DISEASE TRENDS OVER TIME AND CD4^{+}CCR5^{+} T-CELLS EXPANSION PREDICT CAROTID ATHEROSCLEROSIS DEVELOPMENT IN PATIENTS WITH SYSTEMIC LUPUS ERYTHEMATOSUS

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ABSTRACT

INTROUCTION

Patients with Systemic Lupus Erythematosus (SLE) present increased cardiovascular mortality compared to the general population. Few studies assessed long-term development and progression of carotid atherosclerotic plaque in SLE patients. Our aim was to investigate the association of clinical and laboratory markers of disease activity and of classical cardiovascular risk factors (CVRF) with carotid atherosclerosis development in patients with SLE in a prospective 5-year study.

MATERIALS AND METHODS

Clinical history and information on principal CVRFs were collected at basal visit and after 5 years in 40 SLE patients (36 women, mean age 42±9 years; 14.4 ± 7 years of mean disease duration) and 50 age-matched controls. Carotid Doppler ultrasonography was employed to quantify the atherosclerotic burden at baseline and at follow up. Conventional and modified clinimetrics were applied to assess SLE activity over time (SLEDAI). Amongst biomarkers we evaluated the association between basal circulating T cell subsets (including $CD4^+CCR5^+$; $CD4^+CXCR3^+$; $CD4^+HLADR^+$; $CD4^+CD45RA^+RO^-$, $CD4^+CD45RO^+RA^-$ and their subsets) identified by Flow-Cytometry Analysis and plaque development.

RESULTS

During 5-year follow up 32% of SLE patient, developed carotid atherosclerosis compared to 4% of controls. Furthermore, considering SLEDAI changes over time, patients within the highest tertile were those with the increased incidence of carotid plaque independently of CVRF. In addition, increased levels of $CD4^+CCR5^+$ T subsets were independently associated with development of carotid atherosclerosis in SLE.

CONCLUSION

Serial clinical evaluations over time, rather than a single point estimation of disease activity or CVRF burden, are required to define the risk of carotid atherosclerosis development in SLE patients. Specific T cell subsets are associated with long-term atherosclerotic progression and may further be of help in predicting vascular disease progression.

INTRODUCTION

Systemic inflammation contributes to atherosclerotic vessel remodelling and cardiovascular (CV) risk as an independent pathogenic factor [1-4]. Systemic lupus erythematosus (SLE) is a paradigm of how heterogeneous pathophysiologic mechanisms converge towards an increased risk of vascular disease [5, 6] [5, 7-11]. In particular, arterial atherosclerosis is frequent in patients with SLE at any stage [10, 12], and antiphospholipid antibodies (APA), frequent in SLE patients, represent a further independent additional risk factor for thromboembolism [10, 12]. Cross-sectional studies on large cohorts of patients with SLE and with other persistent inflammatory diseases including rheumatoid arthritis and systemic vasculitides document a high prevalence of advanced atherosclerosis as well as of early cardiovascular events [5, 7-11, 13]. By contrast, clear data from cross-sectional and prospective studies based on Intima-Media Thickness (IMT) measurement and patient stratification by baseline clinical characteristics and CVRFs which were aimed at identifying clinically differences between patients and controls in terms of atherosclerosis progression are lacking [7, 10, 13-15]. Disease heterogeneity as well as the effect of immunosuppressive drugs and steroids constitute possible confounders [13, 16-18]. Furthermore, fluctuations of disease activity through time may prompt transient vessel inflammation facilitating rapid plaque development and/or rupture [10, 12, 15]. Accordingly, serial evaluations of disease activity could be more informative than isolated clinical evaluations of the contribution of systemic inflammation to damage accrual [19].

T lymphocytes in particular play a central role in large vessel injury, either of dysmetabolic or autoimmune/autoinflammatory origin [2, 13, 20-25] and autoreactive T-cells are pivotal for the initiation and maintenance of the pathogenic cascade of SLE [6, 26-29]. Among T-cell subsets, $CD4^+$ $HLA\text{-}DR^+$and $CD4^+$ $CCR5^+$ T-cell subsets were expanded in SLE patients; however, little is known about the specific role of these cells as pathogenic actors or diagnostic markers of persistent vascular injury in SLE.

Here we report the results of a five-year longitudinal analysis on cardiovascular risk assessment in a cohort of patients with SLE from a single referral centre, which took into account as potential determinants:

T cell populations signature in the peripheral blood as well as serial evaluation of disease activity through time.

MATERIALS AND METHODS

SLE cohort and study endpoints for disease evolution

Out of 50 SLE previously characterized patients [13], forty (36 women, 42 ± 9 years old, diagnosed according to the 1997 ACR criteria) were followed up longitudinally at S. Raffaele University Hospital, Milan, Italy. All patients gave written informed consent for participation in the study and the Institutional Review Board of the San Raffaele University Hospital (Comitato Etico dell'Ospedale San Raffaele, Milano, Italy) approved the study protocol (protocol "Autoimmuno-mol", PI AAM).

Baseline evaluation included demographics, common CVRF, SLE disease history including antiDNA and antiphospholipid antibody (APA) positivity and concomitance of antiphospholipid syndrome (APAS). SLE disease activity at baseline and at the end of the follow up were estimated by the Physician Global Assessment score (PGA), the SLE disease activity index 2000 (SLEDAI-2K) score and the British Isles Lupus Assessment Group index (BILAG) 2004 score [30, 31]. The SLE-responder index (SRI) was also calculated at the end of follow up with respect to the baseline evaluation [32]. SLE-related damage index as per the Systemic Lupus Erythematosus International Collaborating Clinics (SLICC)/American College of Rheumatology Damage Index score [33] was also recorded at time 0 and after 5 years. Classic Framingham 10 year – cardiovascular risk estimate as well as SLE-specific cardiovascular risk estimate as proposed by Petri and Magder [17] were calculated, based upon the clinical and laboratory data at baseline visit and on the average disease activity over the previous two years. Cardiovascular risk was also estimated by employing the CUORE algorithm, which is more specific for the Italian population and integrates information about fasting glucose and anti-diabetic treatments [34]. During the follow up period, the intensity of disease activity was measured by employing the definitions of Zen et al. [19], which is based on variations in clinical SLEDAI (excluding serological activity) over three annual visits. We also introduced a variant of this index in order to assess the degree of SLEDAI changes (increase/decrease) over follow-up with respect to the basal evaluation. This modified SLEDAI trend was thus calculated as follows:

$$SLEDAI\ trend_{year\ n} = \frac{SLEDAI_{follow-up} - SLEDAI_{basal\ visit}}{SLEDAI_{basal\ visit}}.$$

SLE patients were then divided into tertiles of SLEDAI change to study its correlation with the carotid atherosclerosis development.

We also identified the most represented pattern of disease activity over the time as per Zen et al. [19] and defined four classes of disease evolution ("stable remission", "stable activity", "improving disease", "worsening disease") according to the Clinician's evaluation.

The selection of the control group was described previously [13]; briefly 50 subjects (34 women, 39 + 9 years-old) matched for age, gender, race, and hypertension status were randomly selected from the general population, who had been enrolled in a study on carotid intimal lesions progression, the "PLIC study" [35]. The PLIC study is a large survey of the general population, approved by the Ethics Commitee of the University of Study of Milan, and all participants signed a written informed consent [36].

Clinical, anthropometric and biochemical parameters

Medical history and information about current therapies were obtained for all participants, blood pressure (mmHg) was measured, and body mass index (kg/m^2) and waist/hip ratio were calculated. Blood samples were collected at the basal visit by blood drawn from antecubital vein after at least 10 hours of fasting. After centrifugation at 3,000 rpm for 12 minutes, serum was used for the determination of lipid profile, glucose level and other classical circulating biomarkers [13, 37]. CVD risk and the presence of the metabolic syndrome were assessed, as previously described [37-40]. International guidelines were followed for the diagnosis of diabetes [41] and hypertension [42]. Lifestyle, smoking and dietary habits were recorded, as previously reported [43].

Evaluation of carotid atherosclerosis development

High resolution B-mode ultrasonography with a linear ultrasound probe (4.0-13.0 MHz frequency, 14X48 mm footprint, 38 mm field of view, Vivid S5 GE Healthcare®, Wauwatosa, WI, USA) was performed at basal visit and after follow-up to determine the development of carotid atherosclerosis. The determination were performed by a single expert sonographer, blinded to the subject's identity (intra-class correlation= 0.812, n= 30); in two scans performed on 75 subjects by the same operator, the mean difference in IMT was

0.005±0.002 mm and the variation coefficient was 1.93%. The correlation between two scans was significant with r=0.96 (P<0.0001). All the measurements were done off-line using the software provided by the machinery. The Intima-Media Thickness (IMT) was assessed at the far wall as the distance between the interface of the lumen and Intima, and the interface between the media and adventitia in a standardized number of points. In detail, the protocol involved detection of the Intima at the common carotid artery (CCA) (30 mm proximal to the carotid bulb); then the measurement of IMT at the carotid bulb (CBA) and internal carotid artery (ICA) at both sides was performed to calculate the mean carotid IMT (c-IMT, between CCA, CBA and ICA), as previously described [43]. In the same way, the maximal IMT was recorded and averaged for the left and right sides of the CCA (30 mm proximal to the carotid bulb), the carotid bulb, the ICA. Carotid atherosclerosis development was considered for a) c-IMT progression above the 75th percentile of median age-adjusted values for Caucasian population according to ASE guidelines [44] b) and/or for carotid plaque development, defined for presence of focal thickening (caliper > 1.3 mm in longitudinal resolution, lateral or medial angle) and/or diffusive mean IMT > 1.3 mm (in longitudinal resolution, lateral or medial angle). Ultrasound scanning and analysis of the carotid arteries was performed by a single expert sonographer, using an 8-MHz transducer (Biosound 2000 II sa, Indianapolis, USA) with an axial and lateral resolution of 0.385 and 0.500 mm, respectively as described [18, 20] (see supplemental material and methods). The sonographer was blinded to the subject's identity. B-mode evaluations are obtained from captures of the far wall in the first centimetre of common carotid arteries, proximal to the bulb dilation, in lateral projection. Five standardized points 5, 10, 20, 25, and 30 mm from bulb were measured in both arteries and averaged to calculate the mean IMT for each subject.

Flow- Cytometry Analysis

Flow-cytometry analysis to characterize circulating T cell subpopulations was performed as previously described [22]. Briefly, whole blood was collected in EDTA anticoagulated vacutainer tube. Samples were stained and fixed within the day of collection (differences in the levels of expression were not detected between samples immediately analyzed compared to those analyzed after 24-time room temperature interval post-sampling, as reported [23]. We identified five T-cell subsets, out of total lymphocytes ($CD3^+$: T

cells; $CD3^+CD4^+$: T helper cells; $CD4^+CD45RA^+RO^-CCR7^+$: T naive (T_N) cells; $CD4^+CD45RO^+RA^-CCR7^+$: T Central memory (T_{CM}) cells; $CD4^+CD45RO^+RA^-CCR7^-$ T Effector memory (T_{EM}) cells. Further gating allowed to identify percentages of T_N, T_{CM} and T_{EM} gated on $CD4^+CXCR3^+$, $CD4^+CCR5^+$ and $CD4^+HLA\text{-}DR^+$ therefore separating T-cell subpopulations by means of their combination of the surface markers. Cell viability was >99%, assessed using the Molecular Probes Patented LIVE/DEAD Viability (Invitrogen), according to the manufacturer instructions.

Statistical analysis

Statistical analysis of data was performed using SPSS® v.23.0 for Windows® (IBM Corporation®, Chicago Illinois, USA) program. Shapiro-Wilk test was performed to verify the normal distribution of linear variables. For variables normally distributed, the mean ±standard deviation (SD) was indicated and t-test of comparison was used; for non-normally distributed variables, median and inter-quartile range (IQR) was reported and Mann-Whitney U non-parametric test was performed. For dichotomous variables, $\chi 2$ test and relative risk (95% C.I., confidence interval) assessment was performed. Spearman correlation coefficients (ρ) were reported for univariate correlations between linear variables; standardized regression coefficients (β) were reported when multiple stepwise linear regression models were performed (including all the covariates independently associated with the independent variable at univariate analysis). Multiple stepwise logistic regression model (for comparison of two groups) or Analysis of Co-Variances (ANCOVA) models (for comparison of more than two groups) were built up to compare linear variables among two or more than two groups of patients, respectively. Non-normally distributed variables were log-transformed when included in the multivariable models. To each multivariable regression model test of collinearity was associated to verify presence of redundant variables. Box plots were built up to visualize T subsets distributions by using GraphPad Prism 5® for Windows® (Graphpad Software® Inc., La Jolla, CA, USA). For all analysis, statistically relevant differences were considered for p values < 0.05.

RESULTS

1. Cardiovascular risk and disease activity in SLE patients.

SLE patients and controls were studied and compared in terms of anthropometric and cardio-metabolic parameters (**Table 1**). SLE had a higher waist/hip *ratio* than healthy controls, (0.895 $\pm$ 0.107 vs 0.834 $\pm$ 0.092, p= 0.005) as well as higher LDL-cholesterol levels (133.58 $\pm$ 31.04 mg/dL vs 112.54 $\pm$ 50.44 mg/dL, p= 0.012). Although patients and controls had similar systolic/diastolic blood pressure values, anti-hypertensive drugs were more frequently employed in patients with SLE (p=0.036) to achieve a more effective renal protection.. Estimated 10 year-cardiovascular risk according both to the Framingham's algorithm and the SLE-specific score proposed by Magder and Petri [17] were consistent with higher prevalence of CVRF in the SLE cohort, compared to the general population **(Table 1)**. Nine of SLE patients (22.5%), experienced a history of previous cardiovascular events at time of enrolment, while any were reported for controls.

The age at SLE onset was 28 $\pm$ 9 years and at time of enrolment the mean duration of disease was 14 ± 7 years. Twenty-two SLE patients (55%) had a SLEDAI-2K score ≥4 at basal visit and 11 (27%) had at least one BILAG domain scoring A or B. Damage accrual (SLICC Damage Index $>$ 0) was reported in 30% of the cohort. 19 patients (47.5%) had APA (6 of them were "triple positive", *i.e.* had two serum IgG APA specificities and a functional plasma lupus anticoagulant test, a condition associated with an enhanced thrombo-embolic risk) and 6 (15%) had a diagnosis of APAS **(Supplemental Table 1).** Annual collection of data over 5 years revealed that 21 (52%) SLE patients experienced at least one disease flare, 4 (10%) had a SLEDAI-2K score ≥ 4 at the end of follow up while 5 (25%) active patients at baseline achieved a positive (≥4) SLE-responder index (SRI) at end study visit. Clinical quiescent disease (according to [19]) was the most frequent pattern of activity (62.5%) while a significant proportion of patients (27.5%) had stable or worsening disease. Sixteen patients (40%) had SLICC Damage Index >0 at the end of follow up, with four of them that had no damage at baseline **(Supplemental Table 1).**

At basal evaluation, classical CVRF did not correlate with clinical markers of disease activity or damage accrual, except for BMI, which was increased in patients with low SLEDAI (ρ= -0.429, p= 0.006;

Supplemental Table 2A). Similar data were obtained five years later (**Supplemental Table 2B**), confirming that point evaluation of disease activity through common clinimetrics does not intercept significant associations with classical CVRFs. Therefore, we next tested whether disease evolution rather than point activity status (at baseline and at follow up end) might explain the cardiovascular risk of patient with SLE.

2. Carotid atherosclerosis development characterizes SLE and associates with disease activity over time

Patients with SLE had higher C-IMT values than controls both at the basal visit (0.618 $\pm$ 0.014 mm vs 0.522 $\pm$ 0.012 mm; $p< 0.001$; **Supplemental Table 1**) and at the end of the study (0.619 $\pm$ 0.020 mm vs 0.528 $\pm$ 0.010 mm after follow-up, $p< 0.001$), irrespectively of confounding factors such as age and conventional CVRF ($p<0.001$ at both visits). Antihypertensive treatments or SLE disease related treatments did not affect IMT progression (Supplemental Table 3).

32.5% of SLE patients (13/40) developed carotid atherosclerosis over time, as opposed to 4% (2/50) in the control group ($p= 0.003$ when adjusting for age, gender and basal C-IMT; thus confirming the increased risk of incident atherosclerosis in SLE patients (**Figure 1A**). When the disease course over time was retrospectively analyzed at follow up end, carotid atherosclerosis development was more prevalent among SLE patients with chronically active/worsening disease over 5 years than among those with stable activity/improving disease ($p= 0.011$; **Figure 1B**). When the association between SLEDAI changes over time with atherosclerosis was investigated, patients within the highest tertile of SLEDAI change were those with the highest prevalence of incident atherosclerotic plaques (**Figure 1C**). Of note, trends over time of CVRFs did not correlate with atherosclerosis development (data not shown). These data suggest that the extent and evolution of SLE disease is a risk factor for the progression of subclinical atherosclerosis, independently from CVRFs (**Table 2**) as well as SLE-related auto-antibodies titration (**Table 3**). The autoantibody repertoire was apparently less relevant, since patients with APAS (n=6) – but not those with APA only (n=19) - showed a faster C-IMT progression, which was not statistically significant ($p = 0.068$; not shown). To further investigate potential mechanisms associating the increased immunoinflammatory reactions in SLE patients with atherosclerosis, we also investigated the T cell subsets signature in relation to plaque incidence.

3. Circulating memory T cells are associated with carotid atherosclerosis development.

$CD3^+CD4^+$ T lymphocyte subsets were profiled in SLE patients (**Figure 2**). As expected, all SLE patients had mild lymphopenia and reduced $CD3^+CD4^+$ T cell counts, as well as a different T cell subsets distribution compared to controls **Supplemental Table 4**). When SLE patients were grouped according to presence or absence of subsequent carotid atherosclerosis development, the features of circulating lymphocytes were consistent with a chronically active immune response; among the different subsets investigated, the fraction of $CD4^+CCR5^+$ T cells was significantly higher in SLE patients who developed carotid atherosclerosis compared to those who did not and to controls (**Figure 2B**, **Supplemental Table 4**). Of note this association was independent of the correlation between $CD4^+CCR5^+$ T cells and the markers of disease activity, including SLEDAI-2K, SLICC Damage Index or PGA **(Supplemental Figure 1),** as well as other T cells subsets (**Supplemental Table 5**). The analysis of other subsets in SLE, including $CD4^+CD45RO^+RA^-HLADR^+$ T cells or $CD4^+CD45RO^+RA^-CXCR3^+$ T cells, did not discriminate patients who developed or not carotid atherosclerosis. (**Figure 2C to 2O and Supplemental Table 4**).–No major effects of immunosuppressant therapies, prednisone, aspirine or anti-hypertensive treatments on T cells subsets profiles were observed (**Supplemental Table 6a and 6b**, **Supplemental Table 7**). Altogether, the data indicate that also the expansion of circulating $CD4^+CD45RO^+RA^-CCR5^+$ T cells might contribute to predict the development of carotid atherosclerosis in SLE patients.

DISCUSSION

Thanks to novel therapeutic regimens and consensus guidelines on disease diagnosis and management, morbidity and mortality has been reduced in SLE patients [45]. Nonetheless, comorbidities observed in SLE, such as infections and cardiovascular disease, still constitute a major issue in the daily rheumatology practice. Cardiovascular events, in particular, represent major causes of death in SLE [5, 45].

In this study, we focused on the interplay between systemic inflammation and carotid vascular remodeling in SLE. In line with previous reports, we observed that SLE patients with long disease history had a significant atherosclerosis burden and a high incidence of cardiovascular events, despite the young age and potential protective factors such as the female gender [10, 12, 46, 47]. 22.5% SLE patients developed carotid atherosclerosis after a 5-year follow-up period, suggesting faster carotid intimal thickening over time in comparison with the general population [36]. Focal and low echogenic plaques were more prevalent in SLE patients, possibly reflecting specific pathogenic events occurring at sites of vascular inflammation in systemic autoimmune diseases [7, 48].

Our results also indicate that some classical CVRFs are more prevalent in SLE patients when compared to the general population. While some CVRFs like BMI, cholesterol and hypertension [49] might contribute to the accelerated atherogenesis observed in SLE, none of them appeared to be the real culprit [50]. Lipid metabolism plays a particularly controversial role. In fact, if in one hand increased LDL cholesterol levels have been linked to higher rates of LDL oxidation [51] and atherosclerosis development [52, 53], on the other clinical data do not support a critical role for LDL-C levels in predicting atherosclerosis development in SLE patients [12]. Although we failed to appreciate the impact of each single CVRF on carotid atherosclerosis, SLE patients with metabolic syndrome had higher atherosclerosis development. Of note, SLE patients might present metabolic dysfunction [54] which is often associated to immuno-inflammation [55]; in line with this, SLE patients with metabolic syndrome (MS) presented an increased prevalence of activated T cells subsets compared to SLE patients without MS (Supplemental Figure 2).

Moreover we cannot exclude that alterations in CVRFs profile reflect the long term use of pharmacological treatments. For example, steroid cycles impact LDL cholesterol levels or waist/hip ratio [50, 56, 57].

Thus conventional cardiovascular risk estimation (which is by definition less reliable in secondary prevention [21]) may prove useless in the majority of real-life clinical settings involving patients with SLE.

Markers of inflammation and disease activity are attractive candidate predictors of cardiovascular burden in SLE. Conventional parameters employed to guide immunosuppression and disease monitoring do not correlate with single CVRF and with presence of metabolic syndrome and fail to predict the development of cardiovascular events [12] and carotid atherosclerosis in SLE patients (**Table 2 and 3**); this observation identifies CVRFs and SLE clinical parameters as different sets of the same balance and highlights the need of identifying additional, disease-specific risk factors to classify SLE patients at higher risk of premature cardiovascular disease. Furthermore, while the association between SLE disease activity indexes and initial atherosclerosis development is clear, advanced carotid plaques do not appear to be strictly dependent on SLE disease [58].

The assessment of the trend of disease activity over time is more indicative of the overall cardiovascular risk than scattered evaluations of disease activity and/or common CVRF prevalence at given time points. Most patients who developed carotid atherosclerosis were characterized by persistent or worsening activity during the observation period. This novel information well fits with previous reports showing that SLE duration from diagnosis over years is correlated to the extent of atherosclerosis [49] and to the risk of cardiovascular events [9]. The longitudinal design of this study also reconciles the apparently diverging observations reported in previous cross-sectional and prospective studies on atherosclerosis progression [49, 59-63].

We also report that specific T cells subsets such as $CD4^+$ $CCR5^+$ T cells predict carotid atherosclerosis development in SLE patients. Activated T cell subset, play an important role in the pathogenesis of atherosclerosis [22, 64] and comprise also circulating T follicular helper (Tfh)-like T cells, whose expansion has been previously suggested to reflect an abnormal interaction between T and B lymphocytes in patients with SLE [65], an event that might possibly contribute to accelerated atherosclerosis and enhanced risk of atherothrombosis [66, 67] [68]. Longer follow up studies on larger patients cohorts, and more comprehensive study endpoints are required to verify the possibility that persistence of activated T cells subsets in the blood may be associated with (and possibly responsible of) detrimental vascular effects,

which could be temporally unrelated with clinically detectable disease flares as indicated by the lack of association of T cells subsets with disease activity, vintage, steroids and immunosuppressant treatments as well as SLEDAI changes over follow-up (**Supplemental Tables 5, 6a, 6b**).

Taken together, our data support a model in which sudden surges of vascular inflammation due to concomitant disease flares have still chances to be abrogated without influencing long-term outcomes, if effective therapies are promptly administered. Conversely, mild disease at any time point seems not sufficient to guarantee lower cardiovascular risk if constant, tight control of disease activity is not maintained over time.

Our study has limitations related to the relatively small cohort of patients and set the stage for performing an extensive analysis in larger cohorts where the effect of different treatments on cardiovascular outcome as well as disease trends could be investigated.

CONCLUSIONS

Patients with SLE are characterized by an increased atherosclerotic burden and are more susceptible to cardiovascular events. Common CVRF are highly prevalent in SLE and contribute to the cardiovascular risk in combination with systemic inflammation. Punctual clinical evaluation of disease activity is not sufficient to estimate cardiovascular disease progression over time in contrast to a comprehensive evaluation of disease activity trends during the course of years. Conversely, baseline distribution of specific T-cell subpopulations may predict atherosclerosis progression in the long term.

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FIGURES AND TABLES LEGENDS

Figure 1: SLE disease trend and SLEDAI change over follow-up are associated with carotid plaque development in SLE patients.

(A) Odds ratio (OR) for carotid atherosclerosis development between controls and SLE cohort over 5 years. X axis is in $\log_{10}$ scale; OR with 95% Confidence Intervals (C.I.) are reported on the right side of each group (setting 1 (as reference, "ref.") for Controls group). * indicates $p< 0.001$ according to χ^2 test.

(B) Carotid atherosclerosis development according to SLE disease evolution over follow-up. White bars refer to the proportion of SLE patients who were free from the development of carotid atherosclerosis. Grey bars refer to the proportion of SLE patients who developed carotid atherosclerosis. * indicates $p<0.05$ according to χ^2 test.

(C) Carotid atherosclerosis development according to tertiles of SLEDAI change. White bars refer to the proportion of SLE patients who were free from the development of carotid atherosclerosis. Grey bars refer to the proportion of SLE patients who developed carotid atherosclerosis. * indicates $p<0.05$ according to χ^2 test.

Figure 2: Distribution of circulating $CD4^+$T cells and subsets, according to carotid atherosclerosis development, in SLE patients

The distribution of different T cell subsets, is presented for SLE patients who developed (SLE+) or not (SLE-) carotid atherosclerosis over five years of follow up. Data are presented as total $CD3^+CD4^+$ T cells count (n cell/µL) in panel **(A)** and as percentage of $CD3^+C4^+$ T cells in the subsequent panels. The other panels refer to: **(B)**: % $CD4^+CD45RO^+RA^-CCR5^+$ cells; **(C)**: $CD4^+CD45RO^+RA^-HLADR^+$ cells; **(D)**: % T_N (T naïve): $CD4^+CD45RA^+RO^-CCR7^+$ cells; **(E)**: % T_{CM} (T central memory): $CD4^+CD45RO^+RA^-CCR7^+$; **(F)**: **%** T_{EM} (T effector memory):$CD4^+CD45RO^+RA^-CCR7^-$. **(G,H,I)**: T naïve, T central memory and T effector memory (respectively), gated on $CD4^+CCR5^+$ cells; **(J,K,L)**: T naïve, T central memory and T effector memory (respectively), gated on $CD4^+CXCR3^+$ cells; **(M,N,O)**: T naïve, T central memory and T effector memory (respectively), gated on $CD4^+HLADR^+$ cells. Data are presented as bars with scatter dot plot, reporting mean and confidence intervals (95%) (*indicates $p< 0.05$ from Mann-Whitney non parametric test). Outliers values (from Grubb's test) were removed from analyses.

Table 1: Clinical, anthropometrical and biochemical characteristics of the controls and of the SLE patients.

Mean (±S.D.) values (or median (IQR)) and prevalence (n/total) are reported for the baseline clinical, anthropometrical and biochemical parameters. p are derived from t-student test and χ^2 test.

Table 2: Baseline clinical and classical cardiovascular risk factors of SLE patients grouped according to the presence or absence of subsequent carotid atherosclerosis development.

Mean (±S.D.) values (or median (IQR)) and prevalence (n/total) are reported for all the basal parameters. For linear variables, P are derived from T-student (if normally distributed) (or U- Mann Whitney test if not normally distributed); for dichotomous variables, p are derived from χ^2 test.

"APAS"= Anti-phospholipid syndrome; "HDL-C"= High Density Lipoprotein Cholesterol; "LDL-C"= Low Density Lipoprotein Cholesterol; "CCA-IMT"= Common Carotid Artery Intima-Media Thickness; "BMI"= Body Mass Index; "SLICC"= Systemic Lupus Erythematosus International Collaborating Clinics (SLICC)/American College of Rheumatology Damage Index score; "SLEDAI" = Systemic Lupus Erythematosus Disease Activity Index; "PGA"= Physician Global Assessment.

Table 3: Baseline SLE related features and pharmacological treatments of patients grouped according to presence or absence of subsequent carotid atherosclerosis development.

Mean (±S.D.) values (or median (IQR)) and prevalence (n/total) are reported for all the basal parameters. For linear variables, P are derived from T-student (if normally distributed) (or U- Mann Whitney test if not normally distributed); for dichotomous variables, P are derived from $\chi 2$ test. P for comparison of immunosuppressant drugs is obtained from $\chi 2$ test summing the effect of Azathioprine, Mycophenolate Mofetil and Methotrexate treatment prevalence.

ANA= "Anti Nuclear Antibodies"; APAS= "antiphospholipid syndrome"; Anti-sDNA= "Anti double standed DNA antibodies"; IgM aCL= "anti-Cardiolipin IgM"; IgG aCL= "anti-Cardiolipin IgG"; IgM anti$\beta 2$ GPI= "Anti-$\beta 2$ Glycoprotein I IgM" ; IgG anti$\beta 2$ GPI= "Anti-$\beta 2$ Glycoprotein I IgM".

SUPPLEMENTAL TABLES AND FIGURES LEGENDS

Supplemental Table 1: Characteristics of SLE patients investigated.

Mean (±S.D.) values and prevalence (n (%)) are reported for SLE-related features and activity indexes at baseline. Disease course and outcomes over time are also reported. SLEDAI = "Systemic Lupus Erythematosus Disease Activity Index"; BILAG= "British Isles Lupus Assessment Group index"; SLICC Damage Index= "Systemic Lupus Erythematosus International Collaborating Clinics (SLICC)/American College of Rheumatology damage index score"; Anti-sDNA= "Anti double stranded DNA antibodies"; SRI= "SLE Responder Index"

Supplemental Table 2: Correlation between SLEDAI-2K score, SLICC Damage Index, PGA scores with cardio-metabolic parameters and classical cardiovascular risk factors in SLE patients at baseline and follow-up.

Spearman's univariate correlation coefficients (ρ) and p value are reported for each parameter versus SLEDA-2K score, SLICC Damage Index and PGA. Data are referred to baseline **(A)** or to follow up visit **(B)**. SLEDAI-2K= "SLE disease activity 2000"; SLICC Damage Index = "Systemic Lupus Erythematosus International Collaborating Clinics (SLICC)/American College of Rheumatology damage index score"; PGA= "Physician Global Assessment Score".

Supplemental Table 3: Effects of pharmacological treatments on carotid Intima-Media Thickness in SLE cohort.

Mean (±S.D.) values for Carotid Intima-Media Thickness (CCA-IMT, mm) at basal visit and after follow-up in patients groups according to presence or absence of pharmacological treatments (anti-hypertensives, prednisone, immunosuppressants and hydroxychloroquine). Carotid Intima-Media Thickness change (ΔCCA-IMT / years of follow-up) was calculated for each patient, subtracting the basal CCA-IMT value to that after the follow-up and dividing by years of follow-up.

Supplemental Table 4: Circulating T subsets distribution in controls and SLE patients.

Mean (S.D.) values (or median (IQR), after Shapiro-Wilk test for non-normal distributions). t-tests (for normal distributions) or Mann-Whitney test (for non-normal distribution) were performed and significant differences (for $p < 0.05$) were indicated with: *= Controls vs SLE patients who did not developed atherosclerosis; **= Controls vs SLE patients who developed carotid atherosclerosis; ***= SLE patients who developed carotid atherosclerosis vs SLE patients who did not developed carotid atherosclerosis.

Supplemental Table 5: Correlation between SLEDAI-2K score, SLICC Damage Index and PGA scores and T cells subsets in SLE cohort.

Spearman's univariate correlation coefficients (ρ) and p value are reported for each parameter versus SLEDA-2K score, SLICC Damage Index and PGA. Data are referred separately to baseline or to follow up visit. SLEDAI-2K= "SLE disease activity 2000"; SLICC Damage Index= "Systemic Lupus Erythematosus International Collaborating Clinics (SLICC)/American College of Rheumatology damage index score"; PGA= "Physician Global Assessment Score".

Supplemental Table 6a: Effects of Immunosuppressant and Prednisone treatments on T cells subsets in SLE cohort.

Mean (S.D.) values (or median (IQR), after Shapiro-Wilk test for non-normal distributions). t-tests (for normal distributions) or Mann-Whitney test (for non-normal distribution) were performed and significant differences between each treatment for $p < 0.05$) were indicated ("(*)" in bold).

Supplemental Table 6b: Effects of Hydroxychloroquine and Aspirin treatments on T cells subsets in SLE cohort.

Mean (S.D.) values (or median (IQR), after Shapiro-Wilk test for non-normal distributions). t-tests (for normal distributions) or Mann-Whitney test (for non-normal distribution) were performed and significant differences between each treatment for $p < 0.05$) were indicated ("(*)" in bold).

Supplemental Table 7: Effects of Hydroxychloroquine and Aspirin treatments on T cells subsets in SLE cohort.

Mean (S.D.) values (or median (IQR), after Shapiro-Wilk test for non-normal distributions). t-tests (for normal distributions) or Mann-Whitney test (for non-normal distribution) were performed and significant differences between each treatment for $p < 0.05$) were indicated ("(*)" in bold).

Supplemental Figure 1: CD4+CCR5+ T cell subsets and markers of SLE disease activityCD4+CCR5+ T cell subsets (percentage out of total CD3+CD4+ count) in SLE patients grouped according to disease activity indexes: SLEDAI-2K (at basal visit **(A)** and after follow-up **(B)**), SLICC Damage Index (at basal visit **(C)** and after follow-up **(D)**) and PGA (at basal visit **(E)** and after follow-up **(F)**). SLEDAI-2K= "SLE disease activity 2000"; SLICC Damage Index= "Systemic Lupus Erythematosus International Collaborating Clinics (SLICC)/American College of Rheumatology damage index score"; PGA= "Physician Global Assessment Score". Data are presented as bars with scatter dot plot, reporting mean and confidence intervals (95%) (*indicates $p < 0.05$ from Mann-Whitney non parametric test). Outliers values (from Grubb's test) were removed from analyses.

Supplemental Figure 2: Distribution of circulating $CD4^+$T cells and subsets in SLE patients grouped by presence or absence of Metabolic Syndrome

The distribution of different T cell subsets, is presented for SLE patients with Metabolic Syndrome (SLE MetS+) and those without Metabolic Syndrome (SLE MetS-). Data are presented as total cells counts. The panels refer to: **(A)**: total lymphocytes count (cell/µL); **(B)**: total $CD3^+CD4^+$ T cells (cell/µL); **(C)**: T_N (T naïve) count: $CD4^+CD45RA^+RO^-CCR7^+$ (cell/µL); **(D)** T_{CM} (T central memory) count: $CD4^+CD45RO^+RA^-CCR7^+$ (cell/µL); **(E)** T_{EM} (T effector memory) count: $CD4^+CD45RO^+RA^-CCR7^-$ (cell/µL); **(F)** $CD4^+CD45RO^+RA^-CCR5^+$ count (cell/µL); **(G)**: $CD4^+CD45RO^+RA^-CXCR3^+$ count (cell/µL); **(H)**: $CD4^+CD45RO^+RA^-HLADR^+$ count (cell/µL). Data are presented as bars with scatter dot plot, reporting mean and confidence intervals (95%) (*indicates $p< 0.05$, **$p<0.01$ from Mann-Whitney non parametric test). Outliers values (from Grubb's test) were removed from analyses.

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