Camila de Melo Carvalho Nascimento[1]
Multiuser Laboratory for Research Support in Nephrology and Medical Sciences (LAMAP), Faculty of Medicine, Universidade Federal Fluminense (UFF), Rio de Janeiro – Brazil E-mail: carvalho_camila@id.uff.br

Lilian Alves[2]
Multiuser Laboratory for Research Support in Nephrology and Medical Sciences (LAMAP), Faculty of Medicine, UFF, Rio de Janeiro - Brazil. E-mail: lilian_santos@id.uff.br

Rodrigo Cutrim Gaudio[3]
Reumathology Unit, Hospital Universitário Antonio Pedro, Rio de Janeiro – Brazil E-mail: rodrigogaudio@yahoo.com.br

Alice Ramos Silva [4]
Multiuser Laboratory for Research Support in Nephrology and Medical Sciences (LAMAP), Faculty of Medicine, UFF, Rio de Janeiro - Brazil. E-mail: aliceramos@id.uff.br

Matheus Daudt Lemos[5]
Multiuser Laboratory for Research Support in Nephrology and Medical Sciences (LAMAP), Faculty of Medicine, UFF, Rio de Janeiro - Brazil. E-mail: mdaudt@id.uff.br

Mauro Jorge Cabral-Castro[6]
Multiuser Laboratory for Research Support in Nephrology and Medical Sciences (LAMAP), Faculty of Medicine, UFF, Rio de Janeiro - Brazil.E-mail: maurojorge@id.uff.br

Katia Lino Batista[7]
Reumathology Unit, Hospital Universitário Antonio Pedro, Rio de Janeiro – Brazil. E-mail: linokatia@gmail.com

Ana Luísa Figueira Gouvêa[8]
Department of Pathology, Faculty of Medicine, UFF, Rio de Janeiro - Brazil. E-mail: anafigueira@id.uff.br

Jorge Reis Almeida[9]
Multiuser Laboratory for Research Support in Nephrology and Medical Sciences (LAMAP), Faculty of Medicine, Department of Clinical Medicine, Faculty of Medicine, UFF, Rio de Janeiro - Brazil. E-mail: jorgereis@id.uff.br

Andrea Alice da Silva[10]
Multiuser Laboratory for Research Support in Nephrology and Medical Sciences (LAMAP), Faculty of Medicine, Department of Pathology, Faculty of Medicine, UFF, Rio de Janeiro – Brazil, Rio de Janeiro – Brazil. E-mail: aasilva@id.uff.br

Thalia Medeiros[11]
Multiuser Laboratory for Research Support in Nephrology and Medical Sciences (LAMAP), Faculty of Medicine, Department of Pathology, Faculty of Medicine, UFF, Rio de Janeiro – Brazil, Rio de Janeiro – Brazil. E-mail: thaliamedeiros@id.uff.br

Fabiana Rabe Carvalho[12]
Multiuser Laboratory for Research Support in Nephrology and Medical Sciences (LAMAP), Faculty of Medicine, Department of Pathology, Faculty of Medicine, UFF, Rio de Janeiro – Brazil, Rio de Janeiro – Brazil. E-mail: fabianarc@id.uff.br

THE ASSESSMENT OF DNA DAMAGE BY ALKALINE COMET ASSAY IS NOT ASSOCIATED WITH CLINICAL OUTCOMES IN WOMEN WITH SYSTEMIC LUPUS ERYTHEMATOSUS

A AVALIAÇÃO DO DANO AO DNA PELO ENSAIO COMETA ALCALINO NÃO ESTÁ ASSOCIADA A DESFECHOS CLÍNICOS EM MULHERES COM LÚPUS ERITEMATOSO SISTÊMICO

ABSTRACT

Introduction: In systemic lupus erythematosus (SLE), an inflammatory state leads to oxidative stress and excessive formation of reactive oxygen species, increasing cellular damage, mutations and compromised DNA repair. Therefore, our study aimed to evaluate the performance of the alkaline comet assay to investigate DNA damage in women with SLE.Methods: This is a cross-sectional study that included Brazilian women with SLE attended at an university hospital (Rio de Janeiro, Brazil) from 2015 to 2018. Disease activity was assessed using the SLE Disease Activity Index 2000 (SLEDAI-2K). Diagnosis of CMVand EBV infections were performed by qPCR. DNA damage was determined by alkaline comet assay, which is a electrophoresis techinique used to evaluate DNA fragmentation in leukocytes, which is expressed as arbitrary units (AU). Results: We studied 34 SLE patients with a median age of 34.5 years-old. Hospitalized patients (n=11, 32.3%) presented higher SLEDAI-2K, where 9 (81.8%) had SLEDAI-2K ≥ 5 (p=0.038). We did not identify significant differences between healthy and SLE; however, hospitalized patients showed higher median AU, but with no statistical significance (p=0.095). Also, no differences were in DNA damage according to SLEDAI-2, lupus nephritis, use of immunossupressants, or CMV/EBV infections. DNA damage was not associated with levels of anti-dsDNA. Lastlty, ROC curves demonstrated a poor predictive power of DNA damage to differentiate groups (AUC < 0.7, p > 0,05). Conclusion: Our results indicate that DNA damage measured by alkaline comet assay is not associated with SLE severity and the presence of viral infections.

KEYWORDS: women's mental health; public policy; public policy assessment.

KEYWORDS: Systemic lupus erythematosus, DNA damage, alkaline comet assay.

RESUMO

Introdução: No lúpus eritematoso sistêmico (LES), um estado inflamatório leva ao estresse oxidativo e à formação excessiva de espécies reativas de oxigênio, aumentando o dano celular, mutações e comprometimento do reparo do DNA. Portanto, nosso estudo teve como objetivo avaliar o desempenho do ensaio do cometa alcalino para investigar danos ao DNA em mulheres com LES.Métodos: Trata-se de um estudo transversal que incluiu mulheres brasileiras com LES atendidas em um hospital universitário (Rio de Janeiro, Brasil). de 2015 a 2018. A atividade da doença foi avaliada usando o SLE Disease Activity Index 2000 (SLEDAI-2K). O diagnóstico de infecções por CMV e EBV foi realizado por qPCR. O dano ao DNA foi determinado pelo ensaio cometa alcalino, que é uma técnica de eletroforese usada para avaliar a fragmentação do DNA em leucócitos, que é expressa em unidades arbitrárias (UA). Resultados: Estudamos 34 pacientes com LES com idade mediana de 34,5 anos. Pacientes hospitalizados (n=11, 32,3%) apresentaram maior SLEDAI-2K, onde 9 (81,8%) apresentaram SLEDAI-2K ≥ 5 (p=0,038). Não identificamos diferenças significativas entre indivíduos saudáveis e com LES; entretanto, os pacientes hospitalizados apresentaram mediana de UA maior, mas sem significância estatística (p=0,095). Além disso, não houve diferenças nos danos ao DNA de acordo com SLEDAI-2, nefrite lúpica, uso de imunossupressores ou infecções por CMV/EBV. Os danos no DNA não foram associados aos níveis de anti-dsDNA. Por último, as curvas ROC demonstraram um fraco poder preditivo de danos no DNA para diferenciar grupos (AUC < 0,7, p > 0,05). Conclusão: Nossos resultados indicam que o dano ao DNA medido pelo ensaio cometa alcalino não está associado à gravidade do LES e à presença de infecções virais.

PALAVRAS-CHAVE: Lúpus eritematoso sistêmico, dano ao DNA, ensaio cometa alcalino.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic inflammatory autoimmune disease, characterized by relapsing and remission periods that lead to a wide spectrum of clinical symptoms ranging from mild to life-threatening illness (BASTA et al., 2020). In Brazil, recent studies estimate that 65.000 people live with SLE, 90% being women with 20-45 years-old (ILLESCAS-MONTES et al., 2019; ZUCCHI et al., 2022). SLE etiology is not well understood, but some evidence indicates that genetic, environmental, hormonal, viral infections or even emotional factors can be the triggers for the development this disease (FREIRE; SOUTO; CICONELLI, 2011). The assessment of disease activity is critical to predict clinical outcomes and to evaluate treatment efficacy (ROMERO-DIAZ; ISENBERG; RAMSEY-GOLDMAN, 2011). Thus, the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI-2K) is a useful tool for evaluating SLE activity by concomitantly investigating clinical manifestations and laboratory alterations (FREIRE; SOUTO; CICONELLI, 2011; GLADMAN; IBAÑEZ; UROWITZ, 2002).

Mutations in regulatory segments involved in cellular apoptosis and impaired cellular waste disposal mechanisms are major contributors to SLE, such as upregulation of p53 protein expression and DNA hypomethylation ((WU et al., 2016; ZHANG et al., 2022). Therefore, these abnormalities in the cell death process leads to the production of nuclear autoantigens that stimulate the immune system to produce anti-nuclear antibodies (ANAs), such as Anti-double strand (ds) DNA autoantibodies, which is used for SLE diagnosis and activity assessment (ARNETH, 2019; VINUESA; SHEN; WARE, 2023).

The comet assay, a alkaline-comet electrophoresis techinique, has been applied to assess DNA damage in SLE patients to support the association between oxidative stress and disease (FANG et al., 2015; MICHELI et al., 2021; MONTALVÃO et al., 2012). In this context, a previous study showed that peripheral blood mononuclear cells from patients with lupus nephritis (LN) presented elevated intrinsic DNA damage, which may contribute to systemic autoimmunity (SOULIOTIS et al., 2019). Another study showed that the combined use of N-acetylcysteine and hydroxychloroquine decreased SLE activity and oxidative stress, reducing DNA damage (DOHERTY; OAKS; PERL, 2014; LAI et al., 2012). However, up to this date, we have not found studies that have evaluated levels of DNA damage in women with SLE according to clinical outcomes such as need for hospitalization and cytomegalovirus (CMV) and Epstein-Barr (EBV) infections, since the immunossupression can lead to reactivation of latent viral infections which can trigger and/or worsen SLE activity (FELDMAN et al., 2015; FULKERSON et al., 2021; WANG et al., 2015). Importantly, EBV has been associated with malignant progression of B cells and increased autoantibody production in SLE (ABENAVOLI et al., 2011; HOUEN; TRIER, 2021; ZHU, 2023). Recently, Guta et al. (2023)(GUTA et al., 2023) observed that EBV/CMV coinfection in 6% of patients with SLE. O'Dowd et al. (2012)(O'DOWD et al., 2012), using the comet assay in cultured CMV-infected fibroblasts, observed that infected cells were unable to complete DNA repair process.

Thus, considering the above, this study aimed to assess the performance of alkaline comet assay to investigate DNA damage in women with SLE according to disease activity, the need for hospitalization, levels of anti-dsDNA, use of medications and CMV and EBV infections.

MATERIAL AND METHODS

STUDY DESIGN, PARTICIPANTS AND DATA COLLECTION

This cross-sectional observational study included adult women diagnosed with SLE attended at Hospital Universitário Antônio Pedro (HUAP - Niterói, Rio de Janeiro, Brazil) from March 2019 to February 2020. This project was approved by the Research Ethics Committee of the Universidade Federal Fluminense (UFF, CAAE: 43049215.2.0000.5243) and all activities were performed after obtaining written consent. We included a group of healthy participants (n=12) without preexisting comorbidities for comparison purposes. Exclusion criteria were: pregnancy, smoking, diabetes mellitus, alcohol consumption up to two weeks before the collection, and history of other infections or radiological imaging tests 3 months prior the recruitiment.
Clinical, demographic and laboratory data were obtained from patient's charts. Disease activity was measured by the SLE Disease Activity Index 2000 (SLEDAI-2 K) at the time of sample collection. SLE activity was considered as following: 0 = no activity; 1-4 = mild activity; >5 = moderate/high activity (GLADMAN; IBAÑEZ; UROWITZ, 2002). For the analysis of DNA damage in association with drug use, we considered as immunosuppression the following dosage: prednisone >20mg/day, azathioprine >2mg/kg/day, mycophenolate >1g/day, methotrexate ≥20mg/week, and any dose of cyclophosphamide (BAE et al., 2001).

COMET ASSAY

The alkaline comet assay was performed according to the Minimum Information for Reporting on the Comet Assay guideline (MIRCA) (MØLLER, 2018). Briefly, slides received a layer of normal melting point agarose (1.5%, Sigma-Aldrich, USA) by rapid immersion. Whole blood (5µL) was collected in a heparinized tube (BD Vacutainer®, UK) and posteriorly slowly homogenized with low melting point agarose (0.5%, Sigma-Aldrich, USA) for complete cell distribution. In sequence, the sample was added to the slide and cell lysis was performed overnight at 4ºC protected from light with a lysis buffer (2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris, 1% [w/v] N-lauroylsarcosine sodium salt, 1 % [v/v] Triton X-100 and 10 % [v/v] DMSO, pH 10). The slides were submitted to electrophoresis (300mA for 25 min) in alkaline buffer (NaOH 300mN/EDTA 1mM, pH 13) for 20min in an ice bath (25 V, 0.86 V/m). All slides were covered with a neutralization buffer (3 washes, 0.4 M Tris buffer solution, pH 7.5), fixed with absolute ethanol for 10 min, dried at room temperature overnight, and stained with ethidium bromide (20µg/mL).
Five fields per slide (100 cells/patient) were analyzed using a fluorescence microscope (400× magnification, ZEISS Axio Imager, Germany) using 2 slides (duplicates). Methyl methane-sulfonate (MMS) (160 µM, Aldrich, USA) was used as a positive control. The extension of DNA migration was analyzed by two independent professionals. The intensity of DNA damage was categorized into four classes according to tail size: class 0 - absence of tail (G0); class 1- small tail (G1); class 2 - big tail (G2); class 3 - core completely damaged (G3). DNA damage was expressed in percentage of cells in different classes and number of arbitrary units (AU), according to the formula: AU= [(G0 x 0) + (G1 x 1) + (G2 x 2) + (G3 x 3)] (CARVALHO et al., 2013).

DIAGNOSIS OF CITOMEGALOVIRUS AND EPSTEIN-BARR INFECTIONS

Real-time polymerase chain reaction (qPCR) assays for quantification of CMV (Cat. # XG-CMV-MB Mobius Life Science, Paraná, Brazil) and EBV (Cat. # XG-HHV6-MB Mobius Life Science, Paraná, Brazil) were performed according to the manufacturer's recommended instructions. qPCR reactions were performed in the Applied Biosystems™ 7500 Fast Real-Time PCR Systems (Thermo Fisher Scientific, USA). The detection limit was 0.57 and 0.6 copies/µL for CMV and EBV, respectively. Viral load of CMV > 500 copies/mL and EBV >1000 copies/mL were considered as positive.

STATISTICAL ANALYSIS

Variables were expressed as median with data dispersion evaluated by interquartile range [IQR]. We compared categorical variables using Fisher's exact or chi-square test and continuous variables using the nonparametric Mann-Whitney test. To compare differences between 3 groups, we used Kruskall-Wallis with Dunn's post-test. P values < 0.05 were considered statistically significant. Receiver operating characteristic curves were performed to establish the Youden index cut-off for the comet assay when discriminating groups. Statistical analyses were conducted using the "pROC" package in RStudio (R Core Team - R.3.2.2) and Prism software (GraphPad Software 8.0, San Diego, CA, USA).

RESULTS

COHORT DESCRIPTION

We studied 34 SLE patients with a median age of 36 [25 – 44] years, 88.2% were non-white and 11 (32.3%) were hospitalized. The mean time since the first SLE diagnosis was similar between groups (p=0.08). Thirteen (38.2%) patients presented kidney dysfunction (decline of renal function and/or LN) and 15 (44.1%) performed kidney biopsy. In the healthy group, also composed exclusively by women, the median age was 36 [34 - 42] years and 75% were non-white. Demographic and clinical characteristics of SLE patients and healthy participants are described in Table 1.

Parameters	Healthy (n=12)	SLE (n=34)		*P*-value
		Non-hospitalized (n=23)	Hospitalized (n=11)	
Age (years) (median [IQR])	36.50 [34.75-44.00]	38 [25.50 - 44.00]	4 [20.50 - 42.00]	0.239
Non-white (n, %)	9 (75.0)	20 (87.0)	10 (90.9)	1.0
SLE time (median [IQR])	-	6.50 [4.00 - 10.50]	5.00 [2.00 - 7.50]	0.087
EBV infection (n, %)	-	7 (35.0)	3 (27.3)	0.969
CMV infection (n, %)	-	1 (4.3)	4 (36.4)	0.051
SLEDAI-2K (n, %)	-			
No activity (0)	-	6 (26.1)	0 (0.0)	
Mild (1-4)	-	10 (43.5)	2 (18.2)	**0.038**
Moderate/High (>5)	-	7 (20.6)	9 (81.8)	
Lupus nephritis (n, %)	-	11 (47.8)	5 (45.4)	1.0
Class of DNA damage (n, %)				
G0	98 [95 - 100.00]	99 [93.5 - 100]	95 [94 - 96]	0.192
G1	2 [0 - 3]	1 [0 - 5]	3 [2 - 6]	0.132
G2	0 [0 -0]	0 [0 - 1]	0 [0 - 1]	0.950
G3	0 [0-0]	0 [0 - 0]	0 [0 - 1]	0.210

Table 1. Demographic, clinical, and laboratorial characteristics of SLE patients

As expected, hospitalized patients presented higher median SLEDAI-2K, where 9/11 (81.8%) had SLEDAI-2K ≥ 5 (p=0.038). Regarding the evaluation of laboratory parameters, we observed significant difference between hospitalized and non-hospitalized patients for hemoglobin (p=0.001), leucocytes (p=0.001), erythrocyte sedimentation rate (p=0.048), creatinine (p=0.004), urea (p=0.003) and eGFR (p=0.017) (Supplementary Table).

ASSESSMENT OF DNA DAMAGE BY ALKALINE COMET ASSAY IN SLE PATIENTS

The analysis of DNA damage classes by comet assay showed that the majority of patients, as well as controls, presented G0 and G1 classes. These data are also shown in Table 1. When analyzing AU for DNA damage, we did not identify significant differences between healthy controls and SLE (2.5 [0 – 7] vs. 4 [1 -8], p=0.24). However, we observed that hospitalized patients presented in higher median of DNA damage when compared to non-hospitalized patients, but with no statistical significance (2 [0 – 8.5] vs. 6 [4.5 – 8], p=0.095). Also, no differences were identified when we compared DNA damage according to SLEDAI-2K (inactive vs. mild activity vs. moderate/high activity, p=0.248) and LN (p=0.715). These results are shown in Figure 1.

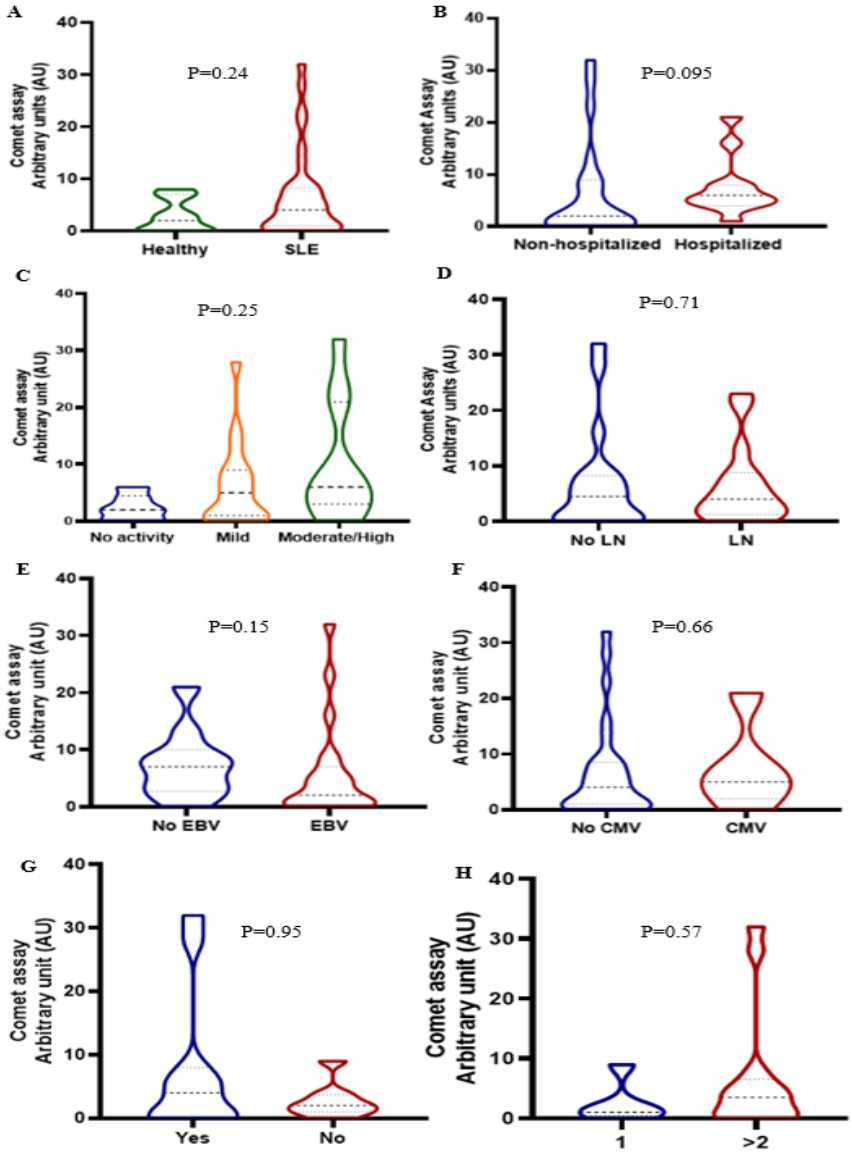

Figure 1: Comet assay and Systemic Lupus Erythematosus (SLE). (A) Comparison of arbitrary units by comet assay in healthy control (HC) and patient with SLE **(B)** SLE patients according to the hospitalization outcome **(C)** SLE patients in activity of disease (SLEDAI-2K) **(D)** SLE patients according to lupus nephritis diagnoses **(E)** SLE patients according to EBV infection and **(F)** CMV infection (G) use of immunosuppressive therapies (H) Number of drugs used in therapy. Differences between groups were assessed using Mann–Whitney test.

DNA DAMAGE ACCORDING TO VIRAL INFECTIONS, IMMUNOSSUPRESSION AND ANTI-DSDNA LEVELS

In our cohort, five (14.7%) patients presented positive qPCR for CMV and 10 (29.4%) patients for EBV. The bivariate analysis showed similar DNA damage (AU) between patients with and without CMV (p=0.66) and EBV (p=0.154) infections. To determine the relationship between viral load and DNA damage (AU), we created a correlation matrix, as illustrated in Figure 2. Once again, we did not observe significant correlations between variables, where Spearman coefficients for DNA damage and viral load were 0.283 (p=0.122) for EBV and 0.285 (p=0.120) for CMV. Of note, in our cohort, one patient

presented a CMV/EBV co-infection with moderate/high SLE disease activity (SLEDAI = 21), LN class IV and 35 of DNA damage (AU).

Our next step was to identify if the DNA damage could be influenced by the use of multiple immunomodulatory/immunossupressant drugs (Figure 1). We identified various combination of drugs with different mechanisms of action, mainly corticoids (prednisone), immunomodulators (hydroxychloroquine), inosine 5′-monophosphate dehydrogenase enzyme inhibitors (mycophenolate), purine antimetabolite immunosuppressant (azathioprine), antimetabolic agents (methotrexate) and alkylating angents (cyclophosphamide). We did not observe any influence of the immunosuppressants use in DNA damage (p=0.95). Besides, we found no differences in DNA damage between patients using one imussupressant and those using multiple grugs (p=0.57).

Moreover, we aimed to asses the correlation between DNA damage (AU) and levels of anti-dsDNA. Is important to mention that 21 (61.8%) patients presented anti-dsDNA above 20 UI/mL. We identified that patient with anti-dsDNA antibody >20UI/mL did not present higher DNA damage (p=0.51). Lastly, we did not observe a significant and direct correlation between levels of anti-dsDNA and DNA damage (r=0.02; p=0.48). These results are shown in Figure 3.

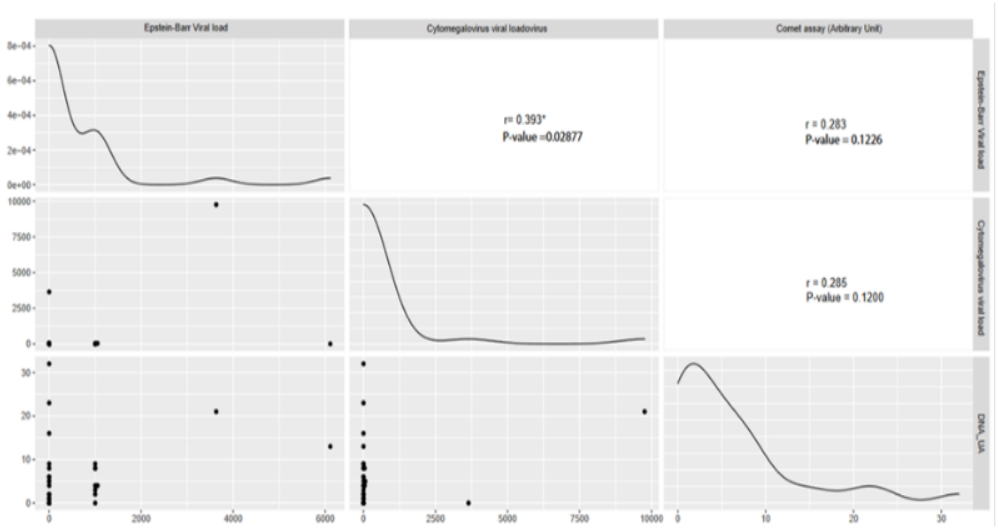

Figure 2: **Correlation matrix**. The distribution of each variable is shown vertically. Scatter plots are provided in the lower triangle of the matrix. The correlation values between each variable are shown at the top of the diagonal. Abbreviations: CMV, cytomegalovirus; EBV, Epstein Barr virus; DNA_UA, DNA damage_ arbitrary unit

ANALYSIS OF THE PREDICTIVE POWER OF DNA DAMAGE (AU)

To evaluate the predictive power of this parameter to discriminate groups according to SLE diagnosis and clinical outcomes (hospitalization, we performed ROC curves. These analysis showed that the comet assay did not presented a good predictive power for distinguish healthy individuals of those with SLE (AUC of 0.61, sensibility of 56.0%, specificity of 67.0%, p=0.24 considering the cut-off 3.5 AU). Next, when analyzing the parameters only in SLE patients, the best performance of DNA damage (AU) was to predicte the outcome “hospitalization”, but the p-value was 0.09 (AUC of 0.52, sensibility of 30.0%, specificity of 91.0%, considerind the cut-off 1.5 AU). These results are demonstrated in Table 2.

Table 2 – Analysis of ROC curves for Comet Assay related parameters in patients with SLE

Parameters	AUC	Cut-off	Sensibility (%)	Specificity (%)	*P value*
Healthy and SLE	0.61	3.50	0.56	0.67	0.24
Hospitalization	0.52	1.50	0.30	0.91	0.09
CMV infection	0.65	5.50	0.45	1.00	0.68
EBV infection	0.61	2.50	0.43	0.80	0.16
Activity	0.73	4.50	1.00	0.52	0.42
Lupus nephritis	0.56	1.50	0.28	0.88	0.72

ROC curves of arbitrary units for the prediction of SLE, hospitalization, activity, infection by cytomegalovirus (CMV), Epstein-Barr virus (EBV) and lupus nephritis in SLE patients. AUC= area under the curve.

DISCUSSION

It is known that DNA alterations contributes to the development of autoimmune disease. Moreover, mutations in DNA repair proteins increases the risk for SLE development and can also contribute to LN stablishment (KAMENARSKA et al., 2019; MIGLANI et al., 2021). Thus, this study aimed to evaluate the degree of DNA damage, using the alkaline comet assay, in women with SLE. We further explored the possible correlations between arbitrary units (AU) of DNA damage and outcomes such hospitalization, disease activity, immunossupression, anti-dsDNA levels and CMV/EBV infection/reactivation. Given the complexity of this disease, it is necessary to search for new laboratory tests that can complement the diagnosis and/or to evaluate SLE activity and therapheutic succes.

Previous studies have demonstrated increased DNA damage by alkaline comet assay in SLE (AKHTER et al., 2016; GAO et al., 2017; SIMONIELLO et al., 2017). In our study, we did not identify increased DNA damage in SLE patients in comparison to healthy individuals. However, in a brief review of the literature, we observed a large heterogeneity in comet assay protocols and inclusion criteria for patients as well as control groups. Some studies compared SLE with controls without excluding smokers or alcohol consumption. It is import to mention that some degree of DNA damage can be observed in healthy individuals, since it can occur due to biological factors such as older age, environmental pollution, occupational exposure, and genetic polymorphisms implicated in DNA damage repair), which can persist until epigenetic control is activated (GIOVANNELLI, 2002; JACKSON; BARTEK, 2009; TRENZ et al., 2002). This may be confounding factors even to define the cut-offs for AU (BONASSI et al., 2003; HOFFMANN; HÖGEL; SPEIT, 2005; POOL-ZOBEL et al., 2004).

When analyzing DNA damage according to SLE activity, no differences were identified according to SLEDAI-2K (inactive vs. mild activity vs. moderate/high activity). SLE activity occurs due to the imbalance between inflammatory and immunoregulatory responses, triggering inflammation and deposition of immune complexes in the vasculature and target organs(BARBER et al., 2021). The prolonged production of ROS induced by the inflammatory process is crucial for disease progression, leading to increased proteins and lipid oxidation, in addition to DNA double-strand breaks (MITTAL et al., 2014), 2014). When intact, the DNA repair process is promoted by the LIG4 and RAD52 genes, which play important roles in repair and homologous recombination (DE AZEVÊDO SILVA et al., 2014; LEE et al., 2023). Here, we can point out that the non-performance of genotyping or micronucleus analysis is a limitation of our work, which could assess the DNA repair capacity.

Some studies observed a relationship between kidney disease and the extension of DNA migration by electrophoresis (COIMBRA et al., 2018; ERSSON et al., 2013). Still, LN, in association with high levels of anti-dsDNA, can lead to higher intrinsic DNA

double-strand breaks (detected by neutral comet assay) and enhanced apoptosis (SOULIOTIS; SFIKAKIS, 2015). In this study, we did not observe differences in DNA damage when comparing patients with and without LN. It is possible that, since the patients included in our study present a short time of disease progression (±5 years), they may still present some DNA repair capacity, mainly through DNA methylation (WANG et al., 2023). Also, we evaluetad the DNA damage by alkaline comet assay, which is capable of detecting DNA double and single-strand sites (PU; WANG; KLAUNIG, 2015).

We have also evaluated if DNA integrity could be affected by the use of immunossupressants in SLE. Martelli-Palomino and colleagues (2017) described that neuthrophil DNA damage are decreased by anti-TNF-α therapy in patients with rheumatoid arthritis. It is known that the cellular damage caused by the association of multiple drugs and their side effects depends on the degree of immunological impairment and genetic heterogeneity (KLEINE SCHAARS; VAN WESTRHENEN, 2023; TABOT et al., 2023). Since SLE is a multisystemic disease, it is difficult to differentiate the damage caused by the disease itself or the treatment. Some studies have observed the protective effect of hydroxychloroquine on SLE development and activity (DOS REIS NETO et al., 2020; FASANO et al., 2023). Other studies have shown that high cumulative dose of prednisone and the presence of anti-dsDNA antibodies were related to permanent organ/system damage (MOLAD et al., 2002). Furthermore, induction of apoptosis has also neen observed in SLE patients using of melphalan or cisplatin in low doses (SOULIOTIS et al., 2019). Recently, the use of azathioprine was associated with the induction of DNA damage in mouse bone marrow cells (MELO BISNETO et al., 2021). In our cohort, 73.7% patients were polytreated (use of ≥ 2 drugs). Overall, we did not observe increased DNA damage associated with immunossupression therapy.

We also aimed to evaluate if CMV and/or EBV infections could affect DNA damage assessed by comet assay in SLE patients, which has not been investigated so far. Infection/reactivation by CMV or EBV leads to clinical manifestations similar to SLE disease activity, and when coexisting, they can contribute to increase morbidity and mortality (SHAO et al., 2023). CMV is a pathogen frequently observed in patients with SLE and its participation in the disease pathogenesis has been discussed (BRUNEKREEF et al., 2021). In our cohort, 44.1% of patients presented infection/reactivation of CMV and/or EBV confirmed by qPCR. Studies have showed these infections can induce genomic instability, decrease DNA repair, and increase genetic mutations (GUTA et al., 2023; SMOLARZ; WILCZYŃSKI; NOWAKOWSKA, 2015; WU et al., 2010, 2023). Genes stimulated by the interferon response to viral infections can affect many cellular processes, including RNA processing, protein stability, and cell viability; directly affecting essential steps in viral replication. Even so, we found no correlation between DNA damage and CMV/EBV infections in the present study.

Determining a cut-off is important by the observer-dependent characteristic of the comet assay (MØLLER, 2018). This work is a pioneer in describing the cut-off by ROC curve analysis for DNA damage measured by alkaline comet assay in SLE; however, in our analysis, the cut-off of 3.5 AU presented low accuracy (specificity of 66.7% and sensitivity of 55.9%) to distinguish SLE from healthy controls. The best performance of DNA damage (AU) in predicting clinical outocomes was for “hospitalization” ($p = 0.09$). Previous studies have evaluated the accuracy of comet assay in different settings. For example, Carvalho *et al.* (2010) showed AUC values < 0.5 when performed ROC curves to distinguish newborns with and without sepsis. In contrast, the comet assay has been shown to be effective in predict male infertility through the analysis of DNA damage in sperm (NICOPOULLOS et al., 2019) and hepatocellular carcinoma in HCV-infected patients with cut-off 248 AU ($r=0.762$; sensitivity of 59% and specificity of 74%) (Shawki et al., 2014).

Once again, several factors can influence in the analysis of DNA damage by alkaline comet assay, ranging from methodological aspects to patient characterization and exclusion criteria. In our study, some limitations could affect these results, including the

small number of patients. Also, our sample is composed exclusively by women mainly because it is characteristic of the disease. A longitudinal cohort could demonstrate if patients present DNA damage before SLEDAI-2K increase or later during remission periods. Moreover, we could not distinguish CMV/EBV primary infection from reactivation due to the absence of serological tests.

CONCLUSION

Our results suggest that DNA damage measured by alkaline comet assay is not associated with disease activity, anti-dsDNA levels, use of immunossupressants or LN in women with SLE. Moreover, even though studies indicate that CMV and EBV can induce genomic instability, we did not observe incresead DNA damage in the presence of these viral infections. Taken together, our results show that the alkaline comet assay shows a poor predictive power for clinical outcomes, and other tests should be considered to assess DNA integrity in SLE patients.

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