Title: Low density granulocytes and monocytes as biomarkers of cardiovascular risk in Systemic Lupus Erythematosus

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Abbreviations list

Endothelial progenitor cells (EPC), low-density granulocytes (LDG), angiogenic-T cells (Tang), Th (T helper) lymphocytes, cardiovascular disease (CVD), interferon (IFN), carotid intima-media wall thickness (cIMT).

ABSTRACT

Objective: To evaluate the most relevant cell populations involved in vascular homeostasis as potential biomarkers of SLE cardiovascular disease (CVD).

Methods: Low-density granulocytes (LDG), monocyte subsets, endothelial progenitor cells (EPC), angiogenic-T cells (Tang), $CD4^{+}CD28^{null}$ and Th1/Th17 lymphocytes, and serum cytokine levels were quantified in 109 SLE patients and 33 controls in relation to the presence of subclinical carotid atheromatosis or CVD. A second cohort including 31 recent-onset SLE patients was also included.

Results: Raised monocytes and LDG counts, particularly those negative for CD16/CD14 expression (nLDG), as well as the ratio of monocytes- and nLDG-to-HDL-molecules (MHR and nLHR, respectively), were present in SLE patients with traditional risk factors or subclinical atheromatosis but not in those CV-free, thus revealing their value in the identification of patients at CV-risk, even at the disease onset. Accordingly, nLDG correlated positively with carotid intima-media thickness (cIMT) and with inflammatory markers (C-reactive protein and IL-6). A bias towards more differentiated monocyte subsets, related to increased IFNα and IL-17 serum levels, was also observed in patients. Intermediate monocytes were especially expanded, but independently of their CV involvement. Finally, $CD4^{+}CD28^{null}$, Th17 and Th1 were increased, the former two associated to cIMT, whereas EPC and Tang levels were reduced in all SLE patients.

Conclusions: Present study highlights the potential use of nLDG- and monocytes-to-HDL ratios as valuable biomarkers of CV-risk in SLE patients, even at diagnosis. The increased amounts of nLDG, monocytes, Th17 and senescent-$CD28^{null}$ subsets, coupled with reduced pro-angiogenic EPC and Tang-cells, could underlie the atheromatosis development in SLE.

Keywords: subclinical atheromatosis; low-density granulocytes (LDG); monocytes; HDL; endothelial progenitor cells; angiogenic T cells; systemic lupus erythematosus.

KEY MESSAGES

- Cells involved in endothelium homeostasis were evaluated as biomarkers of early SLE-CVD.
- $CD16^-$ LDG are increased in SLE patients with subclinical CVD, even at disease onset.
- $CD16^-$ LDG and monocyte-to-HDL ratios could be early CV-risk biomarkers in SLE.

INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by a dysregulation of the immune system that leads to chronic inflammation and subsequent tissue damage (1). In these patients, accelerated atherosclerosis and increased prevalence of cardiovascular disease (CVD) are well-known complications which cannot be completely explained by traditional risk factors or the use of corticosteroids (2,3). Thereby, the active immunological response, dysregulated cytokine profile and altered cell subsets usually observed in SLE have been proposed as important triggers of the premature atherosclerosis process that underlie CV disorders in these patients (2,4). Since endothelial dysfunction is considered an early step of atherogenesis that seems to be occurring in lupus patients before the presence of clinical symptoms of CVD, the identification of altered cells and molecules participating in the endothelial homeostasis at the preclinical stage could be useful for the implementation of preventive therapies. In this line, monocytes have been considered one of the cellular hallmarks of the atherosclerosis (5). Human circulating monocytes constitute a heterogeneous group, being the "classical" monocytes ($CD14^{+}CD16^{-}$) the major subset, whereas "non-classical" ($CD14^{low}CD16^{+}$) and "intermediate" ($CD14^{+}CD16^{+}$) populations represent the 10–20% of all circulating monocytes (6,7) and are considered more mature cells (8). Although $CD16^{+}$ subsets have been associated to various disease states (9,10), little is known about their pathogenic role in the accelerated atherosclerosis observed in SLE. Likewise, a subset of abnormal neutrophils present in the peripheral circulation of SLE patients and described as low-density granulocytes (LDG) has been proposed as an additional factor contributing to SLE disease and its endothelial damage though the production of "neutrophil extracellular traps" (NETs) (11–14). However, LDG represent a heterogeneous population, probably related to different origins. Actually, we have recently reported an enrichment of a specific LDG subset in patients with chronic kidney disease associated with vascular calcification (15). The presence and possible role as CV risk biomarkers of different LDG subsets in SLE are still unknown.

On the other hand, a crucial aspect to preserve endothelial integrity is maintaining the balance between endothelial damage and repair mechanisms. Vascular repair appears to be mediated by the recruitment of bone marrow–derived endothelial progenitor cells (EPC) to the site of endothelial injury (16) and it has been shown that a reduced number of circulating EPC is associated with an increased risk of CVD and preclinical atherosclerosis, hence reinforcing the role of EPC as biomarkers (17–19). Interestingly, EPC function over the endothelium can be modulated by two T cell populations with opposite effects on the vascular endothelium. Thus, functional angiogenic T cells (Tang), characterized as $CD3^{+}CD31^{+}CXCR4^{+}CD28^{+}$ (20), have been described as repairing promoters of damaged endothelium by cooperating with EPCs, so decreased Tang cell frequencies have been associated with vascular outcomes (21–23). By the contrary, $CD4^{+}CD28^{null}$ T cells, linked to immunosenescence, are able to promote direct endothelial damage (24,25). Hence, these cells are expanded in several conditions associated with chronic inflammation (26,27).

Given the role of the endothelium in the development of CV complications of SLE, the present study aims to analyze the balance of a variety of peripheral blood cell populations involved in its homeostasis as possible biomarkers of subclinical atheromatosis in SLE. To this end, cell subsets expected to present a pro-atherogenic effect on the vascular endothelium (monocytes, LDG, $CD4^{+}CD28^{null}$ and Th1/Th17 lymphocytes), as well as anti-atherogenic cells (EPC and Tang) were quantified in patients with established SLE and evaluated in relation to the presence of clinical or subclinical CVD. Finally, an additional cohort of recent-onset SLE patients were analyzed in order to confirm our results as early biomarkers of subclinical atheromatosis in SLE.

MATERIAL AND METHODS

Patients and controls

One hundred-nine SLE patients fulfilling the American College of Rheumatology (ACR) revised criteria for the SLE classification (28) and with at least 2 years of disease duration were sequentially recruited from the outpatient clinic of the Autoimmune Disease Unit (Hospital Universitario Central de Asturias, HUCA). Information on clinical manifestations, traditional CV-risk factors and CV events along the disease course was obtained after a retrospective review of their clinical records. Additionally, parameters of disease activity (anti-dsDNA titer and SLE disease activity index, SLEDAI) and treatments received over the previous 3 months were recorded at the sampling time. Thirty-three sex and age-matched volunteers from the same geographic area, without any pathology or treatment, were enrolled as controls (Table 1).

Additionally, a second cohort of patients at SLE diagnosis or with less than 2 years of disease duration were recruited (recent onset SLE, n=31). Also, a new group of 20 sex and age-matched volunteers participated as healthy controls, whereas 20 individuals with carotid atheromatosis (stenosis recorded by ultrasonography ≥50%) without any autoimmune condition were recruited from the Department of Neurology (HUCA) as disease controls (Supplementary Table 1). This non-autoimmune atheromatosis group was typified as follows: 7 patients (35%) suffered from different condition of ischemic cardiopathy; 2 (10%) peripheral vascular disease; 2 (10%) lacunar stroke; 4 (20%) cardioembolic stroke; 3 (15%) contralateral atherothrombotic stroke.

Fresh blood samples from patients and control groups were tested for cell count and serum lipid analyses (total, high- and low-density lipoprotein cholesterol and triglycerides).

Ethics approval for this study was obtained from the Regional Ethics Committee for Clinical Research (Servicio de Salud del Principado de Asturias), according to the Declaration of Helsinki. All individuals signed a written informed consent prior to participation in the study.

Flow Cytometry

Monocytes, LDG, EPCs, $CD4^{+}CD28^{null}$ Tang and Th1/Th17 lymphocytes were quantified by flow cytometry. Classical, intermediate and non-classical monocytes and LDG quantification was

performed in freshly peripheral blood mononuclear cells. EPCs and the different T cell populations were quantified in fresh blood (see Supplementary material).

Cytokine quantification

Serum samples were maintained at -80ºC until cytokine or chemokine determinations. IFNα, IL-17A, CCL3 (MIP-1α), TNFα, IFNγ, IL-10, IL-6 and BLyS were quantified by immunoassays following the manufacturer's instructions (see Supplementary material).

Doppler ultrasound

Doppler ultrasound was performed in the sonography laboratory of the HUCA. All measures were carried out by the same operator using a Toshiba Aplio XG machine (Toshiba American Medical Systems). The intima-media wall thickness of internal carotid artery (cIMT) was bilaterally measured, according to Mannheim protocol (29). Plaque was defined as a distinct area protruding into the vessel lumen at least 0.5 mm, with 50% greater thickness than the cIMT found in surrounding areas or the presence of cIMT>1.5 mm (30).

Statistical analysis

All data are presented as median (interquartile range, IQR) unless otherwise stated. The Kolmogorov-Smirnov test was used to assess the normal distribution of data. U-Mann–Whitney or Kruskal-Wallis tests, Spearman's rank correlations, χ^2 tests and multivariate regression analysis were used as appropriated. Backward logistic regression analysis performed to determine the risk for subclinical CVD in SLE was carried out including high nLHR or MHR levels, demographic data (sex and age), clinical and therapeutic parameters (SLEDAI, disease duration, ACR clinical criteria, treatments) and the presence of traditional risk factors (dyslipidemia, hypertension, diabetes type II, obesity and smoking habit) as covariables in the analysis; the predictive power of the model is indicated by R^2 of Nagelkerke. Receiver-operating characteristic (ROC) curve analysis was performed to determine the sensitivity and specificity of MHR and nLHR as potential biomarkers of CV-risk (clinical or subclinical CVD) in SLE patients without traditional

risk factors, thus calculating areas under the curve (AUC) with 95% confidence intervals (CI) and p-values. To evaluate the influence of SLEDAI and age to MHR and nLHR as biomarkers, z-scores derived from such variables were summed to create combined indices. A p-value<0.05 was considered statistically significant. Data were analyzed using GraphPad Prism 5 software (GraphPad Software) and SPSS 24 statistical software package (IBM) (see Supplementary material).

RESULTS

LDG and monocyte subsets in SLE

Reduced neutrophil count is a frequent feature of SLE patients (2.47 *vs* 3.17 cells/ml in controls, p=0.032) unless traditional CV-risk factors (tCVR; presence of at least one factor) or CVD were present. Conversely, LDG showed an increased frequency in patients compared to controls [0.28% (0.43) *vs* 0.17% (0.34); p=0.042]. However, LDG from SLE patients is a heterogeneous population, and two different subsets can be detected according to CD16 and CD14 expression (Figure 1A). Interestingly, $CD16^{neg}CD14^{neg}$ LDG displayed the main differences between patients and controls [0.17% (0.32) *vs* 0.10% (0.13), p=0.011], correlated positively with cIMT and CRP, and were increased in patients with carotid atheromatosis (a marker of subclinical CVD) or tCVR but not in those CV-free (Figure 1B). No associations were observed for $CD16^{pos}CD14^{low}$ LDG, thus further analyses we will referred to the $CD16^{neg}CD14^{neg}$ LDG subset, named as nLDG.

In contrast to neutrophils, total monocyte count was increased in SLE patients with clinical or subclinical CVD or tCVR, but not in those CV-free (Figure 1C). Further analysis of monocyte subtypes (Figure 1A) revealed that proportion of the different subsets was also altered in SLE. A bias towards more differentiated populations was observed in patients compared with controls, and the absolute number of intermediate monocytes was significantly increased in all patients groups (Figure 1D). No associations were detected between monocyte subtypes and disease activity, clinical features or treatments.

The analysis of serum levels of several relevant cytokines (Supplementary Table 2) revealed different associations with nLDG and monocytes in SLE patients. IL-6 correlated directly with the amount of nLDG (ρ=0.274, p=0.004) whereas IL-17 seemed to exert an opposite effect (ρ=-0.190; p=0.051), thus suggesting IL-6/IL-17 ratio as a predictive factor for the expansion of this cellular subset (Figure 1E). On the contrary, both IL-17 and IFNα levels correlated inversely with the frequency of classical monocytes (ρ=-0.270, p=0.006; and ρ=-0.303, p=0.002; respectively) and directly with the intermediate and non-classical subsets, suggesting their involvement in the differentiation towards $CD16^{+}$ monocytes. Of note, IL-6 serum levels correlated in patients with

inflammatory mediators (CRP: ρ=0.290; p=0.005) and several cytokines (IL-10: ρ=0.456, p<0.001; TNFα: ρ=0.410, p<0.001; IFNγ: ρ=0.286, p=0.003), whereas IL-17 correlated with IFNα (ρ=0.549; p<0.001) and MIP-1α (ρ=0.483; p<0.001).

Monocyte and LDG-to-HDL ratios as CV-risk biomarkers

According with their immunosuppressive effect, HDL levels in controls were negatively correlated with monocyte, neutrophil and nLDG counts (Figure 2A). Interestingly, monocyte-to-HDL ratio (MHR) was increased in controls with carotid atheromatosis and in SLE with tCVR, clinical or subclinical CVD, but not in CV-free patients (Figure 2B), thus being better predictor of CV-risk in SLE than monocyte-to-lymphocyte ratio (MLR), strikingly increased in all groups of patients (all p<0.001). On the other hand, neutrophil-to-HDL ratio (NHR) and neutrophil-to-lymphocyte ratio (NLR), strongly correlated in controls, are inadequate as inflammatory and CV-risk markers in neutropenic conditions such as SLE, and thus nLDG can be used instead of neutrophils. Remarkably, nLDG-to-HDL ratio (nLHR) was positively correlated with CRP, IL-6 and cIMT in SLE patients (Figure 2C). Moreover, nLHR was increased in patients with subclinical CVD or tCVR but not in those CV-free (Figure 2D), whereas nLDG-to-lymphocyte ratio (nLLR) was increased in all patients groups (data not shown). Accordingly, backward logistic regression analysis including demographic and clinical parameters, traditional risk factors and treatments in the initial model, established high nLHR (>P^{90} in controls) as an independent risk factor for the development of subclinical CVD in SLE (OR=2.69, CI 95%: 1.10-5.97; p=0.015), being age (OR=1.11, CI 95%: 1.04-1.22 p=0.032), hypertension (OR=10.35, CI 95%: 1.11-96.89; p=0.046) and SLEDAI (OR=1.41, CI 95%: 1.04-1.88; p=0.031) significant variables in the model (R^2: 0.607). Similarly, high MHR (>P^{90} in controls) were associated to an increased risk for subclinical CVD in our SLE cohort (OR=4.29, CI 95%: 1.09-16.82; p=0.037), being age (OR=1.12, CI 95%: 1.06-1.20; p<0.001) and hypertension (OR=5.37, CI 95%: 1.43-20.13; p=0.013) significant variables (R^2: 0.587).

Finally, the role of MHR and nLHR as potential biomarkers of CV-risk in SLE was evaluated by ROC analysis (Figure 2E). Interestingly, both indices were adequate, and comparable to cIMT,

for the differentiation of CV-risk and CV-free SLE patients. Moreover, the analyses of combined indices adding the previously observed influence of SLEDAI and age to MHR and nLHR improved their ability to predict CV-risk in SLE patients.

All these data support MHR and nLHR as valuable biomarkers to identify SLE patients with subclinical CVD in absence of traditional risk factors.

T-lymphocytic populations and CVD in SLE

Angiogenic T cells (Tang) collaborate with EPC in vascular repair whereas Th1/Th17 and senescent $CD4^{+}CD28^{null}$ cells promote endothelial damage.

Tang cells and EPC were significantly diminished in SLE patients compared to controls ($p<0.001$), even in those CV-free (Figure 3A). Moreover, Tang reduction seemed to be paralleled by the increase of intermediate monocytes (Figure 3B). Conversely, an increased proportion of $CD4^{+}CD28^{null}$ [10.13% (9.89) *vs* 4.52% (3.31); $p<0.001$)], Th17 [0.16% (0.37) *vs* 0.05% (0.07), $p<0.001$)] and Th1 cells [6.98% (11.06) *vs* 4.31% (4.56), $p=0.007$)] were observed in patients compared to controls. Although no significant differences were detected in SLE groups attending to their CV-affection, frequency of $CD4^{+}CD28^{null}$ and Th17 cells was positively correlated with cIMT in patients (Figure 3C), whereas the frequency of Th1 lymphocytes was positively correlated with NHR ($\rho=0.208$; $p=0.030$) and LHR ($\rho=0.218$; $p=0.025$). Of note, absolute number of $CD4^{+}CD28^{null}$ cells from patients were negatively associated to the frequency of EPC ($\rho=-0.225$; $p=0.026$).

Biomarkers of CV-risk in recent onset SLE patients

Next, in view of our previous results, we aimed to confirm the possible role of monocytes, nLDG and their ratio with HDL as biomarkers of CV-risk in SLE patients at the onset on the disease. Thus, a new cohort of 31 SLE patients with recent diagnosis (rSLE; <2 years of disease evolution), 20 healthy controls and 20 non-autoimmune individuals with carotid stenosis were evaluated (Supplementary Table 1). Of note, compared with patients from the previous cohort, characterized by long-lasting disease (16.37±10.84 years), rSLE patients displayed high disease activity

(SLEDAI: 5.39 ± 5.82 *vs* 2.61±2.76, p=0.028) and a slightly lower prevalence of carotid atheromatosis.

Confirming previous results, MHR levels were significantly increased in SLE patients with subclinical atheromatosis or tCVR compared with those CV-free, in a similar way than patients with carotid stenosis (Figure 4A), thus supporting the value of MHR as an early biomarker of subclinical CVD in SLE.

On the other hand, rSLE patients displayed increased levels of nLDG [0.45 (0.62) %] compared to controls [0.12 (0.15), p<0.001] and the non-autoimmune atheromatosis group [0.20 (0.18), p=0.026], and, intriguingly, higher than the exposed by the previous SLE cohort (p<0.001). This observation could be due to the higher disease activity presented by rSLE patients, since nLDG were strongly correlated with SLEDAI and anti-dsDNA titer in this group (Figure 4B). In fact, rSLE patients with active disease (SLEDAI≥8, n=12) exhibited increased amounts of both nLDG and nLHR independently of their CV-involvement (Figure 4C). In contrast, among patients with moderate/non-active disease (n=19), nLDG, and especially nLHR, were augmented in those with subclinical CVD or tCVR, but not in their CV-free counterparts, thus supporting their value as early biomarkers of CV-risk in SLE. However, nLDG amounts from established SLE was not correlated with SLEDAI (ρ=0.063, p=0.521), not finding differences in their frequency between active and non-active patients (0.16 vs 0.17%, p=0.814).

Finally, ROC analysis confirm MHR as valuable biomarker of CV-risk in rSLE, as well as nLHR when considering the influence of SLEDAI and age (Figure 4D).

DISCUSSION

Systemic inflammatory processes presented in SLE have been proposed as main triggers of the endothelial damage underlying atherosclerosis and CVD, which is the most common causes of premature mortality in these patients (31). A compelling body of evidence highlights the role of altered immune cells or increased levels of cytokines in the SLE pathogenesis. This work analyzed some of the most relevant cellular subsets involved in endothelial homeostasis at once in a SLE cohort, revealing increased amounts of the pro-atherogenic $CD16^{+}$ monocytes, LDG, Th17 lymphocytes and senescent-$CD28^{null}$ subsets, coupled with reduced numbers of vascular repairing EPCs and Tang cells that could tip the balance towards atheromatosis development in these patients.

The most relevant outcome of this work is the value of monocytes and the LDG population negative for CD14 and CD16 (nLDG), but especially their augmented proportion in respect of HDL molecules, as biomarkers of SLE patients with subclinical atheromatosis. HDL has been described as a regulator factor of the pro-inflammatory and oxidant effects of monocytes and neutrophils, being able to control their activation as well as the proliferation of their progenitor cells (32). In line with this, monocyte, neutrophil and nLDG amounts in our control group were inversely correlated with their HDL levels. In fact, monocyte-to-HDL ratio (MHR) has been associated with systemic inflammation and proposed as a marker of CV alterations both in general population and autoimmune conditions (33,34). Interestingly, MHR values were increased in SLE patients with traditional risk factors or carotid atheromatosis, but not in those CV-free, thus allowing the identification of patients at CV-risk. Likewise, we propose, for first time, the ratio between either neutrophil or LDG counts and HDL levels as potential markers of CV-risk. Supporting such proposal, neutrophil-to-HDL ratio (NHR) in our control population is strongly associated to neutrophil-to-lymphocyte ratio (NLR), an established predictor factor of systemic inflammation and CVD (35). However, neither of these two markers can be used in SLE, since neutropenia is a typical condition of these patients. In this scenario, we suggest to replace total neutrophils by nLDG. In fact, the nLDG-to-HDL ratio (nLHR) was positively correlated with

cIMT and associated with subclinical CVD in SLE, in a similar way to MHR. Indeed, our results suggest that both nLHR and MHR could be biomarkers as sensitive and specific as cIMT to identify CV-risk in SLE patients. The availability, feasibility and cost-effectiveness of these indices, especially MHR, make them valuable tools in clinical practice for the prediction of CV alterations. These results highlight the relevance of the HDL dysfunction, usually accompanying chronic inflammation, in the development of premature atheromatosis. Of note, it has been proposed that NETs from lupus LDG can oxidize HDL molecules resulting in a loss of their atheroprotective capacity and the subsequent high-risk of atherosclerosis development (36)**.** The potential value of the presence of increased monocytes- and nLDG-to-HDL ratios as biomarkers of CV-risk in SLE was confirmed in patients at disease onset. Intriguingly, high disease activity seems to expand nLDG in recently diagnosed patients, but not in those with longer disease duration and similar disease activity (SLEDAI≥8), a small group in our cohort (n=7), suggesting that other clinical factors or long-lasting treatments could influence nLDG expansion. Nevertheless, SLEDAI and high nLDG levels were independent risk factors for the development of subclinical-CVD in these patients.

Another interesting finding is the alteration of the different monocyte subsets in SLE, even in those CV-free, probably related to an over-stimulation of classical monocytes in peripheral blood that promotes their differentiation towards $CD16^+$ subsets. Inflammation usually present in SLE seems to be responsible of the characteristic monocyte over-activated state (37) and their migration through the intima-media vascular layer, thus enhancing the endothelial dysfunction. In this backdrop, the monocyte production of soluble mediators, including reactive oxygen species (ROS) and cytokines, such as TNF-α and IL-1β, may cause a vicious cycle between oxidative stress and inflammation promoting endothelial damage. Additionally, the participation of monocytes in antigen presentation for T-cell stimulation and their proangiogenic capacity to neo-vascularize the plaque, represent key elements promoting both chronic inflammation and atherosclerosis (38). Actually, total monocyte count, but also the imbalance among its subsets, have been reported as predictors of atherosclerosis in inflammatory conditions and CVD outcomes (7,39). In line with this, results herein presented strengthen the link between increased

levels of intermediate monocytes and atheromatosis in healthy donors, as previously described (7,40). In contrast, our SLE cohort exhibited augmented amounts of intermediate monocytes regardless of their CV involvement. Thus, given the recognized contribution of the pro-inflammatory and oxidative $CD16^+$ monocytes to CVD (7), they could probably be involved in the vascular dysfunction accompanying SLE. However, results from different studies analyzing the proportions of monocyte subsets in SLE are inconclusive (9,41–46). Of note, our results could find explanation, at least in part, attending to the effect of IFNα and IL-17, two key pathogenic cytokines in SLE, since the presence of higher levels of these molecules appear to promote monocyte differentiation, thus increasing the proportion of pro-atherogenic $CD16^+$ subsets. In line with this, type I IFN may contribute to the transition from classical to non-classical subtype in SLE since CD16 expression has been positively correlated with IFN score (47). Additionally, Rossol *et al* provided a link between the increased frequency of the intermediate monocytes and the expansion of Th17 cells in rheumatoid arthritis (48). Furthermore, the association of circulating Th17 cells with cIMT supports this notion.

An additional key element thought to contribute for atherosclerosis is the recruitment of neutrophils to the lesion site (49). However, as opposed to monocytes, circulating neutrophils are reduced in SLE patients unless tCVR or CVD were present. In this picture, it is worth considering the presence of LDG, known to secrete pro-inflammatory cytokines, such as IL-6, IL-8, IFNα, TNFα or IL-17, as well as matrix metalloproteinases, lytic enzymes or ROS necessary for spontaneous NETosis (50). All these toxic products, and especially the enhanced production of NETs, maintain chronic inflammation and promote endothelial damage responsible of the development of premature atherosclerosis in SLE. NETs can induce endothelial dysfunction directly by activation and damage of endothelial cells, but also by the amplification of local inflammatory immune responses. Furthermore, NETs containing pro-inflammatory molecules, such as elastase or IL-17, have been observed in the atherosclerotic plaques and venous thrombi (51,52). Our findings confirm the presence of an increased frequency of circulating LDG in SLE blood (11). Additionally, we ascertain that LDG in SLE constitutes a heterogeneous population,

including different subsets with probably different origin. Amazingly, such augmented levels were especially significant attending to a specific subset of LDG, double negative for CD14/CD16 and with a characteristic phenotype and nuclear morphology, postulated to represent an immature neutrophil subset. In a recent work, we have characterized $CD14^{-}CD16^{-}CD15^{+}$ LDG by the expression of high levels of early differentiation markers compared to normal density neutrophils and the $CD14^{low}CD16^{+}CD15^{+}$ LDG subset, thus considered terminally-differentiated granulocytes (15). Moreover, the early-stage-specific upregulation of granulocyte-related genes during neutrophil maturation in peripheral blood cells from SLE (53) and their association with nLDG frequency in other inflammatory conditions, such as a chronic kidney disease (15), confirm the immature status of nLDG prematurely released from the bone marrow. Such observation is in agreement with the notion that systemic inflammation can induce an "emergency granulopoiesis" that mobilizes immature precursors into the circulation (54). Accordingly, the frequency of this nLDG subset in SLE seems to be associated to a combination of augmented IL-6 serum levels, a pro-inflammatory cytokine critical in the hematopoiesis stimulation (55), with reduced amounts of the neutrophil activator IL-17 (56). In fact, IL-6 correlated in patients with several cytokines and inflammatory mediators. Moreover, synthesis of IL-6 has been found to be higher in LDG than in normal neutrophils, thus contributing to the amplification of this subset in SLE (57). Therefore, the increased nLDG observed in SLE patients seem to be derived from hematopoietic aberrations in bone marrow that may be associated, at least in part, with the increase in IL-6 and other inflammatory mediators as key inductors of the hematopoiesis stimulation. The most interesting result, however, was that nLDGs were associated with subclinical CVD and positively correlated with cIMT in SLE patients, thus supporting them as biomarkers to identify lupus patients with CV risk**.**

In a scenario like the presented by SLE patients, with amplified amounts of the pro-atherogenic subsets, the activity of EPC and Tang vascular repairing populations represent a crucial aspect to preserve endothelial integrity. However, depleted levels of EPC were observed in the whole SLE population, independently of their CV status, as previously observed by most of the authors (20,22,58–61), with the exception of the study of Castejon *et al* (17). Likewise, Tang cells were

also depleted in SLE patients, mainly in those with CVD, and paralleled to the increase of intermediate monocytes. SLE-Tang diminution could be due to the expansion of the senescent and cytotoxic $CD28^{null}$-Tang cells as previously reported (20), expected to have a pro-atherogenic function in a similar way to the $CD4^{+}CD28^{null}$ subset (24,25). Remarkably, our present results reveal us increased levels of $CD4^{+}CD28^{null}$ cells in SLE that were inversely correlated with EPC, even in those patients without tCVR factors, thus pointing to the presence of these cellular subsets as independent risk factors for CVD in SLE. Certainly, although the frequency of $CD4^{+}CD28^{null}$ cells has been previously related to lupus or atherosclerosis (26,62), the present work offers an evidence of its potential link with cIMT in SLE.

In conclusion, our results support the existence of a strong imbalance between cellular subsets involved in endothelium integrity in SLE. The decreased number of the pro-angiogenic EPC and Tang cells may not be able to repair an enhanced vascular damage due to the increase of endothelium-toxic populations in SLE patients, including LDG, $CD16^{+}$ monocytes, senescent and Th17 cells. Since endothelial damage is theoretically reversible, the identification of potential biomarkers of subclinical atherogenesis represents a valuable tool to propose preventive therapies. In this sense, present study highlights the potential use of monocytes and nLDG to HDL ratios as valuable biomarkers of CV-risk in SLE patients, even at early stages of the disease.

Conflict of interest

Authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1. LDG and monocyte subpopulations in SLE patients and controls according to the presence of subclinical atheromatosis or cardiovascular disease. (A) Gating strategy used to identify monocytes and LDG subsets in PBMC fractions by flow cytometry. Healthy controls or SLE PBMC were stained for markers of the monocyte and granulocyte lineages and analyzed by flow cytometry. Representative dot-plots of a SLE patient are shown. Firstly, total LDG was gated as $CD14^{pos}CD15^{neg/low}$ and total monocyte's population (Mo) as $CD14^{neg/low}CD15^{+}$ cells within the PBMC gate. Analysis of the HLA-DR expression confirmed the distinction between LDG and monocytes. Then, the monocyte population was analyzed on its CD14 and CD16 surface expression to discriminate classical $CD14^{++}CD16^{-}$, intermediate $CD14^{++}CD16^{+}$ and non-classical $CD14^{+}CD16^{+}$ subsets. The expression of CD14 and CD16 in LDG allowed the discrimination of $CD14^{low}CD16^{pos}$ and $CD14^{neg}CD16^{neg}$ (nLDG) subsets. **(B)** nLDG are represented in box-plots where horizontal lines represent median and interquartile range in controls (C) without (-) or with (+) subclinical atheromatosis and in SLE patients grouped according to the presence of traditional risk factors (tCVR), subclinical atheromatosis (sub-CVD) or cardiovascular disease (CVD). Correlation of nLDG with cIMT and CRP values in SLE (Spearman tests). **(C)** Median values (cells/µl) of total monocytes are represented in box-plots according to the presence of tCVR, sub-CVD or CVD in patients and in controls without (-) or with (+) subclinical atheromatosis. **(D)** Proportion of the different monocyte subsets in controls and SLE groups presenting tCVR, subclinical or clinical CVD. Box-plots represent the median values (cells/µl) of classical, intermediate and non-classical monocytes according to the presence of tCVR, sub-CVD or CVD in patients and controls in function of the presence of subclinical atheromatosis. **(E)** Graphs show the correlations between IL-6/IL-17 ratio and nLDG or between IFNα or IL-17 serum levels (pg/ml) and the percentage of $CD16^{+}$ monocytes (intermediate plus non-classical monocytes) in SLE patients (log-transformed values). Correlation analyses were evaluated by Spearman tests

using non-transformed variables (IL-17, IFNα and $CD16^+$ monocytes levels were log-transformed only for the graphical representation).

Statistical differences among groups were evaluated by Kruskal-Wallis test and U-Mann-Whitney test was conducted to determine different medians between each group and controls without atheromatosis (*p<0.05, **p<0.01, ***p<0.001).

Figure 2. Relationship among neutrophils, monocytes and nLDG counts from SLE patients and controls and stablished markers of CV alterations. (A) Correlation graphs among neutrophils, monocytes and nLDG amounts and HDL levels, as well as among the ratio of neutrophils, monocytes and nLDG respect to lymphocytes or HDL levels in controls. **(B)** Monocyte-to-HDL levels (MHR) are represented by box-plots in controls (C) without (-) or with (+) subclinical atheromatosis and in patients grouped according to the presence of traditional risk factors (tCVR), subclinical atheromatosis (sub-CVD) or cardiovascular disease (CVD). **(C)** Correlation diagrams among nLDG-to-HDL ratio (nLHR) and cIMT, CRP or IL-6 levels in SLE. **(D)** Median values of nLHR are represented in box-plots according to the presence of tCVR, sub-CVD or CVD in patients and in controls without (-) or with (+) subclinical atheromatosis. (**E**) The ROC curve analysis for MHR and nLHR in predicting CV-risk in SLE patients.

Horizontal lines represent median and interquartile range. Statistical differences among groups were assessed by Kruskal-Wallis test and U-Mann-Whitney test was conducted to determine different medians between each group and controls without atheromatosis (*p<0.05, **p<0.01, ***p<0.001). Correlation analyses were evaluated by Spearman tests.

ROC, receiver operating characteristic; MHR, monocyte-to-HDL ratio; nLHR, nLDG-to-HDL ratio; AUC, area under the curve; CI, confidence interval.

Figure 3. Progenitor endothelial cells and T-lymphocytic populations and their relation with CV alterations in SLE patients and controls. (A) Frequency of Tang cells ($CD3^+CD31^+CXCR4^+CD28^+$) and EPC in SLE patients according to the presence of traditional risk factors (tCVR), subclinical atheromatosis (sub-CVD) or cardiovascular disease (CVD) and

controls (C) without (-) or with (+) subclinical atheromatosis. **(B)** Correlation between intermediate monocytes and Tang cells in SLE patients. **(C)** Correlation of cIMT with $CD4^{+}CD28^{null}$ cells and Th17 cells frequency in SLE.

Horizontal lines represent median and interquartile range. Statistical differences among groups were assessed by Kruskal-Wallis test and U-Mann-Whitney test was conducted to determine different medians between each group and controls without atheromatosis (*p<0.05, **p<0.01, ***p<0.001). Correlation analyses were evaluated by Spearman tests.

Figure 4. Role of monocytes, nLDG and their ratio with HDL as biomarkers of CV-risk in recent-onset SLE patients. (A) Monocytes levels and MHR in healthy controls (HC), non-autoimmune atheromatosis controls (AC) and SLE patients grouped according to the presence of traditional risk factors (tCVR) and subclinical atheromatosis (sub-CVD). **(B)** Correlation between the percentage of nLDG and SLEDAI or anti-dsDNA titer in SLE patients. **(C)** Frequency of nLDG and nLHR values in HC, AC and active (SLEDA≥8) or non-active SLE patients grouped according to the presence of tCVR or sub-CVD. (**D**) The ROC curve analysis for MHR and nLHR in predicting CV-risk in recent-onset SLE patients.

Horizontal lines represent median and interquartile range. Statistical differences among groups were assessed by Kruskal-Wallis test and U-Mann-Whitney test was conducted to determine differences between each patient group and healthy controls (*p<0.05) or between tCVR or sub-CVD SLE patients and CV-free group (§p<0.05). Correlation analyses were evaluated by Spearman tests.

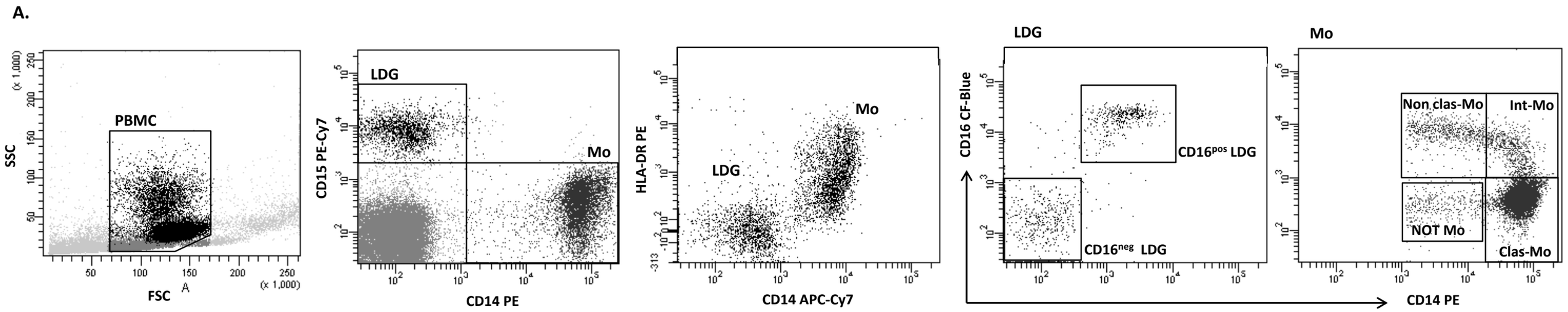
ROC, receiver operating characteristic; MHR, monocyte-to-HDL ratio; nLHR, nLDG-to-HDL ratio; AUC, area under the curve; CI, confidence interval.

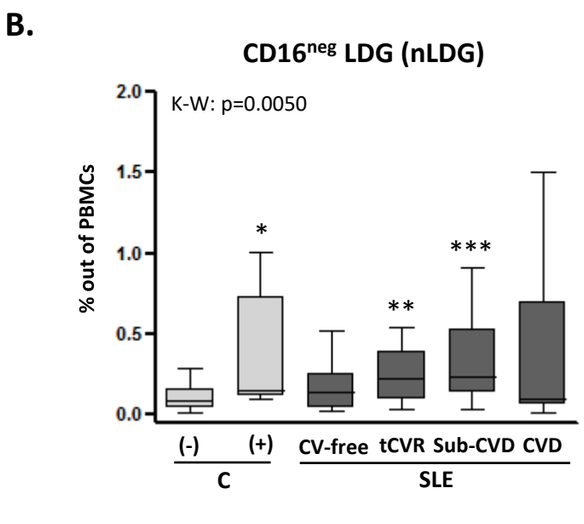
Table 1. Demographic and clinical features of SLE patients

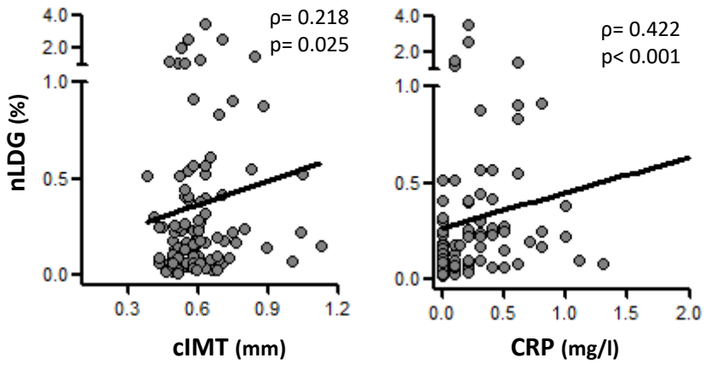
	SLE patients (n=109)	Controls (n=33)
Demographic features		
Sex, n (female/male)	103/6	31/2
Age, years (mean±SD)	47.65±11.35	45.36±11.07
Total cholesterol, mg/dl (mean±SD)	185.93±35.47	189.12±32.37
HDL cholesterol, mg/dl (mean±SD)	61.50±17.42	63.94±13.891
LDL cholesterol, mg/dl (mean±SD)	106.46±30.92	109.00±30.79
Triglycerides, mg/dl (mean±SD)	91.21±46.96	79.42±31.16
Total/HDL-cholesterol ratio	3.22±1.00	3.07±0.87
Traditional CV risk factors, n (%)		
Dyslipidemia	23 (21.10)	3 (9.09)
Hypertension	28 (25.69)*	2 (6.06)
Diabetes (type II)	0 (0.00)	0 (0.00)
Obesity (BMI>30)	16 (14.68)	1 (3.03)
Smoking habit	26 (23.85)	4 (12.12)
Subclinical atheromatosis[a], n (%)	37 (33.94)*	5 (15.15)
CV disease, n (%)	18 (16.51)	
Cerebrovascular disease	4 (3.67)	
Heart disease	9 (8.26)	
Peripheral vascular disease	5 (4.59)	
Clinical manifestations, n (%)		
Age at diagnosis, years (mean±SD)	31.85±11.52	
Disease duration, years (mean±SD)	16.37±10.84	
SLEDAI score (mean±SD)	2.61±2.76	
ACR criteria		
Malar rash	58 (53.21)	
Discoid lesions	36 (33.03)	
Photosensitivity	60 (55.03)	
Oral ulcers	45 (41.28)	
Arthritis	88 (80.73)	
Serositis	28 (25.69)	
Cytopenia	64 (58.72)	
Renal disorder	39 (35.78)	
Neurological disorder	13 (11.93)	
Autoantibodies, n (%)		
ANAs	109 (100.00)	
Anti-dsDNA/titer, U/ml (mean±SD)	86 (78.90)/54.57±103.38	
Anti-SSA	63 (57.80)	
Anti-SSB	22 (20.18)	
Anti-Sm	12 (11.01)	
Anti-RNP	27 (24.77)	
Rheumatoid Factor	21 (19.27)	
Anti-cardiolipin IgG	27 (24.77)	
Anti-cardiolipin IgM	23 (21.10)	
Lupus anticoagulant	24 (22.02)	
Treatment, n (%)		
None or NSAIDs	3 (2.75)	

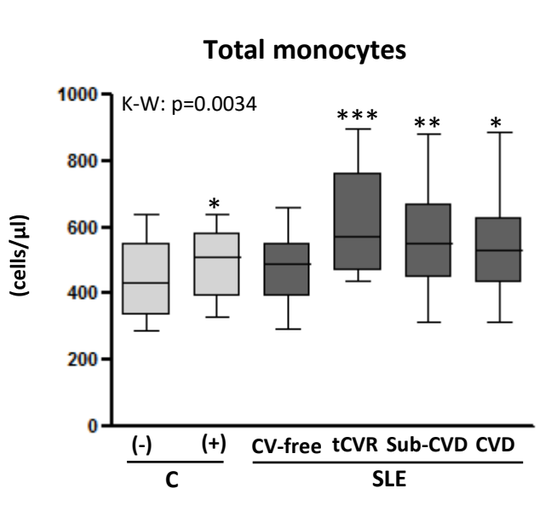
Antimalarial drugs	97 (88.99)
Glucocorticoids	44 (40.37)
Immunosuppressive drugs[b]	37 (33.94)
Statins	18 (16.51)

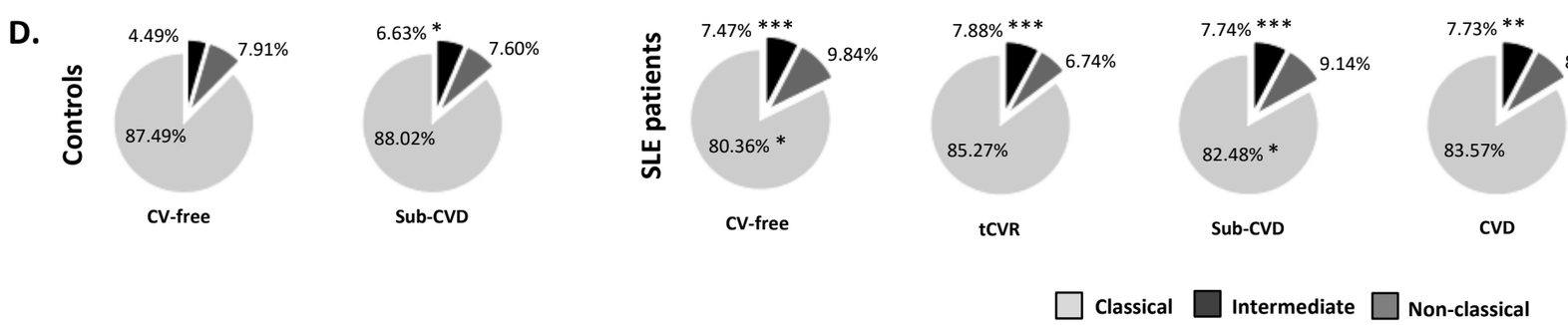
BMI: body mass index; dsDNA: double stranded DNA; RF: rheumatoid factor; NSAID: non-steroidal anti-inflammatory drug.
[a]Subclinical atheromatosis defined as carotid plaque presence or intima media thickness (cIMT)>0.9 mm.
[b]Mycophenolate mophetil, azathioprine.
*Differences among patients and controls were analyzed by χ^2 test ($p<0.05$).

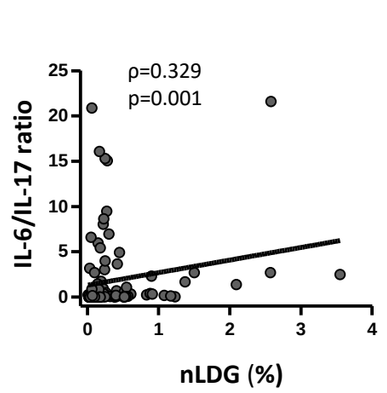

A.
PBMC
SSC
FSC
LDG
Mo
CD15 PE-Cy7
CD14 PE
HLA-DR PE
CD14 APC-Cy7
CD16 CF-Blue
CD16pos LDG
CD16neg LDG
Non clas-Mo
Int-Mo
NOT Mo
Clas-Mo

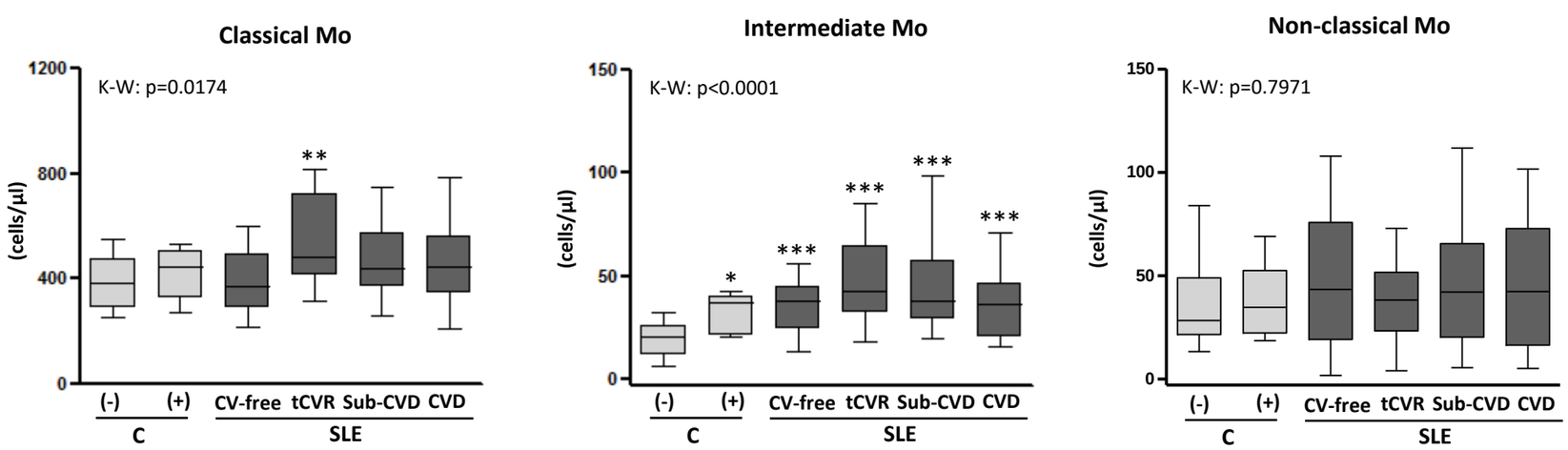

B.
CD16neg LDG (nLDG)
K-W: p=0.0050
% out of PBMCs
C
SLE


ρ= 0.218
p= 0.025
nLDG (%)
cIMT (mm)
ρ= 0.422
p< 0.001
CRP (mg/l)


C.
Total monocytes
K-W: p=0.0034
(cells/µl)
C
SLE


D.
Controls
SLE patients
4.49%
7.91%
87.49%
CV-free
6.63% *
7.60%
88.02%
Sub-CVD
7.47% ***
9.84%
80.36% *
CV-free
7.88% ***
6.74%
85.27%
tCVR
7.74% ***
9.14%
82.48% *
Sub-CVD
7.73% **
8.58%
83.57%
CVD
Classical
Intermediate
Non-classical


E.
ρ=0.329
p=0.001
IL-6/IL-17 ratio
nLDG (%)


Classical Mo
K-W: p=0.0174
Intermediate Mo
K-W: p<0.0001
Non-classical Mo
K-W: p=0.7971
(cells/µl)
C
SLE

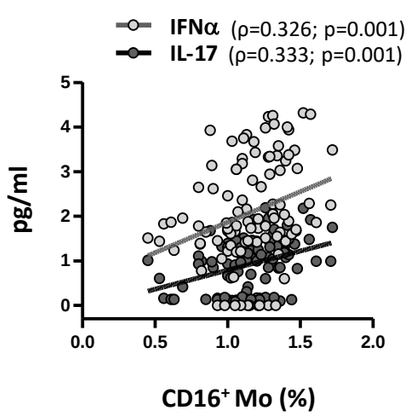

IFNα (ρ=0.326; p=0.001)
IL-17 (ρ=0.333; p=0.001)
pg/ml
CD16+ Mo (%)

Figure 1

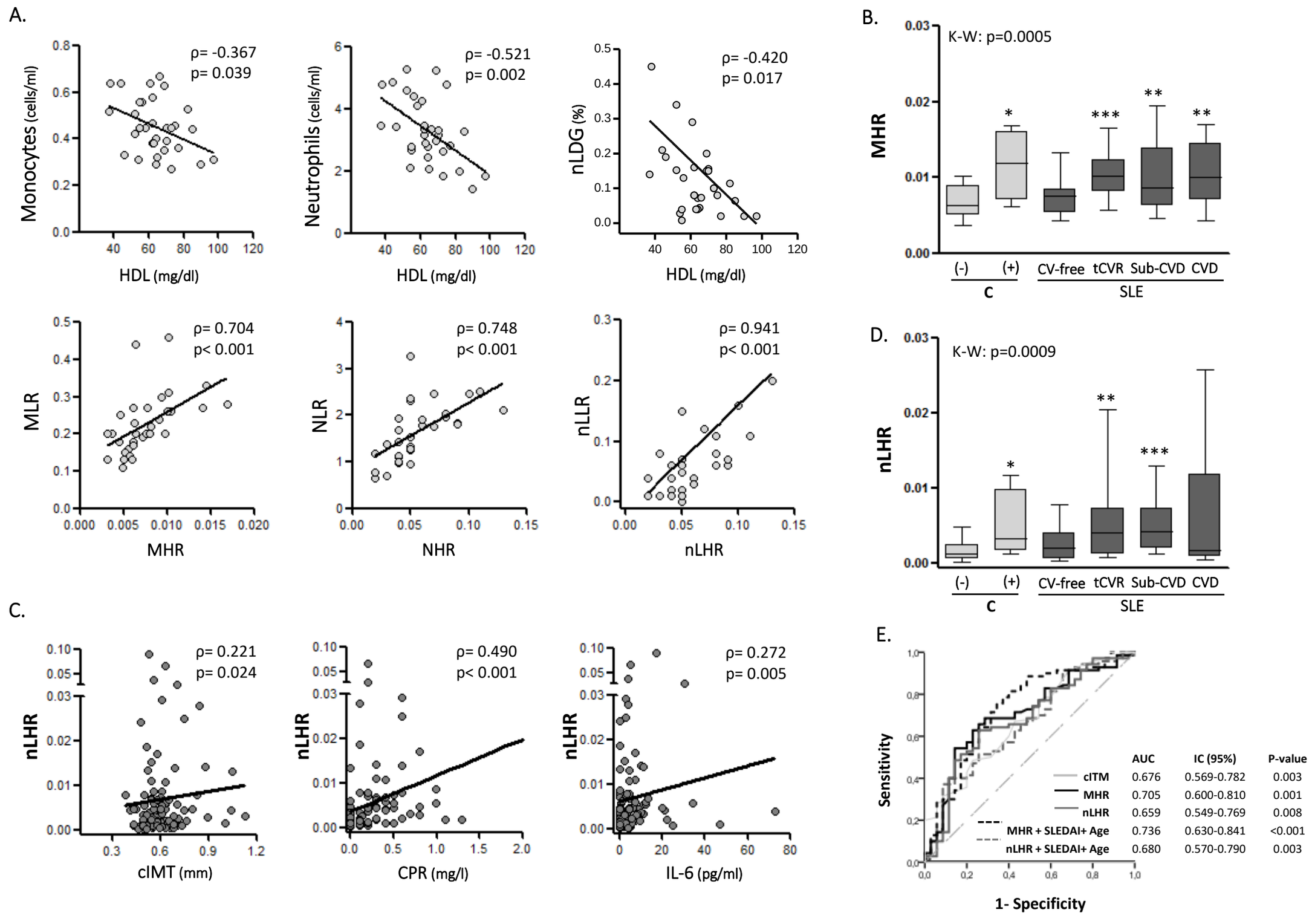

A.
Monocytes (cells/ml)
ρ= -0.367
p= 0.039
HDL (mg/dl)
Neutrophils (cells/ml)
ρ= -0.521
p= 0.002
HDL (mg/dl)
nLDG (%)
ρ= -0.420
p= 0.017
HDL (mg/dl)
MLR
ρ= 0.704
p< 0.001
MHR
NLR
ρ= 0.748
p< 0.001
NHR
nLLR
ρ= 0.941
p< 0.001
nLHR
B.
K-W: p=0.0005
MHR
(-)
(+)
CV-free
tCVR
Sub-CVD
CVD
C
SLE
D.
K-W: p=0.0009
nLHR
(-)
(+)
CV-free
tCVR
Sub-CVD
CVD
C
SLE
C.
nLHR
ρ= 0.221
p= 0.024
cIMT (mm)
nLHR
ρ= 0.490
p< 0.001
CPR (mg/l)
nLHR
ρ= 0.272
p= 0.005
IL-6 (pg/ml)
E.
Sensitivity
1- Specificity
AUC
IC (95%)
P-value
cIMT 0.676 0.569-0.782 0.003
MHR 0.705 0.600-0.810 0.001
nLHR 0.659 0.549-0.769 0.008
MHR + SLEDAI+ Age 0.736 0.630-0.841 <0.001
nLHR + SLEDAI+ Age 0.680 0.570-0.790 0.003

Figure 2

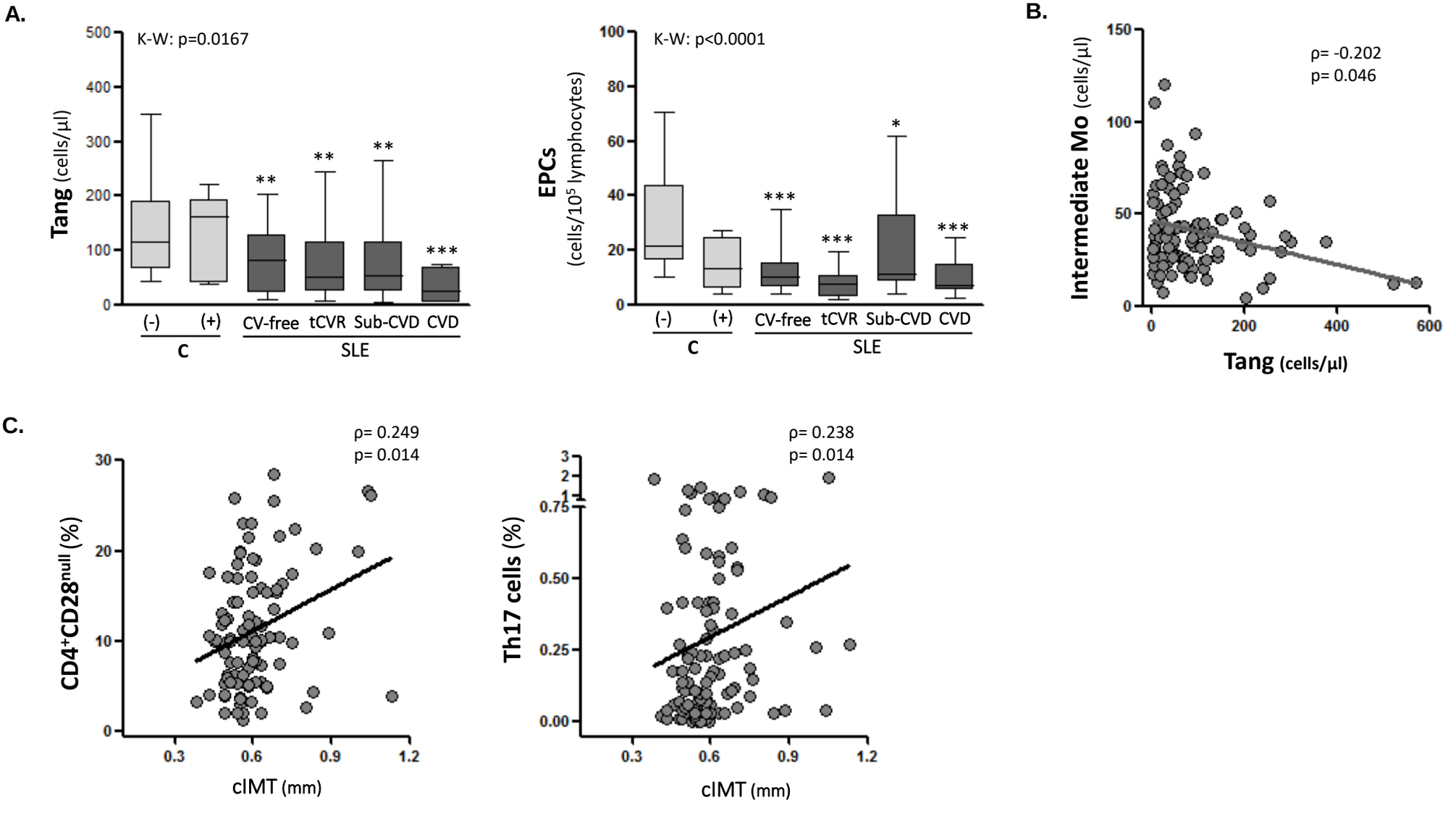

A.
K-W: p=0.0167
Tang (cells/µl)
500
400
300
200
100
0
**
**
**
***
(-)
(+)
CV-free
tCVR
Sub-CVD
CVD
C
SLE
K-W: p<0.0001
EPCs
(cells/10⁵ lymphocytes)
100
80
60
40
20
0
***
***
*
***
(-)
(+)
CV-free
tCVR
Sub-CVD
CVD
C
SLE
B.
ρ= -0.202
p= 0.046
Intermediate Mo (cells/µl)
150
100
50
0
0
200
400
600
Tang (cells/µl)
C.
ρ= 0.249
p= 0.014
CD4+CD28null (%)
30
20
10
0
0.3
0.6
0.9
1.2
cIMT (mm)
ρ= 0.238
p= 0.014
Th17 cells (%)
3
2
1
0.75
0.50
0.25
0.00
0.3
0.6
0.9
1.2
cIMT (mm)

Figure 3

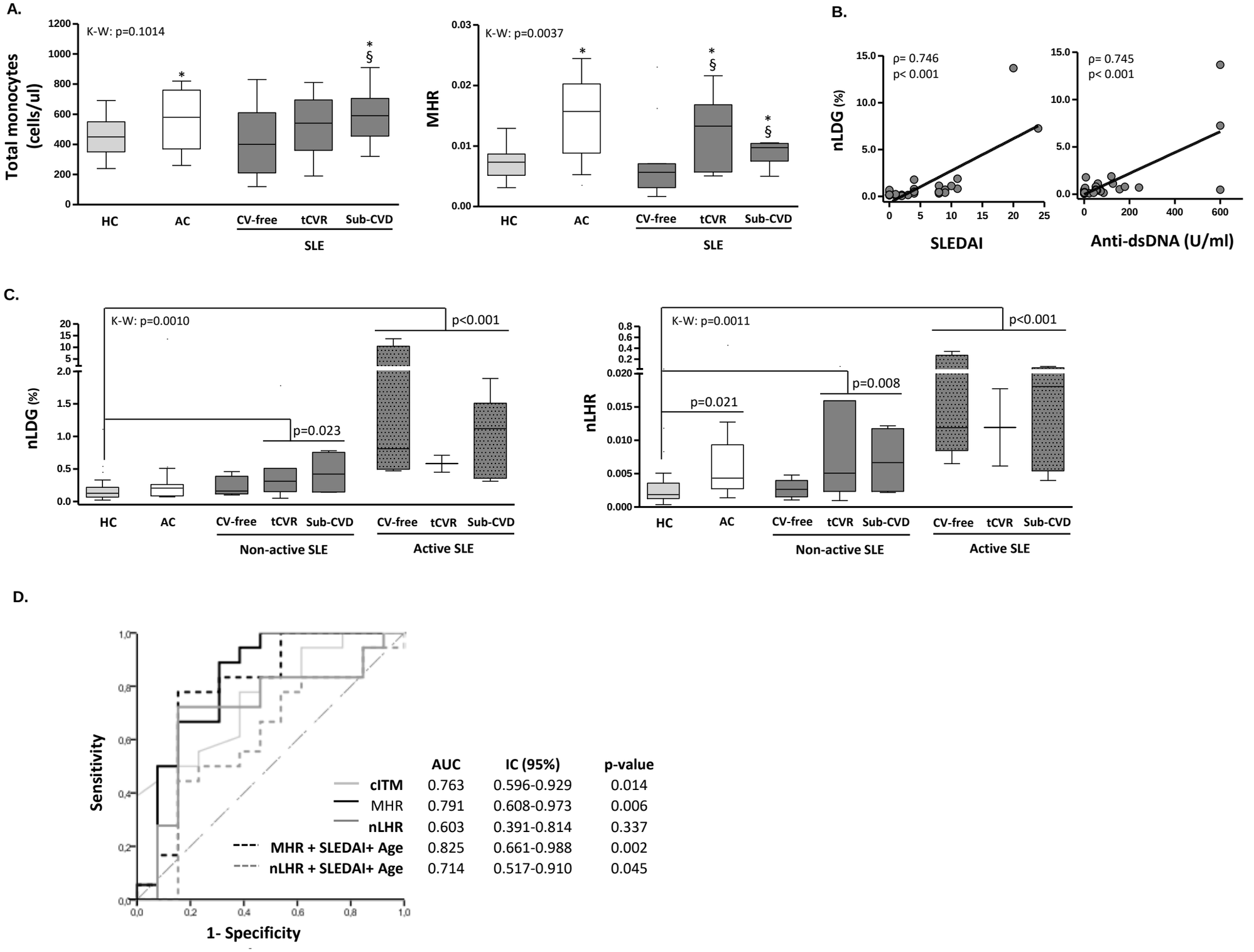

A.
K-W: p=0.1014
Total monocytes (cells/ul)
HC
AC
CV-free
tCVR
Sub-CVD
SLE
K-W: p=0.0037
MHR
B.
ρ= 0.746
p< 0.001
nLDG (%)
SLEDAI
ρ= 0.745
p< 0.001
Anti-dsDNA (U/ml)
C.
K-W: p=0.0010
p<0.001
p=0.023
Non-active SLE
Active SLE
K-W: p=0.0011
nLHR
p=0.021
p=0.008
D.
Sensitivity
1- Specificity
cITM
MHR
nLHR
MHR + SLEDAI+ Age
nLHR + SLEDAI+ Age
AUC
IC (95%)
p-value
cITM 0.763 0.596-0.929 0.014
MHR 0.791 0.608-0.973 0.006
nLHR 0.603 0.391-0.814 0.337
MHR + SLEDAI+ Age 0.825 0.661-0.988 0.002
nLHR + SLEDAI+ Age 0.714 0.517-0.910 0.045

Figure 4

Title: Low density granulocytes and monocytes as biomarkers of cardiovascular risk in Systemic Lupus Erythematosus

SUPPLEMENTARY MATERIAL

Flow Cytometry

Monocytes and LDG quantification was performed in freshly peripheral blood mononuclear cells (PBMC) isolated by density gradient centrifugation and stained with anti-CD14-PE or APC-Cy7 (Immunostep), anti-CD15-PE-Cy7 (Miltenyi Biotec), anti-CD16-CF-Blue (Immunostep), anti-HLA-DR-PE (BD Pharmingen) or with isotype-matched mAb (eBioscience). Total monocytes were defined as $CD14^{+}CD15^{neg/low}$ and total LDG as $CD14^{neg/low}CD15^{+}$ cells within the PBMC gate (Figure 1A). Analysis of the HLA-DR expression confirmed the distinction between monocytes and LDGs. Then, classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$) and non-classical ($CD14^{+}CD16^{+}$) subsets were discriminated among total monocytes, whereas the expression of CD16 and CD14 in LDG allowed the discrimination of $CD16^{neg}CD14^{neg}$ (nLDG) and $CD16^{pos}CD14^{low}$ subsets.

EPCs were analyzed following the recommendations of the European League Against Rheumatism (EULAR) Scleroderma Trials and Research (EUSTAR) [1], as previously described [2]. Briefly, fresh blood was pre-incubated with FcR blocking reagent (Miltenyi Biotech). Then, cells were incubated with anti-VEGFR2-PE (R&D Systems), anti-CD34-FITC (BD Pharmingen), and anti-CD133-APC (Miltenyi Biotech) or with isotype-matched antibodies (eBioscience). EPCs were identified as triple-positive cells for CD34/VEGFR2/CD133 in the lymphocyte gate and total progenitor cells were identified as $CD34^{+}CD133^{+}$ cells.

$CD4^{+}CD28^{null}$ and Tang populations from fresh blood were stained with anti-CD3-PerCP-Cy5.5 (Tonbo biosciences), anti-CD4-PE (Immunostep), anti-CD31-FITC, anti-CXCR4-PE-Cy7 and anti-CD28-APC-Cy7 mAb or with the corresponding isotype-matched antibodies as a negative

control (BD Biosciences). $CD4^+CD28^{null}$ cells were calculated within the $CD3^+CD4^+$ population and Tang ($CD31^+CXCR4^+CD28^+$) among $CD3^+$ cells.

Th1 and Th17 were identified as IFNγ and IL-17 positive cells among $CD4^+$ lymphocytes by using mAb specific for CD4-APC-Cy7, IL-17-APC, IFNγ-PerCP-Cy5.5 and fluorochrome-matched control antibodies (eBioscence). Fresh blood cells were fixed, permeabilized and intracellularly stained with monoclonal antibodies against these cytokines following the manufacturer's instructions ("Fixation/permeabilization buffer set"; eBiosciences).

Acquisition was performed on a BD FACSCanto II flow cytometer. The analysis was based on cells located in a plot-area termed "the living region" which was defined using forward and side-scatter and using FACSDiva Software.

Additionally, the ratio of monocytes- and nLDG-to-HDL-molecules (MHR and nLHR, respectively) were calculated as the absolute number of circulating monocytes/ml or the amount of nLDG/PBMC respect to serum levels of HDL (mg/dl).

Cytokine quantification

Serum samples were maintained at -80ºC until cytokine or chemokine determinations. IFNα, IL-17A and CCL3 (MIP-1α) amounts were quantified by Cytometric Bead Arrays Flex Set, whereas levels of TNFα, IFNγ, IL-10, IL-6 and BLyS were quantified by LEGENDplex (BioLegend), all of them by using a FACS Canto II flow cytometer (BD) and following the manufacturer's instructions. The lower limits of detection were (pg/ml): 1.25 for IFNα, 0.30 for IL-17A, 0.20 for MIP-1α, 1.00 for TNFα and IFNγ, 1.10 for IL-10 and IL-6, 10.40 for BLyS.

Statistical analysis

The Kolmogorov-Smirnov test was used to assess the normal distribution of the data. Differences between control and patient groups in quantitative variables were examined by Mann-Whitney U-test or Kruskal-Wallis test, whereas qualitative parameters were analyzed by χ^2 test. Data from variables were expressed as the median (interquartile range, IQR). Correlation

analyses were examined by Spearman tests. Linear regression analyses adjusted for sex and age and multivariate backward regression models were applied to determine the influence of demographic data (age at diagnosis, disease duration), disease features (SLEDAI, clinical manifestations, therapies) and the different cellular populations or serum factors in the CVD development. To this end, non-normal variables were log-transformed to achieve normal distribution and standardized linear regression coefficients (beta) were used as an estimation of the association. A p-value<0.05 was considered statistically significant. Data were analyzed using GraphPad Prism 5 software (GraphPad Software) and SPSS 24 statistical software package (IBM).

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Supplementary Table 1. Demographic and clinical characteristics of recent onset SLE patients and control groups.

	SLE patients (n=31)	Non-autoimune atheromatosis (n=20)	Healthy controls (n=20)
Demographic features			
Sex, n (female/male)	25/6	4/16	18/2
Age, years (mean±SD)	46.03 ± 17.06	77.15 ± 5.83***	48.25 ± 8.28
Total cholesterol, mg/dl (mean±SD)	181.51 ± 31.33	145.47 ± 31.42***	181.35 ± 30.66
HDL cholesterol, mg/dl (mean±SD)	60.06 ± 20.46	43.23 ± 13.40***	63.70 ± 14.07
LDL cholesterol, mg/dl (mean±SD)	101.87 ± 28.27	75.06 ± 26.14**	100.30 ± 24.59
Triglycerides, mg/dl (mean±SD)	107.19 ± 65.76	145.00 ± 64.15***	86.55 ± 44.96
Traditional CV risk factors, n (%)			
Dyslipidemia	3 (9.68)	13 (65.00)	
Hypertension	5 (16.13)	18 (90.00)	
Diabetes (type II)	2 (6.45)	11 (55.00)	
Obesity (BMI>30)	4 (12.90)	6 (30.00)	
Smoking habit	8 (25.81)	0 (0.00)	
Subclinical atheromatosis[a], n (%)	9 (29.03)	20 (100)	
SLE clinical parameters, n (%)			
Age at diagnosis, years (mean (range))	44.94 (15-77)		
SLEDAI score (mean±SD)	5.39 ± 5.82		
ACR criteria			
Malar rash	5 (16.13)		
Discoid lesions	3 (9.68)		
Photosensitivity	12 (38.71)		
Oral ulcers	8 (25.81)		
Arthritis	17 (54.84)		
Serositis	8 (25.81)		
Cytopenia	16 (51.61)		
Renal disorder	2 (6.45)		
Neurological disorder	2 (6.45)		
Autoantibodies, n (%)			
ANAs	31 (100.00)		
Anti-dsDNA/titer, U/ml (mean±SD)	20 (64.52), 105.80±174.76		
Anti-SSA	10 (32.26)		
Anti-SSB	2 (6.45)		
Anti-Sm	2 (6.45)		
Anti-RNP	1 (3.23)		
Rheumatoid Factor	5 (16.12)		
Anti-cardiolipin IgG	8 (25.81)		
Anti-cardiolipin IgM	4 (12.90)		
Lupus anticoagulant	15 (48.39)		
Treatment, n (%)			
None or NSAIDs	2 (6.45)		
Antimalarial drugs	27 (87.10)		
Glucocorticoids	13 (41.93)		
Immunosuppressive drugs[a]	6 (19.35)		
Statins	0 (0.00)		

BMI: body mass index; dsDNA: double stranded DNA; NSAID: non-steroidal anti-inflammatory drug.
[a] Mycophenolate mophetil, azathioprine.
[b]Subclinical atheromatosis defined as carotid plaque presence or intima media thickness (cIMT) >0.9 mm.
Differences between SLE or non-autoimmune atheromatosis and healthy controls were analyzed by χ^2 test (**p<0.01;***p<0.001).

Supplementary Table 2. Serum levels of inflammatory cytokines

Molecules	Controls (N=33)	SLE patients (N=109)	p-value
IFNα	3.20 (12.41)	7.19 (130.31)	**0.008**
IL-17A	6.94 (11.37)	9.90 (22.83)	0.307
IL-10	1.14 (1.84)	1.98 (2.32)	**0.012**
IL-6	1.21 (4.29)	2.60 (5.80)	0.214
IFNγ	19.84 (32.77)	33.56 (51.32)	0.442
BLyS	520.24 (307.33)	859.02 (1036.23)	**0.005**
CCL3 (MIP1a)	3.22 (8.50)	3.19 (10.72)	0.188

Values represent median (interquartile range). Differences analyzed by U Mann-Whitney test.