**Systemic Lupus Erythematosus: Disease activity may influence the release of endothelial microparticles?**

**Short title: Endothelial microparticles in SLE**

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**ABSTRACT**

**Objectives:** To evaluate blood borne Endothelial Microparticles (EMPs) in women with SLE and correlated these to disease activity as defined by the SLEDAI-2K Score.

**Methods:** The study takes cross-sectional design. A total of 90 age-matched women were recruited including; G1 (healthy volunteers, n=30), G2 (women with SLE and low disease activity (SLEDAI-2K Score ≤4; n=30)) and G3 (women with SLE and moderate/high disease activity (SLEDAI-2K Score > 4; n=30). Blood was collected in 3.2% sodium citrate. Subsequently, the MPs were purified by ultracentrifugation and labeled with anti-CD51/61 and anti-Annexin-V antibodies. Quantification and phenotyping were performed using flow cytometry.

**Results:** The number of EMPs was significantly higher in SLE patients compared to controls (p=0.0178). When SLE patients were stratified according to disease activity, the number of EMPs was significantly increased in women with moderate to high disease activity compared to controls (p=0.0074). We observed a correlation between the number of EMPs and age (r= -0.34; p=0.0123) and between the number of EMPs and SLEDAI-2K Score (r=0.30; p=0.04).

**Conclusions:** Our results suggest that the SLE causes increased EMPs release, especially in patients with SLEDAI-2K Score >4. While measurement of the EMPs could be useful in distinguishing patients with SLE from health controls, they have limited value in differentiating between SLE subtypes.

**Key words:** Systemic Lupus Erythematosus; Endothelial Cells; Microparticles; Flow Cytometry

**INTRODUCTION**

Systemic Lupus Erythematosus (SLE) is an inflammatory multisystemic autoimmune disease. It is characterized by the production of various auto-antibodies and tissue-deposits of circulating antigen-antibody-complexes [1]. Genetic, environmental and hormonal factors are directly involved in the etiopathogesis of SLE, leading to loss of control of the immune balance.

In addition to a wide range of clinical manifestations, patients with SLE are prone to increase risk of arterial disorders and venous thrombosis [2], the main causes of morbidity and mortality in patients with SLE. For example, the incidence of myocardial infarction among SLE patients is reported to be 50% greater than those seen in healthy individuals [3-5]. Interestingly, this was not related to factors such as hypertension, dyslipidemia and diabetes. The mechanisms of accelerated atherosclerotic process in SLE are not fully understood [6]. However, similar to heart disease, changes in vascular endothelium have been implicated; the endothelium is linked to both inflammation and atherogenesis.

The physiological importance of Microparticles (MPs) remains poorly understood, however it is well documented that MPs have a procoagulant surface, express negatively charged phospholipids and tissue factor (TF) and can trigger coagulation activation [7-10]. In addition, MPs carry pro-inflammatory lipids and important membrane components (e.g., inducers of apoptosis). Several disorders with a thrombotic tendency are associated with increase circulating MPs [11,12]. The characteristics of the MPs depend on its origin and mechanism of stimulation [2]. Endothelial Cell MPs (EMPs) can trigger neutrophil activation and activate coagulation [12,13], acting as potent pro-inflammatory inducers, affecting endothelial function. Thus, these fragments may be involved in the regulation of coagulation and vascular function *in vivo*.

A number of studies stressed the clinical importance of identifying biomarkers for early diagnose of SLE, assessing its severity and monitoring response to treatment [14-16]. Increased EMPs numbers have been associated with endothelial damage [17-19]. In the present study, we aim to assess EMPs levels in women with SLE and health volunteers, and investigate whether patients with increased disease activity, as determined by the SLEDAI-2K Score, have higher levels of EMPs.

**METHODS**

**Participants**

Patients were recruited between February 2013 and April 2016 from the Santa Casa Hospital Outpatient Clinic in Belo Horizonte, Brazil. Ethical approval was granted by the Research Ethics Committee of the Federal University of Minas Gerais (Reference: 01928412.8.0000.5149). Informed consent has been sought from all participants. The relevant clinical details and laboratory data were obtained from medical records. The study takes cross-sectional design. A total of 90 age-matched women were recruited. These include three groups; G1 (healthy volunteers, n=30), G2 (women with SLE and low disease activity (SLEDAI-2K Score ≤4; n=30)) and G3 (women with SLE and moderate/high disease activity (SLEDAI-2K Score >4; n=30). They were diagnosed according to the ACR criteria (1997). Azathioprine, Prednisone and Hydroxychloroquine (alone or in combination) were the most commonly used drugs. The epidemiological and clinicodemographic dataare shown in Table 1.

**Inclusion and exclusion criteria**

The control group consisted of healthy women with no family history of rheumatoid diseases, selected through a standard questionnaire. Exclusion criteria common for the SLE groups include patients with other associated conditions, patients taking anticoagulants or medication that are known to affect haemostatic function or anti-inflammatory, patients with immunosuppressive diseases including HIV/AIDS or autoimmunity, and pregnant women.

**Sample collection**

A 5 mL random blood samples were collected from each subject into vacutainer tubes containing sodium citrate (Becton- Dickinson, USA). The samples were then centrifuged twice for 15 minutes at 1027g and platelet-poor plasma was isolated within 2 hours of specimen collection. Aliquots were stored at -80⁰C for batch-wise analysis. For each assay, a previously unthawed aliquot was used.

**Purification of the endothelial microparticles**

Citrated plasma was thawed at room temperature and centrifuged at 12,815g for 10 minutes to obtain platelet-free plasma, the supernatant of which contained the MPs. A 100 μl of the supernatant was removed and placed in another tube containing 300 μl of citrate-heparin (Hepamax-S) solution (1: 3 dilution). The diluted sample was centrifuged at 15,000xg for 90 min at 15°C in a Laborzentrifugen 2K15 centrifuge (Sigma). The supernatant was carefully removed and the MPs pellet was resuspended in 100 μl of the binding buffer.

**Quantification and phenotyping of microparticles**

Quantification and phenotyping of the MPs between the groups were performed using flow cytometry in a "blind" manner and by a single evaluator. The instrument used was the LSR-FORTESSA BD, USA equipped with three lasers (blue, red and violet), which allows the simultaneous evaluation of 16 parameters. The identification of cellular populations of interest, the percentage value of these populations and the sub-populations were performed through DIVA software. Cell population analysis was achieved using Flow Jo software version 8.7 (Tree Star. Ashland, Oregon, USA). The purified and isolated MPs were analyzed according to their size and granularity, being FSC (forward) versus SSC (side) scatter, with dispersion distribution compared to spheres of known size of 0.7 to 0.9 μm (SPHERO ™ Amino Fluorescent Particles-Spherotech Inc. Libertyville, Illinois, US).

In order to determine the cellular origin of the MPs, phenotypic characterization was performed using 100 μL of the plasma containing MPs in the presence of 5 μL of the antibody to endothelial cells (CD51 / PE). 2.5 μL of Annexin V (FITC) was added to each tube. To a separate tube, FITC/PE labeled mouse IgG antibodies were used as control. After the addition of the antibodies, the samples were incubated for 30 minutes in the dark. Subsequently, the MPs were resuspended in 100 μl of the Annexin V binding buffer (BD Pharmingen Sede: Franklin Lakes, Nova Jersey, EUA).

MPs were quantified by flow cytometry using calibration fluorescence microbeads of defined size (0.7 - 0.9 μm; Spherotech Inc. Libertyville, Illinois, US). 10 μl of the beads were added to 100 μl of sterile 1X PBS. The absolute number of MPs per μl was calculated according to the formula of Campos et al., 2010:

**MPs/μl = (N x 400) / (60 x 100)**

Where;

N = Number of events acquired in the MPs region

400 = Total volume in each tube before analysis

60 = Volume of sample analyzed

100 = Original volume of the MPs suspension

**Analysis of microparticles**

The analysis strategy for the evaluation of the MPs was initially based on the (forward-scattered light- FCS) and granularity (Side-scattered light - SSC) followed by the evaluation of the expression of Annexin V by means of the dot plot FSC versus Annexin V. Within the gate exhibiting Annexin V positivity, the percentage of events expressing the CD51PE surface marker of the EMPs was assessed by means of the FSC dot plot versus fluorescence. As a non-specific labeling control, an isotype (anti-IgG) labeled with the same fluorochrome as the CD51 marker was used. This strategy is useful in positioning the gates on the samples labeled with the antibody to EMPs.

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism version 5.01 (GraphPad Prism. San Diego, California, USA).The data was not normally distributed, thus nonparametric tests were performed. Differences between two groups were assessed using the Mann-Whitney test, while differences between more than two groups were evaluated by the Kruskal-Wallis test followed by Dunn's multiple comparison test. The relationship between the studied parameters was assessed by the Spearman’s rho Correlation. The level of statistical significance was set at p<0.05.

**RESULTS**

**Epidemiological and clinicodemographic data**

The epidemiological and clinicodemographic dataare shown in **table 1**.There was a negative and statically significant relationship between EMPs and age (r = -0.3385; p = 0.0123) i.e., younger patients have increased EMPs numbers **(Figure 3)**.

**Quantification of endothelial microparticles in the studied groups**

The number of EMPs was significantly higher in patients with SLE compared to controls (p = 0.0178). However, when the studied groups were stratified according to disease activity, EPMs were significantly increased in SLE women with moderate to high activity compared to the control group (p = 0.0074). There was no significant difference between patients with low / moderate and high disease activity (Figure 2).

**The relationship between endothelial microparticles and the SLEDAI-2K Score**

There is a weak positive but significant correlation between EMPs and the SLEDAI-2K Score (r ​​= 0.2785; p = 0.0377) i.e., high EMPs counts are associated with an increased SLEDAI-2K Score **(Figure 3)**. Arguably such an association might still be important as the SLEDAI-2K Score is calculated based on clinical parameters related to SLE aggravation. A weak negative but significant correlation was also observed between age and SLEDAI-2K Score values in patients with SLE (r= -0.2819; p=0.0291).

**DISCUSSION**

The endothelium has for many years been recognized not only as a physical barrier between blood and vascular wall but also as a strategically located organ with multiple endocrine, autocrine and paracrine functions. Physiologically, the endothelium regulates vascular homeostasis and plays an important role in protecting the blood vessel [20].

In addition to its inflammatory nature, SLE causes an imbalance in the production of mediators that regulate vascular tone, platelet aggregation, coagulation and fibrinolysis. Endothelial dysfunction is also frequently caused by the loss of nitric oxide (NO) bioavailability [21]. Increased inflammation is associated with decreased NO availability, activation of leukocytes and raised serum cytokines, suggesting that inflammatory processes may cause endothelial dysfunction [22-26].

In the present study, patients with SLE showed significantly higher EMPs levels compared to controls **(Figure 2A)**. This indicates that SLE itself, regardless of severity, might be related to endothelial damage. However, hypertension, diabetes mellitus, heart failure and hypercholesterolemia can also cause endothelium injury, resulting into endothelial dysfunction, which is often associated with cardiovascular events [23]. Only two of our patients showed uncontrolled cardiovascular risk factors; one with hypertension and the other with diabetes **(Table 1)**. This finding reinforces the idea that increased EMPs may have a direct relationship with SLE, arguably independent of other factors that could trigger endothelial damage. Thus, one could argue that EMPs could potentially be a suitable biomarker for vascular dysfunction.

It is well recognized that SLE is an inflammatory condition. Thus, one could argue that inflammation and endothelial damage are the cause for the EMPs increase observed in our study. This assumption is supported by previous studies reporting that the reduction of inflammatory processes is associated with a decrease in the number of EMPs [27,28]. Nomura et al., 2004 demonstrated that EMPs carry Tissue Factor and could potential cause coagulation activation and fibrin formation [28]. This may lead to thrombotic manifestations which are frequently observed in patient with SLE.

To quantify EMPs, we used the CD51 marker which is an integral membrane glycoprotein, known as vitronectin receptor α chain, or integrin αV. This complex mediates leukocyte-endothelial adhesion and plays an important role in the initiation of inflammatory processes [29]. It is worth noting that this complex is a marker found not only in endothelial cells but also in monocytes, macrophages, platelets as well as in some B cells. However, Jimenez et al., 2003 observed that CD51/61 is the main antigen expressed in endothelial cells during inflammatory and apoptotic processes and that EMPs, when released from the activated endothelium, carry an increased amount of this antigen [31]. In addition, other studies have used this marker to evaluate EMPs [12,32]. These findings have confirm our choice of CD51/61 to determine the EMPs in our study.

In patients with moderate/high disease, the number of EMPs was not significantly higher than those found in patients with low disease activity **(Figure 2B)**, suggesting that SLEDAI-2K Score may better reflect disease activity in patients undergoing treatment for SLE. One possible explanation is that SLEDAI-2K Score, the main activity scoring system used in SLE, evaluates a set of clinical and laboratory manifestations, whereas EMPs reflect only endothelial damage. However, **Figure 2B** shows a trend towards increased EMPs in patients with SLE with moderate/high disease compared to patients with low disease activity. This suggests that the endothelium is probably being progressively assaulted by an intensifying inflammatory process resulting in an increase in the number of EMPs, which is evidenced by an increase in SLEDAI-2k . In addition, autoimmune diseases are likely to cause endothelial dysfunction and may raise the EMPs. Indeed, Song et al., 2015 showed that intermediate coronary lesions lead to increased EMPs due to endothelial damage [34,36]. An increase in the number of samples tested from patients with low and moderate/high activities may help to elucidate this point further.

Spearman’s correlation analysis showed a weak positive association between the number of EMPs and the SLEDAI-2K Score (r = 0.2785; p = 0.0377). However, a very high correlation between such variables might be impossible in such complex condition, since many factors may contribute to endothelial injury, which are not necessarily linked to those evaluated by the SLEDAI-2K Score. In other words, although the inflammatory process plays a major role in the release of EMPs by the endothelium, inflammation does not affect in an equal way the number of EMPs and the parameters evaluated by the SLEDAI-2K Score. However, our study suggests that SLE (moderate/high activity) manifests into endothelial damage and formation of microvesiculation, giving rise to increased EMPs release. This is a rather worrying finding as it would indicate treatment failure. It should be noted that the patients recruited for this study were on medication.

SLE is a disease that occurs most frequently in women between the ages of 20 and 40, i.e., predominantly of childbearing age [37]. We found an inverse relationship between age and the number of EMPs i.e., the lower the age of the patients, the higher the number of EMPs (r = -0.3385, p = 0.0123). If the number of EMPs reflects the severity of this disease, it could be stated that SLE is more serious in younger women, suggesting some role for sex hormones that act in the reproductive cycle such as estrogen and progesterone, in addition to the pituitary hormones FSH and LH that control the production of these two sex hormones [38]. SLE is considered to be a serious condition in young women. We observed a weak but significant correlation between age and the SLEDAI-2K Score (r=-0.2819; p=0.0291). As an increase in the SLEDAI-2K Score may reflect disease severity, our finding lends further support to the fact that SLE is perhaps both prominent and severe in younger individuals.

The study adds to the exciting body of knowledge in the field. However we recognize that there are some limitations. The subgroups with proteinuria or lupus nephritis have not been explored because of the very limited number. Similarly, the effect of the different therapeutic regimens (azathioprine, prednisone and hydroxychloroquine) on the number of EMPs was not explored. A logistic regression model in which the determinants of active disease can be investigated cannot be performed on the basis of a limited number of cases, a fact aggravated by the absence of many essential data in medical records. Another limiting factor of the study is the fact that there were no significant differences in the number of EMPs between patients with low and moderate/high disease activity. This may be related to the sensitivity of the standardized technique or other intrinsic factor.

Finally, the measurement of other specific markers of endothelial damage such as thrombomodulin and peripheral arterial tonometry (PAT) and their correlation not only with the number of EMPs but also with SLEDAI-2K Score, could contribute to establishment of the real value of the determination of the number of EMPs by flow cytometry.

In conclusion, our result suggest the determination of EMPS by flow cytometric assay could be useful in distinguishing patients with SLE from health controls, especially those with SLEDAI-2K Score >4. However, the numbers of EMPs do not appear to reflect SLE activity as assessed by SLEDAI-2K, hence there was no significant differences between patients with low or medium/high SLE disease activity. Thus, EMPs appear to be of a limited value in stratifying SLE subtypes and hence may not reflect disease severity.

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**LEGENDS TO ILLUSTRATIONS**

**Figure 1:** Comparison of EMPs between healthy patient (control) and SLE patient with low and moderate/high activity. The gates, with double positivity for Annexin V and CD51, show quantitative differences regarding the EMPs of women belonging to the control group or with low or moderate/ high activity.

**Figure 2:** Box-plots of the distribution of EMPs in healthy women (control) versus women with SLE (A) \*Mann Whitney; Healthy women (control) versus women with SLE (low and moderate/high activity) (B).\*Kruskal Wallis.

**Figure 3**: Correlation between the number of endothelial microparticles with age (A) and SLEDAI-2k (B).