

Plasma Interleukin-21 Levels and Genetic Variants Are Associated With Susceptibility to Rheumatoid Arthritis

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Abstract

Background Rheumatoid Arthritis (RA) is a chronic inflammatory condition characterised by the development of autoantibodies and an elevated spectrum of proinflammatory cytokines. Previous reports highlighted a relationship between IL-21 and pathogenesis of RA. Although elevated IL-21 levels have been reported in RA patients, the association of common IL-21 genetic variants with a predisposition to RA development in the Chinese population is lacking.

Materials and methods Five hundred and fourteen Chinese subjects (healthy controls: 303 and rheumatoid arthritis patients: 211) were enrolled in the study. Clinical data of patients were collected from medical records, and patients were treated as per the guidelines. IL-21 level in plasma of RA patients and healthy subjects was measured by ELISA.

Results The plasma level of IL-21 was significantly higher in subjects with rheumatoid arthritis relative to healthy controls. A positive correlation was observed between IL-21 level and DAS28 score, indicating the association of the cytokine with the worsening of the disease. The prevalence of AA genotype (rs2055979) was significantly higher in RA subjects compared to controls. Furthermore, elevated plasma IL-21 was observed in the rs2055979-AA genotype compared to CC type.

Conclusion IL-21 plays a key function in rheumatoid arthritis pathogenesis. IL-21 rs2055979 polymorphism is associated with IL-21 plasma levels and is predisposed to RA development in Chinese population.

Introduction

Autoimmune diseases are characterised by the unregulated activation of the immune system, which attacks and damages various tissues systems. Although various autoimmune disorders are reported worldwide, rheumatoid arthritis (RA) remained the most prevalent one [1]. RA is a systemic autoimmune disease distinguished by the formation of autoantibodies, inflammation, and enlargement of synovial tissues leading to the destruction of bones and cartilages [2]. The involvement of both genetic and environmental factors has been demonstrated with the development of RA [3], and the severity of the diseases depends on several risk factors. Although the etiology of the disease is not fully understood, it is presumed that multiple inflammatory molecules such as cytokines and chemokines play an essential role in disease progression and pathogenesis [4], [5]. Various proinflammatory cytokines such as TNF- α , IL-1 β , IL-6, and IL-17, have been shown to be inducers of the destruction of cartilages, adjacent bone erosions, and increase the severity of the RA pathogenesis [6]. Based on these observations, the regulation of proinflammatory molecules has been a crucial targeting approach for developing a possible therapeutic measure against RA. Mainly, inhibition of these inflammatory mediators using the monoclonal antibody approach is of interest that primarily aimed at hindering the synovial inflammation [7]. However, there are many side effects of these monoclonal antibody-based therapies. Additionally, due to prolonged use, these treatment options become ineffective. Therefore, there is always a quest for developing a newer

therapeutic approach for the treatment of RA and which can achieve by venturing the pathological role several other inflammatory molecules.

Interleukin-21 (IL-21) cytokine is a member of the IL-2 family mainly produced by CD4⁺ T cells and natural killer T cells (NKT) [8]. However, several reports have also highlighted the production of IL-21 by CD8⁺ T cell, B cells, macrophages, monocytes, and dendritic cells[9]. IL-21 plays a vital role in the regulation of both innate and adaptive immune systems [10]. Notably, IL-21 controls the differentiation of Th17 cells, B cell activation and production of immunoglobulins [11], [12], [13]. The role of IL-21 in the pathogenesis of RA is poorly understood. Elevated levels of IL-21 has been demonstrated in the synovial tissue of RA patients [14], [15]. Further, in the experimental arthritis model, the inhibition of IL-21/IL-21 receptor pathways significantly improved disease severity [16], suggesting an important role of IL-21 in disease pathogenesis. Besides, increased IL-21 has also been associated with higher chances of osteoclastogenesis in both humans and mice [15].

In humans, the gene encoding IL-21 is located at the long arm of the fourth chromosome (q26-27). IL-21 gene spans about 8.44kb of DNA and consists of six exons and five introns. Various single nucleotide polymorphisms (SNPs) have been reported (https://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=59067) and association of individual SNPs with autoimmune disorders such as systemic lupus erythematosus[17], graves disease [18]and inflammatory bowel disease [19, 20] have been demonstrated. Various reports have shown a significant association of IL-21 polymorphisms and RA in different populations such as Netherlanders [21], Algerian[22], Columbian [19]. Further, a recent meta-analysis with including nine various studies [23] demonstrated decreased susceptibility of IL-21 rs6822844 polymorphism against the development of RA. Although the association of IL-21 polymorphisms with RA has been studied in different populations, to date, it has not been explored in the Chinese community. The present study is the first of its kind to investigate the possible role of IL-21 polymorphisms in the Chinese cohort.

In the present study, we performed hospital-based case-control research to decipher the role of IL-21 in RA pathogenesis and clinical severity. Furthermore, four common SNPs were genotype and explored a possible association between IL-21 polymorphisms and predisposition to the development of RA in the Chinese population.

Materials And Methods

Study population

A total of 211 rheumatoid arthritis patients (156 females and 55 males) were recruited in the present study from January 2018 to December 2019. All patients visited or admitted in the Department of Rehabilitation, Shanghai Putuo People's Hospital rheumatology division of the hospital and fulfilled the 2010 criteria for American College of Rheumatology/European League against Rheumatism criteria for the classification of RA [24] were enrolled in the study. The mean age of patients was 42.9±13.5 years,

and the duration of diseases was 18.3 ± 9.4 months. The exclusion criteria included subjects with hypo or hyperthyroidism, diabetes, other autoimmune disorders, chronic liver failure, acute/chronic diarrhea, and congestive heart failure. Three hundred three healthy controls hailing from similar geographical areas, with a mean age of 46.1 ± 18.3 years, were included in the study. Various clinical data of RA patients, such as numbers of swollen and tender joints, disease activity score (DAS 28), and swollen joints count (SJC) were collected from medical records. Further, based on DAS28 scores, patients were sub-grouped into low (DAS 28, < 3.2), intermediate (DAS 28, $3.2 - 5.1$), and high (DAS 28, > 5.1), as per the classification criteria for disease activity by European League against Rheumatism (EULAR) [25]. Different biochemical parameters such as C-reactive protein (CRP), rheumatoid factor (RF), erythrocytic sedimentation rate (ESR), and antibodies to cyclic citrullinated peptides (anti-CCP antibodies) were also examined. Out of 211 RA patients, 186 patients were treated with DMARDs disease-modifying anti-rheumatic drugs and the other patients were administered with glucocorticoids (GCs). The study was carried out in compliance with the Declaration of Helsinki on ethical principles for medical research involving human subjects [26]. The study protocol was approved by the Institutional Human Ethical Committee of Shanghai Putuo People's Hospital (PTRMY20200826), and written informed consent was obtained from each participant.

Collection of plasma

At the time of enrollment, about 4 ml of intravenous blood was collected from each participant with anti-coagulant. Plasma was separated after centrifuging blood at 2500 rpm for 15 minutes and stored at -20°C until further use.

Isolation of genomic DNA

Total genomic DNA was isolated from 200ul of whole blood by using Merck blood genomic DNA miniprep kit according to the manufacturer's instructions. Genomic DNA was isolated from all patients and healthy controls and stored at -20 degrees until further use.

Genotyping of IL-21 polymorphisms

A total of four SNPs (rs907715, rs2221903, rs2055979 and rs6822844) were typed by TaqMan SNPs genotyping method. Predesigned SNP genotyping assays kit were procured from Thermo Fisher Scientific (rs907715: C__8949748_10, VIC/FAM-AAAACAGGATTTCTTGTTTTAACT[C/T]GCATTTATGTGATTACTAGGGAGAT; rs2221903: C__16167441_10, VIC/FAM-ACAGACAATGGGGTTTTGTTTTCTT[C/T]TGTTCTGCAAGCAGCAGAGCTGTGT; rs2055979: C__1597496_20, VIC/FAM-CTAACCATAACAGTTAAACAAGGTG[C/A]ATGAGATGCTAGAAATGTATGTTTT; and rs6822844: C__28983601_10, VIC/FAM-CCTGTCTCGCTCTCCATAGCAAAA[G/T]AGAGGACTCTTTTCATGTTGCCACT). Applied Biosystems Realtime PCR system (7900HT) was used for genotyping of IL-21 SNPs as per the manufacturer's instruction.

Enzyme-Linked Immunosorbent Assay

Plasma level IL-21 was measured in patients as well as controls using human IL-21 Duo Set ELISA kit (R&D Systems, Inc, USA) according to the manufacturer's instructions. All plasma samples were measured in duplicate, and the average absorbance value was recorded for a study subject.

Statistical analysis

The statistics analysis was performed by GraphPad Prism version 8.3.0 (GraphPad Software, Inc, La Jolla, CA, USA). The mean IL-21 levels difference in RA patients and healthy controls were carried out by student t-test. Other comparisons with more than two groups were performed with analysis of variance (ANOVA) followed by Tukey's post-test. Further, the relationship between the IL-21 and DAS 28 scores was conducted by Spearman's correlation test. Genotype and allele frequency in RA patients and healthy controls were compared by Fisher exact test. A p-value of less than 0.05 was considered statistically significant.

Results

Baseline characteristics of enrolled subjects

Baseline characteristics of rheumatoid arthritis patients and healthy controls are shown in Table-1. As demonstrated earlier, the RA is most frequent in females compared to males. In our studied cohort, female patients were 2.83 folds higher chance of having RA compared to males. Biochemicals parameters such as levels of ESR and CRP were significantly elevated in RA patients in comparison to healthy controls. Importantly, results for subgrouping of patients based on DAS 28 score revealed that 30.3% of subjects had low disease activity (DAS 28, < 3.2), whereas, 36.9% of subjects had medium (DAS 28, 3.2-5.1) and the remaining 32.8% patients showed a high disease activity (DAS 28, > 5.1). On screening of RA patients' rheumatoid factors, about 63% of patients were found positive for RF, and 62% of patients had antibodies to cyclic citrullinated peptides (CCP).

Table-1 Baseline characteristics of study subjects

Parameters	Rheumatoid arthritis patients	Healthy controls
Total numbers	211	303
Gender (F/M)	156/55	210/93
Age (% Mean)	42.9±13.5	46.1±18.3
Disease duration (Months)	18.3±9.4	NR
Swollen joint counts (0-28)	7.0	NR
Tender joint counts (0-28)	13.0	NR
DAS28 score (%)		NR
< 3.2	30.3	
Between 3.2-5.1	36.9	
> 5.1	32.8	
SJC out of 66	9.4±6.3	NR
ESR (mm at 1 st hour)	37.6±21.4	17.8±11.2
CRP (mg/ml)	18.9±22.4	1.19±13.2
RF positivity (%)	63	NR
Anti-CCP antibody positive (%)	62	NR
Drugs (DMARDs/GCs)	186/25	NR

Data are presented as either mean % or mean % ± SE. DAS – Disease Activity Score. SJC- Swollen Joint Count. ESR – Erythrocytic Sedimentation Rate. CRP – C reactive protein. RF – Rheumatoid Factor. CCP – cyclic citrullinated protein. DMARD – Disease Modifying Anti-rheumatic Drugs. GC – Glucocorticoids. NR – Not required.

RA patients displayed higher plasma IL-21 levels

Plasma levels of IL-21 in RA patients and healthy controls were quantified by ELISA, and results are shown in Figure-1. RA patients (19.6±0.79 ng/ml) displayed significantly higher levels of plasma IL-21 compared to healthy controls (2.12± 0.08 ng/ml) (p<0.0001).

Association of plasma IL-21 levels and DAS28 scores

As the DAS28 scores represent the disease severity of rheumatoid arthritis patients, we hypothesized a possible correlation between DAS28 scores and plasma levels of IL-21. Spearman rank coefficient analysis revealed a significant positive correlation between plasma IL-21 levels and DAS28 scores (spearman r=0.8319, P<0.0001) (Figure-2A).

RA patients were further categorized into three subgroups based on DAS28 scores. As shown in Figure-2B, RA patients with higher disease activity scores (DAS28>5.1) had higher mean plasma IL-21 levels compared to those with medium ($p<0.0001$) and low disease activity score ($p<0.0001$). Furthermore, a significant difference in mean levels of plasma IL-21 was observed among the lower and intermediate disease activity group ($p<0.0001$) (Figure-2B).

Distribution of IL-21 polymorphisms in the healthy Chinese population

A total of 303 healthy Chinese subjects were genotyped for four common SNPs (rs907715, rs2221903, rs2055979, and rs6822844) by TaqMan genotyping method. All subjects were having major genotype (GG) for rs6822844 polymorphism. As shown in Table-2, heterozygous mutants were more frequent in rs907715 and rs2055979 polymorphism followed by wild type and homozygous mutant. Further, for rs2221903 polymorphism, the wildtype remained highly prevalent compared to heterozygous (23%) and homozygous mutant (2%). Distribution of genotypes for three SNPs were in HWE (rs907715: $\chi^2=0.01$, $p=0.90$, rs2221903: $\chi^2=0.04$, $p=0.82$, rs2055979: $\chi^2=0.18$, $p=0.66$).

Table-2 Prevalence of IL21 polymorphisms among controls and RA patients

Polymorphisms	Genotype or Allele	HC (n=303)	RA (n=211)	P-value	OR (95% CI)
rs907715 C>T	Genotype				
	CC	91 (30)	68 (32)	1	ref
	CT	151 (50)	103 (49)	0.682	0.912 (0.613 to 1.366)
	TT	61 (20)	40 (19)	0.698	0.877 (0.525 to 1.444)
	CT+TT	212 (70)	143 (68)	0.628	0.902 (0.622 to 1.319)
	Allele				
	C	333 (55)	239 (57)	1	ref
	T	273 (45)	183 (43)	0.610	0.934 (0.724 to 1.202)
rs2221903 T>C	Genotype				
	TT	227 (75)	154 (73)	1	ref
	TC	70 (23)	49 (23)	0.915	1.032 (0.676 to 1.581)
	CC	6 (2)	8 (4)	0.270	1.965 (0.658 to 5.779)
	TC+CC	76 (25)	57 (27)	0.682	1.106 (0.738 to 1.635)
	Allele				
	T	524 (86)	357 (85)	1	ref
	C	82 (14)	65 (15)	0.415	1.163 (0.822 to 1.655)
rs2055979 C>A	Genotype				
	CC	118 (39)	53 (25)	1	ref
	CA	145 (48)	80 (38)	0.390	1.228 (0.811 to 1.888)
	AA	40 (13)	78 (37)	<0.0001	4.342 (2.623 to 7.219)
	CA+AA	185 (61)	158 (75)	0.001	1.901 (1.301 to 2.796)
	Allele				
	C	381 (63)	186 (44)	1	ref
	A	225 (37)	236 (56)	<0.0001	2.149 (1.662 to 2.766)

Note: Data are no. (%) of participants unless otherwise mentioned , HC: healthy controls, RA: rheumatoid arthritis patients.

Association of IL-21 rs2055979 polymorphism with susceptibility to RA

To test whether common genetic variants in the IL-21 gene are associated with predisposition to the development of rheumatoid arthritis, we genotyped rs907715, rs2221903 and rs2055979 polymorphism in 211 RA patients and 303 healthy controls. As shown in Table-2, the prevalence of homozygous mutant (AA) of rs2055979 polymorphism was significantly higher in RA patients compared to healthy controls ($p < 0.0001$, OR=4.342). The frequency of mutants (CA+AA) was also higher in RA comparison to controls ($p = 0.001$, OR=1.901). Furthermore, the mutant allele (A) was even more frequent in patients than healthy controls ($p < 0.0001$, OR=2.149), indicating an essential genetic susceptible factor on predisposition to RA development.

Functional relevance of IL-21 rs2055979 polymorphism

Plasma levels of IL-21 in RA patients and healthy controls were analyzed among different genotypes of IL-21 polymorphisms (rs907715, rs2221903, and rs2055979) to investigate the possible association plasma IL-21 levels. As shown in Figure-3A, AA genotype of rs2055979 polymorphisms had higher plasma levels of IL-21 compared to other genotypes, i.e., CA demonstrated intermediate levels and CC had the lowest levels of plasma IL-21. Interestingly, similar observations were noticed when the association of IL-21 rs2055979 polymorphism was analyzed in RA patients (Figure-3B) and healthy controls (Figure-3C). For other studied SNPs (rs907715 and rs2221903), no significant association between genotypes and plasma levels of IL-21 was observed (data not shown).

Association of IL-21 rs2055979 polymorphism with DAS28 scores

As DAS 28 and plasma levels of IL-21 were correlated; further, we analyzed the possible association of IL-21 polymorphisms with DAS21 scores. As shown in Figure-4C, we observed a significant association between IL-21 rs2055979 polymorphism with DAS28 scores: subjects with AA genotyped had higher DAS28 scores compared to CA and CC genotypes. However, such association was not observed in rs907715 and rs2221903 polymorphisms (Figure-4A and 4B).

Discussion

The role of different cytokines in mediating pathogenesis rheumatic diseases have been well documented. Prior reports suggested that some cytokines secreted by Th1, Th2 and Th17 cells have been designated as potent biomarkers in the pathogenesis of RA [27]. Studies in Chinese RA patients are limited. A report during 2011-2012 indicated significance of chemokines, pro and anti-inflammatory cytokines RA [28]. In the Chinese population, however, the role of IL-21 in RA pathogenesis has never been critically studied.

In the present investigation, we observed significantly elevated level of plasma IL-21 in Chinese patients with RA as compared to healthy controls. These results are corroborated with previous reports. An earlier hospital based case control study in Chinese patients demonstrated higher serum IL-21 levels in comparison to healthy controls [29]. Similarly, in a longitudinal study in patients with early stage RA, IL-21 level was upregulated in diseased subjects as compared to controls[30]. All of these findings,

including our results, indicated the possible function of IL-21 in the advancement of RA pathogenesis. Nevertheless, controversial results do still occur. There was no substantial difference in serum IL-21 level between subjects with recent RA onset and healthy controls in a study by Sglundaet al. [31]. Furthermore, in rheumatoid arthritis patients with higher disease activity (DAS28 > 5.1) and healthy control levels, IL-21 levels were also comparable. [31]. Although the exact reason for such discrepancy in data is not known, the use of fewer patients (n=51) in the given study may be a contributing factor.

An independent study [31], have highlighted comparable IL-21 levels between high disease activity (DAS28, >5.1) RA patients and healthy subjects. On the contrary, we observed a significantly higher level of IL-21 in the patient group with DAS 28 >5.1 when compared to the other two groups (DAS28 < 3.2 and DAS28 3.2-5.1) as well as healthy controls. In line with these findings, higher plasma levels of IL-6 and IFN- α were recorded in rheumatoid patients with higher disease activity compared with those with lower DAS28 scores [32].

In our current research, a steady rise in plasma IL-21 in the higher disease activity of the patients was observed. This finding led us to investigate further the possible link between the plasma IL-21 levels and DAS 28 scores. Positive association between IL-21 and DAS28 was observed, corroborating with earlier observations [31, 33]. However, another study found no connection between IL-21 and DAS 28 in 126 Chinese RA penitents [29].

The association of IL-21 polymorphisms with a predisposition to the development of RA has been extensively investigated in different populations. In most of the research, the role of rs6822844 polymorphism was investigated in order to find a potential link with the susceptibility to the development of RA. Reports including RA patients from different geographical regions showed the protective role of rs6822844 variant against RA development in the Netherlands [21], Algerian [22], Columbian [19] population. The latest meta-analysis further strengthens individual case-control observation [23]. However, both patients and controls were wild types for rs6822844 polymorphism, similar to an earlier study in the Chinese population [17]. Collectively these observations indicate the absence of rs6822844 variants in the Chinese population.

In this study, we observed a significant role in rs2055979 polymorphism with RA predisposition. Subjects carrying the genotype of AA had a 4.34-fold higher susceptibility to RA. However, the distribution of other common polymorphisms among healthy controls and RA patients was comparable. Earlier research in Chinese systemic lupus erythematosus patients also recorded similar observations: rs2055979 was correlated with susceptibility, whereas rs907715 and rs2221903 polymorphisms did not play a significant role. Similarly, in an earlier study rs907715, polymorphism also failed to display an association with RA susceptibility in Australia's population [34]. In addition, an important functional significance of rs2055979 polymorphism was noted in this report: subjects with AA genotype had higher plasma IL-21 than those with CC genotype. Interestingly, heterozygotes demonstrated intermediate levels of IL-21. Similar association trends have been observed in both healthy control and RA patients. In line with our findings, an earlier study showed a substantial difference in AA and CC genotype plasma IL-21 levels. However,

differences between heterozygous and wild or homozygous mutants could not be detected, likely due to the limited sample size. The mechanism of how the AA genotype is correlated with higher IL-21 levels is not understood. The SNP rs2055979 is located in the intronic region and may have an impact on the splicing process. [35].

In conclusion, IL-21 plasma levels are increased in patients with rheumatoid arthritis, associated with disease severity. Furthermore, IL-21 (rs2055979) mutant is associated with elevated IL-21 plasma levels and predisposed to RA development. However, further studies are required in different populations to validate our findings.

Declaration

Ethics approval and consent to participate: The study protocol was approved by the Institutional Human Ethical Committee of Shanghai Putuo People's Hospital (PTRMY20200826), and written informed consent was obtained from each participant.

Consent for publication: All authors have gone through the final version of the manuscript and approve for the publication.

Availability of data and materials: Data will be available upon request to the corresponding author.

Competing Interest: Authors declare no conflict in interest.

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Contribution statements: **YZ:** Investigation, laboratory experiments, formal analysis; **LX:** Investigation, laboratory experiments; **JX:** Investigation, formal analysis; **ZL:** Investigation, formal analysis; **BY:** Investigation, formal analysis; **MZ:** conceptualization, supervision, writing original draft, review and editing.

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Figures

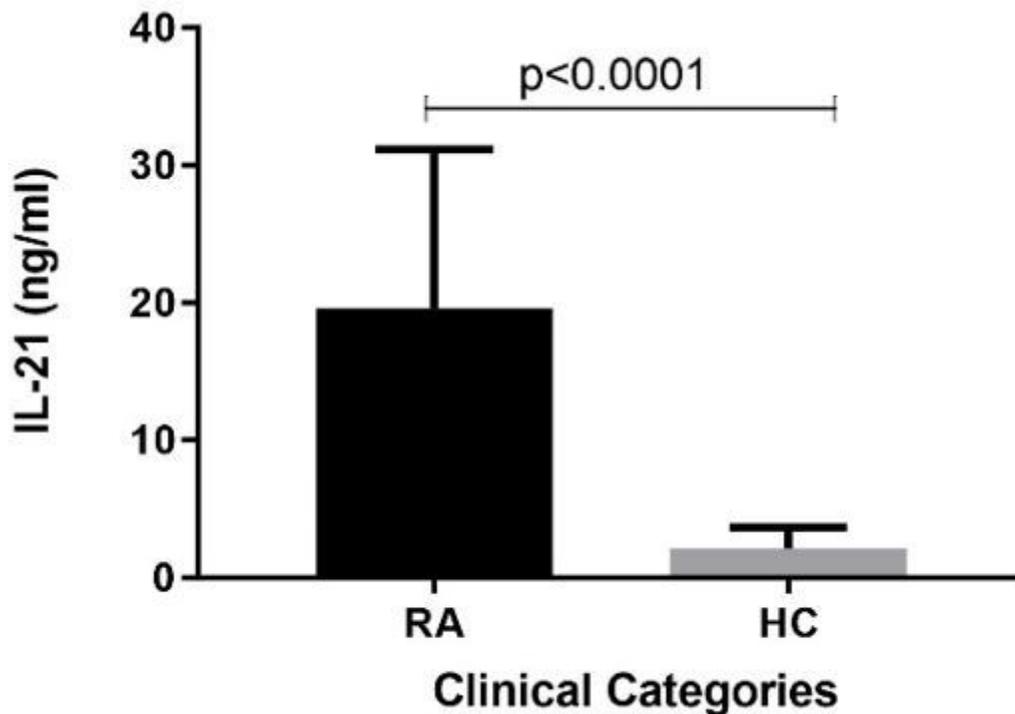


Figure 1

Plasma IL-21 levels in subjects with RA (n=211) and healthy controls (n=303). Data represented the mean IL-21 ng/ml \pm SD and were compared by student's t-test. $P < 0.05$ was deemed to be significantly positive.

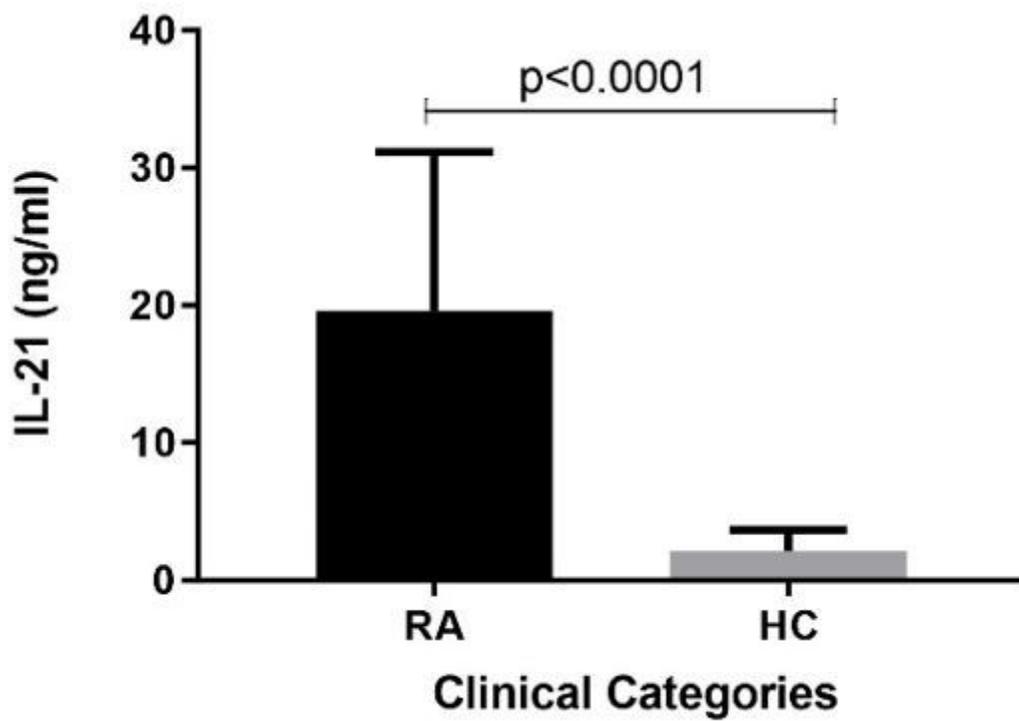


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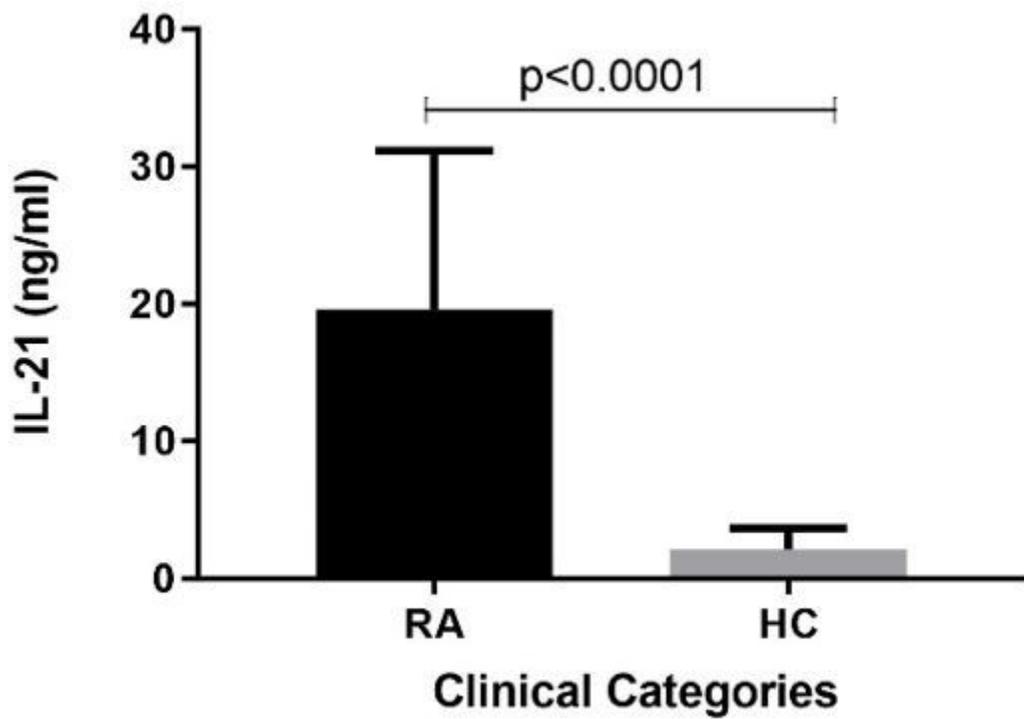


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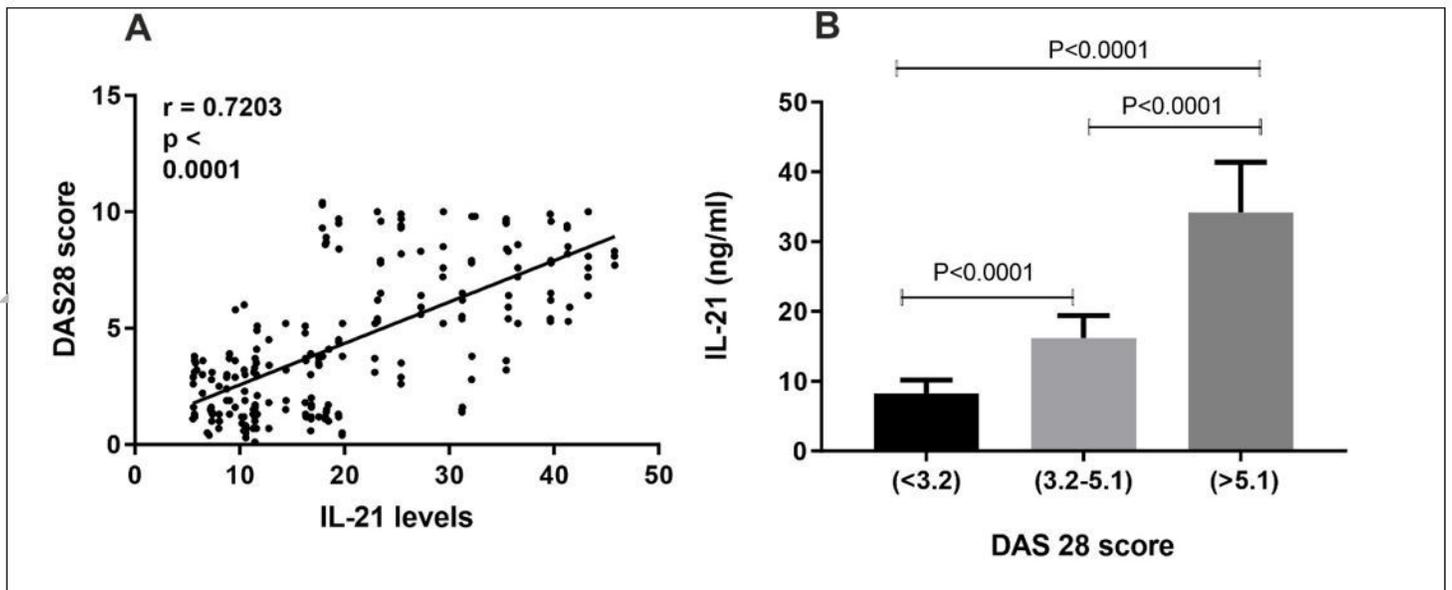


Figure 2

Correlation between plasma IL-21 level with DAS score. IL-21 levels were measured by ELISA in all RA patients and correlated with the DAS score of RA patients. A positive correlation was observed among IL-21 levels and DAS scores (A). Further, RA subjects were subcategorized in to three groups based on DAS scores such as low [DAS 28, <3.2 (n=64)], medium [DAS 28, 3.2-5.1 (n=79)] and high [DAS 28, >5.1 (n=68)]. Data represented the mean of IL-21 levels (ng/ml)± SD and were compared using ANOVA with Tukey's post-test. P value less than 0.05 was taken as statistically significant.

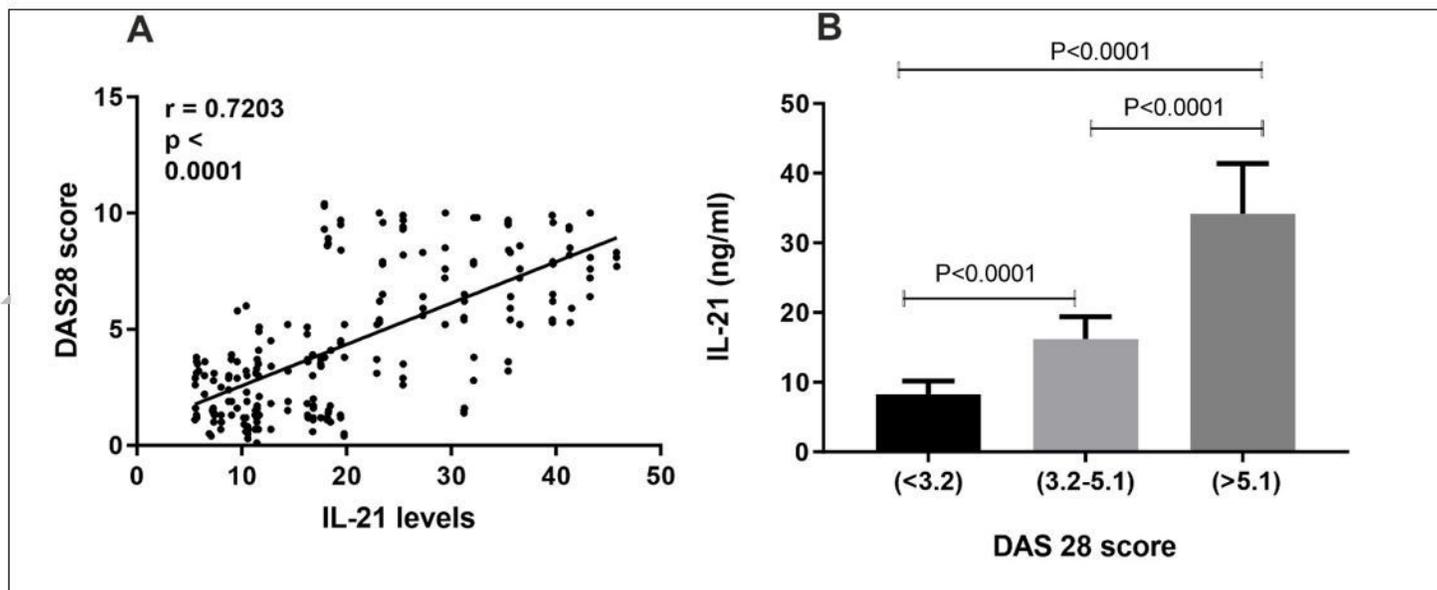


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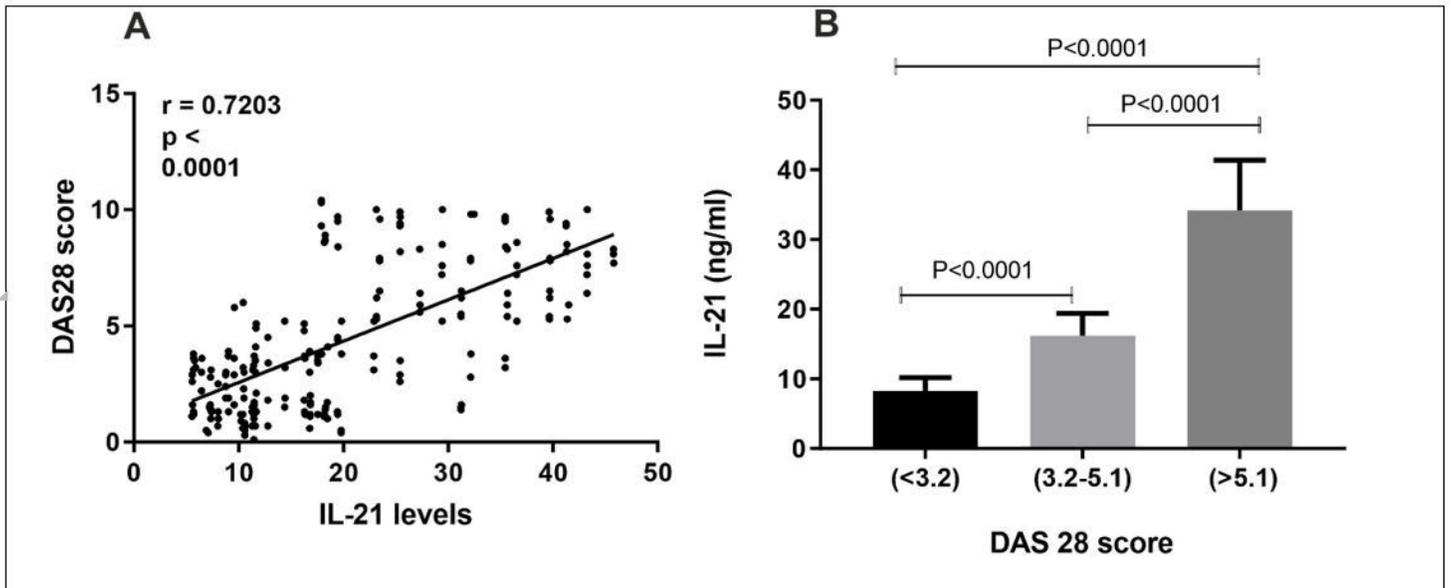


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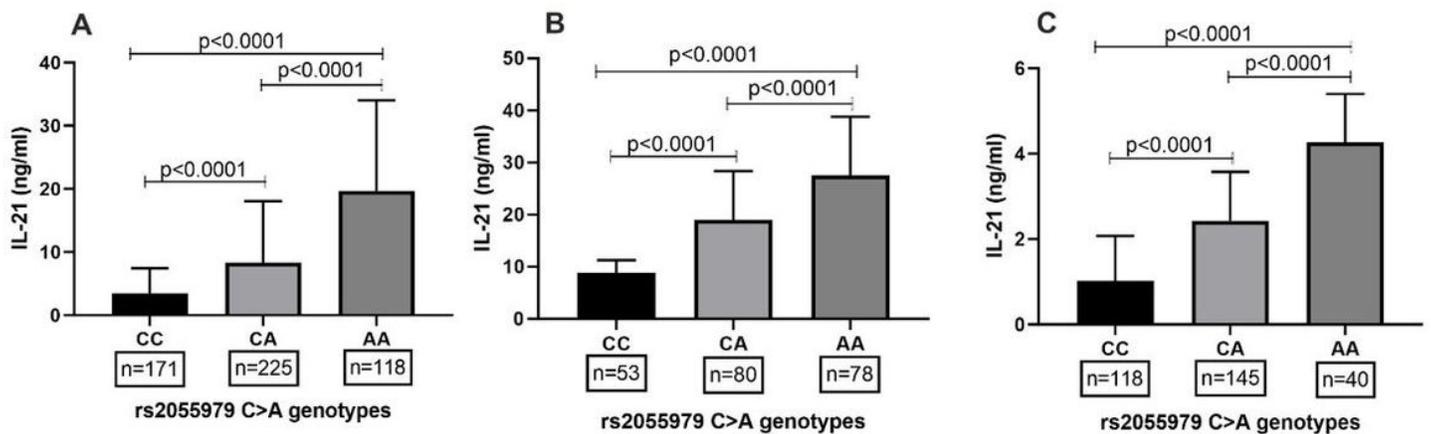


Figure 3

Association of IL-21 rs2055979 polymorphism with plasma IL-21 levels. Plasma levels of IL-21 was quantified by ELISA in both RA patients (211) and healthy controls (n=303). Distribution of Plasma IL-21 in the different genotypes of rs2055979 polymorphism revealed higher levels in AA genotype compared to other genotypes (A). A similar observation was noticed in RA patients (B) and healthy controls (C). Data are represented in mean \pm SD, and plasma levels of IL-21 in different genotypes were compared with ANOVA accompanied by Tukey's post-test. P < 0.05 was deemed to be statistically significant

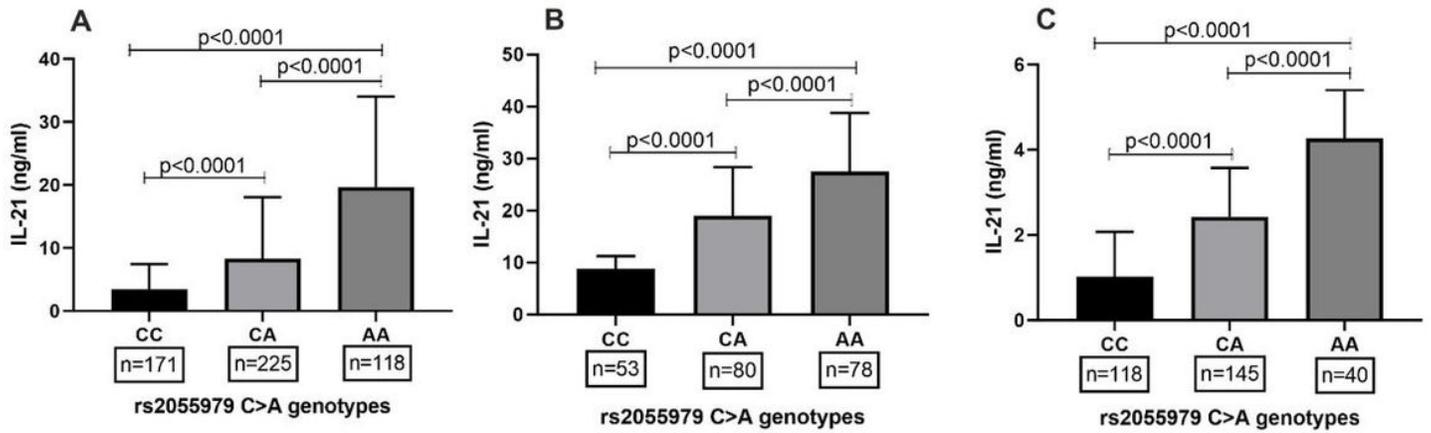


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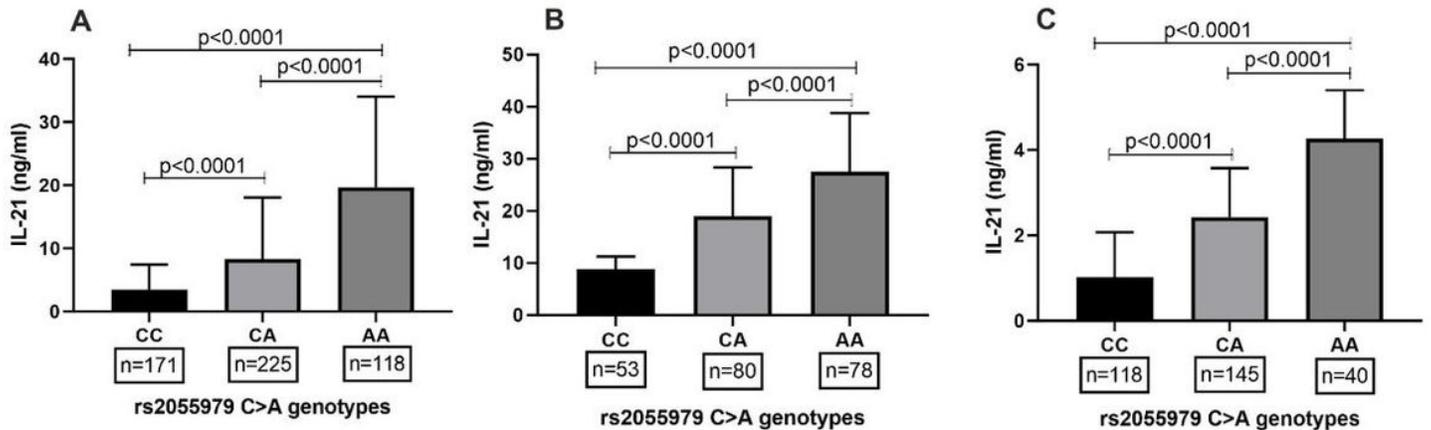


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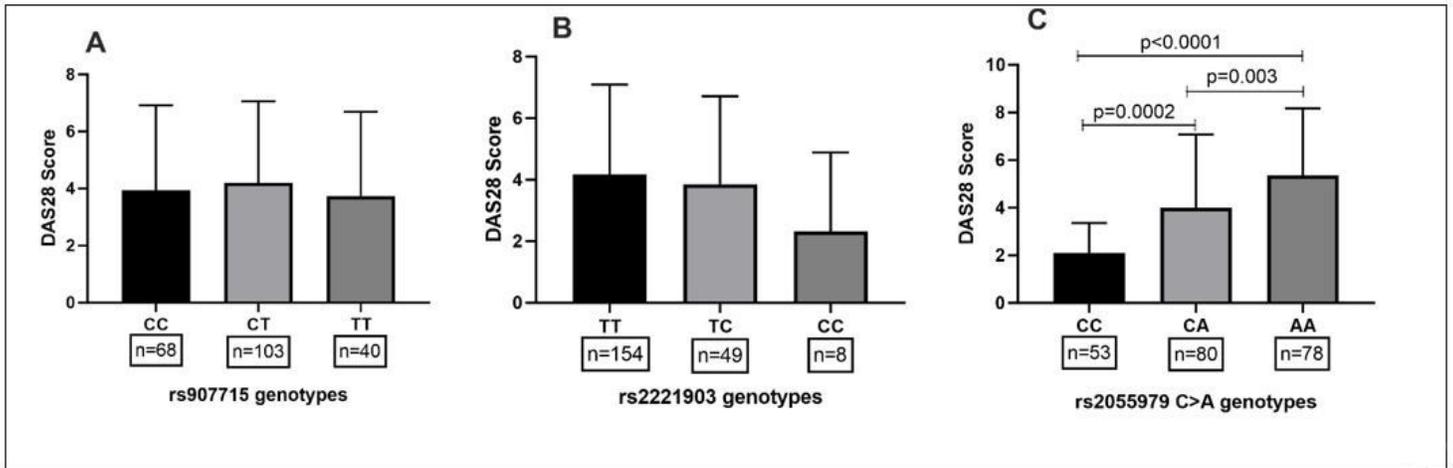


Figure 4

Association of IL-21 gene polymorphisms (rs907715, rs2221903, and rs2055979) with DAS28 scores. The DAS28 score was comparable in different genotypes of rs907715 (A) and rs2221903 (B) polymorphism. A significant association was noticed in rs2055979 polymorphism of the IL-21 gene with DAS-28 scores. AA genotype was associated with higher DAS28 scores compared to other genotypes (C). Data are represented in the mean \pm SD, and plasma levels of IL-21 in different genotypes were compared with ANOVA accompanied by Tukey's post-test. $P < 0.05$ was deemed to be statistically significant.

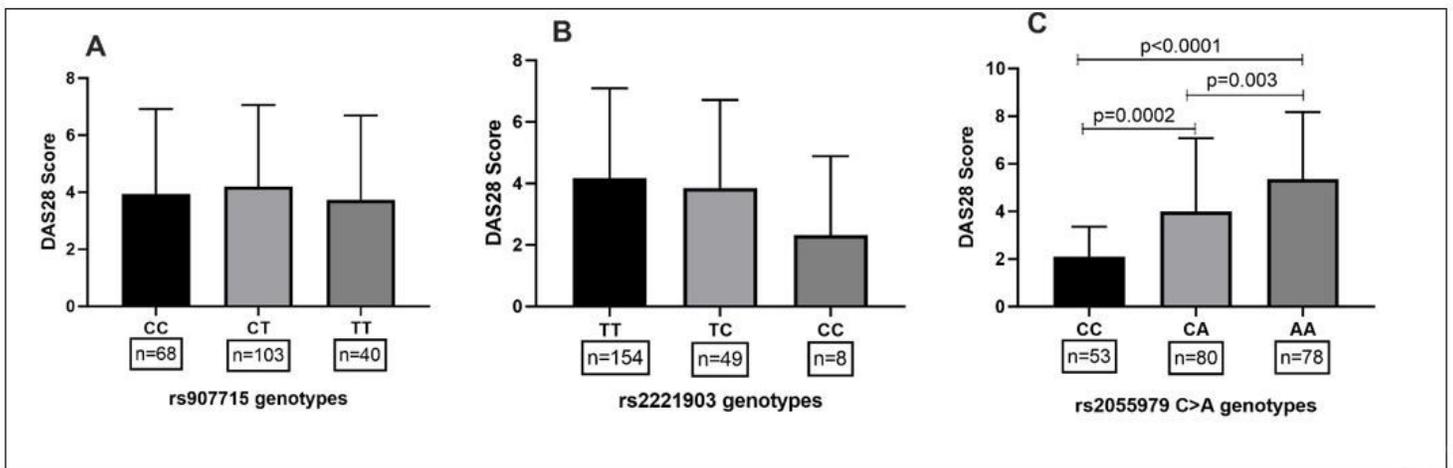


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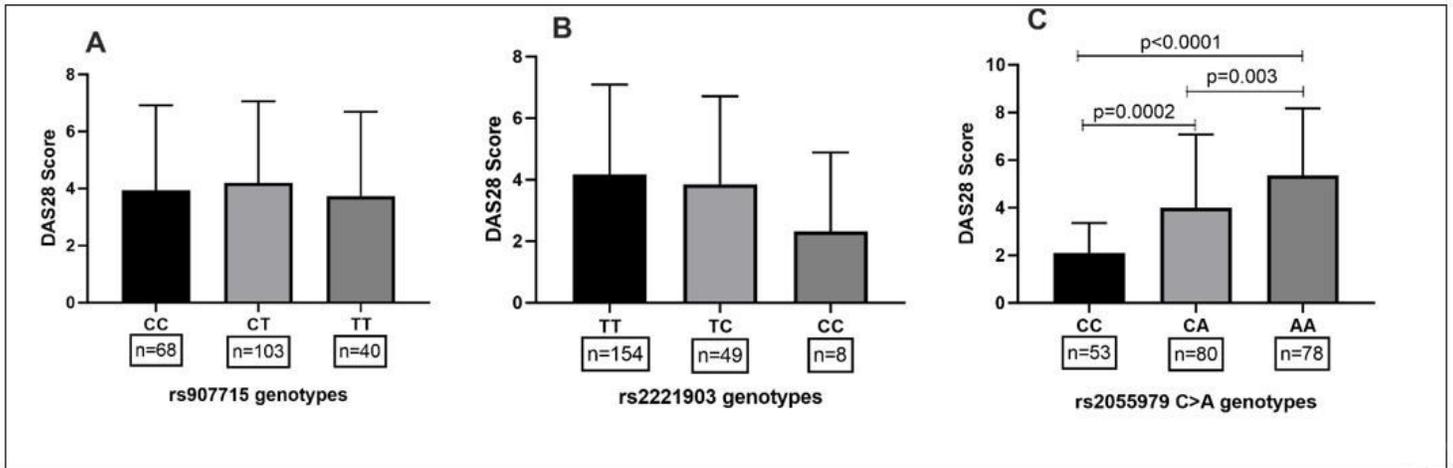


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