UNIVERSIDADE DE BRASÍLIA - UnB
FACULDADE DE CEILÂNDIA - FCE
ENFERMAGEM

RENATA BARBOSA DE ANDRADE

A ASSOCIAÇÃO ENTRE O POLIMORFISMO INTRON 4 VNTR *NOS3* E MANIFESTAÇÕES CLINICAS EM PACIENTES COM LÚPUS ERITEMATOSO SISTÊMICO.

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Trabalho de Conclusão de Curso submetido à Faculdade de Ceilândia da Universidade de Brasília, como parte dos requisitos necessários à obtenção do Grau de Bacharel em Enfermagem.

Orientador: Prof.ª Dra. Silvana Schwerz Funghetto.

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Aprovado em: 22 de Junho de 2017.

CEILÂNDIA, DF

2017

AGRADECIMENTOS

Agradeço, primeiramente, a Deus e a Nossa Senhora de Guadalupe que, de forma singular, conduziram minha vida até esse momento.

Aos meus pais, Roberto Audy e Cida por estimularem sempre a busca pelo melhor, me guiando por esse caminho com muito amor e apoio incondicional.

Ao meu irmão e minha cunhada, Paulo Roberto e Cléo Stuart por estarem presentes em todos os momentos.

À toda minha família, em especial, as minhas madrinhas e padrinho, Pollyanna Reis, Ilma Mesquita e Carlos Alberto por terem contribuído para a minha educação. Obrigado por tudo.

Aos meus melhores amigos, Vanessa, André, Tadeu, Bruna, Graziella e Thaís por estarem comigo há mais de nove anos compartilhando um amor verdadeiramente fraterno.

À minha grande amiga, Ligia Canongia por todos os ensinamentos. Suas virtudes como carisma, paciência e alegria te transformam em uma pessoa extremamente iluminada.

Agradeço às minhas orientadoras, Prof.ª Dra. Silvana Schwerz Funghetto e Izabel Cristina Rodrigues da Silva, pelo direcionamento e aprendizado. Muito obrigado.

Aos colegas do laboratório de Patologia Molecular que trilharam e trilham o árduo caminho acadêmico.

À Universidade de Brasília e ao seu corpo docente pelo aprendizado e dedicação.

E a todos que, direta ou indiretamente, contribuíram para a realização deste trabalho.

The association between intron 4 VNTR of *NOS3* gene and clinical manifestations in patients with systemic lupus erythematosus.

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Case-control study

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ABSTRACT.
Systemic Lupus Erythematosus (SLE) is a multisystem, autoimmune inflammatory disease characterized by antinuclear autoantibodies, tissue destruction, complement system and interferon activation. The etiology of the underlying immune dysregulation seen in SLE remains unknown. However, an increased production of Nitric Oxide (NO) has been well documented in individuals with active SLE. NO is synthesized from l-arginine by the action of nitric oxide synthase (NOS), there are at least three isoenzymes of NOS: inducible NOS, neuronal NOS, and endothelial NOS (NOS3). The 27 bp repeat in intron 4 of *NOS3* gene regulate the plasma levels of NO and could, therefore, play a significant role in the pathogenesis of autoimmune diseases. This study aim to determine the association between the *NOS3* intron 4 variable number of tandem repeats polymorphism and SLE in Brazil´s Federal District group of patients and correlate the results with data of clinical manifestations. Genotyping of intron 4 VNTR of *NOS3* polymorphism was performed in order to obtain a Polymerase Chain Reaction (PCR) product that was later analyzed using the fragment analysis method. The results were evaluated and 4bb genotype was more prevalent than 4aa and 4ab, demonstrating a significant difference in frequencies between the groups ($P<0,05$). Analysis of allelic frequency revealed that the 4b allele was predominant in the case group (68.3%) demonstrating a significant difference in frequencies between SLE patients and healthy subjects ($P<0.001$). Among the manifestations studied arterial thrombosis presented statistical difference ($P = 0.024$). However, other manifestations such as hypertension and vasculitis did not present statistical differences.

Key words: Polymorphism genetic, NOS 3, Systemic Lupus Erythematosus

INTRODUCTION

Systemic Lupus Erythematosus (SLE) is a multisystem, autoimmune inflammatory disease characterized by antinuclear autoantibodies, tissue destruction, complement system and interferon activation. (Harley *et al*., 2008). The American College of Rheumatology (ACR) published, in 1982, a revised criteria for the classification of SLE that consists of 11 criteria: Malar rash, discoid rash, photosensitivity, oral ulcers, arthritis, serositis, renal disorder, neurologic disorder, hematologic disorder, immunologic disorder and antinuclear antibody; and to be classified as having SLE a patient should fulfill 4 or more of the criteria (Tan *et al*., 1982). Epidemiological studies about the incidence and prevalence of SLE show how variable results in different regions of the world are (Vilar *et al*., 2002). A study performed in the UK during 1999-2012 estimated the incidence rate in 4.91 per 100 000 person-years (95% CI 4.73 to 5.09) (Rees *et al*., 2014). Another study performed in a Brazilian population, showed an overall annual incidence rate of 8.7 per 100 000 year (Vilar *et al*., 2002). However, a Spanish study showed an 2.15 per 100 000 year (95% CI: 1.76-2.54/100 000/year) incidence (Lopez *et al*., 2003). The etiology of the underlying immune dysregulation seen in SLE remains unknown (Lehman *et al*., 2016).

However, an increased production of Nitric Oxide (NO) -a gaseous free, potent regulator of the immune response that play a significant role in the inflammatory and autoimmune responses metabolites and endothelial dysfunction- has been well documented in individuals with active SLE (AlFadhli S., 2013). NO is synthesized from l-arginine by the action of nitric oxide synthase (NOS) and there are at least three isoenzymes of NOS: inducible NOS, neuronal NOS, and endothelial NOS (NOS3) (Zhao *et al.*, 2016). The human endothelial NO synthase gene was assigned to the 7q35→7q36 region of chromosome 7 (Marsden *et al.*, 1993). The *NOS3* gene, which encodes NOS3, exhibits a number of polymorphic sites including Single Nucleotide Polymorphisms (SNPs), Variable Number of Tandem Repeats (VNTRs), Short Tandem Repeats (STR), and insertions/deletions (Oliveira-Paula *et al.*, 2016). Among known polymorphisms, eNOS-786 T>C in the promoter region, eNOS+894 G>T in exon 7 and a 27bp VNTR in intron 4 (*NOS3* VNTR 4a/b) have received the greatest attention (Marisi *et al.*, 2016). The 27 bp repeat in intron 4 of *NOS3* gene regulate the plasma levels of NO (AlFadhli S., 2013). Several studies showed a significant association between the intron 4 VNTR and SLE (Serrano *et al.*, 2004, Lee *et al*., 2016, Sandoughi *et al*., 2016). This study aim to determine the association between the NOS3 intron 4 VNTR polymorphism and SLE in Brazil´s Federal District group of patients and correlate the results with data of clinical manifestations.

MATERIAL AND METHODS

Patients and healthy subjects

This study was performed at the general Hospital of Brasília in Federal District, Brazil and included 115 SLE patients and 92 control subjects. To be included, patients had to: be more than 18 years old; be diagnosed with SLE based on the American College of Rheumatology (ACR) criteria (Tan *et al*., 1982) and be under medical supervision at general Hospital of Brasília.

It was selected a control group of individuals who sought the ambulatory clinic for routine exams or who were accompanying patients. To be included, this individuals had to be more than 18 years old; do not present a history of SLE; and do not present autoimmune diseases.

In addition, all those who did not meet the criteria for inclusion of the cases and controls groups were excluded.

For clinical assessment, patients with SLE completed an identification form and authorized access to their medical records to complete the clinical data record. Participants in the control group (without SLE) completed the identification form. Both groups signed a consent form and this study was conducted in collaboration with Ethics Committee in Research of Federal District Health Department.

Genotyping

A blood sample (5 mL) was obtained from each participant and stored at -20°C until use. DNA was extracted using an NucleoSpin® Blood kit (MACHEREY-NAGEL, Düren, Germany) and Genotyping of intron 4 VNTR of NOS3 polymorphism was performed using the primers 5'AGGCCCTATGGTAGTGCCTT-3'(forward) and 5'TCTCTTAGTGCTGTGGTCAC-3'(reverse), in order to obtain a Polymerase Chain Reaction (PCR) product that was later analyzed using the fragment analysis method. Each 25-µL PCR contained 4.0 µL of genomic DNA in a 2.5 ng/µL concentration; 1.5µL each primer; 2.5 µL 10X PCR buffer; 0.5 µL each deoxynucleotide; 0.5 µL $MgCl_2$; 0.5 µL *Taq* polymerase (Ludwig Biotecnology LTDA, Brazil); and ultrapure water to a final volume of 25 µL per reaction. The reactions were conducted in a Life Express® Thermal Cycler, model TC-96/G/H(b).

The thermal amplification program consisted of an initial denaturation at 94ºC (5 min), followed by 30 cycles of 94ºC (30 seconds), 60ºC (30 seconds) and 72ºC (1 min) and a final extension at 72ºC (10 min). After amplification, 5 µL PCR product were analyzed by 3% agarose gel electrophoresis PCR fragment sizes were evaluated a band size of 420 bp indicated five repeats of the 27 bp sequence, while a 323bp band represented four repeats, corresponding to the b and a alleles (AlFadhli S. 2013).

Statistical analysis

The agreement of genotype distribution of the intron 4 VNTR of NOS3 polymorphism with Hardy-Weinberg equilibrium was analyzed by chi-square test. Comparisons of genotypes and alleles between SLE patients and the control group were performed by using chi-square test calculated on 2x2 contingency tables. Odds ratio and 95% confidence intervals were calculated as measurements of the strength of association using the software IBM SPSS Statistics version 23. The association between the haplotypes of cases and controls were verified using the chi-square test with statistical significance defined by $p < 0,05$.

RESULTS

A total of 207 subjects, 115 SLE patients and 92 healthy patients, were genotyped for VNTR intron 4 of NOS3 gene using techniques of PCR. In total, 14 (12.2%), 45 (39.1%), and 56 (48.7%) SLE patients, and 63 (68.5%), 26 (28.3%), and 3 (3.3%) control subjects carried 4aa, 4ab, and 4bb genotypes, respectively. The case and control groups were significantly different ($p<0,05$) (Table 1).

The distribution of the genotypes 4bb and 4ab/4aa at position intron 4 of the NOS3 gene in patients with SLE is also reported in table 1. The genotype 4ab/4aa were more prevalent than

the 4bb genotype, demonstrating a significant difference in frequencies between the groups p<0,05. Analysis of allelic frequency revealed that the 4b allele was predominant in the case group (68.3%) and that the 4a allele was predominant in the control group (82.6%) demonstrating a significant difference in frequencies between SLE patients and healthy subjects (p <0.001, OR=0.10).

Table 1. Genotype and allele frequencies of Intron 4 VNTR of NOS3 gene in SLE patients (N = 115) and healthy control group (N = 92).

Intron 4 VNTR	SLE [N (%)]	Control [N (%)]	P value	OR (95%CI)
Genotype				
4aa	14 (12.2)	63(68.5)	<0.001*	NA
4ab	45(39.1)	26(28.3)		
4bb	56(48.7)	3(3.3)		
Total	115(100.0)	92(100.0)		
Genotype				
4ab+4aa	59(51.3)	89(96.7)	<0.001*	0.03(0.011-0.12)
4bb	56(48.7)	3(3.3)		
Total	115(100.0)	92(100.0)		
Allele				
4a	73(31.7)	152(82.6)	<0.001*	0.10(0.06-0.16)
4b	157(68.3)	32(17.4)		
Total	230(100.0)	184(100.0)		

* Statistical difference. NA = not apply

Association between the NOS3 intron 4 VNTR polymorphism (4bb and 4ab / 4aa) and the 11 clinical criteria adopted by the American College of Rheumatology (ACR) for the diagnosis of SLE were described at table 2 (Tan et al., 1982). The data obtained were: arthritis – ACR (P= 0.169, OR=2.34, CI= 0.68-8.09); malar rash – ACR (P= 0.307, OR=0.68, CI= 0.32-1.42); discoid rash – ACR (P= 0.887, OR=0.92, CI= 0.33-2.59); photosensitivity – ACR (P= 0.740, OR=1.13, CI= 0.53-2.39); serositis – ACR (P= 0.841, OR=1.08, CI= 0.49-2.35); renal disorder – ACR (P= 0.532, OR=0.79, CI= 0.37-1.65); hematologic disorder – ACR (P= 0.887, OR=0.93, CI= 0.35-2.46); oral ulcers – ACR (P= 0.887, OR=1.07, CI= 0.42-2.70); antinuclear antibody – ACR (P= 1); immunologic disorder – ACR (P= 0.217, OR=1.66, CI= 0.74-3.71); neurologic disorder – ACR (P= 0.862, OR=0.91, CI= 0.30-2.70). Therefore, no association between genotype 4bb and the ACR criteria were found.

Table 2. Association between the NOS3 intron 4 VNTR polymorphism (4bb and 4ab / 4aa) and clinical criteria adopted by the American College of Rheumatology (ACR).

Clinical Manifestations		4bb [N (%)]	4ab+4aa [N (%)]	P value	OR (95%CI)
Arthritis - ACR	Yes	52(51.0)	50(49.0)	0.169	2.34(0.68-8.09)
	No	4(30.8)	9(69.2)		
Malar rash - ACR	Yes	26(44.1)	33(55.9)	0.307	0.68(0.32-1.42)
	No	30(53.60)	26(46.4)		

Discoid rash - ACR	Yes	8(47.1)	9(52.9)	0.887	0.92(0.33-2.59)
	No	48(49.0)	50(51.0)		
Photosensitivity - ACR	Yes	34(50.0)	34(50.0)	0.740	1.13(0.53-2.39)
	No	22(46.8)	25(53.2)		
Serositis - ACR	Yes	19(50.0)	19(50.0)	0.841	1.08(0.49-2.35)
	No	37(48.1)	40(51.9)		
Renal disorder - ACR	Yes	30(46.2)	35(53.8)	0.532	0.79(0.37-1.65)
	No	26(52.0)	24(48.0)		
Hematologic disorder - ACR	Yes	46(48.4)	49(51.6)	0.887	0.93(0.35-2.46)
	No	10(50.0)	10(50.0)		
Oral ulcers - ACR	Yes	11(50.0)	11(50.0)	0.887	1.07(0.42-2.70)
	No	45(48.4)	48(51.6)		
Antinuclear antibody - ACR	Yes	56(48.7)	59(51.3)	1	NA
	No	0(0.0)	0(0.0)		
Immunologic disorder - ACR	Yes	42(52.5)	38(47.5)	0.217	1.66 (0.74-3.71)
	No	14(40.0)	21(60.0)		
Neurologic disorder - ACR	Yes	7 (46.7)	8(53.3)	0.862	0.91 (0.30-2.70)
	No	49(49.0)	51(51.0)		

NA = not apply

Other clinical manifestations from patient's records were related to the NOS3 intron 4 VNTR polymorphism (4bb and 4ab/4aa) at table 3. Among the manifestations studied arterial thrombosis presented statistical difference (P = 0.024). However, other manifestations such as hypertension, pericarditis and vasculitis did not present statistical differences.

Table 3 Association between the NOS3 intron 4 VNTR polymorphism (4bb and 4ab / 4aa) and clinical manifestations.

Clinical Manifestations		4bb[N (%)]	4ab/4 aa [N (%)]	P value	OR (95%CI)
Stroke	Yes	6(60.0)	4(40.0)	0.521	1.65(0.43-6.18)
	No	50(47.6)	55(52.4)		
Family Background of Systemic Arterial Hypertension	Yes	29(58.0)	21(42.0)	0.065	2.02(0.95-4.27)
	No	26(40.6)	38(59.4)		
Sjogren´s Syndrome	Yes	1(33.3)	2(66.7)	1	0.51(0.04-5.87)
	No	55(49.1)	57(50.9)		
Vasculitis	Yes	3(33.3)	6(66.7)	0.491	0.50(0.11-2.10)
	No	53(50.0)	53(50.0)		
Pulmonary hypertension	Yes	2(100.0)	0(0.0)	0.234	NA
	No	54(47.8)	59(52.2)		

Pericarditis	Yes	7(53.8)	6(46.2)	0.689	1.26(0.39-4.01)
	No	49(48.0)	53(52.0)		
Myocarditis	Yes	1(100,0)	0(0.0)	0.486	NA
	No	55(48.2)	59(51.8)		
Dyslipidemias	Yes	4(100.0)	0(0.0)	0.053	NA
	No	52(46.8)	59(53.2)		
Hypertension	Yes	18(45.0)	22(55.0)	0.695	0.79(0.36-1.72)
	No	38(50.7)	37(49.3)		
Raynand disease	Yes	22(53.7)	19(46.3)	0.427	1.36(0.63-2.92)
	No	34(45.9)	40(54.1)		
Venous thrombosis	Yes	6(85.7)	1(14.3)	0.056	6.96(0.81-59.78)
	No	50(46.3)	58(53.7)		
Arterial thrombosis	Yes	5(100.0)	0(0.0)	0.024*	NA
	No	51(46.4)	59(53.6)		
Myocardial infarction	Yes	0(0.0)	0(0.0)	1	NA
	No	56(48.7)	59(51.3)		
Angina	Yes	0(0.0)	0(0.0)	1	NA
	No	56(48.7)	59(51.3)		
Gangrene	Yes	1(100.0)	0(0.0)	0.486	NA
	No	55(48.2)	59(51.8)		
Elevation of creatinine	Yes	11(45.8)	13(54.2)	0.751	0.86(0.35-2.13)
	No	45(49.5)	46(50.5)		

* statistical difference. NA = not apply

DISCUSSION

The *NOS3* intron 4 polymorphism is responsible for NO plasma levels and this gaseous free radical mediates vasodilation in the endothelium, inhibits adhesion of platelets and leukocytes and limits oxidation of atherogenic low-density lipoproteins in the vascular endothelium. Therefore, an alteration in its quantity or conformation can generate an endothelial dysfunction, affect vascular homeostasis and, consequently, play a significant role in the pathogenesis of autoimmune diseases, such as SLE (AlFadhli, 2013; Kumar *et al,* 2017). In this study we identified and evaluated the distribution of this polymorphism characterizing the genotypes and alleles in a Brazil's Federal District group of patients correlating the results with data of SLE clinical manifestations.

Oates and colleagues (2008) carried out in their studies an analysis of serum nitrite and nitrate levels correlating this data with disease activity and damage in SLE.

Nagy and collaborators (2010) explains that the NO plays a crucial role in T cell dysregulation in SLE, activation-induced rapid Ca2+ signals are higher in T cells from patients with SLE; in contrast, the sustained Ca2+ signal is decreased in these lupus T cells. As a result the mitochondrial membrane potential is permanently high in lupus T cells and monocytes from lupus patients generate significantly more NO than normal monocytes. To the extent the NO regulates signal transduction by regulating Ca2+ signaling, by regulating the structure of the immunological synapse, or through the modification of intra cellular proteins an alteration in its quantity or conformation could, therefore, generate a dysfunction.

Several studies had as main focus the demonstration of association between intron 4 VNTR of *NOS3* gene and SLE. However, contrary results were found depending on the population studied. Some articles suggested that there was no significant increased risk of SLE associated with *NOS 3* polymorphisms and that this genetic polymorphism differed significantly across ethnic groups (Douglas *et al*., 2004; Tang *et al.*, 2010; Raafat *et al.*, 2013). This result is consonant to our findings, there was a statistical difference, however the data did not indicated an increased risk.

On a contrary current, some articles such as Alfadhli and collaborators (2011) suggested that VNTR4 allele 4b was associated with susceptibility to SLE (OR 1.89, P = 0.023), as was the genotype 4bb (OR 2.41, P= 0.007). Serrano et al (2004) concluded that the intron 4b allele was associated with SLE (OR 2.2, 95% CI 1.29-3.60, P = 0.005) as the 4bb genotype (OR 2.9, 95% CI 1.61-5.33, P = 0.0009). AlFadhli, S. (2013) also concluded that the 4bb genotype was associated with SLE (P = 0.0076, OR = 1.97).

In this study, we found that 14 (12.2%), 45 (39.1%), and 56 (48.7%) SLE patients, and 63 (68.5%), 26 (28.3%), and 3 (3.3%) control subjects carried 4aa, 4ab, and 4bb genotypes, respectively. Analysis of allelic frequency revealed that the 4b allele was predominant in the case group (68.3%) and that the 4a allele was predominant in the control group (82.6%) demonstrating a significant difference in frequencies between SLE patients and healthy subjects (P <0.001, OR=0.10). In general, the genotypic frequencies observed in the present study were similar to those found by other authors, such as the studies carried out in China, Iran and Mexico (SALIMI *et al.*, 2006; NATH *et al.*, 2009; ZHENG *et al.*, 2014).

In our study, arterial thrombosis presented a statistical difference (P = 0.024). Voetsch and collaborators (2014) suggest that the pathogenesis of this disease is complex and involves multiple genetic and environmental factors related to atherosclerosis and thrombosis, as well as their interaction. Despite the plasma increase associated with the 4bb genotype, this association has not been confirmed with the disease in a considerable number of individuals. However, NO-dependent endothelial dysfunction is now accepted as a key initial step in atherothrombogenesis.

In summary, this study demonstrated that 4bb genotype was more prevalent than 4aa and 4ab (P<0,05) and 4b allele was predominant in the case group (68.3%) demonstrating a significant difference in frequencies between SLE patients and healthy subjects (P <0.001).Our result highlights the possible relationship between the intron 4 VNTR, SLE and clinical manifestations such as arterial thrombosis.

ACKNOWLEDGMENTS

Research supported by CAPES-DF with support from Universidade de Brasília – UnB and Fundação de Apoio à Pesquisa do Distrito Federal – FAP-DF. To the entire team involved in the work, all professors, laboratory technicians and laboratory colleagues.

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ANEXO I

GOVERNO DO DISTRITO FEDERAL
SECRETARIA DE ESTADO DE SAÚDE
COMITÊ DE ÉTICA EM PESQUISA

EXERÇA A CIDADANIA E FISCALIZE NO DIA-A-DIA

PARECER Nº 309/2009

PROTOCOLO Nº DO PROJETO: 353/09 – POLIMORFISMOS GENÉTICOS ASSOCIADOS AO LUPUS ERITEMATOSO SISTÊMICO

Instituição Pesquisada: Secretaria de Saúde do Distrito Federal/SES-DF.

Área Temática Especial: Grupo III (não pertencente à área temática especial), Ciências da Saúde.

Validade do Parecer: 03/11/2011

Tendo como base a Resolução 196/96 CNS/MS, que dispõe sobre as diretrizes e normas regulamentadoras em pesquisa envolvendo seres humanos, assim como as suas resoluções complementares, o Comitê de Ética em Pesquisa da Secretaria de Estado de Saúde do Distrito Federal, após apreciação ética, manifesta-se pela **APROVAÇÃO DO PROJETO.**

Esclarecemos que o pesquisador deverá observar as responsabilidades que lhe são atribuídas na Resolução 196/96 CNS/MS, inciso IX.1 e IX.2, em relação ao desenvolvimento do projeto. **Ressaltamos a necessidade de encaminhar o relatório parcial e final, além de notificações de eventos adversos quando pertinentes.**

Brasília, 03 de novembro de 2009.

Atenciosamente.

Maria Rita Carvalho Garbi Novaes
Comitê de Ética em Pesquisa/SES-DF
Coordenadora

Ângela Maria/CEP/SES-DF

ANEXO 2

Termo de Consentimento Livre e Esclarecido

Participantes do Grupo Caso (Pacientes com Lupus)

"Polimorfismos genéticos associados ao Lupus Eritematoso Sistêmico"

Este documento que você está lendo é chamado de Termo de Consentimento Livre e Esclarecido (TCLE). Ele contém explicações sobre o estudo que você está sendo convidado a participar.

Antes de decidir se deseja participar (de livre e espontânea vontade) você deverá ler e compreender todo o conteúdo. Ao final, caso decida participar, você será solicitado a assiná-lo e receberá uma cópia do mesmo.

Antes de assinar faça perguntas sobre tudo o que não tiver entendido bem. A equipe deste estudo responderá às suas perguntas a qualquer momento (antes, durante e após o estudo).

Natureza e objetivos do estudo

- Você está sendo convidado a participar de um estudo pelo fato de apresentar Lupus. Você poderá decidir participar ou não. A decisão é sua.
- Existe uma possibilidade de associação de fatores genéticos com o Lúpus, assim, este estudo tem o objetivo geral de conhecer um pouco melhor como "funciona" o Lúpus, do ponto de vista genético.
- O objetivo específico deste estudo é o de conhecer se determinadas sequências do DNA (material genético que informa como nosso corpo é formado) pode aumentar o risco de pessoas apresentarem Lúpus e suas diferentes características clínicas.

Procedimentos do estudo

- Sua participação consiste em responder um questionário e autorizar que seu os pesquisadores possam ver seu prontuário, para que tenham maior conhecimento de seus exames, tratamento e da história da sua doença.
- Após isso será coletado de você, uma única vez, aproximadamente 10 ml (uma seringa pequena) de sangue, através de uma punção da veia do seu antebraço. O procedimento é o mesmo utilizado para realização de diversos outros tipos de exame de sangue. Serão utilizados equipamentos novos, estéreis e descartáveis.
- Não haverá nenhuma outra forma de envolvimento ou comprometimento neste estudo.

Riscos

- Este estudo possui riscos mínimos que são inerentes do procedimento de coleta de sangue. Medidas preventivas durante a coleta serão tomadas para minimizar qualquer risco ou incômodo.
- Poderá haver pequeno incômodo de dor no momento da introdução da agulha para a retirada do sangue e, eventualmente, a formação de um pequeno hematoma (mancha roxa) no local.

Benefícios

- A sua participação neste estudo poderá proporcionar, no âmbito pessoal, a identificação de algum problema não antes conhecido.
- Os resultados que mostram suas características genéticas e possíveis riscos com as manifestações clínicas do Lúpus estarão sempre disponíveis a você. Caso seja de seu desejo, os resultados serão discutidos com você pela equipe deste trabalho.
- Sua participação poderá ainda ajudar no maior conhecimento sobre o Lúpus, principalmente em relação às causas genéticas da doença.

Participação, recusa e direito de se retirar do estudo

- Sua participação é voluntária e não alterará o seguimento e tratamento da doença que você já está fazendo.
- Você poderá se retirar desta pesquisa a qualquer momento, bastando para isso entrar em contato com um dos pesquisadores responsáveis.
- Caso você decida não participar, isto não afetará o seguimento e tratamento normal nem o seu relacionamento com seu médico.
- Conforme previsto pelas leis brasileiras você não receberá nenhum tipo de compensação financeira pela sua participação neste estudo.

Confidencialidade

- Os seus registros médicos serão sempre tratados confidencialmente.
- Seus dados serão identificados com um número e somente os pesquisadores saberão que número pertence a cada indivíduo.
- Os resultados de seus exames, bem como as informações de seu prontuário, serão acessíveis somente aos pesquisadores envolvidos.
- O seu sangue, coletado no presente estudo, ficará guardado no Centro de Neurociências, no Instituto de Biologia da Universidade de Brasília, no banco de amostras “Lúpus”, sob a responsabilidade da Profa. Dra. Elisabeth Nogueira Ferroni Schwartz.

- Toda nova pesquisa a ser feita com o material guardado será submetida para aprovação de um Comitê de Ética em Pesquisa e, quando for o caso, da Comissão Nacional de Ética em Pesquisa.
- Os resultados deste trabalho poderão ser apresentados em encontros ou revistas científicas, entretanto, ele mostrará apenas os resultados obtidos como um todo, sem revelar seu nome, instituição a qual pertence ou qualquer informação que esteja relacionada com sua privacidade.

Se o Senhor (a) tiver qualquer dúvida em relação à pesquisa, por favor telefone para o médico Dr. Carlos Eduardo Lins, no Hospital de Base de Brasília, telefone (61) XXXXXXXXXXXX, no horário vespertino.

Este projeto foi Aprovado pelo Comitê de Ética em Pesquisa da SES/DF. Qualquer dúvida com relação à assinatura do TCLE ou os direitos do sujeito da pesquisa podem ser obtidos através do telefone: (61) 3325-4955.

Este documento foi elaborado em duas vias, uma ficará com o pesquisador responsável e a outra com o sujeito da pesquisa.

Eu, _____ RG _____, após receber uma explicação completa dos objetivos do estudo e dos procedimentos envolvidos concordo voluntariamente em fazer parte deste estudo.

Brasília, _____ de _____ de _____

_____

Participante

ANEXO 3

Termo de Consentimento Livre e Esclarecido

Participantes do Grupo Controle (Sadios)

"Polimorfismos genéticos associados ao Lúpus Eritematoso Sistêmico"

Este documento que você está lendo é chamado de Termo de Consentimento Livre e Esclarecido (TCLE). Ele contém explicações sobre o estudo que você está sendo convidado a participar.

Antes de decidir se deseja participar (de livre e espontânea vontade) você deverá ler e compreender todo o conteúdo. Ao final, caso decida participar, você será solicitado a assiná-lo e receberá uma cópia do mesmo.

Antes de assinar faça perguntas sobre tudo o que não tiver entendido bem. A equipe deste estudo responderá às suas perguntas a qualquer momento (antes, durante e após o estudo).

Natureza e objetivos do estudo

- Você está sendo convidado a participar deste estudo por não apresentar Lúpus.
- Existe uma possibilidade de associação de fatores genéticos com o Lúpus, assim, este estudo tem o objetivo geral de conhecer um pouco melhor como "funciona" o Lúpus, do ponto de vista genético.
- O objetivo específico deste estudo é o de conhecer se determinadas sequências do DNA (material genético que informa como nosso corpo é formado) pode aumentar o risco de pessoas apresentarem Lúpus e suas diferentes características clínicas.
- Para verificar se determinados segmentos de DNA podem aumentar a chance de indivíduos apresentarem Lúpus, é preciso comparar o DNA de indivíduos com Lúpus com o DNA de indivíduos sem Lúpus, que é o seu caso.

Procedimentos do estudo

- Sua participação consiste em responder uma ficha de identificação e autorizar uma única vez, a coleta de aproximadamente 10 ml (uma seringa) de sangue, através de uma punção de veia periférica no antebraço.
- O procedimento é o mesmo utilizado para realização de diversos outros tipos de exame de sangue. Serão utilizados equipamentos novos, estéreis e descartáveis.
- Não haverá nenhuma outra forma de envolvimento ou comprometimento neste estudo.

Riscos e benefícios

- Este estudo possui os mesmos riscos de uma coleta de sangue para exames comuns. Medidas preventivas durante a coleta serão tomadas para minimizar qualquer risco ou incômodo.
- Poderá haver pequeno incômodo de dor no momento da introdução da agulha para a retirada do sangue e, eventualmente, a formação de um pequeno hematoma (mancha roxa) no local.
- De acordo com a legislação brasileira os participantes de pesquisa não podem receber qualquer remuneração financeira, o que ocorrerá no presente estudo.
- Sua participação poderá ainda ajudar no maior conhecimento sobre o Lúpus, principalmente em relação às causas genéticas da doença.

Participação recusa e direito de se retirar do estudo

- Sua participação é voluntária. Você não terá nenhum prejuízo se não quiser participar.
- Você poderá se retirar desta pesquisa a qualquer momento, bastando para isso entrar em contato com um dos pesquisadores responsáveis.
- Conforme previsto pelas leis brasileiras você não receberá nenhum tipo de compensação financeira pela sua participação neste estudo.

Confidencialidade

- Seus dados serão identificados com um número e somente os pesquisadores saberão que número pertence a cada indivíduo.
- Os resultados de seus exames serão acessíveis somente aos pesquisadores envolvidos e não será permitido o acesso a outras pessoas.
- O seu sangue, coletado no presente estudo, ficará guardado no Centro de Neurociências, no Instituto de Biologia da Universidade de Brasília, no banco de amostras "Lúpus-Sadios", sob a responsabilidade da Profa. Dra. Elisabeth Ferroni Schwartz, professora da Universidade de Brasília e pesquisadora associada do presente estudo.
- Toda nova pesquisa a ser feita com o material guardado será submetida para aprovação de um Comitê de Ética em Pesquisa e, quando for o caso, da Comissão Nacional de Ética em Pesquisa.
- Os resultados deste trabalho poderão ser apresentados em encontros ou revistas científicas, entretanto, ele mostrará apenas os resultados obtidos como um todo, sem revelar seu nome, instituição a qual pertence ou qualquer informação que esteja relacionada com sua privacidade.

Eu, _____ RG _____,
após receber uma explicação completa dos objetivos do estudo e dos procedimentos envolvidos concordo voluntariamente em fazer parte deste estudo.

Se o Senhor (a) tiver qualquer dúvida em relação à pesquisa, por favor, telefone para o médico Dr. Carlos Eduardo Lins, no Hospital de Base de Brasília, telefone (61) XXXXXXXXXXXX, no horário vespertino.

Este projeto foi Aprovado pelo Comitê de Ética em Pesquisa da SES/DF. Qualquer dúvida com relação à assinatura do TCLE ou os direitos do sujeito da pesquisa podem ser obtidos através do telefone: (61) 3325-4955.

Este documento foi elaborado em duas vias, uma ficará com o pesquisador responsável e a outra com o sujeito da pesquisa.

Eu, _____ RG _____,
após receber uma explicação completa dos objetivos do estudo e dos procedimentos envolvidos concordo voluntariamente em fazer parte deste estudo.

Brasília, _____ de _____ de _____.

_____

Participante

ANEXO 4

Instructions for Authors

Genetics and Molecular Research (GMR) publishes Book Review, Brief Note, Case Report, Comment, Correction, Errata, Homage, *In Memoriam*, Letter to the Editor, Methodology, Mini-Review, Obituary, Opinion, Point of View, Research Note, Retraction, Review, Re- view Article, Short Communication, and Thesis Abstract, with regard to genetics, evolution, molecular biology, and bioinformatics. Review articles are normally received by invitation only. If you would like for us to consider a review article, please consult the editor first; send a proposed title, a brief outline and a list of papers relevant to the review published by the author(s). GMR is an exclusively online journal.

The journal is maintained by the not-for-profit scientific foundation Ribeirão Preto Foundation for Scientific Research (FUNPEC-RP) and the articles are open access. **The fee per accepted submission is R$ 1.780,00 for Brazilian authors and US$ 1,060.00 for authors from other countries. The US dollar amount reflects the approximate current foreign exchange rate and is subject to change.** This fee covers part of the expenses for final language and technical revision, for page setup, and for publishing online.

Payment of the publishing fee should be made by the authors only after receiving a preliminary acceptance. After payment is received by our office, the manuscript will be processed further for publication, depending on the final approval of our Editorial Board.

Payment, both from within or outside Brazil, should be made by bank transfer (Banco do Brasil or City Bank).

Please contact the editorial office [gmr@geneticsmr.com] if you have any questions.

All GMR articles must meet the highest standards of scientific quality, both in terms of originality and significance, and the research findings reported should make substantial advances. As it is a journal serving a wide and varied scientific community, article abstracts, introductions and conclusions should be comprehensible to the non-

specialist, stressing any wider implications of the study. However, the papers should not compromise on the scientific rigor and detail demanded by an international research journal. The broad readership that GMR attracts gives authors an opportunity to convey to a large audience, as well as to specialists, the importance of their research. The journal is currently indexed in over 64 services; see [http://www.geneticsmr.com].

Contributions should be sent either by e-mail as attachments to [gmr@geneticsmr.com].

It is a fundamental condition that submitted manuscripts have not been previously published and will not be simultaneously published elsewhere. With the acceptance of a manuscript for publication, the publishers acquire full and exclusive copyright for all languages and countries. The use of registered names, trademarks, etc., in this publication does not imply, even in the absence of a specific statement, that such names are exempt from the relevant protective laws and regulations and therefore free for general use.

All papers should be prepared in U.S. English. An initial evaluation of the language will be made upon receipt of each manuscript. Those that are considered inadequate for initial review will be returned or sent out for correction, at the discretion of the author. The manuscript will be considered officially received when the corrected version is ready to be sent to the referees.

Before final acceptance, a submission letter with the title of the article and names and signatures of all the authors should be sent by e-mail to gmr@geneticsmr.com. Galley proofs will be sent in "pdf" form via e-mail for final revision. All authors are co-responsible for their submissions and they should make every effort to check the paper before this final step to avoid costly reformatting and possible introduction of new errors.

GMR articles have no rigid length restrictions. They should contain sufficient technical detail for an expert reader to understand and assess the methods and results. There is no page limit for GMR articles, but authors should still be concise, for two main reasons. First, our electronic refereeing system relies on e-mail, and very large files occasionally cause problems. Second, lengthy manuscripts can be cumbersome to read and study. Referees tend to dislike them, and they take longer to process. In addition, readers of electronic journals often print articles to read them. Remember that a 10,000-word article takes up around 11 pages.

Editorial policies: GMR is a refereed journal. Only original manuscripts will be considered for publication. Manuscripts will be reviewed by at least two independent reviewers

before a decision is made on publication. The whole process is conducted electronically to speed progress and final publication. Papers will be published (placed online), once were fully processed. Papers accepted in their final form from January 1 to March 31 constitute the first issue of each volume, and so on. There are four issues per year.

Manuscripts (in U.S. English), together with a cover letter from the author responsible for all correspondence, should be submitted to the Editor at [gmr@geneticsmr.com] in electronic format as .doc files saved in Microsoft Word 97 for Windows, or later version. Do not use formatting such as Word's "Heading" or "Style Sheets". Spelling, punctuation, sentence structure, spacing, length, and consistency of usage in form and descriptions should be checked before submission. Please also check references for accuracy. Ensure that all figures and tables are mentioned in the text, and that all references are cited in the text. Figure and table files (see below) should be separate.

Submission information

Authors are required to provide the following information with their electronic submissions: Author submitting the article; article title; authors (full list); article type and session; status of article (e.g., new, revised, etc.); postal address; e-mail address; phone number; fax number; names and types of the files sent.

Brazilian authors should not translate their institutional addresses. These should remain in the original (Portuguese) language.

Revised versions: Authors submitting a revised version of an article must remember to include a list of changes, and replies to the referees (or technical editor). All the files, not just those revised, for the final draft of paper should be sent.

Acknowledgment of electronic submissions: Successful receipt and processing of the author's submission will be acknowledged by e-mail when the submitted manuscript has been checked. If no reply has been received within one week, the author should contact the editor at [gmr@geneticsmr.com].

Review: Articles are reviewed anonymously by independent referees. Authors are encouraged to suggest names of expert reviewers, but selection remains the prerogative of the editors. To facilitate the review process, the authors can send supplementary material, such as cited accepted but not yet published papers, which may be important for assessment of the manuscript.

A review article should contain: an abstract of 250 words or less, no more than six key words, a running title and no more than 60 references. It should be divided into sections with appropriate tittles and subtitles.

Preparation of the manuscript

Order the sections comprising the manuscript as follows: title, running title, author, address, abstract, key words, introduction, material and methods, results, discussion, acknowledgments, and references.

Title Page: The title page should include the title of the article, authors' names (names and initials (only) thinking in indexing services), and authors' affiliation. The affiliation should comprise the department, institution (usually university or company), city, and state (or nation). The title page should include the name and complete mailing address, telephone number, fax number, and e-mail address of the author designated to review proofs. A running title of no more than 60 characters (including spaces) should be provided.

Abstract: An abstract of up to 250 words, single-spaced, is required of research articles and reports and should be arranged in one paragraph. The following information (without headings) should be included: purpose, methods, results (please report numerical data (means ± SE) for significant results), and conclusions. Review articles also require an abstract, which need not include all of these items.

Key words: A list of key words or indexing terms (up to six) should be included.

Text Format: Headings should be bold, and first letters capitalized and left-aligned. All text should be set in Times New Roman font, 12 point, left-aligned, single-spaced. Do not justify the right margin. Leave only one (1) space after periods. Paragraphs should not be indented; there should not be any blank lines between them. Use line returns only at the end of paragraphs. Do not use tabs or spaces to create indents. Use the Symbol font for symbols and special characters. Do not use equation editors or footnoting utilities. Save equations as images. Equations should be numbered consecutively with Arabic numerals in parentheses on the right hand side of the page.

Footnotes: Footnotes should be avoided. When their use is absolutely necessary, footnotes should be numbered consecutively using Arabic numerals and should be placed at the bottom of the page to which they refer. Place a line above the footnote, so that it is set off from the text.

Tables/Charts: Special care should be taken to ensure that all tables are properly formatted. Scientific symbols used should be in Symbol or Times New Roman. Tables should be on a separate page, numbered consecutively (with Arabic numerals) referred to by number in the text and designed to fit the column or page size of the journal. Use tables with cells to separate columns. Do not use spaces, tabs or vertical lines. Left justify the title above the table.

Indicate each table's location within the manuscript.

Illustrations: Illustrations/figures (photographs, drawings, diagrams, and charts) should each be in a single file, numbered in a consecutive series of Arabic numerals in the order in which they are cited in the text. Illustrations must be submitted as separate files. All illustrations are to be supplied in JPEG (jpg) format in either color or black and white. Images must be saved as separate, stand-alone files. The image resolution should be 300 dpi. Do not embed images within the text file. Indicate each figure's location within the text. Do not forget to send the legend in a separate page. The authors should also send, by mail, a printed version of the figures. These should be at least 10 x 15 cm, up to US letter size, so that figures can be scanned (in case the figure files are not adequate) to guarantee good quality for publishing online.

Abbreviations: Try to use abbreviations in the text sparingly. Write out abbreviations in full before the first time they are used in the text. Use the metric system for all measurements without periods (cm, mL, s). Define all symbols used in equations and formulas. Do not abbreviate the word "Figure" or "Table" in titles or text.

Acknowledgments: All acknowledgments (including those for grant and financial support) should be typed in one paragraph directly preceding the reference section. Authors of manuscripts submitted to GMR are requested to state the source of all funding that enabled the described research to be undertaken.

References: References in the text should include the name of the author and the year in parentheses, e.g. (Searle, 1961) or (King and Wilson, 1975). When a reference with more than two authors is cited, only the first author is named, e.g. (Comstock et al., 1958). The references must be cited in the text in chronological order, e.g. (Ideber, 2001; Uetz, 2002; Ottavai, 2004). References to "unpublished results" and "submitted papers" should appear in the text in parentheses following the name(s) of the individual(s). Example: (Pereira KS, Martins PK and Silva TM, unpublished results). **No more than 40 references**

should be cited in a Full-length paper, 20 references in a Short Communication and 60 references in a Review article.

References, under the heading "References", should include only works referred to in the text. This section should be arranged in alphabetical order under the first author's last name. References should be cited as follows: journal papers - names and initials of the first four authors (after that using et al.), year, journal title abbreviated according to PubMed or Web of Science, volume number, first and last page numbers; books - names of authors, year, full title, edition, publishers, address (city); articles published in symposia - names of authors, year, full title of book, name(s) of editor(s) in parentheses, publisher, address (city), first and last page numbers.

The references should consist mainly of articles from indexed journals. References for techniques that are essential for understanding or repeating the methods should always be in easily accessible (indexed) journals.

Reference style: The list of references at the end of the paper should follow the format requested by GMR.