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Study of Serum Level of Neutrophil Extracellular Traps in Rheumatoid Arthritis Patients and Its Correlation with Disease Activity and Associated Cardiovascular Morbidity

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ABSTRACT

Background: An autoimmune disease that impacts both articular and extra-articular organs (e.g., eye, skin, and nervous system), progressive systemic rheumatoid arthritis (RA) is a pathological condition. We aimed to measure the serum level of neutrophil extracellular traps (NETs) in RA patients and to detect the correlation between NET level and RA activity. Also to study the relation between NET level and cardiovascular affection in RA patients by measuring the carotid intima media thickness.

Patients and methods: This study was conducted on 50 RA patients diagnosed according to the American College of Rheumatology and European League Against Rheumatism 2010 diagnostic criteria for RA, and 50 apparently healthy volunteers matched with patients in sex and age for evaluation of the NET level. All patients underwent the following: full clinical assessment, laboratory investigations, radiographic assessment and wrist and measurement of carotid intima media thickness by ultrasound.

Results: There was elevation in total cholesterol, triglycerides, LDL levels and reduction in HDL level in patients. There was increased level of MPO level in RA patients' group with significant difference between the RA and control group ($P<0.05$). There was significant positive correlation between MPO level and clinical, laboratory and radiological findings of the patients ($P<0.05$). **Conclusion:** The serum level of NETs is increased in RA patients and correlated with rheumatoid disease activity detected by MSUS and DAS -28. Also, the serum level of NETs could be considered a useful biomarker for the early prediction of subclinical atherosclerosis and cardiovascular affection in RA patients.

Keywords: Rheumatoid arthritis, NETs, MPO, Carotid intima media thickness, Cardiovascular affection.

INTRODUCTION

An autoimmune disease that impacts both articular and extra-articular organs (e.g., eye, skin, and nervous system), progressive systemic rheumatoid arthritis (RA) is a pathological condition [1].

The risk of developing cardiovascular morbidity is 1.5–2.0 times higher among patients with RA compared to the general population. There is evidence of a high incidence of premature atherosclerosis in both patients with established RA and those with early RA (less than one year of disease duration) [2].

Directly or indirectly, neutrophils contribute to the inflammatory progression of RA through the secretion of chemokines and cytokines, or by releasing neutrophil granules that influence cytokine synthesis [3].

Neutrophil extracellular traps (NETs) consist of a diverse array of constituents, including various enzymes, with MPO being the only discernible contributor. Additionally, MPO is necessary for NETs formation, demonstrating the critical importance of MPO activity in both protective and deleterious neutrophil extracellular trap (NET) - associated processes [3].

Musculoskeletal ultrasound (MSUS) aids in follow-up and diagnosis and of patients with rheumatic diseases by evaluating cartilage, bone surfaces, and soft tissue disease [4].

Aim of study: To evaluate NETs serum level in RA patients and to detect the correlation between NET level and RA activity. Also to study the relation between NET level and cardiovascular affection in RA patients by measuring the carotid intimal thickness.

PATIENTS AND METHODS

This study was conducted on 50 RA patients diagnosed in accordance with the American College of Rheumatology (ACR) and European League Against Rheumatism (EULAR) 2010 diagnostic criteria for RA [5] and 50 apparently healthy volunteers matched with patients in sex and age for evaluation of the NET level. Patients were selected from the outpatient clinic and in-patient section of Rheumatology, Rehabilitation and Physical Medicine Department, Faculty of Medicine, Tanta University Hospitals from March 2021 to June 2023.

Patients underwent:

1) Clinical evaluation: full history taking**,** clinical examination, assessment of disease activity by Disease Activity Score (DAS 28) [6], Functional assessment by using the Modified Health Assessment Questionnaire (MHAQ) [7]
2) Laboratory examinations included complete blood count (CBC), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), rheumatoid factor (RF), anti-cyclic citrullinated protein antibodies (anti-CCP), lipid profile (Triglycerides, LDL, HDL, and total cholesterol). NET level was measured by measuring myeloperoxidase (MPO) by using an enzyme-linked immunosorbent assay (ELISA) [8].
3) Radiological assessment:

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a- Both hands and feet plain X-ray: Using Simplified Erosion and Narrowing Score (SENS) [9].

b- Hands and feet Musculoskeletal ultrasound (MSUS): By using Samsung Medison (UGEO H60) linear array transducers operating at frequencies varying from 10 to 12 MHz. The novel 7-joint ultrasound score (US7) includes MCP (II, III), PIP (II, III), MTP (II, V), and wrist, which are the 7 joints most frequently affected in RA, was used to detect synovitis, synovial thickening, bone erosions and PD evaluation [10].

c-Carotid intimal thickness (IMT) measurement by using ultrasonography 7.5–10 MHz linear phased array transducer. A posterior approach was utilized to examine the common carotid arteries, utilizing both longitudinal and transverse scans. Three points on each side were utilized to determine the IMT: the common carotid artery (10 mm prior to the bulb), the internal carotid artery (10 mm after the flow divider), and the bulb (5–10 mm cranially to the beginning of the bulb). IMT was deemed to be within the normal range when the M-IMT was below 0.9 mm, while values ≥ 0.9 mm were an indication of intima thickening. A carotid plaque was a localized thickening exceeding 1.2 mm in diameter that did not affect the entire artery.

Ethics approval and consent to participate:

In accordance with the Declaration of Helsinki, the study was authorized by the Local Research Ethics Committee of the Faculty of Medicine, Tanta University, under approval code number 34402/1/21. Prior to their participation in this study, every patient provided written informed consent. A special code number was assigned to each patient's file. All results and investigations of the research were used only for scientific purposes with the assurance of patient privacy.

Statistical analysis

SPSS version 20 (Statistical Package for the Social Sciences) was utilized to analyze the data. The results were reported in the form of number (%) and mean ± SD, and range. Quantitative data were compared by independent t-test for parametrically distributed data and by Mann-Whitney test for non-parametrically distributed data. For qualitative variables, Chi-square test was considered. Pearson correlation was used for detection of correlation between two quantitative variables in one group. A p -value <0.05 was considered statistically significant.

RESULTS

There was no statistically significant difference observed between RA patients and the control group regarding gender and age **(Table 1)**.

Table 1: Demographics of RA patients and controls.

	RA patients (n = 50)		Control (n = 50)		P
	No.	%	No.	%	
Sex					
Female	44	88.0	43	86.0	> 0.05
Male	6	12.0	7	14.0	
Age (/years)					
Min. – Max.	24.0 – 59.0		24.0 – 65.0		> 0.05
Mean ± SD.	44.08 ± 9.98		42.38 ± 10.26		

The clinical data and laboratory investigations are summarized in **Table 2**.

Table 2: Clinical and laboratory data for RA patient (n=50)

	Mean ± SD.
Duration of illness (years)	9.90 ± 6.35
Morning stiffness (minutes)	77.90 ± 36.67
No. of tender joints	6.86 ± 3.94
No. of swollen joints	2.74 ± 2.09
DAS 28	4.93 ± 0.86
MHAQ	1.08 ± 0.70
ESR (mm/hr)	44.98 ± 6.53
CRP (mg/l)	11.73 ± 2.43
RF (IU/ml)	85.50 ± 8.47
Anti-CCP (u/ml)	197.7 ± 15.6
Triglycerides (mg/dl	171.08 ± 14.11
Total cholesterol (mg/dl)	189.0 ± 9.55
HDL (mg/dl)	66.02 ± 11.0
LDL (mg/dl)	53.88 ± 6.42

ESR: erythrocyte sedimentation rate, CRP: C-reactive protein, RF: rheumatoid factor, Anti-CCP: Anti-cyclic citrullinated peptide, LDL: low density lipoprotein, HDL: high density lipoprotein.

Radiographic findings including X-ray and MSUS are summarized in **Table 3**.

Table 3: Radiographic assessment of RA patients using X-ray and MSUS

	Mean ±SD.
Joint erosion score	4.50±6.38
Joint space narrowing score	6.46 ±8.48
SENS	14.88 ± 10.13
GS synovitis	9.10 ± 6.16
PD synovitis	4.54 ± 3.83
Erosions	6.18 ± 4.49
GS tenosynovitis	2.40 ± 1.77
PD tenosynovitis	2.64 ± 2.71

SENS: Simplified Erosion and Narrowing Score

Forty-four percent of RA patient have thickened intima media of the carotid artery **(Table 4)**.

Table (4): Percentage of RA patients with thickened Intima media of the carotid artery.

CIMT	No.	%
Normal (≤0.9)	28	56.0
Abnormal (>0.9)	22	44.0

CIMT: carotid intima-media thickness test.

There was significant increased level of MPO level in RA patients' group compared to control group **(Table 5)**.

Table (5): Comparison between the two studied groups according to myeloperoxidase level

MPO	RA patients (n = 50)	Control (n = 50)	P
Min. – Max.	0.13 – 2.36	0.12 – 0.26	<0.05*
Mean ± SD.	0.89 ± 0.53	0.18 ± 0.03	

MPO: measuring myeloperoxidase, *: significant

There was significant relation between MPO level and clinical, laboratory and radiographic findings. There was significant relation between MPO level and medical treatment for RA with best reduction of MPO level in patients receiving monotherapy **(Table 6)**.

Table (6): Correlation between demographic data, clinical data and functional assessment of RA patients and myeloperoxidase level.

MPO	r	P
Age (/years)	0.220	0.125
Duration of illness	0.288	<0.05*
DAS28	0.707	<0.05*
MHAQ	0.579	<0.05*
ESR	0.665	<0.05*
CRP	0.435	<0.05*
Anti CCP	0.311	<0.05*
Triglycerides (mg/dl)	0.503	<0.05*
Cholesterol (mg/dl)	0.524	<0.05*
LDL (mg/dl)	0.403	<0.05*
JSN score	0.392	<0.05*
Bone erosion score	0.746	<0.05*
GS Synovitis	0.534	<0.05*
PD Synovitis	0.496	<0.05*
GS tenosynovitis	0.486	<0.05*
PD tenosynovitis	0.533	<0.05*
IMT	0.758	<0.05*

r: correlation coefficient, MPO: measuring myeloperoxidase, ESR: erythrocyte sedimentation rate, CRP: C-reactive protein, LDL: low density lipoprotein. *: significant.

DISCUSSION

Articular and extra-articular organs, such as the cardiovascular system, are all affected by RA, a systemic autoimmune disease. Premature atherosclerosis is prevalent among both early RA patients with a disease duration of less than one year and established RA patients with a long disease duration [11].

NETs consist of a diverse array of constituents, including various enzymes, with MPO being the only discernible contributor. Additionally, MPO is necessary for NETs formation, demonstrating the critical importance of MPO activity in both protective and deleterious NET-associated processes [12].

This study was conducted to evaluate NETs serum level in RA patients and to detect the correlation between NET level and RA activity. Also to study the relation between NET level and cardiovascular affection.

This trial was conducted on fifty RA patients diagnosed according to the ACR / EULAR 2010 criteria for RA diagnosis and fifty gender and age-matched volunteers who appeared to be in good health were utilized as the control group.

As regards clinical data of RA patients in this study, the mean duration of illness in years was (9.90 ± 6.35). The mean duration of morning stiffness in minutes in patients was (77.90±36.67). The mean of the number of tender joints was (6.86 ± 3.94). The mean of the number of swollen joints was (2.74± 2.09). The disease activity was measured by using DAS-28 with the mean of (4.93± 0.86). RA patients underwent a functional assessment utilizing the MHAQ, which yielded a mean score of (1.08 ± 0. 70).

Regarding lipid profile there was increased level of triglyceride, LDL-C and total cholesterol in RA with the mean of (171.08 ± 14.11), (53.88 ± 16.42) and (189.0 ± 49.55) respectively. Decreased level of HDL-C in RA patients with the mean of (66.02 ± 11.0).

Colunga-Pedraza *et al.* [13] showed that RA patients exhibited decreased levels of TC and LDL-C, according to the report. This recent research suggests that lipoproteins are significantly affected by chronic inflammation in both the early and advanced stages of disease. Furthermore, the management of inflammation via corticosteroids and anti-rheumatic treatments exhibits distinct effects on the extent and configuration of lipid profile alterations.

Weber *et al.* [14] showed that low levels of total cholesterol, triglycerides, and LDL have been reported in RA patients; this phenomenon is commonly known as the lipid paradox.

When assessing the carotid intima media thickness in patients, it was found that 44% of those with RA had an intima media of the carotid artery that was thicker than 0.9 mm. This is similar to the findings of **Saidova** [15], who established carotid intima-media thickness test (CIMT) as a reliable surrogate marker for early atherosclerosis detection using non-invasive ultrasonic technology. CIMT was significantly elevated in RA patients and was associated with a higher incidence of carotid atherosclerotic plaque formation compared to the control group.

Numerous factors attribute to elevated CIMT observed in patients with RA, encompassing an unfavorable lipid profile, heightened homocysteine levels, elevated thrombotic factor concentrations (fibrin

D-dimer, fibrinogen, plasminogen activator antigen and von Willebrand factor), chronic inflammation of the arterial walls, medication-related adverse effects, and reduced daily physical activity [16].

Regarding myeloperoxidase level, it was increased in RA patient group with the mean of (0.89 ± 0.53) while in control group the mean was (0.18 ± 0.03) with significant difference between the two groups (P<0.05).

Wright *et al.* [17] identified elevated concentrations of NETs in the synovial fluid, blood, and tissues of RA patients, and observed a correlation between these increases and systemic inflammatory factors.

Alisik *et al.* [18] reported that NET level was determined to be greater in RA patients than in the control group, as reported using the MPO level as a unit of measurement. Chronic inflammation may also be associated with elevated MPO levels in patients with active disease compared to RA patients in clinical remission.

A significant positive correlation was observed between the level of MPO and the duration of illness, DAS 28, and MHAQ, this aligns with the findings of **Czókolyová *et al*.** [19] who documented the same findings.

There was significant correlation between NET derived protein MPO and anti–CCP level. This is matched with **Mangale *et al.*** [20] who reported increasing serum NET levels, significantly correlated with several citrullinated as during NETosis, neutrophils externalized the citrullinated autoantigens implicated in RA pathogenesis.

A notable positive correlation was observed between the level of MPO and the lipid profile, which included cholesterol, triglyceride, LDL, and HDL. Moreover, by promoting HDL dysfunction, MPO contributes to an atherogenic environment and dysregulated lipid metabolism, in addition to oxidative stress [21].

With respect to joint damage and arthritis among RA patients and MPO levels, a significant correlation was observed between bone erosions and joint space narrowing, which are radiological manifestations of joint damage, and bone erosion, tenosynovitis, and synovitis, which are ultrasonographic findings of MPO. Due to NETs role in rheumatoid-associated bone erosion by promoting osteoclast activation, **O'Neil *et al.*** [22] found a positive correlation between NET level and cartilage damage in RA patients, as indicated by the presence of a high level of NET-derived protein MPO in synovial fluid and serum of patients with active disease compared to those in remission. This correlation was also strong with ultrasonography findings.

NETs are capable of directly degrading synovial cartilage components and enhancing inflammatory pathways in macrophages and FLSs, both of which may lead to joint damage. NETs stimulate the release of peptidylarginine deiminase 2, which enhances the citrullination of cartilage proteins, in addition to their pro-inflammatory effects. FLSs subsequently internalize citrullinated matrix proteins, which can stimulate antigen-specific T cells and induce specific autoantibody responses[23].

Patients afflicted with RA exhibited a significant positive correlation between CIMT and MPO level, as shown in our study. **Cheng *et al*** [24] reported that dysregulation of NETosis-derived products including MPO have a role in subclinical atherosclerosis in RA patients and also correlated with DAS-28 score.

Lipoprotein dysfunction and subclinical atherosclerosis, such as elevated CIMT, are prevalent in RA and correlate strongly with disease activity and duration. Ultrasonography-detected increased media thickness of the carotid intima is considered an early indicator of systemic atherosclerosis. Through oxidation reactions, MPO has been found to significantly reduce the cardioprotective effects of high-density lipoprotein cholesterol; furthermore, there is a correlation between increased enzymatic activity and an increased risk of plaque rupture and formation [3].

NETs also promote thrombosis and endothelial cell dysfunction and apoptosis by forming a fibrin-like base that facilitates platelet activation, adhesion, and aggregation. In addition, NETs facilitate the buildup of prothrombotic molecules, such as fibrinogen and von Willebrand factor, thereby making a significant contribution to the formation of thrombi [25].

CONCLUSION

1- The serum level of NETs is elevated in RA patients.
2- The serum level of NETs is correlated with rheumatoid disease activity detected by MSUS and DAS-28, so it can provide clinical value in the monitoring and prognosis of RA patients.
3- The serum concentration of NETs may function as a biomarker in RA patients, enabling early detection of subclinical atherosclerosis and cardiovascular disease.

- **Competing interests:** The authors declare no conflict of interest.
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