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Cecília Rodrigues Lucas

ASSOCIATION OF INTERLEUKIN-17A/RA AND HLA-G POLYMORPHISMS WITH GESTATIONAL DIABETES MELLITUS

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À minha mãe, Zenilde Rodrigues,
por todo o amor e dedicação.

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“A tarefa não é tanto ver aquilo que ninguém viu, mas pensar o que ninguém ainda pensou sobre aquilo que todo mundo vê”.
(Arthur Schopenhauer)

RESUMO

O Diabetes Mellitus Gestacional (DMG) é um tipo especial de diabetes que definido como qualquer grau de intolerância à glicose com início ou primeiro reconhecimento durante a gravidez, desde que não sendo diagnóstico de diabetes já estabelecido. O aumento de gestações complicadas pela DMG tem sido um grande alerta para a comunidade científica, pois sua fisiopatogenia não está bem descrita e está relacionada a complicações graves da gravidez. O objetivo deste estudo é associar o papel dos polimorfismos rs2275913, rs4819554 e rs66554220 com a predisposição do DMG. O estudo incluiu 79 pacientes com gravidez complicada por DMG e 81 gestantes saudáveis de dois Hospitais Terciários do Rio Grande do Norte. Dados demográficos, clínicos e laboratoriais dos sujeitos foram coletados por formulários e os polimorfismos de rs2275913, rs4819554 e rs66554220 foram determinados a partir de material biológico por Reação em Cadeia da Polimerase por Polimorfismo no Comprimento de Fragmentos de Restrição (PCR-RFLP). A glicemia de jejum e o Índice de Massa Corporal (IMC) foram significativamente maiores no grupo DMG do que no grupo controle ($p < 0,05$; $p = 0,002$, respectivamente). Obesidade e sobrepeso e IMC foram significativamente maiores quando associados ao diagnóstico de DMG ($p < 0,05$, ambos). Obesidade, alto IMC e hiperglicemia foram achados de importância à predisposição ao DMG, além de o polimorfismo da *IL-17A* *rs2275913* (A/A) ter tido significância quando associado à maior probabilidade de diagnóstico de DMG (OR = 5,22; IC95% 1,08-25,29; p = 0,040).

Palavras-chave: Diabetes gestacional. Polimorfismo. Diagnóstico.

ABSTRACT

Gestational Diabetes Mellitus (GDM) is a special type of diabetes that has been defined as any of glucose intolerance with onset or first recognition during pregnancy. The increasing of pregnancies complicated by GDM has been of great warning to the scientific community, as its pathophysiology is not well described and it has been related to severe pregnancy complications. The aim of this study is to evaluate the role of rs2275913, rs4819554 and rs66554220 polymorphisms in pregnancy complicated by GDM. The study comprised 79 patients with pregnancy complicated by GDM, as well as 81 healthy pregnant women (control group) from a High Risk Prenatal Outpatient Clinic. Personal data from the subjects were collected by forms, and polymorphisms of rs2275913, rs4819554 and rs66554220 were determined from subjects biological material by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Fasting glucose and Body Mass Index (BMI) were significant higher in GDM group than in control group ($p < 0.05$; $p = 0.002$, respectively). Obesity and overweight, and high BMI were significant when associated with GDM diagnosis ($p < 0.05$, both). *IL-17A* *rs2275913* (A/A) was associated with an increased likelihood of GDM diagnosis (OR = 5.22; 95% CI 1.08-25,29; $p = 0.040$), and we found no statistical significance to rs66554220 polymorphism, being important to continue further research.

Keywords: Gestational diabetes. Polymorphism. Diagnosis.

LISTA DE ABREVIATURAS

BMI	Body Mass Index
GDM	Gestational Diabetes Mellitus
HLA	Human Leukocyte Antigen
IL17A	Interleukin 17A
IL17RA	Interleukin 17A receptor
MHC	Major Histocompatibility Complex
SNP	Single Nucleotide Polymorphisms
T1DM	Type 1 Diabetes Mellitus
UTR	Untranslated Region

SUMÁRIO

ASSOCIATION OF INTERLEUKIN-17A/RA AND HLA-G POLYMORPHISMS WITH GESTATIONAL DIABETES MELLITUS

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Gestational Diabetes Mellitus (GDM) is a special type of diabetes that has been defined as any of glucose intolerance with onset or first recognition during pregnancy. The increasing of pregnancies complicated by GDM has been of great warning to the scientific community, as its pathophysiology is not well described and it has been related to severe pregnancy complications. The aim of this study is to evaluate the role of rs2275913, rs4819554 and rs66554220 polymorphisms in pregnancy complicated by GDM. The study comprised 79 patients with pregnancy complicated by GDM, as well as 81 healthy pregnant women (control group) from a High Risk Prenatal Outpatient Clinic. Personal data from the subjects were collected by forms, and polymorphisms of rs2275913, rs4819554 and rs66554220 were determined from subjects biological material by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Fasting glucose and Body Mass Index (BMI) were significant higher in GDM group than in control group ($p < 0.05$; $p = 0.002$, respectively). Obesity and overweight, and high BMI were significant when associated with GDM diagnosis ($p < 0.05$, both). *IL-17A* *rs2275913* (A/A) was associated with an increased likelihood of GDM diagnosis (OR = 5.22; 95% CI 1.08-25,29; $p = 0.040$), and we found no statistical significance to rs66554220 polymorphism, being important to continue further research.

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1 INTRODUTION

The gestational period is associated with several changes to women metabolism, some of them have a higher occurrence, as carbohydrate intolerance, which could cause hyperglycemia and insulin resistance and may predispose the development Gestational Diabetes Mellitus (GDM).[1-4] The GDM is defined as a special type of diabetes that has been defined as any of glucose intolerance with onset or first recognition during pregnancy. This definition applies regardless of the use of insulin or if the condition persists after delivery and does not exclude the possibility that glucose intolerance may have preceded pregnancy.[2,4]

In the past years, insulin resistance has been associated with inflammatory responses. Several pro-inflammatory cytokines and inflammatory markers have been associated with the pathophysiology of GDM, especially IL-1, TNF and IL-6.[5-9]

Even though mechanisms of GDM are still unknown, some studies highlight the role of the immune response and have suggested factors as altered functions, and genes polymorphisms to play a role in the pathophysiology.[10-13]

Interleukin (IL) -17 is an inflammatory cytokine produced by T-helper (Th) -17 cells and initially reported to be mainly expressed in autoimmune pathologies. It is known that Th-17 cells have also been shown to participate in successful pregnancy. Recent studies have shown that serum IL-17 levels increase considerably in third trimester of pregnancy suggesting that it might be involved in labor and/or inflammatory process.[14,15]

Since GDM does not have a well-stablished pathophysiology, studies have pointed that IL-17 could also have inhibitory functions, suppressing uterin inflammatory response, what prove it is interesting to start an analysis of biomarkers associated with this balance of immune response.[16-18]

Human leukocyte antigen (HLA)-G is a nonclassical Ib antigen of the major histocompatibility complex (MHC), known to have a 14 bp insertion/deletion (ins/del) polymorphism located in the 3rd untranslated region (3′UTR), and described as a marker for fetal maternal immunoregulation.[19] Recent studies have shown that the frequency of del/del homozygosity and soluble HLA-G concentration is higher in peripheral blood from GDM patients as compared with the control group.[20,21] Despite that, soluble HLA-G levels were significantly lower in women with GDM compared to control group patients, in some cases, showing that the role of HLA-G is not well established in the pathophysiology of GDM.[22]

The aim of this study was to assess the relationship between the IL-17A, IL-17RA and HLA-G 14bp polymorphism in women while GDM.

2 MATERIALS AND METHODS

2.1 Ethical aspects

Research permission was obtained from the Research Ethics Committee of the Onofre Lopes University Hospital, Federal University of Rio Grande do Norte (CEP-HUOL, protocol number: 2,631,092 on May 2, 2018, CAAE: 73305717.2.0000.5292).
We enrolled patients from two terciary teatching hospitais where they were informed about the purpose and methodology of the research, and those who agreed to participate voluntarily, signed an informed consent for further collection of biological material (venous blood) and personal information.

2.2 Study population and Design

This is a case-control study, with prospective data and sample collection. Recruitment of patients with GDM was performed at the High Risk Prenatal in two outpatient clinic of teaching hospitals. Population consists in patients diagnosed with GDM.
DMG diagnosis was based in tests made in the first prenatal visit by fasting blood glucose (92-125 mg/dL) or between 24 and 28 weeks of pregnancy by 75 g 2 h glucose tolerance test (OGTT) to confirm the diagnosis, as suggested by American Diabetes Association (ADA).[2,4]
Were enrolled a total of 160 patients from high risk teaching hospitals, being 79 GDM patients. All individuals studied were born in northeastern Brazil.
Were included in GDM group patients with he following criteria: older than 18 years; fasting glucose (92-125 mg/dL); oral glucose tolerance test (TOTG) one hour ≥ 180 mg/dL or two hours (53 to 199 mg/dL). And excluded patients with any other comorbidities during pregnancy.

2.3 Samples and DNA extraction

For the analysis of genetic polymorphisms, a genomic DNA extraction (gDNA) was performed by the method of salting out, described by Salazar et al (1998)[24], with some modifications. DNA

quality and integrity were analyzed on 1% agarose gel and quantification were performed on Nanodrop spectrophotometry. β-Globin DNA gene was amplified in parallel with each sample and used as internal controls.

2.4 IL-17A/RA gene polymorphisms

The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was used to determine the genotypes of *IL17A* (rs2275913; -197G > A), *IL17RA* (rs4819554; -947A > G) single nucleotide polymorphisms (SNPs). Fragments containing mentioned SNPs were amplified by specific primers. The PCR was carried out by Thermal Cycler B960 Advance (Even) in a total volume of 25 μL containing 0,5μM of each forward and reverse primers, 1,5mM of $MgCl_2$, 0,2mM of dNTP,1 unit of Taq DNA polymerase and 100 ng of DNA template. Primer sequences and PCR protocols are presented in **Table 1**. About 5 μL of PCR products containing IL17A and IL17RA polymorphisms were treated with XagI (Promega, Madison, WI, USA) and PVUII (Thermo Scientific, Waltham, MA, USA) restriction enzymes, respectively. Genotypes of digested mixtures were visualized by 2% agarose gel electrophoresis (Uniscience, São Paulo, SP, Brazil).

10% of all dimensions were retested for results results. Moreover, as the electrophoresis analyzes were performed by three researchers, in order to leave no sign of analytical error.

2.5 HLA-G 14pb gene polymorphisms

The HLA-G 14-bp Ins/Del genotypes in the 3′ untranslated region (UTR) in exon 8 of the HLA-G locus (rs66554220) were analyzed according to the following protocol: 200 ng of genomic DNA was amplified in a 25 μl reaction mixture containing 1,0 μM of each forward and reverse primers, 1,5 mM of $MgCl_2$, 0,2 mM of dNTP, 1 unit of Taq DNA polymerase and 100 ng of DNA template. *HLA-G* 14bp genotyping was done using PCR-RFLP followed by 3.5% agarose gel electrophoresis and fragments size were determined by direct comparison with the molecular weight marker (100bp scale). The presence of a 345-bp fragment correspond to deletion allele, while a 359-bp fragment correspond to 14-bp insertion allele. Primer sequences and PCR protocols are presented in **Table 1**.

10% of all dimensions were retested for results results. Moreover, as the electrophoresis analyzes were performed by three researchers, in order to leave no sign of analytical error.

2.6 Statistical analysis

The Shapiro-Wilk test was performed to test normal distribution. Median and percentiles (25 and 75) were used in the descriptive analysis of variables that did not present normal distribution in either group. Qualitative variables were summarized as counts and percentage. Non-parametric Mann-Whitney test was applied to compare differences for variables which did not show normal distribution. The Chi-square test was used to analyze the association between groups and categorical variables. In situations where the table cells presented expected frequencies below five, Fisher's exact test was applied. Comparisons of different genotypes and alleles between groups were performed using logistic regression and results were reported using odds ratios (OR) and their respective 95% confidence intervals (95% CI). The 5% significance level was used for the analyzes. Hardy-Weinberg equilibrium was verified by chi-square test. A probability value of $P < 0.05$ was considered as statistically significant and all the reported *P*-values were two-tailed.

3 RESULTS

3.1 Clinical Data

We enrolled 160 pregnant, stratified in two groups known as GDM and control. GDM group was composed of 79 (49,4%) women with a mean age of 31 years (26 – 35), as control group was composed of 81 (50,6%) women, with a mean age of 30 years (25 – 35). Demographic and clinical characteristics of patients are summarized in **Table 2**.

3.2 Multivariate analysis

Fasting glucose and BMI were significantly higher in GDM group than in control group ($p < 0.05$; $p = 0.002$, respectively). Obesity and overweight, and high BMI were significant when associated with GDM diagnosis ($p < 0.05$, both). Parity had higher signification when compared GDM to control. Proportion of pregnant with multiparity was higher in GDM group (79.7%) when compared to control group (61.7%) (**Table 2**).

3.3 HLA-G 14bp ins/del, IL17A and IL17RA polymorphisms

Frequency distribution of the IL17A (rs2275913), IL17RA (rs4819554) and HLA-G 14 bp ins/del (rs66554220) genotypes and variant alleles are shown in Table 3.
Data analysis showed that the distribution of rs2275913, rs4819554, and rs66554220 variations in both GDM and control patients were conformed to the Hardy-Weinberg equilibrium. No significant deviations have been found in GDM and control patients, despite deletion alleles have been more significant as risk protectors in both situations.
Odds ratio of GDM to those exposed to IL17A (A/A) was 5.22 (95% CI 1.08-25.29), and the presence of this genotype may be associated with an increased likelihood of diagnosis ($p = 0.04$).

4 DISCUSSION

The present study aimed to determine whether SNPs in *IL-17A* (rs2275913), *IL-17RA* (rs4819554), and *HLA-G 3'UTR* 14-bp insertion/deletion (ins/del) (rs66554220), contribute to the development of GDM and evaluate the relationships between them and the clinical-pathological characteristics.

We know that GDM is a pregnancy-related complication characterized by hyperglycemia and caused by both genetic predisposition and environmental triggers. It's known that hyperglycemia has prominent outcomes that can affect mother and child over the pregnancy and throughout life.[25-27] Some of the risk factors to mothers develop GDM include excessive gestational weight gain, overweight, obesity, hyperglycemia, history of recurrent miscarriage, family history of any kind of diabetes and/or advanced maternal age. In addition, women who had previous GDM are also at increased risk of other comorbidities as cardiovascular diseases and metabolic syndrome. [28-30]

In fact, when exploring biochemical characteristics as fasting blood glucose, and glucose levels, both were higher in GDM group than in control group.[30-32] Biophysical and parity characteristics such as BMI, gestational BMI, and if the subjects had or not children, we found significant differences between groups. Pregnants in GDM group showed higher BMI than control group. Moreover, gestational BMI stratified as overweight and obesity were higher in GDM than control group, corroborating with Abu-Heija et al.[33] who have shown that obesity/overweight is strongly associated with GDM development.[34-37] For those who had children, parity was a marker for increase in the incidences of positive GDM.[35]

In our study, we found a link between gestational BMI, obesity and overweight and the GDM group, which corroborates the findings of Shah et al.,[35] who found similar results and associated it with obstetric complications, including preterm labor, maternal development of type 2 diabetes and macrosomia[36]. In contrast, Page et al.[38] had also shown that children exposed to GDM or maternal obesity were more likely to develop altered hypothalamic response, obesity or metabolic disorder than children not exposed.

Nevertheless, even though there is evidence that parity and gestational age characteristics are important for the development of GDM,[39,40] these data were not significant in our study.

Some studies have pointed to the role of cytokines[41,42,43,44] in the pathophysiology of gestational diabetes, and how the balance between pro and anti-inflammatory cells[45] maintains homeostasis of the immune system to prevent inflammatory diseases, but they do not yet address IL-17 in the associated pathophysiology of GDM.[46,47]

Realizing the lack of association studies that aim to identify polymorphisms in GDM casuistry, this study sought to associate the polymorphism of IL-17 and its receptor, seeking to make a correlation between the gene polymorphism, the pathophysiology presented, and the clinical aspects of gestational diabetes.

Kuzmicki et al.[48] have shown that IL-17 is an important inhibitor of adipocyte differentiation and insulin-induced glucose uptake in adipose tissue. There is also a line that points to IL-17 as responsible for the embryonic allograft rejection mechanisms, which may be the reason for abortions and complications during the gestational period.[49-52] In our study, we detect a relationship between IL-17A (G/G) as being possible marker that may present a role of protection to development of GDM. And that the IL17A (A/A) polymorphism may be, associated with an increased risk of GDM development.

Studies have shown an association of HLA-G del / del polymorphism with the development of Type 1 Diabetes Mellitus (T1DM)[53,54] at an early age, while the presence of an insertion allele would retard this age of T1DM development.

In this sense, association studies between GDM and HLA-G[15,55-60] have shown the role of polymorphism and serum sHLA-G concentration in diabetic pregnant women.

Martinetti et al.[15] have found that HLA-G 14 bp del/del genotype was slightly more frequent in GDM mothers than in healthy ones, which corroborates with Gerasimou et al.[53], that found an increase of deletion allele as a predictor to T1DM. Still, Martinetti et al.[15] found sHLA-G in GDM mother had an increase as their gestational age was increasing, and both mothers and newborns with del/del polymorphisms had a higher risk of complications due to GDM. Regarding HLA-G, in our study we found no statistical significance to associate the HLA-G polymorphism to GDM. Given the scarcity of polymorphism studies associated with GDM, our study attempted to identify the action of IL-17A / RA on complications caused by GDM, as well as the association with the immune balance predicted by the action of HLA-G. Although GDM does not have a known pathophysiological mechanism, it is important that further research be initiated to improve known data.

To date, few association studies have been conducted to identify a possible pathway for elucidating the pathophysiology of GDM. Our study found strong evidence of *IL17A* participation in the pathophysiology of GDM, since its A/A homozygote was a risk association factor for the development of GDM.

The main limitations of this study were the heterogeneity of the subjects studied, and the relatively small number of GDM occurrences in relation to healthy pregnancies. We conducted our study at the referral center for high-risk pregnancies in the state of Rio Grande do Norte, faithfully representing all available cases during the study period. Even so, our group intends to continue the research line, this time analyzing the entire extension of the gene in the 3′UT region.

Authors' contributions: CRL and KTCC conceived of the study and participated in its design. KTCC and JCOCF conducted the literature review and drafted the manuscript. All authors have read and approved the final manuscript.

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TABLES

Table 1 - Primer sets and reaction conditions of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) experiments.

Gene	Primer sequences (foward, reverse)	Cycles	Annealing temperature (°C)	Product size (bp)
IL-17A	F: AGGTACATGACACCAGAAGACC	35	60	514
	R: TGCCCACGGTCCAGAAATAC			
IL-17RA	F: GGAAGAGAGGAGAGGCGAAT	35	60	430
	R: CACCCCTTTGCCTGGTTCTG			
HLA-G	F: TGTGAAACAGCTGCCCTGTGT	30	56	345 (Del)
	R: GTCTTCCATTTATTTTGTCTCT			359 (Ins)

Table 2 - Current pregnancy factors and obstetric history of women with GDM (case) and without GDM diagnosis (control)

Variables	Group		P value[1]	Total
	GDM	Control		
N, %	79 (49,4%)	81 (50,6%)		160 (100,0%)
Current pregnancy				
Age, years	31 (26 – 35)	30 (25 – 35)	0,314	31 (25 – 35)
Age, > 25 anos	61 (77,2%)	57 (70,4%)	0,325	118 (73,8%)
Gestational age, we.	32 (27 – 36)	33 (24 – 36)	0,945	32 (25 – 36)
Fasting glucose, mg/dl	97 (90 – 106)	85 (79 – 91)	**0,001**	90 (82 – 98)
Glucose, ≥ 95 mg/dl	45 (57,0%)	9 (11,1%)	**0,001**	54 (33,8%)
BMI, kg/m2	31,3 (28,2 – 34,7)	28,2 (25,9 – 31,3)	**0,002**	29,5 (26,9 – 33,3)
Gestational BMI, n (%)				
Low weight	4 (5,1%)	8 (9,9%)	**0,005**	12 (7,5%)
Suitable	15 (19,0%)	24 (29,6%)		39 (24,4%)
Overweight	25 (31,6%)	34 (42,0%)		59 (36,9%)
Obesity	35 (44,3%)	15 (18,5%)		50 (31,2%)
Obesity/Overweight, n (%)	60 (75,9%)	49 (60,5%)	**0,036**	109 (68,1%)
Obesity, n (%)	35 (44,3%)	15 (18,5%)	**0,001**	50 (31,2%)
Obstetric background				
Children	63 (79,7%)	50 (61,7%)	**0,012**	113 (70,6%)
Abortion	29 (36,7%)	32 (39,5%)	0,716	61 (38,1%)
GDM diagnosis	6 (7,6%)	2 (2,5%)	0,165	8 (5,0%)
Family history of DM	59 (75,6%)	68 (85,0%)	0,139	127 (80,4%)

Bold values highlight the significant results

Abbreviations: we., weeks; BMI, body mass index; DM, diabetes mellitus; DMG, gestational diabetes mellitus.

Table 3 - Distribution of polymorphisms (IL17A, IL17RA and HLA-G)

Gene	SNP	Allele/Genotype	Group		OR (95% CI)	P value[1]
			GDM	Control		
IL17A	rs2275913	A	38 (24,1%)	25 (15,4%)	1,73 (0,99-3,07)	0,053
		G	120 (75,9%)	137 (84,6%)	Reference	1,0 (Ref.)
		G/G	50 (63,3%)	58 (71,6%)	Reference	1,0 (Ref.)
		G/A	20 (25,3%)	21 (25,9%)	1,11 (0,54-2,27)	0,786
		A/A	9 (11,4%)	2 (2,5%)	5,22 (1,08-25,29)	**0,040**
IL17RA	rs4819554	G	50 (31,6%)	46 (28,4%)	1,17 (0,72-1,89)	0,526
		A	108 (68,4%)	116 (71,6%)	Reference	1,0 (Ref.)
		A/A	39 (49,4%)	45 (55,6%)	Reference	1,0 (Ref.)
		A/G	30 (38,0%)	26 (32,1%)	1,33 (0,68-2,62)	0,408
		G/G	10 (12,7%)	10 (12,3%)	1,15 (0,44-3,06)	0,774
HLA-G	rs66554220	Del	93 (58,9%)	101 (62,3%)	0,86 (0,55-1,36)	0,524
		Ins	65 (41,1%)	61 (37,7%)	Reference	1,0 (Ref.)
		Ins/Ins	15 (19,0%)	8 (9,9%)	Reference	1,0 (Ref.)
		Ins/Del	35 (44,3%)	45 (55,6%)	0,42 (0,16-1,09)	0,074
		Del/Del	29 (36,7%)	28 (34,6%)	0,55 (0,20-1,51)	0,246

Values are expressed as odds ratios (OR) and 95% confidence intervals (95% CI).
Categorical data are expressed as absolute (n) and relative (%) frequency.
Bold values indicates significance at $p < 0.05$.
OR significance and 95% CI by logistic regression adjustment or Chi-square or Fisher test.
Abbreviations: Del., deletion; Ins, insertion.