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Interleukin-38 Level and Polymorphism (Rs 6743376) Associated with Rheumatoid Arthritis

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Abstract

Rheumatoid arthritis (RA) is a multifaceted autoimmune disorder defined by persistent inflammation and joint damage. This study explores the intricate relationship between RA severity and interleukin-38 (IL-38), a cytokine having anti-inflammatory properties. Our research reveals a strong connection between elevated plasma IL-38 levels and the severity of RA, indicating IL-38's crucial role in modulating inflammation. Elevated ESR levels in RA patients highlight active inflammation. Genetic analysis shows specific IL-38 polymorphisms associated with RA susceptibility, emphasizing the disease's genetic basis. These findings position IL-38 as a promising indicator for assessing rheumatoid arthritis RA severity by providing valuable insights into its genetic mechanisms. Understanding IL-38-related pathways could revolutionize RA management strategies.

Introduction

Rheumatoid Arthritis (RA) is a wide spread inflammatory autoimmune disease (Ahmed & Abu-Raghif, 2020). In severe cases, it can lead to damage in various parts of the body, including the skin (Chua-Aguilera, Möller, & Yawalkar, 2017), lungs, eyes, blood vessels, and heart. The

inflammation in RA occurs due to the immune system attacks the body's own tissues (Yan, Su, & Li, 2020). The condition primarily affects the lining of joints, particularly in the knees, wrists, and hands, causing long-term chronic pain, joint instability, and severe complications such as joint deformity and bone erosion (Ebel & O'Dell, 2021; Ungprasert, Ryu, & Matteson, 2019).

The global prevalence of Rheumatoid Arthritis (RA) was approximately 1% in 2020, with higher occurrence observed in individuals aged between 35 and 50 years (Mardanshahi et al., 2020). RA affects diverse populations, with a higher incidence among Native Americans and a lower prevalence among groups like Caribbean individuals. The incidence rate of RA in emerging nations remains unknown (Alamanos, Voulgari, & Drosos, 2006; Widdifield et al., 2019). In Italy, the overall prevalence was 0.33%, with 0.13% in males (Simonsson, Bergman, Jacobsson, Petersson, & Svensson, 1999) and 0.51% in females (Benucci et al., 2016; Simonsson et al., 1999). In Korea, the incidence rate stood at 1.5%, while in France, it was 0.31% (Kim et al., 2020). America had a 0.42% prevalence of RA-affected individuals, and in Barika, it was 0.13% (Park, Le, Slejko, Villalonga-Olives, & Onukwugha, 2019). In 2019,(Naqvi, Hassali, & Aftab, 2019) estimated a spread of 0.142% for RA (Alam et al., 2011)in the Pakistani population (Alam et al., 2011).

Rheumatoid Arthritis (RA) significantly impairs quality of life due to chronic pain and joint deformities (Martinec, Pinjatela, & Balen, 2019). It creates a substantial economic burden through high healthcare costs and work disability (Bevan, 2015). RA raises the risk of comorbid conditions like cardiovascular diseases and osteoporosis, impacting overall health (Ferrucci & Fabbri, 2018). The psychological toll includes depression and anxiety due to persistent pain (Fiest et al., 2017). Public health planning and ongoing research are crucial for effective management, emphasizing the need for a balanced approach in minimizing symptoms and medication side effects (Boselie, 2014).

Interleukin-38 (IL-38), part of the interleukin-1 family (Sims & Smith, 2010) and interleukin-36 subfamily (van de Veerdonk et al., 2012), plays a crucial role in host defense and inflammation (Namba et al., 2021; Tsang, Sun, & Wong, 2020). It shares similarities with IL-1Ra and IL-36 Ra (Conti et al., 2021), and expression is affected in autoimmune conditions like Rheumatoid Arthritis (RA). Produced in various tissues, IL-38 binds to IL-1 receptors, contributing to

dysregulated inflammatory responses and immune activation in diseases such as RA (Xu & Huang, 2018).

Analyzing IL-38 and rs6743376 in the context Rheumatoid Arthritis (RA) is crucial for several reasons (Teufel et al., 2022). Firstly, understanding IL-38's role offers potential therapeutic avenues by regulating inflammatory responses in RA. Secondly, exploring rs6743376, a genetic variant linked to RA susceptibility, aids in identifying high-risk individuals, enabling early intervention and personalized treatment strategies. This research contributes to precision medicine, enhancing treatment efficacy, and minimizes adverse effects. Moreover, it advances scientific understanding, shedding light on the complex interplay of genetics and immune response in autoimmune diseases like RA.

Materials and Methods

Participants

Patients showing rheumatoid arthritis symptoms at Bajwa and Latif Hospital, Lahore, were selected for the study, obtaining informed consent. Demographic data, symptoms, family history, and medical treatment history were recorded through structured questionnaires. Healthy individuals without RA or autoimmune disease history were also interviewed using the same form. A total sample size of 80 participants was determined, with 40 individuals allocated to each group: group I comprising rheumatoid arthritis patients and group II consisting of healthy individuals. The sample size calculation was based on specified relative precision, population fraction, and odds ratio (OR), as per the provided formula:

$$\mathrm{n} = \frac{Z^2_{1-\alpha/2}}{[\log_e(1_{-\xi})]^2}\left[\frac{1}{\dot{\mathrm{p}}_1(1-\dot{\mathrm{p}}_1)} + \frac{1}{\dot{\mathrm{p}}_2(1-\dot{\mathrm{p}}_2)}\right]$$

Measurement of Serum IL-38 level

Tube-1, holding EDTA blood, was frozen at -20°C for subsequent DNA extraction following the method outlined by Iranpur et al., 2002. As for Plain Tube-2, it underwent centrifugation at 2000-3000 RPM for 10 minutes, after which the serum taken into two portions and stored at -80°C (Xu, Su, He, & Huang, 2018). These stored samples later utilized for IL-38 determination via ELISA by using ELISA Kits following the provided guidelines. Every sample was tested in duplicate, ensuring accuracy. Both between and within the assay, the coefficient of variation

values remained below 15%, indicating consistent results. Optical density readings were taken at 450nm by using ELISA reader's computer software (Microplate Manager Version 2.2) for precise measurements. The lower detection limit for the assay was 0.23ng/L according to manufacturer.

Clinical analyses

Patients' erythrocyte sedimentation rates (ESRs) were analyzed, and serum levels of C-reactive protein (CRP), rheumatoid factor (RF), and anti-cyclic citrullinated peptide (anti-CCP) were assessed using scatter turbidimetry (Xu et al., 2018). Serum RF levels exceeding 40 IU/mL classified as RF positive, while serum level of CRP surpassing 8 mg/L considered positive (Xu et al., 2018).

Table 1 Characteristics of Participants in the Experimental and Control Groups. Experimental group (N=40) includes patients with rheumatoid arthritis, and the control group (N=40) comprises healthy individuals. Age, gender distribution, disease duration, presence of other chronic illnesses, positive rheumatoid factor (RF) and anti-cyclic citrullinated peptide (ACCP), erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP) levels are compared between the groups. P-values indicate the significance of differences observed

Characteristics	Experimental group N= 40	Control group N= 40	P-value
Age	45.55±11.30	25.62±6.20 years	0.1
Gender (male/female)	7/33	24/16	0.2
Disease Duration (≤5/ ≥6)	26/14	-	-
Other chronic illnesses	nil	-	-
RF	101 (77.5)	-	-
ESR	64.37±55.05	-	-
ACCP	84.36 (65.4)	-	-

CRP	8.6 (3-15)	-	-

DNA Extraction and PCR

In this DNA extraction procedure, leukocytes were isolated from 500 µl of whole blood using red cell lysis buffer (RCLB). After centrifugation and multiple washes with RCLB, nucleic cell lysis buffer (NCLB) was added, followed by chloroform and sodium chloride. Centrifugation led to the formation of two layers, from which the upper layer containing DNA was collected and mixed with ethanol. DNA strands appeared, followed by centrifugation and removal of the supernatant. The DNA pellet underwent air drying and subsequent rehydration, with water devoid of DNase and RNase enzymes. The solution was preserved at -20°C for potential future applications. The amount of DNA was assessed by measuring the optical density at 260 and 280 nm wavelengths using NanoDrop. A ratio (OD260/OD280) between 1.8 to 2.0 indicated pure DNA, while a lower ratio indicated contamination with proteins or phenols.

Table 2 The sequence of primer for the amplification of alleles

Primers	Sequence	GC (%)	Product Size
Forward Primer for C	$^{5'}$ATACTTCCTAACAGAGGCTTGAA$^{3'}$	39	N=235bp
Forward Primer for A	$^{5'}$ATACTTCCTAACAGAGGCTTGAC$^{3'}$	43	
Reverse Primer	$^{5'}$GAGGAAGTAGGACATGTGGA$^{3'}$	50	

Primers for interleukin-38 polymorphisms were reconstituted and diluted to create working solutions. Allele-specific PCR was performed using DNA, specific primers, and Taq DNA polymerase under specific cycling conditions. PCR products were loaded onto agarose gel with a DNA ladder and electrophoresed. The gel was visualized under UV light for result analysis. The procedure aimed to identify interleukin-38 polymorphisms (C/A, rs674336).

Statistical analysis

Data categorization was based on normality, mean ± SD was used to present normal data, whereas median (interquartile range) was employed for non-normal data. Statistical analyses were conducted employing SPSS. To assess the association of IL-38 SNPs with disease duration, a 2x2 table was constructed. Independent t-test employed to establish a comparison IL-38 levels between experimental group and control group. Odds Ratio use to ascertain the disease risk connected with IL-38 SNPs. The threshold for statistical significance was defined as $p \leq 0.05$, ensuring rigorous analysis.

Results

Il-38 Concentration Comparison between Rheumatoid Arthritis Patients and Controls

Results displayed the mean value of IL-38 concentration in experimental group and controls. Patients exhibited a mean IL-38 value of 26.86±21.70 (with a range of 12.47 to 133.60), while controls had a mean IL-38 value of 19.27±12.92 (ranging from 6.50 to 64.78). While IL-38 levels exhibited an increase in patients in comparison to controls, this difference did not achieve statistical significance (p-value > 0.005).

Table 3 Concentration of IL-38 and comparison (Mean ± SD) between Experimental group and Controls: Assessing Significance and their Odds Ratio

	(IL-38) Concentration Mean ± SD	**P-value**	**Odds Ratio**
Patients n (40)	681.86±21.70	0.061	1.022
Controls n (40)	300.27±12.92		

Initially, the average IL-38 plasma levels in RA patients, were notably exceeded those found in, healthy controls, with an average of 681.00 pg/mL (ranging from 349.7 to 1100 pg/mL) in contrast to 152.04 pg/mL (ranging from 80.7 to 300 pg/mL) for the control group (P<0.001).

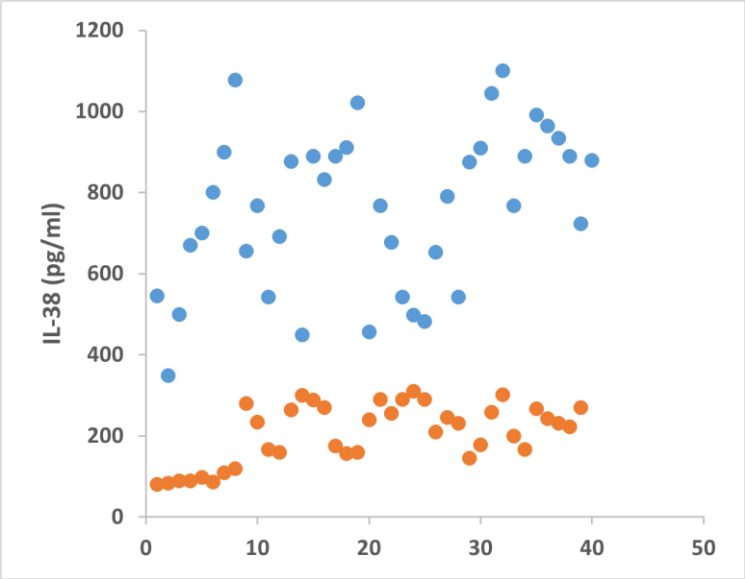

Figure 1 Quantifying Plasma IL-38 Levels: A Comparative Analysis between 40 Rheumatoid Arthritis Patients and 40 Healthy Controls Using the Enzyme-Linked Immunosorbent Assay (ELISA) Method

Among RA patients, those with positive Rheumatoid Factor (RF) exhibited significantly elevated IL-38 expression. The IL-38 levels for positive RF patients averaged at 681.19 pg/mL (range: 345.57–882.00 pg/mL), whereas patients with negative RF had an average IL-38 level of 600.00 pg/mL (range: 71.65–715.06 pg/mL), demonstrating a statistically significant difference ($P = 0.022$). The average Erythrocyte Sedimentation Rate (ESR) among the patients was 64.37 mm/h (±55), while in the control group, it was 8.45 mm/h (±2.85). The disparity in ESR levels between the cases and the control group was highly significant, with a p-value of less than 0.001, indicating a substantial difference in the inflammatory response between the two groups.

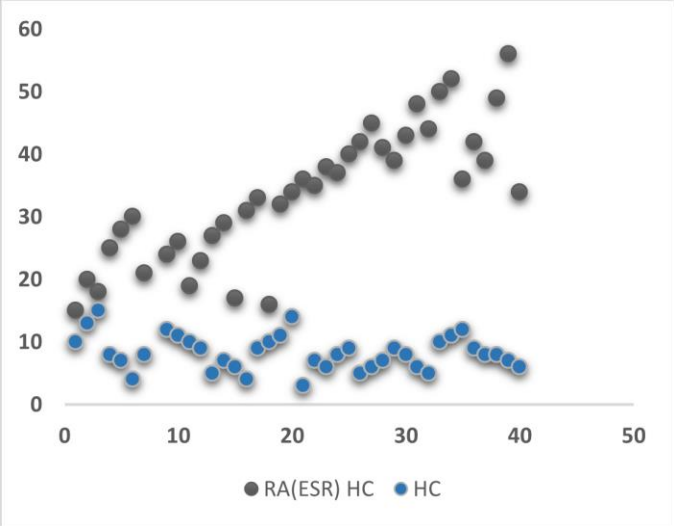

Figure 2 Correlation of IL 38 concentration with ESR

These results highlight the association between IL-38 levels, RF status, and RA, emphasizing the potential significance of IL-38 in understanding the disease and its related factors. The observed elevation in IL-38 levels among RA patients might be correlated with the increased ESR commonly seen in active inflammatory conditions. ESR, a marker of inflammation, tends to rise in response to the presence of inflammatory proteins, including cytokines like IL-38. The elevated relationship between IL-38 and ESR could indicate a direct relation between IL-38 manifestation and the intensity of disease systemic inflammation in RA patients.

Table 4 IL-38 Polymorphisms and Serum Concentrations

		group	**N**	**Mean ± SD**	**p-value**
AA allele	(IL-38 Concentration)	Patients	17	31.16 ± 17.70	0.001
		Controls	3	14.38 ± 1.04	
A/C allele	(IL-38 Concentration)	patients	9	32.51 ± 38.05	0.32
		Controls	16	18.89 ± 14.38	
CC allele	(IL-38 Concentration)	patients	14	18.02 ± 2.32	0.52
		Controls	21	20.27 ± 12.87	

In individuals with the homozygous allele (AA), significant difference seen in mean IL-38 values, compared between patients and controls (p=0.001). Conversely, no significant distinction in IL-38 means was noted between patients and controls with the heterozygous allele (A/C) and homozygous allele (CC) (p>0.005) in accordance with scientific analysis. In the study, 42.5% of patients exhibited the homozygous allele (AA) compared to 7.5% in the control group. A significant association was found between IL-38 SNP (Allele AA) and rheumatoid arthritis (p<0.0001), with an odds ratio (OR) of 9.11. Heterozygous allele (AC) present in both groups. It was 22.5% in experimental group (RA patients) and 40% in healthy persons (controls). This is showing a lack of significant distinction among both groups. Therefore, there was no prominent and notable connection between IL-38 SNP heterozygous allele (AC) and rheumatoid arthritis (p=0.09), (OR= 0.43). Additionally, 35% of patients had the homozygous allele (CC) while 52.5% of controls exhibited the same allele, indicating a non-significant difference. No notable connection was detected regarding IL-38 SNP homozygous allele (CC), rheumatoid arthritis (p=0.012), (OR= 0.48).

Table 5 Association of IL-38 SNPs with rheumatoid arthritis.

	Patients n(40)	**Controls n(40)**	**Chi-Square**	**p-value**	**Odds Ratio (95% CI)**
AA allele n (%)	17(42.5%)	3(7.5%)	13.067	< 0.001	9.11 (2.40-34.58)
A/C allele n (%)	9(22.5%)	16(40%)	2.85	0.091	0.43 (0.16-1.55)
CC allele n (%)	14(35%)	21(52.5%)	2.48	0.12	0.48 (0.19-1.17)

The table displays genetic allele frequencies for the IL-38 in patients (experimental group) (n=40) and among healthy persons (controls) (n=40) with rheumatoid arthritis. In patients, the homozygous AA allele was significantly higher (42.5%) compared to controls (7.5%), indicating a strong association (Chi-Square=13.067, p<0.001, OR=9.11, 95% CI: 2.40-34.58). The heterozygous A/C allele showed no significant difference (22.5% in patients, 40% in controls, Chi-Square=2.85, p=0.091, OR=0.43, 95% CI: 0.16-1.55). Similarly, the homozygous CC allele

did not notably vary between patients (35%) and controls (52.5%) (Chi-Square=2.48, p=0.12, OR=0.48, 95% CI: 0.19-1.17).

Discussions

The notably higher IL-38 levels in patients suffering from RA (Boutet et al., 2016) in contrast to healthy controls suggest a possible function of IL-38 in the development of rheumatoid arthritis. Elevated IL-38 levels at baseline could indicate ongoing inflammation and immune dysregulation in RA patients. The significant difference underscores the importance of IL-38 as a potential indicator for diagnosing and monitoring RA inflammatory disesae. No significant difference in the case of healthy controlled seen (Takenaka et al., 2015). The observed increase in IL-38 expression among RA patients with positive RF signifies a potential link between RF status and IL-38 levels. Positive RF often indicates a more aggressive form of RA. The higher IL-38 levels in these patients suggest a potential involvement of IL-38 in the immune responses associated with RF-positive RA (Xia et al., 2015). This association could be explored further to understand the specific mechanisms underlying this relationship. The findings underscore the clinical relevance of IL-38 in RA. Monitoring IL-38 levels might aid in stratifying patients based on disease severity or predicting the course of the disease (Xiao et al., 2016).

The significantly higher ESR levels in patients compared to controls (64.37±55 vs. 8.45±2.85 mm/h, $p<0.001$) indicate the presence of inflammation and disease activity in rheumatoid arthritis (RA) patients (De Man, Dolhain, Van De Geijn, Willemsen, & Hazes, 2008). ESR, a marker of inflammation, tends to rise in response to the presence of inflammatory proteins, including cytokines like IL-38 (Wang et al., 2016). Elevated ESR is a classic marker of inflammation and disease severity in RA. The substantial difference observed underscores the importance of ESR is pivotal for assessing and tracking the progress of individuals with rheumatoid arthritis (RA) (Del Rincón et al., 2003). The study revealed markedly elevated plasma IL-38 concentrations in RA patients (experimental group) compared to healthy persons (controls) (671 [234.45–926] vs. 142.04 [66.06–216] pg/mL, $p<0.001$) (Hiz et al., 2020; Xu et al., 2018). This increased IL-38 level corroborates with existing literature, highlighting the role of IL-38 in autoimmune diseases, particularly RA (Del Rincón et al., 2003). The high IL-38 levels in RA patients underscore its potential as a biomarker for disease activity and severity. The strong connection between IL-38 and ESR could indicate a direct link between IL-38 expression

and the intensity of the systemic inflammation in RA patients. Combining IL-38 levels with ESR measurements could offer a more comprehensive assessment of the inflammatory status in RA patients. IL-38, being a specific cytokine, might provide insights into the local inflammatory processes, while ESR reflects the overall systemic inflammation. Integrating these markers might enhance the accuracy of disease monitoring, allowing clinicians to better gauge the disease activity and tailor treatment strategies accordingly. While the correlation between IL-38 and ESR is evident (Hensor, Emery, Bingham, Conaghan, & Consortium*, 2010), further studies are essential to dissect the intricate interplay between these markers. Investigating the mechanisms driving their correlation, such as shared inflammatory pathways, could shed light on potential therapeutic targets. Longitudinal studies exploring the fluctuations in IL-38 and ESR levels in response to different treatments and disease stages are necessary to establish their dynamic relationship.

The genetic examination of IL-38 polymorphisms unveiled a notable link between the AA homozygous allele and rheumatoid arthritis (RA) (Abed, Abdulmalek, Yaaqoob, Altaee, & Kamona, 2023; McDowell, Symons, Ploski, FØRre, & Duff, 1995) ($p<0.001$, OR=9.11, 95% CI: 2.40-34.58). This finding indicates that individuals carrying the AA allele are at a significantly higher risk of developing RA. Conversely, the heterozygous A/C allele and homozygous CC allele did not exhibit a notable association with RA mentioned in this study (Wu, Wu, & Liang, 2021). The lack of association with these alleles suggests a specific role for the AA genotype in RA susceptibility. The study demonstrates the multifaceted nature of RA, involving not only traditional markers like ESR but also specific cytokines like IL-38 and their genetic variations. Understanding these complexities aids in early diagnosis, monitoring disease progression, and potentially developing targeted therapies (Paradowska-Gorycka et al., 2010). The identified IL-38 polymorphism (AA allele) could serve as a valuable genetic marker for RA predisposition, guiding further research into the molecular mechanisms underlying RA development and progression. These findings collectively contribute to advancing our understanding of RA pathogenesis and provide potential avenues for personalized therapeutic interventions in the future.

More in depth analysis is essential to clarify the exact function of IL-38 in inflammatory processes developing RA and to investigate its promise as a target for therapy.. Additionally,

studying IL-38 in conjunction with other inflammatory markers could provide a more comprehensive understanding of RA pathogenesis and help in developing targeted treatment strategies. It's crucial to acknowledge the shortcomings of the study, like the sample size and the need for longitudinal studies to establish causality and assess IL-38 dynamics over time. Future research could explore the influence of various factors like treatment regimens, disease duration, and genetic predispositions on IL-38 levels. Investigating IL-38's interactions with other cytokines and immune cells might provide deeper insights into its role in RA pathology.

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