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Investigation of the genetic overlap between Rheumatoid Arthritis and Psoriatic Arthritis in a Greek population

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Complete List of Authors:	Myrthianou, Effie; University of Crete School of Medicine, Laboratory of Molecular Medicine and Human Genetics Zervou, Maria; University of Crete School of Medicine, Laboratory of Molecular Medicine and Human Genetics Budu-Aggrey, Ashley; The University of Manchester , Arthritis Research UK Centre for Genetics and Genomics, Centre for Musculoskeletal Research, Institute for Inflammation and Repair, Manchester Academic Health Science Centre; Manchester Academic Health Science Centre, Manchester, NIHR Manchester Musculoskeletal Biomedical Research Unit, Central Manchester University Hospitals NHS Foundation Trust Eliopoulos, Elias; Agricultural University of Athens, Department of Biotechnology Kardasis, Dimitris; University of Crete School of Medicine, Laboratory of Biochemistry; FORTH, Heraklion, Institute of Molecular Biology and Biotehnology Boumpas , Dimitrios T; Kapodistrian University of Athens, Medical School; FORTH, Heraklion, Institute of Molecular Biology and Biotechnology Kougkas, Nikos; University of Crete School of Medicine, Rheumatology, Clinical Immunology and Allergy, Faculty of Medicine Barton, Anne; University of Manchester, Arthritis Research UK Centre for Genetics and Genomics, Centre for Musculoskeletal Research, Institute for Inflammation and Repair, Manchester Academic Health Science Centre; Manchester Academic Health Science Centre, NIHR Manchester Musculoskeletal Biomedical Research Unit, Central Manchester University Hospitals NHS Foundation Trust Sidiropoulos, Prodromos; University of Crete, Rheumatology, Allergy, Clinical Immunology Goulielmos, GN; University of Crete School of Medicine, Laboratory of Molecular Medicine and Human Geneics; University Hospital of Crete, Department of Internal Medicine
Keywords:	Rheumatoid Arthritis (RA), Psoriatic Arthritis (PsA), genetic association, gene polymorphisms

Investigation of the genetic overlap between Rheumatoid Arthritis and Psoriatic Arthritis in a Greek population

Effie Myrthianou[1], Maria I. Zervou[1], Ashley Budu-Aggrey[2,3], Elias Eliopoulos[4], Dimitris Kardassis[5,6], Dimitrios T. Boumpas[6,7], Nikos Kougkas[8], Anne Barton[2,3,9], Prodromos Sidiropoulos[8] and George N. Goulielmos[1*]

[1] Laboratory of Molecular Medicine and Human Genetics, Department of Internal Medicine, School of Medicine, University of Crete, Heraklion, Greece

[2]Arthritis Research UK Centre for Genetics and Genomics, Centre for Musculoskeletal Research, Institute for Inflammation and Repair, Manchester Academic Health Science Centre, The University of Manchester, Manchester, UK

[3]NIHR Manchester Musculoskeletal Biomedical Research Unit, Central Manchester University Hospitals NHS Foundation Trust, Manchester Academic Health Science Centre, Manchester, UK

[4]Laboratory of Genetics, Department of Biotechnology, Agricultural University of Athens, Athens, Greece

[5]Department of Biochemistry, School of Medicine, University of Crete and Institute of Molecular Biology and Biotechnology of Crete, Heraklion, Greece

[6]Institute of Molecular Biology and Biotechnology, FORTH, Heraklion, Crete,

[7]Faculty of Medicine, University of Athens, Greece

[8]Rheumatology, Clinical Immunology and Allergy, Faculty of Medicine, University of Crete, 71003 Iraklion, Greece

[9]The Kellgren Centre for Rheumatology, Central Manchester Foundation Trust, NIHR Manchester Biomedical Research Centre, Manchester, UK

*****Corresponding author:

George N. Goulielmos, (e-mail:goulielmos@med.uoc.gr); Laboratory of Molecular Medicine and Human Genetics, School of Medicine, University of Crete, Voutes, 715 00 Heraklion, Crete, Greece.

***Abbreviated title*:** Genetics of Rheumatoid and Psoriatic Arthritis in Greece

Abstract

Introduction: Several rheumatoid arthritis (RA) susceptibility loci have also been found to be associated with psoriatic arthritis (PsA), demonstrating that there is a degree of genetic overlap between various autoimmune diseases. We sought to investigate whether single nucleotide polymorphisms (SNPs) mapping to previously reported RA and/or PsA susceptibility loci, including *PLCL2, CCL21, REL, STAT4, CD226, PTPN22* and *TYK2*, are associated with risk for the two diseases in a genetically homogeneous Greek population.

Methods: A total of 392 RA patients, 126 PsA patients and 521 healthy age and sex matched controls from Greece were included. Genotyping of the SNPs was performed with Taqman primer-probe sets. Bioinformatic analysis was performed using BlastP, Pymol and Maestro and Desmond (Schrodinger Inc.).

Results: A significant association was detected between the GC genotype of rs34536443 (*TYK2*) in both the PsA and RA cohorts. The C allele of this SNP was associated with PsA only. Evidence for association with PsA was also found for the GG genotype and G allele of the rs10181656 SNP of *STAT4*. The TC genotype of the rs763361 SNP of *CD226* was associated with PsA only.

Conclusions: Genetic overlap between PsA and RA was detected for the rs34536443 SNP of *TYK2* gene within a Greek population. An association of *STAT4* (rs10181656) with PsA was confirmed whilst *CD226* (rs763361) was associated with PsA but not RA, in contrast to previous reports. The different findings of this study compared to previous ones highlights the importance of comparative studies that include various ethnic or racial populations.

Keywords: Rheumatoid Arthritis (RA), Psoriatic Arthritis (PsA), genetic association, gene polymorphisms

Introduction

Autoimmune diseases affect approximately 5% of the human population; however the genes and mechanisms involved still remain obscure. There is accumulating evidence that common genetic factors might predispose to multiple autoimmune disorders, thus indicating the pleiotropic effect of these gene polymorphisms. Such variants are by definition known as primary causal risk factors. In this framework, it has been documented that common variants of *PTPN22* (1-7), *CTLA-4* (8-12), *STAT4* (13-16), *TRAF1/C5* (17, 18) and *CD40* (19-22) are associated with different autoimmune disorders.

In recent years, a large number of novel genes associated with rheumatoid arthritis (RA) have been identified (23), especially with the use of the Immunochip, which has brought the number of RA-susceptibility loci in Caucasians to 48 (45 non *HLA* loci plus three *HLA* loci) (24) and in all populations to 101 (25). RA is a systemic multifactorial disease that results from a complex interplay between genetic and environmental factors (26). The serum of most RA patients contains various auto-antibodies, such as rheumatoid factor (RF) or anti-citrullinated protein antibodies (ACPAs), the presence of which constitutes one of the new classification criteria for RA (27).

The clinical features of RA resemble those of other autoimmune diseases, especially that of psoriatic arthritis (PsA), an inflammatory arthritis that is associated with Psoriasis (PS). For example, both diseases are characterized by the occurrence of an inflammatory arthritis in peripheral synovial joints. Patients also respond to similar therapies including methotrexate and anti-tumour necrosis factor biologic treatment (28). This observation suggests a possible overlap in the genetic susceptibility between the two diseases. This has been supported by previous studies which have reported a degree of genetic background that is shared between RA and PsA (29). PsA is a seronegative chronic inflammatory joint disease with prevalence rates ranging between 0.3 and 1% worldwide (30). It is interesting to note that 14% of a UK psoriasis cohort has been found to have co-existing PsA in a previous study (31), while 34.85% of a Greek PS cohort in a separate study was found to have PsA (22). The spectrum of PsA symptoms includes inflammatory changes in attachments of articular capsules, tendons, and ligaments to bone surface(32).

A number of candidate gene studies have been carried out in PsA, reporting associations with risk loci such as *RUNX3*, *IL23A* and *TNIP1* (33). Furthermore, a strong correlation between an increased risk of PsA and RA-predisposing genes has also been identified (29). To date, only one GWAS study has been published that has focused solely on PsA (34). However, use of the Immunochip has identified novel genome-wide significant PsA susceptibility loci, including *PTPN22* (rs2476601) (35).

As genetic RA-risk factors tend to overlap with PsA (29, 36), the current study was undertaken to investigate the association of both diseases with suggestive or confirmed RA- and/or PsA-risk genes as well as others that play a key role in the pathogenesis of multiple autoimmune diseases. This study focused on the genetic homogeneous population of the island of Crete (0.65 million), a population sharing a common genetic and cultural background, showing low migration rates and characterized by good genealogical records and uniform environmental influences.

Methods

Patient population and study design

In this case control association study, 392 RA patients and 126 PsA patients followed in the Department of Rheumatology, University Hospital of Heraklion, were included. Only RA patients who met the 2010 ACR/EULAR RA Classification Criteria (37) and PsA patients who fulfilled the CASPAR criteria (38) and also gave their informed consent were eligible for the study. A total of 521 unrelated, age and ethnically matched healthy volunteers from the Department of Transfusion Medicine of the University Hospital of Crete served as controls. The study was performed in the laboratory of Molecular Medicine and Human Genetics of the Medical School of Crete, after obtaining the approval of the research committee of the University Hospital of Heraklion.

Analysis of gene polymorphisms

A panel of single nucleotide polymorphisms (SNPs) mapping to seven previously analyzed RA and/or PsA susceptibility loci were selected for genotyping in Greek cohorts of RA and PsA patients as well as healthy controls. These included *PLCL2 (rs4535211)*, *CCL21 (rs2812378)*, *REL (rs13017599)*, *STAT4 (rs10181656)*, *CD226*

(rs763361), *PTPN22* (rs2476601) and *TYK2* (rs34536443). Genomic DNA was isolated from peripheral blood leukocytes using a commercial kit supplied by INVITROGEN (PureLink® Genomic DNA Mini Kit) according to the manufacturer's instructions. Allelic discrimination of all SNPs was carried out using TaqMan SNP genotyping assays on an Applied Biosystems ViiA™ 7 Real-Time PCR System (catalogue ID: C____481392_10, C__16113556_10, C___2833757_10, C__30530761_10, C___1464836_20 and C__60866522_10, respectively) from Applied Biosystems. Genotyping of rs2476601 SNP of *PTPN22* was performed by RFLP as described elsewhere (39). All allelic discrimination plots were manually reviewed individually for quality. 10% of the samples were amplified twice to ensure accuracy of the results.

Construction of three-dimensional (3-D) model

Bioinformatic analysis was performed using BlastP (for sequence analysis) on the Uniprot sequence database, Pymol (DeLano) for 3D structural positioning and visualisation and Maestro and Desmond (Schrodinger Inc.) for mutations and stability analysis as well as molecular dynamics. The crystal structure of human TYK2 (pdb code 4GVJ) (40) was used as the initial model.

Statistical analysis

Statistical analysis was performed with the GraphPad Prism statistical program (GraphPad Software, San Diego, CA). In case-control comparisons, only unrelated individuals were included. The x^2 test, with one or two degrees of freedom or Fisher's exact test was used to examine differences of genotype and allele frequencies between patients and controls, where all SNPs had a call rate > 98%. A two tailed *P* value less than 0.05 was defined as statistically significant. The gene variants under investigation were evaluated for deviation from Hardy–Weinberg equilibrium (HWE) by comparing observed and expected genotype frequencies by means of chi-squared (x^2) test or Fisher's exact test in the control groups (by using the program named ''Calculate''; Copyright TRG, SR, INMD, 2008). The distribution of genotypes in the case and control groups for all 7 SNPs examined was found to be under HWE ($p > 0.01$). Power analysis was conducted using the Quanto (version 1.2.4) program (41-44).

Results

The average age of the Greek RA cohort was 60.96±12.8 years, and 59.9% of subjects were female. Within the Greek PsA cohort, the average age was 52.12±15.41 and 46% of subjects were female. Unrelated healthy controls (N=521) consisted of 264 women (50.7%) and 257 men (49.3%) of similar ages. The genotyping was performed using TaqMan 5' allelic discrimination technology with predesigned SNP genotyping assays provided by Applied Biosystems. The genotyping success for all the SNPs analyzed was >98%.

Allele and genotype frequencies of the analyzed samples for the *TYK2* polymorphism (rs34536443) are depicted in Tables 1a and 1b. Our results show that the G/C genotype was more frequent in PsA and RA cases than in controls (p=0.019, OR=0.099, 95% CI 0.005-1.65 and p=0.033; OR=0.33, 95% CI 0.123-0.90, respectively), while the C allele was more frequently observed in PsA patients compared to controls (p=0.02, OR=0.10, 95% CI 0.006-1.681). Interestingly, the frequency of this allele did not appear to have any statistically significant difference between RA patients and controls, although previously it was reported to have a potential protective role in RA (45). Bioinformatic analysis revealed that the substitution of a G to C nucleotide caused by rs34536443 SNP generates a Pro1104Ala mutation in the TYK2 protein. In particular the replacement of the Pro^{1104} amino acid residue of TYK2 (an α-helical structure disruptor) with an Alanine (an α-helical former) (Figure 1) may induce to the α-helical segment (residues 1104-1113) a conformational change extending the a-helical segment five more residues (1099-1113). Such a conformational change will probably alter the local 3D structure, affect the folding and the interaction parameters of the molecule and therefore induce changes in the functionality of the molecule. Molecular dynamics studies of the mutant indicate propagation of the folding changes along the molecular structure.

Patients with PsA presented more frequently with the GG genotype and the G allele of the *STAT4* rs10181656 SNP (P = 0.04, OR=4.3, 95% CI 1.01-18.39 and P = 0.03, OR=1.46, 95% CI 1.03-2.08, respectively) (Table 2). These findings support the implication of this SNP in the development of PsA in the Greek population. However, no association was found between RA cases and controls either at the genotypic or the

allelic level (data not shown), contrary to previous results (29). In a first attempt to study the functional consequence of the rs10181656 SNP of *STAT4*, we performed a DNA sequence analysis using the Genomatix Software (Genomatix Software GmbH, "Gene2Promoter" program, Muenchen, Germany). This analysis revealed that the major C allele, but not the minor G allele of the rs10181656 SNP of *STAT4,* is located within a putative binding site for transcription factors Pax-4, PPAR/RXR and ZNF99.

Association with PsA only was also found for TC genotype of the rs763361 SNP of *CD226* (p=0.04, OR=0.61, 95% CI 0.39-0.95) **(**Table 3**)**. In an attempt to clarify the role of this SNP in the development of multiple autoimmune diseases, we analyzed in detail the existing 3-D models of this protein (46). The rs763361 SNP results in a Gly307Ser substitution; this position is located on the amino acid sequence towards the C-terminal end of the CD226 polypeptide. In particular, the Ser^{307} variant alters splicing of the *CD226* transcript, which affects downstream signaling and increases the activation of T and NK cells (47). This region is characterized as the cytoplasmic terminal region after the membrane integrated anchoring α-helical part (46). It is populated by many charged and polar residues and contains several putative phosphorylation sites (Y^{249}, T^{250}, S^{290}, T^{293}, S^{302}, S^{305}, S^{313}, Y^{322}, Y^{325}, S^{329}) conserved between species. Although no definite conclusions could be drawn about a putative structural change, the Ser^{307} residue in the protein's cytoplasmic region is most likely to be involved in polar interactions associated with proximal charged residues or phosphorylation sites.

The distribution of the *PTPN22* 1858C/T alleles in the Cretan population was not significantly different between RA patients and controls (data not shown), while it was found that the minor allele T was more common in controls individuals than in PsA patients. However, the observed difference was not statistically significant when it was evaluated with an x^2 test of independence (*P*=0.056, OR=0.27, 95% CI 0.06-1.14, detailed data not shown).

Interestingly, none of the remaining three SNPs examined (rs4535211 (*PLCL2*), rs2812378 (*CCL21*) and rs13017599 (*REL*) were found to be associated with either RA or PsA (data not shown).

Our study had 80% power to detect effect sizes of 1.3-1.4 for RA and 1.5 to 1.8 for PsA assuming minor allele frequencies of between 10 and 40% at the 5% significance level.

Discussion

The strategy of studying the putative role of RA susceptibility genetic factors in the development of PsA has proven to be highly successful so far (29). Moreover, accumulated evidence indicates that ethnic heterogeneity of genetic factors exists for rheumatic disorders. Thus, understanding allelic homogeneity and heterogeneity among these diseases provides insight into common gene function and pathways. So far, the *TYK2* rs34536443 (24), the *CCL21* rs2812378 (24), the *PTPN22* rs2476601 (24) and the *PLCL2* rs4535211 (48) SNPs have been associated with RA, while the rs13017599 SNP of *REL* has been associated with both PsA (49) and RA (50), attaining a genome-wide level of significance (P<5e-8) in all cases. Moreover, the aforementioned *CCL21*, *PTPN22, PLCL2*, *REL* and *STAT4* (rs10181656) SNPs were found to be overlapping between RA and PsA in a previous study (29), whereas *CD226* rs763361 SNP has been associated with an increased risk for the development of RA and other autoimmune diseases (51).

In the current study, the genetic association of *STAT4* (rs10181656) and *CD226* (rs763361) with PsA but not with RA was confirmed in a Greek population, while a genetic overlap between PsA and RA was detected with the rs34536443 SNP of *TYK2*. The PsA-associated allele of the *STAT4* SNP was protective for RA and not risk, as previously reported in other populations (29). Importantly, to the best of our knowledge, the *CD226* SNP has not previously been associated with PsA (51).

TYK2 is a locus that has been confirmed to be associated with RA at genome-wide significance levels (24). The encoded protein is a tyrosine kinase in the STAT signaling pathway, important for signaling by type I IFN and induction of Th1 cell differentiation upon antigen stimulation of dendritic cells (52). A meta-analysis of Immunochip, Exomechip and targeted exon-sequencing data recently demonstrated that the minor allele of rs34536443 SNP of *TYK2* was protective against RA, Systemic Lupus Erythematosus (SLE) and Inflammatory Bowel Disease (IBD) (45), while the same variant has been previously reported as conferring risk for PS) (53) and Juvenile Idiopathic Arthritis (JIA) (54). Interestingly, we found the minor allele to

increase the susceptibility for RA as well as PsA in the Greek population. However, the possibility that the results of our present study are spurious should be considered. The location of the Pro1104Ala mutation on the 3D structure of TYK2 may affect structural and dynamical elements of the molecule. The introduction of a helical former alanine residue to replace the rather constrained helical breaker proline influences the secondary structure of this region with an α-helical conformation extension, (residues 1099-1113), that may alter folding properties as well as dynamic aspects of the molecule which in turn affect its functionality.

STAT4 encodes a transcription factor that transmits signals induced by several key cytokines, including interleukin-12, interleukin-23 and type 1 interferons. STAT4-dependent signaling by interleukin-12 receptors plays a critical role in the development of a Th1-type T-cell response (13). Our bioinformatic analysis showed that the minor G allele of the rs10181656 SNP of *STAT4* may destroy a putative binding site of the heterodimer of transcription factors PPARα/RXRα. PPARα is a nuclear receptor expressed in macrophages, granulocytes and lymphocytes (55, 56) and plays an important role in suppressing autoimmune diseases by regulating Th2 cytokine production (57). Notably, it has been demonstrated that PPARα ligands can inhibit T-cell proliferation or the production of IFN-γ by T cells stimulated by antigens (58). Thus, it can be hypothesized that the minor allele of rs10181656, which was found to have increased frequency in PsA, may suppress T cell activation via the disturbance of the binding of the PPARα/RXRα dimer.

CD226 is a type 1 membrane protein that belongs to the Ig-superfamily and is involved in the adhesion and co-stimulation of T cells and NK cells (59-61). The non-synonymous rs763361 polymorphism of *CD226*, resulting in a Gly307Ser substitution, was suggested to alter the exon-splicing silencer sequence, which may affect gene expression. The rs763361 SNP has been found to be associated with RA (51), JIA (62), SLE (63), T1D (51), MS (51), Wegener's Granulomatosis (WG) (64), MS (65), Autoimmune Thyroid Disease (AITD) (51) and, for the first time, we present its association with PsA. Based on a detailed analysis of the existing 3-D models of this protein (46), we propose that the Ser^{307} residue is most likely to be involved in polar interactions associated with proximal charged residues or phosphorylation sites. By affecting the proximal phosphorylation sites or protein-protein subunit recognition, this mutation could alter the signaling cascade (51).

A lower rate of the MAF of *PLCL2* (rs4535211), *CCL21* (rs2812378) *REL* (rs13017599) and *PTPN22* (rs2476601) SNPs has been observed in the healthy individuals from Greece compared to those previously described by Bowes et al (29), a finding that delineates the role of some existing population-specific differences in the frequency of these alleles. A North-South gradient in the frequency of the *PTPN22* minor allele across Europe has been widely reported previously, ranging between 15.5% and 2.1% (36), and will impact on the power to detect association; thus, there is still the possibility that increasing the size of the cohort, an association of the rs2476601 *PTPN22* SNP with RA and/or PsA could be detected. Considering the low MAF of the *PTPN22* variant in Greek control samples (2.7-3%), more than 5,000 patient and control samples would be required to detect even a large effect at this locus (i.e. a 50% increase in risk with 80% power at p=0.05).

An advantage of our study was the ethnic matching of the cohort and control group. As a consequence, the results are unlikely to be biased by sampling. A clear weakness of the study is the limited sample size, especially for PsA. The lack of consistency between studies of various RA- and PsA susceptibility loci may be due to the lack of power of individual studies to detect the modest effect sizes reported previously at most loci.

Whilst multiple ethnic populations share some of the enormous genetic variation detected after the completion of the human genome sequence, a substantial variability has been seen in the frequency of many alleles. The different findings of this current study from previous ones highlights the importance of comparative studies that include different ethnic or racial populations in any attempt to confirm genetic associations detected.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

GNG and AB participated in study design and analysis and interpretation of data. EM, MIZ, AB-A, EE, GG, NK, DK and TS participated in acquisition of data and data analysis. DTB and PS participated in analysis and interpretation of data. All authors were involved in drafting the manuscript or revising it critically for important intellectual content, and all authors approved the final version to be submitted for publication.

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Authors' information

[1]Laboratory of Molecular Medicine and Human Genetics, Department of Internal Medicine, School of Medicine, University of Crete, Heraklion, Greece. [2]Arthritis Research UK Centre for Genetics and Genomics, Centre for Musculoskeletal Research, Institute for Inflammation and Repair, Manchester Academic Health Science Centre, The University of Manchester, Manchester, UK. [3]NIHR Manchester Musculoskeletal Biomedical Research Unit, Central Manchester University Hospitals NHS Foundation Trust, Manchester Academic Health Science Centre, Manchester, UK. [4]Laboratory of Genetics, Department of Biotechnology, Agricultural University of Athens, Athens, Greece. [5]Department of Biochemistry, School of Medicine, University of Crete and Institute of Molecular Biology and Biotechnology of Crete, Heraklion, Greece. [6]Institute of Molecular Biology and Biotechnology, FORTH, Heraklion, Crete, Greece. [7]Faculty of Medicine, University of Athens, Greece. [8]Rheumatology, Clinical Immunology and Allergy, Faculty of Medicine, University of Crete, 71003 Iraklion, Greece. [9]The Kellgren Centre for Rheumatology, Central Manchester Foundation Trust, NIHR Manchester Biomedical Research Centre, Manchester, UK.

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Figure legends

Figure 1: 3D ribbon diagram of the TYK2 native structure (PDB code 4GVJ) containing the position of the P1104A polymorphism. Helical regular segments are represented in cyan and beta strands in magenta color. Diagram created using program PyMol (DeLano et al. Schrodinger Inc).

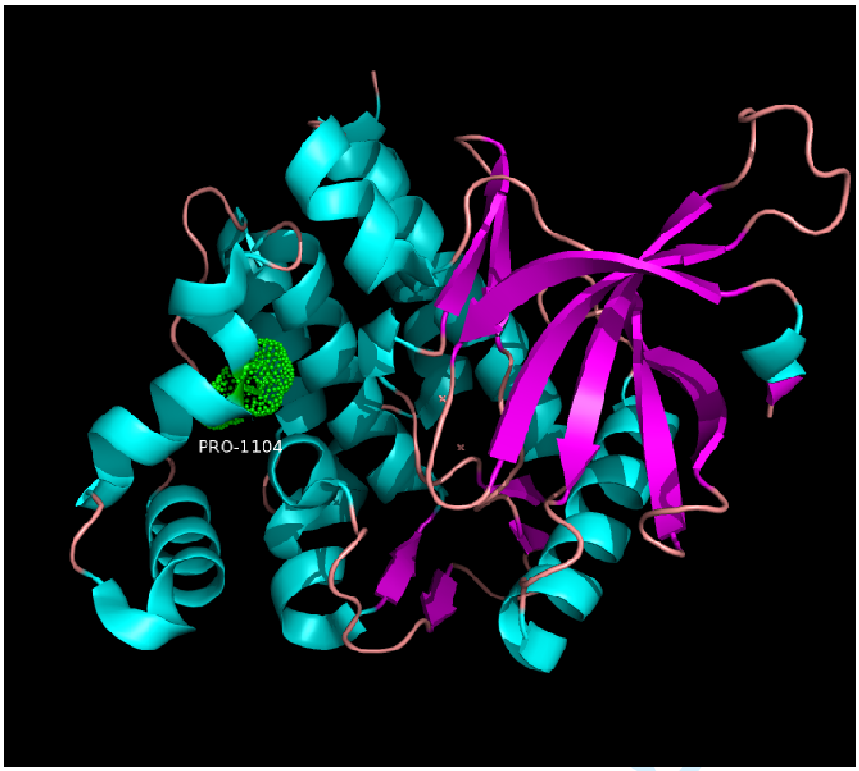

Table 1a. Genotypes and alleles frequency of the *TYK2 G/C* rs34536443 polymorphism analyzed in 392 RA patients and 507 healthy controls.

GENOTYPES **rs34536443**	**RA N=392**	**Controls N=507**	***p*-value**	**OR (95% CI)**
G/G	385 (98.2%)	488 (96.3%)		
G/C	5 (1.3%)	19 (3.7%)	**0.0347**	0.33 (0.123-0.90)
C/C	2 (0.5%)	0 (0%)	0.1953	6.33 (0.3-132.5)
ALLELES	**N=784**	**N=1014**		
G	775 (98.9%)	995 (98.1%)		
C	9 (1.1%)	19 (1.9%)	0.25	0.60 (0.27-1.352)

Table 1b. Genotypes and alleles frequency of the *TYK2* G/C rs3453443 polymorphism analyzed in 126 PsA patients and 507 healthy controls.

GENOTYPES **rs34536443**	**PsA N=126**	**Controls N=507**	***p*-value**	**OR (95% CI)**
G/G	126 (100%)	488 (96.3%)		
G/C	0 (0%)	19 (3.7%)	**0.0194**	0.099 (0.005-1.65)
C/C	0 (0%)	0 (0%)		
ALLELES	**N=252**	**N=1014**		
G	252 (100%)	995 (98.1%)		
C	0 (0%)	19 (1.9%)	**0.0203**	0.10 (0.006-1.681)

Table 2. Genotypes and alleles frequency of the *STAT4* C/G rs10181656 polymorphism analyzed in 126 PsA patients and 521 healthy controls

GENOTYPES **rs10181656**	**PsA N=126**	**Controls N=521**	***p*-value**	**OR (95% CI)**
C/C	82 (65.08%)	295 (56.62%)		
C/G	42 (33.33%)	195 (37.43%)	0.13	3.34 (0.77-14.5)
G/G	2 (1.59%)	31 (5.95%)	**0.04**	4.3 (1.01-18.39)
ALLELES	**N=252**	**N=1042**		
C	206 (81.75%)	785 (75.34%)		
G	46 (18.25%)	257 (24.66%)	**0.03**	1.46 (1.03-2.08)

Table 3. Genotypes and alleles frequency of the *CD226* T/C rs763361 polymorphism analyzed in 126 PsA patients and 521 healthy controls.

GENOTYPES	**PsA N=126**	**Controls N=521**	***p*-value**	**OR (95% CI)**
rs763361				
T/T	48 (38.1%)	148 (28.4%)		
T/C	52 (41.3%)	260 (49.9%)	**0.0388**	0.61 (0.39-0.95)
C/C	26 (20.6%)	113 (21.7%)	0.23	0.71 (0.41-1.21)
ALLELES	**N=252**	**N=1042**		
T	148 (58.7%)	556 (53.4%)		
C	104 (41.3%)	486 (46.6%)	0.14	0.80 (0.61-1.06)

Table 1a. Genotypes and alleles frequency of the *TYK2 G/C* rs34536443 polymorphism analyzed in 392 RA patients and 507 healthy controls.

GENOTYPES rs34536443	**RA N=392**	**Controls N=507**	***p*-value**	**OR (95% CI)**
G/G	385 (98.2%)	488 (96.3%)		
G/C	5 (1.3%)	19 (3.7%)	**0.0347**	0.33 (0.123-0.90)
C/C	2 (0.5%)	0 (0%)	0.1953	6.33 (0.3-132.5)
ALLELES	**N=784**	**N=1014**		
G	775 (98.9%)	995 (98.1%)		
C	9 (1.1%)	19 (1.9%)	0.25	0.60 (0.27-1.352)

Table 1b. Genotypes and alleles frequency of the *TYK2* G/C rs3453443 polymorphism analyzed in 126 PsA patients and 507 healthy controls.

GENOTYPES rs34536443	**PsA N=126**	**Controls N=507**	***p*-value**	**OR (95% CI)**
G/G	126 (100%)	488 (96.3%)		
G/C	0 (0%)	19 (3.7%)	**0.0194**	0.099 (0.005-1.65)
C/C	0 (0%)	0 (0%)		
ALLELES	**N=252**	**N=1014**		
G	252 (100%)	995 (98.1%)		
C	0 (0%)	19 (1.9%)	**0.0203**	0.10 (0.006-1.681)

Table 2. Genotypes and alleles frequency of the *STAT4* C/G rs10181656 polymorphism analyzed in 126 PsA patients and 521 healthy controls

GENOTYPES **rs10181656**	**PsA N=126**	**Controls N=521**	***p*-value**	**OR (95% CI)**
C/C	82 (65.08%)	295 (56.62%)		
C/G	42 (33.33%)	195 (37.43%)	0.13	3.34 (0.77-14.5)
G/G	2 (1.59%)	31 (5.95%)	**0.04**	4.3 (1.01-18.39)
ALLELES	**N=252**	**N=1042**		
C	206 (81.75%)	785 (75.34%)		
G	46 (18.25%)	257 (24.66%)	**0.03**	1.46 (1.03-2.08)

Table 3. Genotypes and alleles frequency of the *CD226* T/C rs763361 polymorphism analyzed in 126 PsA patients and 521 healthy controls.

GENOTYPES rs763361	**PsA N=126**	**Controls N=521**	***p*-value**	**OR (95% CI)**
T/T	48 (38.1%)	148 (28.4%)		
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ALLELES	**N=252**	**N=1042**		
T	148 (58.7%)	556 (53.4%)		
C	104 (41.3%)	486 (46.6%)	0.14	0.80 (0.61-1.06)

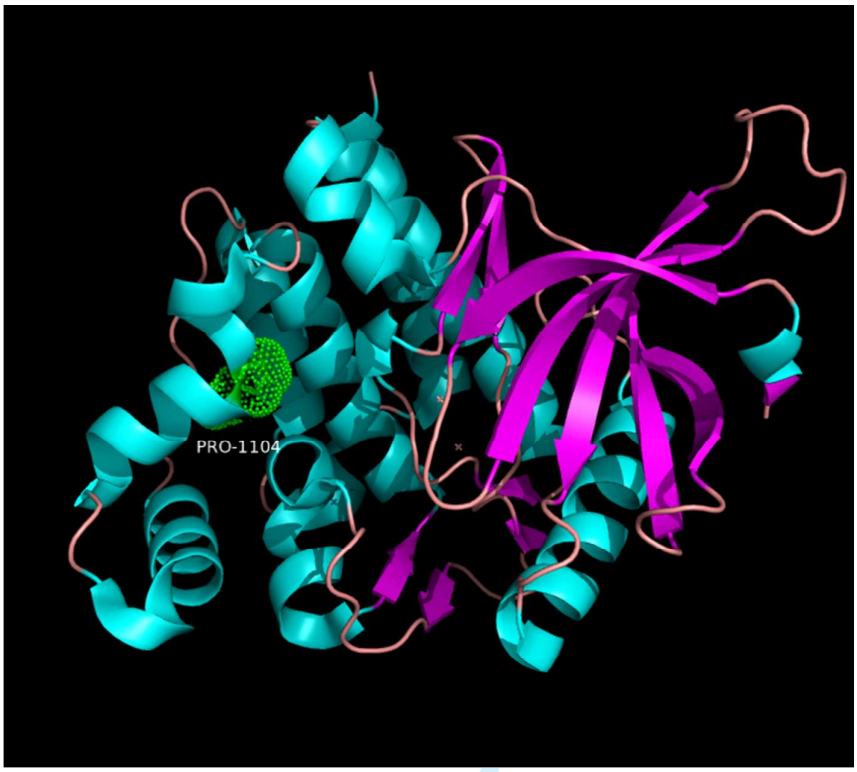

Figure 1: 3D ribbon diagram of the TYK2 native structure (PDB code 4GVJ) containing the position of the P1104A polymorphism. Helical regular segments are represented in cyan and beta strands in magenta color. Diagram created using program PyMol (DeLano et al. Schrodinger Inc).