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Neutrophil extracellular traps and common carotid intema media thickness as predictors of cardio vascular risk in patients with systemic lupus erythematous

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Abstract
Background: Neutrophil extracellular traps (NETs) are chromatin complexes of cell-free DNA and histones, with attached neutrophil granular proteins which trap and kill infectious microbes. NETs are essential in host defense, and also linked to inflammation and autoimmunity, including systemic lupus erythematosus (SLE) and recently emerged as contributor to arterial and venous thrombosis. Common carotid intema media thickness (CIMT) used commonly as indicator for subclinical atherosclerosis and myocardial infarction in rheumatic diseases. In the current study, we investigated, the role of NET and CIMT in human SLE and their association with disease activity and cardiovascular complications.
Methods: Levels of NETs (human myeloperoxidase-DNA complexes) were analyzed in plasma from 40 SLE patients and 40 healthy individuals using MPO ELISA kit, and subclinical cardiovascular risk was assessed in these groups by using CIMT.
Results: Comparison between both groups showed significant difference in the level of NETs (human MPO) and CIMT ($p< 0.01$), and positive correlation with disease activity and cardiovascular complications.
Conclusion: NETs and CIMT could be good predictors for disease activity and cardiovascular risk in SLE patients.

Keywords: systemic lupus erythematous, neutrophil extracellular traps, carotid intema media thickness, cardiovascular risk

1. Introduction
Systemic lupus erythematosus (SLE) is an autoimmune disease of unknown etiology. Its incidence has been constant in recent decades, with about 4-5 per 100,000 people affected each year, with a female: male ratio is about 9:1 in adulthood [1].
Common hypotheses about SLE immunopathogenesis suggest that environmental triggers, such as infectious agents, operate in the context of genetic and epigenetic influences, resulting in aberrations in antigen presentation, lymphoid signaling, apoptosis, epitope modification, and antigen and immune complex (IC) clearance [2].
Inadequate clearance of dead cells seems to be the main mechanism that triggers autoimmunity in (SLE) patients [3].
In SLE, neutrophils extracellular traps (NET) have been suggested as an important source of autoantigens, with SLE patients having high levels of antibodies binding to NET, including anti-dsDNA and anti-histone antibodies. The released NET engage DNA receptors, Toll-like receptor (TLR) and cyclic GMP-AMP synthase (cGAS) to induce type I interferons (IFN) and the key cytokines in SLE pathogenesis [4].
NETosis is a cell death process in which DNA is expelled together with cytosolic and granular content in a web-like structure to trap and eliminate extracellular pathogens [5].
The more prominent cause of mortality in SLE is cardiovascular complications making up 30% of mortality in the first 5 years after diagnosis [6].
Cardiovascular disease pathogenesis in lupus was explained by either atherosclerosis and/or subclinical vasculitis. Ultrasonographic carotid intima-media thickness, as a proven predictor of myocardial infarction events in lupus, was used as surrogate of atherosclerosis [7].

In recent years, neutrophils were implicated in the inflammation observed in the pathogenesis of both athero-embolic disease and SLE pathophysiology. Understanding how neutrophils mediate atherosclerosis may provide an understanding of actionable pathways in SLE subjects and, ultimately, could contribute to improving patient management and clinical outcomes [8].

The aim of this study was to evaluate the serum level of neutrophil extracellular traps (NETs) and Carotid intema media thickness (CIMT) in patients with systemic lupus erythematosus and their association with cardiovascular risk and clinical and laboratory parameters of disease activity.

Patients and Methods

This prospective randomized controlled study was carried out at the Department of Rheumatology, Rehabilitation and Physical Medicine, Tanta University Hospitals from June 2021 to May 2023.

Subjects were randomly allocated to two groups: Test group (n = 40): SLE patients with age varying from 15-50 years old, clinically diagnosed SLE according to the American College of Rheumatology (ACR)/ European League Against Rheumatism (EULAR) 2019 criteria for diagnosis of systemic Lupus erythematosus, and Control group (n = 40): apparently healthy, age and sex matched volunteers.

Concerned patients who had co-morbid diseases that could affect serum NET levels (Patients with a history of atherosclerosis or previous cardiovascular disease, smokers, patients suffering from condition that affect lipid profile as Diabetes Mellitus, hypothyroidism, liver or kidney disease, Cushing syndrome, obesity (body mass index > 30), patients with dyslipidaemia or receiving medications affecting lipid metabolism, such as lipid lowering drugs, beta blockers, oral contraceptive, thyroxin and vitamin E, patients with other autoimmune diseases) were excluded from the study

Written informed consent was obtained from all the participants of the study. The study was approved by the Ethics Committee of Faculty of Medicine, Tanta University with approval code: 34556/3/21**.**

All tests were explained to patients and control subjects before having their written informed consents to participate in this study.

All patients were subjected to: Complete history taking, Complete Musculoskeletal examination including assessment of disease activity by SLEDAI Score, complete laboratory investigations including (Antinuclear antibody (ANA) by ELISA, Anti double stranded DNA antibodies (anti-ds DNA) by ELISA, Complement C3, and C4 by radial immunodiffusion technique, ESR by westergreen method, Complete blood count, Urine analysis. Especially for proteinuria, RBCS casts, Lipid profile (LDL.HDL, total cholesterol, triglycerides).

Neutrophil extracellular trap measurement: by sandwich ELISA, utilizing human MPO ELISA kit [9].

Common Carotid intema media thickness(CIMT) is measured by Using a B mode ultrasound with 7.5-10 MHz linear phased transducer with the patients lying supine and their neck extended and turned away from the side being examined [10].

Statistical analysis

Statistical analysis was done by using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp) Qualitative data were described using number and percent. The Shapiro-Wilk test was used to verify the normality of distribution Quantitative data were described using range (minimum and maximum), mean, standard deviation, median and interquartile range (IQR). Significance of the obtained results was judged at the 5% level [11].

Results

Regarding patients' characteristics, Comparison between both groups showed insignificant differences as regard to demographic data. Regarding SLE- DAI, all patients were active with very high activity found in 57.5% of patients. Table 1

There was significant difference between both groups as regard ANA, Anti-dsDNA, C3, C4, ESR (1st hour), CRP, 24 hour protein in urine, urine albumin-creatinine ratio, urinary casts, HB level, TC, TG and LDL, while there was no significant difference between both groups as regard leucocytes, platelets and HDL. Table 2

Serum level of NET and CIMT were significantly higher in SLE patients comparative to controls. Table 3

Fig. (1-2)**:** showed CIMT in a case of control group (normal) and in SLE patients (increased)**.**

There was significant correlation between NET and duration of the disease, TG and SLEDAI in SLE patients. Table 4 and significant correlation between IMT and age, duration of the disease, TC, NET and SLEDAI in SLE patients. Table 5.

Table 1: Demographic and clinical data of SLE patients and control

	Patients n=(40)		Control n=(40)		t	P
Age						
Min.-Max.	18.0-55.0		26.0-50.0		0.599	0.552
Mean ± SD	32.08 ±9.32		33.10±5.52			
Sex	No.	%	No.	%	x^2=	0.166
Male	4	10.0	0	0.0	4.211	
Female	36	90.0	40	100.0		
BMI (kg/m2)						
Min-Max.	24.20-29.0		20.50-29.0		1.803	0.077
Mean ± SD	26.85±1.18		26.09±2.36			
Duration of the disease						
Min-Max.	1.0-12.0		-			
Mean ± SD	6.21±3.59		-			
Total SLEDAI Score						
Min-Max.	8.0-32.0					

Mean ± SD.	19.08 ± 5.86				
Grades of activity					
Mild	0	0	-		
Moderate	1	2.5	-		
High	16	40.0	-		
Very high	23	57.5	-		

BMI: Body mass index
SLE-DAI: Systemic lupus erythematous disease activity index

Table 2: Laboratory data of SLE patients and control

	Patients (n=40)		**Control (n=40)**		**IT**	P
ANA (Iu/m1):						
Min-Max.	80.0-216.0		٣,٥٠–7.35		0.0	<0.001*
Mean ± SD	91.35 +24.11		6.23+1.26			
Anti-dsDNA (Iμ/m1)						
Min-Max.	3.60-300.0		5.50-22.10		0.0	<0.001*
Mean ± SD	137.62+98.80		13.27+5.46			
C3(mg/d1):						
Min-Max.	1.10-177.0		89.90-136.50		246.50	<0.001*
Mean ± SD	75.34+39.90		113.69+14.83			
C4(mg/d1)						
Min-Max	0.32-43.10		17.90-33.0		124.0	<0.001*
Mean ± SD	13.18+8.33		27.83+4.26			
ESR						
Min-Max.	12.0-170.0		6.0-14.0		6.0	<0.001*
Mean ± SD	65.43+37.79		8.23+1.95			
CRP						
Min-Max.	6.0-14.0		1.0-5.0		6.0	<0.001*
Mean ± SD	8.23+1.95		3.40+0.62			
24 hour protein in urine						
Min-Max.	80.0-1500.0		30.0-120.0		111.0	<0.001*
Mean ± SD	454.29+363.05		84.33+22.15			
Urine A/C ratio						
Min-Max.	L80-637.30		012-0.18		0.0	<0.001*
Mean ± SD	N, 1+104.26		0.15+0.02			
Urinary casts	**No**	**Vo**	**No**	**%**	**X2**	
Nil	29	72.5	34	85	3.353	0.001*
Granular +	7	17.5	5	12.5		
Granular+2		5	1	2.5		
Hyaline +2	'	5	0	0.0		

HB (g/dl)			U	
Min-Max.	7.60 - 13.0	10.90 -14.40	7.466	<0.001*
Mean ± SD	10.45+1.52	12.54±0.91		
WBCs (cells/mm^3)				
Min.-Max.	1.0 -12.40	4.60 - 10.90	0.158	0.875
Mean ± SD	6.1919 75	6.20±1.88		
Platelet cells/mm^3				
Min-Max.	35.0 - 430.0	164.0 - 448.0	1.038	0.303
Mean ± SD	250.88*86.05	269.48173.81		
Total cholesterol (up to 200 mg/d1)				
Mm-Max.	190.0 - 332.0	98.0 -182.0	U=141.50	<0.001*
Mean ± SD.	170.76 37.92	122.51121.65		
Triglycerides (60-169 mg/d1)				
Min-Max.	50.0 - 302.0	66.0 -142.0	U=544.0	0.014*
Mean ± SD.	123.08 ± 53.7S	92.381 15.28		
LDL-C (less than 130mg/d1)				
Min-Max.	39.40 - 251.30	78.0 -177.0	U=156.0	<0.001*
Meant ± SD.	156.67 t 55.21	92.92 ± 18.58		
HDL-C(35-55 mg/d1)				
Min-Max.	40.50 - 60.90	41.0 - 65.0	t=.0.593	0.555
Mean ± SD.	51.76 t 5.81	52.64 ± 7.34		

ANA: Anti-nuclear antibody. Anti-ds DNA: anti double stranded DNA antibody. C*:* complement. ESR: erythrocyte sedimentation rate. CRP: C reactive protein. A/C: albumin/ creatinine, HB: haemoglobin, WBC: white blood cells LDL: low density lipoprotein. HDL: high density lipoprotein.

Table 3: Comparison between the two studied groups according to NET (HMP) and CIMT

NET (HAM)	Patients (N =40)	Control (N =40)	U	P
Min-Max	2.16-36.11	0.18-5.60	4.203	< 0.0001*
Mean ± SD.	12.13 ± 10.76	2.77 ± 10.76		
IMT (< 0.9 MM)				
Min-Max	0.50-10	0.40-0.70	4.203	< 0.0001*
Mean ± SD.	0.71 ± 0.16	0.59-0.09		

NET: Neutrophils Extracellular Traps HMP: Human Myeloperoxidase
IMT: Intema media thickness

Table 4: Correlation between NET (HMP) and different parameters in patients group (n= 40)

NET (HMP)		
	r	**p**
Age (years)	0.294	0.066
BMI (kg/m2)	-0.018	0.912
Duration of disease	0.322*	0.042*
UP	0.011	0.944
WBCs	0.056	0.732
Platelets	0.098	0.591
ESR	0.0965	0.561
CRP	0.0626	0.701
ANA	-0.0487	0.077
Anti-dsDNA	-0.0167	0.919
C3	-0.0716	0.661
C4	-0.111	0.496
Urine A/C ratio	-0.085	0.601
24hour protein in urine	-0.192	0.236
Casts	0.026	0.874
T.0	0.067	0.681
T.G	-0.420'	0.007*
HDL-C	0.156	0.336
LDL-C	0.062	0.702
SLEDAI	0.486'	0.001*

HB: haemoglobin, WBCs: White blood cells, ESR: erythrocyte sedimentation rate, CRP: C reactive protein, ANA: anti-nuclear antibody, Anti dsDNA: anti double stranded DNA, C: complement. TC: total cholesterol. TG :triglycerides. HDL: high density lipoprotein. LDL: low density lipoprotein, SLEDAI: Systemic lupus erythematous disease activity index.

Table 5: Correlation between IMT and different parameters in patients group (n= 40)

IMT		
	r	**p**
Age (years)	0.510'	0.001*
BMI (Kg/m2)	0.101	0.536
Duration of disease	0.322*	0.043*
Up,	-0.205	0.204
WBCs	0.181	0.264
Platelets	-0.170	0.295
ESR	0.055	0.735
CRP	-0.098	0.547
ANA	-0.034	0.833
Anti-dsDNA	0.0120	0.728
C3	-0.121	0.458
C4	0.034	0.837
Urine A/C ratio	-0.010	0.952
24 hour protein in urine	0.056	0.749
Casts	0.168	0.298
T.0	0.481	0.002*
T.0	-0.080	0.623
HDL-C	-0.083	0.611
LDL-C	0.069	0.674
NET (HMP)	0.385*	0.014*
SLEDAI	0.411*	0.008*

HB: haemoglobin, WBCs: White blood cells, ESR: erythrocyte sedimentation rate, CRP: C reactive protein, ANA: anti-nuclear antibody, Anti dsDNA: anti double stranded DNA, C: complement. TC: total cholesterol. TG :triglycerides. HDL: high density lipoprotein. LDL: low density lipoprotein, SLEDAI :systemic lupus erythematous disease activity index.

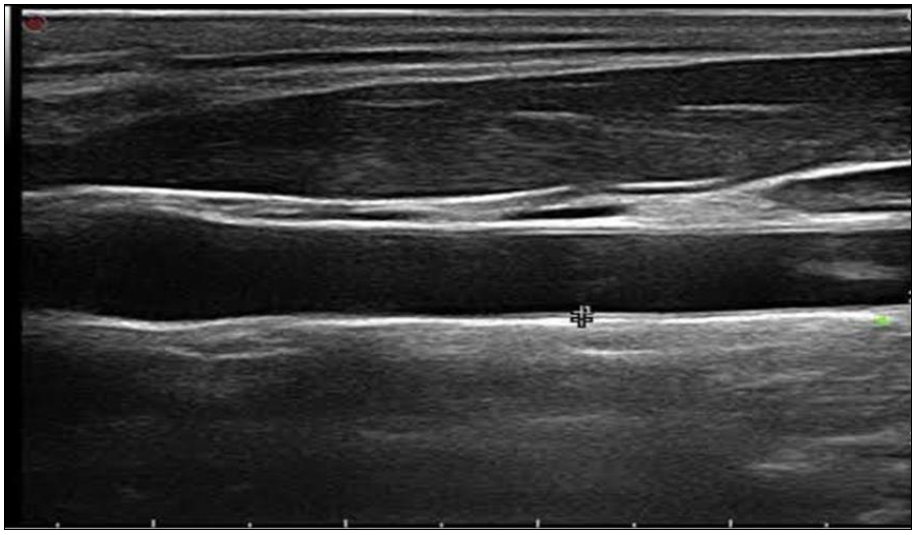

Fig 1: Carotid ultrasonography of a control case showing normal IMT (IMT =0.60 mm).

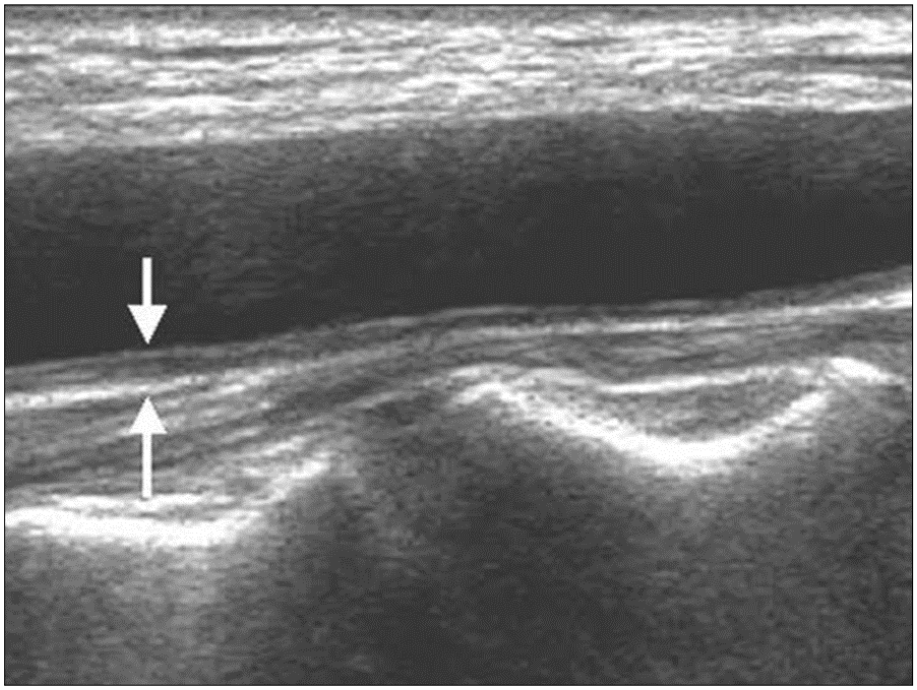

Fig 2: Carotid ultrasonography of a patient with systemic lupus showing increased IMT (IMT =1.1 mm)

Discussion:

Neutrophils extracellular traps (NET) have been suggested as an important source of autoantigens, with SLE patients having high levels of antibodies binding to NET, including anti-dsDNA and antihistone antibodies. The released NET engage DNA receptors, Toll-like receptor (TLR) and cyclic GMP–AMP synthase (cGAS) to induce type I interferons (IFN) and the key cytokines in SLE pathogenesis [12].

In our study, there was significant difference between SLE patients and controls as regard NET (HMP) (p<0.001) with mean NET (HMP) (12.13±10.76). (Table 3)

This is in agreement with Stanley *et al*. (2020) [13], who assessed true levels of NET in plasma samples from 4 well characterized cross-sectional SLE cohorts, as well as 1 longitudinal cohort and confirmed that levels of NET were markedly elevated in patients with SLE as compared to healthy individuals even at the time of LDA. The occurrence of neutrophil activation and cell death even at LDA is intriguing, and suggests that current treatment strategies, while reducing clinical symptoms, may not be sufficient to prevent low grade chronic inflammation and subsequent organ damage.

Also Ivica *et al*. (2019) [14], demonstrated that SLE patients have decreased NETolytic activity, leading to increased levels of various NETs-associated markers (DNAse I, MPO activity, anti-MPO antibodies and cfDNA), which correlate with anti-dsDNA antibodies in drug-naïve SLE patients.

On the same context Telles *et al*. (2010) [15], showed elevated MPO levels in several infammatory conditions.

Leffler *et al*. (2013) [16], found that a decreased ability to degrade NETs was a rather common feature of SLE with 41% of the patients at least once, but commonly more than once, displaying a reduced ability to degrade NETs.

Denny *et al*. (2010) [17], described in details that, the phenotype of a low-density neutrophil subset that seems to be present in higher numbers in SLE patients with distinct clinical manifestations. LDGs have preserved neutrophil function overall, but they display impairments in phagocytic potential, have a pro inflammatory phenotype, and induce vascular damage, suggesting that they may contribute to the

accelerated atherosclerosis observed in SLE patients.

Leffler *et al*. (2012) [18], studied 94 SLE patients and showed that a substantial fraction of SLE patient sera failed to degrade NETs. This phenomenon appeared to be a variable characteristic of each patient, as failure to degrade NETs was more common during flare than when the same patients were in remission.

Hakkim *et al*. (2010) [19], showed in the German cohort that NET degradation activity was impaired in a subset of lupus patients because of the presence of DNase1 inhibitors or anti-NET antibodies, and NET non-degraders had significantly higher anti-dsDNA antibody titers than NET degraders

Maarten *et al*. (2018) [20] showed that plasma of SLE, SLE+APS patients induces NET release, which is associated with ANAs in PAPS and anti-dsDNA autoantibodies and the IFN signature in SLE patients. They observed a rapid release of NETs (within 30min) upon exposure to patient plasma in both SLE and PAPS patients. They speculate that the content of NETs could differ between SLE and APS, since NET release was differentially associated with the IFN signature in SLE and APS.

In contrast to our results Morgan *et al*. (2005) [21] demonstrated MPO levels were significantly decreased in SLE patients compared with controls. A trend toward decreased MPO levels with increasing disease activity, as indicated by low versus high SLEDAI scores and by negative versus positive anti-dsDNA antibody levels, was observed, but these differences were not statistically significant**.**

These contrasting results could be attributed to the size of the samples, the type of study (prospective or cross-sectional), differences in the disease type (established or early), or to differences in the disease activity. Patients in remission or with controlled disease compared to patients with active disease. The observed discrepancy in the above study could be attributable to their small number of patients and controls (19 SLE and 11 controls).

In our study there was significant difference between SLE patients and control as regard IMT, which reflect sub clinical cardiovascular disease and risk for thrombosis (P <0.001) with mean IMT (0.71±0.16) (Table 3)

This is coincide with Ammirati *et al*. (2014) [22], who studied 50 SLE patients without any disease relapse within the last 3 months and 50 control, a mean CCA-IMT increase of 0.05 mm was observed in SLE patients when compared to a sex- and age-matched population, and assuming a 0.0079-mm physiological annual increase in CCA-IMT would suggest that SLE patients appear to be approximately 6 years older in terms of the mean CCA-IMT. This difference, which is > 4 standard deviation (SD), coupled with a recent meta-analysis of individual participant data, showing an odds ratio of 1.16 of MI and stroke per1-SD increasing CCA-IMT, supports the potential clinical significance of this finding.

This is also in agreement with Ahmad *et al*. (2007) [23] who studied 200 women with SLE and 100 controls and found that sub clinical atherosclerosis develops at an earlier age in SLE patients. As well as risk factors identified, they also noted that plaque developed at a lower IMT in SLE patients. This supports the hypothesis that the accelerated process of plaque development may be driven by different factors than those that chiefly determine IMT progression.

In our study, there was strong significant positive correlation between NET and SLEDAI as our patients were all active, with (P <0.001) with mean NET in moderate and high SLEDAI (6.37±5.08), mean NET in very high SLEDAI (6.39±11.91) and mean NET in total SLEDAI (19.07±5.86) (Table 4)

Our results were matched with Stanley *et al*. (2020) [13]. Although they were unable to find any direct correlation between SLEDAI and levels of NET, they assessed that NET levels reflect a propensity of patients with SLE to develop a high SLEDAI, levels of NET were surprisingly not associated with concurrent disease activity. Instead, levels of NET associated with worsening of disease within 3 months. This observation is significant, as it could provide opportunities for preventive treatment and/or closer monitoring of patients at high risk of flare. It also provides insight into the pathogenesis of SLE, suggesting that NET formation may be an early event, occurring prior to apparent clinical disease. Therefore NET formation may be an ideal therapeutic target, inhibiting disease at an early stage.

In contrast to our study, Telles *et al*. (2010) [15] could not find a correlation between MPO serum levels and SLE activity measured by SLEDAI score.

Bruschi *et al*. (2020) [24], demonstrated that serum NETs did not correlate with parameters of lupus activity, SLEDAI-2k.

In our study, there was significant correlation between duration of the disease, arthritis, TG and NET (HMP) but there was no significant correlation between NET and the rest of parameters. (Table 4).

Our results were matched with Hannes *et al*. (2021) [25] who showed relationship between high neutrophil cell count and NET and high concentrations of blood triglycerides as a predisposition of the immune overreaction in critical illness due to COVID-19. They also identified the enzyme CDK6 as a potential drug target to prevent in high risk individuals with high neutrophil cell count and triglycerides the immune overreaction in critical illness due to COVID-19.

In our study, there was significant correlation between age, duration of the disease, TC and IMT, there was no significant correlation between IMT and the rest of parameters. (Table 5)

On the same context Caie *et al*. (2014) [26] demonestrated that carotid IMT was significantly correlated with age and total cholesterol levels (p=0.017).

In our study there was strong positive correlation between NET and IMT. (Table 5).

This is in agreement with Stanley *et al*. (2020) [13], who assessed that levels of NET were associated with CVD, in particular arterial thrombosis. NET have been recognized as proatherogenic and prothrombotic in their interaction with, and activation of, platelets and endothelium **.**Further, released NET and NET-containing enzymes (e.g., MPO) oxidize high-density lipoprotein to accelerate atherosclerotic processes.

This also in agreement with Fuchs *et al*. (2010) [27] who showed that NETs interact closely with fibrin strands in the thrombus, thus potentially influencing thrombus organization and stability. Given the procoagulant activity of nucleic acids and polyphosphates.

Conclusion

1. NETs have been involved in SLE immunopathogenesis and correlated with clinical and laboratory parameters of disease activity.
2. NETs and CIMT could be attributed as good predictors

for subclinical cardiovascular risk, that may contribute to prevent cardiovascular disease by timely treatment in SLE patients.

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