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Association of IL-10 gene promoter polymorphisms with food allergy susceptibility and serum IL-10 level in a pediatric Caucasian population

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BACKGROUND: Interleukin 10 has been shown to play a critical role in the regulation of the immune responses in allergic diseases.

AIM: To investigate if genetic polymorphisms in the promoter region of the IL-10 gene are associated with food allergy (FA) susceptibility in Caucasian pediatric patients with concomitant allergic diseases and IL-10 levels.

METHODS: The single nucleotide polymorphisms (SNPs) at -1082A>G (rs1800896), -819 T>C (rs1800871), -592A>C (rs1800872) of 62 pediatric patients with IgE-mediated FA were analyzed and correlated with clinical parameters, serum IgE and IL-10 levels. The results were compared with those of 92 healthy controls without FA, personal and/or family history of atopy.

RESULTS: Analysis and comparison of genotype distributions, allele frequencies and haplotypes showed that none of the genotypes confers an increased risk of FA. The genotype -1082 AA in FA patients was associated with moderate to severe symptoms of FA, the development of atopic asthma and higher levels of IL-10. In a linear regression study, we confirmed that the genotype -1082 AA acts as an independent factor for the higher levels of IL-10. A positive association was also observed between -819 T/C and -592 A/C SNPs and later onset of FA.

CONCLUSION: Polymorphisms in the promoter region of the IL-10 gene are not associated with FA susceptibility in our cohort. In FA patients -1082 A/G SNPs seem to influence the production of IL-10, the severity of FA symptoms and the development of atopic asthma in this population.

Key messages

To our knowledge, this is the first study investigating the role of single nucleotide polymorphisms (SNPs) in the promoter region of the IL-10 gene in food allergy in pediatric Caucasian children. The study provides insight into the association between -1082 A/G, -819 T/C and -592 A/C SNPs and food allergy susceptibility, clinical and surrogate parameters of the disease in this population. A secondary aim of the study is to correlate the studied SNPs with the development of other atopic diseases in FA patients.

Conflict of interest: The authors have no conflict of interest to declare.

Keywords: Interleukin 10, IL-10 gene promoter polymorphisms, food allergy, children.

Introduction

Growing evidence suggests that food allergy (FA) has been affecting an increasing proportion of the population worldwide over the past decades ¹. Despite advances in research, the pathophysiology of food allergy has not yet been fully elucidated and it seems that the complex pathways leading to T- helper cell type 2 (Th2) immune responses against food antigens can't be attributed to a single driving force. Current research is focusing on genetic and epigenetic regulations and the role of the microbiome and dysbiosis in food allergy ^{1,2}.

In order to understand the pathophysiology of food allergy it is crucial to delineate factors that disturb the innate surveillance system promoting oral tolerance (OT). OT is a complex process mediated by antigen-specific regulatory T cells (Tregs) resulting in active suppression of immune responses to antigens first encountered in the gastrointestinal tract. Tregs seem to play a central role in the maturation of the immune system and the onset of immune- mediated diseases. Two inhibitory cytokines, interleukin 10 (IL-10) and transforming growth factor β (TGF- β), contribute to the ability of Tregs to induce a tolerant immune response to antigens ³.

IL-10 is a pleiotropic cytokine and the regulation of its expression is complex and delicately controlled at both transcriptional and post- transcriptional level ⁴⁻⁶. The IL-10 gene is located on the long arm of chromosome 1 (*q31-q32*) and is composed of four introns and five exons. The production of the cytokine is associated with two loci at

positions -1082A/G and -627A/C that lie on the putative transcription factor-binding sites^{7,8}. Three common single nucleotide polymorphisms (SNPs), -1082 A/G (*rs1800896*), -819 T/C (*rs1800871*), -592 A/C (*rs1800872*) have been identified within the promoter region of the IL-10 gene and in recent years special interest has been drawn to their impact on the susceptibility of atopic diseases^{9,10}.

The primary aim of this prospective cross-sectional study was to investigate for the first time if the three SNPs in the promoter region of IL-10 gene are associated with FA susceptibility and IL-10 levels in pediatric Caucasian children. The studied SNPs were also correlated with clinical/ surrogate parameters and the development of concomitant allergic diseases.

Materials and methods

Subjects' characteristics

One hundred fifty four Caucasian children- 62 with IgE- mediated FA and 92 healthy controls- were recruited between November 2012 and December 2016 and 136/154 were followed up until December 2019. Children with FA were recruited from the Pediatric Allergy and Gastroenterology Clinics in Hippokration Hospital, Aristotle University of Thessaloniki.

Children who visited the Outpatient Pediatric Surgical and Haematology Clinics in Hippokration Hospital for elective follow up were asked to participate in the study as healthy controls. To enter the study, the participants of this group needed to fulfill the following criteria: a) no personal or family history of atopic diseases (FA, asthma, hay fever, atopic dermatitis), b) negative specific IgE antibodies for the most common food antigens (milk, soya, egg, wheat, fish) and IgE level within the normal range. History of chronic or immune-mediated disorders was an exclusion criterion for both groups.

The study was approved by the ethics committee of Aristotle University of Thessaloniki in Greece (No 32, 1/8-11-2012) according to the Helsinki declaration. All parents/legal guardians provided written informed consent for blood sampling and genetic testing.

Definition of IgE-mediated FA

According to the guidelines for the diagnosis of IgE-mediated FA^{11,12} participants with the diagnosis of food allergy showed evidence of allergic sensitization and clinical symptoms in keeping with food allergy. All pediatric patients with IgE- mediated food allergy developed specific signs and symptoms on the exposure to the food antigen(s) along with antigen- specific IgE titer ≥ 0.35 kU/L and/or positive skin prick test(s) to the offending food antigen(s). Oral food challenge was deemed necessary for the diagnosis of FA in 10 cases and in all 10 cases the challenge was positive. The severity of FA symptoms by organ/system was classified as mild, moderate and severe according to the classification by the group Anvari et al ¹³. Specific IgE against food antigens and serum IgE level in all cases were determined by the ImmunoCAP100 system (Phadia, Uppsala, Sweden).

Genotyping

Peripheral blood was obtained from all participants under aseptic conditions. Genomic DNA was isolated from leukocytes from EDTA- anticoagulated whole blood with PureLink Genomic DNA Kit (Qiagen & Invitrogen) according to manufacturer's instructions. Genotyping was performed by Sanger sequencing using the forward primer 5'-GGGTTCTCATTCGCGTGTT-3' and reverse primer 5'-CAGGTAGAGCAACACTCCTCG-3' that were designed according to information collected from Oligo, SnapGene Viewer and NCBI Blast programs. The sequencing of the polymerase chain reaction (PCR) products was performed with the use of ABI Prism BigDye and analysed by the ABI 3100 Sequencer. The sequencing of the promoter region of the IL-10 gene was compared with the wild type sequence ENST00000423557 (ensembl database). The analysis of the sequencing chromatograms was carried out by a member of the research team blinded to clinical data with the use of SNAP GENE VIEWER and lalign programs.

Level of Serum IL-10

The serum level of IL-10 was determined from 86 randomly selected participants (42 with FA, 44 controls). All selected participants met the following criteria a) no history of infection/febrile illness or vaccination within the last 3 months, b) no febrile illness 15 days post blood sampling.

Blood samples were collected into serum separator tubes (SST), were allowed to clot for 30 minutes at room temperature and centrifuged for 10 minutes at 1500g. Supernatant

serum was separated, aliquoted and stored at -70°C . Commercially available enzyme-linked immunosorbent assay (ELISA) was used for IL-10 serum level measurement according to the manufacturer's instructions (R&D Systems Inc., Minneapolis, USA). All samples were measured in duplicates. The serum level of IL-10 was expressed as picograms per milliliter (pg/ml). The intra assay precision coefficient of variation was 10%. R^2 - values for calibration curves were over 0.98. The lower limit of detection was 1pg/ml. The biologist who performed the ELISA was blinded to the clinical details of the participants.

Statistics

Standard descriptive analyses were performed both for the qualitative and the quantitative variables. Comparisons between qualitative and qualitative variables were performed using the Fisher's exact test, Mann-Whitney U, Kruskal-Wallis and ANOVA as appropriate. Multiple linear regression analysis was employed to assess the association between different predictors and the IL-10, treated as the dependent variable. To test the null hypothesis that Hardy-Weinberg equilibrium holds, a chi-squared based method was used, including the computation of an unadjusted chi-Squared test statistic and of the respective p-value using a simulation method with 10,000 permutations. The level of significance was set at $p=0.05$ in all cases. All calculations were performed using SPSS V.25 and R programming language V.4.01.

Results

Demographic and clinical data of the participants are shown in Table 1. The mean age of FA patients and controls at recruitment was 2.6y and 3.9y, respectively ($p<0.0001$) with a mean follow up 4.5y and 3.6y respectively. The manifestations of FA at diagnosis most commonly involved the skin (erythema and facial/body edema) and gastrointestinal tract (vomiting and diarrhea). Milk (50/62, 80%) and egg (36/62, 58%) were the commonest offending food antigens and the majority of patients were allergic to one or two antigens. Concomitant atopic diseases were diagnosed in 77.4% (48/62) FA patients during the course of the disease.

Association of IL-10 gene polymorphisms with the risk of FA

Genotype distributions, allele frequencies and haplotypes of the three IL-10 polymorphisms (-1082 A/G, -819 T/C, -592 A/C) in FA children and healthy controls are summarized in table 2. The Hardy- Weinberg equilibrium was satisfied for both groups with regards to each of the three polymorphisms. No significant difference between the two groups was observed. The haplotype GCC was the most frequent for both FA patients and controls in our cohort. A difference in percentages of the genotypes -1082 GG and -1082 AG between FA children and controls was noted (32.3% vs 25% and 38.7% vs 46.7%, respectively), although this did not reach the level of significance.

Association between IL-10 gene polymorphisms and clinical/surrogate parameters

In the FA group, we further investigated the association between the studied SNPs and clinical/surrogate parameters, including age of FA onset, number and type of food antigens, severity of FA symptoms, concomitant atopic diseases (eczema, asthma, allergic rhinitis), serum IgE and IL-10 levels. The significant associations are summarized in table 3. The genotype -1082 AA was associated with moderate to severe symptomatology of FA ($p= 0.0487$, OR= 3.518, 95% CI: 1.074-11.764). The results of our study showed that the genotypes -819 CC and -592 CC were associated with later onset of FA. More specifically, with regards to SNP -819 T/C, the age of FA onset for genotype CC vs TT+TC was 9.2 ± 9.6 months vs 5 ± 4.7 months ($p= 0.04$), and regarding SNP -592 A/C the age of onset was 8.9 ± 9 vs 4.5 ± 4 months ($p= 0.03$). The AA homozygosity compared with at least one G-allele at loci -1082 was correlated with a significant difference with regards to the development of concomitant atopic asthma in FA patients [(AA vs AG+GG: 35.3% vs 9.3%), $p=0.0237$, OR=5.318, 95% CI:1.271-22.258]. We didn't find any other association with the level of IgE or other parameters of interest.

IL-10 Analysis

The serum level of IL-10 from 42 FA children and 44 age- and sex-matched healthy controls was determined (Table 1). No difference in IL-10 levels was observed between the two groups (Figure 1a). However, in the food allergy group the genotype -1082 AA was associated with the highest mean value of serum IL-10 (AA vs AG vs GG: 8.6 ± 5.4 vs 5.4 ± 7.3 vs 3 ± 1.4 pg/mL, respectively, $p=0.0014$, Figure 1b). In addition, a marginally significant association ($p=0.0596$) was identified between the increased level of IL-10 in children with FA and concomitant asthma. In order to investigate whether the genotype -1082 AA is an independent factor for the IL-10 levels, we further conducted a multiple regression analysis model. The analysis confirmed that the genotype -1082 AA is an independent predictor of the serum IL-10 levels after adjusting for other confounders, such as -819 T/C and -592 A/C SNPs and other factors that could influence the level of IL-10, such as the age, presence of asthma and disease activity (table 4).

Discussion

The pathogenesis of FA is multifactorial and its development is attributed to the imbalance between allergen-specific Type 1 regulatory (Tr1) cells and Th2 cells. Tregs seem to play a central role in the maturation of the immune system. The suppression of the Th2-responses is mediated by the induction of allergen specific CD4⁺CD25⁺Foxp3⁺ Tregs by dendritic cells (DC) that produce IL-10^{14,15}. The regulatory effect of IL-10 in food allergy includes its ability to decrease IgE synthesis and the survival of eosinophils^{16,17}.

In recent years, the research has focused on candidate genes and the association with specific variants to investigate if these can influence the course of the disease and identify patients at risk that might benefit from early interventions. Several polymorphic sites in the IL-10 gene have attracted the researchers' interest.

In our study, we aimed to investigate if three SNPs in the promoter region of the IL-10 gene are associated with the risk of developing FA in pediatric Caucasian patients and we didn't confirm that the studied SNPs confer an increased risk. The group of Campos et al reported a marginal association between the presence of A allele at -1082 and FA in atopic Japanese children¹⁸. Another cross-sectional study in Brazil showed that the presence of the G allele at -1082 conferred a twofold risk for persistent cow's

milk allergy ¹⁹. SNPs in *-1082 A/G* and *-592 A/C* were associated with FA in a study in Taiwan ²⁰. The inconsistent results from different populations could be attributed to interethnic genetic variations and heterogeneity in atopic stratification.

We further evaluated if the studied SNPs were correlated with clinical and surrogate parameters of FA and we found that the presence of A- allele at *-1082* is associated with moderate to severe symptoms of FA, whereas SNPs in *-819T/C* and *-592 A/C* with later onset of FA in our population. A previous study by Negoro et al suggested that the severity of food allergy should be attributed to a combination of SNPs, laboratory parameters and environmental factors ²¹. The existing data relating to the correlation between specific genotypes and clinical parameters of food allergy are limited to date and therefore further prospective studies are deemed necessary to provide the evidence in order to clarify whether our observations could represent a causal association. Even though this wasn't the primary aim of our study, we also found that the genotype *-1082 AA* was correlated with higher prevalence of atopic asthma in children with FA. This is in keeping with the findings of a recent systematic analysis showing that the overall population did not show a significant association between *IL-10 -1082A/G* polymorphisms, however, the subgroup analysis based on ethnicity did reveal a significant association between *-1082 AA* homozygosity and pediatric asthma in non-Asian populations ²².

Evidence has shown that IL-10 production is influenced by genetic variation in the promoter region of the IL-10 gene. Experiments in vitro demonstrated that the genotype *-1082 GG* downregulates the expression of IL-10 ²³, whereas Eskdale et al reported that individuals with the G allele at loci *-1082* produce less IL-10 than individuals with the A allele ²⁴. These findings support what is observed in our study; we report that genotype *-1082 AA* in FA patients is associated with higher level of IL-10. This, however, is in contrast with other studies that found an association between *-1082 AA* homozygosity and lower serum IL-10 ^{20,25}. In our study, we also did not observe a significant difference in the level of IL-10 between children with food allergy and healthy controls. This could be possibly attributed to the fact that the majority of the FA patients were on elimination diets at the time of IL-10 level determination and close to achieving a tolerant state. In order to evaluate the possible predictive value of IL-10 level in FA, it may be useful future studies

to take into account the different stages of the disease at which the cytokine is determined (i.e diagnosis, elimination diet, tolerant state) along with the genetic variants.

To our knowledge, this is the first study investigating the association between three IL-10 gene promoter polymorphisms (-1082A/G, -819T/C, -592A/C) and the risk of FA and IL-10 serum level in Caucasian pediatric patients. A limitation of our study is the relatively small number of FA patients that potentially did not allow some associations to reach the level of significance. We also did not evaluate the serum IL-10 level longitudinally that could provide more information for the predictive value of the cytokine during the course of the disease. One of the strengths of our study is the long follow up (up to 7.5y) for both groups. For the study group this proved beneficial in order to investigate if the patients developed other atopic diseases over time. With regards to the healthy controls we managed to ensure that none of the participants in this group developed food allergy or other atopic or immune-mediated diseases until the completion of the study. Another strength is that we obtained a detailed clinical history from all participants to ensure that at the time of IL-10 determination, other factors that could influence the cytokine level were excluded.

In conclusion, our study suggests that SNPs in the promoter region of the IL-10 gene are not associated with FA susceptibility in our cohort, but they are correlated with clinical parameters of the disease. We observed that SNPs at -1082 A/G influence the levels of IL-10 in FA patients and further studies are needed to delineate the mechanisms that regulate the expression of this critical cytokine at different stages of the disease.

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- Accepted Article
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	Total population		IL-10 analysis subgroups	
	Food allergy n=62	Controls n=92	Food allergy n=42	Controls n=44
Age [years; mean±SD (range)]*	2.6 ±1.48 (1-5)	3.9 ±1.7 (1.5-7.5)	3.4 ± 2.3 (1-8)	4.13±1.9 (1-8)
Male sex [n (%)]	41 (66.1)	62 (67)	28 (66.6)	29 (66)
Duration of follow up [years]	4.54 ±1.8(2-7.5)	3.6± 0.9 (1-6)	4.77±1.7(2-7.5)	3.6±1 (1-6)
System involved [n (%)]				
Skin	50 (80)	n/a	37 (88)	n/a
GI tract	25 (40)	n/a	15 (36)	n/a
Respiratory	6 (9.7)	n/a	5 (12)	n/a
Cardiovascular	3 (4.8)	n/a	2 (5)	n/a
Anaphylactic reaction	6 (9.7)	n/a	2 (5)	n/a
Severity of reaction [n (%)]				
Mild	30 (48)	n/a	18 (43)	n/a
Moderate	21 (34)	n/a	15 (36)	n/a
Severe	11 (18)	n/a	9 (21)	n/a
Food antigens [n (%)]				
Milk	50 (80)	n/a	32 (76)	n/a
Egg	36 (58)	n/a	24 (57)	n/a
Fish	7 (11)	n/a	4 (9)	n/a
Nuts	7 (11)	n/a	6 (14)	n/a
Legumes	2 (3)	n/a	2 (5)	n/a
Wheat	2 (3)	n/a	2 (5)	n/a
Sesame	2 (3)	n/a	1 (2)	n/a
Number of antigens [n (%)]				
1	26 (42)	n/a	18 (43)	n/a
2	24 (39)	n/a	15 (36)	n/a
≥3	12 (19)	n/a	9 (21)	n/a
Specific IgE antibody titer (kU/L)				
0.7-3.49	18 (29)	n/a	10 (24)	n/a
3.5- 17.49	17 (27)	n/a	12 (29)	n/a
17.5- 49.99	9 (15)	n/a	7 (17)	n/a
50-100	8 (13)	n/a	6 (14)	n/a
>100	4 (6)	n/a	4 (10)	n/a
Skin prick test [n (%)]				
Positive	22/22 (100)	n/a	18/18 (100)	n/a
Oral Food challenge [n (%)]				

Positive	10/10 (100)	n/a	4/4 (100)	n/a
Concomitant atopic diseases				
[n(%)]				
Eczema	41 (66)	n/a	30 (71)	n/a
Asthma	10 (16)	n/a	8 (19)	n/a
Allergic Rhinitis	11 (18)	n/a	8 (19)	n/a

* p<0.0001 for total population, p=0.1355 for IL-10 analysis subgroups, n/a: not applicable

Table 1. Demographics and clinical characteristics of patients with food allergy and healthy controls in the total population and IL-10 analysis subgroups

	Food allergy N=62 %	Controls N=92 %	p- value	Odds ratio (95% CI), p-value
IL-10 -1082 A>G (rs1800896)				
Genotype				
AA*	18 (29)	26 (28.3)		1
AG	24 (38.7)	43 (46.7)	0.528	0.806 (0.369-1.761), 0.689
GG	20 (32.3)	23 (25)		1.256 (0.538-2.935), 0.668
Allele frequencies				
A	60 (0.484)	95 (0.516)	0.642	1
G	64 (0.516)	89 (0.484)		1.138 (0.721- 1.795), 0.642
IL-10 -819 T>C (rs1800871)				
Genotype				
TT*	5 (8.1)	10 (10.9)		1
TC	26 (41.9)	39 (42.4)	0.863	1.333 (0.409-4.351), 0.772
CC	31 (50)	43 (46.7)		1.442 (0.448-4.639), 0.580
Allele frequencies				
T	36 (0.29)	59 (0.321)	0.616	1
C	88 (0.71)	125 (0.679)		1.154 (0.702- 1.895), 0.616
IL-10 -592 A>C (rs1800872)				
Genotype				
AA*	3 (4.8)	3 (3.3)		1
AC	21 (33.9)	32 (34.8)	0.914	0.656 (0.121-3.564), 0.679
CC	38 (61.3)	57 (62)		0.667 (0.128- 3.479), 0.887
Allele frequencies				
A	27 (0.218)	38 (0.207)	0.887	1
C	97 (0.782)	146 (0.793)		0.935 (0.536- 1.631), 0.887
Haplotypes				

GCC*	41 (66.1)	59 (64.1)		1
ACC	15 (24.2)	23 (25)		0.938 (0.437-2.012), 1
GTC	3 (4.8)	7 (7.6)		0.616 (0.150- 5.998), 1
ATA	2 (3.2)	3 (3.3)	0.835	0.959 (0.153-5.998), 1
ACA	1 (1.6)	0 (0)		-

Table 2 IL-10 genotypes, allele frequencies and haplotypes for the gene polymorphisms -1082 A>G (*rs 1800896*), -819 T>C (*rs1800871*) and -592 A>C (*rs1800872*)

*: reference category for odds ratio calculation

IL-10 SNPs	p- value			
	Age of FA onset	Severity of FA symptoms	Asthma	IL-10 level
-1082 A/G				
AA vs AG vs GG	ns	ns	ns	0.0014
AA vs AG+GG	ns	0.0487	0.0237	0.0005
-819 T/C				
CC vs TT+TC	0.030*	ns	ns	ns
-592 A/C				
CC vs AA+AC	0.040**	ns	ns	ns

* mean±SD (range) age (m): 9.2±9.6 (0.5-54) vs 5 ± 4.7 (0.2-18)

** mean±SD (range) age(m): 8.9±9 (0.5-54) vs 4.5±4 (0.2-14)

ns: non- significant (p>0.05)

Table 3. Significant associations between genotypes and clinical or surrogate parameters of interest

Independent Variables	B	Sig.	95% Confidence Interval	
			Lower Bound	Upper Bound
Intercept	3,019	,342	-3,355	9,393
Age at measurement	,159	,669	-,592	,910
Absence of asthma	-,052	,982	-4,813	4,709
Presence of asthma	0 (ref)	.	.	.
FA tolerant state	-3,016	,217	-7,895	1,863
FA active disease	0 (ref)	.	.	.
-1082- AA	7,308	,017	1,397	13,218
-1082- AG	4,936	,081	-,639	10,510
-1082-GG	0 (ref)	.	.	.
-592- AA	-4,850	,364	-15,593	5,893
-592-AC	-3,101	,430	-11,005	4,803
-592-CC	0 ^a	.	.	.
-819C-TT	-3,323	,475	-12,695	6,049
-819C-TC	-,214	,948	-6,826	6,398
-819C-CC	0 (ref)	.	.	.

ref:reference group

Table 4. Linear regression analysis. Genotype -1082 AA is an independent predictor of serum IL-10 level (p 0.017) after adjusting for other confounders.

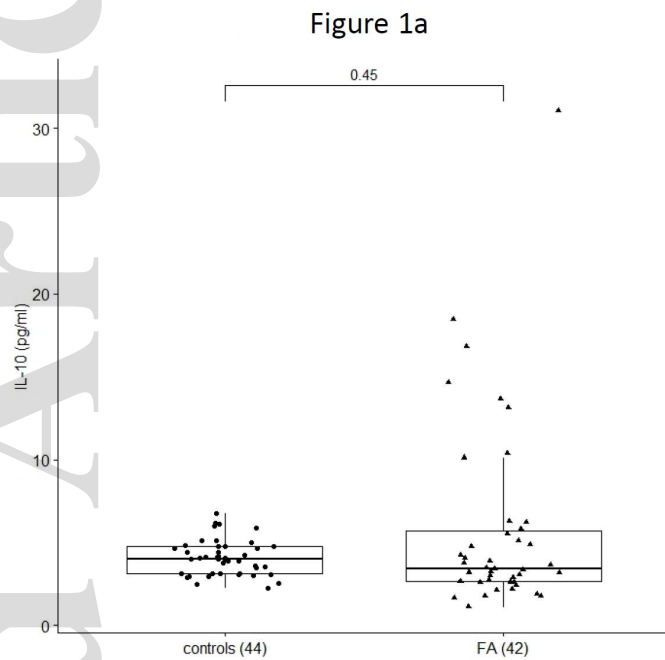


Figure 1a. Serum IL-10 level in controls and FA patients.

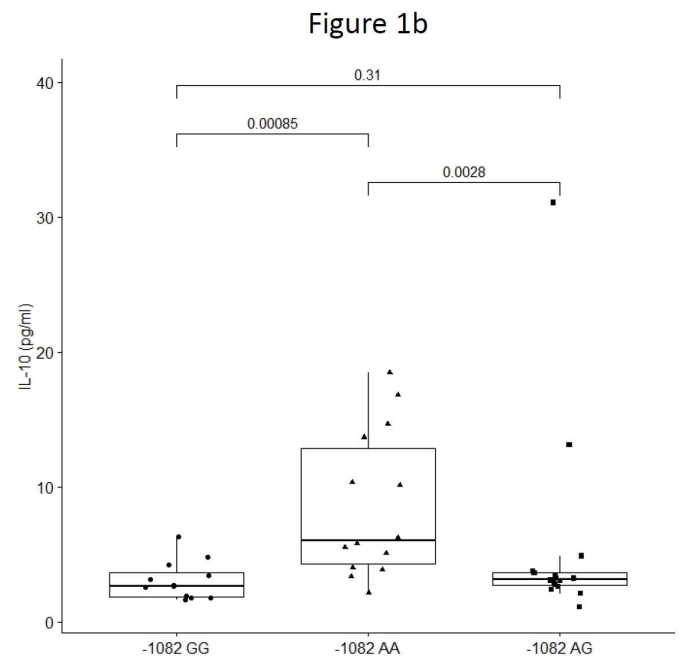


Figure 1b. Serum IL-10 level in FA patients. The genotype *-1082AA* is associated with higher IL-10 level.