

ESTIMATION OF THE CONCENTRATION OF SOME BIOMARKERS IN SERUM OF WOMEN WITH RHEUMATOID ARTHRITIS

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Abstract. *The current study was conducted on women with rheumatoid arthritis (RA) in Basrah city, Iraq. The study aimed to evaluate the relationship between some biomarkers such as adiponectin hormone, anti-citrullinated protein antibody (ACPA), acute phase C-reactive protein (CRP) and vascular endothelial growth factor (VEGF) in the serum of female patients. In this study, 80 serum samples were collected from women and distributed as follows: 56 serum samples from women with RA and 24 serum samples from non-infected women who were considered a healthy group. The patient samples were divided into three categories depending on age, disease severity and duration. The samples were obtained from Basrah General Hospital. The results of the current study showed a significant increase in the concentration of adiponectin, ACPA, CRP and VEGF in RA patients compared to healthy subjects. In addition, there were significant differences in the concentration of these biomarkers between some of the three age groups and between the disease severity and duration. The study concluded that all the biomarkers under investigation had increased their concentrations in the serum of women suffering from RA.*

Key words: *Adiponectin, ACPA, CRP, VEGF, Rheumatoid Arthritis, Biomarkers*

INTRODUCTION

Rheumatoid arthritis (RA) is one of the common inflammatory joint diseases. This autoimmune disease can be caused by a range of genetic and environmental factors. It affects people at any age stage but peaks between the ages of 30-50. The injury caused by this disease leads to the destruction of joints and impedes movement [1]. Inflammatory cells infiltrate into the synovial membrane in the RA joint, where the endothelial cells of the synovium swell with the formation of many new blood vessels. The inflamed synovium is called Pannus, and its formation leads to erosion of cartilage and bone, laxity and destruction of ligaments and tendons [2].

It has been observed that some physiological and immunological markers (hormones, interleukins, growth factors, etc.) have changed due to RA, and their change is a negative indicator of other body functions. Therefore, it is necessary to use some biomarkers to detect and monitor the disease before it develops, to know the extent of the relationship of these criteria to the disease, and to evaluate the functions of the body. All these are, accordingly, considered predictive criteria for the occurrence of the disease [3].

Adiponectin is a fat-derived hormone, a secretory protein produced by adipocytes and consists of 244 amino acids [4]. It is considered the most

abundant in human blood among all adipokines. Adipokines are known as soluble media in the form of biologically active polypeptides (hormones, cytokines, extracellular matrix proteins (ECM), growth factors, etc.) It is produced by fat cells and preadipocytes or by immune cells filtered into the adipose tissue. It plays a major role in various physiological functions, including inflammatory ones [5].

The molecular forms of adiponectin in the blood vary. Therefore, its biological functions vary as well, as it has three forms of different molecular weights [6]. The forms with high and medium molecular weight constitute the majority of the adiponectin circulating in the blood and play an initiating role in inflammation, while low molecular weight adiponectin is found in very low concentrations in the blood plasma and has an anti-inflammatory role [7]. Adiponectin reduces the concentrations of fatty acids in the blood and the levels of triglycerides in the liver and muscles. Moreover, adiponectin is mainly involved in modulating the inflammatory response [8].

The anti-citrulline antibody ACPA is a group of autoantibodies which are also called an anti-cyclic citrullinated peptide (anti-CCP) and isotypes (IgG, IgA, IgM), which recognize non-essential amino acids citrulline – in proteins [9]. Many citrulline proteins are distinguished by ACPAs, which include Fibrinogen, Vimentin, α -enolase, Collagen type II, Keratin,

Fibronectin, Histones and others [10]. Anti-cyclic citrulline proteins (Anti-CCP) were detected in the serum of RA patients at 60-80% with a specificity of 85-99% [11]. Thus, ACPA alone has sufficient predictive power to accurately distinguish individuals at risk of developing RA from the rest of the population [12].

CRP is one of the types of blood plasma proteins, and its concentrations rise in response to inflammation, tissue damage, or when cancerous transformations occur in normal cells in the host. It is a member of the family of proteins called Pentraxins [13]. CRP is mainly produced by the liver in response to inflammation, infection, trauma, necrosis and tissue damage, malignancy, and allergic reactions. The designation of acute phase response is used to express increased concentrations of plasma proteins, including CRPs [14].

CRP is associated with various chronic inflammatory processes such as rheumatic diseases, cancerous diseases, cardiovascular diseases, chronic hepatitis, etc. [15]. In addition to the beneficial (protective) effects, CRP possesses harmful effects. This is because it contributes to atherosclerosis by activating endothelial cells to increase the expression of adhesion molecules, facilitating the adhesion of platelets and leukocytes and their aggregation, and the production of Monocyte chemoattractant protein-1 (MCP-1) that attracts monocytes to the vessel wall, and the increase in the production of IL-8 that leads to the aggregation of neutrophils and their adhesion to endothelial cells, and all these roles lead to the expansion of inflammation and the development of sclerosis Arteries [16].

VEGF represents one of the angiogenic factors important for stimulating the process of generating new blood vessels, or angiogenesis, which is a complex physiological process. This process includes the division and migration of endothelial cells and the selective dissolution of the vascular basement membrane. During this process, new blood vessels are formed from pre-existing blood vessels, as in adults or the angiogenesis and growth during embryonic development [17]. The signaling protein VEGF is produced by cells that stimulate angiogenesis when hypoxia is present [18]. VEGF performs direct inflammatory activities; it exerts anti-apoptotic effects and causes the development of synovitis through hyperplasia and the formation of new blood vessels in the joints of RA patients [19]. Moreover, VEGF increases endothelial cell division, migration, survival and differentiation. It activates MMPs, attracts macrophages and granulocytes, and indirectly dilates blood vessels by releasing NO; increasing

the release of these oxides enhances vascular permeability [20].

The current study aims to evaluate the concentrations of some immunological and physiological biomarkers in the serum of sick women and the relationship of these parameters to RA.

MATERIAL AND METHODS

The patients' group

A total of 56 serum samples were obtained from women with RA, and the samples were divided based on age Categories into 18 serum samples for women aged 20-35 years, 19 serum samples for women aged 35-50 years, and 19 serum samples for women whose ages ranged between 50-65 years. The samples were also divided according to the severity of the disease into (severe, moderate and mild infection) and based on (Disease Activity Score-28). The disease duration was divided into two periods, the first from a month to 3 years and the second from 3 years to 20 years. The samples were collected from the joint consultants at Basrah General Hospital.

The healthy group

Twenty-four serum samples were obtained from healthy women after ensuring they did not have RA.

Preparation of serum

From each woman, 5mL of venous blood was drawn from the vein of the elbow using a clean medical syringe; then, the blood was collected in a gel tube and left for 20 minutes. Afterwards, the tube was placed in a centrifuge at a rate of 3000 rpm for 15 minutes to obtain the blood serum. The obtained serum was divided and collected in 1.5 mL Eppendorf tubes, and the samples were stored at -80°C in deep freeze until tests were carried out.

Estimation of the concentration of biomarkers

The concentrations of some biomarkers (Adiponectin, ACPA, CRP, VEGF) were estimated by using the well-known immunoassay (Enzyme-Linked Immuno Assay (ELISA)) using ELISA reader, which is of American origin and the biomarker kits supplied by the American company MyBioSource, and according to the Leaflet attached to each kit.

Statistical Analysis

Statistical analysis of the data was carried out using the T-test and the analysis of variance (ANOVA) for the least significant difference LSD at the probability level of $P < 0.05$. The standard deviation was calculated using the SPSS program version 21.

RESULTS

The results of the current study showed a significant increase at the probability level $P < 0.05$ in the concentration of adiponectin hormone in patients with RA (68.30 ± 12.21 ng/mL) compared to its concentration in healthy women (51.20 ± 13.97 ng/mL). The study also showed that significant differences were found in the concentration of this hormone among the three age Categories and between moderate and mild disease severity, and the statistical analysis did not record significant differences in the concentration of (Adiponectin) according to the first and second disease duration, as shown in Table 1.

With regard to the ACPA antibodies, the results showed that a significant increase in the concentration of the ACPA antibodies was found in patients (207.94 ± 69.06 ng/mL) compared to its concentration in healthy women (17.95 ± 7.06 ng/mL). The results also showed significant differences in the concentration of this biomarker according to the three

age categories, the disease severity and duration, as shown in Table 2.

The results of the current study showed a significant increase in the concentration of acute phase protein CRP in rheumatic patients (1186.89 ± 318.16 pg/mL) compared to its concentration in healthy control (867.58 ± 271.03 pg/mL). The current study also found significant differences in the concentration of CRP among the three age categories and the severity of the disease. However, the study did not report significant differences in the concentration of this protein in the two duration of illness, as shown in Table 3.

The results of the current study showed a significant increase in the concentration of VEGF in RA patients (207.58 ± 44.45 pg/mL) compared to its concentration in healthy control (17.50 ± 6.40 pg/mL). The statistical analysis showed significant differences in the concentration of this biomarker among the categories according to age, the severity of the disease and the two duration of illness, as shown in Table 4.

Table 1. Serum concentration of Adiponectin hormone

Variables		No.	Mean \pm SD	P value
Participants	Rheumatoid arthritis	56	68.30 ± 12.21 ng/mL*	0.0001
	Healthy control	24	51.20 ± 13.97 ng/mL	
Age (years)	20-35	18	59.27 ± 6.36 a	0.0001
	35-50	19	71.89 ± 9.78 b	
	50-65	19	73.26 ± 10.19 b	
Severity	Severe	16	67.37 ± 10.99	0.036
	Moderate	32	70.81 ± 10.64 a	
	Mild	8	60.12 ± 6.64 b	
Disease duration	1 Month – 3 years	26	69.38 ± 14.46	0.581
	3 years – 20 years	30	67.36 ± 12.74	

* Significant difference at the probability level of $P < 0.05$ in the group of patients compared to the group of healthy subjects. The different letters (a, b) indicate that there were significant differences between the Categories at ($P < 0.05$)

Table 2. Concentration of ACPA

Variables		No.	Mean \pm SD	P value
Participants	Rheumatoid arthritis	56	207.94 ± 69.06 ng/mL*	0.0001
	Healthy people	24	17.95 ± 7.06 ng/mL	
Age (years)	20-35	18	184.61 ± 43.92 a	0.014
	35-50	19	215.63 ± 33.33 b	
	50-65	19	222.36 ± 42.81 b	
Severity	Severe	16	284.06 ± 41.61 a	0.0001
	Moderate	32	243.69 ± 37.67 b	
	Mild	8	189.37 ± 44.07 c	
Disease duration	1 Month – 3 years	26	138.80 ± 20.97 *	0.0001
	3 years – 20 years	30	267.86 ± 27.66 *	

*Significant difference at the probability level of $P < 0.05$ in the group of patients compared to the group of healthy. The different letters (a, b, c) indicate that there were significant differences between the Categories at ($P < 0.05$)

Table 3. Concentration of CRP

Variables	No.	Mean \pm SD	P value
Participants	Rheumatoid arthritis	56	1186.89 \pm 318.16 pg/mL*
	Healthy people	24	867.58 \pm 271.03 pg/mL
Age (years)	20-35	18	1052.88 \pm 136.40 ^a
	35-50	19	1289.36 \pm 292.56 ^b
	50-65	19	1211.36 \pm 253.39 ^b
Severity	Severe	16	1214.37 \pm 155.22 ^a
	Moderate	32	1211.06 \pm 160.84 ^a
	Mild	8	1035.25 \pm 151.38 ^b
Disease duration	1 Month – 3 years	26	1188.07 \pm 233.91
	3 years – 20 years	30	1185.86 \pm 294.27

*Significant difference at the probability level of $P < 0.05$ in the group of patients compared to the group of healthy subjects. The different letters (a, b) indicate that there were significant differences between the Categories at ($P < 0.05$)

Table 4. Concentration of VEGF

Variables	No.	Mean \pm SD	P value
Participants	Rheumatoid arthritis	56	207.58 \pm 44.45* pg/mL
	Healthy people	24	17.50 \pm 6.40 pg/mL
Age (years)	20-35	18	184.61 \pm 26.42 ^a
	35-50	19	215.63 \pm 40.87 ^b
	50-65	19	212.36 \pm 23.32 ^b
Severity	Severe	16	282.81 \pm 43.43 ^a
	Moderate	32	168.90 \pm 33.32 ^b
	Mild	8	176.87 \pm 35.48 ^b
Disease duration	1 Month – 3 years	26	138.80 \pm 25.65 *
	3 years – 20 years	30	261.20 \pm 32.54 *

*Significant difference at the probability level of $P < 0.05$ in the group of patients compared to the group of healthy. The different letters (a, b) indicate that there were significant differences between the Categories at ($P < 0.05$)

DISCUSSION

The adipokines, including adiponectin, are important inflammatory mediators that play a critical role in autoimmune diseases. Their importance results from their many biological properties, including their interaction with the immune system. Adiponectin exerts a pro-inflammatory activity in the joints and stimulates their erosion and breakdown mechanisms despite its strong anti-inflammatory effects and improving insulin sensitivity [21]. The results of the current study showed a significant increase in the concentration of adiponectin in patients with RA (68.30 \pm 12.21ng/mL) compared to healthy women (51.20 \pm 13.97 ng/mL), and our results agreed with the results of other studies [22, 23]. The reason for the high concentration of adiponectin may be attributed to the roles that adiponectin plays in regulating the inflammatory response through in-

hibiting the production of adhesion molecules, suppressing macrophage function, and inhibiting NF- κ B signaling [24]. Or the reason for the increase may be explained by looking at the anti-inflammatory regulatory function of adiponectin, which counteracts the pro-inflammatory effects of inflammatory cytokines, inhibits them and stimulates the release of anti-inflammatory factors [25].

Regarding the effect of age on the concentration of adiponectin, the results of our study showed significant differences in the concentration of this hormone among the three age categories. These results agree with the study by Bucci et al. [26]. The reason for the increase may be due to the increase in the production of this hormone from adipose tissue and other tissues such as the liver and skin at aging. With regard to the effect of disease severity on adiponectin concentration in patients' serum, sig-

nificant differences were obtained between moderate and mild disease severity only. Adiponectin has been identified as a factor associated with disease severity and joint destruction in several previous studies [27, 28, 29]. The study by Liu et al. [30] reported that the concentration of adiponectin increases significantly in the serum of patients with active RA compared to healthy patients or patients with inactive RA, and the researchers noticed a positive association of adiponectin with the disease severity scale, ESR and RF. As for the disease duration and its effect on the concentration of this biomarker, we did not notice any significant differences between the two courses of the disease.

The results of the current study showed a significant increase in the concentration of ACPA antibody in the serum of patients (207.94 ± 69.06 ng/mL) compared to healthy women (17.95 ± 7.06 ng/mL), and these results were consistent with the findings of other studies [31, 32]. The reason for the high concentration of ACPA may be due to the formation of immune complexes as a result of the association of the types of ACPAs produced by B cells with citrullinated proteins in the synovial membrane of the joint, which leads to the activation of macrophages to produce many cytokines that initiate inflammation. Moreover, the production of ACPAs promotes the development of osteoclasts and, thus, joint destruction by causing erosion of bone and cartilage [33]. ACPA affects immune cells and enhances the production of more inflammatory and oxidative indices in monocytes, lymphocytes, and neutrophils. It was found that it activates platelets, which may contribute to atherosclerosis through the accumulation of pro-inflammatory mediators, endothelial tissue damage, and increased vascular permeability [34].

The results of the current study also showed significant differences in the concentration of ACPA depending on age. Our results agreed with the results of previous studies [35, 36]. The reason why the concentration of ACPA remains elevated with age may be due to the fact that it may appear several years before the onset of disease symptoms and before the onset of inflammation, and then it increases with the onset of disease symptoms and joint destruction [37].

The results also showed significant differences in the concentration of ACPA in all types of disease severity (severe, moderate and mild), and the highest concentration was recorded in severe disease. The results of our study agreed with the results of the study by Roos Ljungberg et al. [38]. This study revealed an increase in the concentration of ACPA-IgA antibody by 12% in saliva and by 45% in the serum of

RA patients, and this increase was associated with a higher activity of the disease. The reason for this can be attributed to the fact that the production of citrulline proteins increases with the increase in the severity of the disease in the inflamed tissues of patients, which leads to an increase in autoantibodies, especially ACPA [39]. As for the effect of the disease duration on the concentration of ACPA, the results showed a significant increase in the concentration of this biomarker between the two periods of illness, and this was consistent with the results of the study by Kastbom et al. In addition, they found significant differences in the concentrations of ACPAs, which were associated with the long duration of the disease [31]. This may be due to the effect of the concentration of ACPA on anti-inflammatory treatments.

The results of the current study showed a significant increase in the concentration of acute phase protein CRP in rheumatic patients (1186.89 ± 318.16 pg/mL) compared to its concentration in healthy women (867.58 ± 271.03 pg/mL). This is because the acute phase proteins show a marked increase under different conditions such as bacterial infections, connective tissue disorders, abscesses, necrosis, tumors, etc. Therefore CRP is an important diagnostic tool for detecting inflammation [13]. The results of our study are in agreement with the findings of other studies [40, 41, 42]. The high concentration of this protein in the serum of patients may be due to the sex difference, as some studies indicated that the concentration of CRP was higher in females than in males [43, 44]. The reason for this may be due to genetic differences, hormonal factors, or the prevalence of obesity in women, which stimulate the production of more (IL-6), which in turn works to regulate the secretion of acute phase proteins [45].

Regarding the effect of age on acute phase protein concentration in patients, we found significant differences in CRP concentration among the age groups studied. These results agree with the results of previous studies that showed a significant association between getting old and increasing CRP concentration [45, 46]. The reasons for the increase in protein concentration with age may be due to differences in lifestyle and weight gain in women, as the increase in body mass stimulates the production of IL-6, which leads to an increased CRP production, as well as changes in hormonal factors after menopause or the occurrence of some immunity changes and metabolism changes [47]. As for the effect of disease severity on increasing CRP concentration in patients, the results showed significant differences in protein concentration between severe, moderate

and mild infections. Several studies confirmed the close relationship between an increase in CRP concentration and an increase in disease severity and the contribution of the protein to the pathogenesis of rheumatic disease [48, 49]. The reason for the rise in the concentration of CRP may be related to the activity or severity of the disease as a result of the large role that this protein plays in stimulating osteoclast cells and the resulting increase in joint destruction in severe disease [50].

The results of the current study showed a significant increase in the concentration of VEGF in patients with RA (207.58 ± 44.45 pg/mL) compared to its concentration in healthy women (17.50 ± 6.40 pg/mL), as many studies [51, 52, 53] reported similar results to what we found. The reason for the high concentration of this biomarker in the serum of patients may be attributed to the fact that rheumatoid disease is an inflammatory disease that causes the occurrence of tissue hyperplasia and the formation of fibrous vascular tissue or the so-called Pannus, which leads to a lack of oxygen and in turn stimulates the production of more vascular growth factors that work to generate blood vessels. This process promotes the delivery of inflammatory cells to the site of damage. Thus, inflammation-initiating cytokines are produced and released from inflammatory cells; therefore, VEGF acts as a functional bridge between angiogenesis and inflammation [54, 55].

As for the effect of age on the concentration of VEGF, the results showed significant differences in concentration according to the different age categories. These results agreed with the findings of Smets et al., who reported an increase in the concentration of VEGF in the serum with the advancing age of patients [56]. This increase indicated an exacerbation of the inflammatory condition in patients and was associated with an increase in acute phase reactants and the number of platelets. With regard to the effect of disease severity on the concentration of VEGF, the results showed significant differences in the concentration of this factor between severe infection with both moderate and mild infection, and many studies showed a higher concentration of vascular factors as the severity of the disease increased [57, 58, 59]. It was also observed that the concentration of VEGF in the serum increases with the increase in disease activity, the rise in CRP, and the number of swollen joints; therefore, the researchers suggested that the level of this factor expresses the activity and severity of the disease [51]. Thus, it is normal to increase the concentration of vascular factors when the severity and activity of the disease increase due to the in-

creased production of inflammatory cytokines, which stimulate cells to produce more VEGF.

As for the effect of the disease duration on the concentration of VEGF, the results showed significant differences between the periods, and the results of our study agreed with the results of previous studies [60, 61]. The reason for the rise in vascular growth factors in the late stages of the disease (the second time period) may be attributed to the poor therapeutic control of the disease.

CONCLUSIONS

The current study concluded that all the biomarkers under investigation had increased their concentrations in the serum of women suffering from RA, which indicates the association of these biomarkers with the inflammatory reactions that occur in patients with RA. These high concentrations of these parameters can help predict the therapeutic effects and can be adopted as biomarkers for early detection of RA.

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