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Association between ß2-adrenergic receptor polymorphisms and rheumatoid arthritis in conjunction with HLA-DRB1 shared epitope

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Abstract

PURPOSE: The frequency of inherited polymorphisms of the β2-adrenergic receptor (β2AR) gene and the association with rheumatoid arthritis (RA) as well as HLA-DRB1 alleles was examined. METHODS: An allele-specific polymerase chain reaction was used to determine the common variants of the β2AR at position 16, 27, and 164 in patients with RA (n= 310) and ethnically matched healthy controls (n= 305) from Germany. HLA-DRB1 genotyping was performed by oligonucleotide hybridisation of enzymatically amplified DNA allowing low-resolution HLA-DRB1 genotyping comprising specificities DRB1*01 to DRB1*17. RESULTS: Arginine (Arg) at codon 16 was present in 278 RA patients (89.7 %) compared to 202 controls (66.2%; OR 4.43, 95 % CI 2.81 to 7.02, p=0.00001). Homozygosity for Arg16 was found in 107 RA patients (34.5 %) compared to 14 controls (4.6 %; OR 10.9, CI 5.9 to 20.5, p=0.00001). Stratifying patients for their HLA-DR status revealed that homozygosity for Arg16 exhibited the greatest risk for RA in combination with HLA-DRB1*04 (OR 17.1, 95% CI 1.71 to 414.4, p=0.004). Interestingly, patients with the Arg16 allele have a younger age at disease onset compared to patients without Arg16 (46.1±2.0 versus 53.1±2.7 respectively, p<0.05). Furthermore, 93.3% patients with homozygosity for Arg16 are positive for anti-CCP antibodies versus 75% patients with homozygosity for Gly16 (p<0.05).

CONCLUSION: Between RA patients and controls there was a highly significant distortion in the distribution of β2AR polymorphisms at codon 16 contributing together with the HLA-DR alleles to the genetic background of RA.

Key words: ß2-adrenergic receptor, polymorphisms, German population, rheumatoid arthritis, HLA-DR

Introduction

A disturbed interaction of the autonomic nervous system (ANS) and the immune system is supposed to contribute to the pathogenetic process in rheumatic diseases (1). The anatomical compartmentalisation of noradrenergic innervation in lymphoid organs suggests the existence of sympathetic neural interactions via the neurotransmitter norepinephrine (NE) with lymphoid and accessory cells of the immune system (2). Further evidence for such interactions can be drawn from the discovery of the expression of β_2-adrenergic receptors (β2AR) on various immune cells (3-4). Cells of the immune system express β2AR with a varying density due to the activation status and the developmental stage of the cells. The influence of the ANS on lymphocyte function via activation of adrenergic receptors plays an important (patho-) physiologic role. It could be shown that lymphocytes of RA patients exhibit a decreased expression of β2AR on the cell surface which is negatively correlated with the systemic disease activity (5). Further studies revealed that cytokines modulate the expression of β2AR on peripheral blood mononuclear cells (PBMC) (6). However, various polymorphisms in the β2AR gene could be demonstrated leading to a variation of β2AR expression on the cell surface (7-8). After positional cloning of the β2AR gene to chromosome 5q31-32 (9) several polymorphisms could be detected in the coding region as well as in the 5'regulatory region (10-13). Four of nine polymorphisms lead to a change in the amino acid sequence of the protein as well as to functional changes of β2AR characteristics. In particular the Gly16 receptor downregulates following exposure to an agonist to a much greater extent than the Arg16 form in both transfected cell systems and in primary cultured human airway smooth muscle cells (14). Population studies could demonstrate differing allele frequencies in various populations (15-17) and an association of β2AR gene polymorphisms with various diseases, e.g. myasthenia gravis, asthma, obesity, and arterial hypertension (18-21). Furthermore, the distribution of β2AR gene polymorphisms were associated with immune effector mechanisms indicating a modulation of functions of the immune system by β2AR variants (19, 21). In this respect, growing evidence supports the hypothesis that alterations of the interaction between ANS and immune system can be an important factor for initiation and perpetuation of RA (23-24). It is also known that RA can be regarded as an autoimmune disease on a permissive genetic background. The class II human leukocyte antigens (HLA) are important for predisposition as well as the rate of progression of joint destruction in this autoimmune disease and contribute approximately 30 to 40 % to the genetic background of RA (25). To test the hypothesis that β2AR gene variations function as an additional genetic factor for RA susceptibility we studied β2AR polymorphisms in RA patients from eastern Germany and in healthy controls from the same area.

Material and methods

Study groups

The frequency of β2AR polymorphisms in RA patients was studied in DNA of 310 patients from the eastern parts of Germany and in DNA from 305 ethnically matched healthy donors from the same area from a blood donor bank. In a subgroup of 169 RA patients we could obtain clinical data as outlined in Table 1.

Determination of the β2R polymorphisms

Genomic DNA was extracted from EDTA preserved whole blood leukocytes by a standard proteinase K digestion and phenol/chloroform method.
After DNA extraction from leukocytes the three most common polymorphisms resulting in a modification of the amino acid sequence (at positions 16, 27, and 164) were studied. For each polymorphism an allele-specific PCR was established as described earlier (20, 27).
Amplifications were done in 50 µl total volume containing 1 µl (300 ng) genomic DNA. The PCR reaction conditions were: 100 µM of each dNTP; 10 pmol of each primer; 0,5 U Taq polymerase (Perkin Elmer, Norwalk, CT) and 0,5 M Betaine (Sigma).

PCR reactions were in generally started with denaturation at 94 °C for 5 minutes, and stopped by a final extension step at 72 °C for 5 min. 15 µl of the PCR product were separated by size on 2% agarose gel. Each gel was stained with ethidium bromide, visualised under UV light, photographed and analysed with Alpha Innotech Corporation Software Chemilmager 4400, U.S.A.

PCR amplifications of the DNA segment containing codon 16, 27 and 164 of the ß$_2$AR gene
Depending on the amplified fragment we added 2.5 mM $MgCl_2$ (codon 16), 5 mM $MgCl_2$ (codon 27 and 164), respectively.
To delineate the polymorphism at nucleic acid position 46 (amino acid 16), the primer pairs were: 5`-CTTCTTGCTGGCACCCAAT<u>A</u>-3` (sense) und 5`-CCAATTTAGGAGGATGTAAACTTC-3`(antisense) or the same antisense primer and 5`-CTTCTTGCTGGCACCCAAT<u>G</u>-3` (sense). Temperature cycling was 94°C for 45 sec, 61°C for 45 sec and 72°C for 60 sec for 30 cycles (Perkin Elmer, PCR GeneAmp PCR System 9600 Norwalk, CT, USA). The generated PCR product size using these primers is 913 bp.
Primer pairs for delineating the polymorphism at nucleic acid position 79 (amino acid 27) were 5`-GGACCACGACGTCACGCAG<u>C</u>-3` (sense) and 5`-ACAATCCACACCATCAGAAT-3` (antisense), or the same antisense primer and 5`- GGACCACGACGTCACGCAG<u>G</u>-3` (sense). The initial denaturation step was followed by 35 cycles for denaturation (94°C for 30 sec), annealing (62°C for 45 sec) and extension (72°C for 45 sec). The generated PCR product exhibited a molecular size of 442 bp.
For detection of the polymorphism at nucleic acid position 491 (amino acid 164) primer pairs were 5`-TGGATTGTGTCAGGCCTTA<u>C</u>-3` (sense) and 5`-CACAGCAGTTTTCTTT-3` (antisense), or the same antisense primer and 5`- TGGATTGTGTCAGGCCTTA<u>T</u>-3` (sense).
Temperature cycling was 94°C for 30 sec, 59°C for 45 sec and 72°C for 45 sec for 30 cycles. The PCR product contained of 662 bp.

Identification of polymorphism in the 5`-leader cistron (LC) of the human ß$_2$AR gene (-47, T→C)
We added 4 mM $MgCl_2$ to PCR reaction to amplify fragments from the 5` LC of the ß$_2$AR gene. The primer pairs were: 5`-AATGAGGCTTCCAGGCGTC-3` (sense) and 5`-AGACATGACGATGCCCATGC-3` (antisense). After an initial denaturation, 30 cycles of 94°C for 30 sec, 62°C for 30 sec, and 72°C for 45 sec were carried out, followed by final extension. The generated PCR product size using these primers is 225 bp. An aliquot of the respective PCR product was separated by size on a 2% agarose gel, visualised under UV light by ethidium bromide staining to confirm the proper amplification. The remaining PCR product was purified with a commercial kit (PCR Purification Kit, Qiagen GmbH Hilden, Germany). Restriction fragment length polymorphism (PCR-RFLP) was carried out using 25 µl of each purified product digested with *MspA1I* (New England BioLabs, Frankfurt, Germany) und visualised by agarose gel electrophoresis. Proper enzyme function of MspA1I was checked by digestion of commercial Lambda DNA (New England Biolabs). The T→C substitution leads to the destruction of the recognition site for *MspA1I.*
The results were confirmed by representative DNA sequencing on an ABI Prism 310 automated Sequencer (Applied Biosystems) using DNA Sequencing Kit, Big DyeTM Terminator Cycle Sequencing (Applied Biosystems, Warrington, United Kingdom).

HLA genotyping
0.5 µg of genomic DNA was PCR amplified using two primers specific for the second exon of DRB1 as described previously (28). HLA-DRB1 genotyping was performed by oligonucleotide hybridisation of the PCR products. The method allowed low-resolution HLA-DRB1 genotyping comprising specificities DRB1*01 to DRB1*18 and the identification of alleles pertinent to the assessment of the shared epitope and the DERAA-positive DRB1 alleles. The DRB1 PCR products were hybridized in a dot-blot format in the presence of digoxigenin-11-ddUTP-labeled oligonucleotides. After the stringent wash, nonradioactive detection was carried out using

antidigoxigenin antibody-alkaline phosphatase conjugate (Boehringer Mannheim, Mannheim, Germany) and CSPD (disodium 3-(4-methoxyspiro(1,2-dioxetane-3,2-(5'-chloro)tricyclo [3.3.1.13,7]decan)-4-yl)phenyl phosphate; Tropix, Bedford, USA) as chemiluminescent substrate. For DRB1*04 subtyping, primers and oligonucleotides were again used as published previously (28). In cases of ambiguity, direct sequencing of enzymatically amplified DNA was carried out. Anti-CCP antibodies were determined by a commercially available ELISA according to the manufacturer's instructions (Euroimmun, Germany).

Statistical analysis

Agreement between the observed genotypes and those predicted by the Hardy-Weinberg equilibrium was assessed by χ^2 test. A χ^2 test with Yates correction was used for comparing the prevalence of different single nucleotide polymorphisms. Stratification analysis (Mantel-Haenszel) was used to detect relative influences of various beta-polymorphisms types. Logistic regression analysis was performed by stepwise removal of independent variables using the likelihood ratio test. Odds ratios and 95% confidence interval (95% CI) for relative risks were calculated using Fisher's exact test when necessary (Programme EPIINFO, USA). A p value < 0.05 was considered to be significant. SPSS (SPSS Inc., Chicago, IL, USA) was used for the statistical calculations.

Results

The ß2AR polymorphisms at Arg16Gly, Glu27Gln and Thr164Ile in RA patients as well in controls were in Hardy-Weinberg equilibrium.

Frequencies of ß2AR polymorphisms at amino acid position 16, 27, 164 are presented in Table 2. A positive association between Arg16 and Gln27 could be demonstrated indicating linkage disequilibrium (RA patients: OR 3.07, 95 % CI 1.35-6.9, p < 0.005; controls: OR 2.92, 95 % CI 1.62-5.25, p = 0.00018). Homozygosity for Arg16 was significantly increased in RA patients compared to controls (OR 10.9, CI 5.9 to 20.5, p=0.00001 and OR 1.55, CI 1.06 to 2.26, p=0.023, respectively) in contrast to homozygosity for Gly16 that was significantly decreased in RA patients (Table 2). Combining for the compound alleles revealed that two alleles Arg16ArgGln27GlnThr164Thr and Arg16ArgGln27GluThr164Thr promote the highest risk for RA (Table 2).

There was a significant distortion in the Arg16 allele frequency and in the Arg16 carrier status between RA patients and controls (Table 3). Furthermore, stratification of patients and controls for gender showed an association of the Arg16 allele frequency with RA in both male (OR 6.47, CI 2.69 to 15.9, p=0.000034) and female (OR 4.2, CI 2.29 to 7.72, p=0.00001) patients.

Stratifying RA patients for Arg16 revealed that there was no independent effect of Gln27 on RA susceptibility.

As well as determining susceptibility and resistance to a disease, gene polymorphisms may modulate the age at onset within a predisposing polygenic background. To examine this possibility, we stratified the patients according to β2AR allele and examined age at onset of disease. RA patients with the Arg16 allele have a younger age at disease onset compared to Arg16 negative patients (46.1±2.0 versus 53.1±2.7 respectively, p<0.05).

Anti-CCP antibodies were positive in 83.6% of RA patients. In addition, with respect to anti-CCP antibodies there was a significant difference between homozygosity for Arg16 (93.3% of patients anti-CCP positive) versus homozygosity for Gly16 (75% of patients anti-CCP positive, p<0.05).

A multiple logistic regression analysis with various clinical parameters of RA such as gender, disease duration, RF factor, X-ray stadium, vascular complications of RA, arterial hypertension and HLA-DR status was carried out. Interestingly, RA patients with the compound alleles Arg16ArgGlu27GluThr164Thr and Arg16ArgGln27GluThr164Thr, respectively, have an increased risk to develop arterial hypertension (OR 3.67, CI 1.27 to 10.67, p=0.01 and OR 3.09, CI 1.01 to 9.73, p=0.04, respectively).

Comparable to previous reports our data demonstrated an increased risk for RA in DRB1*04 and DRB1*01 positive individuals (OR 3.15, CI 1.72 to 5.83, p=0.0008 and OR 1.75, CI 1.0 to 3.0, p=0.05 respectively). There was an association between the carrier status of Arg16 and HLA DR*04 and 01 (Table 4). The combined effect of Arg16 together with DRB1*04 and DRB1*01, respectively, increased the OR for RA (OR 4.24, CI 2.08 to 8.82, p=0.000015 and OR 2.76, CI 1.43 to 5.39, p=0.001). Finally, homozygosity for Arg16 in combination with DRB1*04 as well as DRB1*01 lead to a further increased risk for RA indicating a gene dosage effect (Figure 1).

Discussion

The following major points emerged from our study: 1. There is a distortion in the distribution of the β2-adrenergic receptor (β2AR) polymorphism at codon 16 (Arg and Gly, respectively) between RA patients and healthy controls. 2. An association between the β2AR status and HLA DR alleles was observed leading to an increased risk to develop RA. 3. Of clinical importance, β2AR polymorphisms at codon 16 modulate age at onset of RA and, as a compound allele with further β2AR polymorphisms at codon 27 and 164, promote an increased risk for developing arterial hypertension in RA patients.

Since sympathetic dysregulation is considered to be one of the pathophysiological triggers for RA development (23) and peripheral blood mononuclear cells of RA patients exhibit a decreased density of β2AR (34) as well as a reduced catecholamine response (35) we studied β2AR polymorphisms in conjunction with the HLA DR status and markers of disease severity. Analyses of β2AR identified a total of nine different polymorphisms (14); four polymorphisms resulting from single nucleotide base changes were identified that altered the amino acid sequence of the receptor protein. Three of these polymorphisms (amino acid 16, 27, 164) appear to alter the functional properties of the receptor. As concerning amino acid 16, which can be either arginine (Arg) or glycine (Gly), we found that Arg16 promote an increased risk for RA confirming a recent study from northern Sweden (36) supporting the close interplay between the autonomic nervous system and the immune system in RA (23,37). After agonist activation β2AR undergo processes that limit function termed desensitisation. These events are critical for the cell to integrate the signals being received and for adaptation to physiologic and pathologic states (7)**.** It has been shown that the ability of β2AR to desensitise is markedly influenced by the presence of Gly at position 16 (14). The Gly16 receptor downregulates following exposure to an agonist to a much greater extent than the Arg16 form in both transfected cell systems and in primary cultured human airway smooth muscle cells while Arg16 β2AR exhibits to a greater extent tachyphylaxis (17). Recent studies have demonstrated that homozygosity of Gly16 is associated with severe asthma as indicated by dependency on corticosteroids or immunotherapy and an increased risk of nocturnal symptoms of asthma (11,19-20,37). Taking these and our studies together a picture emerges that the amino acid 16 polymorphism of β2AR differentiates between a Th2 disease, i.e. Asthma with Gly16 association, and a Th1 disease, i.e. RA with Arg16 association. Hence, it is hypothesised that the β2AR status at codon 16 is a major factor in shifting the immune response toward a Th1 or Th2 response (37-38). Furthermore, our results add information to a recent study demonstrating time dependent opposing effects of the sympathetic nervous system (SNS) in experimental arthritis and in RA (39). From our results one can conclude that decreased desensitisation of the Arg16 receptor facilitates pro-inflammatory effects of the SNS at initiation of the disease emphasizing the role of neuroendocrine interactions with the immune system in physiologic and pathologic states. In this respect, the association between the Arg16 polymorphism and younger age at onset of RA underlines the clinical relevance of this finding. On the other hand, in later stages of RA increased tachyphylaxis of the Arg16 form might prohibit anti-inflammatory effects of the SNS leading to a more severe disease.

HLA is a genetic risk factor for rheumatoid arthritis (RA) that was consistently observed in different populations (28-30). Although HLA-DRB1 is the main genetic factor in RA, the HLA locus contributes only about 30% of the total familial risk. Genetic epidemiological studies support the existence of a major susceptibility gene and of several minor susceptibility genes (30-33).

At present, the number and importance of these genes are unknown. Several of the possible minor-gene combinations may be capable of conferring susceptibility. Thus, the obtained association between β2AR polymorphisms and HLA DR status might be a new gene combination to promote the development of RA. The determination of SE-positive DRB1*04 alleles and anti-CCP antibody positively facilitates the prediction of disease course and prognosis at the time of initial presentation (40). In this respect, the higher prevalence of anti-CCP antibodies in Arg16 positive RA patients further underlines the association of the β2AR polymorphisms and the clinical course of the disease. Moreover, it can be speculated that Arg16 facilitates presentation of β2AR peptides by HLA-DRB1*0401 since Arg fits in one pocket of HLA-DRB1*04 which is significantly enhanced by conversion of arginine to citrulline (41).

Further analysis of the compound alleles revealed an increased risk for arterial hypertension in RA patients exhibiting the haplotypes Arg16ArgGln27GluThr164Thr and Arg16ArgGlu27GluThr164Thr. A similar association of β2AR genotype with arterial hypertension was found in previous studies with the Arg16 allele promoting a significant risk (18, 42). Our findings about the association between β2AR haplotypes and arterial hypertension are of interest since arterial hypertension is a clinical problem for RA patients (43). Furthermore, it was shown that RA is associated with increased cardiovascular mortality for reasons which are insufficiently understood. As in the general population, cardiovascular disease is the single most common cause of death in RA patients. Although epidemiological data have suggested an association between disease activity and cardiovascular mortality in RA the cause of such a link is unclear. Hence, one can conclude that the Arg16 allele of the β2AR acts as a susceptibility factor for RA and contributes to the cardiovascular morbidity of RA patients due to an increased frequency of arterial hypertension.

In conclusion, our data point to complex interactions of the autonomic nervous system and the immune system in patients with RA. Further studies are warranted to determine the diagnostic relevance and to open possible new therapeutic avenues.

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Table 1
Characteristics of RA patients

	Number of patients n=310	
	n	%
Female/Male	210/100	67,7/32,3
Age of disease onset (years)	46,7±6,3	
Duration of disease (years)	18,0±3,45	
	Number of patients n=169	
X-ray stadium 0-I	25	14,7
X-ray stadium II	41	24,2
X-ray stadium III	35	20,7
X-ray stadium IV	68	40,2
Definitive erosions	108	63,6
Rheumatoid factor positive	117	87,3
Cardiovascular complications	67	40
Arterial hypertension	40	24

Table 2
Distribution of various genotypes at amino acid positions 16, 27, and 164 in patients with rheumatoid arthritis and in healthy controls

β2AR genotype	**RA n=310**		**controls n=305**		**Odds ratio**	**95% confidential interval**	**p**
	n	**%**	**n**	%			
Arg16Arg	107	34.5	14	4.6	10.9	5.9-20.5	0.00001
Arg16Gly	171	55.1	188	61.7	0.77	0.55-1.07	0.12
Gly16Gly	32	10.3	103	33.7	0.23	0.14-0.36	0.00001
Gln27Gln	94	30.3	67	22	1.55	1.06-2.26	0.023
Gln27Glu	165	53.2	171	56	0.53	0.64-1.24	0.53
Glu27Glu	51	16.4	67	22	0.7	0.46-1.07	0.1
Thr164Ile	35	11.2	21	6.9	1.72	0.95-3.15	0.07
Thr164Thr	275	88.8	284	93.1	0.58	0.32-1.06	0.075
Compound alleles							
Arg16ArgGln27GlnThr164Thr	33	11	3	1.2	11.9	3.47-49.5	0.00008
Arg16ArgGln27Glu Thr164Thr	43	14.1	6	2	8.03	3.21-21.3	0.00001
Arg16GlyGln27GluThr164Thr	86	28.2	112	36.7	0.66	0.46-0.94	0.021
Gly16GlyGln27GlnThr164Thr	5	1.7	35	11.5	0.13	0.04-0.34	0.00001
Gly16GlyGln27GluThr164Thr	14	4.6	42	13.8	0.3	0.15-0.57	0.00011

Table 3
Allele frequency and carrier status for polymorphisms at amino acid positions 16 and 27

Characteristics	RA n=310		controls n=305		Odds ratio	95% confidential interval	p
	n	%	n	%			
Allele frequency							
Arg 16	385	62	216	35.4	2.99	2.35-3.8	0.00001
Gly	235	38	394	64.6	0.37	0.34-0.41	0.00001
Gln 27	353	57.9	305	50	1.32	1.05-1.67	0.01
Glu	267	42.1	305	50	0.76	0.6-0.95	0.01
Thr 164	275	88.8	284	93.1	0.58	0.32-1.06	0.075
Ile	35	11.2	21	6.9	1.72	0.95-3.15	0.07
Carrier							
Arg	278	89.7	202	66.2	4.43	2.81-7.02	0.00001
Non-Arg	32	10.3	103	33.8	0.23	0.14-0.36	0.00001

Table 4
Frequency of HLA DRB1 alleles in combination with β2AR polymorphisms at position 16 and 27, respectively, in RA patients and controls.

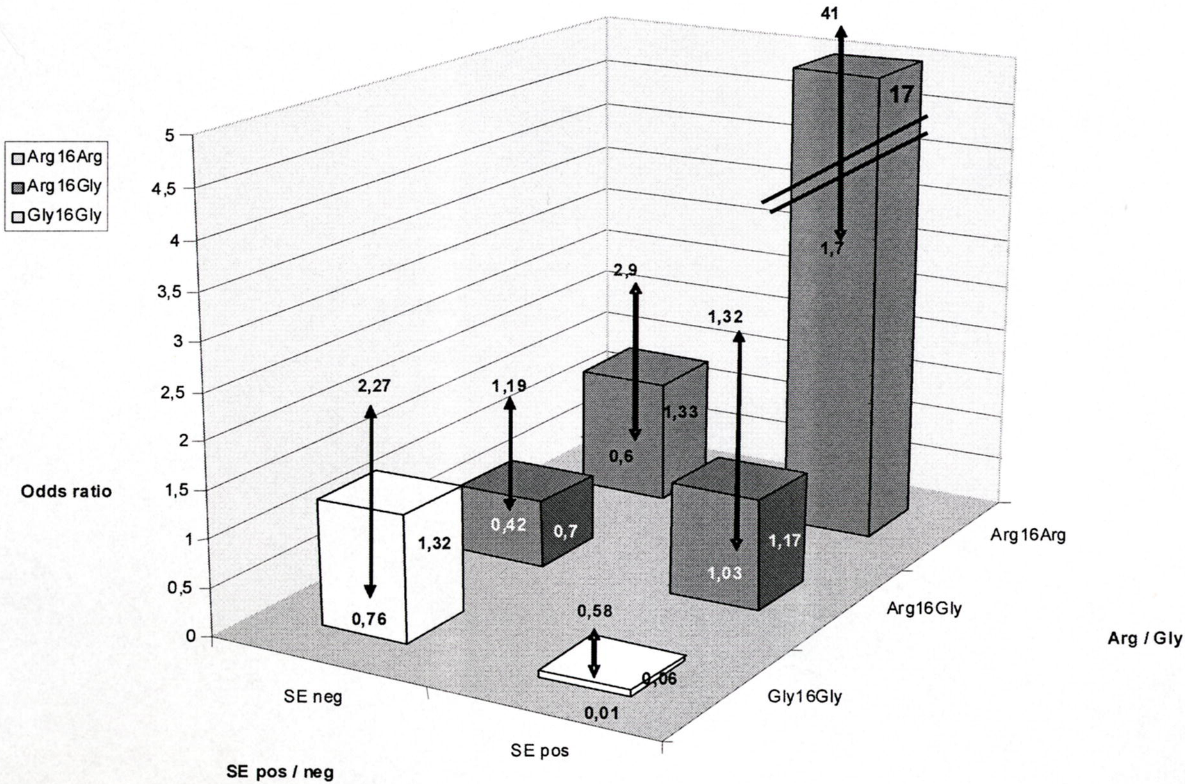
Carrier status	**HLA DRB1**					
	***01**			***04**		
	RA patients (n)	Controls (n)	OR	RA patients (n)	Controls (n)	OR
Arg16	78	14	5.57 95% CI 1.57-20.0 p =0,004	87	11	3.65 95% CI 1.0-13.0 p<0,05
Non Arg16	8	8		13	6	
Gln27	65	19	ns	85	14	ns
Non Gln27	19	5		15	3	

ns- not significant
OR – odds ratio

Figure 1.

Legend:

Numbers depict OR for each combination with the arrows between the numbers indicating the 95 % confidential interval. SE = shared epitope; Arg = Arginine, Gly = Glycine at position 16 of the β2AR.


Arg16Arg
Arg16Gly
Gly16Gly
Odds ratio
5
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
41
17
1,7
2,9
1,33
0,6
1,32
1,17
1,03
2,27
1,32
0,76
1,19
0,42
0,7
0,58
0,06
0,01
SE neg
SE pos
SE pos / neg
Arg16Arg
Arg16Gly
Gly16Gly
Arg / Gly

Association between ß2-adrenergic receptor polymorphisms and rheumatoid arthritis in conjunction with HLA-DRB1 shared epitope

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