Gender differences in multiple sclerosis epidemiology and treatment response

Melinda Magyari

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Tutor(s): Nils Koch-Henriksen & Per Soelberg Sørensen

Official opponents: Kjell-Morten Myhr & Maria Trojano

Correspondence: Department, Danish Multiple Sclerosis Center, Department of Neurology, Copenhagen University Hospital, Rigshospitalet, 2100 Copenhagen, Denmark

E-mail: melinda_magyari@dadlnet.dk

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INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease [Compston and Coles 2002] of the central nervous system (CNS) that typically affects young people and is a major cause of disability development in the young and middle-aged. The first description of MS dates back to the 14th century, but it was Charcot who first described the correlations between the clinical features of MS and the pathological changes noted post-mortem [Lublin 2005].

The aetiology of MS is not fully elucidated, but the evidence points towards MS being a multifactorial disease where genetic [Compston 2000;Nielsen et al. 2005], and environmental factors interact.

There is an unequal distribution of MS regarding geography and gender [Milo and Kahana 2010]. Concerning the geographical distribution, it is widely accepted that the prevalence is higher in temperate zones. However the latitudinal worldwide distribution has been reappraised, challenging the traditional view of a North-South gradient in Europe and North America [Koch-Henriksen and Sorensen 2010;Zivadinov et al. 2003]. It is also well known that MS, presumed to have an autoimmune pathogenesis similar to many other inflammatory diseases, has female prevalence preponderance [Nussinovitch and Shoenfeld 2012]. Recent serial cross-sectional assessments have reported an increasing incidence of MS in women [Alonso and Hernan 2008]. In Denmark the MS incidence rates have been monitored since 1948. Data from the Danish Multiple Sclerosis Registry indicate an increase in the female to male ratio from 1.3:1 in 1950 to 1.5:1 in 1977 and to 2.02:1 in 1990 [Bentzen et al. 2010]. A new update is on the way.

Historically, in the beginning of the 20th century, MS was considered a male predominant disease [Brain WR. 1930] as it was believed to be more important that men being the breadwinner of the family, remained capable of functioning and were therefore diagnosed more frequently, also the misdiagnosis of hysteria in women was not uncommon at the same time.

In the 1940s the sex ratio started to reverse, with studies describing a ratio of 1:1 [Talley 2005]. Later, articles from many different countries including Canada [Orton et al. 2006], Sardinia [Pugliatti et al. 2009], Japan [Osoegawa et al. 2009] and Iran [Maghzi et al. 2010], began to document an evidence of female excess. A comparison of the changes in sex ratio among MS populations from different geographical areas reported a sex ratio increase over time in relapsing remitting multiple sclerosis (RRMS) and also demonstrated a latitudinal gradient of this increase [Trojano et al. 2012]. Few studies, such as those from Nord-Trøndelag County, Norway [Dahl et al. 2004], and Greater Hobart in Australia [Simpson S Jr et al. 2011], failed to document any disproportionate changes in the sex ratio, and a study from Olmsted County in the US reported a plateau of the incidence increase in both genders [Mayr et al. 2003]. Previously studies from The Swedish multiple sclerosis register reported an increase in MS incidence, but a stabile sex-ratio [Bostrom et al. 2013b], however a recent update of the sex ratio since the 1930's showed an increasing tendency [Bostrom et al. 2013a].

The underlying cause of the growing female preponderance is not yet clear, but the speed of the changes suggests environmental factors acting at the population level as well as possible epigenetic modification of HLA-DRB1*1501 [Chao et al. 2009;Chao et al. 2011].

The increasing incidence in women was reported for RRMS, but was not seen for the primary progressive multiple sclerosis (PPMS) [Celius and Smestad 2009;Debouverie et al. 2007]. This observation suggests that environmental factors trigger pro-inflammatory mechanisms. Regarding paediatric MS the female preponderance is reported mostly after the age of 10, suggesting

that the environmental factors may play a role in the pre-puberty [Mikaeloff et al. 2006].

In Denmark the risk of developing MS in women has doubled since the 1970's, whereas only a small increase for men has been observed [Bentzen, Flachs, Stenager, Bronnum-Hansen, and Koch-Henriksen2010]. The genetic composition of the population is considered constant over generations, supporting the role of the exogenous factors in the disease aetiology and could be derived from women's changing lifestyles over the past 50 years.

The difference in MS incidence trends between the sexes could indicate that women may be more sensitive to hypothesised environmental changes. It cannot currently be ruled out that men have been subjected to the same lifestyle changes, but because of possible biological gender differences they are not affected to the same degree by the same factors.

The putative changing environmental factors may be related to the lifestyle of Western females, which have changed over the last half century. Examples of this are cigarette smoking, changes in dietary habits, obesity, early age of menarche, oral contraceptive use, hormone replacement therapy, later and fever childbirths, changes in type of occupation, and better housing conditions.

A number of environmental factors have been investigated, such as infections, the use of oral contraceptives, smoking, obesity and dietary habits. Several environmental factors have been found to be associated with MS:

Late infection with the Epstein-Barr virus (EBV) causing mononucleosis is a predisposing factor to developing MS by a factor 2-3 [Ascherio and Munger 2007;Nielsen et al. 2007]. Having mononucleosis along with HLA-DRB1*15, increases the risk of MS by a factor of 7 [Nielsen et al. 2009].

Several studies have shown that smoking is a risk factor for MS. A recent Swedish study has shown that smoking increases the risk of MS in both sexes, and the risk increases with the cumulative dose of smoking [Hedstrom et al. 2009]. It is known that particularly women's tobacco consumption has increased since the second half of the 20th century.

The inverse correlation between daily dose of sunlight and the MS prevalence is documented by several epidemiological studies [Orton et al. 2011]. Sunlight influences the level of Vitamin D in the blood; however, high actinic-skin damage caused by sunlight and Vitamin D levels were independently associated with the risk of MS [Lucas et al. 2011]. Changing sun-habits cannot explain the MS incidence increase in women, because there is nothing to suggest that Danish women's exposure to sunlight has been decreasing. The last 30 years the number of Danes diagnosed with skin cancer is doubled [Fuglede et al. 2011].

Epidemiological and experimental studies suggest that high serum vitamin D levels reduce the risk of MS [Spach and Hayes 2005]. There are also studies suggesting that vitamin D has greater immunomodulatory effects in women and thus a better protective effect against developing MS [Kragt et al. 2009].

Urbanisation, probably associated with some of the above mentioned circumstances, has proven to be a significant co-factor for the increased female incidence of MS [Kotzamani et al. 2012], However growing up in an urban centre was shown to be associated with a lower risk of developing MS in a case-control study conducted in Berlin [Conradi et al. 2011], and in a Sardinian province the prevalence was significantly higher in rural more traditional areas than in urban territories where the "westernisation" process is more noticeable [Sotgiu et al. 2003].

Studies from Iran [Maghzi, Ghazavi, Ahsan, Etemadifar, Mousavi, Khorvash, and Minagar2010] and Kuwait [Alshubaili et al. 2005] have reported increasing female incidence in spite of probably less pronounced changes in women's lifestyle.

One of the major changes in the contemporary woman's life is the tendency to have fewer children and have them later in life than their grandmothers. Because of the temporary immunosuppression during pregnancy [McCombe and Greer 2012], pregnancy may exert a protective effect against MS in women, and a higher age at giving birth to the first child or fewer pregnancies may have its share in the increasing incidence of MS in women.

Based on the available evidence it is not easy to explain the gender differences from a genetic aspect [Harbo et al. 2013]. Regarding the clinical features of MS, there are differences between men and women. Females have an earlier onset, fewer of them have PPMS [Runmarker and Andersen 1993], and general less progression to disability [Debouverie 2009]. Longitudinal follow-up studies of large patient populations reported that sex is a prognostic variable [Runmarker and Andersen1993;Weinshenker et al. 1991]. Male patients had a shorter time to reach a requirement for assisted walking devices and a higher risk of primary-progressive disease, both of which are factors associated with a poorer prognosis [Weinshenker, Rice, Noseworthy, Carriere, Baskerville, and Ebers1991]. Regarding gender effects on treatment efficacy, the results are conflicting, some studies showing no significant gender-related differences [Rudick et al. 2011], while others report significant gender effect on treatment response to Interferon-beta (IFN-β) therapy [Trojano et al. 2009].

It could be hypothesised that the gender differences in incidence changes may also be reflected in the disease course and probably in treatment response.

The question whether the incidence increase in women is reflected in higher disease activity or a different response to immunomodulatory treatment is not sufficiently elucidated.

Revealing and understanding the factors that contribute to the epidemiological changes and revealing gender differences in treatment response could provide an insight into the pathogenesis and may give us a chance to prevent this devastating disease. Because every Danish citizen has a unique personal identification number, linkage between population registers and registers on diseases is possible. This provides us the possibility to investigate a number of demographic, physical, social and health related factors that could play a role in the increasing incidence of MS in women. According to the Act on Processing Personal Data (Registerloven), working with individual level data requires full confidentiality and anonymity.

OBJECTIVE

The aim of the study was to investigate various aspects of gender differences of MS, focusing on exogenous factors that may influence the risk of MS, which are specific to females and can be examined at the individual level.

Hypotheses of article I

Maternal age at first childbirth and the number of births has an effect on the risk of developing MS in women.

Hypotheses of article II

Occupational physical exposures, level of education, and housing conditions in early adulthood influence the risk of developing MS differently in women and men.

Hypotheses of article III

The incidence increase of MS in women is reflected in a higher female risk of other autoimmune diseases.

Hypotheses of article IV

There are gender differences in the response to IFN-β therapy.

MATERIAL AND METHODS

STUDY POPULATION

A cohort of all MS patients with definite MS according to the criteria of McDonald 2001 [McDonald et al. 2001] with onset from 2000 to 2004 was drawn from The Danish Multiple Sclerosis Registry. The distribution regarding disease course was: 16.3% (241) PPMS and 83.7% (1242) RRMS. We chose all MS patients with clinical onset within the age interval 15-55 years. This study population consisted of 1,403 MS cases and 35,045 matched controls with a female: male ratio of 2.02:1. The mean age at clinical onset of MS was 35.3 years, (36.1 years for men and 34.9 years for women).

For each MS case, 25 control persons were drawn at random from The Danish Civil Registration System, individually matched for sex, year of birth and residential municipality at the reference year, defined as January 1st in the year of onset of the first symptom of the matched MS case. The large sample was chosen to obtain more accurate parameter estimates, which leads to greater precision and higher power to correctly find true associations between exposure and disease. Each case/control pair then had identical values on the matching factors for control of confounding. Patients and control subjects are below referred to as index persons. Matching was not performed for social belonging, as it would eliminate the chance of determining social differences between patients and controls. This provides the chance of estimating a more recent association between MS and social factors, although it has previously been shown that there are no socio-economic differences between MS patients and the general population in Denmark [Koch-Henriksen 1989a].

Index persons not born in Denmark were excluded from the study because of their different genetically susceptibility to MS and environmental exposures other than those common in Denmark could lead to confounding. Men were included as a reference for both the case and control groups. The total population consisted of all index persons, children and household members.

STUDY DESIGN

The studies I-III was designed as a case-control study with MS/non-MS as the outcome variable and the different environmental factors as the exposition. Study IV was an observational cohort study including all patients with RRMS who started treatment with IFN-β from 1996 to 2003.

Data Sources

Danish registers contain information on many important health and social issues on the individual level. All data are obtained by independent registries.

MS cases were identified through The Danish Multiple Sclerosis Registry and control persons from The Danish Civil Registration System via Statistics Denmark. The possibility of linking data from different registers and databases by the unique CPR-number made it possible to analyse the effect of different exposures on the disease. We mined individual data for each year up to 25 years back in time, and for some variables prospectively up to 10 years after clinical onset, from the following registries.

The Danish Multiple Sclerosis Registry was started in 1949 as a nationwide prevalence survey, but it was formally established in 1956. Since 1948 the Registry has collected data on all MS cases from multiple sources [Koch-Henriksen et al. 2001]. At present there are more than 20.000 cases with a proven diagnosis registered.

From 2001 the criteria of McDonald were used. All of the cases included in the study were classified by the same criteria. During the existence of The Danish Multiple Sclerosis Registry, the records of suspected MS cases have been reviewed by one of only three neurologists: Dr. Kay Hyllested; Dr. Nils Koch-Henriksen and Dr. Egon Stenager, which ensures certain homogeneity. The Danish MS registry has an estimated validity of 94% and a completeness of >90% [Koch-Henriksen and Hyllested 1988].

The Multiple Sclerosis Treatment Registry was established in 1996 when the first immunomodulatory treatment was approved in Denmark [Koch-Henriksen and Sorensen 2000]. The task of the registry is to monitor the quality of the immunomodulatory treatment of MS in all neurological departments in Denmark and to collect data for research projects. Prospective clinical parameters are reported for all MS patients in Denmark who receive immunomodulatory or immunosuppressive treatment. Among the reported data are: dates of relapses, neurology clinical status assessed by the Expanded Disability Status Scale (EDSS), side effects, and the presence of neutralizing antibodies (NAbs) against IFN-β [Sorensen et al. 2006a].

The Danish Civil Registration System is a nationwide civil register, established in 1968. All citizens who were alive in 1968 or have been born since are registered with a unique and life-long 10-digit code (CPR- number). The codes are used by all local or governmental institutions including the tax authorities and the banking system. The purposes are to administer general personal data reported from the national registration offices [Pedersen 2011]. Names, addresses, marital status, birth registration place and other basic information has been systematically registered for every person with residence (present as well as past) in Denmark or Greenland. The Civil Registration System includes references to parents and spouses, making it possible to establish the family unit. The 35,045 control persons were drawn by random from this database by the matching criteria.

The Fertility Database (FTDB) covers the total population aged 15-49 years on January 1st every year (since 1980) and contains information about parity measures [Blenstrup and Knudsen 2011]. Information about pregnancy complications, pregnancy losses, induced abortions and infertility was linked to the study database from this registry. Data about infertility was supplemented from The Danish Infertility Cohort where infertility data were recorded between 1963 and 1993.

The Danish National Patient Register (NPR) was established in 1977 and contains information about hospital admissions since 1977 [Lynge et al. 2011]. Outpatient-hospital contacts have also been included since 1995. From 1977 to 1993 the diagnoses were made in accordance with The International Classification of Diseases version 8 (ICD 8) and from 1994 according to the International Classification of Diseases version 10 (ICD 10). The analysis in Study III about autoimmune comorbidity was based on the registered diagnosis enrolled from NPR. Index persons were classified with a diagnosis with autoimmune disease if they had been admitted into a hospital or had been treated in an outpatient clinic with the main- or secondary discharge diagnosis of an autoimmune disease.

The National Prescription Register is an individual-level register containing data on all prescription drugs sold in Danish community pharmacies since 1994 [Kildemoes et al. 2011]. We obtained information about the use of oral contraceptives, and antidiabetic and asthma medications in individuals.

The Register of educational programmes was established in 1970 and contains information for each subject on all educational programmes as: Ongoing education, a completed education, final

leaving level measured by the minimum number of months at school from 1. class to the completion of the education and a measure of the kind of qualifications which the education gives. The Register provided data about the highest obtained educational level at each year and about the length of the education received.

Register of Buildings and Dwelling contains a systematically registration of housing and building conditions dating back to 1977. The registry provided information about dwelling size, sanitary installations, size and type of households for each year.

The Danish Industrial Classification Database contains sociodemographic information on the entire Danish population, including each individual's attachment to the labour market and workplaces. Data on a person's occupation are available through the DISCO, a Danish version of the International Standard Classification of Occupations (ISCO). The information is obtained from the employers obligatory reports of the employees' occupation to the Salary Statistics with the use of the DISCO codes. Each Danish firm has a unique identification number which makes a link between the firm and the employees possible. Information about the index-persons' occupation in different registered industries was obtained from this database. In 1995 the database was reorganised and new categories were defined. We chose to determine on the occupational exposure only until 1995 because our attempts at grouping different exposures into the same categories yielded uncertain results.

Personal Income Statistics contain information about average total income, personal income, gross income (income liable to general taxation calculated by Statistics Denmark), taxable income, taxes and the like since 1977. We obtained information about the mean yearly total pre-tax income for each year.

The Household and family statistics illustrate households, families and also personal characteristics regarding household and family. The basis for the statistics consists solely of CPR data on sex, age, marital status, references to spouses and parents, and address specification. The database provided information about the marital status, type of family, status in family, type of household, number of persons in the family, number of children in the family and in the household, and information on whether the person lives in the same household as his/her father and/or mother.

Midwife records for data regarding smoking during pregnancy. The only database in which smoking is documented is in midwife records. The registering of smoking data started in 1989, but because of the extent of missing data we chose not to draw any conclusions based on this data.

ESTABLISHING THE STUDY DATABASE

The study database applied in Study I-III was established by combining many types of data relevant for the studies from health and social registers. We linked various datasets by the CPR-number allocated to each person. Data from the different sources were combined, managed, and analysed using SPSS algorithms, developed by the author for the purpose.

Denmark has a long history of collecting information on demographic factors, health and social conditions. The majority of the registers have been developed from already existing administrative registers. This requires a detailed understanding of how the study data have been generated in order to define the final variable which contains the exact information needed in the analysis.

Statistics Denmark was the main provider of register data. A remote access was established to all individual level data that were necessary to carry out the analysis. The CPR- numbers from the various databases were encrypted and anonymised by Statistics Denmark. The encrypted ID's were the same for all databases so that they could be linked. The datasets were stored at Statistics Denmark and we constructed the linkage between the datasets using a remote online access.

The data from the different registries were stored in annual datasets. We ended up with more than 300 separate databases linked together by an anonymised ID number. Because the datasets only contained year-specific data with differences in coverage, we had to define final explanatory variables by using more than 70 different programs ("SPSS syntax files") for management and data analyses, developed by the author in SPSS programming language.

The resulting working database was used to perform the statistical analysis. The same study database was used in Study I-III, to analyse the influence of different risk factors on the risk of MS.

MISSING DATA

The period in which the occupational exposure was determined ranged from 1981 to 1995. In this period the frequency of missing values amounted to 6.4% on average.

Regarding the highest achieved educational level, data was missing in 9.0% in cases and 8.4% in controls.

We used the dwelling area and the number of persons in the household to define the variable "areal per person-year in the puberty". Missing values made it impossible to calculate this variable for 9.9% of cases and 10.2% of controls.

Data about average yearly income were missing for 9.7% of cases and 9.5% of control persons.

Data were missing mostly because of the limited time between establishment of the various registers and the reference dates between 2000 and 2004, mainly for subjects of higher age for whom no data on their childhood and youth are available.

STATISTICAL ANALYSIS

In Study I-III, conditional logistic regression was applied to estimate unadjusted and adjusted odds ratios (OR) with 95% confidence intervals (CI), using the SPSS Coxreg procedure for paired analysis, with inclusion of a number of possible confounders.

For comparing means Student's t-test was used.

Statistical significance was defined at $p<0.05$

For groups of variables defined a priori, correction for the false discovery rate was employed using the Bonferroni method [Bland and Altman 1995] in study II and [Benjamini H 1995]-Hochberg method in study III

For variables not defined a priori, we separated out a pilot material that contained 20% of the randomly selected cases and the 25 matched controls for each case. The purpose of this pilot cohort was to induce hypotheses that were not defined a priori. If an outcome was significant and meaningful, the analyses should be repeated on the project cohort that contained the remaining 80% of the index persons.

All analysis in Study I-III was performed using the statistical package SPSS version 19.

In Study IV, Poisson regression analysis was performed using the GENMOD procedure in SAS v. 9.3.

METHODOLOGICAL CONSIDERATIONS

The patient group was selected with clinical onset between 2000 and 2004 to ensure that:

1) There would be a sufficient long retrospective observation period to find exposures back in time in relation to the establishment of other registers and some follow-up years.

2) The Danish Multiple Sclerosis Registry has a sufficient degree of completeness for the selected onset period to avoid selection bias because of delayed registration of benign cases. Early diagnosis after onset is often associated with a short time between the initial relapses. Assuming a more recent onset cohort will, for that reason, result in certain bias in favour of patients with more disease activity.

Persons younger than 15 or older than 55 years at disease onset were excluded because of the small number of cases in this age interval. Patients with onset age >55 and their control counterparts could not be followed far enough back in time because most of the other registries were only established in 1977 or later. Furthermore, with a first demyelinating event below the age of 15 the diagnosis may be confused with acute disseminated encephalomyelitis (ADEM).

ETHICS

The study was approved by the Danish Data Protection Agency. The Danish Multiple Sclerosis Registry is assigned as a public scientific registry in, and linkage to other public registries is solely permitted for the purpose of research. Statistics Denmark linked the study database from the Danish MS Registry with each of the external registers and returned databases with encrypted civil registration numbers, anonymising the subjects but enabling recognition across the databases. All analyses are based on this number, and identifying an individual person is not possible.

SUMMARY OF OWN STUDIES

SUMMARY OF ARTICLE I: REPRODUCTION AND THE RISK OF MS

Background

It this article, we aimed to investigate an important factor that might underlie the growing discrepancy between the sexes in terms of incidence: the tendency for women to give births to fewer children and have them at a higher age than generations before.

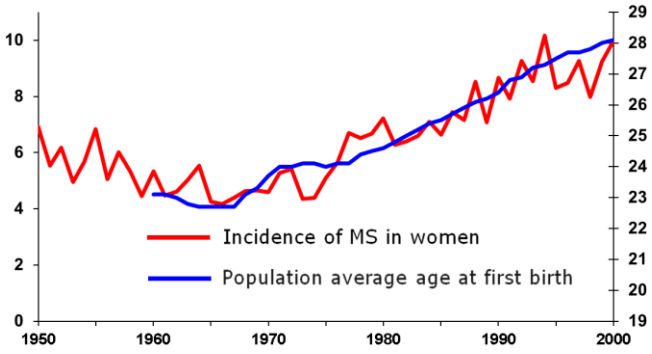

Figure 1

Incidence of MS onset in Danish women and Danish women's average age when giving birth to the first child.

The increasing incidence of MS in women occurred at around the same time as the age of women at the birth of their first child increased. According to Statistics Denmark, the age at giving birth to the first child has increased in Denmark from 23.7 years in 1970 to 29.1 in 2011. Figure 1 shows the parallel increase of these two tendencies.

To control for possible confounders affecting the parental age and the number of children we undertook a broad investigation of reproductive factors.

Results

Parental age at first childbirth

We found no significant difference between the mean age at giving birth to the first child between cases and controls.
The mean age at giving birth to the first child was 26.08 years for female cases and 26.19 years for female controls (Students t-test: p=0.07). For control of the possible role of non-biological mechanisms, we performed a similar analysis for male patients and male controls using the paternal age at the birth of the first child, but neither here there was any differences between cases and controls (28.46 years for male cases and 28.67 years for male controls, p=0.74).

The mean age at the first childbirth was influenced by the educational level, but was not found to be different between cases and controls. The association between the educational level and maternal age at first childbirth is presented in Figure 2.

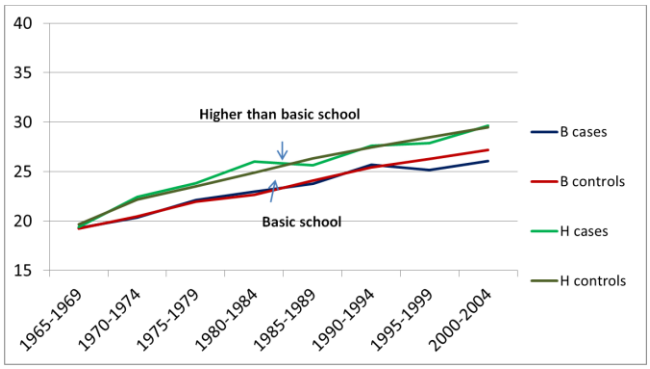

Figure 2

Mothers age at first childbirths and the educational level over time.

We also analysed the mothers age at first childbirth (in five-year age groups) in conditional logistic analysis with subject type (case or control) as a response variable with the inclusion of calendar year of the birth of first child and educational level (basic school or higher educational level) as co-variates but still we found no statistical significance or even a trend.

Number of childbirths

The occurrence of stillbirths was very low and without any significant difference in both cases and controls (1.1% cases, 0.7% controls). For this reason, stillbirths were excluded from the analysis.

Number of children was broken down into three categories:

no children
one child
more than one child

The time from birth to clinical onset was divided into three periods:

more than ten years before clinical onset
from ten to five years before clinical onset
within the last five years before clinical onset

More female cases were childless or had fewer childbirths than controls before clinical onset (p=0.018), the full effect could exclusively be attributed to childbirths the last five years preceding onset (p<0.0001).

Thus the probability of childbirths within five years before clinical onset was lower in MS patients than in controls with an OR=0.44 (95% CI 0.31-0.61, p<0.001) for one child and an OR=0.71 (95% CI 0.57-0.88, p=0.002) for more than one child. The association persisted after adjusting for maternal age at first childbirth, the use of oral contraceptives and educational level.

The risk of MS was not influenced by parity in any of the periods in men.

Both cases and controls with an educational level higher than basic school had fewer children compared with persons with basic school as the highest education level, independent of gender.

Abortions

Induced abortions within five years before clinical onset were also less common in cases than in controls (4.6 % versus 6.4 %). It seems that even early terminated pregnancies within five years before clinical onset reduces the risk of MS with an OR=0.70 (95% CI 0.51-0.86, p=0.026). The analysis was performed by including onset age, education and use of oral contraceptives as covariates.

There was, however, no difference between cases and controls as to the occurrences of non-induced abortions (4 % versus 4.7 %, p=0.37).

Pregnancy complications

Pregnancy complications such as hyperemesis, gestational hypertension and preeclampsia were not more common in cases than in controls.

Infertility

Among women, 85 cases (9.1%) and 1824 (7.8%) control persons were diagnosed with female infertility (p=0.15), but only one female case (0.1%) and 36 (0.2%) female controls were registered to have undergone artificial insemination.

Fewer male index persons were diagnosed with infertility; 11 cases (2.4%) and 197 (1.7%) controls had a diagnosis of male infertility (p=0.27).

Infertility treatment in women before clinical onset had no influence on the risk of MS (p=0.71).

Use of oral contraceptives

We found no difference in oral contraceptive use between female cases and controls. During the last five years before clinical onset, 8.7% of all cases and 7.6% of controls had used oral contraceptives for four years or more (p=0.20).

Educational level

The length of education may also influence the decision regarding parenthood. The educational level was expressed in two categories: basic school and higher than basic school. These groups did neither differ between cases and controls nor between women and men.

Stability of partnerships

Being in a relationship is an important factor in the decision regarding parenthood. Establishing and maintaining a relationship is also an indicator for social behaviour changes before clinical onset.

The Family and Household Statistics distinguish between: married couples, registered partnerships, consensual unions, and cohabiting couples; the comparison for stability partnership between cases and controls was therefore possible.

The frequency of persons living as a couple was the same among cases and controls and the frequency of cessation of partnership was not significantly different in women or men.
Mean annual pre-tax income (adjusted by general price index for the specific calendar year to correct for inflation)

Income is another useful social indicator for changes in energy or feeling sick. Cases and controls had the same mean income in the five year period before the year of clinical onset when compared within the genders.

Discussion

The main question we sought to answer in this study was: Do more childless years increase a women's risk of MS?
We found that cases have significantly less childbirths than controls in the five year period before clinical onset and more of them were childless. Our results support a similar observation in an Australian multicentre case-control study, which found strong associations between the number of pregnancies and the risk of a first clinical demyelinating event [Ponsonby et al. 2012], but we could not confirm the effect of maternal age at first childbirth found in that study or in a Swedish study [Holmqvist et al. 2010].

The parallel increase in age of first motherhood in the Danish female population and of the female incidence of MS proved to have no causal relation when studied on the individual level. A Danish historical prospective study showed that the number of children and age of first motherhood had a certain protective effect against MS later in life, but that the effect was virtually equal for men and women indicating social rather than biological factors to be at play [Nielsen et al. 2011]. A Swedish population-based case-control study reported similar associations for both sexes between reproductive history and MS risk [Hedstrom et al. 2013]. We performed the analysis in men to control for possible biological mechanisms and to distinguish from a social effect and found that parity did not influence the risk of MS in men in our study.

The association between parity and MS risk only in women in our study suggests a biological effect of pregnancy, probably due to modulation of the immune system by pregnancy.

The biological onset of the disease may in some cases precede clinical onset with years. Preclinical cognitive impairments and a vague feeling of the yet unnamed disease could influence the decision of motherhood. Therefore the possibility of reverse causality cannot be excluded, where reduced numbers of childbirths are the results of MS rather than the opposite. Social variables that could represent an evidence for social behaviour changes in the years before clinical onset were investigated for this reason. We considered the possibility of reverse causation by looking at social indicators that could also be influenced by pre-clinical disease activity like fatigue and mild cognitive impairment. Investigation of income, stability of partnerships and differences in the level of education did not suggest reverse causality. We found no difference between the age and the level of the highest obtained education for cases and controls. Broken partnerships in the period 0-

5 years before the first MS symptoms could be signs of changed social behaviour, which could also result in fewer births. However, there was no significant difference between cases and controls in women, or in men before onset of the disease, although it has been shown that MS patients have a higher risk of partnership cessation after clinical onset [Pfleger et al. 2010].

These proxy variables of cognitive and social behaviour do not support that fewer births in the 5 years prior to onset could be result of reverse causation.

The fact that women have fewer relapses during pregnancy [Confavreux et al. 1998] and the association between parity and MS risk suggests that oestrogens, progesterone, or other pregnancy-related hormones may play an immune stabilising role in pregnant women. Pregnancy is considered to be an immunomodulated state [Voskuhl and Gold 2012], by suppressing the mother's immune system to prevent the rejection of the foetus, which is considered to have foreign antigens [Tafuri et al. 1995].

Pregnancy causes hormonal changes in the level of oestrogens, progesterone, α- fetoprotein, prolactin, and early pregnancy factor. High levels of oestrogens and progesterone during pregnancy promotes type 2 helper T cells (Th2) deviation [Garay et al. 2008]. Early pregnancy factors also showed a beneficial effect in experimental autoimmune encephalomyelitis (EAE) models [Zhang et al. 2000]. Sex hormones influence type 1 helper T cells (Th1) or Th2 differentiation and starts cross-inhibition between these responses. In MS, the immune response switches to Th2 response when female sex hormones increase during pregnancy [Kipp and Beyer 2009], due to the increase of Th2 and Regulatory T cell (Treg) populations and a decrease in Th1 and Th17 cells [Saito et al. 2010]. Estradiol is probably responsible for the increase of the Treg cells [Tai et al. 2008]. Oestrogens have a biphasic dose effect with low doses facilitating an immune response and high doses suppressing it [Gilmore et al. 1997]. High doses of oestrogens down-regulate TNF-alpha and IFN-gamma with an increase in IL-10 and other suppressor cytokines [Kipp and Beyer2009]. Both oestriol and oestradiol showed a favourable immunosuppressive effect on the demyelinating process in EAE models [Brann et al. 2007;Gatson et al. 2011]. High concentrations of progesterone in animal models induce the anti-inflammatory Th2 response by producing IL-4 and IL-5[Giatti et al. 2012]. Pregnancy initiates downregulation in inflammatory genes that remain upregulated in the non pregnant MS women [Gilli et al. 2010].

The treatment of non pregnant women with estriol resulted in a decrease in the size and number of brain lesions on MRI, and an increased protective immune response in patients with RRMS [Sicotte et al. 2002].

In genetically susceptible individuals exposed to an unknown environmental factor, alterations in sex hormones could modulate the immune response. There is evidence that hormonal changes in women affect the risk and the course of the disease. The positive association between early menarche and the risk of MS [Ramagopalan et al. 2009b] may also be attributed to hormone changes. A later age at menarche appears to be associated with a slower disability progression, both in women with RRMS and in those with SPMS; and women with at least two pregnancies had a reduced risk of reaching EDSS 6 compared with nulliparous women [D'hooghe et al. 2012].

The fact that even early terminated pregnancies with induced abortions diminished the risk of MS suggests a biological mechanism. However, it cannot be excluded that the fewer induced abortions in cases before clinical onset could be explained by fewer pregnancies due to the influence of the already underlying disease, but reverse causation is unlikely, because in the five years period after clinical onset we found no difference in the frequency of induced abortions between cases and controls (2.2% versus 2.8%, p=0.31).

The use of the oral contraceptive pill is one of the major changes in the modern woman's life. The pill provided women with an opportunity for education, work and economic independence. It also gave them the freedom to plan childbirths. Earlier studies have acquitted the oral contraceptive pill with regard to increasing the risk of MS [Alonso and Clark 2009], and our study supports this observation. The lower number of childbirths in female cases in our study could not be attributed to the oral contraceptive pill. There was no evidence that more female cases were diagnosed, or were seeking treatment for infertility in this period. However a possible underlying factor, independently reducing fertility and increasing the risk of MS, cannot be completely ignored. A prospective study from Finland reported that more MS patients than controls had undergone artificial insemination [Jalkanen et al. 2010], although this was after the diagnosis.

Pregnancy, by its temporary immunosuppressive effect, may diminish the risk of developing MS. This is in keeping with evidence of the beneficial effects of pregnancy in patients with MS [Keyhanian et al. 2012;Runmarker and Andersen 1995;Tsui and Lee 2011;Verdru et al. 1994].

Also we cannot exclude that the tendency to childlessness in Denmark in the latest few decades together with other lifestyle factors which may affect the hormone balance, can have its share in the increasing incidence of MS in women.

SUMMARY OF ARTICLE II: PHYSICAL AND SOCIAL ENVIRONMENTAL FACTORS AND THE RISK OF MS

Background

In this study we investigated whether environmental factors deriving from the modern lifestyle have affected the genders differently and have contributed to a higher risk for developing MS in women.

One of the interesting explanations for the increased risk of MS is the hygiene hypothesis [Leibowitz et al. 1966]. This theory proposes that the risk of MS may be higher in individuals with a high level of sanitation and thereby reduced bacterial, viral and parasitic infections during their childhood.

Over the past century Danish people have experienced improvements in household amenities, smaller family sizes, and higher standards of personal cleanliness, hygiene and sanitation that have reduced the likelihood of cross-infections among family members.

Since the hypothesis was proposed, numerous studies have tried to clarify the role of the infectious burden in early life in the protection of allergic and autoimmune diseases [Fleming and Cook 2006;Bach 2002], but the immunological mechanism is not yet fully understood [Bach 2005]. The immunological explanation so far is based on an unbalanced reciprocal downregulation of Th1 by Th2 cytokines, but there are several observations [Weiss 2002] pointing towards a more complex yet unrevealed picture.

Puberty is the supposed key risk period of life, where the environmental factors exert their influence on the disease development, particularly in females [Chitnis 2013]. For this reason, we chose to investigate the available hygiene indicators on the risk of MS between the ages of 10 and 15.

To separate the effect of pregnancies/childbirths on the MS risk (as shown in article 1) from a possible "social" or physical effect of sharing home with children, we investigated whether the presence of children in the homes of index persons, who had never given birth themselves (or fathered, if male), and thereby greater exposition to infections itself had an influence on the risk of developing MS. The evidence of a beneficial effect of childbirths on the risk of MS is growing, but because some studies reports similar beneficial effect of childbirths on both men and women [Nielsen, Jorgensen, Stenager, Jensen, Pedersen, Hjalgrim, Kjaer, and Frisch2011;Hedstrom, Hillert, Olsson, and Alfredsson2013], it has been discussed whether the influence is due to other biological mechanisms, reverse causation, or social factors.

Denmark is one of the countries where the biological effects of childbirths and the effects of environmental exposure to children in the household can be investigated separately, because more than one-third of marriages or relationships end in divorce or broken partnerships. In the majority of cases the parents share custody, with the children often spending alternate weeks with each parent. For this reason, even childless persons living in a relationship with a divorced parent may be exposed to children.

Today, the female position is no longer based on the husband´s position; many women achieve a higher education, and these changes have occurred rapidly in the latest decades. Over the last 50 years women have been employed in various industrial sectors which were previously reserved for men; therefore investigating the influence of the occupational exposure on the risk of MS in women was a topic of much interest.

An association between the risk of MS and exposure to organic solvents has been found in some studies [Amaducci et al. 1982;Landtblom et al. 1996], but no association was found in other studies [Riise et al. 2011]. Outdoor activities in the summer have also shown an association with the risk of MS [Kampman et al. 2007], although the study mentioned focused on childhood and adolescence.

We investigated the occupational exposure grouped with two different rationales. First we investigated if employment in occupational sectors, integrated into five main categories, affected the risk of MS. Besides, independently from the first categorisation, we defined dichotomous variables and included outdoor occupational activities to investigate the effect of exposure to ultraviolet sunlight, exposure to organic solvents or chemical substances.

Results

Educational level

We classified educational level into four categories defined as basic school, secondary school, short-term on-the-job training or post-secondary school,bachelor's degree or moderate-term on-the-job training and long-term education

The educational level was the same for cases and controls, and showed no differences for men or women. There were differences between the distribution of the highest achieved educational level before clinical onset between the genders, with men being represented more in the basic school and long term education category, while women in the medium-term education category, but the distribution was the same for cases and controls and did not have an effect on the risk of MS.

The distribution of the highest achieved educational level before clinical onset in cases and controls is presented in Figure 3.

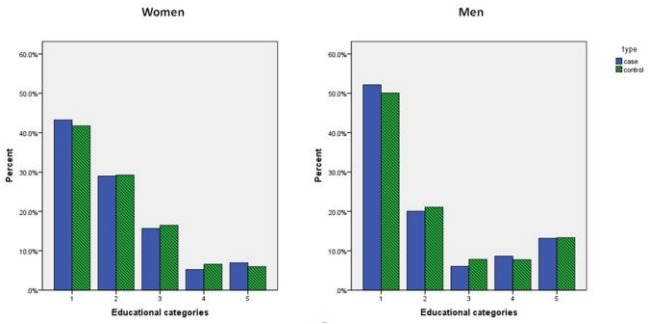

Figure 3

Educational categories:

1. Basic school
2. Secondary school, short-term on-the-job training or/and postsecondary school
3. Bachelor's degree or moderate-term on-the-job training
4. Long-term education
5. Missing

Children in the household

A child was defined as a person under the age of 18, belonging to a family living on the same address. A person was categorised as being in a relationship if he/she was married or had been living in a relationship for more than eight years in the observation period. We did not distinguish between a partner's children (or young siblings) when we defined cohabitation with children.

In women after the age of 25 years and up to the age at clinical onset of MS, 86.1% (N=310) of childless female cases and 86.9% (N=7193) of nulliparous female controls had been exposed to children less than the age of 25 years for more than 3 years in their household during any period (p=0.66). There was no difference between the frequency of cases and controls living in a relationship after the age of 25 years.

The conditional logistic regression analysis, including educational level and onset age as covariates did not show a significant effect of children in the household in nulliparous women (OR 0.70, 95% CI 0.27-1.80, p=0.45) or men without their own children (OR 1.49, 95% CI 0.45-4.90, p=0.52).

As we in our previous analysis found that pregnancies have a protective effect against developing MS lasting up to five years, we investigated if cohabitation with children within the five year period before clinical onset affects the MS risk.

In the five years preceding the occurrence of first symptom 70.9% (N=558) of childless female MS cases and 71.9 % of (N=13064) of nulliparous female controls have shared their household with children. Sharing a home with non-biological children in the five years period before clinical onset did not influence the risk of MS: OR 1.03 (95 % CI 0.73-1.45, p=0.88). The presence of children in the household also did not influence the risk of MS the OR being 0.70 (95 % CI 0.47-1.05, p=0.08).

Housing condition

Our analysis showed that very few index persons were living in a household with deficient sanitary conditions. Only 0.4% female cases vs. 0.4% female controls and 0.4% of male cases vs. 0.3% of male controls lived without sufficient housing amenities, such as without toilet or bathroom, between the ages 10 to 15. There was no difference between cases and controls (p=0.48 for men and p=0.97 for women).

We defined a coefficient as living space/person within the 10-15 age interval, to see if the living in a crowded home and therefore probably exposed to more infections had an influence on the risk of MS.

$$\text{Living space pr. person} = \frac{\sum \text{areas of the housing spaces over the respective 5 years in m}^2}{\sum \text{persons living in the households over these years}}$$

The ratios were then logarithmic-transformed to obtain a normal distribution and the independent sample t-test was performed. The analysis showed no statistically significant difference between the square meters available per person between the age 10-15 for cases and controls, in any of the sexes.

The mean space per person in the household over these years was:

female cases: 37.79 m²
female controls: 40.15 m²
male cases: 39.42 m²
male controls: 40.47 m²

We found no association between the living space earlier in life and MS in either of the sexes. The results of the independent sample t-test on logarithmic transformed coefficients were: for men: t=0.031, df=3813, p=0.97; for women: t=-1.44, df=9328, p=0.15).

Occupational exposures

Based on the original 303-item classification occupations integrated into five main categories:

agriculture
craftsman work
health sector
workers in chemical industries
work without specific physical exposure (working in retail, finance sector, insurance, education, social services, etc; and this group was chosen as the reference category)

Of the index persons, 7422 were recorded to have an occupation in the Danish Industrial database between 1982 and 1995. Persons without employment in this period were excluded.

We defined exposure as 3 or more years spent in the respective occupational field.

We found a slight, albeit statistically significant excess of female cases (but only based on 6 cases and 1 control) who had been employed in agriculture. Working in agriculture increased the risk of MS with an OR=3.52 (95% CI 1.38-9.00, p=0.008). The result remained significant after correction with the Bonferroni method p=0.04. We found no evidence that craftsman work, employment in the health sector or in the chemical industry had any influence on the risk of MS.

There was no significant correlation between any of the five occupational categories and the risk of MS in men.

Independent of the classification into the five occupational categories, we also created three dichotomous variables of physical exposure so that all index persons could be classified as exposed or not exposed to each of the following three factors:

chemical substances
organic solvents
outdoor work

Only outdoor work was correlated with an increased risk of MS in women: (OR 1.94, 95% CI 1.06-3.55, p=0.048). The results were based on just 12 female MS-cases, however, and after correction for multiple significance the result was not significant p=0.09.

There was an overlap between these and the six female MS patients employed in agriculture. There was no difference between male MS cases and controls for any of the three variables.

Discussion

Our study did not support the hygiene hypothesis. We could not prove that sanitation in the puberty could influence the risk of developing MS, because cases and controls were exposed to the same good level of hygiene between the ages 10-15 years. Nor did our study show a correlation between more frequent exposures to infections due to cohabitation with children in adulthood.

Previous studies have shown that environmental factors may modify the risk of MS [Koch et al. 2013]. The role of infections or treatment of these on the risk of developing MS is not yet clear. The influence of childhood infections [Hernan et al. 2001] and birth order [Isager et al. 1980] is supported by some studies and ruled out by others [Bager et al. 2004;Bager et al. 2006;Koch-Henriksen 1989b]. A nationwide case-control study from Denmark reported a positive association between the use of antibiotics and the risk of MS [Norgaard et al. 2011].

Denmark is not a country where social inequalities are predominant; therefore cases and controls have both been exposed to better socioeconomic conditions than earlier generations and the risk of MS does not seem to be associated with measurable social factors.

One could argue that any social differences would be mitigated by our matching for residential municipality, as the greatest social disparities in the country are reflected in the region where they live. On the other hand, major cities contain a wide range of the social spectrum, why a social difference will not be masked.

Socio-economic indicators are sometimes strongly linked to lifestyle and can vary by country and geographical area, which can further complicate epidemiological studies. It seems that in the case of MS, the epidemiological picture is more complicated.

The cause of MS is not entirely environmental, because ethnic different groups living together do not have the same rates of incidence rates; however migration studies support a changing epidemiology in populations migrating to another MS risk area [Gale and Martyn 1995]. Migration studies also corroborate the role of puberty [Poser 2006]. Puberty occurs when hormones signal the development of organs related to sexual reproduction. The importance of what happens during this period with regard to the risk of MS is further supported by the associations between early menarche [Ramagopalan et al. 2009c] and childhood obesity [Munger et al. 2009] and the risk of MS.

The correlation between lifestyle and the risk of MS is reflected by the positive correlation between gross national product and the incidence of MS [Kurtzke 2000]. A recent Danish study disproved associations between childhood socioeconomic situation and the risk of MS [Nielsen et al. 2013], suggesting that the distribution of social and economic factors in the Danish population is relatively equal both in early life and in the adulthood, making it difficult to reveal a causative association. Therefore there must be other factors contributing to the sexual dimorphism observed in post-pubertal sex ratio, which is not that accentuated in pre-pubertal cases.

Our study did not have the statistical power to show a strong association between occupational exposures and the risk of MS. A relative high frequency of MS was recently reported among persons working in the agricultural sector, especially dairy operators [Horwitz et al. 2013], and farming and exposure to livestock

showed to increase the risk for the first demyelinating symptom [Valery et al. 2013]. When investigating exposure to outdoor work, we found the opposite of what we expected, but further investigations pointed to an overlap between exposure to outdoor work and the agricultural sector.

SUMMARY OF ARTICLE III: GENDER AND AUTOIMMUNE COMORBIDITY IN MS

Background

Based on our current knowledge MS is considered to be an autoimmune disease [Ramagopalan et al. 2009a]. Most of the autoimmune diseases are more prevalent in women than in men [Gleicher and Barad 2007]. The reasons for the sex bias in MS and other autoimmune diseases are unclear but may include such factors as sex-related differences in immune responsiveness, response to infections, sex steroid effects, and sex-linked genetic factors. It has been shown that autoimmune diseases occur more frequently in family members of patients with MS than in the general population [Barcellos et al. 2006;Nielsen et al. 2008], therefore a common genetic and environmental background for susceptibility to autoimmunity is presumed. Genome-wide association studies (GWAS) have shown an overlap in the genetic risk foci for MS and some other core autoimmune diseases [Lettre and Rioux 2008]. Recently, ImmunoChip analyses disclosed overlapping of approximately 22% of MS signals with at least one other autoimmune disease signal, most frequently with inflammatory bowel disease and less frequently with rheumatoid arthritis (RA) and autoimmune thyroid disease [Beecham et al. 2013].

This article focuses on the gender differences in the co-occurrence of other autoimmune diseases in MS cases compared with controls. The presumed altered pro-inflammatory mechanism triggered by changes in the environment may be expressed in a higher clustering of autoimmune diseases in women compared to men. Autoimmune comorbidity in MS could be one of the factors that differentiates male from female MS patients.

Bronchial asthma is not considered an autoimmune disease, but its coexistence with MS is a discussed topic [Bergamaschi et al. 2009;Tremlett et al. 2002], therefore we additionally aimed to investigate this correlation in the Danish population.

Results

Our study found a relatively low prevalence of autoimmune diseases in both cases and controls, but there were some significant differences between the genders.

The follow-up for cases and controls was the same: from 1977 to 2010. We chose the 18 most common autoimmune diseases to determine their association with MS .

Since some milder forms of type 1 diabetes mellitus (DM1) are treated by general practitioners, we completed the data with information from The National Prescription Registry. The identification of the type of medication used was based on the second-level of the Anatomical Therapeutic Classification (ATC). For insulin and insulin analogs we used the ATC code A10. A person who started insulin treatment below the age of 41, and had been treated with insulin preparations for more than two years, and had been registered in the NPR with the diagnosis DM1 was considered to have the diagnosis of DM1. For other diseases we accepted one occurrence of the diagnoses as a main diagnosis, but for RA two occurrences were required.

Separate analyses were performed for each autoimmune disease and each sex. To determine the diagnostic sequences we assessed the first year of the first recorded diagnosis of autoimmune diseases relative to the reference year.

In men 3.66% of MS cases and 3.11% of controls had a coexisting autoimmune disease (p=0.59), while 10.12% of female MS cases and 9.42% of female controls had an autoimmune diagnosis (p=0.47). Eight male MS cases (1.72%) and 51 male controls (0.44%) experienced comorbidity with more than one autoimmune disease. Among women, 14 (1.49%) MS cases and 322 controls (1.37%) had experienced more than one autoimmune disease within the same time frame. The difference was statistically significant for men (p=0.002) but not for women (p=0.92). The presence of more than one autoimmune disease (other than MS) was typically associated with DM1.

The mean age at diagnosis of the autoimmune comorbidities was 38.79 for cases and 39.69 for controls, (p=0.34).The OR for male MS cases to have DM1 compared with male controls was 3.34 (95% CI 1.40-7.02, p=0.008).

Women with MS did not show a significantly higher probability of having DM1 then controls (p=0.81).

A statistically significant association between the coexistence of inflammatory bowel diseases and the risk of MS was only found in men. Crohn's disease occurred at a higher rate in male MS cases in the period preceding the clinical onset of MS with an OR=5.03 (95% CI 1.18-16.10, p=0.03), but this association was not seen in female MS cases (p=0.82). For ulcerative colitis there was a trend for a higher MS risk when considering the whole observational period in men, the OR being 2.22 (95% CI 0.93-4.59, p=0.057), but not in female MS cases (p=0.64).

Having systemic lupus erythematosus (SLE) prior to MS raised the risk of developing MS only in men with an OR=12.55 (95% CI 1.62-69.95, p=0.02), however, this result was only based on two MS cases and four control persons.

When assuming a p-value limit of 0.0083 after correcting the standard value of 0.05 for false discovery rate, the increased OR for DM1 in male MS cases with a p-value limit of 0.008 remained statistical significant.

Because autoimmune diseases are rare and few diseases were not represented in either the case or control group, we calculated the upper 95% confidence limit for the zero ORs, but none of the autoimmune diseases were found to have a protective effect for developing MS.

Multiple sclerosis and allergic bronchial asthma

As for the DM1 we supplemented the data from NPR with the ATC code R03 if a person was treated with these medications for more than two years. Persons with the diagnosis chronic pulmonary obstructive disorder were excluded.

We found neither positive nor negative association between MS and allergic asthma; the OR was 0.96 (95% CI 0.76-1.20, p=0.72) for female MS cases and 1.10 (95% CI 0.66-1.80, p=0.71) for male MS cases.

Discussion

Our case-control study showed that some specific autoimmune diseases seemed to be associated with MS, but only in men. Despite the fact that twice as many women have autoimmune diseases, there is a significant clustering of autoimmune diseases in men. However, only the significance for DM1 survived correction for multiple testing by the Benjamini-Hochberg method [Benjamini H1995]. The coexistence of MS with inflammatory bowel diseases are reported by other studies [Gupta et al.

2005;Bernstein et al. 2005;Midgard et al. 1996]. Although these diseases are generally more common in women than in men, the association with MS was only significant for men for Crohn's disease and ulcerative colitis.

A higher co-occurrence of DM1 in MS patients and in their first-degree relatives, but without significant gender differences, has been reported by a Danish study [Nielsen et al. 2006]. Our results confirm the finding of this study designed to obtain a long follow-up, but the coexistence with MS was only significant in men in our study, as the main part of our study was looking at the time before onset of MS.

The weakness of this study is the size of our MS cohort, which was limited to onset in the years 2000–2004, because the main hypotheses of the whole study were related to the time before clinical onset. This choice, however, may also have caused underestimation of autoimmune diseases with a higher age of onset than MS, e.g. RA and reduce the statistical power to detect rarer autoimmune disease combinations. Another weakness is that until 1995 hospital out-patients were not included in the NPR, and patients in private practice were not included at all, which may explain the low frequency of autoimmune diseases often treated in general practice alone, like autoimmune thyroid disease and psoriasis , but this filter applies to MS patients as well as to controls. This limitation may apply to the other comorbidities studied and suggests the possibility that our findings are biased towards more severe diseases. If MS patients are more liable than controls to be hospitalized for comorbidities, differences will be biased away from the null-hypothesis. Nevertheless, in Denmark practicing specialists only cover a small part of the activity, and in most cases more serious diseases are diagnosed and treated in highly specialized hospital clinics, meaning that the probability of a disease being recorded in central hospital registers is relative high.

Another limitation could be the reclassification of the diagnostic codes for autoimmune diseases from ICD 8 to ICD 10 with a shift in January 1994. The ICD 8 system did not distinguish between types 1 and type 2 DM up to 1988, which may have caused some diagnostic uncertainty.

We required at least two hospital contacts with the diagnosis rheumatoid arthritis as the main diagnosis, because a validity study of rheumatoid arthritis in NPR pointed to a higher validity of the registered diagnosis for inpatient contacts and for more than one hospital registrations with rheumatoid arthritis [Pedersen et al. 2004]. Otherwise with ulcerous colitis and Crohn's disease, where the validity as well as the completeness of the NPR was > 90% without a requirement for repeated diagnoses [Fonager et al. 1996]. The increase in specificity by requiring several contacts for a diagnosis to be confirmed would be at the expense of sensitivity, and a balance point may differ between the specific diseases. Another even more significant and bias-causing problem with requiring multiple contacts would be that cases with MS tend to have more hospital contacts than controls, and hence, their comorbidities could be noted repeatedly as secondary diagnoses in the registers even if they had been treated outside hospitals.

All patients and controls were Danish born and alive at the time of onset of the MS patient. So, immortal time bias up to the time of onset of MS in the retrospective part of the study is non-existing. However, in the 5-10 years after clinical onset there is a possibility that some MS patients have died early and have provided less observation time than the controls, as the seven-year cumulative survival after onset of MS was 97.9%, and in a matched background population it was 98.4%. (Koch-Henriksen, personal communication).Thus, the bias caused by not taking into account different survival in MS patients and controls within the time frame of the prospective part of the study is negligible.

Autoimmune diseases are individually rare, but their impact on the MS population is of importance. The estimated combined prevalence of 31 autoimmune diseases in Denmark using hospital data was 5.3% [Eaton et al. 2007].

Knowledge about comorbidities in MS patients is important, as it may affect clinical features, treatment decisions and responses, health outcomes, and inclusion in clinical trials. An improved understanding of the autoimmune comorbidity in MS may give a novel insight into shared pathophysiology, genetic predisposition and etiological risk factors for these diseases.

STUDY IV: GENDER DIFFERENCES IN THE TREATMENT RESPONSE TO IFN-B

Background

Gender appears to play a role in the disease course of MS, but there are only few reports about gender differences in the response to disease modifying therapy (DMT) [Kalincik et al. 2013]

Natural history studies suggest that men progress more rapidly [Debouverie2009;Confavreux, Hutchinson, Hours, Cortinovis-Tourniaire, and Moreau1998], and have a greater risk of developing persistent Tl-hypointense lesions "black holes" [Pozzilli et al. 2003]. PPMS occurs more frequently in males [Runmarker and Andersen1993]; however if the age of onset is around the age of menopause in women, the disease is more often associated with a progressive course [Bove et al. 2012].

Women are predisposed to a higher frequency of relapses [Held et al. 2005;Tremlett et al. 2008], but they seem to recover better after relapse than men [Scott and Schramke 2010].

The first approved disease modifying drug for the treatment of RRMS was IFN-β, approved in Denmark in 1996. One major problem in treating with IFN-β is the development of neutralising antibodies (NAbs) It has been shown that the presence of NAbs in high titres abrogate the biological activity of IFN-β and leads to a higher disease activity [Hesse et al. 2009;Sorensen et al. 2003].

The aim of this study was to investigate whether men and women respond differently to IFN-β treatment in terms of relapse activity. We have no placebo group for comparison, but patients can, in high-titre neutralizing antibody (NAb) - positive periods, be regarded as being without treatment.

A significant difference in the relapse activity changes will allow an assessment of whether the treatment response to IFN-β is different between the genders.

Material and methods

MS patients treated in Danish neurological clinics with IFN-β in the period 1996-2003 were identified from the nationwide and virtually complete Multiple Sclerosis Treatment Registry.

For all MS patients in Denmark who receive immunotherapy (including IFN-β) clinical parameters are prospectively reported to the Multiple Sclerosis Treatment Registry. MS patients treated with IFN-β are clinically examined at baseline and after 3 and 6 months of treatment, then every 6 month and the Multiple Sclerosis Treatment Registry is notified after each visit. Among the reported data are: dates of relapses, neurological clinical status assessed by the EDSS, side effects, and the presence of NAbs against IFN-β. NAbs are measured at 6, 12 and 24 months after starting treatment with IFN-β and subsequently in some cases then at 6 month intervals until 48 months.

We included only patients treated before 1.06.2003, because before that time the importance of NAbs on treatment efficacy was uncertain, and tests were only made for research. Furthermore, the neurological departments were blinded to the NAb results. From the end of 2003, when the detrimental effect of NAbs was documented, treating neurologists were informed about the individual NAb-results and normally took the consequence by discontinuing IFN-beta treatment in NAb-positive patients and shifted to non-IFN-beta medication.

Patients treated with any IFN- β therapy were included, because NAbs have been shown to be cross-reactive [Khan and Dhib-Jalbut 1998] and the clinical effect of NAbs against IFN- β is independent of the type of IFN- β used for patients with RRMS [Koch-Henriksen et al. 2009].

Neutralising capacity is measured as the percentage of added IFN- β neutralised by NAbs.

It was generally accepted that samples with a neutralising capacity ≥ 20% were regarded as NAb-positive, as was the lowest concentration previously shown to have a clinical importance [Rudick 2003]. Because neutralising capacity between 20 and 79% is defined arbitrary as low-level NAb-positivity and high-level NAb-positivity as >80% [Sorensen et al. 2006b], we performed the analysis using two different definitions for NAb-positivity and NAb-negativity.

We defined NAb-positive and NAb-negative periods for all observational periods by using two different cut-off values of NAb.

a) NAb-positive periods, defined as the time period following immediately after a blood test with the presence of antibodies with neutralising capacity either of ≥ 80 % or of ≥ 20 %. In such periods, patients are considered not to have treatment effect.

b) NAb-negative periods, defined as the most recent time period before and up to the NAb test, showing neutralizing capacity of either <20% or <80% in the two set-ups. These NAb levels are considered not to affect the treatment effect. This time distinction was made because NAbs tend to last during the subsequent period whereas a NAb-negative state will probably have been present in most cases in the full period before the NAb test. The clinical effect of the intermediate NAbs range from 20 to 79% neutralising capacity is uncertain, therefore we deemed NAb tests within this range and their corresponding observation periods to be missing and they were ignored in the regression analysis.

The number of relapses in the NAb-positive and negative periods were summed up over all patients. The sum of relapses is assumed to be Poisson distributed.

Only data from the first treatment series and from the first 8 visits were included. Control periods without a test for NAbs were ignored.

A number of variables were drawn from The Multiple Sclerosis Treatment Registry:

Age and year at onset, and of treatment start

Duration of MS

Number of relapses the last 24 months prior treatment start

Baseline neurological status expressed in EDSS

Number of relapses between each visit and their dates

The results of all NAb tests and the blood sample dates

Some of these variables were includes as covariates in the regression analysis.

Period-specific ARR were calculated from these raw data.

When performing the Poisson regression analysis, the observations used were individual patient periods rather than patients. Each of these observations were characterized by its NAb state, number of relapses in the period, the patient ID, sex, age and certain clinical variables for the person to whom it belonged, and the duration of the period. Sex, NAb status and their interaction were regarded as the main effects to be estimated, and the logarithm to the duration served as the offset in the Poisson model. The adjusted model also included age at onset, EDSS at onset, age at treatment start, and time since treatment start. Age at onset, EDSS, and time since treatment start were treated as continuous variables. Age was treated as continuous variable.

The effects of sex and Nab status were estimated when the interaction term was excluded from the model.

Results

Data from 2034 patients (639 men and 1395 women) were included.

Baseline characteristics including EDSS at treatment start and the number of relapses 24 month prior treatment initiation were similar for men and women. Baseline variables are presented in Table 1.

Analysis of relapse rates in NAb-positive and -negative periods

For all three set-ups of definition of NAb-positivity and NAb-negativity the ratio of ARR in NAb-positive and NAb negative periods was between 1.30-1.48, confirming that NAbs abrogates the treatment effect of IFN- β. The rate-ratios for all three set-ups are shown in Table 2

Analysis of factors affecting the number of relapses

To find out how disease activity is affected by different variables including sex and NAb status we performed a Poisson regression analysis, enabling us to include covariates. Each of the 4140 time periods was regarded as a “case". Instead of using period-specific relapse rates as response variables, we used number of relapses assumed to be Poisson-distributed, and included period length as a covariate.

We only performed the analysis with the set-ups that ignores intermediate NAb values 20-79.

In the unadjusted model sex and NAbs influenced independently the relapse activity.

Table 1
Baseline variables

		Male	Female	All
Age at start of treatment	Mean Median (min-max)	38.2 38 (14 – 68)	37.8 38 (13 – 67)	37.9 38 (13 – 68)
Duration of MS	Mean Median (min-max)	5.82 4 (0 – 51	5.81 4 (0 – 44)	5.82 4 (0 – 51)
Baseline EDSS	Mean Median (min-max)	2.80 2.5 (0 – 6.5)	2.58 2.5 (0 – 7.5)	2.65 2.5 (0 – 7.5)
24 months pre-treatment relapses	Mean Median (min-max)	2.50 2 (0 – 9)	2.57 2(0 – 9)	2.55 2 (0 – 9)

Table 2
Relapse rates in NAb-negative and NAb-positive periods according to the three different definitions (ARR: annualized relapse rates)

	∑relap-ses in NAb-positive periods	∑observa-tion time in NAb posi-tive periods (years)	ARR in NAb- posi-tive peri-ods	∑relap-ses in NAb-nega-tive periods	∑observa-tion time in NAb nega-tive periods (years)	ARR in NAb- ne-gati-ve pe-riods	Pos/neg relapse rate ratio	95 % CI	p-value
NAb definition: 0-19 = negative; 20+ = positive									
Men	69	140.07	0.49	241	636.12	0.38	1.30	0.98-1.71	0.054
Women	214	338.49	0.63	606	1,265.21	0.48	1.32	1.12-1.55	0.0005
All	283	478.55	0.59	847	1,901.33	0.45	1.33	1.16-1.52	<0.0001
NAb definition: 0-79 = negative; 80+ = positive									
Men	50	95.29	0.52	270	678.94	0.40	1.32	0.96-1.79	0.071
Women	172	240.62	0.71	666	1,356.63	0.49	1.46	1.22-1.72	<0.0001
All	222	335.90	0.66	936	2,035.57	0.46	1.44	1.24-1.67	<0.0001
NAb definition: 0-19 = negative; 80+ = positive; 20-79 ignored									
Men	50	95.28	0.52	241	636.12	0.38	1.39	1.00-1.89	0.035
Women	172	240.62	0.71	606	1,265.21	0.48	1.49	1.25-1.77	<0.0001
All	222	335.90	0.66	847	1,901.33	0.45	1.48	1.27-1.72	<0.0001

There was a strong effect of sex (female/male relapse ratio was 1.21; 95% CI; 1.04-1.40; p = 0.013) and NAb-status (positive/negative=1.59; 95% CI; 1.29 – 1.97; p < 0.0001) on the relapse count. Age at start of treatment was also a strong predictor of relapse activity which decreased with a factor 0.98 (95% CI; 0.97-0.99; p < 0.0001) per year of increasing age.

Time after treatment start, decreased the relapse count with a factor 0.78 per year (95% CI; 0.73-0.83; p < 0.0001) and EDSS at start of treatment: a factor 1.086 for each EDSS 1.0 point increase (95% CI; 1.04-1.14; p = 0.0009) .

The number of relapses in 24 months preceding starts of IFN-β treatment also had a significant effect on the number of relapses (p < 0.0001).

The main hypothesis of this study was, whether the interaction between gender and NAb- status had a significant influence on the relapse activity.

The strong effect of both sex and NAb-status proved to be insignificant both when tested alone with the main effects sex and NAbs (p = 0.29) or with the other significant covariates included: age at start of treatment and number of relapses in 24 months preceding start of IFN-β treatment (p = 0.30). Thus the detrimental effect of NAbs on relapse activity was the same for both sexes, meaning that the effect of IFN-β treatment is not associated with sex.

Discussion

We found that NAbs affects the IFN-β treatment efficacy both in women and men, without any gender differences.

As all the included patients in our study were treated with IFN-β, we used an alternative approach to compare treatment with no treatment, as treatment periods with NAb positive test could be regarded as equivalent to periods without treatment. This is justified by the well-known negative effect of NAbs on treatment effect [Hesse, Sellebjerg, and Sorensen2009;Khan and Dhib-Jalbut1998;Koch-Henriksen, Sorensen, Bendtzen, and Flachs2009;Rudick2003;Sorensen, Ross, Clemmesen, Bendtzen, Frederiksen, Jensen, Kristensen, Petersen, Rasmussen, Ravnborg, Stenager, and Koch-Henriksen2003].

The number of relapses was independently influenced by gender, and the presence of NAbs, but the presence of NAbs did not affect the treatment effect differently in women and men.

Interestingly, the ARR was higher in women than men in both NAb -positive and NAb-negative periods, despite the relative small difference in the number of relapses 24 months before treatment start. The results support a higher relapse rate in women reported in a previous study based on data from the Multiple Sclerosis Treatment Registry [Sorensen, Koch-Henriksen, Ravnborg, Frederiksen, Jensen, Heltberg, Schaldemose, Deth, Kristensen, Worm, Stenager, Hansen, Sivertsen, and Torring2006a]. Higher relapse rate in women compared with men was also reported by other studies [Tremlett, Zhao, Joseph, and Devonshire2008;Kalincik, Vivek, Jokubaitis, Lechner-Scott, Trojano, Izquierdo, Lugaresi, Grand'Maison, Hupperts, Oreja-Guevara, Bergamaschi, Iuliano, Alroughani, Van, V, Amato, Slee, Verheul, Fernandez-Bolanos, Fiol, Spitaleri, Cristiano, Gray, Cabrera-Gomez, Shaygannejad, Herbert, Vucic, Needham, Petkovska-Boskova, Sirbu, Duquette, Girard, Grammond, Boz, Giuliani, Rio, Barnett, Flechter, Moore, Singhal, Bacile, Saladino, Shaw, Skromne, Poehlau, Vella, Spelman, Liew, Kilpatrick, and Butzkueven2013] without differentiating between treated and untreated patients or between treatment with different immunomodulatory drugs. However, a large Italian multicenter study concluded that a higher percentage of men than women had cognitive impairment after treatment with IFN-β, suggesting a better response for women for this outcome[Patti et al. 2009].Pooled data from five clinical trials with IFN-β could not detect a gender difference in treatment efficacy expressed in annual relapse rate (ARR), time to disability progression or the number of gadolinium enhanced lesions. [Rudick, Kappos, Kinkel, Clanet, Phillips, Herndon, Sandrock, and Munschauer, III2011], but the effectiveness of a treatment estimated in post marketing observational studies is closer to the daily clinical practice. An Italian multicenter observational study with a seven years follow-up suggested that the responsiveness to IFN-β was different between the sexes, with men having lower risk of a first relapse, and a higher risk of progression on the EDSS scale [Trojano et al. 2008]

A possible explanation for the gender dimorphism can be differences in the immune response mediated by sex hormones. The beneficial effect of pregnancy on the relapse rate [Confavreux, Hutchinson, Hours, Cortinovis-Tourniaire, and Moreau1998] indicates the influence of sex hormones through the immune response, also documented by changes in MRI activity during hormonal changes [Bansil et al. 1999].

There have been few studies investigating predictors of relapses, and our findings confirm some of these results. A large retrospective cohort investigating predictors of relapses after inclusion in clinical trials concluded that the on-study relapse rate was higher for younger and for female patients, and the best predictors for the on-study relapse rate were the relapse number prior to entry into clinical trials together with disease duration [Held, Heigenhauser, Shang, Kappos, and Polman2005].

In conclusion, it seems that sex matters, and sex-specific differences in immune functions may be reflected in different immunomodulatory responses, but the sex differences do not include differences in clinical effects of IFN-beta treatment.

CONCLUSIONS

In this thesis I have reviewed the four underlying studies where we investigated various registry based reproductive and life style factors that may affect men and women's risk for developing MS differently. We also investigated the gender differences in autoimmune comorbidities and in the treatment response to IFN-β.

Based on the studies, the main conclusions are:

Reproductive factors' influence the risk of MS

Childbirths, up to five years before clinical onset, reduces the risk of MS in women

Pregnancies, even ended in induced abortions in the period five years before the first MS symptom also decrease the risk of MS

Pregnancy, by its temporary immunosuppressive effect, may diminish the risk of developing MS for up to five years

Pregnancy complications, diagnosed infertility or its treatment did not affect the risk of MS

Physical and social environmental factors and the risk of MS

Educational level has no influence on the risk of MS in Denmark

Our study could not support the hygiene hypothesis in the Danish population

The relative protection against MS, exerted by pregnancies, as shown in study 1 (see above) could not be attributed to the domestic physical environment caused by children in the household We only found excess numbers among female MS cases working in agriculture or working outdoors, but this was based on small numbers and could not contribute quantitatively to the incidence of MS in women.

Gender and autoimmune comorbidity in MS

Although women in the population have a higher occurrence of autoimmune diseases than men, male MS patients had a higher occurrence of other autoimmune diseases then female MS patients.

Women have a lower risk of the co-occurrence of MS and DM1 than men.

None of the investigated autoimmune diseases were, on the other hand, found to be less common in MS patients than in the population.

Gender differences in the treatment response to IFN-β

The presence of NAbs in IFN-beta treated patients has a significant detrimental influence of the efficacy of the treatment.

The effect of NAbs on the relapse activity was not different for men and women, which indicates that the treatment response to IFN-β is not affected by gender.

Age at treatment start and the number of relapses in the last 24 months are independent predictors of the relapse rate. Women had higher frequency of relapses than men.

FUTURE PERSPECTIVES

The incidence of MS in women is increasing, but we do not have the full explanation. A multifactorial explanation is likely and the increasing incidence of MS in women cannot be attributed to one single factor. It is also possible that the risk factors are various in different geographical areas with different living conditions and standards.

Our findings have contributed to approach the clarification of the role of the investigated factors for MS aetiology; however some of them are only applicable to the Danish population.

Epidemiological studies suggest a strong correlation between lifestyle changes and the incidence of MS, especially in women, but cannot provide an answer for a causal relationship.

Potential epidemiological factors that can contribute to an explanation of the diverging sex ratio include lifestyle choices and environmental triggers and one interesting target of research is hormonal changes and gene-environment interactions. Beside the investigated lifestyle factors, smoking, nutrition, EBV infection, exposure to sunshine and vitamin D level are the major candidates, which may have contributed to the changing sex ratio and all have capacity to effect epigenetic changes. Future studies on gene-environmental interactions inducing epigenetic changes may help to identify factors that affect MS susceptibility.

We found no evidence for sex related treatment response to IFN-β, but given the limited evidence, about this issue, the gender effect in treatment response to other drugs should be investigated.

Strengthening the epidemiological evidence by investigating the accumulation and clustering of exposures and possibly the interaction between exposures over time may bring us closer to an answer. Further studies with a more detailed collection of occupational data are needed to confirm a possible increased risk of MS from agricultural or outdoor working, and in particular from exposure to pesticides.

The unique possibilities of register-based research in Denmark facilitate verification of different hypotheses, but final conclusions needs collaboration with the biological disciplines. More studies clarifying the immunological mechanisms involved in pregnancy might provide a better insight to the pathogenesis and risk factors of MS onset

SUMMARY

There is an increasing incidence of MS in women in Denmark and Danish women's risk of developing MS has more than doubled in 25 years, while it has remained virtually unchanged for men. The explanation for these epidemiological changes should be sought in the environment, as genetics only explain a small part of the MS risk as the changes are too rapid to be explained by gene alterations. The rapid increase of MS incidence likely reflects unidentified changes in the environment and probably gene-environmental interactions.

My PhD thesis work was conceived and designed to investigate the relevant exposures in different periods of life that may have contributed to the increasing female to male ratio of cases of multiple sclerosis in Denmark. To study this, we investigated

the effect of numerous biological, social, physical and chemical environmental factors available from population based registries in a case-control approach.

Pregnancy may have a biological protective effect against developing MS in women, lasting for about 5 years. The protective effect is probably due to the modulation of the immune system by pregnancy. Our data on social behaviour changes regarding educational level, income, and relationship stability did not indicate reversed causality as a significant contributor to the lower number of childbirths in the five years before onset. Fewer pregnancies are one possible explanation we found for the increasing incidence of MS in women in our study. The trend towards fewer childbirths in the female population over decades may contribute to the increasing sex ratio and female incidence of MS.

Socio-economic status and lifestyle expressed in educational level and the sanitary conditions in youth are not associated with the risk of MS, and cannot contribute to the increasing epidemiological disparity between the genders over the last decades. A greater likelihood to be exposed to common infections did not show any effect on the MS risk neither in puberty nor in adulthood. The apparent protective effect of childbirth does not appear to reflect postnatal child exposure.

The only factor that may show association with a higher MS risk in women is working in agriculture but it was based on very small numbers and cannot contribute quantitatively to the incidence of MS in women.

Women are generally more prone to autoimmune diseases than men, but significant increased occurrence of some other autoimmune diseases was only found in male MS cases in the period before clinical onset. None of the investigated autoimmune diseases occurred less frequently in MS patients than in control persons.

Treatment response to Interferon-β, expressed in relapse rate was independently influenced by gender and the presence of NAbs, but it seems that the presence of NAbs does not affect the treatment effect differently in women and men. The results indicate that men's and women's treatment response to Interferon-β is similar.

Females had a higher frequency of relapses than males.
Our study did not reveal only one reason for the incidence increase, but as MS is multifactorial it is presumed that the incidence increase is caused by more than one factor, because women's lifestyle has undergone tremendous changes in the last half century. Our study contributes to clarification of this issue, with the role of pregnancies on the risk of MS. It is accepted that sex hormones have a clear immunologic involvement in the female predominance in MS, but there is no knowledge yet to explain the changes over time.

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