inner arm, smoking prior to MS onset and sun exposure after disease onset slightly decreased the magnitude of the risk estimates for males (OR 0.61 {0.12—3.19}, but the difference between females and males was still not significant (test for interaction, $p=0.51$). Thus, the small differences in prevalence and risk estimates of low actinic damage between females and males, although in the right direction, are not likely to explain an important part of the female excess of MS.

For younger siblings, self-reported history of teenage IM and high IgG antibody levels against EBV, no differences were observed in exposure between female and male controls (Table 8) and the estimates of the relative risk were also similar for females and males (Table 9). Thus, these factors also do not seem to explain part of the female excess of MS.

10.5 DISCUSSION

10.5.1 Female to male ratio

The female to male ratio of our case sample was 2.1:1. This ratio was slightly higher for subjects who had an early onset of MS compared to those who were diagnosed at a later age. This has been observed in many other population-based studies for MS[3, 4] and other autoimmune diseases[2] and has been suggested to be related to the protective effects of testosterone in males[30] and the reproductive system in females.[46]

10.5.2 Puberty development and risk of MS

We found some evidence that having a later puberty development was positively associated with MS. Female cases reported more often an age of menarche of 12 years than an earlier age of menarche, but there was no dose-response trend for those who had an age of menarche later than 12 years, and no difference was observed in the mean age of menarche between cases and controls. One study found a lower age of menarche for cases,[7] while two other case-control studies found no difference.[8, 9] We also observed a positive, although non-significant, association between being an average or late developer compared to an early developer. The association was stronger for males compared to females. The potential mechanism could be that having higher levels of sex hormones for a longer duration is beneficial. The stronger effect for males could then reflect a stronger protective effect of testosterone compared to estrogen, as has been reported in the literature.[30] However, differences in puberty development might also reflect in behavioural differences, such as the tendency to spend more or less time outdoor or participate in sport, or differences in susceptibility to particular infections.

Unfortunately, there are no other studies that have assessed sex differences in puberty development. In our study, puberty development was assessed by only using two basic questions due to burden and time limitations of the participants. However, it seems warranted to examine this issue in more detail by including a higher number and/or better questionnaire measures and possibly biomarkers of puberty development in newly designed epidemiological studies.

10.5.3 Childbirth and breastfeeding history and risk of MS

In our sample, among women, having had children prior to MS onset was associated with a strong decreased risk of MS. This effect did not seem to be mediated through or modified by breastfeeding. The finding is in accordance with Runmarker and Anderson[14] who used an incident cohort of MS patients (1950-1964) and found that people with MS were more often childless prior to MS onset ((74 childless women out of 153 women with a diagnosis of definite or probable MS) compared to what was expected (50.9/153) based on the general population. In addition, one cohort study found a protective effect for having had three or more pregnancies compared to none (rate ratio 0.4 {0.2—1.4}), but the confidence interval was wide due to the low numbers and no dose-response trend was observed.[13] In contrast, two prospective cohort studies and two case-control studies showed no association between parity and MS.[7, 10-12] Our epidemiological findings are consistent with other work that show that pregnancy (especially the third trimester) decreases the number of relapses of people with relapsing remitting MS[15-19] and that breastfeeding does not seem to have an independent effect.[19] The decrease in relapse rate during pregnancy is thought to be related to the shift from Th_1 to Th_2 mediated responses which promotes foetal survival by decreasing Th_1

responses involved in rejection of the foetus as an allograft.[36, 37] Although a number of studies[15-18] found no overall effect of pregnancy short term, by comparing the progression before and after pregnancies, there is evidence to suggest that long-term progression is influenced by pregnancies after MS onset.[14, 18]

As has been mentioned in chapter 8, our total case sample appeared similar to other populations with MS of North European ancestry with regard to disease-related features such as the type of MS, age at diagnosis and sex ratio.[47-50] Participation rates for cases and controls were high, reducing non-response bias, but it is conceivable that some selection bias may have occurred. Measurement error was likely to be low for simple questions such as the number of children, the date of birth of the subject and children, and whether the subject breastfed each child. The reliability has been shown to be lower for more complex questions (see chapter 6). From experience of the research assistants, the duration of breastfeeding seemed to be a more complex question. A weakness of this study was the relative small size of the sub-sample of women on which we had information on the history of childbirth and breastfeeding. This limited the accuracy of the risk estimates. For most characteristics, the sub-sample appeared similar in structure as the total sample of women of the study, although some sampling error seemed to have occurred as female cases tended to have a slightly higher weight and lower average levels of sun exposure at age 6-15 years and actinic damage.

If this association is a true effect, then what would be the underlying causal mechanism of the association between having had children and MS? In line with what is known about the effects of pregnancy on the immune system, a long-term effect could be expected from the pregnancy related endocrine effects that cause a shift from Th_1 to Th_2 mediated responses.[32, 33] However, having children not only mediates an endocrine effect, but is also accompanied with lifestyle changes, such as less smoking, less employment and higher infection load through contact with other children. If the pregnancy and its related endocrine effects is not the underlying causal factor of the association between having had children and MS, but simply a proxy of another causal factor, then adjusting for the causal factor should decrease or eliminate the association. We adjusted for childhood and early adolescence sun exposure, which slightly increased the magnitude of the risk estimate for having had children prior to MS onset and MS. Further adjustment for other factors associated with MS did not influence the results. We did not have information on specific exposures of the first years after the pregnancy. It is possible that unmeasured exposure factors, such as infection load in the first years after pregnancy, might underlie the association found in this study.

Future studies could be designed more specifically to answer this question. Firstly, comparing females and males could be one way to separate pregnancy-related endocrine effects from child-related lifestyle and exposure changes. Secondly, specific exposure factors could be measured around the period of childbirth, such as childcare attendance of the child and number of people in the household as a measure of infection load, and assessment of factors prior to and after birth so change in lifestyle factors can be assessed (dietary intake, tobacco smoking, employment and sun exposure behaviour).

10.5.4 Sex differences in prevalence and strength of MS risk factors

The protective effect of having children does, however, not explain the excess of MS among women. We examined whether there were sex differences in prevalence of exposure and risk estimates for factors associated with MS such has higher summer sun exposure at age 6-15 years, actinic damage, younger sibling number, history of teenage IM and IgG antibody levels against EBV. We found no differences in prevalence of exposure and in risk estimates between females and males for younger sibling number, history of teenage IM and IgG

antibody levels against EBV. Although females had low levels of sun exposure more often at age 6-15 years, the magnitude of risk estimates were significantly lower than that for men. An evaluation of their combined effect using population attributable fraction showed that sun exposure at age 6-15 years could not explain part of the female excess. For low actinic damage, another sun exposure measure, its prevalence was higher and the risk estimate stronger in females, but the effects were too small to explain an important part of the female excess of MS. As we discussed in chapter 2 and the previous chapter, the only environmental factor with sufficient evidence to conclude that this factor might be causally related to MS is the involvement of EBV. None of the studies, however, have evaluated whether there are any sex differences in prevalence and/or risk of EBV-related variables such as history of IM, seropositivity against EBV antibodies and EBV antibody titers, even though there is evidence that immunological responsiveness to infections might be different between the two sexes.[41] In addition, genetic factors may also contribute to the excess of females in MS. It is, for example, possible that females are more sensitive to low sun exposure and/or low vitamin D. Evidence has been found for an association between vitamin D receptor gene polymorphisms and alleles of the class II region of the human leukocyte antigen.[51] Two other studies have observed that, compared to males with MS, females with MS more often have the susceptibility haplotype in the class II region (DRB1*1501 – DQB1*0602) of the human leukocyte antigen,[52, 53] but neither of these studies used a control group and could therefore not assess whether the risk of this susceptibility haplotype might also differ by sex.

10.5.5 Conclusions and future research

Similar to other MS samples, we observed a female excess of MS that was slightly higher for subjects who had an early onset of MS compared to those who were diagnosed at a later age. The question is what might be causing this difference in onset between females and males. We found some evidence that having a later puberty development was positively associated with MS, which was stronger in males than in females. Also, among women, having had children prior to MS onset was associated with a strong decreased risk of MS, and this effect did not seem to be mediated through or modified by breastfeeding. Having children, however, not only mediates an endocrine effect, but is also accompanied with lifestyle changes, such as less smoking, less employment and higher infection load through contact with other children. It will be important to separate endocrine effects of pregnancies and lifestyle related changes. We also examined whether there were sex differences in prevalence and strength of particular risk factors observed in this case-control study. No significant sex differences in prevalence and risk estimate were found for younger sibling number, EBV-related factors and actinic damage. Although females more often had low levels of sun exposure at age 6-15 years in summer during weekends and holidays, their risk estimate was lower compared to that of males and the combined effect could not explain part of the female excess.

For most aspects examined in this chapter, there is a lack of data. Future analytical studies could be conducted that not only replicate some of our results, but also improve them by increasing the sample size and by including multiple and/or better measures. Puberty development and risk of MS can be assessed by using questionnaire measures and/or biomarkers. The issue of having children requires separating pregnancy-related endocrine effects from child-related lifestyle effects. This can be accomplished by, for example, comparing females and males, including specific exposure factors around the period of childbirth (e.g. childcare attendance of the child, number of people in the household as a measure of infection load), and assessing factors prior to and after birth so change in lifestyle factors can be assessed (dietary intake, tobacco smoking, employment and sun exposure behaviour). In addition, future or even previous studies that examine(d) environmental or genetic factors that have substantial evidence to be causally related to MS, could conduct a

separate analysis by sex to examine whether this factor might contribute to the female excess of MS.

10.6 SUMMARY

The excess of MS among women is a striking epidemiological feature. Although animal research has examined differences between females and males, epidemiological studies have mainly focused on women by studying the effects of different stages in the reproductive system on disease risk and progression, and by examining the effects of medications such as oral contraceptives and hormone replacement therapy on MS risk and progression. Both types of studies provide some evidence for involvement of sex-related hormones such as testosterone and estrogen. In this chapter we used the data of the Tasmanian MS case-control study to examine the female to male ratio by age, the association between puberty development and MS, the association between having had children prior to MS onset among women and MS, and differences in exposure levels and risk estimates for past summer sun exposure during weekends and holidays at age 6-15 years, actinic damage, younger sibling number and Epstein-Barr antibody titers between females and males. We found some evidence that having a later puberty development was positively associated with MS, which was stronger in males than in females. Among women, having had children prior to MS onset was associated with a strong decreased risk of MS, and this effect did not seem to be mediated through or modified by breastfeeding. No significant sex differences in prevalence and risk estimates were found for younger sibling number, Epstein-Barr virus-related factors and actinic damage. Although females more often had low levels of sun exposure at age 6-15 years in summer during weekends and holidays, their risk estimate was lower compared to that of males and the combined effect could not explain part of the female excess. Future analytical studies could replicate some of these findings, and improvements could be made by including multiple and/or better measures.

10.7 POSTSCRIPT

The last chapter will discuss the main conclusions of the thesis, future research that might assist in the causal inference of the finding regarding UVR exposure and the public health implications that this finding might have in the future.

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Chapter 11

Main findings, future research and public health implications

11.1 MAIN FINDINGS CONCERNING UVR AND MS

The primary goal of this thesis was to examine whether past sun exposure played a protective role in the development of Multiple Sclerosis (MS). New data were generated by using an ecological approach. We found that the regional variation in MS prevalence in the continent of Australia could be closely predicted by regional ultraviolet radiation (UVR) levels. More importantly, the case-control study, that was conducted as part of this thesis, found that higher sun exposure during childhood and early adolescence, and actinic damage – a biomarker of sun exposure – were both associated with a reduced risk of MS. These findings are compatible with UVR having a protective role against MS.

11.2 CAUSAL INFERENCE WITH REGARD TO UVR AND MS

Throughout the thesis we have provided different types of evidence, either consistent or inconsistent with the hypothesis of UVR having a protective role against MS. Criteria of Hill,[1] proposed in 1965, are useful to evaluate the credibility that the relationship between past sun exposure and MS is causal.[2] We will discuss a number of these criteria (written in italics) in relation to the association between past sun exposure and MS, and will discuss where future research can contribute to greater knowledge about the causality of the association.

The review of UVR and MS (chapter 3) showed that there is good biological plausibility for the association, because studies on UVR and immune function and laboratory-based evidence using animal models of MS suggest that UVR seems to play a protective role in MS. There is a certain amount of coherence in the descriptive epidemiological data of UVR and MS such as the gradient of MS prevalence with increasing latitude, and some seasonal variation in month of birth, relapses and number of active lesions in people with MS. The coherence is also supported by the finding observed in this thesis (chapter 4) that the regional variation in MS prevalence in the continent of Australia could be closely predicted by regional UVR levels. Our case-control study found (chapter 8) a strong (strength) association between past sun exposure during childhood and early adolescence, measured by questionnaire and calendar, and MS with evidence of a dose-response relationship (biological gradient). An association of similar strength and dose-response was found between a biomarker of sun exposure, actinic damage, and MS. This finding is consistent (consistency) with the only other case-control study on sun exposure and MS, although this study was different in that it used MS mortality as their outcome factor and sun exposure was estimated by evaluating residential UVR levels and type (indoor or outdoor) of occupation. Although there is some analogy with the finding of a protective effect between vitamin D supplementation in the first year of life and subsequent onset of type 1 diabetes mellitus, another disease which is thought to be a Th_1-mediated autoimmune disease, the amount of data from analytical studies is insufficient to fully satisfy the criteria of consistency, where repeated observation of an association in different populations and different circumstances strengthens the causal inference. In addition, a prevalent case-control study is not the strongest design to resolve the criteria of temporality, the necessity that the cause precedes the effect in time. Although the calendar data provides

us with information on timing of sun exposure, recall bias could have affected the measures. In addition, the objective marker of actinic damage also included sun exposure after disease onset and adjusting for sun exposure after disease onset might not have completely removed the effect. Further, among cases, we did not find a temporal pattern between sun exposure during childhood and early adolescence or actinic damage and age at onset.

The criterion of specificity has not been regarded as terribly important in the past.[2] It referred to the idea that the more broadly an exposure is associated with a variety of outcomes, the less likely it is to be causally associated with any one of them. In 2001, however, Weiss proposed a modified version of the concept.[3] There might be a good basis for predicting that only some outcomes are related to a given exposure, or that only one or several types of exposure are related to a given outcome, or that only persons with a certain characteristic have the relationship between exposure and outcome. When the observations made in epidemiological studies conform to those expectations, the credibility we assign to a causal hypothesis increases.[3] Conversely, when a causal hypothesis predicts specificity, but that is not what is observed, the credibility of that hypothesis is diminished.[3] We can apply this to our situation. In our study, we found, with prior expectation, an inverse association between sun exposure during childhood and early adolescence and MS, but not for sun exposure during adulthood or sun exposure at particular years prior to MS onset.

There is currently no experimental evidence for the association between sun exposure and MS, because intervention studies are usually mounted when the evidence for causality is very strong. For the future, an intervention study (randomised clinical trial) by using, for example, a group of people that are of high risk of developing MS and influencing their childhood sun exposure behaviour does not seem a feasible ethical option. The administration of vitamin D supplements might be slightly more feasible, but still, such a study is difficult to conduct given the large variation in age of onset, and the consequent long follow up that is required, as well as the rarity of the disease. Similar problems occur when a prevention program is aimed to be used to assist on causal inference.

11.3 FUTURE RESEARCH WITH REGARD TO UVR AND MS

Future research should focus on a replication of results, preferably using a design that can improve causal inference. A cohort study would be the preferred option, because it assesses exposure prior to disease onset and addresses the temporality criteria. There are some cohorts in the United States and the United Kingdom that have been followed for many years and include people diagnosed with MS. However, they do not seem to have collected data on sun exposure prior to MS onset. As an alternative marker of sun exposure, vitamin D serum levels could be assessed if serum samples were stored. According to the website of the US MS Society, serum vitamin D levels are currently being analysed in samples taken prior to disease onset in at least one of the cohorts in the United States.[4]

An incident case-control study would be another alternative. This type of study would avoid problems associated with post-disease phenomena. We are currently conducting a multicentre incident case-control study in four locations across Australia: Brisbane (27°S), Newcastle (32°S), Geelong (38°S) and Tasmania (43°S). This study will not only examine whether past sun exposure is associated with MS (at four different locations) using similar measures to those used in the Tasmanian MS case-control study, but will also re-examine the latitudinal gradient of increasing MS incidence with increasing latitude, and will assess whether there is potentiation between high sun exposure behaviour and low residential latitude with regard to reducing the risk of MS. That is, according to the results of the only other case-control study conducted on sun exposure and MS,[5] the protective effect of outdoor activity might be more

evident in a high residential UVR location such as Brisbane (27°S) than lower UVR locations such as Geelong (38°S) and Tasmania (43°S).

Another area of future research might be the examination of interactions between past sun exposure and genes. MS is a multifactorial disease where both environmental and genetic factors influence the development of MS and also interact with each other to cause disease. In 1987, Khoury et al.[6] stated that in the presence of gene-environment interaction, quantifying the main effects of environmental factors alone or genetic factors alone can lead to mis-specification of the causal model, and may miss important clues to the aetiology of disease. Despite this long standing awareness of these aspects of disease risk, the ability to investigate the combined effects of exposure and genotype on the risk of disease, has, until recently, been limited and a clear picture of these relationships is yet to emerge. A full understanding of the combined effect of genotype and environmental exposure requires the accurate quantification of the risk of disease associated with exposures of interest, for individuals with and without a particular genotype, and quantification of the effect of genotype on the risk of disease in people with and without particular exposures.

Examining interactions for MS between past sun exposure and genotype may help to elucidate the mechanism underlying the inverse association and may also contribute to the credibility of the causal relationship of past sun exposure and MS through the criteria of specificity. If results conform to the prior expectation that the association between past sun exposure and MS is present or stronger in a group with a particular genotype and absent or weaker in a group without that genotype, then that increases the credibility that the association between past sun exposure and MS is causal. For example, a study in a Japanese population found that individuals with particular vitamin D receptor gene allelic variants were at increased risk of MS.[7, 8] We might expect that the effect of past sun exposure is stronger in those with these particular vitamin D receptor gene allelic variants compared to those without these variants.

We have the opportunity to screen for gene-environment interactions in the study described in this thesis. The concurrent genetic study included the same cases as the Tasmanian MS case-control study. It used a different source of controls. Because neither set of controls had both environmental and genetic measures, we cannot examine gene-environment interaction using a traditional case-control approach. Several non-traditional approaches have been developed to detect gene-environment interaction.[9] One of these approaches is the case-only design.[10] This method assumes that the exposure and genetic factor occur independently in the general population.[10] The advantage of this design is that it has a higher power than the traditional case-control approach, but the disadvantages are that it cannot estimate the genetic and environmental main effects and that it could miss interactions that were present within an additive model.[9] Umbach and Weinberg have proposed an incomplete-data case-control design which uses a log-linear model.[11] It requires both genotype and environmental exposure data from the cases but only environmental exposure or only genotype data from two distinct groups of controls.[11] We will apply this method to our data, but ultimately, when interactions are found, the assumption of independence between exposure and genotype in the general population should be tested by obtaining exposure data on the controls for whom we already have genetic data, or alternatively, by obtaining genetic data of the controls for whom we already have environmental data. In addition, the Australian multicentre incident case-control study will also have the opportunity to examine gene-environment interactions in the near future.

Other types of research might supplement the causal inference by improving the plausibility, coherence and/or specificity. Laboratory based studies on animals could further assess the effects of administration of UVR (and/or vitamin D) on EAE onset, progression and immune

response. In specimens from people with MS or with a first demyelinating event, associations could be assessed between UVR-related markers (such as serum 25(OH)D and calcitonin related peptide), immune response (such as interleukin-10 and interferon-γ) and antibody levels against myelin proteins. Having antibodies against myelin proteins has recently been shown to be predictive in patients with a first demyelinating event of conversion to clinically definite MS.[12] We are currently conducting a study examining the effect of recent sun exposure on the progression of MS in our clinical cohort, and work is in progress in the United States examining the relationships between vitamin D supplementation and immune response in people with MS using a randomised double blind design.[4, 13]

11.4 PUBLIC HEALTH IMPLICATIONS OF THE INVERSE ASSOCIATION BETWEEN UVR AND MS

If the association between past sun exposure and MS proves to be causal, it has potentially important public health implications. In our study, sun exposure in winter seemed more important than sun exposure in summer. A large part of the risk reduction exists already for rather low levels of sun exposure: 1-2 hours of winter sun during weekends and holidays provided a significant reduction in risk compared to less than 1 hour a day. As was outlined in the chapter 3 and 8, an increased risk for low levels of UVR and/or vitamin D levels has also been implicated for various cancers (e.g. prostate, colon, breast, ovary, non-Hodgkins lymphoma), type 1 diabetes mellitus, tuberculosis, schizophrenia and bone related diseases (rickets, fractures).

Current public health messages are aimed to reduce disease due to excessive exposure to UVR such as skin cancer.[14] This is important as at least 90% of melanoma is estimated to be attributable to excessive UVR exposure.[15] Behaviour changes have a large impact on personal UVR exposure. A 100-fold difference in personal UVR exposure has been shown to be related to behavioural factors.[16] Current public health messages on skin cancer, however, do not take into account regional differences, seasonal variation and skin pigmentation. There are large regional differences in Australia. For example, the mean daily effective UVR mid-winter is 18.2 mean erythemal doses (MEDs) in Darwin (12°S), 7.9 MEDs in Brisbane (28°S) and 1.6 MEDs in Hobart (43°S). In addition, the mid-summer to mid-winter ratio of daily effective UVR, a measure of seasonal variation, is much lower for Darwin (1.1) and Brisbane (3.0) than for Hobart (12.8).[17] The amount of vitamin D produced in the body also differs according to the latitude and time of the year. In Boston (42°N), the conversion of 7-dehydrocholesterol to precholecalciferol (previtamin D_3) was greatest in June and July, gradually declined after August, and between November and February exposure to sunlight on a cloudless day for five or less hours a day did not result in any significant production of precholecalciferol.[18] In Edmonton, Canada (52°N), this period of ceased production of precholecalciferol lasted from mid-October until mid-April, while for Los Angeles (34°N) and San Juan, Puerto Rico (18°N), the cutaneous production of precholecalciferol occurred throughout the year.[18] Deeply pigmented skin is estimated to have a minimal erythemal dose that is 33-fold higher than the fairest skin. Put in other terms, deeply pigmented skin is estimated to have a sun protection factor of 13.4, almost equivalent to a fair-skinned person constantly wearing SPF 15 sunscreen applied to the whole body. Compared to fair skin, the dose required for vitamin D synthesis increases substantially with increasing skin pigmentation— a sixfold increase in exposure time for very black skin, and two fold increase for Asian skin.[19] Other factors that influence the production of vitamin D_3 in the body are time of the day, altitude, ozone air pollution and aging of the skin.[20]

The public health messages aimed to reduce excessive UVR exposure and prevent insufficient UVR exposure do not need to contradict each other. There is a wide gap between

the UVR dose that is required to synthesise sufficient vitamin D cutaneously and the higher dose that will cause skin and eye disorders. For example, exposure of 6-10% of the body surface (face, hands and arms) to sunlight at noon in Boston (42°N) in spring, summer and autumn for five minutes two to three times a week is thought to be sufficient to maintain optimal vitamin D levels in a person with moderately fair complexion.[21] In contrast, at a higher ambient UVR location, Geraldton Australia (29°S), a person with maximal risk for basal-cell carcinoma may have been receiving as much as 14 hours of sun exposure per week for every week of life.[22] Thus, for any individual, it should be possible to obtain the beneficial effects of adequate vitamin D levels from appropriate UVR exposure, without risking skin and eye disease from excess exposure.

If vitamin D proves to be one of the pathways through which UVR mediates its effect on MS, then vitamin D supplementation could become part of the public health program to reduce MS. There is currently debate about the recommended daily intake of vitamin D_3 and the maximum amount that can safely be given. There is evidence to suggest that the current recommended daily intake for vitamin D_3 is too low (200-400 IU (5-10 μg)/day for infants, 200 IU (5 μg)/day for adults up to age 50 years, 400 IU (10 μg)/day for adults age 51-70 years, 600 IU (15 μg)/day for adults over age 70 years).[23] In addition, supplements in Australia contain vitamin D_2 rather than vitamin D_3. Vitamin D_3 has not been approved in Australia and vitamin D_2 seems only half as effective as vitamin D_3.[23]

There is a need to fully understand the spectrum of related health effects so that appropriate advice can be given with regard to personal UVR exposure and vitamin D supplementation. Our work will hopefully contribute to this difficult task.

11.5 OTHER MAIN FINDINGS OF THIS THESIS

Apart from the inverse association between UVR and MS, the Tasmanian case-control study also showed positive associations between infectious mononucleosis, high IgG antibody titers to Epstein-Barr nuclear antigen and high IgG antibody titers to Epstein-Barr virus capsid antigen and MS. These data are consistent with other studies and adds to the growing body of information that the Epstein-Barr virus is causally related to MS.

We found some novel results by conducting a detailed analysis of sibship structure and MS. Having younger siblings, but not older, was strongly associated with a reduced risk of MS in a dose response manner, and the effect was strongest when the interval to the next sibling was small. This might indicate that not primary infections in the first year of life of the subject are of critical importance but rather that repeated stimulation of the immune system through early life infections of the younger siblings are important. Also, among cases, an age at onset pattern was observed in that younger sibling number was associated with a later onset of MS.

Table 1 shows a model that includes the most important factors found in this study (Table 1). Based on the analyses in this thesis, it is reasonable to consider them as independent predictors. It shows that serum IgG antibody titers to EBNA, having younger siblings, sun exposure at age 6-15 years and melanin density at the upper inner arm are the strongest predictors of MS, while smoking and education levels are no longer predictors of MS. For risk at an individual level, many different situations can occur, depending on whether someone has or does not have the individual risk factors. For example, the risk of MS of somebody with both high antibody titers to EBNA and low levels of sun exposure at age 6-15 years is 53.3 times (11.19 * 1/0.21) higher compared to someone with low antibody titers to EBNA and higher levels of sun exposure at age 6-15 years.

Other new data that arose from the case-control study was that among women, having had children prior to MS onset was strongly associated with a decreased risk of MS, and this effect did not seem to be mediated through or modified by breastfeeding.

We examined the reliability of a large number of measures used in the case-control study. Importantly, there was no evidence that the reliability for different exposure measures was lower in cases compared to controls, and among cases there were no differences in reliability by subgroups of disease related factors such as disability (EDSS score), disease duration or type of MS. This suggests that, even if there was some degree of cognitive dysfunction among cases, it was unlikely to affect the results of the Tasmanian MS case-control study. These findings might also be applicable to other case-control studies.

Table 1. Univariate and multivariate results for Multiple Sclerosis and the most important factors found in the Tasmanian MS case-control study.

	Univariate results Unadjusted OR {95% CI}	Multivariate results Adjusted OR {95% CI}
Sun exposure at age 6-15 years (≥2-3 hrs vs <2-3 hrs per day)	0.39 {0.22—0.70}	0.21 {0.06—0.69}
Actinic damage (grade 4-6 vs grade 3)	0.39 {0.17—0.90}	0.32 {0.09—1.20}
Melanin density at the upper inner arm (<2% vs ≥2%)	1.59 {0.99—2.55}	2.87 {1.19—6.91}
Having younger siblings	0.47 {0.30—0.74}	0.21 {0.08—0.56}
Self-reported history of teenage IM	3.88 {1.88—7.99}	2.37 {0.60—9.32}
Serum IgG antibody titers to EBNA (>2.50 vs ≤2.50 units)	5.69 {3.33—9.71}	11.19 {4.07—30.72}
Serum IgG antibody titers to VCA (>2.00 vs ≤2.00 units)	1.97 {1.15—3.40}	2.19 {0.72—6.70}
Ever smoker prior to the age of diagnosis	1.53 {0.99—2.36}	1.17 {0.53—2.58}
Education level (per category increase)	1.24 {0.99—1.55}	1.27 {0.81—1.98}

Notes

1. The multivariate analysis shows the adjusted odds ratios using a model that includes all factors shown in the table.
2. IM = infectious mononucleosis; EBNA = Epstein Barr virus Nuclear Antigen; VCA = Viral Capsid Antigen
3. Education levels: No formal education, primary school; Secondary school until year 10; Higher secondary school, TAFE, trade and apprenticeship; University

In a separate study using 104 healthy volunteers, we found that the spectrophotometric assessment of skin type at the upper inner arm was somewhat affected by the season in which the measurement was taken, and at the buttock, the measurements were influenced by body hair among men. Both sources of error can be reduced by careful attention to key aspects of study design, and the effect of these two types of error was estimated to be small in our case-control study.

11.6 Overall limitations of the Tasmanian MS case-control study

As was outlined in chapter 5, the Tasmanian MS case-control study was carefully designed and conducted. Interviewer bias was limited by using a standardised questionnaire and interview protocol. The participation rates for cases and controls were high, reducing non-response bias, although it is conceivable that some selection bias may have occurred. Simple questions and objective measures were used where possible, aiming to reduce measurement error. A possible weakness of the study was that prevalent, not incident cases were studied, and recall bias and disease-related changes could have influenced the results. Although these

weaknesses are inherent to the type of study, we were able to examine the effect of possible recall bias (by using a question on the subjects' view on the importance of a number of factors as a possible cause of MS) and disease-related changes in behaviour on our findings in the analysis stage. The sample size of 136 cases and 272 controls was sufficient to determine modest main effects, which was the primary aim of this study. However, the study power was limited to determine significant interactions. A large number of factors were included in this study. Although the effect of sun exposure, infections and sibling structure on MS were the main hypotheses chosen prior to the start of this study, it is possible that some other positive findings might have occurred by chance. For example, the finding that having had children was associated with MS was not a specific a-priori hypothesis, we did not observe a strong dose-response relationship and the size of the sub-sample of women on which we had information on the history of childbirth and breastfeeding was relatively small.

11.7 THE FUTURE OF EPIDEMIOLOGICAL RESEARCH OF MS

This thesis might have contributed to finding one or more possible causal factors of MS. As was already mentioned in a previous section, future research should focus on a replication of results, preferably by using a design that can improve the causal inference. This is important for the association between past UVR and MS, the apparent protective effect of younger sibling number and MS, and the negative association between having had children prior to the age of onset and MS. In addition, the underlying biological mechanism of younger sibling number deserves attention, because there might, for example, be a link between the negative association between having younger siblings and MS and the positive association between the Epstein-Barr virus and MS.

With the already known genetic and environmental risk factors, it seems that the aetiology of MS is starting to unravel. New developments in areas such as diagnostic precision, genetic information, computer technology and improved measures for epidemiological research will remain important for finding new environmental and genetic causal factors. At the same time, it will be important to examine the combined effects of (putative) causal factors, because MS is a multifactorial disease where both environmental and genetic factors influence the development and also interact with each other to cause the disease. Interactions between environmental factors should be assessed, between genetic factors and between both genetic and environmental factors. By examining those interactions and by integrating immunological and pathological knowledge of MS, a more complete picture of the aetiology of Multiple Sclerosis will hopefully emerge.

11.7 REFERENCES

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