Research article

Toll-Like receptor-2 (Arg753Gln), Toll-like receptor-4 (Asp299Gly), and CD14 (C/T-159) Polymorphisms in Tunisian patients with atopic asthma

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Abstract

Background: According to hygiene hypothesis, the development of allergic disease may be influenced by exposure to microbial agents. Toll-like receptor 2 and 4 (TLR-2 and TLR-4) and their co-receptor CD14 link innate and adaptive immunity through PAMPs recognition. Polymorphisms (SNPs) in TLR-2, TLR-4 and CD14 genes may modify qualitatively and/or quantitatively their expression, thus they could influence the susceptibility and/or severity of allergic disease.

Patients and methods: TLR-2 (Arg753Gln), TLR-4 (Asp299Gly) and CD14 (C/T -159) SNPs were genotyped using polymerase chain reaction in 213 patients with atopic asthma and 323 healthy controls matched in age and gender.

Results: TLR-2*G/A genotype and TLR-2*A allele were significantly more frequent in patients (14.5%, 0.072) comparatively to controls (0%, 0), (p<10^-7, OR(95% CI)= 2.77 [2.47-3.12]; p<10^-7, OR(95% CI) = 2.64 [2.44-2.85]; respectively). CD14*T/T genotype and CD14*T allele frequencies were higher in patients but the differences failed to reach significance; [(24.4% and 0.481) vs (17.6% and 0.436), p=0.056 and p=0.15 respectively]. Inversely, we found no evidence to support a significant association between TLR-4 SNP and asthma susceptibility.

Analysis of these SNPs according to clinical and biological features showed that TLR-2*G/A genotype were associated to a higher mean of onset age (23.93 ± 15.77 vs 16.13 ± 13.67), p=0.01. Otherwise, CD14*C/T and CD14*T/T genotypes were significantly correlated to elevated serum levels of both total and specific IgE, p=0.028 and p=0.011 respectively. Nevertheless, none of the 3 investigated SNPs was associated to asthma severity.

Conclusion: TLR-2 Arg753Gln SNP might play a role in asthma predisposition, while CD14 C/T -159 polymorphism seems to influence IgE synthesis.

Keywords: Polymorphisms; TLR2; TLR4; CD14; Atopic; Asthma

1. Introduction

Atopic asthma is a chronic inflammatory respiratory disorder characterized by variable airway obstruction, bronchial hyper-responsiveness (BHR), elevated type 2 T-helper (Th2) cytokines secretion, and high IgE levels [1]. Epidemiological studies have shown a lower prevalence of asthma and allergic sensitization in both children
and adults who lived in a rural area during childhood [2-7]. That led to the hygiene hypothesis which suggests that the development of allergic disease might be influenced by exposure to a variety of bacterium and viruses which are abundantly present in a farm environment [8]. The immunological concept proposes that a lack of microbial exposure leads to a Th2 deviation with impaired regulatory T cell (Treg) activity [9].

Toll-like receptors (TLRs) are pattern-recognition receptors (PRRs) that are expressed in antigen-presenting cells (APCs) and recognize both microbial and endogenous antigens. Following binding to diverse pathogen-associated molecular patterns (PAMPs), TLRs initiate intracellular signaling pathways, influence T-cell polarization and development [10], and modulate Treg function [11]. CD14 is an accessory receptor which shepherds both bacterial lipoproteins and endotoxin (LPS) to TLR-2 and TLR-4 respectively. Besides, it is well known that LPS modulates airway inflammation. Nevertheless, there are contradictory findings, reported by studies suggesting protective role for LPS through weakening Th2 responses while others showed exacerbating effects on asthma [9, 12].

All these data suggest that TLR-2, TLR-4 and their co-receptor the CD14 are obviously candidate genes to allergy and asthma susceptibility, and in which several single nucleotide polymorphisms (SNPs) were described. The most studied SNPs in asthma and atopic diseases were: 1) The TLR-2 Arg753Gln SNP that affects the intracellular TIR (Toll Interleukin 1 receptor) domain which had been correlated to a lower NF-κB nuclear translocation following lipoproteins binding [13]. 2) The TLR-4 Asp299Gly SNP altering the extracellular LRR (Leucine Rich Repeat) domain that was associated to a significant decrease of response to LPS [14, 15]. 3) The CD14 C/T -159 SNP modifying the affinity of transcription factors (Sp1, Sp2 and Sp3) to the promoter leading to enhanced activity and raised expression of both membranous (mCD14) and soluble (sCD14) forms [16].

In the present study we aimed to assess the impact of TLR-2, TLR-4 and CD14 SNPs on atopic asthma susceptibility and severity in a Tunisian population.

2. Materials and methods

2.1 Patients

This study included 213 patients with atopic asthma and 323 healthy voluntary blood donors from the same geographic origin. Patients were visiting both pneumonology department of the Charles Nicolle Hospital in Tunis and pediatrics department of Tahar Sfar Hospital in Mahdia and were diagnosed according to the Global Initiative for Asthma (GINA) guidelines 2006 [17]. Clinical and biological features of patients are recorded in Table 1.
Controls were healthy subjects matched for age, gender and ethnicity. None of the healthy subjects had any evidence of personal or family history of asthma and/or atopy.

All patients and controls gave written informed consent to participate in the study, and patient anonymity was preserved using documents and methods approved by the local Ethics committee of Charles Nicolle Hospital.

2.2 Methods

Genomic DNA was extracted from peripheral blood using salting-out procedure [18]. TLR-2 Arg753Gln polymorphism was conducted by a tetraprimer amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) [19] with specific primers [TLR-2-F 50-TATGGTCCAGGAGCTGGAGA, TLR-2-R 50-TGACATAAGATCCCCACTAGAC, TLR-2-G 50-GGTCTTGGTTGTTATTATCTTTCT] and TLR-2-A 50-GGTCTTGGTTGTTATTATCTTTCT (metabion® international AG, Lena-Christ-strasse 44I, D-82152 Martinsried, Deutshland)]. The primers TLR-2-F, TLR-2-R, TLR-2-G were used for detection of G allele and TLR-2-F, TLR-2-R, and TLR2-A were used for detection of A allele of TLR-2 Arg753Gln SNP.

Identification of TLR-4 (Asp299Gly) and CD14 (C/T -159) SNPs was performed by PCR using specific primers [(TLR4*F: 5´-GAT TAG CAT ACT TAG ACT ACT ACC TCC ATG -3´ and TLR4*R: 5´-GAT CAA CTT CTG AAA AAG CAT TCC CAC-3´) and (CD14*F: 5´- GTG CCA ACA GAT GAG GTT CAC-3´ and CD14*R: 5´-GCC TCT GAC AGT TTA TGT AAT C-3´) (metabion® international AG, Lena-Christ-strasse 44I, D-82152 Martinsried, Deutschand)], followed by a digestion of amplification products using NcoI and AvaII respectively (Restriction fragment length polymorphism: RFLP).

2.3 Statistical analysis

The results of continuous variables (Age, Onset Age and Total IgE level) are expressed as means ± SD, and the means of groups were compared by ANOVA-test (SPSS 11 Inc. Chicago, Illinon, USA). For qualitative variables, univariable analysis was performed using chi-square test or fisher’s exact test for small numbers (Epi-info Stat 6.04 program CDC, Atlanta). Probability (p) values were corrected for the number of tested alleles (pc). Values < 0.05 were considered to be statistically significant. Frequencies of genotypes and alleles were analyzed by chi-square test. In order to evaluate the strength of associations, the odds ratios (OR) together with 95% confidence intervals (CI) were calculated. Logistic regression models were built according to age, gender to estimate adjusted ORs.

3. Results
3.1 TLR-2 Arg753Gln analysis

The results of TLR-2 genotyping in asthma patients are summarized in Table 2. Frequencies of both TLR-2*G/A genotype and TLR-2*A allele were significantly higher in patients (14.4%, 0.072) comparatively to controls (0%, 0); \( p < 10^{-7} \), OR (95% CI) = 2.77 [2.47-3.12] and \( p < 10^{-7} \), OR (95% CI) = 2.64 [2.44-2.85]; respectively.

Interestingly, TLR-2*G/A genotype was correlated to a lower frequency of early onset of asthma (28%) comparatively to TLR-2*G/G genotype (56.4%); \( p = 0.008 \), OR(95% CI) = 0.3 [0.119-0.758]. In fact, the mean of onset age was in the same way significantly higher in case of TLR-2*G/A genotype (23.93 ± 15.77) than in patients with TLR-2*G/G (16.13 ± 13.67); \( p = 0.01 \).

Nevertheless, analysis of TLR-2 with clinical and biological features showed no significant impact on asthma control or both total and specific IgE synthesis.

3.2 TLR-4 Asp299Gly analysis

There were no significant differences in TLR-4 Asp299Gly genotypes and alleles frequencies between patients and controls (Table 2). Moreover, TLR-4 SNP did not seem to influence the age of onset, and was not correlated to any associated atopic manifestations (rhinitis and/or conjunctivitis) or to a family history of allergy and/or asthma. Besides, asthma control status was quite similar between TLR-4 genotypes. Biologically, there were no statistical associations of both total and specific IgE positivity with TLR-4 Asp299Gly SNP, and the mean level of total was comparable in both TLR-4 genotypes.

3.2 CD14 C/T -159 analysis

The CD14*T/T genotype and CD14*T allele were more frequent in patients with asthma (24.4% and 0.481) comparatively to controls (17.6% and 0.436); but the differences failed to reach the threshold of significance; \( p = 0.056 \) and \( p = 0.15 \), respectively (Table 2).

Examination of CD14 SNP according to clinical features of asthma showed no association with onset age, or an associated atopy or a family history of atopy and/or asthma. Similarly, there were no significant differences in asthma control between the three genotypes of the CD14. Inversely, the CD14*T mutated allele (CD14*C/T and *T/T) was significantly correlated to the positivity of both total and specific IgE; \( p = 0.028 \), OR(95% CI) = 2.23 [1.02-4.89] and \( p = 0.011 \), OR(95% CI) = 3.19 [1.14-8.94], respectively.

4. Discussion
Since previous studies had suggested that asthma is a multifactorial disease influenced by genetic and environmental factors about 30 years ago [20], a bundle of genome-wide screens for asthma were performed [21-25]. About 6 regions (2q, 5q, 6q, 11q, 12q and 13q) were found to be highly linked with asthma and extensively replicated in many studies [21-25]. Among these linked regions, the 5q31.1 holds many candidate genes including IL-4, IL-5, IL-9, IL-13, GM-CSF and CD14. In the beginning, IL-4 was well chosen as the candidate gene as no other cytokine except IL-13 can induce germline transcription and isotype switching to IgE [26]. It was therefore reasonable to think that genetic variations in IL-4 and IL-13 were the source of the linkage signal spotted on chromosome 5q. Unexpectedly, some years and many studies later, the probability that IL-4 and IL-13 genes play a major role in allergy and asthma pathogenesis is fading away.

Likewise, the CD14 gene located in this region encodes a receptor that conveys PAMPs and more specifically lipoproteins and LPS to both TLR-2 and TLR-4 respectively. As it has been suggested by hygiene hypothesis [9], exposure to microbial agents in early life is mostly shielding against atopy and asthma, thus a genetic variation in TLR-2, TLR-4 and their co-receptor the CD14 could influence their susceptibility and explain the 5q spot. TLR-2 Ligation in APCs signals T-cell activation through several cytokines secretion. If bacterial lipopeptides induce IL-12 synthesis and Th1 polarization, other TLR-2 ligands such as peptidoglycans and lipoteichoic acids rather promote IL-6 secretion [27]. Yet again, if the pattern of T-cell polarization is very important in host defense against invaders, it is equally the fulcrum upon which atopy susceptibility turns. Literature data concerning the role of TLR-2 in predisposition/protection against allergy are conflicting: 1) First TLR-2 predisposing: In fact, in murine model ligation of TLR-2 with LPS/R848 induced a shift from Th1 to Th2 response [28], activation of this receptor with Pam3-Cys-OH in C57BL/6 mice resulted in IL-13 and IgE synthesis [29]. In human, both TLR-2 mRNA in sputum [30] and membranous TLR-2 expression in PBMC (Peripheral Blood Mononuclear Cells) CD14^{high} [31] were significantly higher in asthmatic patients comparatively to controls. 2) Second TLR-2 protector: Actually, administration of TLR-2 agonists to BALB/c mice sensitized with ovalbumin resulted in an extinction of eosinophils and T-cells recruitment in sputum and a significant decrease of both IL-13 and IgE leading to a drastic reduction of BHR [32]. Moreover, invalidation of TLR-2 [33] and MyD88 [34] genes led to a stepping up of asthma onset and abolishment of the protection induced by bacterial exposure. Whether, predisposing or protecting TLR-2 expression is genetically determined, and above the numerous polymorphisms in its gene the Arg753Gln (G→A +2258) SNP was correlated to a lower NF-κB activity and a decrease in cytokines production [13]. Besides, the mutated allele TLR-2*A was associated to several bacterial and viral infections [13, 19, 35-38] and predisposed to septic shock [13].
Consequently, these acute and chronic severe infections could wield a natural selection which may explain the low prevalence of the mutated allele TLR-2*A and the almost absence of the TLR-2*A/A homozygous genotype in the mainstream of studied populations [19, 39-42]. Unfortunately, only few studies investigated the impact of this SNP in asthma and/or atopy. A study performed on 210 Tunisian asthmatic children and 224 controls, showed no association with asthma susceptibility [42]. This was similarly the case in the study of Hussein et al [40], but interestingly the mutated allele TLR-2*A was associated to partly controlled and uncontrolled forms of the disease. Inversely, in our study we found that TLR-2*A was correlated to asthma susceptibility without any impact on asthma control or on IgE levels. This peculiar result corroborates the hygiene hypothesis, in fact by altering signaling the TLR-2*A allele may prevent the protective role of early exposure to microorganisms. Nevertheless, this original finding must be confirmed by other studies on independent cohorts.

TLR-4 which was the first member of TLR family to be discovered, has many exogenous (LPS) and endogenous ligands. TLR-4 ligation can activate both Myd88 and TRIF pathways, and results in secretion of TNFα, IFNβ and IL-18 that lead to a Th1 adaptive response [43]. Nevertheless, Dabbagh et al [44] showed that membranous expression of TLR-4 in dendritic cells (DC) was required for Th2 cytokines (IL-4 and IL-5) synthesis. These paradoxical effects provide to TLR-4 a critical role in host defense and obviously in allergy predisposition. Investigating TLR-4 role in allergy led again to contradictory conclusions: 1) First TLR-4 predisposing: indeed TLR-4 gene invalidation in sensitized mice with ovalbumin resulted in vanishing of both bronchial inflammation and eosinophilia [45]. Moreover, degranulation of mast cells after LPS binding which was lacking in these mice was fully restored in mice TLR-4+/+ [45-46]. In those mice TLR-4+/+, stimulation of mast cells with LPS induced Th2 cytokines (IL-4, IL-5 and IL-13) secretion [45]. In human, asthmatic patients had significantly higher expression of TLR-4 in sputum [30] and in PBMC-CD14high [31]. 2) Second TLR-4 protector: In fact, sputum eosinophilia was radically annihilated in ovalbumin-sensitized mice by TLR-4 agonists [32]. Furthermore, Conrad et al [33] found that invalidation of TLR-4 gene in mice was significantly predisposing to asthma. In human, expression of TLR-4 was statistically lower in PBMC from asthmatic patients comparatively to normal subjects [47]. Those conflicting effects of TLR-4 are influenced by genetic polymorphisms and more specifically the Asp299Gly SNP which alters the extracellular domain and that have been associated with a significant decrease of response to LPS [14-15]. Additionally, this impaired response to LPS was associated to a variety of bacterial and viral infections [48-51]; and to sepsis with important secretion of inflammatory cytokines (TNFα and IL-6) [52-53]. Therefore, it is reasonable to think that infections could
exert a selective pressure on the mutated allele TLR-4*G which may explain its low frequency in all investigated populations [40, 42, 54-57]. In the present study, TLR-4 alleles and genotypes frequencies in patients and controls were similar, thus corroborating results found in previous studies in Tunisian [42] and Egyptian [40] populations. Nevertheless, Hussein et al [40] found the mutated allele TLR-4*G was correlated to more severe forms of asthma which is in agreement with a preceding study performed on 341 British sibling-pairs in which the homozygous TLR-4*A/A wild genotype was predictive to a less severe disease with a higher FEV (forced expiratory volume) [58]. The lack of association of the TLR-4 SNP must be considered with caution because of the low prevalence of the mutation which can hide any probable impact on asthma susceptibility.

Both TLR-2 and TLR-4 are assisted by CD14, a molecule expressed and secreted by myeloid cells [26]. CD14-cells such as epithelial and endothelial cells become responsive to PAMPs in the presence of sCD14 [26]. A part from a preeminent role during infections, the impact of CD14 on allergy isn’t yet well defined and faces the disparity of literature data: 1) First CD14 protective: Actually, a decrease of sCD14 in sera was found to be highly predictive of atopy [58-60]. Moreover, low levels of sCD14 in either amniotic fluid or maternal milk were at a high risk for later atopy occurrence [61]. Additionally, sCD14 interfered with CD40 signaling in B cells, inhibited IL-6 production and marked inhibition of IgE production [62]. 2) Second CD14 predisposing: Indeed, it has been proved that mRNA of CD14 was significantly increased in asthmatic patients [30]. Besides, 18 hours after allergen challenge sCD14 levels considerably increased and were correlated to IL-13 concentration in bronchial fluid [63]. Finally, inhaled corticosteroids significantly decreased sCD14 levels even with a concomitant intrabronchial interaction with LPS [64]. Apart from these conflicting data, the potential influence of CD14 on allergy and/or asthma predisposition is most certainly controlled by its functional genetic variability. The C/T -159 SNP in the CD14 gene is of the most interest as it has been correlated to a significant increase of its expression [16]. This could lead to a higher response to LPS, in fact the CD14*T mutated allele was correlated to endotoxin-induced sepsis and to subsequent mortality [65]. Although, an enhanced response to LPS can theoretically prevent allergy and asthma, it would rather be pejorative by promoting inflammation after allergic disease occurrence. In the present study, the CD14 C/T -159 SNP was not correlated to asthma susceptibility, thus agreeing with the results of a meta-analysis that included 11 eligible studies [66]. However, in a previous study [42] that included only children with a mean age of onset of about 4.2 years; considerably lower than in our patients (17.17); the CD14*T mutated allele was found to be protective. This is precisely in agreement with the hygiene hypothesis, indeed the protective role of microbial exposure and induction of CD14

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synthesis would be effective only in toddlers before allergen sensitization. Inversely, later and after allergen sensitization this protective impact is offset by exacerbation of bronchial inflammation. In another hand, we found that CD14*T allele was associated to the positivity of both total and specific IgE. This result corroborates the study of Sackesen et al [67] in which PBMC from asthmatic patients with CD14*T/T genotype had a significant increase of in vitro IgE synthesis comparatively to those from CD14*C/C patients. In reverse, in other studies performed in juvenile groups [68-69] the IgE positivity was inversely correlated to the CD14*T mutated allele. Therefore, all these data analyzed altogether would constitute a strong argument for the hygiene hypothesis. Indeed, an early exposure concomitant with a high production of CD14 would be protective against IgE production and by corollary atopy. Inversely, after allergen sensitization, hyper-production of CD14 would rather promote IgE secretion and worsen atopic manifestations.

Conclusion

TLR-2 Arg753Gln SNP might play a role in asthma predisposition, while CD14 C/T -159 polymorphism seems to influence IgE synthesis.

Acknowledgements

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Competing interests

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

Authors’ contribution

Pr Gorgi Yousr proposed the study and wrote the first draft. Tarak dhaouadi analyzed the data. All authors contributed to the design and interpretation of the study and to further drafts. Pr Gorgi Yousr is the guarantor of the integrity of this study.

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Table 1: Clinical and biological features of patients

<table>
<thead>
<tr>
<th>Features</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age ± SD (years)</td>
<td>22.62 ± 13.76</td>
</tr>
<tr>
<td>Sex ratio (Males / Females)</td>
<td>0.83 (97/116)</td>
</tr>
<tr>
<td>Onset age ± SD (years)</td>
<td>17.17 ± 14.17</td>
</tr>
<tr>
<td>Early onset before 16 years old</td>
<td>52.6%</td>
</tr>
<tr>
<td>Exposure to tobacco</td>
<td>31.45%</td>
</tr>
<tr>
<td>Associated atopy (rhinitis and/or conjunctivitis)</td>
<td>64.5%</td>
</tr>
<tr>
<td>Family history of asthma and/or allergy</td>
<td>34.9%</td>
</tr>
<tr>
<td>Asthma classification (GINA 2006)</td>
<td></td>
</tr>
<tr>
<td>Controlled</td>
<td>84%</td>
</tr>
<tr>
<td>Partly Controlled</td>
<td>10.6%</td>
</tr>
<tr>
<td>Uncontrolled</td>
<td>5.3%</td>
</tr>
<tr>
<td>Allergen identified</td>
<td>84%</td>
</tr>
<tr>
<td>Total IgE &gt; 200 IU/ml</td>
<td>58.1%</td>
</tr>
<tr>
<td>Average level of Total IgE ± SD (IU/ml)</td>
<td>389.56 ± 363</td>
</tr>
<tr>
<td>Specific IgE positive</td>
<td>81.3%</td>
</tr>
</tbody>
</table>
Table 2: Results of TLR-2 Arg753Gln, TLR-4 Asp299Gly and CD14 (C/T -159) SNPs genotyping in patients and controls

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Controls n=323</th>
<th>Patients n=213</th>
<th>(p)</th>
<th>OR(95% CI)</th>
</tr>
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<tbody>
<tr>
<td><strong>TLR-2 Arg753Gln</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotypes</td>
<td>G/G</td>
<td>323 (100%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>G/A</td>
<td>0</td>
<td>31</td>
<td>14.5%</td>
</tr>
<tr>
<td>Alleles</td>
<td>G</td>
<td>1</td>
<td>0.927</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>0</td>
<td>0.072</td>
<td>&lt;10(^{-7}) †</td>
</tr>
<tr>
<td><strong>TLR-4 Asp299Gly</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotypes</td>
<td>A/A</td>
<td>294 (91%)</td>
<td>196</td>
<td>92%</td>
</tr>
<tr>
<td></td>
<td>A/G</td>
<td>29 (9%)</td>
<td>17</td>
<td>8%</td>
</tr>
<tr>
<td>Alleles</td>
<td>A</td>
<td>0.955</td>
<td>0.96</td>
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</tr>
<tr>
<td></td>
<td>G</td>
<td>0.045</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td><strong>CD14 C/T -159</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotypes</td>
<td>C/C</td>
<td>98 (30.3%)</td>
<td>60</td>
<td>28.1%</td>
</tr>
<tr>
<td></td>
<td>C/T</td>
<td>168 (52%)</td>
<td>101</td>
<td>47.4%</td>
</tr>
<tr>
<td></td>
<td>T/T</td>
<td>57 (17.6%)</td>
<td>52</td>
<td>24.4%</td>
</tr>
<tr>
<td>Alleles</td>
<td>C</td>
<td>0.563</td>
<td>0.518</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>0.436</td>
<td>0.481</td>
<td>0.15</td>
</tr>
</tbody>
</table>

* \(p\) comparing TLR-2*G/A genotype prevalence between patients and controls

† \(p\) comparing TLR-2*A allele frequencies between patients and controls