Polymorphisms in CD28, *CTLA-4*, *CD80* and *CD86* genes may influence the risk of multiple sclerosis and its age of onset

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ABSTRACT

CD28/CTLA-4–CD80/CD86 molecules play an important role in the regulation of T cells activation. Defects in proteins involved in this pathway may lead to the development of autoimmune diseases in which T cells are involved.

In this case–control study (336 multiple sclerosis (MS) patients and 322 controls) we investigated the possible association of eleven polymorphisms in *CD28*, *CTLA-4*, *CD80* and *CD86* genes with susceptibility to MS and/or its progression. We also took into account *HLA-DRB1*15:01* status. Moreover, this study aimed to determine the possible gene-gene interactions between examined SNPs associated with the susceptibility to MS and its outcome.

Our investigation revealed that in *HLA-DRB1*15:01* negative individuals, G allele in rs231775A > G of *CTLA-4* gene was associated with higher risk of multiple sclerosis. Additionally, the association of rs2715267T > G of *CD86* gene with MS susceptibility was detected. In details, carriers of G allele at this polymorphic site possessed higher risk of MS in comparison to TT homozygotes. On the other hand, the lower risk of MS was observed in individuals carrying A allele at the rs1599795T > A polymorphic site of *CD80*. Furthermore, the analysis revealed an interaction between three polymorphisms: rs3116496T > C (*CD28*), rs6641T > G (*CD80*) and rs17281995G > C (*CD86*), associated with the age of MS onset.

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1. Introduction

The optimal activation of T lymphocytes requires two signals: the first results from the interaction of T cell receptor (TCR) with antigen presented by MHC on the surface of antigen presenting cell (APC); the second is delivered from the interaction of costimulatory receptors on T lymphocytes with ligands presented on the surface of APCs. The absence of second signal may lead to anergy and/or apoptosis of T lymphocytes, which may contribute to failure in initiation of an effective immune response (Chitnis and Khoury, 2003). CD28–CD80/CD86 pathway provides the strongest costimulatory signal, whereas CTLA-4 is a negative regulator of T cell activation. The balance between activation (CD28–CD80/CD86) and inhibition (CTLA-4–CD80/CD86) processes is of great importance to the maintenance of immune tolerance. Defects in protein involved in these pathways may lead to the development of

autoimmune diseases in which T cells are involved, such as multiple sclerosis (MS) (Sansom, 2000).

MS is a chronic disease of the central nervous system leading to demyelination and axonal damage in the brain and the spinal cord (Pender and Greer, 2007). It is estimated that the disease affects around 2.5 million people worldwide. MS most commonly starts in young adults, between 20 and 40 years of age. Moreover, women are affected twice as often as men, which is characteristic of many autoimmune diseases (Sawcer et al., 2014). The pathomechanism of MS is complex and its course and progression can be various. About 85% of patients experience relapsing–remitting (RR) course of the disease characterized by relapse followed by recovery period (Inglese, 2006). Most of them evolve into a secondary progressive (SP) phase characterized by a steady increase in disability within 25 years (Inglese, 2006, Weinshenker et al., 1989). About 10–15% of patients experience primary progressive (PP) form, defined by the accumulation of disability from the onset of the disease (Inglese, 2006).

Here we investigated the possible association of eleven polymorphisms in *CD28*, *CTLA-4*, *CD80* and *CD86* genes with susceptibility to multiple sclerosis and/or progression of this disease. Moreover, this

study aimed to determine the possible gene-gene interactions between examined SNPs associated with the susceptibility to MS and its outcome. We also took into account *HLA-DRB1*15:01* status, which was determined in our earlier studies (Wagner et al., 2013, Wisniewski et al., 2013) According to the literature data stratification for this well-established MS risk factor is expected to help to reveal the associations of other polymorphisms with MS susceptibility (Bronson et al., 2010, Gyllenberg et al., 2014). This fact was also confirmed in our earlier study (Wagner et al., 2013).

2. Materials and methods

2.1. Study population

336 patients — 226 females and 110 males, with clinically definite MS according to the McDonald criteria (Polman et al., 2011) were included in this study. All patients were under the charge of Department of Neurology, Wroclaw Medical University. Degree of disability and the rate of its progression were scored using Kurtzke's Expanded Disability Status Scale (EDSS) and MS Severity Score (MSSS), respectively (Kurtzke, 1983, Roxburgh et al., 2005). 250 patients presented relapsing remitting course of multiple sclerosis, while 86 secondary progressive form of the disease. We did not include patients with primary progressive course, since we did not collect sufficient number of samples to perform powerful statistical analysis. The detailed characteristics of patients are presented in Supplementary Table 1.

Controls were 322 blood donors (138 females and 184 males) with no history of inflammatory disease. The study was approved by the ethics committee of Wroclaw Medical University and written informed consent was obtained from all participants.

2.2. Selection of single nucleotide polymorphisms

SNPs examined in this study were selected on the basis of the literature data and in silico analysis performed using SNPinfo Web Server (Xu and Taylor, 2009) (please see Supplementary Table 2). This analysis encompassed the gene-transcribed sequences and 3000 bp upstream and 3000 bp downstream regions. Prediction of SNP's function was performed on the basis of data for the CEU population (Utah residents with Northern and Western European ancestry) from the International HapMap Project and for the European population from the NCBI dbSNP database. Moreover, in order to choose TagSNPs, linkage disequilibrium (LD) blocks were estimated.

2.3. DNA isolation and genotyping

Genomic DNA was isolated from whole blood using Invisorb Blood Midi Kit (Stratec Molecular) according to the manufacturer's protocol.

Nine of the examined SNPs were genotyped by applying polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP). Primer sequences, annealing temperatures and restriction enzymes used in this study are listed in Supplementary Table 3. Genotyping of rs3116496T > C of *CD28* gene and rs1129055G > A of *CD86* gene was carried out using allelic discrimination method with the TaqMan SNP Genotyping Assays (Life Technologies, C_25922478_10 and C_7504226_10, respectively).

*HLA-DRB*15:01* status was determined by genotyping the rs31355588 G > A, as described by Wisniewski et al. (Wisniewski et al., 2013). These data were generated in our earlier studies (Wagner et al., 2013, Wisniewski et al., 2013) and they were taken into consideration in the analyses performed here.

Accuracy of genotyping methods for all SNPs was verified through direct sequencing of few samples representing homozygotes of two types and heterozygotes for each investigated SNP. These samples were used as the reference samples in following genotyping experiments.

2.4. Statistical analysis

Chi-square test, χ^2_{df}, was used to test the null hypothesis that cases and controls have the same distribution of genotype counts. In case of small numbers the distribution of the test statistics was estimated numerically. *Odds ratio* (OR) and confidence interval for it at $1-\alpha = 0.95$ level (CI95%) was computed as the measure of effect size. Homogeneity of two odds ratios was tested with Breslow–Day test. Median and mean were used as the location parameters. In case of median S_n statistics was computed as the measure of variability: $S_n = med\{med|x_i - x_j|; j = 1 \dots n\}$ (Rousseeuw and Croux, 1993). Additionally 1st and 3rd quartile, minimal and maximal observation were reported. Departure from Hardy–Weinberg equilibrium (HWE) was tested with chi-squared test and measured as $f = \frac{p_{CC} - p_C^2}{p_C(1 - p_C)}$, where p_C and p_{CC} are allele C and genotype CC frequencies while $f < 0$ and $f > 0$ corresponds to deficiency and excess of homozygotes, respectively, and $f = 0$ in case of HWE. Haplotype frequencies (HFs) among SNPs were estimated with *maximum likelihood* function (Excoffier and Slatkin, 1995). Measure for the estimation of pair-wise linkage disequilibrium (LD) was squared correlation between two SNPs, r^2 (Abdallah et al., 2003). For two SNPs $r^2 = \sum_i^2 \sum_j^2 \frac{D_{ij}^2}{p_i q_j}$, where p_i and q_j are the population allele frequencies of the ith allele on locus A and the jth allele on locus B, $D_{ij} = x_{ij} - p_i q_j$, and x_{ij} is the frequency of the haplotype with alleles i and j on loci A and B, respectively. Chi-square statistic was calculated to test that $D_{ij} = r^2 = 0$ between two SNPs: $\chi^2_{df=2} = \sum_i^2 \sum_j^2 \frac{n D_{ij}^2}{p_i q_j}$. Likelihood ratio statistics, LRS ∼ χ^2, was used to test for differences in haplotype frequencies between cases and controls, $LRS = 2(LL_{Cases} + LL_{Controls} - LL_{Combined})$.

To control type I error in case of many tests for differences between SNPs genotypes of cases and controls global (omnibus) chi-square test was performed first to test the hypothesis, that there were no differences between cases and controls in any SNP, opposite to the alternative, that genotype frequencies in cases and controls were different at least in one SNP. Because of correlation between SNPs distribution, global chi-square statistics was estimated numerically. MS progression and its association with genetic factors was modeled as two-dimensional variable $\mathbf{x}_i = (EDSS_i, MSSS_i) \in \Re^2$ and tested with λ-Pillai statistic. Age of onset was described by mean, standard deviation (SD) and range [min-max]. Linear regression was used to test relations between selected polymorphisms and age of onset including interactions between them. *Bootstrap* approach (B = 5999) was employed to estimate model's coefficients (differences between the means) and 95% confidence intervals for them. Determination coefficient R^2 is total variation of response variable explained by the model.

Table 1

Genotypes distribution of *CTLA-4* rs231775A > G polymorphism in multiple sclerosis patients and controls in dependence on the presence of *HLA-DRB1*15:01* allele.

	Cases		Controls		OR	CI95%
	N	%	N	%		
CTLA-4 rs231775A > G in *HLA-DRB1*15:01* (−) individuals						
AA	52	29.5	97	39.6	1[a]	
AG	87	49.4	109	44.5	**1.49**	**0.96; 2.31**
GG	37	21.0	39	15.9	**1.77**	**1.01; 3.10**
∑	176	100.0	245	100.0		
CTLA-4 rs231775A > G in *HLA-DRB1*15:01* (+) individuals						
AA	55	34.4	21	27.3	1[a]	
AG	80	50.0	42	54.5	0.73	0.39; 1.36
GG	25	15.6	14	18.2	0.68	0.30; 1.55
∑	160	100.0	77	100.0		

$\chi^2_{df=2} = 6.934$; p = 0.031.
The statistically significant values are bolded.
 [a] Reference group.

Four hypotheses (H1–H4) considering relation between polymorphisms in two ligands, one receptor and age of onset, a priori formulated, were tested with F statistic for contrasts. As the measures of effect size two correlation coefficients were used: $r_{alerting}$ and $r_{efect\ size}$. First of them is Pearson correlation coefficient between group means and weights of contrast. The second one is the correlation adjusted for two additional sources of variation: variation within group and noncontrast variation between groups:

$$r_{efectsize} = \sqrt{\frac{\overline{F}_{contrast}}{\overline{F}_{contrast} + \overline{F}_{noncontrast} \times (ss_{noncontrast}) + ss_{within-group}}}$$

Null hypothesis, H0, stated that there were no differences in mean of age of onset between eight groups.

3. Results

3.1. Polymorphisms and MS susceptibility

In this study, we investigated eleven polymorphisms in *CD28*, *CTLA-4*, *CD80* and *CD86* genes. We also took into consideration the presence of *HLA-DRB1*15:01* allele.

We found no evidence that genotypes frequencies of all examined SNPs were different from those expected from Hardy–Weinberg equilibrium both in controls and cases.

The result of the global (omnibus) test allowed us to reject the hypothesis about lack of association of all examined SNPs with MS risk and to assume that the genotype distribution in the patients group is different than in the control group in at least one polymorphism ($\chi^2_{df=31}$ = 51.325; p = 0.0201). The detailed analysis of particular SNPs is presented below.

3.2. CD28 polymorphisms

We studied two polymorphisms in *CD28* gene: rs35593994G > A and rs3116496T > C and observed that neither of them was associated with MS susceptibility (Supplementary Table 4). The lack of the association of genotypes in rs35593994G > A as well as in rs3116496T > C with MS risk was found in both *HLA-DRB1*15:01*– and *HLA-DRB1*15:01* + individuals (data not shown). Next, we analyzed the frequencies of haplotypes in the controls and the patients, however, we did not find any differences in their distribution between compared groups (LL $_{df=3}$ = 1.538; p = 0.6735) (Supplementary Table 5). The examined *loci* were not in LD with each other in controls as well as in patients.

3.3. CTLA-4 polymorphisms

In our study we also examined three polymorphisms of *CTLA-4* gene rs5742909C > T, rs231775A > G, rs11571302G > T. No association was found between any of these SNPs and susceptibility to multiple sclerosis (Supplementary Table 6). However, further analysis revealed that the association of rs231775A > G with MS risk should not be considered without taking into account the presence of *HLA-DRB1*15:01* allele ($\chi^2_{df=2}$ = 6.934; p = 0.0312) (Table 1). We observed that G allele in rs231775A > G of *CTLA-4* gene was associated with higher MS susceptibility but only in *HLA-DRB1*15:01*-individuals ($\chi^2_{df=1}$ = 4.66; p = 0.0309). In detail, heterozygotes AG had 1.49 higher risk of MS in comparison to AA homozygotes (OR = 1.49; CI95%[0.96; 2.31]). Taking into consideration the additive model of association, GG individuals should have had over 2-fold higher risk of MS than those with AA genotype (expected OR = 2.22). The results of our study showed that this risk for GG homozygotes was 1.7 higher than for AA individuals (OR = 1.77), however the confidence interval CI95%[1.01; 3.1] allowed us to accept the assumption about additive model of association. As mentioned earlier, the above-described relation concerned only *HLA-DRB1*15:01*-individuals. In the group of individuals possessing *HLA-DRB1*15:01* allele, such association probably did not exist. The analysis of *CTLA-4* haplotypes did not reveal any differences in their distribution between patients and controls (LL$_{df=5}$ = 4.64; p = 0.46) (Supplementary Table 7). Moreover, only moderate LD was found between rs231775 and rs11571302. Both in patients and controls the coefficients of determination of two alleles was about r^2 = 0.5, what means that the presence of specific allele in rs231775 was determined in about 50% by the presence of specific allele in rs11571302 (and vice versa).

3.4. CD80 polymorphisms

Of the three *CD80* polymorphisms tested in our study (rs6641T > G, rs1599795T > A, rs16829980T > C) only rs1599795T > A was associated with MS risk ($\chi^2_{df=1}$ = 4.41; p = 0.036) (Table 2a). In detail, individuals with TA genotype in this *locus* had 1.5-fold lower risk of multiple sclerosis in comparison to those with TT genotype (OR = 0.65; CI95%[0.46; 0.92]), while for AA homozygotes this risk was 1.2 times lower as compared to the TT homozygotes (OR = 0.85; CI95%[0.35; 2.08]). We also observed that above-described association, as well as the potential relation of remaining *CD80* SNPs with MS, were not dependent on the presence of *HLA-DRB1*15:01* allele ($\chi^2_{df=2}$ = 0.12; p = 0.94). The genotypes distribution of rs6641T > G and rs16829980T > C, which were not associated with MS susceptibility, are shown in

Table 2

Genotype distribution of *CD80* rs1599795T > A and *CD86* rs2715267T > G polymorphisms in multiple sclerosis (MS) patients and controls.

	Cases		Controls		OR	CI95%	
	N	%	N	%			
a) CD80 rs1599795T > A							
TT	247	73.5	209	64.9	1[a]		$\chi^2_{df=1}$ = 4.41; p = 0.036
TA	79	23.5	103	32.0	0.65	0.46; 0.92	
AA	10	3.0	10	3.1	0.85	0.35; 2.08	
∑	336	100.0	322	100.0			
H-W equilibrium	χ^2 = 1.38; p = 0.19 f(CI95%) = 0.064 (−0.05; 0.18)		χ^2 = 0.40; p = 0.60 f(CI95%) = −0.035 (−0.14; 0.07)				
b) CD86 rs2715267T > G							
TT	115	34.2	134	41.6	1*		$\chi^2_{df=1}$ = 4.41; p = 0.036
TG	164	48.8	146	45.3	1.31	0.94; 1.83	
GG	57	17.0	42	13.1	1.58	0.99; 2.53	
∑	336	100.0	322	100.0			
H-W equilibrium	χ^2 = 0.01; p = 0.91 f(CI95%) = −0.006 (−0.12; 0.1)		χ^2 = 0.05; p = 0.90 f(CI95%) = 0.013 (−0.1; 0.12)				

f-Departure from Hardy–Weinberg equilibrium.

[a] Reference group.

Supplementary Table 8. Analysis of haplotypes frequencies, which results are shown in Supplementary Table 9, revealed that their distribution differed between controls and patients (LL $_{df=7}$ = 17.755; p = 0.013). The most frequent haplotype T T T (rs6641 rs1599795 rs16829980) was almost 10% more frequent in MS patients than in controls (the ratio: haplotype frequency in patients/haplotype frequency controls was RR = 1.08). Moreover, we found that in patients haplotypes with A allele in rs1599795 (T A T, T A C, G A T and G A C) were less frequent than in controls. The analysis of linkage disequilibrium showed that two of the investigated SNPs of *CD80*, rs6641 and rs16829980, were in moderate LD (r^2 = 0.445 for patients and r^2 = 0.421 for controls).

3.5. CD86 polymorphisms

We also investigated three polymorphisms of *CD86* gene: rs2715267T > G, rs1129055G > A and rs17281995G > C. One of them - rs2715267T > G was associated with MS susceptibility (χ^2 $_{df=1}$ = 4.41; p = 0.036) (Table 2b). We noticed that individuals with TG genotype in this *locus* showed 1.3-fold higher risk for MS in comparison to those with TT genotype (OR = 1.31; CI95%[0.94; 1.83]), while the risk of the disease for GG homozygotes was 1.58 times higher than for TT individuals (OR = 1.58; CI95%[0.99; 2.53]). The genotypes distribution in remaining CD86 SNPs is shown in Supplementary Table 10. Our study also revealed the difference in haplotypes frequencies between the patients and the controls (LL$_{df=7}$ = 18.054; p = 0.012) (Supplementary Table 11). We observed that the most frequent haplotype T G G (rs2715267 rs1129055 rs17281995) was almost 10% less frequent in MS patients than in controls (the ratio: haplotype frequency in patients/haplotype frequency controls was RR = 0.89). The results of genotype analysis indicated that the presence of G allele in rs2715267 was associated with the increased risk of MS. Data presented in Supplementary Table 11 confirmed this result, since haplotypes possessing allele G in rs2715267 were more frequent in the group of patients than in controls. Furthermore, it is worth paying attention to haplotype G G C (rs2715267 rs1129055 rs17281995), which was 1.4 times more frequent in patients than controls as well as to haplotype T G C (rs2715267 rs1129055 rs17281995), which was 2-fold less frequent in individuals with MS than in healthy subjects. The analysis of linkage

disequilibrium revealed that the investigated *loci* were not in LD with each other in controls as well as in patients.

3.6. Polymorphisms in CD28, CTLA-4, CD80 and CD86 genes and clinical MS data

In the next step, we analyzed the possible association between all studied here polymorphisms and clinical MS data such as gender, age of onset, duration of RR course, EDSS and MSSS. We assumed the dominant model of association due to the low frequencies of minor alleles in the case of some polymorphisms. Thus, the minor allele carriers were compared to the group of common homozygotes.

We found no evidence for association between analyzed polymorphisms of *CD28*, *CTLA-4*, *CD80* and *CD86* genes and duration of relapsing-remitting course of the disease ($\chi^2_{df=11}$ = 15.15; p = 0.18).

Moreover, none of the examined SNPs was associated with disease progression measured on the scales of disability - EDSS and MSSS (λ_{Pillai} = 0.10; p = 0.06).

However, we identified an interaction of three polymorphisms: rs3116496T > C of *CD28*, rs6641T > G of *CD80* and rs17281995G > C of *CD86* associated with the age of onset. The mean values of the age of onset, 95% confidence intervals, standard deviations and the size of groups of individuals divided according to the combination of genotypes at those three polymorphic sites are presented in Table 3.

The mean value of the age of onset in the group H, that is, in the group of individuals who were simultaneously carriers of at least one C allele in rs3116496 (*CD28*) and at least one G allele in rs6641 (*CD80*) and at least one C allele in rs17281995 (*CD86*) was 23.89 and it was lower as compared to the mean age of onset of the rest of individuals (total mean for groups A-G: 29.34 years). Fig. 1 shows box-and-whiskers plot of age of onset in eight groups, according to Table 3.

The detailed description of this interaction is showed in Table 4. Although *HLA-DRB1*15:01* seemed not to be associated with the age of onset, we took it into consideration as a key genetic factor associated with multiple sclerosis. Thus, *HLA-DRB1*15:01*-individuals who were simultaneously wild (common) homozygotes at all three polymorphic sites were treated as a reference group. Table 4 presents the differences between the age of onset in groups of individuals with specific genotype (or specific combination of genotypes) and the age of onset in the reference group. Due to the significant interaction between rs3116496, rs6641 and rs17281995 polymorphisms, their association with the age of onset has to be considered together. The mean value of the age of onset for carriers of minor alleles in these three *loci* was 24.65 CI95%[19.42; 29.88]. This estimation is not very precise due to the small number of individuals possessing all three minor alleles at considered sites. However we cannot exclude that the mean value of age of onset was about 20 years, that is, 10 years lower than the age of onset of the rest of individuals.

It is worth mentioning that the best regression model, described in Table 4, explains only about 3.5% observed variability in the age of onset (R^2 = 0.036). This means that the age of onset is influenced also by many other variables which were not analyzed in our study.

In order to further investigate the relation (revealed by regression analysis, please see Table 4) between age of MS onset and rs3116496 (*CD28*), rs6641 (*CD80*) and rs17281995 (*CD86*) polymorphisms in ligand–receptor system, four a priori formulated hypotheses were tested (Table 5).

First hypothesis (H1) presumed that the presence of at least one minor allele in each of three *loci* (in gene encoding *CD28* receptor as well as in genes encoding ligands, *CD80* and *CD86*) may cause changes leading to abnormal activation of T cells, and thereby influence the age of MS onset. Therefore, this hypothesis presumed that two groups of individuals should be considered. Individuals who were common (wild) homozygotes in three examined *loci* belonged to the first group, while the second group consisted of individuals who were

Table 3
Statistics describing the groups of individuals divided according to the combination of genotypes at three analyzed polymorphic sites.a

		CD80 rs6641T>G			
		TT	**TG | GG**		
CD28 rs3116496T>C	**TT**	m = **28.4**; SD = 8.36 **A**[a] n = 109 CI95%(26.8; 30.0)	m = **28.51**; SD = 7.73 **B** n = 68 CI95% (26.6; 30.4)	**GG**	CD86 rs17281995 G>C
		27.88; 6.97 **C** 40 CI95% (25.6; 30.1)	**29.95**; 6.98 **D** 21 CI95% (26.8; 33.1)	**GC/CC**	
	TC/CC	**31.0**; 8.51 **E** 46 CI95% (28.5; 33.5)	**30.56**; 6.42 **F** 23 CI95% (27.8; 33.3)	**GG**	
		34.25; 7.29 **G** 20 CI95% (30.8; 37.7)	**23.89**; 6.95 **H** n = 9 CI95% (18.5; 29.23)	**GC/CC**	

The table presents the mean values of the age of onset, 95% confidence intervals, standard deviations and the size of groups of individuals divided according to the combination of genotypes at rs3116496 > C (*CD28*), rs6641T > G (*CD80*) and rs17281995G > C (*CD86*) polymorphic sites.
m — mean values of the age of onset.
SD — standard deviation.
n — number of individuals in the group.
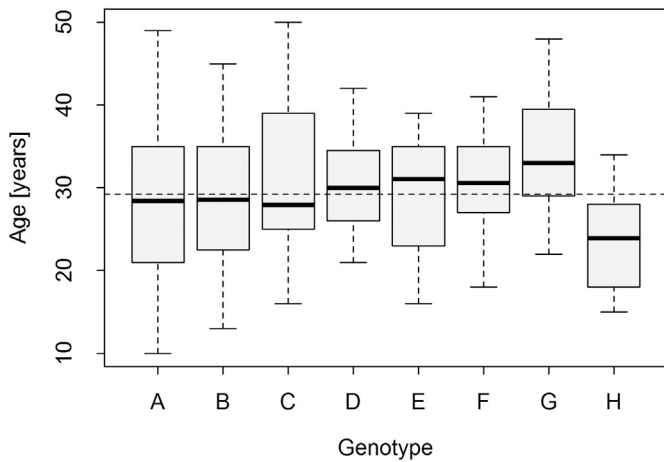[a] The groups were encoded with letters A–H.

Fig. 1. The median age of onset of multiple sclerosis (MS) in dependence on genotypes at rs3116496 (*CD28*), rs6641 (*CD80*) and rs17281995 (*CD86*) polymorphic sites. Box-and-whiskers plot of age of MS onset in eight groups, according to Table 3. Dashed line indicates the mean value of age of MS onset in all groups.

Table 5
Results of testing of the four a priori formulated hypotheses.

Hypothesis	A	B	C	D	E	F	G	H	$r_{alerting}$	$r_{effect\ size}$	$F_{1,328}$	p
H1	+	−	−	−	−	−	−	−	−0.112	0.0435	1.24	0.27
H2	+	+	+	+	−	−	−	−	−0.222	0.0789	1.38	0.24
H3	+	+	+	−	+	+	+	−	0.4941	0.1753	4.137	0.049
H4	+	+	+	−	−	−	−	+	−0.7562	0.2683	9.689	0.002

Hypotheses considering the relation between age of MS onset and rs3116496 (*CD28*), rs6641 (*CD80*) and rs17281995 (*CD86*) polymorphisms in ligand–receptor system.
The capital letters from A to H indicate groups of individuals divided according to the combination of genotypes at those three polymorphic sites (please see Table 3).
The signs (+) and (−) present which groups (from A to H) are treated as one group (+) and which are treated as another (−) according to tested hypothesis.
Crucial statistics, $r_{alerting}$ and $r_{effect\ size}$ show how each hypothesis is supported by data.

minor allele carriers in at least one of the three studied *loci*. It means that in this hypothesis {A} vs {B, C, D, E, F, G, H} was tested.

Second hypothesis (H2) presumed that it is only polymorphism in gene encoding receptor that may influence the level of T cells activation (leading to changes, disturbances, in function or expression of CD28) and in this way contribute to the changes in the age of onset. Therefore, this hypothesis presumed that individuals who were wild homozygotes (TT) in rs3116496 (CD28), that is, individuals who belonged to the groups A, B, C, D had similar age of onset and this age was different from the mean age of onset of individuals from the rest of the groups (E, F, G and H). Therefore, in this hypothesis {A, B, C, D} vs {E, F, G, H} was tested.

Third hypothesis (H3) presumed that only polymorphisms in genes encoding ligands (i.e. rs6641 in *CD80* and rs17281995 in *CD86*) may alter the expression and/or function of encoded molecules and thereby contribute to changes in the age of MS onset through the abnormal T cells activation. At the same time, the redundant role of CD80 and CD86 molecules was assumed. This means that the proper expression and function of only one of these two ligands is sufficient to the optimal T cells costimulation. Therefore, in this hypothesis individuals were divided into two groups. First group was composed of the individuals who were wild homozygotes in rs6641 of *CD80* (A, C, E, G) or in rs17281995 of *CD86* (A, B, E, F) or in both of them (A, E). The rest of the individuals was included in the second group. Therefore, in this hypothesis {A, B, C, E, F, G} vs {D, H} was tested.

Fourth hypothesis (H4) presumed that polymorphism in gene encoding receptor as well as polymorphisms in genes encoding ligands may cause the disturbances in expression levels and/or function of these molecules. In consequence it may lead to the abnormalities in T cells activation and thereby influence on the age of onset of multiple sclerosis. In this hypothesis, like in the third one, the redundant function of two ligands (CD80 and CD86) was assumed. Therefore, two groups were considered. The first group was composed of individuals who were wild homozygotes in rs3116496 in gene encoding receptor CD28 and simultaneously wild homozygotes at one or two polymorphic sites in genes encoding ligands, that is in rs6641 of *CD80* and/or rs17281995 of *CD86* (groups A, B, C). Individuals who belonged to H group were also included in the first pool, since in this hypothesis we assumed that the presence of minor allele in rs3116496 in *CD28* and simultaneously the presence of minor alleles at both polymorphic sites in genes encoding ligands cause such a significant abnormalities in CD28, CD80 and CD86 expression and/or function, that other costimulatory molecules take over their function and thereby the optimal T cells activation is achieved (by eliminating negative effect caused by the presence of minor alleles in these three *loci*). The second group consisted of individuals who had minor allele in rs3116496 in *CD28* gene (encoding receptor) and simultaneously who were wild homozygotes in one or two *loci* in genes encoding ligands (rs6641 in *CD80* and/or rs17281995 in *CD86*) (groups E, F, G). In this pool we included also the individuals from group D, even though they were wild homozygotes in rs3116496 in *CD28* gene. We were allowed to do that, since in this hypothesis an assumption was made that altered expression and/or function of both ligands simultaneously (as a result of possessing minor alleles in both polymorphisms rs6641 in *CD80* and rs17281995 in *CD86*) may lead to disturbances in T cells activation, what, in consequence, may result in changes in age of onset. Therefore, in this hypothesis {A, B, C, H} vs {E, F, G, D} was tested. It is worth noticing that this hypothesis (H4) is nothing more than the encoding of receptor-ligand interaction.

The summary of these four hypotheses as well as the results of their testing are presented in Table 5. We noticed that observed mean values of age of onset in groups of individuals divided according to genotypes in the following *loci* rs3116496 in *CD28*, rs6641 in *CD80* and rs17281995 in *CD86* best supported fourth hypothesis (H4). This observation confirms the significance of the interaction between polymorphisms in gene encoding CD28 receptor and genes encoding CD80

Table 4
Regression of the age of onset of multiple sclerosis (MS). Variables and interactions between them associated with the age of onset of MS.

Group	Difference in age of onset [years]	CI95%		p
*HLA-DRB1*15:01 −/CD28* rs3116496 TT/*CD80* rs6641 TT/*CD86* rs17281995 GG[a]	28.92[b]	27.13	30.60	–
*HLA-DRB1*15:01* +	−1.14	−2.82	0.49	0.18
CD28 rs3116496 TC/CC	2.67	−0.24	5.64	0.07
CD80 rs6641 TG/GG	0.13	−2.26	2.55	0.92
CD86 rs17281995 GC/CC	−0.43	−3.05	2.25	0.75
CD28 rs3116496 TC/CC × *CD80* rs6641 TG/GG	−0.74	−5.22	3.59	0.74
CD28 rs3116496 TC/CC × *CD86* rs17281995 GC/CC	3.61	−1.20	8.39	0.14
CD80 rs6641 TG/GG × *CD86* rs17281995 GC/CC	1.93	−2.58	6.00	0.38
CD28 rs3116496 TC/CC × *CD80* rs6641 TG/GG × *CD86* rs17281995 GC/CC	−11.44	−19.09	−3.32	0.005
$F_{df=8;327} = 2.573$; p = 0.010 R^2 = 0.036				

[a] Reference group.
[b] Mean age of diagnosis in the reference group.

and CD86 ligands, associated with the age of onset of multiple sclerosis (Table 4).

For the fourth hypothesis the correlation coefficient between contrast weights and means in groups was as high as $r_{alerting} = -0.7562$, 0.7562, which points to good fit of group means to the assumed hypothesis describing the relation between examined polymorphisms in receptor-ligand system and age of onset. After taking into consideration the variability within groups and the differences between groups which cannot be explained by this hypothesis, the coefficient was $r_{effect\,size} = 0.2683$. This means that the age of onset is influenced also by many other variables not necessary connected with genetic factors.

The observed values of means were also better explained by the H3 than by the hypothesis H0 which assumed that in fact there are no differences between eight groups (p = 0.043). However, mean values in groups were less correlated with contrast weights ($r_{alerting} = 0.4941$ (the sign of the coefficient is not important), $r_{effect\,size} = 0.1753$) than in the case of H4. So, the observed means of age of onset were worse explained by the third hypothesis. However, the question arose if the fourth hypothesis better explained the observed means of age of onset than the third hypothesis indeed or if it was only an accidental effect. H3 was a simpler hypothesis than H4. If it had turned out that H4 fitted the observed data better (than H3) by chance, the third hypothesis (H3) would have been adopted as the most reliable (according to parsimony principle). Nevertheless, the results of the analysis revealed that, in fact, H4 better explained the observed age of onset in groups of individuals divided according to the genotypes in the examined *loci* ($F_{1;328} = 9.46$, p = 0.002). Therefore the fourth hypothesis was adopted.

4. Discussion

CD28/CTLA-4–CD80/CD86 pathway plays a crucial role in regulation of T cells activation, and in this way in maintenance of immune tolerance against self-antigens. The results of both functional and genome screening studies show that genes encoding molecules which belong to this pathway may contribute to the risk of development of autoimmune diseases and may be considered as excellent positional candidate genes for MS (Teutsch et al., 2004).

In the literature there is a number of published researches considering the association of genetic variants in *CTLA-4* gene with the risk of multiple sclerosis and the outcome of this disease. However the results of these investigations are inconsistent and ambiguous. Some of them did not show the influence of *CTLA-4* on MS (Dyment et al., 2002, Greve et al., 2008, Roxburgh et al., 2006). On the contrary, the results of other studies reported the association of this gene with multiple sclerosis susceptibility (Harbo et al., 1999, Kantarci et al., 2003, Ligers et al., 1999). Moreover, some of them suggest also the role of *CTLA-4* polymorphisms in modifying the disease outcome (Heggarty et al., 2007, Malferrari et al., 2005).

Here we investigated the association of three *CTLA-4* polymorphisms rs5742909C > T (−319C > T), rs231775A > G (+49A > G), rs11571302G > T (Jo31G > T) with the risk of MS and the disease outcome on well-defined Polish population. The patients group included the individuals, who had been studied earlier by Karabon et al., however our group was enlarged by more than 100 MS patients. The investigation mentioned above (Karabon et al., 2009) indicated the association of rs11571302 G > T with MS susceptibility. In that work it was shown that individuals with G allele in this *locus* had higher risk of the disease in comparison to TT homozygotes (OR = 1,75; CI95%[1,08; 2,84]). Moreover, individuals possessing T allele at this polymorphic site had shorter duration of relapsing-remitting course of the disease. Unfortunately, extended study did not confirm relations described above. On the contrary, in presented here investigation, association of G allele in rs231775 with increased MS susceptibility was found, but only in *HLA-DRB1*15:01*-individuals. This observation confirms that the stratification for *HLA-DRB1*15:01* may help to reveal new associations of polymorphisms with MS. In contrast to our observation,

recently performed meta-analyses did not identify the association of rs231775 with MS risk (Gyu Song and Ho Lee, 2013, Liu and Zhang, 2014). However, the lack of such association in these studies, as well as in the study performed by Karabon et al., may result from the fact that samples were not stratified for *HLA-DRB1*15:01* allele.

Polymorphism rs231775 (+49A > G), located in the first exon of *CTLA-4* gene, influences the structure of the encoded molecule. The adenine to guanine substitution results in Thr > Ala change in amino acid sequence and appearance of hydrophobic amino acid in highly conserved region of the signal (leader) peptide (Dallos and Kovacs, 2005). Despite the fact that the signal peptide is cleaved before the molecule is transported to the T lymphocyte surface, it plays an important role in directing the synthesized peptide to the endoplasmic reticulum (ER) membrane and ensures its transmission to the ER lumen. Therefore, even small changes in the structure of signal peptide may alter intracellular transport of CTLA-4 molecule and in consequence, influence its accessibility on the cell surface (Anjos et al., 2002, Dallos and Kovacs, 2005). It was shown that Thr > Ala amino acid change in the signal peptide results in altered inhibition of T cells activation through CTLA-4 molecule (Kouki et al., 2000). Therefore, G allele in rs231775 could contribute to decreased inhibition of autoreactive T cells and in consequence lead to development of multiple sclerosis.

Polymorphisms in *CD28*, *CD80* and *CD86* genes and their association with the risk and progression of multiple sclerosis were not investigated as broadly as in the case of *CTLA-4*. In our study two polymorphisms of *CD28* gene were investigated: rs35593994G > A located in the promoter region in putative binding site for transcription factor (as it was shown by in silico analysis) and rs3116496T > C, which according to literature data is located near the splice acceptor site and therefore may contribute to aberrant mRNA splicing (Chen et al., 2012). Similarly to Veen et al. (van Veen et al., 2003), we did not observe the association of rs3116496T > C with MS susceptibility. Moreover, we did not identify the differences in the genotypes and alleles distribution at rs3559394G > A polymorphic site between controls and patients, what is in contrast to observation made by Teutsch et al. (Teutsch, Booth, 2004). However, there are some limitations of their analysis - there was no need to test differences between alleles proportions when two populations were in Hardy-Weinberg equilibrium and there were no differences in frequencies of genotypes. Therefore further studies on different population are necessary, in order to check whether rs3116496T > C in *CD28* gene could be considered as MS genetic risk factor.

Of the three polymorphisms examined in our study in *CD80* gene, encoding molecule being a ligand for CD28 and CTLA-4, only rs1599795 T > A was associated with the risk of multiple sclerosis. This risk was lower in the individuals possessing the minor allele in this *locus*. In accordance with in silico analysis performed using SNPinfo Web Server (Xu and Taylor, 2009) this SNP is located in putative miRNA binding site, so thereby it may alter the expression of *CD80* gene. To the best of our knowledge, to date there is no data considering the association of this polymorphism with MS susceptibility. Thus it will be important to confirm this association in follow up studies. Moreover, research on verification of the functionality of the putative miRNA binding site and studies aimed at explanation in what way the minor allele in this *locus* may decrease the risk of MS are needed.

CD86 gene (encoding molecule which is, apart from CD80, the ligand for CD28 and CTLA-4) also seems to be the good candidate for studies on diseases in which T lymphocytes are involved. Nonetheless, to date only Teutsch et al. investigated the possible association of polymorphisms in this gene with susceptibility to MS. They analyzed polymorphism in the exon 8 (A304T) and AAG deletion in the first intron of *CD86* (−359delAAG), however they did not find the association of examined *loci* with MS. Interestingly, study performed by Abdallah et al. (Abdallah et al., 2006) indicated polymorphism rs2715267T > G (−3479 T > G) in *CD86* as a predisposing factor to another disease in which T lymphocytes are involved − systemic sclerosis. This group showed the

association of G allele at this polymorphic site with higher risk of this disease. The results of our study revealed that the same allele in this *locus* was associated with multiple sclerosis. Polymorphism rs2715267T > G is located in the promoter region of *CD86*. According to Abdallah et al. T allele in this *locus* contains putative binding sites for transcription factor GATA and TATA-binding protein, whereas G allele does not. Moreover, this group analyzed DNA protein-binding activity of the rs2715267T > G polymorphism in vitro using electromobility gel shift assays (EMSA) and showed that the G allele in this *locus* had less binding affinity for nuclear proteins in comparison to T allele (Abdallah et al., 2006). It will be essential to determine in further studies the transcription factors which bind to this *locus* and to establish whether this polymorphism contributes to increased or decreased *CD86* expression.

It is well known that an important role in the susceptibility to MS, similarly to other multigenic diseases, may be played by the interactions between polymorphisms which individually exert only modest effects on disease risk. Taking above into consideration, we analyzed the interactions between SNPs in *CD28*, *CTLA-4*, *CD80* and *CD86* genes and their association with multiple sclerosis. We did not observe the interaction associated with the risk of MS, however the analysis revealed the interaction between rs3116496T > C (*CD28*), rs6641T > G (*CD80*) and rs17281995G > C (*CD86*) associated with the age of MS onset.

In the literature there are only a few reports considering the function of these polymorphisms. SNP rs3116496T > C is located in the third intron of *CD28* gene, and as it was mentioned above, it is found near the splice acceptor. Therefore it may contribute to aberrant mRNA splicing. In consequence it could influence immune homeostasis by disturbing balance between different isoforms of CD28 protein (Chen et al., 2012). In fact, the study performed by Ledezma-Lozano et al. (Ledezma-Lozano et al., 2011) revealed that in group of rheumatoid arthritis patients, TC genotype in rs3116496T > C was associated with the decreased level of soluble CD28 form in comparison to TT. As far as rs6641T > G polymorphism is concerned, in the literature there is no data considering its function and association with MS susceptibility. As for rs17281995G > C polymorphism, it was shown that this SNP can modulate the expression of CD86 by the influence on the binding different miRNAs to 3'UTR region. Five miRNAs which may be influenced by rs17281995G > C polymorphism were predicted: miR-337, miR-582 and miR-200a*, which bind weaker to C allele in this *locus*, and miR-184, miR-212, which bind stronger in case of this allele (Landi et al., 2011).

Due to the low number of literature data and reports considering the effects of these polymorphisms on regulation of expression and/or function of CD28, CD80 and CD86 molecules, it is hard to predict in what way they may influence the age of MS onset. For polymorphisms rs6641 of *CD80* and rs17281995 of *CD86* the in silico analysis predicted that they are located in the potential miRNA binding site. No function was predicted for rs3116496 of *CD28* (Supplementary Table 2). Based on our results we adopted the hypothesis which states that individuals possessing at least one C allele in rs3116496T > C in *CD28* gene and simultaneously being wild homozygotes in one of the two polymorphisms in genes encoding ligands (rs6641T > G (*CD80*) or rs17281995G > C (*CD86*)) (groups: E, F, G) as well as the individuals being wild homozygotes in rs3116496T > C in *CD28* gene and possessing at the same time the minor allele in two SNPs in genes encoding ligands rs6641T > G and rs17281995G > C (group D) developed MS later than the rest of individuals. The cause of this higher age of MS onset may be the insufficient costimulation of T lymphocytes triggered (caused) by the lower expression or altered function of CD28, CD80 and CD86 molecules. Insufficient costimulation may contribute to the appearance of T lymphocyte anergy, what may result in lack of immune response against self-antigens. In the literature it has been suggested that costimulatory pathways other than CD28/CTLA-4–CD80/CD86 may provide the necessary second signals for complete T cells activation (Yamada et al., 2002). Therefore we assumed that individuals

possessing minor allele in three examined *loci* (group H) did not have higher age of onset since other costimulatory molecules take over their function and the optimal T cells activation is achieved (by eliminating negative effect caused by the presence of minor alleles in these three *loci*). The major limitation of our study is the lack of biochemical and functional data and it seems to be essential to evaluate in follow-up studies how rs3116496T > C, rs6641T > G and rs17281995G > C polymorphisms can modulate the expression and/or function of CD28, CD80 and CD86, respectively, and to verify the above hypothesis.

In conclusion, our study revealed that genetic variations within genes encoding molecules belonging to CD28/CTLA-4–CD80/CD86 pathway may influence not only risk of multiple sclerosis but also its age of onset. It will be important to investigate in further study the mechanism by which these polymorphisms may be associated with the susceptibility and the age of MS onset. Our data indicated also that stratification for *HLA-DRB1*15:01* allele is of great importance to studies on MS risk factors.

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Conflict of interest

The authors declare no conflict of interest.

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