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Confirmation of association between Multiple Sclerosis and CYP27B1

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Abstract

Multiple Sclerosis, MS (OMIM #126200) is a complex inflammatory disease, characterised by lesions in the central nervous system. Both genes and environmental factors influence disease susceptibility. One of the environmental factors that has been implicated in MS and autoimmune disease such as type 1 diabetes is vitamin D deficiency, where patients have lower levels of 25-OHD₃ in blood than controls. Previtamin D₃ is produced in the skin, and in the liver turned into 25-OHD₃. In the kidney, skin and immune cells 25-OHD₃ is turned into the bioactive 1,25(OH)₂D₃ by the enzyme coded by CYP27B1 on chromosome 12q13.1-3. 1,25(OH)₂D₃ binds to the Vitamin D receptor, expressed in T cells and antigen presenting cells. 1,25(OH)₂D₃ has a suppressive role on the adaptive immune system, decreasing T cell and dendritic cell maturation, proliferation and differentiation, shifting the balance between Th1 and Th2 cells in favor of Th2 cells, and increasing the suppressive function of Tregs. Rs703842 in the 12q13-14 region was associated to MS in a recent study by the ANZgene consortium. We show associations to three SNPs in this region in our Swedish material (2158 cases, 1759 controls) rs4646536, rs10877012, rs10877015 (p=0.01, 0.01 and 3.5 x 10⁻³, respectively). We imputed rs703842 and performed a joint analysis with the ANZgene results, reaching a significant association of rs703842 (p = 5.1 x 10⁻¹¹ odds ratio 0.83 (0.79 – 0.88 95% confidence interval). Because of its close association with 25-OHD₃ our results lend further support to the role of vitamin D in MS pathology.

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Keywords: Multiple Sclerosis, CYP27B1, Association study, vitamin D, Case control study, meta analysis.

Introduction

Multiple Sclerosis, MS (OMIM #126200) is an inflammatory disease, characterised by demyelinating lesions in the brain or spinal cord, axonal loss and progressive build-up of sclerotic plaques. It has a complex disease etiology where both genetic and environmental factors influence disease susceptibility. To date the strongest genetic factor found is the HLA-DRB1*15 haplotype (DQB1*0602, DQA1*0102, DRB1*1501, DRB5*0101). Many other genes with more modest effects on disease risk have been found through candidate gene approaches and genome wide association studies.

There is an apparent geographical pattern in MS prevalence, with increasing prevalence with increasing latitude. This has been observed both in the US, Europe, and Australia¹. The level of solar radiation and UV radiation has been implicated in causing this difference in prevalence^{2,3}. There is also a month-of birth effect in MS susceptibility, with fewer cases born during the autumn months and more cases born in the spring compared to healthy controls^{4,5} which could be linked to differences in climate during the year, including sun exposure.

Recently the Australian and New Zealand Multiple Sclerosis Genetics Consortium, ANZgene, published their findings from a genome wide association study in a total of around 2900 cases and 5700 controls. Besides confirming the association of a number of previously known genes, they also found associated SNPs on chromosomes 12q13-14 and 20q13. The 12q13-14 region includes 17 known genes, including *CYP27B1* (cytochrome P450 family 27

subfamily B peptide 1). In the combined cohort, rs703842, which lies 1.76 kb upstream of *CYP27B1* was associated with MS ($p = 5.4 \times 10^{-11}$)⁶.

Association to *CYP27B1* has been seen in autoimmune diseases; rs4646536 (+2838T>C) which is in high LD with rs703842, and rs10877012 (-1260C>A) have previously been associated to type 1 diabetes^{7,8}, Hashimoto's thyroiditis, and Graves' disease⁹. Rs4646536 and rs703842 have been associated to 25(OH)D₃ levels in Canadian twins where at least one of the twins suffered from MS¹⁰.

Vitamin D3 is naturally produced in the skin after UV B radiation, but can also be supplied in the diet. The enzyme 25-hydroxy-vitamin D-1 alpha hydroxylase, coded by the gene *CYP27B1*, hydroxylates the inactive form of vitamin D3, 25-hydroxyvitamin D3, into its bioactive form, 1,25-dihydroxyvitamin D3. This means that the final level of active vitamin D3 is dependent on both environmental factors, such as sun exposure and diet, and genetic factors such as the *CYP27B1* gene. Bioactive vitamin D3 can bind to the vitamin D receptor (VDR) and has a regulatory role in calcium metabolism, as well as in immune functions.

Vitamin D3 stimulation leads to decreased expression of MHC class II molecules and co-stimulators in B-cells, decreased proliferation, differentiation and cytokine production and immune response in T-cells^{11,12}. A theory is that the production of CYP27B1, and activation of vitamin D3 by T-cells and DCs in extra-lymphoid tissues, might increase the T-cell programming of antigen-specific response, so called T-cell homing¹³. Overall, the findings support a suppressive role of vitamin D3 on the adaptive immune system.

Low levels of vitamin D in serum are associated with a higher risk for developing MS¹⁴ and several studies show that MS patients have lower levels of 25-OHD₃ compared to controls¹¹. A recent study showed that high circulating 25-OHD₃ levels among MS patients was associated with improved regulatory T cell function. It also had an impact on the T helper cells, shifting the balance in favor of Th2 cells¹⁵.

Based on the collective previous data on vitamin D, sun exposure, and inflammatory diseases, we decided to study if variants in the *CYP27B1* gene were associated to MS in a Swedish population.

Materials and methods

Dataset

Swedish Cohort 1: Swedish cohort 1 consisted of 1013 subjects (727 female) and 1215 blood donor controls (768 female). Patients were recruited from Danderyds Hospital and Karolinska University Hospital in Huddinge and in Solna, in the Stockholm County. Controls were blood donors that visited any of three blood donation facilities in the Stockholm area in 2001 and 2004/2005. Blood was consecutively sampled and re-sampling avoided by collection under a period of three month (under which blood donors are advised not to donate a second time) in 2001 or by explicitly asking if sample had previously been obtained (in 2004 and 2005). Controls were not screened for MS. Information about the study, along with questions regarding previous participation and ethnic origin of parents, was presented to donors both orally and in written form¹⁶.

Swedish Cohort 2: These cases and controls are part of an ongoing population based case-control study called EIMS (Epidemiological Investigations in Multiple Sclerosis), described previously¹⁷. 664 patients (472 females, 1 unknown sex) and 544 controls (406 females) were available for genotyping. Patients and controls are recruited from all over Sweden. Controls are matched for age, sex, and residential location.

Swedish Cohort 3: Patients (n=481, 347 female) recruited at Karolinska University Hospital Solna and from clinics all over Sweden.

In total, 2158 cases (1546 females) and 1759 controls (1174 females) were included and available for genotyping. All cases fulfilled the MacDonald criteria. Possible overlaps between the cohorts were excluded using the national personal identification number for cases. All participants were of Scandinavian ancestry. Oral or written informed consent was obtained from all participants. The Ethical Committees at Karolinska University Hospital Huddinge and Karolinska University Hospital Solna approved the study.

DNA extraction

DNA was extracted from whole blood using standard procedures. DNA concentrations were measured using Picogreen™ fluorescence or with a ND-1000 Spectrophotometer (NanoDrop Technologies Inc, Wilmington, DE, USA).

SNP selection

SNP markers were selected for genotyping by running the tagging algorithm¹⁸ implemented in Haploview 3.32 based on data from the HapMap project using $r^2=0.9$ and LOD 3.0. (<http://www.hapmap.org/>, release 21/phaseII, NCBI B35 assembly, dbSNP b125). Two additional SNPs were included because of either reported association to type 1 diabetes (rs10877012) or because of a proposed involvement in MS susceptibility (rs4646536)^{8,10}. A complete list of included SNPs is shown in table 1.

Genotyping

Two methods were utilized for allelic discrimination in the Swedish cohorts (see table 1). Rs10877012 and rs4646536 were genotyped using Taqman SNP ® assays from Applied Biosystems as described previously¹⁹. 6 SNPs, including rs4646536 were genotyped in all cohorts using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Sequenom Inc., San Diego, CA, USA), as described previously¹⁶. Primer sequences are available upon request. One of the tagSNPs (rs11172327), which only tagged itself, was excluded from the analysis because of low success rate <75%, however this marker was also monomorphic in our samples.

Imputation and statistical analysis

Association of genotypes in the Swedish samples was tested using the model command in PLINK v1.06 (<http://pngu.mgh.harvard.edu/~purcell/plink/index.shtml>). Imputation was performed using Mach 1.0²⁰ accessed through the HapMap Genome Browser (Phase 3 - genotypes, frequencies and LD). The CEU population was used as reference population; the region was set to chromosome 12, 56,430,000 kb to 56,470,000 kb. rs8176345 was

excluded from the imputation because it was not in Hardy-Wienberg equilibrium among controls. All other settings were kept at default. A fixed effects (Mantel-Haenszel) meta-analysis was performed using the *rmeta* package and the *meta.MH* command in R 2.8.0, a joint p-value was obtained from Unphased using study population as a covariate²¹.

Results

Seven SNPs were genotyped in the Swedish cohorts, described in more detail in table 1 (also see supplementary table 1 for detailed information for each of the three cohorts). All SNPs, except rs8176345 were in Hardy-Weinberg equilibrium among the unaffected controls. Three SNPs genotyped in the Swedish cohorts were associated to MS, rs4646536, rs10877012 and rs10877015, with p-values of 0.01, 0.01 and 3.5×10^{-3} , respectively. They all had a reduced minor allele frequency among cases, with odds ratios of 0.88, 0.88 and 0.87, respectively for the minor allele. When we looked at the linkage disequilibrium in the region (figure 1), and defined the blocks either by the four gamete rule or solid spine of linkage disequilibrium there were two associated haplotypes, a CTACGA-haplotype ($p = 8.5 \times 10^{-3}$) and a CTGCTG-haplotype ($p = 7.8 \times 10^{-3}$). Defining the blocks by the confidence interval method gave similar results.

Imputed SNP and combined analysis

The most associated SNP from the ANZgene study located in the 12q13-14 region was rs703842, at position 55,449,006, between rs10877012 and rs10877015, run in our own samples. Genotypes from 1759 controls and 2158 Swedish cases were imputed. Other SNPs found in the ANZgene paper could not be imputed in our material. Rs703842 was found to

be significantly associated (table 1), with a p-value of 0.02, and an odds ratio of 0.89 (0.81 – 0.98 95% CI) for the minor allele, showing the same protective effect found in the ANZgene datasets.

For the combined analysis, genotypes from a total of 6032 cases and 7482 controls were available after imputation (see table 2). The results from the meta-analysis can be seen in figure 2. The combined OR for rs703842 was 0.83 (0.79 – 0.88 95% CI). The overall association was significant, $p = 5.1 \times 10^{-11}$.

Discussion

In this study we tested the association of the *CYP27B1* gene to MS in a Swedish population. We replicate the previously identified association in the Australian and New-Zeeland population (ANZgene 2009). In a joint analysis the association of rs703842 to MS show an OR of 0.83 (0.79 – 0.88 95%CI), reaching a significance 5.1×10^{-11} lending further evidence to the involvement of CYP27B1 and vitamin D3 in MS.

The candidate region on chromosome 12q13-14 studied by the ANZgene consortium contains 17 genes. *CYP27B1* is the most likely MS susceptibility candidate, given its importance in vitamin D3 activation and the important role of vitamin D3 in immunological functions. We have in this study confirmed the association between MS and the *CYP27B1* gene. We cannot, however, exclude the possibility that our association to *CYP27B1* is caused by linkage disequilibrium with some other marker in the 12q13-14 region such as

polymorphisms in the *MTTL1* gene. This study was a case-control study, although it has been performed in the fairly homogeneous Swedish population it is possible, although unlikely, that our observed association is due to population stratification between cases and controls. This could be corrected for by performing an association analysis in a family based material or by using genomic controls which unfortunately were not available for the included individuals²².

Several studies show that MS patients have lower levels of 25-OHD₃ compared to controls¹¹. In an Australian study, low levels of 25-OHD₃ could be linked to increased disability, likely caused by decreased sun exposure due to disability²³. It has also been shown that there is an association between SNPs in the *CYP27B1* region and 25(OH)D₃ levels¹⁰.

Lower levels of 25-OHD₃, and, 1,25(OH)₂D₃ have also been seen in type 1 diabetes patients, and they also had a lower expression of CYP27B1 mRNA compared to controls. This reduction in expression could be linked to the GG variant of the rs10877012 polymorphism²⁴. In our own material there was a significant difference in the number of GG homozygotes between cases and controls (cases frequency: 0.46, controls frequency: 0.42, $p = 0.02$). We would then expect a similar result in terms of CYP27B1 expression in our own material.

One of the important immune genes regulated by vitamin D is the HLA class II locus, where there is a vitamin D receptor response element in the HLA-DRB1 promoter region, and

highly conserved in the HLA-DRB1*1501 haplotype, which is the most important risk gene in MS. It was also shown that vitamin D3 had an effect on HLA-DRB1*1501 expression *in vitro*. However, the exact mechanism for the interaction between vitamin D3 and HLA and in which tissues or cells it takes place remains unknown²⁵.

Lower exposure to sunlight and low expression of CYP27B1 mRNA will likely influence the amount of available active vitamin D3 in the body. In individuals with other genetic risk factor(s) and possibly exposed to other environmental factor(s), this could, through the effects of vitamin D3 on the immune system, lead to the development of MS or other autoimmune disease.

The results from studies on type 1 diabetes and CYP27B1 genotype and expression levels show that CYP27B1 expression could play a role in autoimmune disease. This should be replicated in MS patients, as well as studying the relationship between genotype and 25-OHD₃ levels. It will also be of value to study possible genetic interactions between *CYP27B1*, *VDR* and the HLA-complex, in particular HLA-DRB1*1501.

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Conflict of interest (flyttas till före referenserna)

Ms Sundqvist reports no disclosures.

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Dr Kockum reports no disclosures.

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TABLES

Table 1: Summary of the SNPs genotyped and imputed in the Swedish material

SNP	Alt.name	Position*	Alleles		Method	Cases			Controls			HWE (p)		Association, allelic test	
			Minor	Major		Total N	MAF	No.	Total N	MAF	No.	Controls	P-value	OR	95 % CI
rs1048691		56439215	T	C	MT-MS	1938	0.222	760	1722	0.226	699	0.21	NS		
rs4646537		56443548	G	T	MT-MS	2072	0.038	161	1723	0.041	141	0.36	NS		
rs4646536	+2838T>C	56444255	G	A	Taqman/ MT-MS	2064	0.324	1118	1733	0.352	1008	0.79	0.01	0.88	0.80 – 0.97
rs8176345		56444825	T	C	MT-MS	2102	0.029	121	1751	0.030	101	0.02	NS		
rs2307096		56445011		C	MT-MS	2040	0.00		1708	0.00		1.00	NA		
rs10877012	-1260C>A	56448352	T	G	Taqman	1973	0.326	1075	1682	0.354	982	0.92	0.01	0.88	0.80 – 0.97
rs10877015		56454055	G	A	MT-MS	2086	0.321	1129	1741	0.353	1016	0.68	3.5x10 ⁻³	0.87	0.79 – 0.95
rs703842		56449006	G	A	Imputed	2158	0.330	1209	1759	0.355	1037	0.32	0.02	0.89	0.81 – 0.97

Total number of cases and controls genotyped, the minor allele frequency for each group and the number of individuals

carrying the minor allele *NCBI build 36.3, MT-MS = MALDI-TOF MS.

Table 2: The different study populations

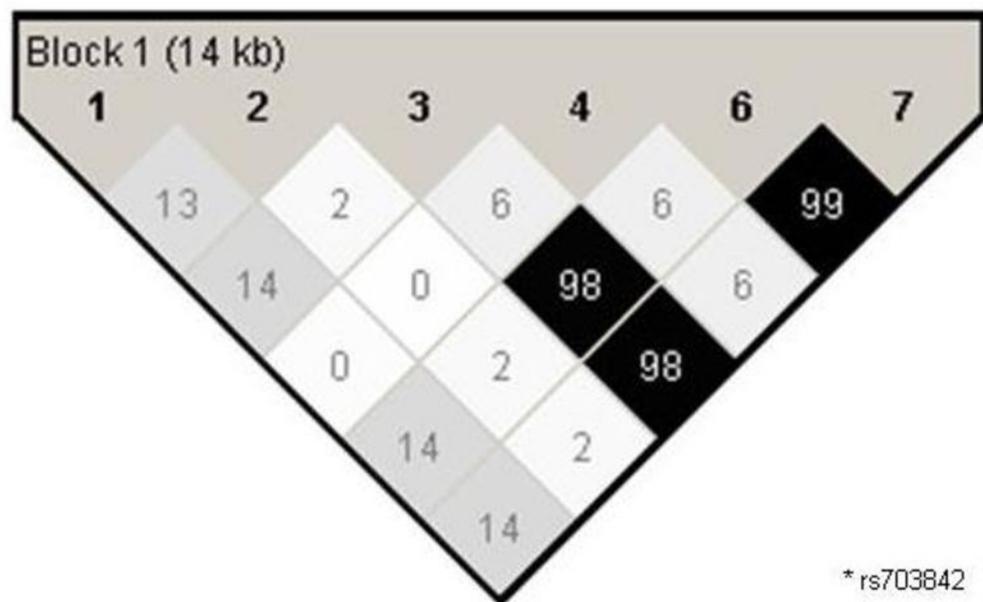
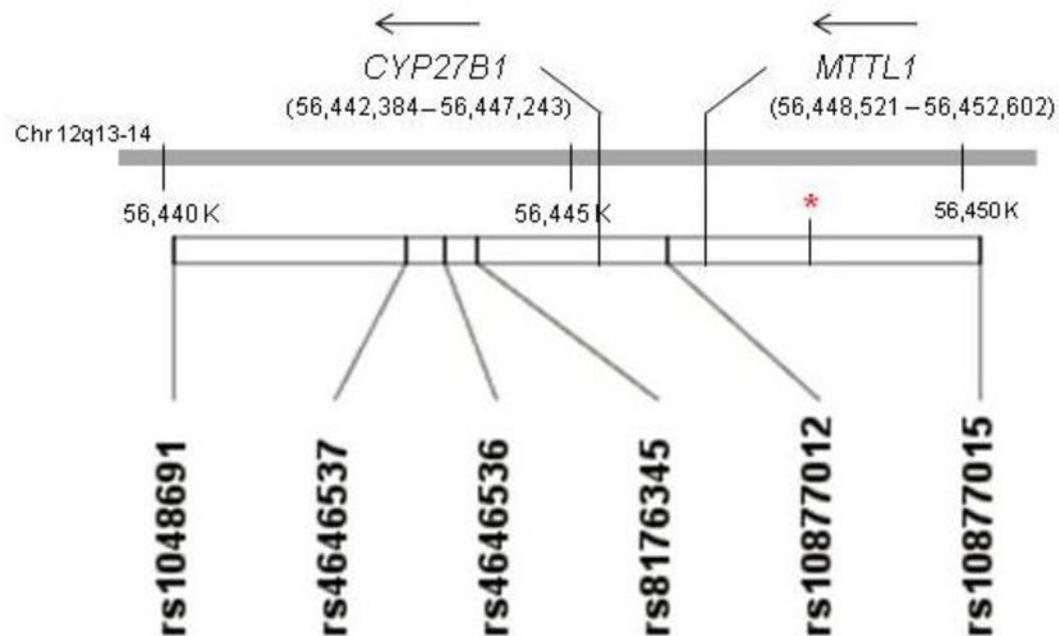
Study	Cases	Controls	Genotyped/Imputed
ANZgene GWAS	1618	3413	Genotyped
ANZgene replication	2256	2310	Genotyped
Sweden	2158	1759	Imputed
<i>Total N</i>	<i>6032</i>	<i>7482</i>	

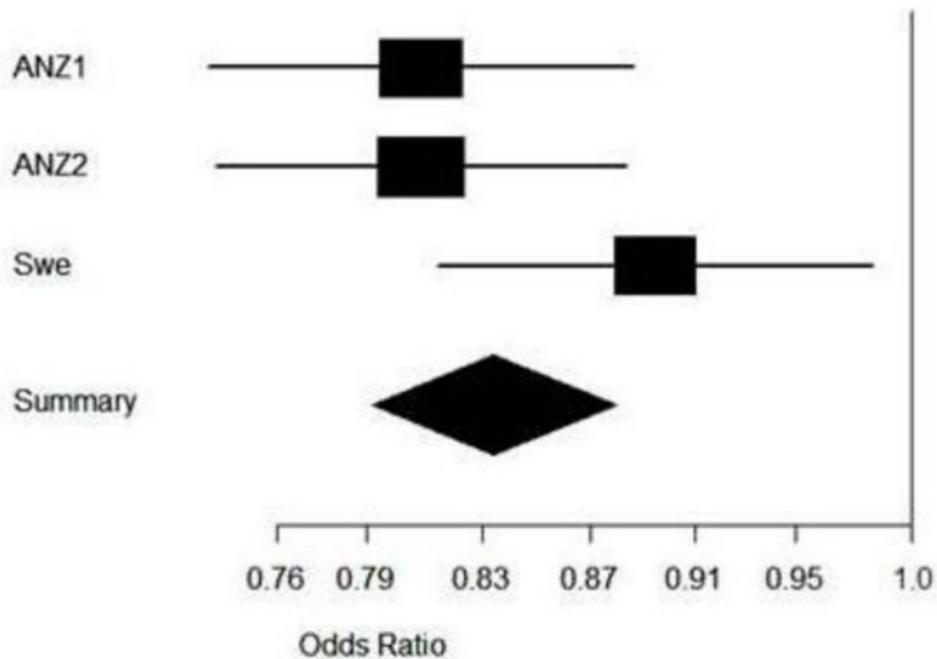
The three different study populations used in the combined analysis. Partially adapted from ANZgene. 2009.

Supplementary Table 1. Allele and haplotype frequencies in different Swedish cohorts

SNP	Cohort 1			Cohort 2			Cohort 3			
	Total N	MAF		P-value	Total N	MAF		Total N	MAF	
		<i>Cases</i>	<i>Controls</i>			<i>Cases</i>	<i>Controls</i>		<i>Cases</i>	
rs1048691	2022	0.218	0.227	NS	1093	0.225	0.224	NS	453	0.225
rs4646537	2140	0.033	0.043	NS	1181	0.042	0.036	NS	466	0.041
rs4646536	2093	0.322	0.352	0.04	1175	0.334	0.352	NS	469	0.316
rs8176345	2180	0.032	0.030	NS	1200	0.028	0.030	NS	473	0.024
rs2307096	2105	0.00	0.00	NS	1190	0.00	0.00	NS	453	0.00
rs10877012	2074	0.325	0.356	0.04	1121	0.339	0.350	NS	460	0.311
rs10877015	2175	0.321	0.353	0.03	1182	0.327	0.352	NS	470	0.313
rs703842	2228	0.332	0.354	NS	1208	0.335	0.357	NS	481	0.318
Haplotype	Total N	Frequency		P-value	Total N	Frequency		P-value		
		<i>Cases</i>	<i>Controls</i>			<i>Cases</i>	<i>Controls</i>			
CTACGA	2175	0.456	0.418	0.01	1197	0.437	0.424	NS	470	0.457
CTGCTG	2175	0.289	0.323	0.01	1197	0.304	0.320	NS	470	0.287

Total number of cases and controls genotyped in each of the Swedish datasets. For each SNP and haplotype data over total samples genotyped, the minor allele frequency for cases and controls, p-values. There were no significant differences between the three groups.





	Cases (alleles)	Controls (alleles)	OR
ANZ1	3236	6826	0.81
ANZ2	4512	4620	0.81
Swe	4316	3518	0.89

Titles and legends to figures

Figure 1: LD-plot

A plot showing the linkage disequilibrium structure of the six markers genotyped in the Swedish cohorts in the 12q13-14 region. The location of the imputed SNP, rs703842, is marked with an asterisk. The linkage disequilibrium in the region of the tested genes is given as r^2 , and analysed using the four gamete rule.

Figure 2: Meta-analysis of the two ANZgene cohorts combined with the Swedish cohorts.

Meta-analysis of the two ANZgene cohorts combined with the Swedish cohorts, with the allele count for cases and controls, and the odds ratio for each group. The combined odds ratio is 0.83 (0.79 - 0.88 95%CI).