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# Global DNA methylation pattern and correlation with complement system proteins in Brazilian patients with systemic lupus erythematosus

## *Padrão global de metilação do DNA e correlação com proteínas do sistema complemento em pacientes brasileiros com lúpus eritematoso sistêmico*

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### Abstract

**Introduction:** Nucleic acid methylation may have major effects on gene expression patterns and, by consequence, on the development of autoimmunity, like Systemic Lupus Erythematosus (SLE). **Objective:** To investigate the pattern of global DNA methylation in SLE patients and compare this pattern with laboratory parameters. **Methods:** Genomic DNA was isolated from SLE patients with non-active disease (SLEDAI<6), SLE patients with active disease (SLEDAI>6), and healthy individuals. Global DNA methylation was evaluated by digestion of genomic DNA with *HpaII* and *MspI* and compared with laboratory parameters. **Results and conclusion:** A statistical difference in DNA global methylation was observed when SLE patients were compared to healthy individuals. A positive correlation was observed between the frequency of global methylation and C3 and C4 serum levels for SLE patients with SLEDAI<6. These results suggest that the relative amount of DNA methylation is increased in SLE patients, and differential methylation of genes related to the complement pathway alters gene expression involved in autoimmune response in SLE patients.

**Key words:** Autoimmune diseases; DNA methylation; Genetic; Proteins; Systemic lupus erythematosus.

### Resumo

**Introdução:** Metilação do ácido nucleico pode alterar a expressão gênica e favorecer o desenvolvimento de autoimunidade, como lúpus eritematoso sistêmico (LES). **Objetivo:** Investigar o padrão de metilação global do DNA em pacientes com LES e comparar com parâmetros laboratoriais. **Métodos:** DNA genômico foi isolado de pacientes com LES com doença não ativa (SLEDAI <6), pacientes com doença ativa (SLEDAI > 6) e indivíduos saudáveis. Metilação do DNA global foi avaliada por digestão do DNA genômico com *HpaII* e *MspI* e comparados com parâmetros laboratoriais. **Resultados e conclusão:** Foi observada diferença estatística na metilação global do DNA em pacientes com LES. Verificou-se correlação positiva entre a frequência de metilação global e níveis séricos C3 e C4 em pacientes com SLEDAI <6. Estes resultados sugerem que a quantidade relativa de metilação do DNA está aumentada em pacientes com LES, e a metilação de diferentes genes relacionados com o sistema complemento podem alterar a expressão de genes envolvidos no LES.

**Palavras-chave:** Doenças autoimunes; Genética; Lúpus eritematoso sistêmico; Metilação de DNA; Proteínas.

## Introduction

Epigenetics consist of the study of changes in gene expression excluding changes in DNA sequence<sup>1</sup>. The role of epigenetic modifications in regulating tissue-specific expression, genomic imprinting, or X-chromosome inactivation is widely recognized. These reversible modifications include DNA methylation, histone acetylation, and RNA interference<sup>2</sup>. Epigenetic imprinting by methylation can exert effects on normal and abnormal immune response, influencing disease susceptibility and severity. DNA methylation consists of the addition of a methyl group to the fifth carbon of cytosine residues, converting these to 5-methylcytosines. This reaction involves specific enzymes called DNA methyltransferases (DNMT) and a methyl group donor, S-adenosylmethionine (SAM).

In mammalian genomes, DNA methylation occurs mostly at CpG islands, that are regions exceeding 500 base pairs (bp) with CpG content higher than 55%<sup>3</sup>. CpG islands have important regulatory functions, and can be found in promoter regions of about half of all genes<sup>4</sup>. Altered CpG island methylation may change chromatin structure, typically being able to modulate the fine promoter-transcription factor interactions within the transcription machinery<sup>5</sup>. The consequence is the maintenance of the DNA sequence with repression of certain genes, since methylation prevents the binding of transcription factors to those methylated gene targets. Aberrant DNA methylation is affected by age, gender, nutritional disorders, lifestyle characteristics, and infections<sup>6</sup>. Particular attention has been focused on the study of epigenetic alterations in cancer; and, more recently, epigenetic changes in cardiovascular, neurological, and autoimmune disorders have also started to emerge<sup>7</sup>. Nucleic acid methylation may have major effects on gene expression patterns and, as a consequence, on the development of autoimmunity<sup>8</sup>.

Systemic Lupus Erythematosus (SLE) is a systemic, multiorgan autoimmune disease with diverse immunological and clinical manifes-

tations characterized by an autoantibody response to nuclear and/or cytoplasmic antigens, forming immunocomplexes that are deposited in the walls of the circulatory system and in different organs, causing inflammation. Primary or secondary deficiencies affecting the complement system are thought to be the most frequent forms of serum protein deficiency associated with SLE<sup>9</sup>. However, the association between epigenetic changes and autoimmune disease to the complement system is not well established. Based on the above-mentioned concepts, we investigated the pattern of global DNA methylation in SLE patients with active and non-active disease and made comparative analyses with inflammatory laboratory parameters (erythrocyte sedimentation rate, C reactive protein), the complement system (C2, C3, and C4), and complement activity by CH100.

## Patients and methods

### Patients

Patients with SLE (n=100) from the outpatient clinic of autoimmune rheumatic diseases at the Federal University of São Paulo (UNIFESP, Brazil) were diagnosed according to updated criteria from 1997 of the American College of Rheumatology (ACR)<sup>10</sup>. Patients underwent a detailed clinical evaluation, with emphasis on SLE clinical manifestations, recurrent infections, current and previous medications, age at SLE onset, other autoimmune diseases, family history, and determination of both the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI)<sup>11</sup> and the Systemic Lupus International Collaborating Clinics-Damage Index (SLICC-DI)<sup>12</sup>. One hundred SLE patients were evaluated in this study. These patients were separated into two groups, following SLEDAI criteria: patients with non-active disease (SLEDAI<6) (n=50, 1 male and 49 females, mean age of 39.78±11.59 years) and patients with active disease (SLEDAI>6) (n=50, 50 females, mean age of 40.7±13.30 years). Fifty healthy blood do-

nors were recruited as healthy controls (n=50, 2 males and 48 females, mean age of 37.28±10.09 years). Only blood donors with no evidence of autoimmune disease, as determined by a structured medical interview, were included in this study. Patients and controls were 18 years old or older and signed the Informed Consent Form, previously approved by the Unifesp Ethical Committee.

## Blood sampling and laboratory analysis

Blood samples were collected from all subjects. The complement system was evaluated by determination of total hemolytic complement using standard immuno-hemolytic assays by (CH100), C2 (immuno-hemolytic assay), C3 (turbidimetry), and C4 (immunonephelometry). Inflammatory laboratory parameters were evaluated by measuring C reactive protein (CRP) and erythrocyte sedimentation rate (ESR) using routine procedures at the Clinical Analysis Laboratory of the Hospital São Paulo of EPM – Unifesp.

## Measure of DNA methylation

Genomic DNA was isolated from 50 SLE patients with non-active disease (SLEDAI<6), 50 SLE patients with active disease (SLEDAI>6), and 50 healthy individuals, matched by gender and age. Global DNA methylation was evaluated by digestion of genomic DNA with *HpaII* and *MspI* in duplicate. *MspI* enzyme digests genomic DNA with methylation CPG islands, and *HpaII* enzyme digest genomic DNA with non-methylation CPG islands (Figure 1). Two µg of genomic DNA were incubated with 2 µl of each enzyme in separate reactions at 37 °C for 16 hours, and after this period 1 µl of each enzyme was added and the tubes kept at 37 °C for one more hour. Samples were then resolved onto 0.8% agarose gel stained with ethidium bromide. The intensity of the band corresponding to intact genomic DNA in different samples was determined us-

ing ImageJ software. The percentage of methylation was calculated using the following formula: relative global methylation content = (*HpaII*-*MspI*)×100/genomic DNA. This analysis was performed in the Genomic Laboratory of the Department of Immunology of IBS – USP.

## Statistical analysis

Data are presented as mean±SD. For comparisons between groups, the Wilcoxon rank sum test was used. Nominal variables were tested using the  $\chi^2$  test. Spearman's rank correlation analysis was used to determine association with the *HpaII*/*MspI* ratio and selected laboratory biomarkers. The *p* values were considered significant for *p*<0.05. All statistical analyses were performed using the statistical software GraphPad Prism version 5 for Windows (GraphPad-USA).

## Results

A statistical difference of DNA global methylation was observed when SLE patients with active and inactive disease were compared to healthy individuals; however, no difference was found in DNA methylation between SLE patients with active disease and those with inactive disease (Figure 2). In the terms of laboratory biomarkers, a statistical difference was found between the SLE groups in the evaluation of C2, C4 and CH100, but not to ESR, CRP and C3 (Table 1).

**Table 1: Laboratory biomarkers**

Laboratory biomarkers	SLEDAI<6 (n=50)	SLEDAI>6 (n=50)	<i>p</i> value
ESR (mm)	30,74±26,78	30,27±22,32	0,5971
CRP (mg/dL)	5,135±6,295	10,19±19,86	0,8392
C2 (mg/dL)	94,20±19,91	62,61±39,63	0,0001*
C3 (mg/dL)	104,7±22,47	96,54±28,82	0,1320
C4 (mg/dL)	18,76±10,11	13,38±8,595	0,0024*
CH100 (%)	93,42±23,80	68,78±33,51	0,0002*

C reactive protein=CRP; erythrocyte sedimentation rate=ESR; data presented as mean±SD; \* *p*<0.05.

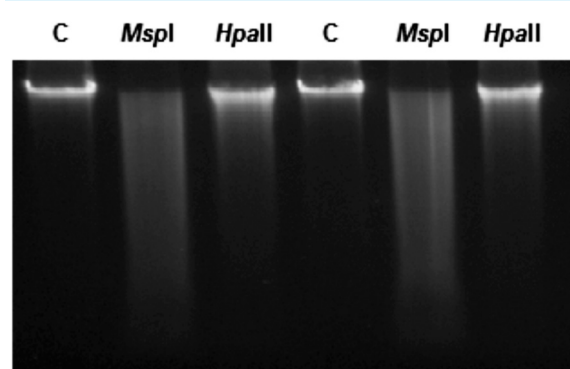
When the values of laboratory biomarkers were compared with the control group, SLE patients with non-active disease (SLEDAI<6) and SLE patients with active disease (SLEDAI>6), presented a statistical difference for C2. When the values of C4 were compared between the SLE group with SLEDAI<6 and the control with the SLE group with SLEDAI>6 (Figure 3A), a significant statistical difference was found. For CH100 the comparison between the control and SLE groups with SLEDAI<6 (Figure 3B) and SLEDAI>6 demonstrated a statistical significant difference (Figure 3D).

A positive correlation by Spearman's rank correlation analysis was observed between the frequency of global methylation and serum levels of C3 ( $r=0.3020$ ,  $p=0.0331$ ) and C4 ( $r=0.3252$ ,  $p=0.0212$ ) (Figure 4) for the SLE group with SLEDAI<6 (Table 2). No correlations were observed for C2 or activity hemolysis by CH100 and inflammatory laboratory parameters, like CRP and ESR, common, but non-specific, inflammatory markers.

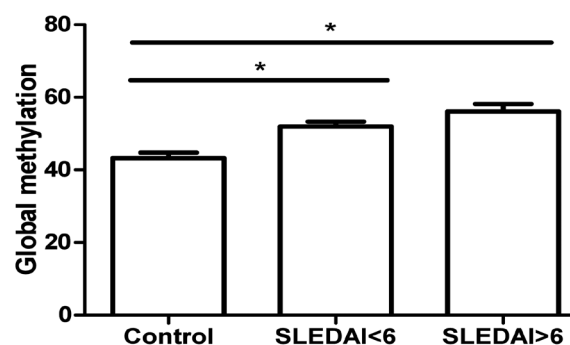
**Table 2:** Correlation between *HpaII*/*MspI* ratio, inflammatory markers and complement

Correlation	Spearman r	Significance
<b>SLEDAI&lt;6 (n=50)</b>		
	<i>HpaII</i> / <i>MspI</i> ratio	
CRP (mg/dL)	-0,07618	$p=0,5990$ (ns)
ESR (mm)	0,06276	$p=0,6650$ (ns)
C2 (mg/dL)	-0,1108	$p=0,4437$ (ns)
C3 (mg/dL)	0,3020	$p=0,0331$ (*)
C4 (mg/dL)	0,3252	$p=0,0212$ (*)
CH100 (%)	0,1053	$p=0,4666$ (ns)
<b>SLEDAI&gt;6 (n=50)</b>		
	<i>HpaII</i> / <i>MspI</i> ratio	
CRP (mg/dL)	0,04811	$p=0,7427$ (ns)
ESR (mm)	0,02633	$p=0,8575$ (ns)
C2 (mg/dL)	0,2244	$p=0,1210$ (ns)
C3 (mg/dL)	0,03896	$p=0,8668$ (ns)
C4 (mg/dL)	-0,1899	$p=0,4096$ (ns)
CH100 (%)	0,1294	$p=0,4263$ (ns)

Both the group with SLEDAI<6 and the one with SLEDAI>6 were composed of 50 patients each. CRP=C reactive protein; ESR= erythrocyte sedimentation rate; ns=not significant; \*  $p<0,05$ .



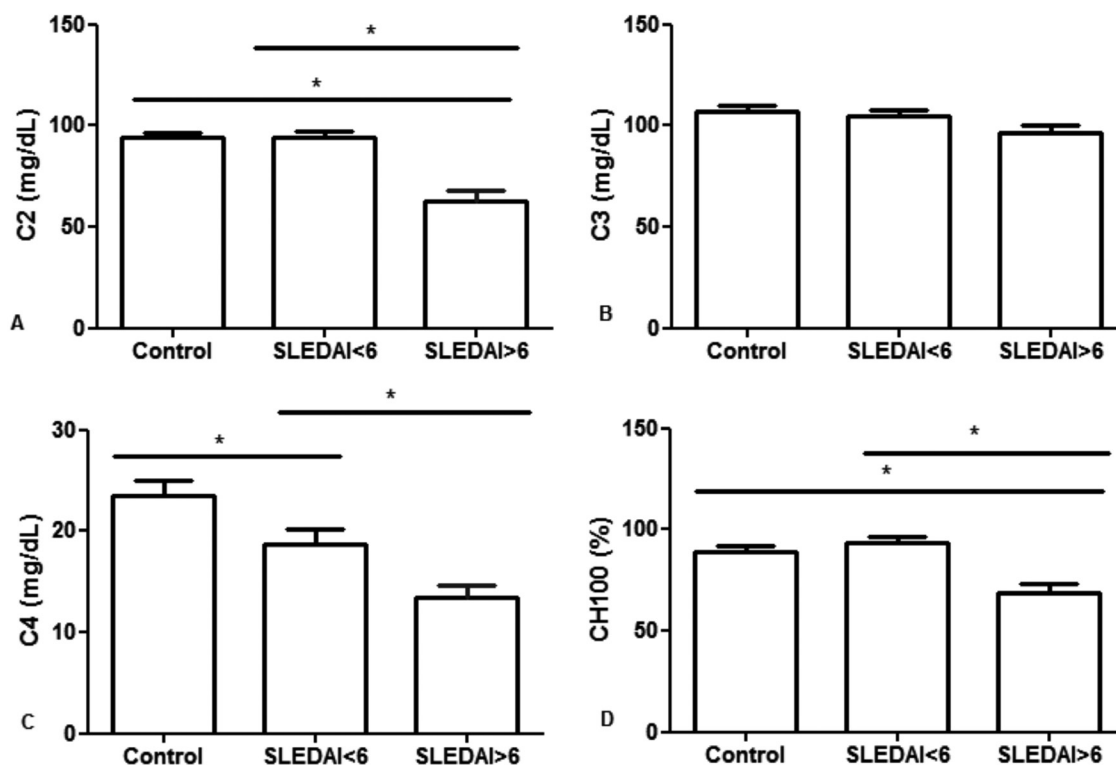
**Figure 1:** Global DNA methylation was evaluated by digestion of genomic DNA with *HpaII* and *MspI*. Figure represents an example of genomic DNA digestion with *HpaII* and *MspI* in 0.8% agarose gel stained with ethidium bromide. Digestion of DNA was performed in duplicate. C= product of DNA control not digested with enzymes; *MspI*= product of genomic DNA digests with *MspI* enzyme; *HpaII*= product of genomic DNA digests with *HpaII* enzyme



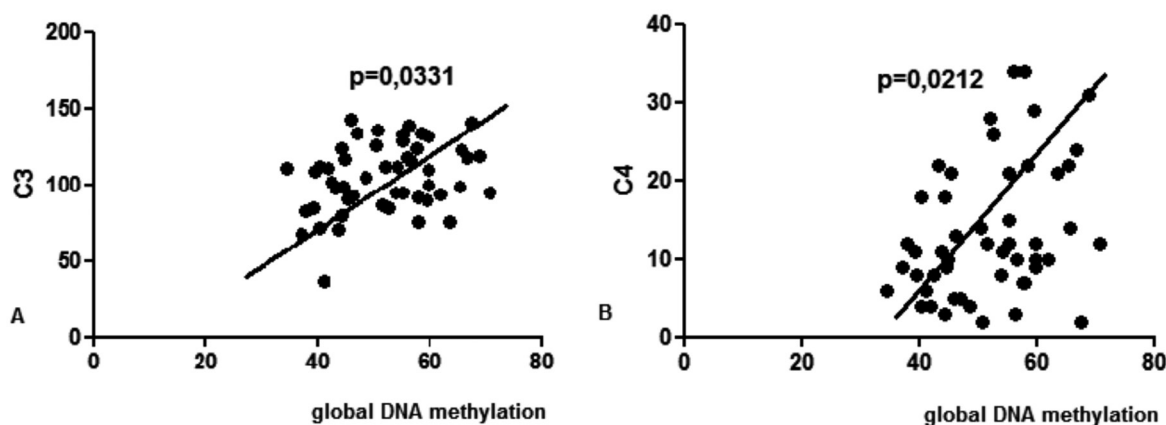
**Figure 2:** Comparison of global DNA methylation between healthy controls and SLE group with non-active disease (SLEDAI<6) and SLE group with active disease (SLEDAI>6). Groups were composed of 50 patients with SLEDAI<6, 50 patients with SLEDAI>6 and 50 healthy individuals

## Discussion

In this study, a statistical difference in DNA global methylation was observed when SLE patients with active and inactive disease were compared to healthy individuals. Using a



**Figure 3:** Comparison of complement proteins and complement activity. The control group (n=50) and the SLE group with non-active disease (n=50) present higher levels of C2 (A), C4 (B) and CH100 (D) than the SLE group with active disease (n=50)



**Figure 4:** Correlation between global DNA methylation and complement. A correlation by Spearman's rank correlation analysis was observed between global DNA methylation and C3 ( $r=0.3020$ ,  $p=0.0331$ ) (A) and C4 ( $r=0.3252$ ,  $p=0.0212$ ) (B) in the SLE group with non-active disease (n=50)

molecular approach (digestion of genomic DNA with *HpaII* and *MspI*), we observed that the relative amount of DNA methylation is significantly increased in SLE patients when compared to

healthy individuals regardless of disease activity. A positive correlation was observed between the frequency of global methylation and C3 and C4 serum levels for SLE patients with SLEDAI<6.



Accumulating epidemiological, clinical, and experimental evidence supports the conclusion of a critical role of epigenetic factors in immune programming<sup>13</sup>. Epigenetics signifies stable and heritable changes in gene expression without changes in the genetic code. There is a wealth of emerging evidence for such processes in promoting autoimmunity. The first clue is that inhibition of DNA methyl transferases (DNMT) induces SLE in animals. Similar immune-mediated disorders have been generated by injecting normal T cells incubated with DNMT inhibitors into healthy mice, and the autoreactivity has been demonstrated with cloned and polyclonal human and murine CD4<sup>+</sup> T cells<sup>14</sup>.

SLE is a systemic, multiorgan autoimmune disease with different immunological and clinical manifestations characterized by an autoantibody response to nuclear and/or cytoplasmic antigens. Different blood cell populations of SLE patients are characterized by a global loss of DNA methylation. Among the identified targets undergoing demethylation are genes involved in auto reactivity (ITGAL), osmotic lysis and apoptosis (PRF1, MMP14 and LCN2), antigen presentation (CSF3R), inflammation (MMP14), B- T-cell interaction (CD70 and CD40LG), and cytokine pathways (CSF3R, IL-4, IL-6 and IFNGR2)<sup>15</sup>. Hypomethylation of specific genes overexpressed in lupus T cells such as ITGAL (CD11a), CD40LG (CD40L), TNFSF7 (CD70), KIR2DL4 and PRF1 (perforin), and CD5 in lupus B cells seems to play an important role<sup>16</sup>.

The literature focuses on specific methylation of CD4<sup>+</sup> T cells in pathogenesis of SLE<sup>17</sup> and describes global DNA hypomethylation in CD4<sup>+</sup> T cells as having been detected in SLE, suggesting their link to pathogenesis. We have also found in this study an inverse association between low DNA methylation and high expression of two methyl CpG-binding domain (MBD) proteins, MBD2 and MBD4, from purified CD4<sup>+</sup> T cells of SLE patients<sup>18</sup>. Other studies suggest that aberrant regulation of DNA methylation in CD4<sup>+</sup> T cells is associated with the development of subacute cutaneous lupus erythematosus<sup>19</sup>.

Liu et al.<sup>20</sup> observed that the global methylation level of DNA was significantly decreased in SLE patients in comparison with that in the controls, but the global methylation levels of DNA were measured by the ELISA method. Using flow cytometry, Zhu et al.<sup>21</sup> found a global DNA hypomethylation of PBMC in SLE patients. Qin et al.<sup>22</sup> observed that SLE patients had significantly lower global DNA methylation levels than controls, and the global DNA methylation was inversely correlated with the SLE disease activity index (SLEDAI). The global DNA methylation levels in CD4<sup>+</sup>T cells were evaluated by the Methyflash DNA methylation quantification kit<sup>22</sup>. Nakamura et al.<sup>23</sup> compared the DNA methylation levels from CD3<sup>+</sup>CD4<sup>+</sup> T and CD3<sup>+</sup>CD8<sup>+</sup> T lymphocytes of SLE patients infected with human retrovirus (HERV)-E and HERV-K using bisulfite restriction analysis, in combination with interspersed repetitive sequences (COBRA-IRS). The hypomethylation of HERV-E LTR2C in CD3<sup>+</sup>CD4<sup>+</sup> T lymphocytes was positively correlated with lymphopenia in active SLE, whereas the hypomethylation of HERV-K was significantly correlated with complement activity and the SLE Disease Activity Index score. In summary, for each lymphocyte subset in patients with SLE, IRS hypomethylation was found to be type-specific<sup>23</sup>.

However, a Japanese study demonstrates that global DNA hypermethylation is associated with elevated levels of the inflammatory marker Procalcitonin (biomarker of inflammation induced by bacterial infection) in Japanese incident dialysis patients. Global DNA methylation was evaluated in peripheral blood DNA using the *HpaII/MspI* ratio by the luminometric methylation assay method<sup>24</sup>. In our study, when we correlated global methylation status according to activity or inactivity of disease with complement protein – complement activity and inflammatory markers having been determined by Spearman's rank test – we could find statistically significant differences between DNA hypermethylation in the SLE group with non-active disease with C3 and C4. Complement classical

pathway components are important in clearance of apoptotic cells, and defects or deficiencies in this pathway confer susceptibility in development of SLE<sup>25</sup>. In a retrospective study, Boeckler et al.<sup>26</sup>, emphasizes that the usual measurements of CH50, C3 and C4 levels are not adequate to detect a C4 and/or C2 deficiency in patients with SLE, but Ceribelli et al.<sup>27</sup> detected a significant link between the phases of lupus activity and the reduction of the complement proteins. In another study, Complement C3 and C4 levels were significantly reduced in patients with SLE compared with those in the control group, and disease activity of SLE was negatively correlated with complement C3 and C4<sup>28</sup>.

The molecular approach used in the present study demonstrated that the relative amount of DNA methylation is significantly increased in SLE patients when compared to healthy individuals regardless of disease activity. Correlation analysis suggests that differential methylation of genes related to the complement pathway alters gene expression involved in autoimmune response in SLE patients. Thus, DNA methylation could be involved in the pathogenesis of SLE.

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