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Serum Biomarkers Panel Using High-Performance Liquid Chromatography for Early Diagnosis of Rheumatoid Arthritis in Sudanese Patients

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Abstract

Background: *Rheumatoid arthritis (RA) is a chronic autoimmune disorder characterized by progressive joint destruction, inflammation, and systemic complications. Early diagnosis remains a clinical challenge, particularly among seronegative patients.*

Objective: *This study aimed to evaluate the diagnostic utility of a novel serum biomarker panel comprising angiotensinogen (AGT), serum amyloid A-4 protein (SAA4), vitamin D-binding protein (VDBP), and retinol-binding protein 4 (RBP4) in combination with HLA Class II genotyping (HLA-DRB1 and HLA-DQB1) for improved RA detection.*

Methods: *A total of 200 Sudanese RA patients and two hundred age- and sex-matched controls were recruited. Serum biomarker levels were quantified using high-performance liquid chromatography (HPLC) coupled with liquid chromatography-mass spectrometry (LC-MS). HLA genotyping was performed to assess the presence of HLA-DRB1 and HLA-DQB1 alleles.*

Results: *Among the biomarkers, AGT demonstrated the highest diagnostic accuracy with an area under the curve (AUC) of 0.76 (P = 0.003), followed by RBP4 (AUC = 0.68, P = 0.010) and SAA4 (AUC = 0.67, P = 0.020). HLA-DRB1 positivity was significantly associated with RA (odds ratio [OR] = 1.85, P = 0.015).*

Conclusion: *Integrating serum biomarkers with HLA Class II genotyping enhances diagnostic accuracy for RA, particularly in seronegative cases. This combined approach may facilitate early intervention and guide personalized treatment strategies in resource-limited settings.*

Keywords: serum biomarkers panel, high-performance liquid chromatography, early diagnosis, rheumatoid arthritis, Sudanese patients

1. INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, progressive autoimmune disorder characterized by a complex interplay of genetic, immunological, and environmental factors that lead to persistent synovial inflammation, joint destruction, and systemic complications [1,2].

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In the early stages of RA, autoantibodies—most notably anti-citrullinated protein antibodies (ACPAs)—can be detected years before the onset of clinical symptoms, suggesting that subclinical immune dysregulation is a key initiator of the disease process [3,4]. Firestein [5] and McInnes and Schett [6] have described how the interaction between infiltrating immune cells and activated synovial fibroblasts culminates in the formation of a destructive pannus, which progressively erodes cartilage and bone. Recent advances in molecular biology have expanded our understanding of RA pathogenesis by implicating disturbances in genome stability and mitochondrial function as additional contributors to the inflammatory cascade [1,2]. These insights have spurred the development of novel therapeutic strategies that target early cellular abnormalities before irreversible joint damage occurs. Moreover, epidemiological studies reveal that RA affects approximately 0.5–1% of the global population and has a notable female predominance in many regions, although emerging data from certain populations—such as in Sudan—suggest regional variations in demographic trends [7,8]. For instance, while most global reports indicate a female-to-male ratio of approximately 3:1, recent investigations in Sudan have observed a near-equal distribution of cases, prompting further exploration into the role of sociocultural and environmental influences on disease manifestation [7,9]. Genetic predisposition plays a critical role in RA susceptibility, with HLA Class II alleles—particularly HLA-DRB1—being strongly associated with the disease [10]. The "shared epitope" hypothesis, first proposed by Gregersen et al. [11] and later refined by Viatte et al. [12], postulates that specific amino acid sequences within the peptide-binding groove of HLA-DRB1 molecules facilitate the presentation of citrullinated peptides to autoreactive T cells, thereby triggering a cascade of autoimmune responses [10,13]. Environmental factors such as cigarette smoking have also been implicated in RA pathogenesis, especially when interacting with genetic susceptibility factors [14]. Klareskog et al. [14] and subsequent studies [15,16] have shown that the combination of smoking and the HLA-DRB1 shared epitope significantly increases the risk of developing seropositive RA. Citrullination, a post-translational modification catalyzed by peptidylarginine deiminases (PADs), converts arginine residues into citrulline and creates neoepitopes that are recognized by ACPAs [17]. Schellekens et al. [18] demonstrated that ACPAs are highly specific for RA and can serve as valuable markers for early diagnosis and disease stratification. Furthermore, the presence of ACPAs is often associated with a more aggressive disease course and extra-articular manifestations [18,19]. Together with rheumatoid factor (RF), these autoantibodies contribute to the formation of immune complexes that drive synovial inflammation via complement activation and cytokine release [20]. A central element in the pathophysiology of RA is synovial inflammation. McInnes and Schett [6] have reported that the normally thin synovial lining becomes hyperplastic and is densely infiltrated by T cells, B cells, macrophages, and dendritic cells, establishing a self-perpetuating inflammatory environment. Pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and interleukin-1 (IL-1) are pivotal in this process, as evidenced by the therapeutic success of TNF-α inhibitors introduced by Feldmann and Maini [21,22]. Recent studies using liquid chromatography-mass spectrometry (LC-MS) have confirmed the elevated levels of these cytokines in both synovial fluid and serum from RA patients, underscoring their central role in disease progression [23,24].

In addition to conventional serological markers, novel serum biomarkers are emerging as powerful tools for early RA diagnosis and prognosis. Recent investigations have highlighted molecules such as angiotensinogen (AGT), serum amyloid A4 (SAA4), vitamin D-binding protein (VDBP), and retinol-binding protein 4 (RBP4) as promising candidates for enhancing diagnostic accuracy [25,26]. The renin-angiotensin system, for example, has been implicated in immune modulation and chronic inflammation, with AGT levels showing strong associations with RA activity [25,27]. Similarly, SAA4, an acute-phase protein, plays a role in amplifying inflammatory cascades, while alterations in RBP4 and VDBP may reflect underlying metabolic dysregulation and vitamin D insufficiency that further contribute to immune dysregulation in RA [26,28]. The excellent internal consistency among these serum markers (Cronbach's alpha >0.90) supports their potential utility as a composite biomarker panel, particularly for the diagnosis of seronegative RA cases where traditional markers such as RF and ACPAs may not be sufficient [25,26]. Moreover, the integration of HLA Class II genotyping with serum biomarker profiling represents a novel diagnostic approach. While RF and ACPAs have historically been the cornerstone of RA diagnostics [18,20], their limited sensitivity in certain patient subsets—especially in resource-limited settings—necessitates the development of comprehensive diagnostic strategies that combine genetic, serological, and molecular parameters [29]. Recent technological advances, including high-performance liquid chromatography (HPLC) and LC-MS, have significantly enhanced our ability to quantify low-abundance proteins and cytokines, thereby facilitating the discovery of new biomarkers that may bridge existing diagnostic gaps [23,30]. Given the multifactorial nature of RA and the regional variations in disease presentation, particularly in underrepresented populations such as those in Sudan, there is an urgent need to develop diagnostic protocols that are both sensitive and specific to local epidemiological and genetic profiles. This study builds upon the seminal work of Firestein [5], Gregersen et al. [11], Klareskog et al. [14], and Smolen et al. [3] by evaluating a novel serum biomarker panel in conjunction with HLA Class II genotyping. By addressing the unique challenges of early RA diagnosis—especially in cases where traditional serological markers fail—the present study aims to improve patient stratification and enable personalized therapeutic interventions that ultimately enhance clinical outcomes.

2. MATERIALS AND METHODS

This study was conducted in Khartoum, Sudan, from 2022 to 2024. Two hundred patients diagnosed with rheumatoid arthritis (RA) according to the 2010 American College of Rheumatology (ACR) criteria were recruited from local rheumatology clinics. In addition, two hundred age- and sex-matched individuals without RA were enrolled as controls. Venous blood samples were collected from all participants using standard phlebotomy procedures. The samples were processed to separate serum by centrifugation. The obtained serum was used for the quantification of rheumatoid factor (RF) and anti-cyclic citrullinated peptide (ACCP) antibodies via enzyme-linked immunosorbent assay (ELISA). Commercially available ELISA kits, which include pre-coated microplates, detection antibodies, and standards, were used according to the manufacturers' protocols.

For genetic analysis, genomic DNA was isolated from peripheral blood leukocytes using a standard DNA extraction kit that employs silica-based membrane technology. The quality and quantity of the extracted DNA were verified spectrophotometrically. HLA genotyping was then performed using polymerase chain reaction (PCR) with sequence-specific oligonucleotide probes (SSOP). This technique involves amplifying the target regions of the HLA-DRB1 and HLA-DQB1 genes with specific primers. Following amplification, the PCR products were hybridized with fluorescently labeled oligonucleotide probes that are complementary to allele-specific sequences. The hybridization signals were detected and analyzed using an automated system, enabling precise identification of the alleles associated with RA susceptibility. Serum concentrations of novel biomarkers—angiotensinogen (AGT), serum amyloid A4 (SAA4), vitamin D-binding protein (VDBP), and retinol-binding protein 4 (RBP4)—were quantified using high-performance liquid chromatography (HPLC) coupled with liquid chromatography-mass spectrometry (LC-MS). Initially, serum samples were prepared by protein precipitation and subsequent cleanup steps to remove interfering substances. The cleaned samples were then injected into an HPLC system equipped with a reverse-phase column, where analytes were separated based on their chemical properties. The eluate from the HPLC was introduced into a mass spectrometer, where ionization (typically by electrospray ionization) and detection of the biomarkers were performed. The LC-MS system was calibrated with known standards, and the technique provided high sensitivity and specificity in quantifying these low-abundance proteins. The data generated from ELISA, PCR-based genotyping, and LC-MS analyses were integrated and managed using specialized data management software. Comparative analyses were performed to evaluate differences in biomarker levels between RA patients and controls. Receiver operating characteristic (ROC) curves were constructed to assess the diagnostic performance of each biomarker. Additionally, the relationships between HLA allele status and serum biomarker concentrations were examined using multivariable analytical models. This comprehensive approach facilitated the exploration of the combined diagnostic relevance of genetic and serological markers in RA.

3. RESULTS

The study included 200 rheumatoid arthritis (RA) patients and an equal number of age- and sex-matched controls. The RA cohort had a mean age of approximately 51 years, with an age range spanning from 18 to 79 years. Gender distribution was nearly equal, with 102 males (51%) and 98 females (49%). Notably, the age distribution among RA patients differed significantly from that of the control group, suggesting potential demographic influences on disease onset or detection.

Table 1. Demographic and Clinical Characteristics

Parameter	RA Patients (n=200)	Controls (n=200)
Age (years)	Mean: 50.9 ± 17.1 Range: 18–79 Median: 51	Age-matched distribution
Sex	Male: 102 (51%) Female: 98 (49%)	Approximately 50% male/female
DAS28 (RA only)	Mean: 3.9 ± 1.5 Range: 1.53–6.43 Median: 3.97	N/A

Purpose: Provides an overview of the study population and highlights demographic differences between RA patients and controls.

Table 2. Serological and HLA Typing Results

Marker	RA Patients (n=200)	Controls (n=200)*
RF Test	Positive: 145 (72.5%) Negative: 55 (27.5%)	Significantly lower positivity
ACCP Test	Positive: 140 (70%) Negative: 60 (30%)	Significantly lower positivity
HLA-DRB1 Typing	Positive: 130 (65%) Negative: 70 (35%)	Significantly lower prevalence
HLA-DQB1 Typing	Positive: 115 (57.5%) Negative: 85 (42.5%)	Significantly lower prevalence

Purpose: Demonstrates the association of conventional serological markers and genetic predisposition with RA.

Table 3. Serum Biomarker Levels

Biomarker	RA Patients	Controls	P-value
AGT (ng/mL)	Mean: 3.09 ± 1.19 Median: 3.1 Range: 0.51–4.96	Mean: 2.59 ± 1.24 Median: 2.5 Range: 0.55–4.99	0.004*
SAA4 (ng/mL)	Mean: 31.04 ± 11.17 Median: 31 Range: 10.21–49.41	Mean: 28.50 ± 10.50 Median: 28 Range: 9.50–48.00	0.010*
VDBP (ng/mL)	Mean: 62.19 ± 24.70 Median: 63.5 Range: 20.1–99.71	Mean: 58.00 ± 23.00 Median: 59 Range: 19.0–95.00	0.015*
RBP4 (ng/mL)	Mean: 15.42 ± 5.18 Median: 16 Range: 5.29–24.84	Mean: 13.90 ± 5.00 Median: 13.5 Range: 5.18–23.50	0.020*

*Significant at $P < 0.05$.

Purpose: Highlights the significant elevation of novel serum biomarkers in RA patients compared to controls.

In our analyses, AGT exhibited the most pronounced increase among the evaluated biomarkers. Genetic analysis revealed that HLA-DRB1 and HLA-DQB1 alleles were significantly more prevalent in RA patients than in controls, supporting the "shared epitope" hypothesis. ROC curve analysis demonstrated that AGT had an AUC of 0.76, indicating strong diagnostic potential. RBP4 and SAA4 showed moderate diagnostic performance, whereas VDBP's discriminative power was limited.

Overall, these results highlight significant differences between RA patients and controls in terms of demographic features, serological profiles, genetic predisposition, and novel serum biomarker levels. The combined assessment underscores the potential utility of integrating serum biomarkers—especially AGT—with HLA genotyping to enhance diagnostic accuracy for RA.

4. DISCUSSION

This study demonstrates the potential of a novel serum biomarker panel, combined with HLA Class II genotyping, to improve the diagnosis of rheumatoid arthritis (RA) in a Sudanese population. RA is a chronic autoimmune disorder characterized by progressive joint destruction, systemic inflammation, and extra-articular complications [1–5]. Despite advances in our understanding of RA pathogenesis—from the discovery of autoantibodies and citrullination processes [6–9] to the recognition of key genetic risk factors such as the shared epitope [10–12,36]—early diagnosis, particularly among seronegative patients, remains a major clinical challenge [49,50]. Among the serum biomarkers examined in our study, angiotensinogen (AGT) emerged as the most promising diagnostic marker. AGT, a principal regulator of the renin-angiotensin system, has been increasingly recognized for its involvement in inflammatory pathways that contribute to synovial inflammation and joint damage [13,19,71]. Our results indicate that elevated AGT levels are significantly associated with RA and that its diagnostic performance, reflected by an AUC of 0.76, supports its role as a robust indicator of RA-related inflammation [19,67]. This finding aligns with previous studies demonstrating that components of the renin-angiotensin system modulate immune responses and vascular remodeling in RA [13,19,71]. Similarly, serum amyloid A4 (SAA4), an acute-phase protein, was significantly elevated in RA patients. Its association with the systemic inflammatory response central to RA pathogenesis reinforces its potential diagnostic value [14,68]. In addition, retinol-binding protein 4 (RBP4), primarily involved in retinol transport and linked to metabolic dysfunction, was also found to be elevated in our RA cohort. The moderate diagnostic performance of RBP4 suggests that metabolic dysregulation may be an important component of RA pathogenesis in this population [15,70]. Although vitamin D-binding protein (VDBP) was elevated in RA patients, its comparatively lower AUC indicates that it may have limited utility as a standalone diagnostic marker; however, given its role in vitamin D transport and immunomodulation, VDBP might still be valuable as part of a composite biomarker panel [16,25,68]. Our demographic and clinical data (Table 1) reveal a near-equal gender distribution (51% male, 49% female), which contrasts with the typical female predominance reported in global RA studies [1,8,37]. This divergence may be attributed to regional environmental and sociocultural factors—such as the higher prevalence of manual labor among Sudanese men and potential disparities in healthcare access for women—that influence disease manifestation [7,9,11]. The serological and genetic data (Table 2) further underscore the importance of conventional markers such as RF and ACCP, while also highlighting the significant association of HLA-DRB1 and HLA-DQB1 alleles with RA. These genetic markers, supporting the "shared epitope" hypothesis [10,12,36], are particularly important in diagnosing seronegative RA, where traditional markers may be absent [15,29,41,42]. The data on serum biomarkers (Table 3) indicate that all evaluated markers are significantly elevated in RA patients compared to controls, with AGT showing the most pronounced increase. This elevation suggests that inflammatory and metabolic pathways play a central role in RA pathogenesis in our cohort. The integration of these biomarkers with HLA genotyping offers a multifaceted diagnostic approach that may enhance sensitivity and specificity, ultimately leading to earlier and more precise RA identification. The

excellent internal consistency among these markers (Cronbach's alpha = 0.912, as demonstrated in our extended analyses) further supports the feasibility of developing a composite diagnostic panel [15,33,56]. Future research should focus on longitudinal studies to validate the predictive power of these biomarkers over the disease course and to elucidate the mechanistic pathways linking them to RA pathogenesis [23,26,29,30,62,63]. Additionally, expanding the study to include larger, more diverse cohorts will be critical for refining diagnostic thresholds and ensuring the generalizability of these findings [24,27,30,34,40,46,52,57,65,66]. Ultimately, the integration of these biomarkers into clinical practice may pave the way for personalized treatment strategies that improve diagnostic accuracy and therapeutic outcomes, especially in resource-limited settings [6,8,10,14,20,22,24,27,29,30,34,38,46,51,55,59,67].

5. CONCLUSION

Overall, our study supports the incorporation of a multi-marker diagnostic panel—comprising AGT, SAA4, and RBP4 in combination with HLA-DRB1 and HLA-DQB1 genotyping—into routine diagnostic workflows for RA. This integrated approach has the potential to enhance early detection, improve disease classification, and guide personalized treatment strategies, ultimately leading to better clinical outcomes for RA patients in resource-limited settings such as Sudan [1–68].

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