Cardiovascular disease and serum defensin levels in systemic lupus erythematosus

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Abstract

Objectives

To analyse if defensins, immunomodulatory peptides involved in angiogenesis and elevated in the sera of systemic lupus erythematosus (SLE) patients, relate to cardiovascular disease in SLE.

Methods

Serum levels of the defensins human beta defensin 2 (hBD2) and human neutrophil peptide (HNP) of 72 SLE patients were determined by ELISA at baseline. Cardiovascular risk factors and the occurrence of cardiovascular events (CVE: stroke, claudication, angina pectoris, myocardial infarction) were recorded over 6 years. Intima media thickness of the carotid arteries (CIMT) was measured by ultrasound in 42 patients at baseline and at 4 years. Normally distributed log-transformed defensin levels (log-hBD2 and log-HNP) were used for statistical analysis.

Results

SLE patients who experienced a CVE had significantly higher log-hBD2 values and a likelihood-ratio for CVE of 2.23 when levels increased above 3.3 log(ng/ml). Using binary logistic regression analysis, log-hBD2 significantly contributed to a model also incorporating the number of traditional cardiovascular risk factors (dyslipidemia, hypertension, positive family history, age, smoking) as explanatory variables for the incidence of cardiovascular events. Moreover, SLE patients with progressive CIMT showed increased log-hBD2 and log-HNP values. Both defensin-levels also showed some correlation to the plaque stadium at baseline (hBD2: r^2 0.10; HNP r^2 0.12). Neither log-hBD2 nor log-HNP were correlated to traditional cardiovascular risk factors.

Conclusion

HNP and especially hBD2 may be indicators of progressive cardiovascular disease in SLE.

Key words

systemic lupus erythematosus, cardiovascular disease, antimicrobial peptide, defensin

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Introduction

Systemic lupus erytheamtosus (SLE) is a complex autoimmune disorder characterised by the presence of autoantibodies and various clinical manifestations like rashes, photosensitivity, arthritis, and haematologic abnormalities (1). Despite the occurrence of serious organ involvement like glomerulonephritis in a number of patients, cardiovascular mortality and morbidity due to arteriosclerosis become more important with increasing duration of the disorder (2). In fact, after a 5-year observation in the "Euro-Lupus" Project, cardiovascular events (CVE) became the main cause of death in SLE patients (3). In order to develop strategies to lower cardiovascular mortality in SLE, it is desirable to identify patients with an increased risk for CVE. Next to traditional cardiovascular risk factors (CVRF), SLE inherent mechanisms which are currently largely unknown contribute to arteriosclerosis (4-6). In an attempt to identify such a factor, we focused on the defensins human neutrophil peptides (HNP) and human beta defensin 2 (hBD2). Defensins are small cationic peptides and constitute an important part of the innate immune system by exerting a direct bactericidal effect (7). Furthermore, they modulate various functions of the adaptive and innate immune system (8, 9), many of which are thought to be involved in SLE pathogenesis (1, 10). While HNP promotes apoptosis, hBD2 and HNP attract dendritic cells and render antigen presentation more effective by upregulation of costimulatory molecules in both antigen-presenting cells and T-cells. Additionally, T-cells are chemoattracted and IFNγ-release by T-cells is augmented by HNP and hBD2 (8, 9). Consequently, specific antibody-production was shown to be more effective when delivery of antigens was accompanied by HNP or hBD2 in a mouse model (11). In SLE patients, hBD2 and HNP are increased in the serum and show some correlation to disease activity (12, 13). Their involvement in arteriosclerosis has been recently suggested by the findings that elevated HNP levels in patients with peripheral arterial disease and intermittent claudication confer a higher risk for cardiovascular death (14) and that hBD2 promotes angiogenesis *in vitro* by a mechanism independent from vascular endothelial growth factor (15). Thus, we analysed serum levels of hBD2 and HNP in relation to cardiovascular disease progression in SLE patients, represented by the occurrence of CVE during follow-up (*e.g.* stroke, new claudication, angina pectoris, myocardial infarction), the degree of atherosclerotic plaques and progression of the intima-media-thickness of the carotid arteries (CIMT).

Material and methods

Patients and assessment

Eighty SLE patients according to the 1982 ACR criteria were prospectively enrolled into the study and followed up for 6 years. Traditional CVRF (dyslipidemia, hypertension, positive family history, age, smoking), defined according to recommended cardiovascular risk stratification by the Adult treatment panel III (ATPIII) and national guidelines (16, 17), weight, and routine laboratory parameters were assessed initially and every 2 years during regular visits to our SLE outpatient clinic. The occurrence of CVE (stroke, new claudication, angina pectoris, myocardial infarction) was recorded according to a standardised questionnaire and confirmed by medical records. At baseline and at 4 years, ultrasound of the carotid arteries was carried out with MyLabTM Gold (Esaote, Köln, Germany) using a 10-MHz linear-array probe by an experienced physician (OS) blinded to the patients' hBD2 and HNP values. Initial measurement and 4-year follow-up measurements were available from 42 individuals. Patients lay supine in a dark and quiet room with the head in midline position. The probe was adjusted to parallel the near and far wall, obtaining a maximised diameter of the lumen. High resolution B-mode pictures were digitally recorded. The extent of the plaques was scored according to their length along the vessel wall (0=no plaque; 1=≤3 mm; 2=3–6 mm; 3=<6 mm). CIMT was assessed at 1 and 3 cm distance from the bulbus on both sides and averaged. On the basis of previous observations about

Table I. Patient characteristics.

Patient characteristics		
n.	72	
Age (years ± SD)	37.46 ± 1.4	
% female	86.1%	
Years since diagnosis	8.65 ± 7.3	
Laboratory values	mean ± SD	normal range
RBC (/µl)	4.4 ± 0.47	3.9–5.0
WBC (/µl)	5.4 ± 2.12	4.0–11.0
Thrombocytes (/nl)	230 ± 102	150–400
CRP (mg/dl)	0.68 ± 1.23	<0.5
ANA-IF (median)	1:640	<1:80
dsDNA-ELISA (U/l)	81.45 ± 166.41	<80 U/l
C3c (mg/dl))	88.87 ± 24.47	90–180
C4 (mg/dl)	13.38 ± 7.71	10–40
Disease activity/damage parameters (mean ± SD)		
SLAM	7.43 ± 4.86	
SLICC	1.54 ± 1.83	
Organ involvement (n (%))		
Renal	30 (41.7)	
Central nervous system	15 (20.8)	
Arthritis	11 (15.3)	
Vasculitis	7 (9.7)	
Thrombocytopenia	4 (5.6)	
Rash	2 (2.8)	
Haemolysis	1 (1.4)	
Myositis	1 (1.4)	
Immunosuppressive medication (n. [%] or mean ± SD)		
Glucocorticosteroids	43 (59.7)	
Current prednisone dose	11.10 ± 10.00 mg	
Average prednisone dose	7.19 ± 12.69 mg	
Antimalarials	38 (52.8)	
Azathioprine	15 (20.8)	
Cyclophosphamide	9 (12.5)	
Mycophenolate mofetil	5 (6.9)	
Cyclosporine	3 (4.2)	
Cardiovascular risk (mean ± SD or frequency: n[%])		
Current SBP (mmHg)	120.82 ± 16.32	
Current DBP (mmHg)	77.21 ± 11.42	
Weight (kg)	69.14 ± 17.81	
Cholesterol (mg/dl)	195.25 ± 41.39	
LDL (mg/dl)	134.36 ± 32.86	
TG (mg/dl)	140.96 ± 111.03	
HDL (mg/dl)	58.07 ± 16.00	
CIMT (mm)	0.701 ± 0.1753	
Hypertension	23 (31.9)	
Smoking	13 (18.1)	
Dyslipidemia	12 (16.7)	
Diabetes	3 (4.2)	
Family history	11 (15.3)	
Total CVRF	0.86 ± 1.05	

Characteristics, laboratory values and disease extend of patients with systemic lupus erythematosus at baseline. Organ involvement was defined according to ACR criteria. RBC: red blood cell count; WBC: white blood cell count; CRP: C-reactive-protein; ANA-IF: antinuclear antigen immunofluorescence; dsDNA: double-stranded DNA; SLAM: Systemic Lupus Erythematosus Activity Measure; SLICC: Systemic Lupus International Collaborative Clinics Damage index; renal: lupus nephritis; average prednisone over study period; SBP: systolic blood pressure; DBP: diastolic blood pressure; LDL: low density lipoprotein; TG: triglycerides; HDL: high density lipoprotein; CIMT: carotid intima media thickness; CVRF: traditional cardiovascular risk factors.

the reliability of CIMT measurements and having excluded inter-observer variances by study design, an increase in CIMT of 0.05 mm was considered clinically meaningful and labelled as "progressive" (18). Measurement of CIMT is a well established surrogate parameter for atherosclerosis and predictor of CVE (19), and has been used as an endpoint in some studies (20, 21). Eight patients had to be excluded due to incomplete data recording during follow-up. All patients gave their informed consent and the study was approved by the local ethics committee.

Measurement of defensins

Serum samples of all patients were collected at baseline and frozen at -20°C prior to the assay. Human HNP1-3 ELISA Test Kit (Hycult biotechnology, Uden, The Netherlands, lower detection limit 165 pg/ml) and human β-defensin 2 ELISA Kit (Phoenix Pharmaceuticals, Burlingame, California, USA, lower detection limit 7.815 pg/ml) were used to measure serum concentrations of HNP and hBD2, respectively, as described before (12).

Statistical analysis

All tests were performed by IBM SPSS Statistics Version 19 (SPSS, Chicago, Illinois, USA). The logarithms of HNP and hBD2 (log-HNP and log-hBD2) showed normal distribution according to Kolmogorov-Smirnov test. Hence, log-transformed values were used for further analysis instead of HNP and hBD2, which showed skewed distribution. Bivariate linear regression analysis with log-HNP and log-hBD2 was conducted by Pearson's correlation coefficient for parametric data and Spearman's rank correlation for non-parametric data as appropriate. To analyse the contribution of log-defensin levels to the prediction of CVE, binary logistic regression analysis was used (22). For comparison of patients regarding CVE or progressive CIMT, independent *t*-test was applied. Receiver-operating-characteristics (ROC) analysis was used to estimate the discriminative function calculating the area under the curve (AUC) and a cut-off point with maximum likelihood-ratio for CVE. In all tests, $p<0.05$ was considered significant.

Results

Patient characteristics

After the exclusion of 8 patients in whom CVE were not recorded according to the study protocol, 72 patients were included in the analysis. Patient

Table II. Patients with cardiovascular events.

Patient	Sex	Cardiovascular event	Age at event (years)	Years since SLE diagnosis
1	F	Angina pectoris	45	16
2	F	Myocardial infarction, TIA	41	26
3	F	Claudication	39	4
4	M	Claudication	37	7
5	M	Angina pectoris	63	15
6	F	Stroke, TIA, angina pectoris	57	17
7	F	Stroke	30	9
8	M	Angina pectoris	49	5
9	M	Angina pectoris	55	7
10	F	Angina pectoris	40	7
11	F	Claudication	22	5
12	F	TIA	26	3
13	F	Myocardial infarction	40	16

characteristics, laboratory values, organ involvement, current immunosuppressive therapy and known CVRF are summarised in Table I. In order to analyse if log-defensin levels may be related to any of the continuous or ordinal parameters, including the disease activity/damage parameters SLAM (Systemic Lupus erythematosus Activity Measure) and SLICC (Systemic Lupus International Collaborative Clinics damage index), bivariate regression was used. Thereby, log-hBD2 correlated to the CRP-value (r^2 0.228, p=0.045), and log-HNP to the variable "time since diagnosis" which describes the number of years from SLE diagnosis to the point of inclusion into the study (r^2 -0.249, p=0.030). Of note, there was no correlation to the number of traditional CVRF, lipid-levels, weight. Thirteen patients with a CVE were identified (Table II). The mean age at the occurrence of CVE was 41.8±12 years. CVE occurred after a mean period of 10.5±6.8 years after the diagnosis of SLE.

Defensins and cardiovascular events

In the next step, we determined if patients with a new CVE had higher defensin-levels at baseline. While there was no difference in the levels of log-HNP, log-hBD2-levels in SLE patients with a CVE (n=13) were significantly higher (3.73 *vs*. 3.40 log[ng/ml], p<0.05, Fig. 1a). According to ROC analysis, log-hBD2 was moderately discriminative (AUC 0.68). At the cut-off point (3.3 log[ng/ml]), the likelihood-ratio for CVE was 2.23 with a specificity of 85% and sensitivity of 34%. (data not shown). Non-log-transformed values for hBD2 and HNP (median [range]) were 4,866 pg/ml (869–22,824) and 282 ng/ml (73–632) in patients with CVE and 2,831 pg/ml (31–19,134) and 252 ng/ml (79–1,227) in patients without CVE, respectively.

Next, we hypothesised that log-hBD2 values could contribute to the recommended risk stratification of the ATPIII and national guidelines consisting of the number of traditional CVRF (16, 17). Since the occurrence of new CVE is a dichotomous variable, binary logistic regression analysis was employed. As demonstrated in Table III, a model was created expressed in the equation: predicted logit of (CVE) =-8.953 +0.775*CVRF+1.835*log-hBD2. The likelihood-ratio test demonstrates that the model incorporating both CVRF and log-hBD2 is more effective in predicting CVE than the null model. Furthermore, Wald's χ^2 statistic shows that both CVRF and log-hBD2 are significant predictors for CVE within the model. The Hosmer and Lemeshow test was insignificant, suggesting that the logistic model fits the actual outcome (*i.e*. CVE) well. In order to illustrate the impact of log-hBD2 and CVRF on the probability of a CVE, SLE patients were divided into 4 groups according to the number of traditional CVRF and the estimated probability of a CVE was plotted against log-hBD2 of the patients (Fig. 1b). In the present cohort, no additional benefit was derived by including either lipid-levels, weight, CIMT, anti-β2-glycoprotein-antibodies, anti-cardiolipin-antibodies, the mean dose of steroids, or baseline SLAM or SLICC into the model (data not shown).

Subsequently, the validity of the predicted probabilities of the model for a CVE was assessed against the actually observed CVE in the cohort using a cut-off of 0.50. As can be seen in Table IV, 81.9% of events or non-events were correctly predicted, which underlines a distinct improvement over the chance level. Furthermore, the prediction of SLE patients who did not experience a CVE was more accurate than that for those who did (sensitivity 30.77% and specificity 93.22%).

Defensins and carotid ultrasound

CIMT is an established surrogate parameter for CVE and arteriosclerosis (20, 21). We therefore analysed if patients with a progression in CIMT showed elevated defensin-levels, as suggested by the above results. By this means, both log-HNP and log-hBD2 were elevated in SLE patients with progressive CIMT (Fig. 1c). However, no correlation was noted between log-defensin levels and CIMT-measurements.

In 14 patients, manifest plaques were detected in carotid ultrasound at baseline, a known risk factor for CVE (23). Thus, we analysed the relation between defensin levels and the extent of plaques. Bivariate regression analysis was employed and both log-HNP and log-hBD2 showed some correlation to the stadium of plaques (p<0.05, r^2 0.12 and 0.1, respectively).

Discussion

Autoimmune disorders are increasingly recognised to confer an increased risk for cardiovascular events due to increased arteriosclerosis. Patients with SLE, for instance, were estimated to have a relative risk for arteriosclerosis of 6.0 (5). The pathogenesis is multifactorial. Besides traditional CVRF, like smoking and hypertension, chronic inflammation and a range of disease, inherent mechanisms contribute to the excess observed risk (4). Recently, the defensins hBD2 and HNP have been repeatedly shown to be elevated in the sera of SLE patients (12, 13, 24, 25). On the one hand, defensins function as

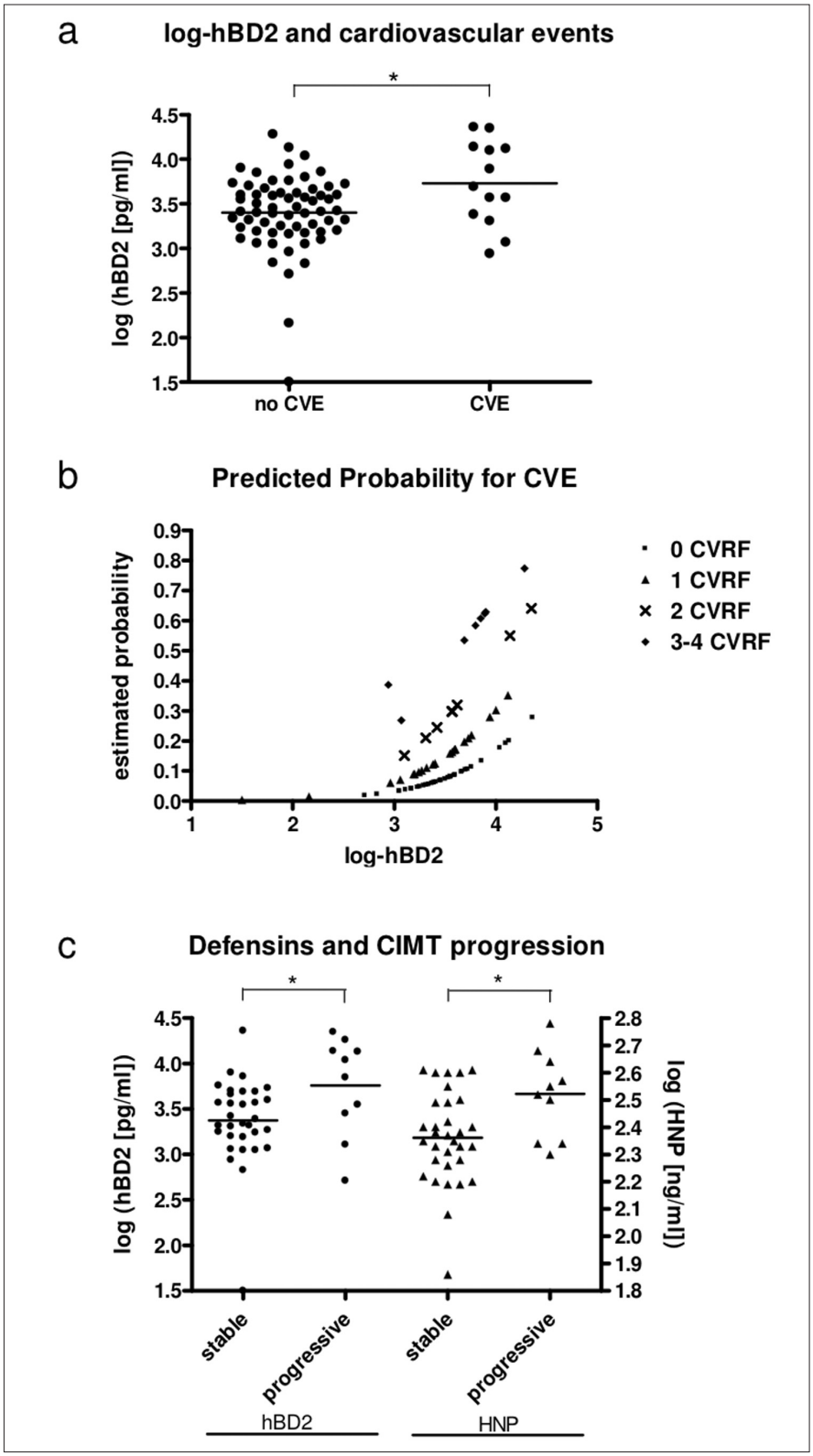

Fig. 1. a. Comparison of log-transformed beta-defensin 2 levels (log-hBD2) in serum of SLE patients who did (n=13) and did not (n=59) experience a cardiovascular event (CVE) during a 6-year follow-up. * $p<0.05$ by *t*-test. **b**. Impact of log-transformed beta-defensin 2 levels (log-hBD2) and the number of cardiovascular risk factors (CVRF) on the predicted probability of cardiovascular events (CVE) according to a model derived by binary logistic regression. **c**. Comparison of log-transformed beta-defensin 2 (log-hBD2) and human neutrophil peptide levels (log-HNP) between SLE patients who did (n=14) or did not (n=28) show progressive carotid intima media thickness (IMT) in ultrasonographic evaluation in the course of 4 years. *, $p<0.05$ by *t*-test.

immunomodulators connecting innate and adaptive immune responses and increase antibody production, possibly by enhancing effective antigen-presentation (8, 9). On the other hand, there is evidence that both HNP and hBD2 affect angiogenesis or endothelial cell function *in vitro* (15, 26). Moreover, HNP has already been shown to be a risk factor for CVE in patients with peripheral arterial disease (14). Thus, we arrived at the hypothesis that defensins are potential pathogenetic factors contributing to increased atherosclerotic processes observed in SLE patients.

The present results indeed show that patients with an increased hBD2 value at baseline are more likely to experience a CVE. Moreover, hBD2 and the number of traditional CVRF could be combined into a model that predicted the presence or absence of CVE correctly in almost 82% of cases. Given the high specifity (93.22%) and low sensitivity (30.77%), the present model suggests that log-hBD2 may especially be useful to identify SLE patients at a lower risk for CVE (*e.g.* those with a log-hBD2 of 3 or less, Fig. 1c).

In the present study, the definition of traditional CVRF was based on recommendations of the ATP III (16) as opposed to a broader interpretation. However, the inclusion of other risk factors (*i.e.* weight, CIMT, anti-cardiolipin antibodies, anti-β2-glycoprotein antibodies, CRP-values) into the model derived by binary logistic regression, did not significantly contribute to better predictions in our cohort. Interestingly, log-hBD2 correlated to CRP values, albeit with a small magnitude of effect (r^2 0.228). Chronic inflammation is now thought to be an important mechanism of ateriosclerosis (4). Indeed, CRP was identified to be a strong predictor of CVE (27). Increased hBD2 may likely reflect the activation of innate and adaptive immune responses in SLE with consequent inflammation which might explain its relation to the CRP-values. However, CRP did not independently correlate with CVE and could not substitute log-hBD2 in our model. In the present study, log-hBD2 retained its statistical association with the occurrence of CVE after the inclu-

Table III. Binary logistic regression analysis of 72 SLE patients for the incidence of cardiovascular events.

Predictor	β	SE β	Wald's χ^2	df	*p*-value	e^{β} (odds ratio)
Constant	-8.953	3.440	6.772	1	0.009	NA
CVRF	0.775	0.294	6.944	1	0.008	2.170
log-hBD2	1.835	0.932	3.880	1	0.049	6.266
Model evaluation			χ^2	df	*p*-value	
Likelihood ratio test			13.556	2	0.001	
Hosmer and Lemeshow test			6.11	8	0.635	

Table IV. Observed and predicted cardiovascular events by logisitic regression.

	Observed		Predicted
	Yes	No	% Correct
Yes	4	9	30.8
No	4	55	93.2
Overall % correct			81.9

Sensitivity = 4/(4+9)%=30.77%, specificity = 55/(4+55)%=93.22%, false positive = 4/(4+4)%=50%, false negative = 9/(9+55)%=14.06%. Cut-off: 0.50.

sion of traditional CVRF in our model and we found no correlation of log-hBD2 to CVRF, possibly implying an independent effect. The present results warrant follow-up studies with greater patient numbers enabling adjustment for more risk factors in order to unequivocally confirm the notion of log-hBD2 as an independent risk factor for the occurrence of CVE in SLE.

In addition to CVE, surrogate parameters were also assessed. Patients with an increase in CIMT had higher log-hBD2 and -HNP values, supporting the above observations. This finding is hampered by the fact that we surprisingly did not find a correlation between CIMT or CIMT-progression and CVE that might have been expected from larger studies in non-SLE patients (19). Furthermore, even though based on published data (18, 28), we somewhat arbitrarily classified patients as progressive with an increase in CIMT of >0.05 mm. This decision was based on the observations that an CIMT increase of 0.1 mm already augments the risk for acute myocardial infarction by 11% (28) and that differences in CIMT progression of <0.02 mm/years can be detected with 90% power (18). Although the extent of plaques is a less well studied surrogate parameter for CVE (23, 29, 30), we compared the size of plaques to log-defensin values and found some correlation to both log-hBD2 and -HNP, even though the magnitude of effect was small. This finding is consistent with the above notion of higher defensin-values in patients with an increased cardiovascular risk.

Taken together, hBD2, and to a lesser extent, HNP correlate to CVE and ultrasonographic surrogate parameters of arteriosclerosis in SLE. Their determination may permit to further stratify SLE patients according to the risk of future cardiovascular events.

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