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Faculdade de Ciências Médicas

Deborah Conte Santos

Alelos de classe II do sistema maior de histocompatibilidade HLA e marcadores INDELs de ancestralidade genômica e sua associação com a retinopatia diabética: estudo multicêntrico de pacientes com diabetes tipo 1 no Brasil

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Orientadora: Prof.ª Dra. Marilia de Brito Gomes

Rio de Janeiro

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Rio de Janeiro
2020

DEDICATÓRIA

Dedico este trabalho aos meus pais, minha irmã, minha sobrinha e meu marido por sempre estarem ao meu lado.

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Existem muitas hipóteses em ciência que estão erradas. Isso é perfeitamente aceitável, eles são a abertura para achar as que estão certas.

Carl Sagan

RESUMO

SANTOS, Deborah Conte. **Alelos de classe II do sistema maior de histocompatibilidade HLA e marcadores INDELs de ancestralidade genômica e sua associação com a retinopatia diabética: estudo multicêntrico de pacientes com diabetes tipo 1 no Brasil**. 2020. 100f.Tese (Doutorado em Fisiopatologia Clínica e Experimental) – Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, 2020.

Este estudo tem como objetivo avaliar os alelos HLA de classe II e a ancestralidade genômica de pacientes portadores de diabetes tipo 1 (DM1) no Brasil e sua associação com a retinopatia diabética (RD). Os pacientes estudados fazem parte de um estudo multicêntrico brasileiro que envolveu 1760 pacientes. Dados clínicos e laboratoriais foram avaliados e a genotipagem para o loci HLA-classe II (DRB1, DQB1, DQA1) foi realizada pelas técnicas de NGS e SSO. A ancestralidade genômica foi determinada utilizando um painel com 46 AIMs . Os pacientes foram submetidos à oftalmoscopia indireta para classificação da RD em: ausente, RD não proliferativa leve, moderada, severa e RD proliferativa. Esta tese apresenta seus resultados em formato de artigos científicos. O primeiro artigo avalia a prevalência dos alelos HLA na população portadora de DM1 comparada a um grupo de indivíduos saudáveis retirados da base de dados dos doadores de medula óssea (REDOME) e ainda faz uma avaliação dos principais alelos em subgrupos de cor autodeclarada. Este artigo nos mostra que os principais alelos de risco e proteção associados aos DM1 são, respectivamente, o DRB1*03 e o DRB1*07, com pouca variação relacionada à cor autodeclarada. O segundo artigo analisa o perfil HLA associado à RD, comparando indivíduos portadores de DM1 com RD grave e portadores de DM1 sem RD, pareados por tempo de duração de doença. Após correção de Bonferroni, não foi encontrada associação para qualquer alelo do sistema HLA de classe II. O terceiro artigo mostrou associação positiva da ancestralidade africana com a presença de RD grave mesmo após ajustes para variáveis clínicas e sócio demográficas. O quarto artigo mostra que a ancestralidade africana influenciou negativamente a qualidade de vida relacionada à saúde nos pacientes portadores de DM1 se mostrando como um importante determinante social. Em conclusão, a população brasileira portadora de DM1, apesar de altamente miscigenada, se assemelha às populações caucásicas em relação aos alelos e haplótipos de risco e proteção mais prevalentes. Foi observado ainda que os alelos HLA de classe II não se associam à RD, porém houve influência da ancestralidade africana na RD. Uma possível hipótese para explicar este fato se relaciona a fisiopatologia da RD que parece estar mais ligada às vias inflamatórias associadas ao estresse oxidativo. Possivelmente os genes que predispõem essa importante complicação crônica do diabetes devem estar localizados em outras regiões do genoma. Portanto, mais estudos são necessários para melhor estabelecer a relação da genética com o desenvolvimento da RD. E por fim, foi observada a importância da abordagem multidisciplinar no manejo do DM1, levando em consideração fatores sócios culturais como a etnia do paciente para atingir melhor controle da doença.

Palavras chave: Retinopatia diabética. Diabetes tipo 1. HLA. Ancestralidade. Etnia. Qualidade de vida relacionada à saúde.

ABSTRACT

SANTOS, Deborah Conte. C**lass II alleles and genomic ancestry and their association with diabetic retinopathy: a nationwide study with type 1 diabetes patients in Brazil** 2020. 100f. Tese (Doutorado em Fisiopatologia Clínica e Experimental) – Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, 2020.

This study's objective is to analyze the class II alleles profile and the genomic ancestry of patients with type 1 diabetes (T1D) in Brazil and its relationship with diabetic retinopathy. Patients belong to a nationwide study involving 1760 individuals from all over the country. Clinical and laboratory data were collected and HLA-class II (DRB1, DQB1, DQA1) genotyped by either the NGS or SSO method. A panel of 46 AIMs was used for the determination of genomic ancestry. All patients had indirect ophthalmoscopy performed and were classified as absent, mild, moderate or severe non-proliferative DR or proliferative DR. This thesis presents the results in the form of scientific articles. The first article analyses the prevalence of HLA alleles in patients with T1D compared to a healthy population from the bone marrow donors' database of Brazil (REDOME). In addition, evaluation of frequencies of HLA alleles between groups of self-reported color/race was performed. In summary, this article shows that the most frequent alleles associated with risk and protection are, respectively, DRB1*03 and DRB1*07. The second article studies the relationship of HLA class II alleles and severe DR. It compares T1D individuals with and without severe DR matched by the duration of diabetes. After Bonferroni correction, no association was found. The third study shows a positive association of African genomic ancestry and the presence of severe DR even after adjusting for clinical and socio-demographic variables. The fourth (and last) article shows that health-related quality of life in T1D patients is influenced by African genomic ancestry. We can conclude that the Brazilian population that has T1D, although highly admixed, is similar to Caucasian populations in relation to its genetic profile. Evidence pointed out that DR was not associated with class II alleles, but it was associated with African genomic ancestry. This might be explained due to the fact that the pathogenesis of DR is more related to inflammatory pathways linked to oxidative stress, and so, other loci might be responsible for the genetic influence on DR. More studies are needed to better understand the genetic mechanism associated to the development of DR, especially in admixed populations. The results also highlight the importance of a multidisciplinary approach in T1D patients, considering socio-cultural-factors, like ethnicity, to achieve better clinical and psychological control.

Keywords: Diabetic retinopathy. Type 1 diabetes. HLA. Ancestry. Ethnicity. Health-related quality of life.

LISTA DE ABREVIATURAS E SIGLAS

AIMs	Marcadores de ancestralidade
HbA1c	Hemoglobina glicada
DCCT	*Diabetes Control and Complications Trial*
DCCT/EDIC	*Diabetes Control and Complications Trial/Epidemiology of diabetes Interventions and Complications Study*
DM1	*Diabetes Mellitus* tipo 1
DNA	Ácido desoxirribonucleico
EROs	Espécies Reativas de Oxigênio
ETDRS	*Early Treatment of Diabetic Retinopathy Study*
GWAS	*Genome Wide Association Studies*
HDL	*High Density Lipoprotein*
HLA	*Human Leukocyte Antigen*
HPLC	*High Performance Liquid Cromatography*
IBGE	Intituto Brasileiro de Geografia e Estatística
IDF	*International Diabetes Federation*
IL-6	Interleucina-6
INDELs	Polimorfismos de inserção/deleção
IRMA	Dilatações segmentares irregulars dos capilares retinianos
LDL	*Low density Lipoprotein*
NGS	*Next Generation Sequencing*
PKC	Proteina Kinase C
RD	Retinopatia Diabética
RDNP	Retinopatia Diabética não Proliferativa
SNPs	*Single Nucleotide Polymorphism*
SSO	*Sequence-Specific Oligonucleotide*
SUS	Sistema Único de Saúde
TNF-α	Fator de necrose tumoral alfa
UERJ	Universidade do Estado do Rio de Janeiro
VEGF	Fator de crescimento endotelial vascular
WESDR	*Wisconsin Epidemiologic Study of Diabetic Retinopathy*

LISTA DE SÍMBOLOS

%	Porcentagem
α	Alfa
±	Mais ou menos
cm	Centímetro
cm^3	Centímetros cúbicos
dL	Decilitro
Kg	Kilograma
L	Litro
m	Metro
m^2	Metro quadrado
min	Minuto
mg	Miligrama
ml	Mililitro
mmol	Milimol

SUMÁRIO

O diabetes tipo 1 (DM1) é uma doença autoimune crônica na qual o sistema imune ataca as células beta pancreáticas produtoras de insulina levando a ausência ou redução significativa de insulina. Sua etiologia ainda não está completamente elucidada, porém parece estar relacionada à combinação de fatores genéticos e ambientais. A maioria dos casos tem seu diagnostico ainda na infância ou adolescência. Atualmente, o DM1 é considerado a doença crônica mais prevalente na infância (1). A incidência global do DM1 varia entre 5 a 60 por 100.000 na faixa etária infanto-juvenil (0 a 14 anos), sendo maior em países nórdicos como Finlândia e Suécia. No Brasil a taxa de incidência chega a 7,6/100.000 habitantes/ano (1). É importante observar que Índia, Estados Unidos e Brasil concentram a maior parte de pacientes com DM1 em termos absolutos de prevalência segundo estimativa da IDF (*International Diabetes Federation*) (1).

A região do HLA responde por aproximadamente 50% da predisposição genética ao DM1, principalmente os alelos de Classe II(2, 3), O DRB1*03 e DRB1*04 são os principais alelos de risco e estão presentes em quase todas as populações. Porém, alguma diferença é observada entre os países e entre diferentes etnias.

A hiperglicemia crônica pode levar a complicações crônicas micro e macrovasculares. Retinopatia, nefropatia e neuropatia (periférica e autonômica) compreendem as complicações microvasculares enquanto que as doenças cardiovasculares como infarto, insuficiência arterial periférica e AVC compreendem as complicações macrovasculares. Estudo recente em uma coorte de pacientes DM1 atendidos em serviço terciário no Brasil mostrou que a taxa de mortalidade chega a 6,7%, sendo doença macrovascular a segunda maior causa de óbito (19,4%) (4).

A estimativa global da prevalência de complicações crônicas microvasculares relacionadas ao DM1 chega a cerca de três a cada 10 pacientes. A retinopatia diabética (RD) é a principal causa de perda visual em população jovem em todo mundo. No Brasil, a prevalência de RD foi de 35,7%, sendo que 12% apresentavam retinopatia grave, com risco de perda visual (5). A fisiopatologia da RD ainda não está completamente compreendida. Dentre os fatores de risco clássicos associados à RD estão tempo de duração do diabetes, controle glicêmico e a hipertensão arterial (6). Porém, observam-se na prática clínica alguns pacientes que apesar da longa duração de doença e/ou controle glicêmico inadequado não evoluem para RD enquanto outros

pacientes a desenvolvem logo nos primeiros anos de doença e às vezes com glicemias não tão elevadas. Uma possível justificativa seria a presença de uma predisposição genética associada à doença.

Apesar do papel já estabelecido da genética relacionado à predisposição ao desenvolvimento de DM1, estudos genéticos relacionados à RD mostram resultados conflitantes.

A etnia também é um fator importante no estudo genético do DM1 e da RD além de poder influenciar em diversos aspectos relacionados à qualidade de vida e tratamento da doença, principalmente no âmbito das inequidades do acesso à saúde. A relação da ancestralidade com a presença e/ou progressão da RD também merece atenção. Estudos realizados em populações específicas de pacientes DM2 nos Estados Unidos mostraram influência da ancestralidade ameríndia apenas em uma subpopulação Latina, mas não encontraram associação da ancestralidade africana na subpopulação de Africano-americanos (7, 8).

O principal objetivo desta tese é identificar os principais fatores genéticos, tanto de HLA quanto de ancestralidade genômica, associados ao DM1 e à RD na população Brasileira. Com isso, esperamos contribuir com o conhecimento prévio para conseguirmos no futuro uma identificação precoce dos pacientes mais suscetíveis a fim de atingir melhor controle da doença e prevenir desfechos desfavoráveis destas importantes patologias.

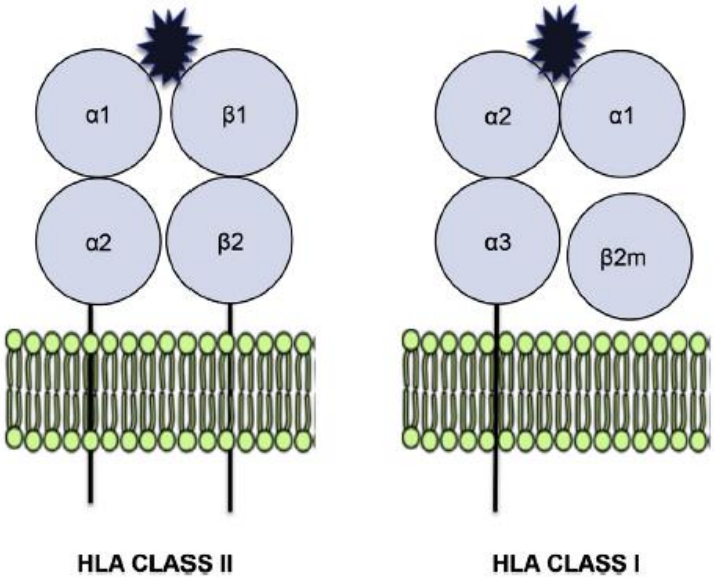
Nos próximos capítulos apresentarei uma breve revisão da literatura relacionada aos temas expostos acima e uma sequência de quatro artigos, publicados ou submetidos à publicação, como resultado deste estudo.

1. REVISÃO BIBLIOGRÁFICA

1.1 HLA

O sistema HLA, presente no braço curto do cromossomo 6, faz parte de um complexo de genes responsáveis pela resposta imunológica, o chamado MHC (“Major Histocompatibilty Complex). Duas classes moleculares de glicoproteínas de superfície celular fazem parte do HLA e se diferenciam em relação a estrutura, função e distribuição tecidual. As moléculas de classe I, formadas por uma cadeia simples de polipeptídio (alfa), forma heterodímeros com a b2-microglobulina quase invariavelmente e é expressa virtualmente em todas as células. Já as moléculas de classe II, formadas por 2 cadeias polipeptídicas (alfa e beta), extremamente polimórficas, são expressas nos leucócitos B, células dendríticas, macrófagos e linfócitos T ativados. A figura 1, retirada do artigo de revisão da Noble (2), mostra de forma esquemática a estrutura das moléculas de HLA de classe I e II.

Figura 1 - Representação esquemática das moléculas HLA de classe I e II. (2)



A primeira descrição do papel do sistema HLA em doenças autoimunes data da década de 70, sendo inicialmente descrita com a associação do HLA-B*27 e espondilite anquilosante (9). Na mesma década, diversos estudos mostraram a associação do HLA

com diabetes (10, 11) e outras doenças autoimunes como artrite reumatoide, doença celíaca e lúpus eritematoso sistêmico (12-14). Hoje em dia, após décadas de estudos, apesar da reconhecida associação das moléculas HLA de classe I e outros genes não-HLA, as moléculas HLA de classe II são as mais fortemente associadas à predisposição ao DM1 (15, 16).

1.1.1 HLA e DM1

Os principais haplótipos HLA que conferem maior risco de desenvolver o DM1 são o DRB1*03 (HLA-DRB1*03:01~DQA1*05:01~DQB1*02:01) e o DRB1*04 (HLA-DRB1*04:01/02/04/05~DQA1*03:01~DQB1*03:02) (2, 3). A maioria dos estudos é baseada em populações caucásicas. Apesar da presença do DRB1*03 e do DRB1*04 em praticamente todas as populações, alguma variação é observada. Por exemplo, nos estudos realizados em pacientes Japoneses há ausência do DRB1*03, mas apresenta o DRB1*09 como alelo de risco (17). Nos Estados Unidos, em populações afro-americana, há também a presença do DRB1*09 (18). Estudos brasileiros regionais mostram o DRB1*03 e DRB1*04 como principais haplótipos de risco, porém até o momento não temos um estudo verdadeiramente representativo da população brasileira como um todo (19-22). As tabelas 1 e 2, adaptadas do livro Diabetes tipo 1 no Brasil (23), mostram respectivamente a distribuição de risco e proteção dos principais alelos HLA de classe II associados ao DM1 e as populações onde já foram descritos.

Tabela 1 - Principais haplótipos de risco para o diabetes tipo 1, de acordo com a etnia*

DR	DRB1	DQB1	DQA1	OR	Etnia	OR
			Risco			**São Paulo**
DR3					Presente em quase todas as etnias (baixa frequência em asiáticos). Ausência no Japão?	
DR3	03:01	02:01	05:01	2,38-9,5	Caucasiana, africana, Oriente Médio, miscigenada**	
DR3	03:01	02:01			Miscigenada **	4,64
DR4					Presente em todas as etnias	
DR4	04:05	03:02	03:01	3,0-14,0	Caucasiana, africana, asiática, Miscigenada**	
DR4	04:05	03:02			Miscigenada**	7,03
DR4	04:05	02:01	03:02	10,24	Afro-americana	NO
DR4	04:01	03:02	03:01	6,3 -15,8	Caucasiana, africana, asiática, miscigenada**	2,79
DR	**DRB1**	**DQB1**	**DQA1**	**OR**	**Etnia**	**OR**

			Risco			São Paulo
DR4	04:02	03:02	03:01	3,2 -7,1	Caucasiana, africana, asiática, miscigenada**	4,02
DR4	04:02	03:01			Miscigenada***	5,6
DR4	04:04	03:02	03:01	2,0 -12,5	Caucasiana, afro-americana, miscigenada**	
DR4	04:04	03:02		2,05	Miscigenada***	2,05
DR7	07:01	02:01	03:01	4,3	Afro-americana	0,42
DR8	08:02	03:02		5,67	Asiática (Japão)	
DR9	09:01	02:01/02	03:01	3,17-9,0	Africana, asiática, afro-americana, miscigenada***	NO
	09:01	02:02		4,27	Miscigenada***	4,27
DR9	09:01	02:01	05	15,5	Asiática (China)	
	09:01	03:03		2,2-17,0	Asiática (Japão,Korea,Tailândia) miscigenada***	

**Consideramos apenas os haplótipos com OR com significado estatístico.*
***Caucasiana com africana ou com ameríndia.*
****Caucasiana com africana.*
*****Caucasiana com ameríndia.*
 # Caucasiana,Africana e Oriente Médio

NA: não avaliado
Tabela adaptada do livro "Diabetes tipo 1 no Brasil", 2019.

Tabela 2 - Principais haplótipos de proteção para o diabetes tipo 1, de acordo com a etnia*

DR	DRB1	DQB1	DQA1	OR	Etnia	OR
			Proteção			São Paulo
DR2	DR15:01	06:02/ 06:01	01:02	0,04 -0,1	Caucasiana, asiática, africana, miscigenada**	0,1
DR3	03:02	04:02		-	Miscigenada**	0,29
DR3	03:02	04:02	04:01	0,16	Afro-americana, miscigenada***	0,29
DR4	04:03	03:02	03:01	0,06-0,37	Caucasiana (Sardenha,Finlândia) ,Miscigenada (Egito) #	
DR4	04:03	03:02			Asiática (Norte da India)	
DR4	04:07	03:01	03:01		Miscigenada***	
DR7	07:01	02:02	02:01	0,54	Miscigenada ***	*NO*
DR7	07:01	02/01/02:02		0,25-0,49	Miscigenada ***	0,42-0,49
DR	**DRB1**	**DQB1**	**DQA1**	**OR**	**Etnia**	**OR**
			Proteção			São Paulo

DR7	07:01	03:03		0,09-0,1	Caucasiana	
DR7	07:01	03:03	02:01	0,09-0,1	Miscigenada***	
DR8	08:03	03:03	03:03	0,9	Asiática (China)	
DR8	08:03	06:01	01:03	0,1	Asiática (Tailândia)	
DR8	08	06:01			Asiática (Korea)	
DR8	08:02	04:02	04:01		Miscigenada***	
DR8	08	04:02			Miscigenada***	0,49
DR8	08:04	04:02	04:01		Miscigenada***	
DR8	08:04	03:01	04:01		Afro-americana	
DR10	10		05			
DR10	DR10	05:01			Miscigenada***	0,31
DR10	10:01	05:01	01:01	0,61-0,70	Miscigenada***, afro-americana, miscigenada (Egito)[#] ?	
DR11	11	03:01		0,23-0,31	Miscigenada***, Irã	0,47
DR11	11:01	03:01	05:01	0,12/ 0,29	Caucásica (Rússia) ,China Miscigenada***, Miscigenada (Egito)[#]	
DR11	11:01	06:02	01:02		Miscigenada***	
DR11	11	06:02			Miscigenada***	0,02
DR12	12:02	03:01	06:01	0,28	Asiática (China)	
DR12	12:01/02			0,05/0,1	Tailândia	
DR13	13:02	06:03	01:03		Miscigenada***	
DR13	13:01	06:03	01:03	0,2	Turquia	
DR13	13:01	06:03	01	0,05	Miscigenada (Egito)[#]	
DR13	13	06:02/08	01:03	0,16	Rússia	
DR13	DR13	06:03			Miscigenada***	0,19
DR14	14	03:01	05		Miscigenada**	
DR14	DR14:01	05:03	01:01	0,1-0,6	Afro-americana, Caucasiana, Turquia	
DR14	DR14:01	05:02	01:01	0,09-0,29	Asiática (China,Japão)	
DR14	DR14:02/ DR14:54	05:01/05:03	03:01/ 01:01		Miscigenada *** Miscigenada **	NO
DR14	DR14:01	05:03		0,03-0,18	Caucasiana (Finlândia) ,Miscigenada ***	0,18

**Consideramos apenas os haplótipos com OR com significado estatístico.*
***Caucasiana com africana ou com ameríndia.*
****Caucasiana com africana.*
*****Caucasiana com ameríndia.*
 # Caucasiana,Africana e Oriente Médio

NO: Não Observado
Tabela adaptada do livro "Diabetes tipo 1 no Brasil", 2019.

1.1.2 HLA e RD

A RD é uma importante complicação microvascular do DM1 cujos principais fatores de risco associados são a hiperglicemia, tempo de duração da doença e hipertensão arterial. Entretanto, alguns pacientes desenvolvem esta complicação ainda nos primeiros anos após o diagnóstico, enquanto outros pacientes permanecem sem RD apesar da longa duração da doença e hiperglicemia. Diante deste fato, a hipótese de uma relação entre RD e fatores genéticos vem sendo estudada nas últimas décadas. A relação do HLA com a RD apresenta resultados conflitantes. Em uma coorte familial, encontrou-se associação positiva do DRB1*03:01 como protetor para RD, assim como o haplótipo DQA1*05:01-DQB1*02:01 em desequilíbrio de ligação. Além disso, mostrou que o DRB1*04:01 só apresentava risco na ausência do DRB1*03 (24). Em contraste, um estudo realizado também em população Caucásica, na Suécia, não encontrou associação dos alelos HLA de classe II com a RD (25). Mesmo a análise de um mesmo grupo de pacientes mostrou resultados discordantes, ocorrido na coorte WESDR. Em seu estudo inicial, de corte transversal, sem realizar ajuste para tempo de duração da doença, foi encontrada associação positiva entre HLA-DR4 e presença de RD (26). Porém, em sua publicação após 14 anos de acompanhamento, nenhuma relação foi encontrada entre alelos HLA e RD (27).

1.2 **Fisiopatologia da RD**

A fisiopatologia da RD ainda não está completamente elucidada, porém as alterações estruturais e alguns de seus mecanismos de dano como a inflamação e o estresse oxidativo já foram descritos (28).

Uma das alterações mais precoces da RD é o espessamento da membrana basal e redução do número de pericitos, os quais são células responsáveis por manter a integridade dos vasos (29). Com isso, há alteração da estrutura do vaso e distensão de sua parede, levando a formação de microaneurismas e proliferação das células endoteliais por diversos estímulos, entre eles o VEGF. Além do espessamento da membrana basal, ocorre ainda lesão da barreira hemato-retiniana interna, também por estimulo do VEGF, levando a um aumento da permeabilidade vascular e edema local

(30). O edema local por sua vez pode levar à oclusão das arteríolas pré-capilares gerando hipóxia progressiva associada a pequenos infartos nas camadas de fibras nervosas, formando as chamadas manchas algodonosas (31). A manutenção do estimulo oxidativo e progressão do mecanismo patológico levam a formação de neovasos e formação de tecido conjuntivo na parte posterior do vítreo. A contratura desse tecido conjuntivo por fibrose pode levar às alterações graves que causam perda da visão como o descolamento de retina e hemorragia vítrea (31, 32).

O principal estímulo às modificações observadas na RD é a exposição à hiperglicemia. O excesso de glicose leva a diminuição do oxido nítrico biodisponível e consequente aumento das espécies reativas de oxigênio (EROs), levando ao estresse oxidativo e ativação do NF-KB. Na realidade, diferentes processos moleculares correlatos estão envolvidos na produção das EROs, entre eles a inflamação, o acúmulo de produtos de glicação avançada da via dos polióis, o estimulo da via das hexosaminas e ativação da PKC. Esses mecanismos parecem estar relacionados à superprodução mitocondrial das EROs (33).

O acúmulo dos produtos de glicação avançada (AGEs) e a ativação da PKC levam a aumento da produção do VEGF, que exerce importante papel na patogênese da RD (34). Além disso, os AGEs também são responsáveis pela apoptose dos pericitos (35) e ativação de macrófagos com consequente liberação de citocinas inflamatórias como a IL-6 e o TNF-α (36, 37).

1.3 **Estudos genéticos em RD**

Conforme já descrito anteriormente, estudos de avaliação de possíveis genes relacionados ao HLA para RD têm apresentado resultados controversos. A inflamação local e o estresse oxidativo parecem ser os principais mecanismos na patogênese da RD e diversos biomarcadores genéticos vem sendo estudados nos últimos anos na tentativa de localizar novas regiões do genoma relacionadas à RD (38).

Dentre os diversos estudos de genes candidatos realizados, se destacam os relacionados ao VEGF e aldose redutase (AKR1B1) por apresentarem resultados mais consistentes e replicáveis. A aldose redutase é uma enzima que participa no metabolismo glicídico e sua ativação induz alterações metabólicas e bioquímicas que levam ao desenvolvimento precoce da RD (39, 40). Em uma metanálise com 20 genes

candidatos na RD, as variantes do gene da aldose redutase foram os que se mostraram mais associados à RD (41). Estudos de diversos polimorfismos do gene do VEGF foram realizados, mas o único com resultado conclusivo encontrado na metanálise foi a mutação do alelo C no SNP rs2010963 e apenas para retinopatia não proliferativa (41, 42).

Estudos de GWAS também foram realizados para RD, porém com uma pequena amostra de indivíduos e em três populações distintas: Mexicano-americanos, Chineses e caucásicos (43-46). Nenhum deles encontrou associações robustas relacionadas à RD.

Novos estudos, especialmente em populações mistas, são necessários para enriquecer nosso conhecimento do mecanismo fisiopatológico e identificar possíveis genes associados para ajudar na prevenção do dano vascular e proteção da visão.

1.4 **Ancestralidade e cor autodeclarada**

O Brasil é um dos países com maior diversidade genética e racial do mundo, resultado de 500 anos de cruzamentos étnicos originário basicamente de três continentes: Europa (colonizadores), África (escravos), e América (ameríndios nativos). Na tentativa de elucidar a contribuição dos diferentes fluxos migratórios na formação da população brasileira, alguns estudos foram realizados nas últimas décadas.

Apesar da classificação em raça ser equívoca do ponto de vista genético, ela é muito utilizada em censos demográficos. Com o estudo da ancestralidade genômica, fica ainda mais notável a superficialidade da classificação baseada em cor/raça. Em estudo publicado em 2006, foram avaliados 120 indivíduos classificados como brancos e 50 como mulatos derivados da miscigenação entre brancos e pretos. Foram analisados marcadores de sequências repetidas de cromossomo Y e investigados polimorfismos. Essas sequências eram constituídas de material quase que exclusivamente de origem europeia tanto em brancos como mulatos. Já na análise do DNA mitocondrial dos brancos, mostrou-se o mesmo peso de contribuição das ancestralidades europeia, africana e ameríndia (47).

Um estudo brasileiro de 2005 comparou cor autodeclarada com análise de DNA mitocondrial e mostrou também diferenças entre o fenótipo e a ancestralidade. Dos 492 indivíduos avaliados, 74.6% se declararam brancos, 13.8% pardos e 10.4% pretos. A maior contribuição na ancestralidade era de fato de origem europeia (em média 57.4%).

Porém, mesmo entre os que se declararam brancos, havia contribuição importante de ancestralidade africana: 37.6% apresentavam DNAmt africano, enquanto os que se declararam pretos ou pardos, 80.4% e 69.1% tinham DNAmt africano. Já a contribuição ameríndia tinha distribuição semelhante entre os três tipos de etnia autodeclarada (48). Em outro estudo, Manta et. al. avaliaram amostras mais amplas correspondentes às cinco regiões geográficas do Brasil (Norte, Nordeste, Centro-Oeste, Sul e Sudeste). Foram coletadas 798 amostras de indivíduos sem parentesco, de 12 diferentes populações, incluindo amostras aleatórias de 10 das 27 unidades federativas do país além de uma comunidade ameríndia (Terena) e de uma pequena comunidade na Amazônia (Santa Isabel do Rio Negro). As amostras foram analisadas com 46 marcadores de ancestralidade (AIMs). Foi observada maior variabilidade genética nas populações urbanas, com contribuição das três principais etnias, ao contrário das populações que apresentam parentesco, como, por exemplo, a população de Terena, constituída essencialmente de ancestralidade dos nativo-americanos (>75%). Também com menor diversidade genética está a população rural de Santa Isabel, que teve pouca exposição a europeus e africanos e assim, também tem predomínio de ancestralidade de nativo-americanos. Em geral, a população brasileira urbana tem maior contribuição de ancestralidade europeia (>70%), embora no Norte, a ascendência de nativo-americanos tenha maior peso que no restante do país e nas regiões Nordeste, Centro-Oeste e Sudeste, a contribuição africana é a segunda mais prevalente (49).

Outro grande estudo que avaliou ancestralidade na população brasileira foi o EPIGEN Brazil Initiative, que envolveu análise de coortes das três regiões brasileiras mais populosas: Nordeste, Sudeste e Sul, representadas respectivamente pelas cidades de Salvador/BA (SCAALA - Social Changes, Asthma and Allergy in Latin America Program), Bambuí/MG (The Bambuí Aging Cohort Study) e Pelotas/RS (Pelotas Birth Cohort Study). Foram analisados 5871 indivíduos, com análise de mais 370,000 SNPs. No EPIGEN, foi confirmado o maior peso da ancestralidade africana (em torno de 50%) na população do Nordeste, ao passo que a população das regiões Sul e Sudeste tem mais de 70% de contribuição de ancestralidade europeia e apenas 6-8% da ancestralidade é derivada de ameríndios (50).

A ancestralidade genômica aplicada nesta população miscigenada de brasileiros portadores de DM1 nos possibilita descobrir e correlacionar a verdadeira herança genética de risco para desenvolvimento de complicações microvasculares.

1.5 **Ancestralidade e DM1**

A variabilidade genética do DM1 pode estar relacionada a diferenças no perfil de ancestralidade e miscigenação das diferentes populações. Um estudo realizado nos Estados Unidos com pacientes afro-americanos portadores de DM1 mostrou maior diversidade de haplótipos HLA relacionados ao diabetes neste grupo de pacientes quando comparado a estudos em populações caucásicas (51). É importante observarmos também as diferentes taxas de incidência entre diferentes populações. De acordo com dados da IDF, as maiores incidências da doença estão presentes em países de origem ancestral Caucásica, como Finlândia e Suécia aonde a incidência chega a mais de 40/100.000hab/ano. Em contraste, países como China, Índia, Peru, Colômbia e Japão apresentam incidências menores de 5/100.000hab/ano (1). Dados americanos mostram que a prevalência de DM1 na população jovem pertencente a minorias étnicas é menor quando comparado a prevalência em caucásicos (52), porém sua incidência vem crescendo, especialmente nos Hispânicos (53). No Brasil, estudo realizado em Bauru, mostrou aumento de incidência nas últimas três décadas principalmente em crianças caucásicas (54).

Estudo recém-publicado (55) realizado na mesma população que analisaremos neste presente trabalho, analisou a ancestralidade genômica de 1698 pacientes portadores de DM1 através de 46 AIMs comparando com um grupo de 936 participantes saudáveis de todo o Brasil. Os portadores de DM1 apresentaram predominância de ancestralidade Europeia, assim como os controles, porém com proporções significativamente maiores (67,8% versus 56,3%, respectivamente). Além disso, se considerarmos um ponto de corte para definição de paciente não miscigenado sendo >95% de uma única ancestralidade genômica, neste estudo, isso só foi observado em 6,2% dos pacientes e apenas para a ancestralidade Europeia. Outro fator importante foi a baixa proporção de ancestralidade africana observada em nossa população (22,5%) quando a comparamos com a população Americana que apesar de possuir um perfil migratório semelhante, apresenta maior histórico de segregação racial (56, 57).

1.6 **Ancestralidade e RD**

O perfil de ancestralidade e migração das diferentes populações também pode estar relacionado ao papel da genética no desenvolvimento de complicações como a RD. Estudos mostram resultados distintos em diferentes populações.

A maioria deles foi realizado em populações com diabetes tipo 2 de origem Caucásica e utilizando a cor autodeclarada para determinar etnias (58-62). Entre eles, algumas coortes multiétnicas incluindo Brancos, Hispânicos, Pretos, Sul-asiáticos e Chineses realizadas nos EUA (61), Reino Unido (60) e Singapura (62) não mostraram associação entre etnia e RD, porém a etnia esteve associada à deficiência visual nas coortes do Reino Unido (Sul-asiáticos e Pretos) e de Singapura (Indianos).

Outros dois estudos realizados em uma população de latinos e outra de africanos-americanos nos EUA utilizaram ancestralidade genômica para definir etnia e avaliaram sua associação a RD. A ancestralidade ameríndia esteve associada a RD na população de latinos mesmo após ajustes para os fatores de risco clássicos da RD (7). Em contraste, o estudo com africanos-americanos encontrou associação da proporção de ancestralidade africana com RD proliferativa apenas na análise inicial, a qual não se manteve após analise multivariada (8).

Há escassez de dados em populações miscigenadas e em DM1, portanto uma análise da população brasileira poderia enriquecer o conhecimento da influência genética na fisiopatologia das complicações microvasculares, em especial a RD.

1.7 **Classificação da RD**

Com base nos estudos Early Treatment of Diabetic Retinopathy Study (ETDRS) e no Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR) (63, 64), a RD pode ser classificada em:

a) Retinopatia ausente;

b) Retinopatia diabética não proliferativa (RDNP) leve: apenas presença de microaneurismas;

c) RDNP moderada: microaneurismas e mais alterações como: hemorragias intra-retinianas, exsudatos duros, manchas algodonosas, porém em pouca quantidade;

d) RDNP severa: mais de 20 hemorragias nos 4 quadrantes da retina, perolização venosa definitiva (*beading* venoso) em 2 ou mais quadrantes ou presença de IRMA em 1 ou mais quadrantes;

e) Retinopatia diabética proliferativa: presença de neovasos, hemorragia vítrea ou hemorragia pré-retiniana.

Qualquer estágio da retinopatia diabética pode estar associado a edema macular.

1.8 **Qualidade de vida e DM1**

Outro aspecto importante no tratamento do DM1 está relacionado à qualidade de vida relacionada à saúde. Devido ao caráter crônico da doença e suas complicações potencialmente debilitantes, o aspecto social e psicológico e principalmente a percepção do paciente em relação ao seu estado de saúde tem sido estudada.

Estudos anteriores demonstram que pacientes portadores de DM1 possuem menor qualidade de vida relacionada a saúde (65). Além da baixa qualidade de vida, os estudos também demonstram mudança no estilo de vida dos familiares dos pacientes e ainda, maior grau de ansiedade e depressão (66).

A qualidade de vida relacionada à saúde representa uma percepção do paciente em relação ao seu estado físico e mental. Diversos questionários foram desenvolvidos e validados para a população brasileira, entre eles o SF-6D (Short Form-6 Dimensions) (67), e o EQ-5D (EuroQoL-5 Dimensions) (68). O SF-6D é mais voltado para avaliação da produtividade e impacto nas atividades diárias, enquanto que o EQ-5D avalia outros aspectos como mobilidade, autocuidado, atividades usuais, dor e desconforto e, ansiedade e depressão. Além disso, o EQ-5D possui ainda uma segunda avaliação que consiste em uma escala visual numerada de 0 a 100 na qual o paciente deve atribuir uma nota para o seu estado de saúde naquele momento. A partir de ambos os questionários, se pode calcular um escore que permite comparar diferentes populações.

Diversos fatores relacionados à diabetes influenciam na qualidade de vida, como o nível da HbA1c, presença de complicações microvasculares, sobrepeso, obesidade e sedentarismo (69-74). Porém fatores socioeconômicos, escolaridade e etnia também parecem estar associados (75-79). Estudo realizado em uma coorte de pacientes idosos não diabéticos mostrou que os pacientes que se autodeclaravam não brancos e aqueles

que possuíam maior percentual de ancestralidade africana, tinham menor nota em relação à qualidade de vida (80).

Portanto, diversos são os fatores influentes no manejo clínico do paciente portador de diabetes, entre eles a etnia. Com o estudo da ancestralidade genômica se torna possível uma investigação mais precisa desta variável e sua influência nas diversas nuances do manejo clinico dos pacientes.

2. OBJETIVOS

Os principais objetivos desta tese são:

a) Identificar o perfil HLA de classe II dos pacientes portadores de DM1 no Brasil comparando-o a uma população saudável e sua distribuição de acordo com a cor autodeclarada;
b) Identificar possíveis associações do perfil HLA de classe II com a retinopatia diabética em pacientes portadores de DM1 no Brasil;
c) Avaliar a associação entre ancestralidade genômica e retinopatia diabética em pacientes portadores de DM1 no Brasil;
d) Avaliar a influência da ancestralidade genômica na qualidade de vida dos pacientes portadores de DM1 no Brasil.

3. **MÉTODOS**

Apesar dos artigos apresentados como resultados nesta tese terem seus próprios métodos detalhados em cada estudo, nesta seção será apresentada a metodologia geral utilizada na obtenção dos dados do estudo multicêntrico brasileiro (Brazdiab1SG) da qual retiramos os pacientes e seus respectivos dados utilizados em todos os artigos aqui apresentados. Diante do extenso volume de dados, dividimos a análise em quatro artigos, todos com delineamento transversal e observacional, multicêntrico. Primeiro foi realizado um estudo mais abrangente comparativo entre pacientes portadores de DM1 e indivíduos saudáveis a fim de identificarmos possíveis marcadores HLA de risco e proteção relacionados à doença. Em seguida, realizamos a análise destes mesmos marcadores e sua relação com a retinopatia diabética, utilizando um delineamento caso-controle aninhada a esta coorte de pacientes. O terceiro artigo avalia ainda em um desenho de estudo caso-controle, a influência da ancestralidade genômica na RD. E por fim, apresentamos um estudo transversal, com amostragem mais abrangente avaliando a influência da ancestralidade genômica na qualidade de vida relacionada à saúde destes mesmos pacientes.

O Brazdiab1SG foi o estudo brasileiro multicêntrico, de corte transversal, realizado sob coordenação da Prof. Dra. Marilia Brito Gomes, com o objetivo principal de avaliar a prevalência de complicações crônicas nos pacientes portadores de DM1 no Brasil. A coleta de dados foi realizada em 13 centros distribuídos nas cinco regiões do país, no período de 2011 a 2014. Foram incluídos 1760 pacientes atendidos pelo SUS em centros secundários e terciários localizados em áreas urbanas.

3.1 **Critérios de inclusão**

Para serem incluídos no estudo os pacientes deveriam ter idade maior que 13 anos, acompanhamento mínimo de 6 meses em cada centro e diagnóstico clínico de DM1 realizado por um endocrinologista baseado em sintomatologia clínica clássica (poliúria, polidipsia, polifagia, perda ponderal) associada a necessidade de insulinoterapia desde o diagnóstico.

3.2 **Critérios de exclusão**

Foram excluídas gestantes, lactantes ou ainda pacientes que apresentaram, nos 3 meses antecedentes à seleção, quadro de cetoacidose diabética ou infecção aguda.

3.3 **Cálculo da amostra**

O cálculo amostral foi baseado em estimativas prévias de prevalência de DM1 e dados do censo de 2000 realizado pelo IBGE(81) mantendo a proporção entre os centros.

3.4 **Avaliação clínica e laboratorial**

Os pacientes responderam a um questionário e foram submetidos a uma avalição clínica realizada por um médico endocrinologista no momento da seleção do estudo. O questionário aplicado fornecia informações clínicas e sócio demográficas, como idade, gênero, cor autodeclarada, nível sócio econômico, escolaridade, profissão, renda familiar, comorbidades, dieta, grau de atividade física, hábitos tabágicos, medicações em uso, além dos questionários relacionados à qualidade de vida (EQ-5D, EQVAS e SF-6D).

A avaliação clínica consistia em aferição de peso, altura e pressão arterial, pesquisa de neuropatia periférica e autonômica, além de uma avaliação oftalmológica (fundoscopia).

Os pacientes também forneciam coleta de urina e amostras de sangue para as análises laboratoriais, as quais foram todas realizadas no laboratório da unidade de diabetes do Hospital Universitário Pedro Ernesto (HUPE). A HbA1c foi dosada pela técnica *High Performance Liquid Cromatography* (HPLC, Bio-Rad Laboratories, Hercules, California, USA). Glicemia de jejum e colesterol (total, HDL e LDL e triglicerídeos) foi determinada por técnicas enzimáticas.

3.4.1 Determinação dos alelos HLA e marcadores INDELs de ancestralidade

O DNA dos participantes foi extraído de amostra de sangue periférico utilizando o Kit comercial *SP QIA symphony* conforme orientações do fabricante (Qiagen, USA).

Os alelos HLA de classe II (DRB1, DQA1 e DQB1) foram analisados pela técnica NGS (Next Generation Sequencing) (GenDx, Ultrech, Netherlands) em 46,7% das amostras para os locus HLA- DRB1 e DQB1 e 12,1% dos pacientes foram sequenciados pelo Kit *Holotype HLA Assay* (Omixon, Inc., Budapest, Hungary) para os locus HLA –DRB1, -DQB1, - DQA1. O restante dos pacientes teve sua determinação dos alelos HLA de classe II realizados pela técnica de média-alta resolução PCR-SSO (LabType SSO, One Lambda Inc., West Hills, USA). A definição alélica foi baseada na versão 2.0 da lista CWD (Common and Well Documented) e as ambiguidades foram resolvidas por métodos de sequenciamento. Os alelos HLA – DQA1 foram imputados em 31,5% dos pacientes a partir do critério de desequilíbrio de ligação com base nos resultados do sequenciamento dos nossos pacientes. As ambiguidades em alelos de classe II que se encontravam dentro de um mesmo grupo G, isto é, grupos de alelos que possuem sequências nucleotídicas idênticas nos éxons codificadores de domínios ligantes de peptídeos, foram designadas como ‘g’ em letra minúscula (DRB1*12:01g = 12:01/12:10; DQA1*01:01g = 01:01/01:04/01:05; DQA1*03:01g = 03:01/03:02/03:03; DQA1*05:01g = 05:05/05:09; DQB1*03:01g = 03:01/03:09/03:19).

A determinação da ancestralidade genômica foi realizada através da técnica de marcadores INDELs de ancestralidade, utilizando um painel de 46 AIMs com o protocolo descrito por Pereira et al.(82). A genotipagem foi realizada por dois analistas independentes usando o GeneMapper Analysis Software v.4.1 (Applied Biosystems) e os resultados foram comparados para consistência. O software Structure v.2.3.3 foi utilizado para estimar a ancestralidade e o painel HGDP-CEHP subgrupo H952 foi utilizado como referência de populações ancestrais (83). Foram designadas três populações (K=3), sendo elas: europeia, africana e ameríndia.

3.4.2 Avaliação da retinopatia diabética

Os pacientes foram submetidos à fundoscopia realizada por oftalmologista treinado em cada centro. Todos os oftalmologistas receberam treinamento prévio

centralizado para padronização. O exame de fundo de olho foi realizado sob midríase (colírio de Tropicamida 1%), por oftalmoscopia indireta com oftalmoscópio Eyetec (Eyetec- São Carlos- SP, Brasil) e lente de 20 dioptrias (Volk Optical, Mentor, OH,EUA). Os pacientes foram classificados em: RD ausente, RD não proliferativa leve, moderada e severa, RD proliferativa e presença de edema macular, baseado no pior olho e conforme recomendação da Academia Americana de Oftalmologia (63).

4. RESULTADOS

4.1 HLA class II genotyping of admixed Brazilian patients with type 1 diabetes according to self-reported color/race in a nationwide study. (artigo publicado)

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HLA class II genotyping of admixed Brazilian patients with type 1 diabetes according to self-reported color/race in a nationwide study

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The HLA region is responsible for almost 50% of the genetic risk of type 1 diabetes (T1D). However, haplotypes and their effects on risk or protection vary among different ethnic groups, mainly in an admixed population. We aimed to evaluate the HLA class II genetic profile of Brazilian individuals with T1D and its relationship with self-reported color/race. This was a nationwide multicenter study conducted in 10 Brazilian cities. We included 1,019 T1D individuals and 5,116 controls matched for the region of birth and self-reported color/race. Control participants belonged to the bone marrow transplant donor registry of Brazil (REDOME). HLA-class II alleles (DRB1, DQA1, and DQB1) were genotyped using the SSO and NGS methods. The most frequent risk and protection haplotypes were *HLA~DRB1*03:01~DQA1*05:01g~DQB1*02:01* (OR 5.8, p < 0.00001) and *HLA~DRB1*07:01~DQA1*02:01~DQB1*02:02* (OR 0.54, p < 0.0001), respectively, regardless of self-reported color/race. Haplotypes *HLA~DRB1*03:01~DQA1*05:01g~DQB1*02:01* and *HLA~DRB1*04:02~DQA1*03:01g~DQB1*03:02* were more prevalent in the self-reported White group than in the Black group (p = 0.04 and p = 0.02, respectively). The frequency of haplotype HLA~*DRB1*09:01~DQA1*03:01g~DQB1*02:02* was higher in individuals self-reported as Black than White (p = < 0.00001). No difference between the Brazilian geographical regions was found. Individuals with T1D presented differences in frequencies of haplotypes within self-reported color/race, but the more prevalent haplotypes, regardless of self-reported color/race, were the ones described previously in Europeans. We hypothesize that, in the T1D population of Brazil, although highly admixed, the disease risk alleles come mostly from Europeans as a result of centuries of colonization and migration.

Type 1 diabetes (T1D) is a chronic polygenic disease that arises from the combination of multiple genetic and environmental factors[1]. The HLA region on chromosome 6p21 is known to be responsible for almost 50% of the genetic risk, and it has been associated with diabetes since the 1970s[2]. Even though HLA Class I genes and non-HLA genes also contribute to T1D risk, Class II alleles such as DR and DQ demonstrate the strongest associations with the disease[1,3].

Susceptibility to T1D is mainly associated with haplotype *DRB1*04~DQA1*03:01~DQB1*03:02*, followed by DRB1*03:01~*DQA1**05:01~*DQB1**02:01[3]. More than 90% of patients carry one of those two haplotypes, and 30% carry both of them, while the prevalence in the general population is 2%[4–6], including previous data from regional populations and familial studies from Brazil[7–9].

In recent years, several risk scores for the diagnosis and risk assessment of T1D have been proposed, using growing and extensive knowledge about T1D genetics[1]. Most of them are based on the presence of high-risk HLA alleles described in previous studies[10–12].

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The highest prevalence of T1D is observed in the European population[13], and most of the studies are concentrated on homogeneous populations[6,14–16]. However, previous data have shown that the frequency of HLA haplotypes, as well as their effects on T1D risk or protection, could vary among populations[17]. With the advancement of genetic risk scores for the diagnosis and prediction of T1D, it is critical to account for ethnic differences in the genetics of T1D that may impact clinical outcomes such as chronic complications. Haplotypes that denote risk for one population might have a phenotype of protection on another. For instance, the haplotype *DRB1*07:01~DQA1*03:01~DQB1*02:02* appears to be protective for the European population and denotes susceptibility for African Americans[18].

Brazil has a large multiethnic population as a result of centuries of miscegenation since Portuguese colonization in 1500. The Brazilian population is formed by basically three principal ancestry roots: European (EUR), sub-Saharan African (AFR), and Native American (NAM). The country was originally populated by NAM. With the colonization, and later de slavery traffic, the EUR (particularly Portuguese) and the AFR ancestries started the miscegenation of the population, spreading gradually to the internal part of the country, explaining the substantial Brazilian genetic variability[19–21].

There is a scarcity of data on the genetics of the T1D population in Brazil, characterized as highly admixed. In this study, the primary objective was to evaluate the HLA class II genetic profile of Brazilian individuals with T1D and its relationship with self-reported color/race (CRsr) in comparison to a sample of individuals without T1D that belonged to the bone marrow transplant donor's registry of Brazil (REDOME), matched by region of birth and self-reported color/race. Second, we aimed to analyze regional geographic differences in HLA class II risk distribution of individuals with T1D in Brazil, a country with continental proportions.

Research design and methods

Study design and population. This analysis derives from a nationwide multicenter cross-sectional study conducted between August 2011 and August 2014 in 14 public clinics located in 10 Brazilian cities. The methods have been described previously[22]. Briefly, subjects received health care from the National Brazilian Health Care System (SUS) and were included in the study if they had been diagnosed by the presence of typical clinical presentation of T1D, including variable degrees of hyperglycemia, weight loss, polyuria, polydipsia, polyphagia and the need for continuous insulin use since the diagnosis with at least six months of follow-up evaluations in each center. From the initial cohort of 1,760, we randomly selected 1,019 individuals by region of birth and CRsr. The comparison between the selected group and the initial group showed no differences regarding principal clinical and demographic variables (data not shown). The institutional ethics committee of Pedro Ernesto University Hospital (State University of Rio de Janeiro) and each center's local ethics committee approved the study. All participants or their representatives signed written informed consent for the study. A standardized questionnaire was also used during a clinical visit to evaluate clinical and demographic data such as gender, current age, birthplace, self-reported color/race, age at diagnosis and duration of diabetes.

We also included information on HLA typing, region of birth and CRsr from 5,116 REDOME entries matched for the region of birth and CRsr at a 5:1 ratio. Inclusion criteria as a donor at REDOME are 18 to 55 years of age, good health status, and no infection, hematological or immunological disease. Individuals who had a diagnosis of cancer or diabetes with the use of insulin or other injectable medication are also excluded from REDOME[23]. We provide a supplemental figure with a chart flow of the selection process of patients with and without T1D (Supplemental Fig. S1). Each center's local ethics committee approved the study. All participants or their representatives signed written informed consent for the study. A standardized questionnaire was also used during a clinical visit to evaluate clinical and demographic data such as gender, current age, birthplace, self-reported color/race, age at diagnosis, and duration of diabetes.

Self-reported color/race. Participants categorized themselves into one of the five CRsr groups used for the Brazilian population censuses: White ("branca"), Brown ("parda"), Black ("preta"), Asian (yellow "amarela"), and Indigenous ("indígena") as recommended by IBGE[24].

DNA extraction. Genomic DNA was extracted from peripheral blood with the commercial SP QIA Symphony Kit by automation with QIA Symphony equipment, following the manufacturer's instructions (Qiagen, USA).

HLA genotyping. HLA-class II alleles (*DRB1*, *DQA1*, and *DQB1*) from 1,019 individuals with T1D were genotyped. Genotyping was performed using PCR-RSSO (LabType SSO2B1 High resolution, One Lambda Inc., West Hills, USA) in 543 (53.3%) participants with T1D and 476 (46.7%) had their DNA typed by next-generation sequencing (NGS). Of those, 352 were amplified at loci *HLA-DRB1* and *HLA-DQB1* by long-range PCR using primers from the NGSgo.v2 (GenDx, Utrecht, the Netherlands) Library Preparation Kit and 124 with Holotype HLA Assay (Omixon Inc., Budapest, Hungary) for *HLA-DRB1*, *HLA-DQB1* and *HLA-DQA1*, according to the manufacturer's instructions. These primers cover exons 2, 3, and 4. *HLA-DQA1* allele was imputed in 31.5% of the samples from the group of T1D individuals (n = 321) using the linkage disequilibrium criteria, based on the results found by NGS.

The HLA genotyping results of the group of participants without T1D were obtained at high resolution in DRB1 and DQB1 loci in 2,201 REDOME entries. The class II alleles assigned in any loci with NMDP codes were defined based on Common and Well Documented, version 2.0 (n = 2,915). *HLA-DQA1* alleles were typed in all control samples with PCR-RSSO (LabType SSO2B1 High resolution, One Lambda Inc., West Hills, USA).

Ambiguous HLA class II alleles within the P or G group were designated by a lower case 'g' (*DRB1*12:01g* = 12:01/12:10; *DQA1*01:01g* = 01:01/01:04/01:05; *DQA1*03:01g* = 03:01/03:02/03:03; *DQA1*05:01g* = 05:05/05:09; *DQB1*03:01g* = 03:01/03:09/03:19). After validating the HLA dataset via an EM

	T1D Individuals (1019)	Individuals without T1D (5116)	p-value
Self-reported color/race			0.37
White	520 (51.0%)	2625 (51.3%)	
Brown	382 (37.5%)	1948 (38.1%)	
Black	94 (9.2%)	464 (9.1%)	
Asian	11 (1.1%)	48 (0.9%)	
Indigenous	12 (1.2%)	31 (0.6%)	
Region			0.99
Midwest	81 (7.9%)	397 (7.8%)	
Northeast	318 (31.2%)	1587 (31.0%)	
North	44 (4.3%)	223 (4.4%)	
Southeast	429 (42.1%)	2173 (42.5%)	
South	147 (14.4%)	736 (14.4%)	
Gender			<0.001
Female	563 (55.3%)	2304 (45%)	
Male	456 (44.7%)	2812 (55%)	
Age	30.2 ± 11.8	29.4 ± 8.8	0.02
Diabetes duration	15.3 ± 8.9		
HbA1c %	8.9 ± 2.4		
HbA1c mmol	74.7 ± 25.0		

Table 1. Population characteristics. Data are represented as number (%) or mean ± SD (standard deviation). T1D = type 1 diabetes.

algorithm for resolving allelic ambiguities and determining both allele and extended haplotype frequencies despite some missing loci data, this imputation was manually performed according to the haplotype results from Arlequin output data for both groups (individuals with and without T1D), according to race and region.

Three-locus haplotype frequencies (*DRB1~DQA1~DQB1*) were estimated for each of the races and regions for both groups (individuals with and without T1D), resolving phase and allelic ambiguity using the expectation-maximization (EM) algorithm[25,26]. Deviations from Hardy-Weinberg equilibrium (HWE) were assessed at the allele-family level (first nomenclature field) using a modified version of the Guo and Thompson algorithm[27] as implemented in Arlequin software v.3.5[28].

The most frequent haplotypes associated with risk for T1D (OR > 3.0) were compared among Brazilian regions.

Statistical analysis. Categorical variables such as self-reported color/race, geographical region of birth and gender were presented as frequencies (percentages). All normally distributed continuous variables, such as age, duration of diabetes, and HbA1c values, were given as the mean ± standard deviation (SD). We used chi-square and Fisher's tests to compare categorical data; Student's t-test and analysis of variance (ANOVA) were used for comparisons between groups with numeric variables when indicated. Samples were divided into two groups (individuals with and without T1D) for population comparison testing. Arlequin software was used to calculate FST genetic distance, and the exact test for population differentiation test results was performed via allele frequency extrapolations[28]. Tests were then repeated after dividing the two populations into smaller groups according to self-declared ethnicity (White, Black, and Brown, when n > 30) and region to detect potential ancestry or regional related biases.

Bonferroni correction was applied for multiple tests. We used the Statistical Program for Social Sciences version 17.0 (SPSS, Inc., Chicago, Illinois). A two-sided p-value of less than 0.05 was considered significant. Haplotype frequencies between cases and controls were compared using a Pearson chi-square test. Odds ratios (ORs) and 95% CIs were calculated.

Results

Population characteristics. Population characteristics are shown in Table 1. Half of the participants declared themselves as White. Individuals with T1D were older than healthy participants ($p = 0.02$). The group of individuals without diabetes had more male individuals than the group of individuals with T1D ($p < 0.001$).

Overview of the risk and protective alleles and/or haplotypes of the HLA system in individuals with and without T1D. The frequencies of the HLA-DRB1, HLA-DQB1, and HLA-DQA1 alleles are shown in Tables 2–4, respectively. The *HLA-DRB1* alleles associated with the risk of T1D were *DRB1*03* (OR 4.03, CI 3.60–4.51, $p < 0.0001$) and *DRB1*04* (OR 2.98, CI 2.66–3.33, $p < 0.0001$) and *DRB1*09* (OR 2.43, CI 1.84–3.19, $p < 0.001$). The most frequent protective *HLA-DRB1* alleles were *DRB1*13* (OR 0.56, CI 0.47–0.66, $p < 0.001$), *DRB1*07* (OR 0.49, CI 0.41–0.59, $p < 0.0001$) and *DRB1*11* (OR 0.26, CI 0.20–0.33, $p < 0.p < 0.001$). Only the *HLA-DQA1*03* allele conferred risk (OR 3.24, $p < 0.001$). The two *HLA-DQB1* alleles that were associated with the risk of T1D were *HLA-DQB1*02* (OR 2.93, CI 2.64–3.24, $p < 0.0001$) and *DQB1*03* (OR 1.38, CI

*DRB1**	Individuals without T1D N (%)	Individuals with T1D N (%)	OR	CI	P-value
01	1,012 (9.89%)	151 (7.41%)	0.73	0.61–0.87	0.000
03	1,014 (9.91%)	626 (30.72%)	4.03	3.60–4.51	0.000
04	1,283 (12.54%)	610 (29.93%)	2.98	2.66–3.33	0.000
07	1,321 (12.91%)	139 (6.82%)	0.49	0.41–0.59	0.000
08	644 (6.29%)	67 (3.29%)	0.51	0.39–0.65	0.000
09	163 (1.59%)	77 (3.78%)	2.43	1.84–3.19	0.000
10	202 (1.97%)	24 (1.18%)	0.59	0.39–0.91	0.015
11	1,211 (11.84%)	69 (3.39%)	0.26	0.20–0.33	0.000
12	162 (1.58%)	19 (0.93%)	0.58	0.36–0.94	0.026
13	1,397 (13.65%)	165 (8.10%)	0.56	0.47–0.66	0.000
14	415 (4.06%)	15 (0.74%)	0.17	0.10–0.29	0.000
15	1,027 (10.04%)	44 (2.16%)	0.2	0.15–0.27	0.000
16	381 (3.72%)	32 (1.57%)	0.41	0.28–0.59	0.000

Table 2. *HLA-DRB1* alleles distribution in individuals with type 1 diabetes and without T1D. T1D = type 1 diabetes mellitus; n = number of individuals; OR = odds ratio; CI = confidence interval; DRB1*01 included 01:01, 01:02, 01:03 and 01:28; DRB1*03 included 03:01, 03:02, 03:05, 03:07, 03:11, 03:12, 03:15, 03:37, 03:52 and 03:61; DRB1*04 included 04:01, 04:02, 04:03, 04:04, 04:05, 04:06, 04:07, 04:08, 04:09, 04:10, 04:11, 04:14, 04:29 and 04:50; DRB1*07 included 07:01, 07:11 and 07:15; DRB1*08 included 08:01, 08:02, 08:03, 08:04, 08:06, 08:07 and 08:10; DRB1*09 included 09:01 and 09:10; DRB1*10 included 10:01 and 10:03; DRB1*11 included 11:01, 11:02, 11:03, 11:04, 11:06, 11:11, 11:13, 11:18, 11:19, 11:34 and 11:37; DRB1*12 included 12:01, 12:01 G, 12:02, 12:05 and 12:38; DRB1*13 included 13:01, 13:02, 13:03, 13:04, 13:05, 13:11, 13:15, 13:23, 13:31, 13:40, 13:42, 13:49 and 13:56; DRB1*14 included 14:01, 14:02, 14:03, 14:04, 14:06, 14:07, 14:13, 14:17, 14:21, 14:54 and 14:81; DRB1*15 included 15:01, 15:02, 15:03, 15:18 and 15:20; DRB1*16 included 16:01 and 16:02.

*DQB1**	T1D Individuals N (%)	Individuals with T1D N (%)	OR	CI	P value
02	1,969 (19.24%)	837 (41.07%)	2.93	2.64–3.24	0.000
03	2,873 (28.08%)	715 (35.08%)	1.38	1.25–1.53	0.000
04	892 (8.72%)	64 (3.14%)	0.34	0.26–0.44	0.000
05	1,901 (18.58%)	243 (11.92%)	0.59	0.51–0.68	0.000
06	2,597 (25.38%)	179 (8.78%)	0.28	0.24–0.33	0.000

Table 3. *HLA-DQB1* alleles distribution in individuals with type 1 diabetes and without T1D. T1D = type 1 diabetes mellitus; n = number of individuals; OR = odds ratio; CI = confidence interval, DQB1*02 included 02:01, 02:02, 02:03; DQB1*03 included 03:01 G, 03:02, 03:03, 03:04, 03:05, 03:14, 03:34, 03:40/03:110/03:141/03:155, 03:10/03:139/03:186 and 03:41; DQB1*04included 04:01, 04:02 and 04:04; DQB1*05 included 05:01, 05:02, 05:03, 05:04, 05:05, 05:07, 05:11 and 05:47/05:165; DQB1*06 included 06:01, 06:02, 06:03, 06:04, 06:05, 06:08, 06:09, 06:10, 06:11, 06:19, 06:26 N, 06:27, 06:33, 06:38, 06:49 and 06:72.

*DQA1**	Individuals without T1D N (%)	Individuals with T1D N (%)	OR	CI	P-value
01	4,491 (43.89%)	415 (20.36%)	0.33	0.29–0.37	0.000
02:01	1,351 (13.20%)	134 (6.58%)	0.46	0.38–0.56	0.000
03:01 g	1,417 (13.85%)	698 (34.25%)	3.24	2.91–3.61	0.000
04	873 (8.53%)	72 (3.53%)	0.39	0.31–0.50	0.000
05	1,974 (19.29%)	718 (35.23%)	2.28	2.05–2.53	0.000
06:01	126 (1.23%)	1 (0.05%)	0.04	0.01–0.28	0.000

Table 4. HLA-DQA1 alleles distribution in patients with type 1 diabetes and without T1D. T1D = type 1 diabetes mellitus; n = number of individuals; OR = odds ratio; CI = confidence interval; DQA1*01 included 01:01 g, 01:02, 01:03, 01:07 and 01:13; DQA1*04 included 04:01, 04:02, 04:03 and 04:04; DQA1*05 included 05:01 g, 05:02, 05:03, 05:04, 05:08 and 05:10.

1.25–1.53, p < 0.001). *HLA-DQA1*03* (OR 3.24, CI 2.91–3.61, p < 0.0001) and *DQA1*05* (OR 2.28, CI 2.05–2.53, p < 0.0001) alleles conferred risk in our population of T1D.

Table 5 shows the frequencies of DRB1/DRB1 genotypes in both groups. The *HLA-DRB1*03/DRB1*04* genotype presented the highest risk (OR 12.1, CI 9.64–15.20, p < 0.0001) in 23.6% of the T1D participants, followed by

Genotypes	T1D Individuals with T1D N (%)	Individuals without T1D N (%)	OR	CI	p value
*DRB1*01/DRB1*XX*	95 (9.32%)	1862 (36.40%)	0.18	0.14–0.22	0.00000
*DRB1*03/DRB1*01*	40 (3.93%)	99 (1.94%)	2.07	1.42–3.01	0.00010
*DRB1*03/DRB1*03*	100 (9.81%)	52 (1.02%)	10.6	7.52–14.92	0.00000
*DRB1*03/DRB1*04*	240 (23.55%)	127 (2.48%)	12.1	9.64–15.20	0.00000
*DRB1*03/DRB1*09*	28 (2.75%)	16 (0.31%)	9.01	4.85–16.71	0.00000
*DRB1*03/DRB1*XX*	117 (11.48%)	668 (13.06%)	0.86	0.70–1.07	0.16924
*DRB1*04/DRB1*01*	47 (4.61%)	128 (2.50%)	1.88	1.34–2.65	0.00022
*DRB1*04/DRB1*04*	60 (5.89%)	82 (1.60%)	3.84	2.73–5.40	0.00000
*DRB1*04/DRB1*09*	13 (1.28%)	21 (0.41%)	3.13	1.56–6.28	0.00068
*DRB1*04/DRB1*XX*	191 (18.74%)	843 (16.48%)	1.17	0.98–1.39	0.07762
*DRB1*09/DRB1*09*	2 (0.20%)	0 (0.00%)			
*DRB1*09/DRB1*XX*	25 (2.45%)	121 (2.37%)	1.04	0.67–1.61	0.86596
*DRB1*XX/DRB1*XX*	61 (5.99%)	1109 (21.68%)	0.23	0.18–0.30	0.00000

Table 5. *HLA-DRB1/DRB1* genotypes distribution in individuals with type 1 diabetes and without T1D. T1D = type 1 diabetes mellitus; n = number of individuals; OR = odds ratio; CI = confidence interval; p required for significance after Bonferroni correction 0.004. DRB1*XX = any haplotype other than DRB1*03, DRB1*04 or DRB1*09.

*DRB1*03/DRB1*03* (OR 10.6, CI 7.54–14.92, $p < 0.0001$) in 9.8% and *DRB1*03/DRB1*09* (9.01, CI 4.85–16.71, $p < 0.0001$) in 2.7%.

Frequencies of the full haplotype (*DRB1~DQA1~DQB1*) for both groups are shown in Table 6. Considering a p-value of 0.0007 after Bonferroni correction, 21 of the 66 haplotypes showed a statistically significant association with T1D (positive or negative). The most frequent risk haplotypes found in our population were *DRB1*03:01~DQA1*05:01g~DQB1*02:01* (OR 5.8, CI 5.13–6.57, $p < 0.00001$), *DRB1*04:05~DQA1*03:01g~DQB1*03:02* (OR 5.34, CI 4.37–6.51, $p < 0.00001$), and *DRB1*04:02~DQA1*03:01g~DQB1*03:02* (OR 3.43, CI 2.74–4.31, $p < 0.00001$). The most prevalent protection haplotypes were *DRB1*07:01~DQA1*02:01~DQB1*02:02* (OR 0.54, CI 0.44–0.65, $p < 0.0001$), *DRB1*13:01~DQA1*01:03~DQB1*06:03* (OR 0.30, CI 0.22–0.42, $p < 0.00001$) and *DRB1*01:02~DQA1*01:01g~DQB1*05:01* (OR 0.45, CI 0.34–0.60, $p < 0.00001$). Table 6 shows haplotypes that were seen at least 18 times total in the T1D participants and the healthy control group. Other less frequent haplotypes were grouped as others.

HLA class II distribution by self-reported color/race. Figures 1 and 2 present a bar plot with the distribution of the most prevalent risk and protection alleles in the T1D group, respectively, by self-reported color/race. Tables with the haplotype frequencies in both groups stratified by CRsr (White, Black, Brown, Asian and Indigenous) appear in the supplemental material. *HLA-DRB1*03:01~DQA1*05:01~DQB1*02:01* was the most frequent risk haplotype in all self-reported color/race groups, and haplotype *HLA-DRB1*07:01~DQA1*02:01~DQB1*02:02* was the haplotype with the highest frequency of protection in all groups but did not show statistical significance in the Black, Asian and Indigenous groups. Haplotypes *HLA-DRB1*03:01~DQA1*05:01g~DQB1*02:01* and *-DRB1*04:02~DQA1*03:01g~DQB1*03:02* were significantly more prevalent in the self-declared White group than in the Black group ($p = 0.04$ and $p = 0.02$, respectively). Individuals self-reported as Black had a statistically higher prevalence of the haplotype *HLA-DRB1* 09:01~DQA1*03:01g~DQB1*02:02* compared to White and Brown groups ($p = <0.00001$ and $p = 0.008$, respectively). This haplotype presented a higher frequency in the Brown group than in the White group ($p = 0.001$). Figure 3 shows the distribution of the self-reported color/race for the most prevalent risk and protection alleles for all participants. Frequent haplotypes associated with T1D risk grouped by Brazilian regions are shown at Supplemental Table S6. No statistical difference was observed.

Discussion

In general, our results are in accordance with previous studies in the European population as well as with the last regional studies in Brazil. The most frequent haplotype in all CRsr groups and geographical regions was *HLA-DRB1*03:01~DQA1*05:01g~DQB1*02:01*, which is also the most prevalent risk haplotype described in European populations. This demonstrates that although highly admixed, the Brazilian population seems to have greater genetic influence from European populations. The miscegenation process in Brazil is relatively recent, beginning only 500 years ago with the entrance of the Portuguese colonizers (European ancestry). The native Brazilian population was originally formed by indigenous populations, who share some similar HLA alleles and haplotypes with Native Americans[29]. Almost two centuries later, with the beginning of slavery traffic, African ancestry began to contribute to the miscegenation of the Brazilian population. The roots of these three ancestries (European, Native Amerindian, and African) are the basis of our admixed population. Our colonization history described above might explain higher degrees of European ancestry in our population, as demonstrated in previous studies[19]. Although our study design cannot confirm the hypothesis that, in the highly admixed T1D Brazilian population, the disease risk alleles appear to come mostly from Europeans as a result of centuries of colonization and migration, data from two Brazilian previous studies showed that the incidence of T1D was greater in patients self-reported as White[30,31].

Haplotypes (*DRB1*~*DQA1*~*DQB1*)	T1D Individuals N (%)	Individuals without T1D N(%)	OR	CI	p value
01:01–01:01 g–05:01	75 (3.68%)	315 (3.08%)	1.2	0.93–1.55	0.16472
01:02–01:01 g–05:01	54 (2.65%)	578 (5.65%)	0.45	0.34–0.60	**0.00000**
01:03–01:01 g–05:01	4 (0.20%)	67 (0.66%)	0.3	0.11–0.82	0.00970
03:01–05:01 g–02:01	590 (28.95%)	671 (6.56%)	5.8	5.13–6.57	**0.00000**
03:02–04:01–04:02	10 (0.49%)	306 (2.99%)	0.16	0.08–0.30	**0.00000**
04:01–03:01 g–03:01 g	11 (0.54%)	74 (0.72%)	0.74	0.39–1.41	0.45982
04:01–03:01 g–03:02	102 (4.99%)	81 (0.79%)	6.6	4.91–8.87	**0.00000**
04:02–03:01 g–03:02	130 (6.39%)	199 (1.94%)	3.43	2.74–4.31	**0.00000**
04:03–03:01 g–03:02	7 (0.34%)	33 (0.32%)	1.06	0.47–2.41	0.83218
04:04–03:01 g–03:02	69 (3.39%)	100 (0.98%)	3.55	2.60–4.84	**0.00000**
04:04–03:01 g–04:02	1 (0.05%)	19 (0.18%)	0.26	0.03–1.97	0.23192
04:05–03:01 g–02:02	11 (0.52%)	17 (0.17%)	3.26	1.52–6.98	0.00352
04:05–03:01 g–03:02	205 (10.06%)	210 (2.05%)	5.34	4.37–6.51	**0.00000**
04:06–03:01 g–04:02	2 (0.10%)	36 (0.35%)	0.28	0.07–1.16	0.07690
04:07–01:03–06:03	0 (0.00%)	20 (0.20%)			
04:07–03:01 g–03:01 g	3 (0.15%)	58 (0.57%)	0.26	0.08–0.83	0.00924
04:07–03:01 g–03:02	9 (0.44%)	37 (0.36%)	1.22	0.59–2.54	0.55330
04:08–03:01 g–03:01 g	5 (0.25%)	20 (0.19%)	1.26	0.47–3.35	0.59360
04:11–03:01 g–03:02	14 (0.69%)	258 (2.52%)	0.27	0.16–0.46	**0.00000**
04:11–03:01 g–04:02	1 (0.05%)	21 (0.20%)	0.24	0.03–1.78	0.15815
07:01–02:01–02:02	125 (6.12%)	1112 (10.87%)	0.54	0.44–0.65	**0.00000**
07:01–02:01–03:03	4 (0.20%)	166 (1.63%)	0.12	0.04–0.32	**0.00000**
08:01–04:01–04:02	30 (1.47%)	171 (1.67%)	0.88	0.59–1.30	0.56217
08:02–04:01–04:02	6 (0.29%)	139 (1.36%)	0.21	0.09–0.49	**0.00004**
08:03–06:01–03:01 g	1 (0.05%)	68 (0.66%)	0.07	0.01–0.53	**0.00012**
08:04–04:01–03:01 g	9 (0.44%)	66 (0.64%)	0.68	0.34–1.37	0.35046
08:04–04:01–04:02	6 (0.29%)	84 (0.82%)	0.36	0.16–0.82	0.01335
08:07–04:01–04:02	4 (0.20%)	52 (0.51%)	0.38	0.14–1.07	0.06935
09:01–03:01 g–02:02	58 (2.86%)	61 (0.60%)	4.88	3.40–7.02	**0.00000**
09:01–03:01 g–03:03	11 (0.53%)	82 (0.80%)	0.67	0.36–1.26	0.26463
10:01–01:01 g–05:01	23 (1.13%)	189 (1.84%)	0.61	0.39–0.94	0.02799
11:01–01:02–05:02	1 (0.05%)	51 (0.50%)	0.1	0.01–0.71	0.00210
11:01–01:02–06:02	8 (0.39%)	327 (3.19%)	0.12	0.06–0.24	**0.00000**
11:01–01:02–06:11	0 (0.00%)	22 (0.22%)			
11:01–05:01 g–03:01 g	19 (0.93%)	324 (3.16%)	0.29	0.18–0.46	**0.00000**
11:01–05:10–03:01 g	0 (0.00%)	18 (0.18%)			
11:02–05:01 g–03:01 g	12 (0.59%)	150 (1.46%)	0.4	0.22–0.72	0.00155
11:02–05:10–03:01 g	1 (0.05%)	24 (0.23%)	0.21	0.03–1.54	0.10735
11:03–05:01 g–03:01 g	5 (0.25%)	50 (0.49%)	0.5	0.20–1.26	0.14891
11:03–05:10–03:01 g	0 (0.00%)	21 (0.20%)			
11:04–05:01 g–03:01 g	16 (0.79%)	119 (1.16%)	0.67	0.40–1.14	0.16619
12:01 g–01:01 g–05:01	4 (0.20%)	58 (0.57%)	0.34	0.12–0.95	0.02621
12:01 g–05:01 g–03:01 g	12 (0.59%)	35 (0.34%)	1.73	0.89–3.33	0.11396
12:02–01:02–06:02	0 (0.00%)	20 (0.20%)			
12:02–06:01–03:01 g	0 (0.00%)	35 (0.34%)			
13:01–01:02–05:01	6 (0.29%)	32 (0.31%)	0.94	0.39–2.25	0.99990
13:01–01:03–06:03	43 (2.11%)	676 (6.60%)	0.3	0.22–0.42	**0.00000**
13:01–03:01 g–03:03	2 (0.10%)	17 (0.17%)	0.59	0.14–2.56	0.75702
13:02–01:02–05:01	9 (0.44%)	36 (0.35%)	1.26	0.60–2.61	0.54621
13:02–01:02–05:02	3 (0.15%)	15 (0.15%)	1.00	0.29–3.47	0.99480
13:02–01:02–06:04	57 (2.80%)	248 (2.42%)	1.16	0.86–1.55	0.31273
13:02–01:02–06:09	15 (0.74%)	122 (1.19%)	0.61	0.36–1.05	0.08778
13:02–01:03–06:03	1 (0.05%)	34 (0.34%)	0.15	0.02–1.08	0.02212
13:03–02:01–02:02	0 (0.00%)	34 (0.33%)			
13:03–05:01 g–03:01 g	11 (0.54%)	81 (0.79%)	0.68	0.36–1.23	0.26351

Continued

Haplotypes (*DRB1~DQA1~DQB1*)	T1D Individuals N (%)	Individuals without T1D N(%)	OR	CI	p value
14:02–05:01 g–03:01 g	1 (0.05%)	227 (2.22%)	0.02	0.003–0.15	**0.00000**
14:04–01:01 g–05:03	4 (0.20%)	18 (0.18%)	1.12	0.38–3.30	0.77609
14:06–05:01 g–03:01 g	3 (0.15%)	43 (0.42%)	0.35	0.11–1.13	0.07289
14:54–01:01 g–05:03	1 (0.05%)	79 (0.77%)	0.06	0.009–0.45	**0.00001**
15:01–01:02–05:02	2 (0.10%)	18 (0.18%)	0.56	0.13–2.40	0.55981
15:01–01:02–06:02	16 (0.79%)	414 (4.04%)	0.19	0.11–0.31	**0.00000**
15:02–01:03–06:01	2 (0.10%)	45 (0.44%)	0.22	0.05–0.92	0.01755
15:03–01:02–06:02	18 (0.88%)	503 (4.92%)	0.17	0.11–0.28	**0.00000**
16:01–01:02–05:02	17 (0.83%)	212 (2.07%)	0.4	0.24–0.65	**0.00014**
16:02–01:02–05:02	6 (0.29%)	70 (0.68%)	0.43	0.19–0.99	0.04314
16:02–05:01 g–03:01 g	4 (0.20%)	84 (0.82%)	0.24	0.09–0.65	0.00213
Others	159 (7.80%)	660 (6.45%)	1.23	1.03–1.47	0.02560

Table 6. Distribution of the *HLA-DRB1~DQA1~DQB1* haplotypes in individuals with type 1 diabetes mellitus and without T1D. T1D = type 1 diabetes mellitus; n = number of individuals; OR = odds ratio; CI = confidence interval; sixty-eight haplotypes with total number in patients plus controls greater than 18 were included (0.3%). P required for statistical significance after Bonferroni correction for multiple tests <0.00074. Rare alleles were included in others.

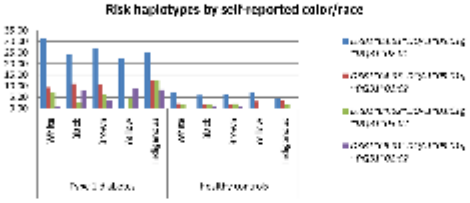

Figure 1. Most relevant risk haplotypes by self-reported color/race.

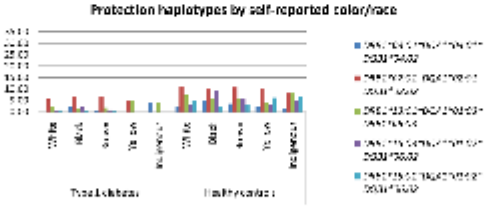

Figure 2. Most relevant protection haplotypes by self-reported color/race.

*DRB1*03* and *DRB1*04* alleles are known to be the most prevalent high-risk alleles between individuals with T1D, with individual allele frequencies varying between 20 and 30%[32]. The highest frequencies are shown in European populations, but they have also been described in African Americans[18]. In Brazil, the frequencies of those alleles are as high as 28%[7], similar to those found in our sample (28.9%). Up to 63.3% of our type 1 participants carry *DRB1*04* and/or *DRB1*03*, and 39.2% carry both (either in homozygosis or heterozygosis) compared to 5.1% of the control group. It is important to note that the most frequent haplotypes in our analysis were not always the ones with the most significant effect. For instance, although the *DRB1*03:01~DQA1*05:01 g~DQB1* 02:01* haplotype was the most frequent in individuals with T1D (28.9%), the haplotype with the largest effect was *DRB1*04:01~DQB1*03:01 g~DQA1*03:02* (OR 6.6, CI 4.91–8.87, p-value <0.000001).

The commonly described protection alleles are *DRB1*03:02, DRB1*07, DRB1*10, DRB1*11*, DRB1**13*, DRB1**14, and* DRB1**15*. Frequencies of those alleles vary among populations[1]. Haplotype *HLA-DRB1*07:01~D QA1*02:01~DQB1*02:02/03:03* was more prevalent in our control group, with a frequency up to 12.5% compared to 6.3% of the T1D group. This haplotype has been described as protective in previous studies in Brazil[32] as well as in European populations[33], but it was shown to be associated with risk in the African population[18]. The same situation occurred with *DRB1*13*. Although the Brazilian population originates mainly from three ancestral roots, with African being one of them, it has lower degrees of sub-Saharan African genomic ancestry than populations

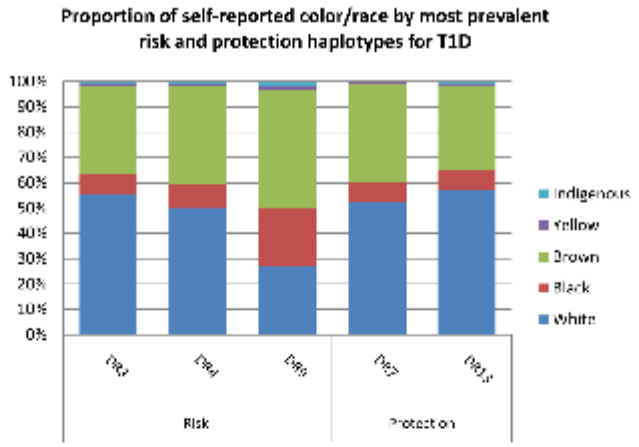

Figure 3. Proportion of self-reported color/race by most prevalent risk and protection haplotypes for T1D. DR3 = DRB1*03:01~DQA1*05:01 g~DQB1*02:01, DR4 = DRB1*04:05~DQA1*03:01 g~DQB1*03:02, DR9 = DRB1*09:01~DQA1*03:01 g~DQB1*02:02, DR7 = DRB2*07:01~DQA1*02:01~DQB1*02:02, DR13 = DRB1*13:01~DQA1*01:03~DQB1*06:03, T1D = Type 1 diabetes.

presented in the USA, as demonstrated in previous studies from our group[19]. It is important to highlight that up to 51% of our T1D population declared themselves White as opposed to only 9% reported as Black.

Genotype *DRB1*03/DRB1*04* presented the highest risk in our study, with an OR of 12.1 (CI 9.64–15.20, $p < 0.000001$), followed by *DRB1*03/DRB1*03* (OR 10.6, CI 7.52–14.92, $p < 0.00001$). The *DRB1*09* allele only presented risk when accompanied by one of the high-risk alleles (*DRB1*03* or *DRB1*04*), and this combination was present in 4% of the individuals with T1D. This result is similar to previous studies in Brazil[7]. A study with the African American population shows *DRB1*09* as a risk allele even when not associated with *DRB1*03* or *DRB1*04*[18]. This might be explained by the very low rates of Asian or African ancestry in our population, as discussed above and demonstrated in previous studies[19]. One possible conclusion is that in admixed populations, such as that in Brazil, the disease was brought over by populations of European ancestry, with a stronger presence of *DRB1*03* and *DRB1*04* among those self-declared as White and the presence of *DRB1*09* in those self-reported as Black. Nonetheless, although a frequency variation of the haplotype *DRB1*09:01~03:01 g~02:02* was found among the Brazilian regions within the T1D group (1.4% South vs. 7.8% Northeast), it did not present statistical difference.

It is also important to highlight the rates of homozygosity found in our T1D population, where 9.81% of the individuals with T1D were homozygous for *DRB1*03*, 5.89% for *DRB1*04* and only 0.2% for *DRB1*09*, similar to a previous study in Brazil[7]. Noble *et al.*'s study in African Americans found similar rates for the *DRB1*03* genotype but higher rates for *DRB1*09* homozygosity[18], probably due to the above-cited explanation with a population of higher degrees of African ancestry.

Although we found differences in gender proportions between groups, HLA risk assessment usually does not differ between males and females. One study in children at risk of T1D found an association between gender and HLA risk alleles *DRB1*03/DRB1*04* and islet autoimmunity[34]. This is probably not relevant in our population, as we included only individuals with T1D older than 13 years.

In our study, we analyzed only Class II HLA alleles. Although Class I alleles and non-HLA genes also contribute to T1D risk, Class II alleles such as DR and DQ demonstrate the strongest associations with the disease[1]. Recently, several risk scores for diagnosis and risk assessment of T1D have been proposed, and the vast majority of them are based on the presence of high-risk class II HLA alleles[10–12].

The present study is the first multicenter study in T1D including all five geographical regions of the country with a large multiethnic sample. Additionally, we had a large number of controls matched by region of birth and CRsr, adding strength to our results. Another strength is that we used a uniform, standardized recruitment protocol in all participating centers and the three genotyped loci *HLA-DRB1*, *-DQA1*, -and *-DQB1*). REDOME comprises HLA types from all regions and with representative entries of the distinct CRsr, and the allele frequency distributions vary both per region and CRsr[21]. To minimize these differences, a randomized selection included information available in the REDOME database in a pair-matched CSsr and region basis.

Our study has some limitations. First, autoantibodies and C peptide levels were not measured. The diagnosis of diabetes was made based on typical clinical presentation and the need for insulin since diagnosis. Although some individuals with other types of diabetes might have been included, it is important to emphasize that 96.5% of them were diagnosed before 30 years of age, which reinforces the high probability that they most likely have T1D. Second, although T1D participants were from urban areas, patients who receive primary attention care and live in rural areas represent the minority of patients with T1D under treatment in Brazil.

Conclusion

Regarding the most prevalent risk alleles, such as *DRB1*03* and *DRB1*04*, our findings are in accordance with previous studies both in European and admixed populations. It is important to note that the *DRB1*07* allele, which is usually protective only in European populations, was also protective in our population. Additionally, the *DRB1*09:01* allele conferred risk only when accompanied by a high-risk allele such as *DRB1*03* or *DRB1*04*. This could be explained by a characteristic of the Brazilian population, in that, although highly admixed, it has a greater contribution of the European ancestry, as demonstrated in previous studies. Therefore, we can conclude that in Brazil, the disease risk allele comes mostly from Europeans. Future studies are needed to better understand the genetics of T1D in admixed populations, especially regarding other genetic loci that might be associated.

Ethical standard. All procedures performed in the study were in accordance with the ethical standards of the institutional ethics committee of all centers as listed below and with the 1964 Helsinki declaration and its later amendments.

List of Ethics committee

Ethics Committee of Pedro Ernesto University Hospital
Ethics Committee of Clementino Fraga Filho University Hospital
Ethics Committee of Clinic's Hospital of São Paulo's University Medical School
Ethics Committee of Faculty of Medical Sciences of Campina's State University
Ethics Committee of Federal University of São Paulo
Ethics committee of the Municipal Health Department of Bauru
Ethics Human Research Committee of João de Barros Barreto University Hospital, Federal University of Pará
Research Ethics Committee of the Diabetes and Endocrinology Center of Bahia
Research Ethics committee of the Federal University of Ceará
Research Ethics committee of the Municipal Health Department of Distrito Federal
Research Ethics Committee of Clinic's Hospital of Porto Alegre
Ethics Human Research Committee of Federal University of Parana Clinic Hospital
Research Ethics Committee of Walter Cantídio University Hospital

Informed consent. Informed consent was obtained from all the study participants included in the study, which has done according to the national guidelines of ethical standards and in keeping with the Helsinki Declaration of 2008 (ICH GCP).

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Author contributions

D.C.S., L.C.P. and M.B.G. conceived and planned the study. R.V.O., D.S. and L.H. carried out the H.L.A. sequencing. L.H.M. contributed to sample preparation. D.C.S., L.C.P., M.B.G., M.H.P., B.S.V.B., L.G.N.M. and D.A.S. contributed to the analysis and interpretation of the results. D.C.S., L.C.P. and M.B.G. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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4.2 **HLA gene profile of patients with severe diabetic retinopathy in patients with type 1 diabetes from an admixed population: a nested case-control study in Brazil (artigo submetido)**

HLA gene profile of patients with severe diabetic retinopathy in patients with type 1 diabetes from an admixed population: a nested case-control study in Brazil.

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Abstract

Diabetic retinopathy (DR) is a common diabetes microvascular complication and the leading cause of blindness in the adult population. The study of its multifactorial pathogenesis and risk factors is crucial to achieving early diagnosis and high treatment efficacy. Although the well-stablish role of the HLA genes on the predisposition of type 1 diabetes, its contribution to the development and progression of diabetic retinopathy is still unclear, especially in admixed populations. We aimed to study the relationship between HLA alleles and severe diabetic retinopathy in a highly admixed population of type 1 diabetes patients.

Methods: This was a nested case-control study based on a cross-sectional, nationwide survey conducted in all 5 geographic regions of Brazil. We included 117 patients with severe diabetic retinopathy (severe non-proliferative or proliferative diabetic retinopathy) and 117 random controls composed of type 1 diabetes patients from the same cohort without retinopathy, matched for diabetes duration by a range of five years. HLA-class II alleles (DRB1, DQA1, and DQB1) were genotyped using the SSO and NGS methods.

Results: Haplotypes HLA-DRB1*04:05~DQA1*03:01g~DQB1*03:02 (OR 1.75, CI 0.97-3.16, p-value 0.058) and HLA-DRB1*13:02~DQA1*01:02~DQB1*06:04 (OR

5.18, CI 1.12-23.09, p-value 0.019) were more prevalent on the severe DR group but they did not present statistically difference after Bonferroni correction. The most frequent haplotype on both groups was HLA-DRB1*03:01~DQA1*05:01g~DQB1*02:01 (29.6% on severe DR and 33.33% on the control group).

Conclusions: Our study showed no influence of HLA alleles on the development of DR. Further longitudinal data is needed to better understand the role of genetic factors on this multifactorial significant microvascular complication.

Keywords: Type 1 diabetes; Retinopathy; HLA; Human leukocyte antigen

INTRODUCTION

Type 1 diabetes (T1D) is a chronic polygenic disease that arises from the combination of multiple genetic and environmental factors [1]. Almost 50% of the genetic risk is linked to the HLA region on chromosome 6p21, and it has been associated with the predisposition of diabetes since the 1970 decade [2].

A prevalence of 30% of microvascular complications is observed in T1D patients from different populations [3]. Diabetic retinopathy (DR) is one of the most frequent diabetes-related chronic complications that has an estimate global prevalence of 35 %, and is considered the leading cause of blindness in the adult population resulting in a significant social and financial burden [4]. The Diabetes Control and Complications Trial (DCCT) and Epidemiology of Diabetes Interventions and Complications (EDIC) studies [5] showed that adequate glycemic control is essential to avoid or postpone diabetes-related chronic complications, including DR.

Besides glycemic control and diabetes duration, HLA-DR4/ DR3 have been linked to the development and progression of DR with controversial results [6-12]. The WESDR cohort with T1D caucasian patients showed a positive association of HLA-DR4 with DR in its first cross-sectional analysis with no adjustment for disease duration [13]. In contrast, in its 14-years-follow-up report, no differences were found[6]. Another study also conducted in the caucasian population showed a protective effect of HLA-DR3 and a risk effect of HLA-DR4 in familial-base linkage analysis[14].

The controversial results found in previous studies could be even more controversial in a highly admixed population, such as the Brazilian. Previuos HLA study in Brazilian type 1 diabetes patients showed similar haplotypes frequencies for

DR3 and DR4 as the ones described in the Caucasian population. However, it also demonstrated the DR9 haplotype as conferring susceptibility which is mostly expressed in the African-American population [15]. The heterogeneous Brazilian population is composed of a highly admixed combination of three principal ancestral roots: Native Amerindians (NAM), Europeans (EUR), and Africans (AFR). The process of miscegenation initiated with the European colonization (particularly Portuguese) by the coast, spreading gradually to the internal part of the country that was initially populated by NAM. Afterward, with the heavy slavery traffic (particularly from Africa), even more migration and miscegenation have occurred, explaining the substantial Brazilian genetic variability.

We aimed to study the relationship between HLA alleles and severe diabetic retinopathy in a highly admixed population of type 1 diabetes patients.

METHODS AND SUBJECTS

Study design and population

This was a nested case-control study based on a cross-sectional, nationwide survey conducted in all 5 geographic regions of Brazil between August 2011 and August 2014. The original study included 1,760 patients with T1D, and the methods have been described previously [16]. Briefly, all patients received health care from the Brazilian National Health Care System (SUS). The diagnosis of type 1 diabetes was made based on the presence of typical clinical presentation (hyperglycemia, weight loss, polyuria, polydipsia, polyphagia, and the need for continuous insulin use since the diagnosis). Inclusion criteria were: at least 13 years of age and minimum follow-up at each center of at least six months.

For the present study, we included 117 cases, defined as patients with severe diabetic retinopathy (severe non-proliferative or proliferative diabetic retinopathy) and 117 random controls defined as patients from the same cohort without retinopathy, matched for diabetes duration by a range of five years. The Ethics Committee of the University Hospital of Pedro Ernesto approved the study. Written informed consent was obtained from all participants or their representatives.

Participants responded to a standardized questionnaire during a clinical visit to evaluate clinical and demographic data such as current age, age at diagnosis, gender, duration of diabetes, years of formal education and socio-economic status, and self-reported color/race. They were also submitted to clinical evaluation to assess the presence of complications and measurement of clinical data. Hypertension was

considered if self-reported or in the presence of a previous diagnosis made by a health practitioner on at least two separate occasions. Glomerular Filtration Rate (GFR) was estimated by the CKD-EPI equation [17] in adults and by the Schwartz formula [18] in adolescents and was expressed in milliliters per minute per 1.73m2 (ml/min). Blood sampling was collected for determination of HbA1c levels (by high-performance liquid chromatography-HPLC, Bio-Rad Laboratories, Hercules, California, USA) and for genetic analysis.

Evaluation of diabetic retinopathy

Mydriatic binocular indirect ophthalmoscopy with an Eyetec Ophthalmoscope (Eyetec, São Carlos-SP,Brazil) and a 20-diopter lens (Volk Optical, Mentor, OH, USA) was performed by an experienced retinal specialist in each center. Patients had both eyes examined. Mydriasis was obtained with 1% tropicamide drops. All ophthalmologists who performed the fundoscopy were trained by the same retinal specialist and followed the same protocol. For patient classification, we considered the eye with the most severe classification of DR (absent, mild non-proliferative, moderate non-proliferative, severe non-proliferative and proliferative DR according to the American Academy of Ophthalmology guidelines [19]).

DNA extraction and HLA genotyping

Genomic DNA was extracted from peripheral blood with the commercial kit SP QIA symphony by automation with QIA symphony equipment, following manufacturer's instructions (Qiagen,USA).

HLA-class II alleles (DRB1, DQA1, and DQB1) were genotyped either by Next Generation Sequencing (NGS) or Medium to high-resolution PCR-RSSO (LabType SSO, One lambda Inc. West Hills, USA) method. We used the Common and Well Documented, version 2.0 (CWD2) to assign ambiguities in DRB1 and DQB1 alleles. Three-locus haplotype frequencies (DRB1~DQA1~DQB1) were estimated using the expectation-maximization (EM) algorithm [20,21]. Deviations from Hardy-Weinberg equilibrium (HWE) were assessed at the allele-family level (first nomenclature field) using a modified version of the Guo and Thompson algorithm [22] as implemented in the software Arlequin v.3.5 [23].

Ambiguous HLA class II alleles within G group (i.e. groups of alleles that have identical nucleotide sequences across the exons encoding the peptide binding domains) were designated by a lower case `g` (DRB1*12:01g = 12:01/12:10; DQA1*01:01g = 01:01/01:04/01:05; DQA1*03:01g = 03:01/03:02/03:03; DQA1*05:01g = 05:05/05:09; DQB1*03:01g =.03:01/03:09/03:19).

Statistical Analysis

Categorical variables were presented as frequency (percentage). All normally distributed values were given as the mean ± standard deviation (SD), and all other values were given as the median (IQR). We used Chi-squared and Fisher's tests to compare categorical data; the Student t-test and analysis of variance (ANOVA) were used for comparisons between groups with numeric variables when indicated. Samples were divided into two groups (patients with T1D and severe DR and patients with T1D without DR) for population comparison testing. Arlequin software was used to calculate FST genetic distance, and the exact test for population differentiation test results was performed via allele frequencies extrapolations [23]. Bonferroni correction was applied for multiple tests. We used the Statistical Program for Social Sciences version 17.0 (SPSS, Inc., Chicago, Illinois). A two-sided p-value of less than 0.05 was considered significant. Haplotype frequencies in cases and controls were compared using a Pearson x2 test. Odds ratios (ORs) and 95% CIs were computed.

RESULTS

Population characteristics

Table 1 shows the study population characteristics. Patients with severe DR were older, predominantly male gender, with lower GFR, higher levels of HbA1c, and were more prone to hypertension than patients without DR.

Overview of the risk and protective alleles and/or haplotypes of the HLA system in patients with severe DR and controls

Allele frequencies and distribution between groups are described in table 2. HLA-DRB*13:02 and DRB1*04:05 were more frequent in patients with severe DR. HLA-DRB1*03:01 and DRB1*07:01 had higher frequencies in the group without DR. DRB1*13:02 showed a tendency to a higher risk which was no longer statistically significant after Bonferroni correction for multiple tests. None of the HLA-DRB1, DQA1 and DQB1 alleles showed statistically significant differences between groups.

Table 3 shows the distribution of the HLA-DRB1~DQA1~DQB1 haplotypes in patients with severe DR and patients without DR. The most frequent haplotype on both groups was HLA-DRB1*03:01~DQA1*05:01g~DQB1*02:01 (29.6% on severe DR and 33.33% on patients without DR group). Haplotypes HLA-DRB1*04:05~DQA1*03:01g~DQB1*03:02 (OR 1.75, CI 0.97-3.16, p-value 0.058) and HLA-DRB1*13:02~DQA1*01:02~DQB1*06:04 (OR 5.18, CI 1.12-23.09, p-value

0.019) were more prevalent on the severe DR group but they did not present statistically difference after Bonferroni correction.

HLA DR/DR genotypes are demonstrated in table 4. The most frequent genotype on both groups was DR3/DR4 (26.5% on each group). The DR13/X was more prevalent in the severe DR group (OR 5.37, CI 1.15-25.09, p-value 0.019). Although DR9/X and DR3/X were more frequent in the group of patients without DR, this difference was not statistically significant.

DISCUSSION

Our study, conducted in a highly admixed population with T1D, showed no influence of HLA alleles on the development of DR. All the possible risk associations such as those with the HLA-DR13 allele and with the haplotype HLA-DRB1*13:02~DQA1*01:02~DQB1*06:04 as well the protection association such as that with the haplotype HLA-DRB1*04:01~DQA1*03:01g~DQB1*03:02 were no longer significant after Bonferroni correction. Independent of the retinopathy status, HLA DR3 and DR4 were the most prevalent alleles.

The association between the HLA region and diabetes has been studied for decades [24-27]. Although the well-established role in the predisposition to the development of the disease, its association with the development of diabetic microvascular complications, especially retinopathy, demonstrated to be controversial [6-9,12-14,28]. Several studies from different populations, including Brazilians, showed that DR3 and DR4 are the most frequent alleles in patients with type 1 diabetes [15,27]. This also reflects in its higher prevalence when studying subsets of patients with diabetic retinopathy. Our study, as well as other studies in diabetic retinopathy, demonstrated the HLA-DR3 and DR4 alleles as the most prevalent. Although some studies found the HLA-DR4 allele to be associated with the risk of developing DR and the HLA-DR3 allele to be related to protection[6], others found no association when comparing patients with severe DR and patients without severe DR [11-13]. Our study found no association between groups after Bonferroni corrections. It is important to note that the majority of the previous studies did not adjust for critical classic factors associated with DR, such as duration of diabetes and were conducted in Caucasian populations.

Duration of diabetes is a critical factor in the development of DR, as demonstrated in previous data from several studies including a survey from our group [29]. It is hypothesized that HLA-related influence on DR might be significant only in patients that develop DR in the early course of T1D [12]. In our study, as we matched patients and controls by the duration of diabetes in a 5-years range, therefore, excluding

this potential confounder from our analysis, this time-influenced association might be diminished. However, this hypothesis is yet to be proved by further extensive longitudinal data.

In addition, the lack of association of the HLA genes and the development of DR might relay on the basis of the pathogenesis of DR. The pathogenesis of DR is multifactorial and still not yet completely understood. One well-established mechanism is the endothelial lesion mediated by hyperglycemia or hypoxia. High glucose levels induce oxidative stress mediated by the reduced effect of NO and activation of macrophages and the productions of inflammatory cytokines [30]. The TNF-α has its gene located at the same region of HLA and has been implicated in the pathogenesis of DR [31], but genetic studies of its relation with DR predisposition are yet to be proved [32,33]. Different genes have been studied in the search of the genetic predisposition of DR but the only ones that showed relevant results were the polymorphisms of aldose reductase (AKR1B1) and VEGF genes [33].Recent studies have demonstrated neurodegeneration as an early factor in the pathogenesis of DR and some other studies have concentrated on the role of epigenetics, especially in mitochondrial DNA [34]. Despite several treatment options for severe DR, some patients do not achieve a satisfactory response to treatment. Therefore, further research is needed to better elucidate novel mechanisms related to this multifactorial disease.

Our study was strengths and limitations. One particular strength is the population-based ascertainment of diabetes cases in a highly admixed population. Also, we adjusted for the duration of diabetes, one of the most important risk factor for DR, at selection, matching controls by range of five years. Our study was based only in patients assisted by the public health system in urban areas of the country. Although this could have led to some selection bias, in Brazil, the majority of T1D patients are treated on the public health system or on both public and private health care. Another limitation is that autoantibodies and C-peptide levels were not measured. This bias of misdiagnosis might be mitigated by the fact that 96.5% of the patients included were diagnosed before 30 years of age. In addition, the diagnosis of DR was assessed by binocular indirect ophthalmoscopy for all participants. Despite the inherent limitations of this method, a previous study from our group showed a substantial agreement between binocular indirect ophthalmoscopy and digital retinography [35].

In conclusion, our study, performed in a highly admixed population comparing patients with severe DR to T1D without DR matched for diabetes duration, found no

association between HLA Class II allele and severe DR. Further longitudinal data is needed to better understand the role of genetic factors on this multifactorial significant microvascular complication.

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Tables

Table 1. Characteristics of the study participants.

Variable	Patients with severe DR, *n* = 117	Patients without DR, *n* = 117	*P* value

Sociodemographic			
Gender, N (%)			**0.01**
Male	57 (48.7)	38 (32.5)	
Female	60 (51.3)	79 (67.5)	
Age, y Mean (SD)	38.8 (11.68)	34.56 (12.32)	**0.007**
Economic status, N (%)			0.32
High	4 (3.4)	7 (6)	
Medium	52 (44.4)	49 (41.9)	
Low	52 (44.4)	58 (49.6)	
Very low	9 (7.7)	3 (2.6)	
Years of schooling, y Mean (SD)	11.98 (3.99)	12.5 (3.68)	0.3
Diabetes-related variables			
HbA1c (%), Mean (SD)	8.89 (1.87)	8.40 (1.74)	**0.043**
HbA1c mmol/mol, Mean (SD)	73.64 (20.41)	68.38 (19.01)	**0.043**
Duration of diabetes, y Mean (SD)	23.42 (9.28)	22.18 (8.32)	0.28
GFR	66.62 (24.35)	83.04 (25.6)	**<0.001**
Arterial hypertension, y, n (%)	50 (42.7)	21 (18.1)	**<0.001**
Self-reported ethnicity, N (%)			0.24
Caucasian	62 (53.0)	65 (55.6)	
Black	15 (12.8)	9 (7.7)	
Mullatos	36 (30.8)	41 (35)	
Native Americans	4 (3.4)	1 (0.9)	
Yellow	0	1 (0.9)	

y = years, data are present as number (percentage), mean ± SD (standard deviation) or median (IQR or range).

Table 2. HLA-DRB1, DQB1, and DQA1 alleles distribution in patients with severe DR and patients without DR.

	Patients with severe DR		Patients without DR				
	N	%	N	%	OR	CI	P VALUE
HLA-DRB1							
01:01	6	2.56%	8	3.42%	0.74	0.25-2.18	0.59
01:02	5	2.14%	6	2.56%	0.83	0.25-2.76	0.76
03:01	68	29.06%	80	34.19%	0.79	0.53-1.16	0.23
04:01	8	3.42%	15	6.41%	0.52	0.21-1.24	0.13
04:02	13	5.56%	14	5.98%	0.92	0.42-2.01	0.84
04:04	8	3.42%	10	4.27%	0.79	0.31-2.05	0.63
04:05	35	14.96%	24	10.26%	1.54	0.88-2.68	0.13
07:01	18	7.69%	23	9.83%	0.76	0.40-1.46	0.41
09:01	4	1.71%	10	4.27%	0.39	0.12-1.26	0.1
13:02	15	6.41%	4	1.71%	3.94	1.29-12.05	0.01
Others	54	23.08%	40	17.09%	1.45	0.92-2.30	0.11

HLA-DQA1							
01:01g	17	7.26%	21	8.97%	0.79	0.41-1.55	0.49
01:02	24	10.26%	15	6.41%	1.67	0.85-3.27	0.13
01:03	5	2.14%	3	1.28%	1.68	0.40-7.12	0.48
02:01	17	7.26%	22	9.40%	0.75	0.39-1.46	0.4
03:01g	74	31.62%	77	32.91%	0.94	0.64-1.39	0.77
04:01	12	5.13%	6	2.56%	2.05	0.76-5.57	0.14
04:02	1	0.43%	0	0.00%			NA
05:01g	82	35.04%	90	38.46%	0.86	0.59-1.26	0.44
05:10	2	0.85%	0	0.00%			NA
HLA-DQB1							
02:01	71	30.34%	81	34.62%	0.82	0.56-1.21	0.32
02:02	23	9.83%	35	14.96%	0.62	0.35-1.09	0.09
03:01g	22	9.40%	11	4.70%	2.10	1.0-4.44	0.047
03:02	60	25.64%	58	24.79%	1.05	0.69-1.59	0.83
04:02	11	4.70%	5	2.14%	2.26	0.77-6.61	0.13
05:01	17	7.26%	20	8.55%	0.84	0.43-1.64	0.61
06:04	10	4.27%	4	1.71%	2.57	0.79-8.30	0.1
Others	20	8.55%	20	8.55%	1.00	0.52-1.91	1

N = number of individuals; OR = odds ratio; CI = confidence interval; Rare alleles were included in others

Table 3. Distribution of the HLA -DRB1~DQA1~DQB1 haplotypes in patients with severe DR and patients without DR.

	Patients with severe DR		Patients without DR				
Haplotype	N	%	N	%	OR	CI	P VALUE
03:01~05:01g~02:01	68	29.06%	78	33.33%	0.82	0.55-1.21	0.32
04:05~03:01g~03:02	33	14.10%	20	8.55%	1.75	0.97-3.16	0.06
07:01~02:01~02:02	16	6.84%	21	8.97%	0.74	0.38-1.46	0.39
04:02~03:01g~03:02	13	5.56%	13	5.56%	1.00	0.45-2.21	1
04:01~03:01g~03:02	5	2.14%	13	5.56%	0.37	0.13-1.06	0.05
04:04~03:01g~03:02	7	2.99%	9	3.85%	0.77	0.28-2.11	0.61
09:01~03:01g~02:02	4	1.71%	8	3.42%	0.49	0.15-1.65	0.24
13:02~01:02~06:04	10	4.27%	2	0.85%	5.18	1.12-23.90	0.02
01:01~01:01g~05:01	5	2.14%	6	2.56%	0.83	0.25-2.76	0.76
Others	73	31.20%	64	27.35%	1.2	0.81-1.80	0.36

N = number of individuals; OR = odds ratio; CI = confidence interval; Rare alleles were included in others

Table 4. HLA-DRB1/DRB1 genotypes distribution in patients with severe DR and patients without DR.

	Patients with severe DR		Patients without DR				
DR/DR	n	frequency	n	frequency	OR	CI	P VALUE
DR3/DR3	13	11.11%	15	12.82%	0.85	0.38-1.87	0.69
DR3/DR4	31	26.50%	31	26.50%	1.00	0.56-1.79	1.00
DR3/X	10	8.55%	16	13.68%	0.59	0.27-1.36	0.21
DR4/DR4	6	5.13%	5	4.27%	1.21	0.36-4.09	0.76
DR4/X	20	17.09%	20	17.09%	1.00	0.51-1.97	1.00
DR9/DR3	3	2.56%	4	3.42%	0.74	0.16-3.40	0.70
DR9/DR4	0	0.00%	1	0.85%			NA
DR9/DR9	0	0.00%	0	0.00%			NA
DR9/X	1	0.85%	5	4.27%	0.19	0.02-1.68	0.10
DR13/13	0	0.00%	0	0.00%			NA
DR13/DR3	3	2.56%	1	0.85%	3.05	0.31-29.78	0.31
DR13/DR4	7	5.98%	5	4.27%	1.42	0.44-4.63	0.55
DR13/X	10	8.55%	2	1.71%	5.37	1.15-25.09	0.02
DRX/DRX	13	11.11%	12	10.26%	1.09	0.48-2.51	0.83

DR =Diabetic Retinopathy; N = number of individuals; OR = odds ratio; CI = confidence interval; p required for significance after Bonferroni correction .004 DRX= any haplotype other than DR3, DR4, DR9 or DR13.

4.3 Genomic ancestry as a risk factor for diabetic retinopathy in patients with type 1 diabetes from an admixed population: a nested case-control study in Brazil. (artigo publicado)

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ORIGINAL ARTICLE

Genomic ancestry as a risk factor for diabetic retinopathy in patients with type 1 diabetes from an admixed population: a nested case–control study in Brazil

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Abstract

Aims The influence of genetic factors on the development and progression of diabetic retinopathy is still unclear. Previous studies showed controversial results. We aimed to characterize the relationship between genomic ancestry and self-reported color/race with severe diabetic retinopathy in patients with type 1 diabetes belonging to a highly admixed population.

Methods This study was a nested case–control based on data collected from a large cross-sectional, nationwide survey conducted in clinics from all five geographic regions of Brazil. For the present study, we included 414 individuals. Cases (n = 176) were considered if they had severe non-proliferative or proliferative diabetic retinopathy, and controls (n = 238) were type 1 diabetes patients without retinopathy, matched for diabetes duration by a range of 5 years. Indirect ophthalmoscopy was performed, and individual genomic ancestry was inferred using a panel of 46 ancestry informative markers.

Results The backward stepwise logistic regression analysis showed that African genomic ancestry (OR 3.9, p = 0.045), HbA1c (OR 1.24, p = 0.001), glomerular filtration rate (OR 0.98, p < 0.001) and hypertension (OR 2.52, p < 0.001) were associated with severe diabetic retinopathy after adjusting for clinical and demographic data. Self-reported color/race was not statistically associated with diabetic retinopathy.

Conclusions Genomic ancestry, as well as clinical variables such as hypertension, impaired glomerular filtration rate and poor diabetes control (HbA1c), was important risk factor for the development of severe diabetic retinopathy. Further studies are needed, especially in highly admixed populations, to better understand the role of genomic ancestry and possible genes that might be associated with the development and/or progression of diabetic retinopathy.

Keywords Type 1 diabetes · Genomic ancestry · Ethnicity · Retinopathy

Introduction

Type 1 diabetes (T1D) is a chronic disease with a worldwide increasing incidence that can lead to chronic complications which result in high direct and indirect costs for the health-care systems [1]. One of the most frequent diabetes-related chronic complications is diabetic retinopathy (DR) which is considered the leading cause of blindness in the adult population, representing a significant social and financial burden [2]. Obtaining and keeping adequate glycemic control is fundamental to avoid or postpone diabetes-related chronic complications, including DR as demonstrated by the Diabetes Control and Complications Trial (DCCT) and Epidemiology of Diabetes Interventions and Complications (EDIC) studies [3]. Besides glycemic control and diabetes duration, other factors such as socioeconomic status, ethnicity, hypertension and genetic factors could influence the development of DR [4, 5]. The influence of ethnicity on the development and progression of DR is still unclear. So far, most studies have been performed in patients with type 2 diabetes (T2D) in homogeneous populations [6, 7] or formed

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mostly by Caucasian patients [8, 9]. In general, these studies have used self-reported color/race instead of genomic ancestry (GA) to stratify participants according to ethnicity, which can result in some biases. Recently, two studies using GA to evaluate ethnicity were performed in Latino [10] and African-American patients with T2D [11], both populations with low to medium admixture rate [12]. These studies showed controversial results. Only the one performed with Latino patients showed an association between Native American ancestry and DR after adjusting for traditional risk factors for DR [10]. Other studies conducted in multiethnic cohorts comprising white, Hispanic, Black, South-Asians and Chinese individuals mostly with T2D in the USA [13], UK [9] and Singapore [14] using self-reported color/race, showed that ethnicity was not an independent predictor of any type of retinopathy [13], but it was a predictor of visual impairment in the UK (South-Asians and Blacks) [9] and in Singapore (Indians) [14]. The controversial results found in previous studies using either GA or self-reported color/race could be even more controversial in a highly admixed population such as the Brazilian [15]. However, it is important to emphasize that so far there is no cutoff point for GA to define either a non-admixed or an admixed population [10, 11].

Brazil has a heterogeneous population, formed basically by three ancestral roots that are highly admixed: native Amerindians (NAM), Europeans (EUR) and Africans (AFR). The colonization started by the coast, with Europeans (particularly Portuguese) and Africans, spreading gradually to the internal part of the country that was originally populated by NAM. Afterward, migration and miscegenation have occurred, explaining the strong Brazilian's genetic variability. Since 1991, self-reported color/race has been used for Brazilian population censuses and is divided into five color/race groups: White, Black, Brown (*parda*), Asian (*amarela*) and Indigenous (*indígena*) [15].

The development of methodologies on the inference of genomic ancestry, like AIMS, can minimize the risk of misclassification on population studies by providing more knowledge on genes' structure and diversity in highly admixed populations such as the Brazilian [16, 17].

We aimed to characterize the relationship between GA, self-reported color/race and DR in addition to other traditional risk factors for this chronic complication in patients with T1D belonging to a highly admixed Brazilian population.

Methods and subjects

This was a nested case–control study, using data collected from a cross-sectional, nationwide study, conducted between August 2011 and August 2014 in 14 public clinics located in 10 Brazilian cities, from all five geographic regions (North, Northeast, Midwest, Southeast and South). The original study included 1,760 patients with T1D, and the methods have been described in detail previously [18]. Briefly, T1D individuals received health care from Brazilian National Health Care System and have been diagnosed by the presence of typical clinical presentation of T1D, including variable degrees of hyperglycemia, weight loss, polyuria, polydipsia, polyphagia and the need of continuous insulin use since the diagnosis. Included individuals had at least 13 years of age and were followed at each diabetes center for at least six months. Each clinic provided data from 50 consecutive or more individuals with T1D who regularly attended this center.

This study was approved by the Ethics Committee of Pedro Ernesto University Hospital (Rio de Janeiro State University) and by the local ethics committee of each center. Written informed consent was obtained from all participants or their parents.

A standardized questionnaire was applied during a clinical visit to evaluate clinical and demographic data such as gender, current age, birthplace, self-reported color/race, age at diagnosis, duration of diabetes, years of formal education and socioeconomic status. Hypertension was considered if self-reported or in the presence of a previous diagnosis made by a health practitioner on at least two separate occasions. Blood sampling was collected for determination of HbA1c levels (by high-performance liquid chromatography (HPLC), Bio-Rad Laboratories, Hercules, California, USA) and for genetic analysis. Glomerular filtration rate (GFR) was estimated by the CKD-EPI equation [19] in adults and by the Schwartz formula [20] in adolescents and was expressed in milliliters per minute per 1.73 m^2 (ml/min).

Sample calculation

The sample size of the present study was based on the Brazilian Multicenter Type 1 Diabetes Study, described elsewhere [18]. The number of patients needed to be enrolled in each region was calculated based on the estimated prevalence of T1D and DR in Brazil [21] combined with the overall population density of each geographic region reported by the 2010 Brazilian Institute of Geography and Statistics Census (IBGE) [15]. Economic status was defined according to the Brazilian Economic Classification Criteria [22]. The following economic status categories were considered for this analysis: high, middle, low and very low.

Evaluation of diabetic retinopathy

Each patient had both eyes examined and underwent mydriatic binocular indirect ophthalmoscopy with an Eyetec Ophthalmoscope (Eyetec, São Carlos-SP, Brazil) and a 20-diopter lens (Volk Optical, Mentor, OH, USA) by an experienced

retinal specialist in each center. Mydriasis was obtained with 1% tropicamide drops. All ophthalmologists who performed the fundoscopy were trained by the same retinal specialist and followed the same protocol. Each eye was classified as absent, mild non-proliferative, moderate non-proliferative, severe non-proliferative and proliferative DR according to the American Academy of Ophthalmology guidelines [23]. For patient classification, we considered the eye with the most severe classification of DR.

For the present study, we included 414 patients. Cases ($n=176$) were considered if they had severe non-proliferative or proliferative DR, and controls ($n=238$) were patients from the same cohort where retinopathy was absent and matched for diabetes duration by range of 5 years.

DNA extraction and AIM-Indel genotyping

Genomic DNA was extracted from peripheral blood with the commercial kit SP QIA symphony by automation with QIA symphony equipment, following the manufacturer's instructions (Qiagen, USA).

The global and individual GA was inferred using a panel of 46 AIM-INDEL, with a protocol described by Pereira et al. [24]. Genotyping was carried out independently by two analysts using GeneMapper analysis software v.4.1 (Applied Biosystems), and results were compared for consistency. We used the Structure V.2.3.3 software to estimate ancestry and the HGDP-CEHP diversity panel (Sub-Set H952) as reference date of ancestral populations [25]. Structure ran with 100,000 burning steps followed by 100,000 Markov chain Monte Carlo (MCMC) interactions using the "Admixture model" default correlating allele frequencies and the number of populations ($K=3$), designated as European (EUR), African (AFR) and native Amerindians (NAM).

Statistical analysis

Categorical variables were presented as frequency (percentage). All normally distributed values were given as the mean ± standard deviation (SD), and all other values were given as the median and interquartile range (IQR). Chi-squared and Fisher's tests were used to compare categorical data; the Student's t test and analysis of variance (ANOVA) were used for comparisons between groups with numeric variables when indicated. We performed two models of binomial backward stepwise logistic regression analysis with the presence of severe DR (proliferative and severe non-proliferative) as the dependent variable. The first one included AFR GA among the independent variables, and the second included self-reported color/race. They were studied separately due to the presence of high collinearity. Other variables included in all the analysis were those with statistical significance at the exploratory analysis and those of clinical relevance for this study, like sociodemographic variables and classical risk factors for DR. Therefore, the following variables were included: gender, age, economic status, years of study, HbA1c, hypertension and GFR. In all models, collinearity among variables was evaluated. For the second multivariate analysis of DR and self-reported color/race, patients self-reported as Asians or Indigenous were excluded due to their small number ($n=7$).

We included only AFR GA in the analysis because proportions of AFR, EUR and NAM ancestries are complementary measures, whose sum equals 1. Residuals were inspected for each of the models' results using graphs to consider how well the model performed. A two-sided $p<0.05$ was considered statistically significant. Data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 20.0 (IBM, Chicago, IL, USA).

Results

Overview of the studied population and univariate analyses

For this study, 414 T1D participants were enrolled. Table 1 shows the demographic data of the enrolled population. AFR GA ($p=0.04$), age ($p=0.04$), years of schooling ($p=0.02$), GFR ($p<0.001$) and the presence of hypertension ($p<0.001$) were significantly different between the two groups. Figure 1 shows the proportions of GA in the two groups.

Multivariate analyses with African genomic ancestry

Table 2 shows the binomial logistic regression results including AFR GA as one of the independent variables. The backward stepwise method was applied. The final logistic regression model was statistically significant, $\chi^2(4)=76.51$, $p<0.0005$. The model explained 23.4% (Nagelkerke $R2$) of the variance and correctly classified 71.8% of cases. AFR GA, HbA1c, GFR and hypertension were associated with severe DR in the final model. None of the variables included in the analysis presented collinearity.

Multivariate analyses with self-reported color/race

When assessing the relationship of self-reported color/race with severe DR, no association was found. Table 3 shows the result of the binomial logistic regression analysis with the backward stepwise method applied. The final model was statistically significant, $\chi^2(4)=68.84$ $p<0.0001$. The model explained 21.6% (Nagelkerke $R2$) of the variance and correctly classified 71.2% of the cases. The variables that were associated with severe DR were: HbA1c, hypertension and

Table 1 Characteristics of the study sample

Variable	Controls, $n=238$	Cases, $n=176$	p value
Proportion of genomic ancestry			
African	0.13 [0.23]	0.15 [0.28]	0.04
European	0.72 [0.30]	0.67 [0.32]	0.03
Native Americans	0.10 [0.13]	0.11 [0.16]	0.33
Self-reported ethnicity			0.14
Caucasian	147 (61.8)	91 (51.7)	
Black	12 (5.0)	20 (11.4)	
Mulattos	76 (31.9)	61 (34.7)	
Native Americans	0	4 (2.3)	
Yellow	3 (1.3)	0	
Sociodemographic			
Gender			
Male	94 (39.5)	76 (43.2)	
Female	144 (60.5)	100 (56.8)	0.45
Age (y)	35.5 (12.5)	37.9 (11.1)	0.04
Economic status			
High	11 (4.6)	5 (2.8)	
Medium	114 (47.9)	78 (44.3)	0.1
Low	107 (45.0)	82 (46.6)	0.06
Very low	6 (2.5)	11 (6.2)	0.06
Years of schooling	12.9 ± 4.0	12.0 ± 4.2	0.02
Diabetes-related variables			
HbA1c (%)	8.4 ± 1.7	8.9 ± 1.9	0.008
HbA1c (mmol/mol)	68.9 ± 19.1	74.2 ± 20.5	0.008
Duration of diabetes (y)	22.4 ± 8.9	23.7 ± 9.2	0.17
LDL cholesterol	110.3 ± 2.3	110.8 ± 3.0	0.9
GFR	81.3 ± 1.7	63.3 ± 1.9	< 0.001
Arterial hypertension, yes	41 (17.2)	77 (43.8)	< 0.001

Data are present as number (percentage), mean ± SD (standard deviation) or median [IQR]

y, years; GFR, glomerular filtration rate

GFR. None of the variables included in the analysis presented collinearity.

Discussion

To the best of our knowledge, this is the first study to evaluate the relationship between GA and DR in patients with T1D from a highly admixed population like the Brazilian. AFR GA but not self-reported color/race was statistically associated with severe DR after controlling for demographic and clinical factors. Other variables that were significantly associated with DR were: the presence of hypertension, higher levels of HbA1c and lower levels of GFR.

AFR GA, in our study, was the only social determinant statistically associated with DR at the multivariate analysis. It is important to note that although AFR GA was statistically associated with severe DR in our study, this association should be interpreted with caution due to the wide confidence interval found, and therefore, further studies are needed to clarify these data. Previous studies conducted on specific T2D populations in the USA showed an influence of Native American ancestry in DR only among the Latino population [10], but no association of AFR GA on African–American participants after adjustment for clinical, demographic and socioeconomic factors was found [11]. It is important to highlight that although NAM ancestry in the Americas comes from a common ancestral root [26], the USA had a history of less miscegenation than Brazil, which could explain the higher degrees of AFR ancestry among their studied population [11]. This could justify the different results found in the present study. In our study, the population was more admixed, with only 5% having more than 50% of AFR ancestry. For instance, none of the participants had AFR GA or NAM GA higher than 90% and only 76 (17.6%) individuals had EUR GA higher than 90%.

The association of ethnicity and DR is controversial [27]. Some studies suggest differences in prevalence and progression of DR between different ethnical groups [28, 29], and others found no association after adjustments for socioeconomic factors [13, 30, 31]. It is important to highlight that most of the studies were with patients with T2D and on a homogeneous population. There are few studies with T1D, especially on admixed populations [28, 30, 31]. A study from a South African population showed that the prevalence of DR was higher in Blacks compared to Indians (55.6 vs 45.5%, respectively) [30]. Another study found that

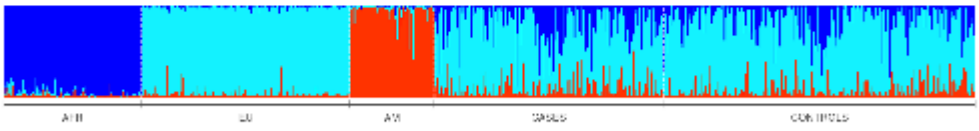

Fig. 1 Ancestry proportion for each individual considering reference population $k=3$

Table 2 Multivariate association results with African genomic ancestry

Variables	Model 1			Model 2			Model 3			Model 4			Final model		
	OR	IC 95%	*p* value	OR	IC 95%	*p* value	OR	IC 95%	*p* value	OR	IC 95%	*p* value	OR	IC 95%	*p* value
African genomic ancestry	3.84	1–14.77	0.051	3.6	0.95–13.65	0.06	3.64	0.96–13.80	0.058	3.9	1.03–14.74	0.045	3.9	1.03–14.69	0.045
GFR	0.97	0.96–0.98	< 0.001	0.97	0.96–0.98	< 0.001	0.97	0.97–0.98	< 0.001	0.97	0.97–0.98	< 0.001	0.98	0.97–0.99	< 0.001
Hypertension, yes	2.57	1.50–4.38	0.001	2.52	1.48–4.27	0.001	2.44	1.47–4.07	0.001	2.5	1.51–4.16	< 0.001	2.52	1.52–4.18	< 0.001
HbA1c (%)	1.23	1.07–1.40	0.003	1.22	1.07–1.39	0.004	1.23	1.07–1.40	0.002	1.24	1.09–1.41	0.001	1.24	1.09–1.41	0.001
Gender, female	1.43	0.90–2.25	0.13	1.44	0.91–2.27	0.12	1.44	0.91–2.27	0.12	1.46	0.93–2.30	0.11			
Years of study (y)	0.96	0.90–1.03	0.25	0.97	0.92–1.03	0.33	0.97	0.92–1.03	0.36						
Age (y)	0.99	0.97–1.02	0.6	1	0.98–1.02	0.67									
Economic status															
Very low	0.8	0.14–4.51	0.8												
Low	0.84	0.24–2.93	0.79												
Medium	1.05	0.32–3.49	0.94												
High	0[a]														

Model 1: all variables (ENTER model)

Model 2: Economic status did not persisted in the model

Model 3: Economic status and age did not persisted in the model

Model 4: Economic status, age and years of study did not persisted in the model

Final model: Economic status, age, years of study and gender did not persisted in the model

y, years; GFR, glomerular filtration rate; [a], reference group

Table3 Multivariate association results with self-reported color/race

Variables	Model 1			Model 2			Model 3			Model 4			Model 5			Final model		
	OR	IC 95%	*p* value	OR	IC 95%	*p* value	OR	IC 95%	*p* value	OR	IC 95%	*p* value	OR	IC 95%	*p* value	OR	IC 95%	*p* value
GFR	0.97	0.96–0.98	<0.001	0.97	0.96–0.98	<0.001	0.97	0.96–0.98	<0.001	0.97	0.96–0.98	<0.001	0.98	0.97–0.98	<0.001	0.98	0.97–0.99	<0.001
Hypertension, yes	2.372	1.38–4.10	0.002	2.34	1.36–4.01	0.002	2.31	1.37–3.88	0.002	2.38	1.42–3.98	0.001	2.54	1.53–4.23	<0.001	2.55	1.53–4.23	<0.001
HbA1c (%)	1.231	1.08–1.41	0.003	1.22	1.07–1.40	0.003	1.22	1.07–1.40	0.003	1.24	1.09–1.41	0.001	1.27	1.11–1.44	<0.001	1.26	1.11–1.44	<0.001
Gender, female	1.389	0.88–2.20	0.16	1.4	0.88–2.22	0.15	1.4	0.88–2.22	0.15	1.42	0.90–2.25	0.13	1.44	0.91–2.26	0.12			
Self-reported color/race†			0.19			0.23			0.22			0.21						
Caucasian	0ª																	
Black	2.135	0.88–5.18	0.09	2.07	0.85–5.01	0.11	2.08	0.86–5.03	0.1	2.07	0.86–5.00	0.11						
Mulattos	1.32	0.80–2.17	0.27	1.26	0.77–2.05	0.35	1.27	0.78–2.05	0.33	1.28	0.79–2.10	0.31						
Years of study (y)	0.96	0.90–1.02	0.18	0.97	0.91–1.02	0.26	0.97	0.91–1.02	0.27									
Age (y)	1	0.98–1.02	0.78	1	0.98–1.02	0.85												
Economic status			0.81															
High	0ª																	
Medium	0.93	0.28–3.10	0.93															
Low	0.72	0.21–2.54	0.62															
Very low	0.74	0.13–4.24	0.74															

Model 1: all variables (ENTER model)

Model 2: Economic status did not persisted in the model

Model 3: Economic status and age did not persisted in the model

Model 4: Economic status, age and years of study did not persisted in the model

Model 5: Economic status, age, years of study and self-reported color/race did not persisted in the model

Final model: Economic status, age, years of study, self-reported color/race and gender did not persisted in the model

y, years; GFR, glomerular filtration rate; 0ª, reference group

†Native American and Yellow categories were excluded for this analysis ($N=7$)

African–Americans presented a higher prevalence of proliferative diabetic retinopathy (PDR) than Whites, but after adjustments, ethnicity was not an independent risk factor for PDR [31].

Socioeconomic and genetic factors might be associated with the observed difference in the prevalence of DR among different populations [4, 5]. Some studies have shown a positive association between low socioeconomic status and poor metabolic control [18, 32] which is an important predictor of DR. This fact might also be related to other social and behavioral factors, including inequalities in the access to health care [33].

Disease duration and glycemic control are well-known risk factors for DR and were also important risk factors in our population. As demonstrated by DCCT/EDIC studies, glycemic control, especially if achieved on early stages of the disease, is important for the prevention of development of microvascular complications [3].

Hypertension is another important risk factor for DR [34]. Black patients are more likely to develop earlier onset hypertension than other ethnical groups and have a higher prevalence of the disease [35]. A recent study showed that patients at the highest quartile of AFR GA were more likely to have hypertension. However, the adjustment for education, income and wealthy explained over one-third of this disparity [36]. Considering the above-mentioned studies, we can hypothesize that social determinants could also be considered important factors in the risk of developing DR.

Some studies performed in patients with T1D showed that DR precedes the development of diabetic nephropathy [37, 38]. In our sample, as we studied patients with severe retinopathy, it was expected that renal function would be an important risk factor. Low GFR has also been associated with higher AFR GA in a study conducted in a hospital-based population in USA [39], supporting that a genetic component might be involved in the prevalence of chronic kidney disease.

Particular strengths of our study are the population-based ascertainment of diabetes cases in a large, high admixed sample of Brazilian T1D individuals; also, all participating centers followed a uniform and standardized protocol. In our study, due to the important relationship between diabetes duration and development of DR, we preferred to adjust for it on the study selection.

One limitation of our study may be the characteristics of our studied sample which included only patients assisted by the public health system in urban areas of the country. Although we could have had some selection bias, T1D patients assisted by the private health system or from rural areas probably represent the minority of patients with T1D under treatment in Brazil. Although autoantibodies and C-peptide levels were not measured, 96.5% of the patients included were diagnosed before 30 years of age, which mitigates the probability of misdiagnosis. The diagnosis of DR was assessed by binocular indirect ophthalmoscopy for all participants. Despite the inherent limitations of this method, a previous study from our group showed a substantial agreement between binocular indirect ophthalmoscopy and digital retinography [40].

In conclusion, our study showed that besides classical known risk factors (such as hypertension, GFR and HbA1c), AFR GA but not self-reported color/race was also associated with severe DR. Further studies are needed, especially in highly admixed populations, to better understand the role of GA on DR and to investigate possible genetic markers for DR that could be related to GA in different populations.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standard This study was approved by the Ethics Committee of Pedro Ernesto University Hospital (Rio de Janeiro State University) and by the local ethics committee of each center.

Informed consent Written informed consent was obtained from all participants or their parents.

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4.4 Does ancestry influence health-related quality of life in type 1 diabetes patients? A nationwide study in Brazil. (artigo publicado)

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ORIGINAL ARTICLE

Does ancestry influence health-related quality of life in type 1 diabetes patients? A nationwide study in Brazil

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Abstract

Aims The aim of the present study was to evaluate the relationship between self-reported color/race and genomic ancestry with HRQoL of patients with type 1 diabetes in a highly admixed population.

Methods This was a nationwide, cross-sectional study conducted with 1760 patients with type 1 diabetes from 2011 to 2014 at public clinics in all five Brazilian geographical regions. Information on HRQoL was obtained from two self-completed questionnaires: Short Form-6 Dimensions (SF-6D) and EuroQol-5 Dimensions (EQ-5D) with a visual analogue scale (EQ-VAS). Genomic ancestry was assessed using a Multiplex PCR methodology. Utility scores generated from the questionnaires were analyzed with multivariate logistic regression models.

Results We included 1698 patients. Those patients who self-reported as black had lower EQ-VAS scores compared to the patients who self-reported as white (67.46 ± 18.45; 72.37 ± 16.44, respectively, $p = 0.02$). In a linear regression model, each 1% increase in African ancestry resulted in a 9.5 point decrease in EQ-VAS score ($p < 0.001$). In a multivariate logistic regression, after adjusting for demographic, socioeconomic status and diabetes-related variables, African ancestry remained associated with lower EQ-VAS scores.

Conclusion A higher level of African ancestry implicates on lower quality of life even after adjustments for sociodemographic and diabetes-related data. Gender, physical activity and diabetes-related microvascular complications were strongly associated with low HRQoL in all three questionnaires used. This fact highlights the importance of social aspects when assessing quality of life, as well as the need for regular practice of physical activity and prevention of chronic complications to improve patients' quality of life.

Keywords Ancestry · Type 1 diabetes · Health-related quality of life · Ethnicity

Introduction

Type 1 diabetes mellitus is a chronic disease with rising incidence worldwide [1]. The disease is associated with chronic complications that are responsible for elevated mortality and morbidity rates and are strongly correlated with glycemic control and disease duration [2]. Patients with diabetes have

Managed by Massimo Federici.

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a reduced health-related quality of life (HRQoL) compared with the general population [3].

Due to its chronic nature, the severity of complications and the treatment needed to achieve good glycemic control, diabetes is a costly disease both to individuals and to healthcare systems. The indirect costs of diabetes are primarily related to productivity losses due to disability, early retirement, early mortality, unemployment and absenteeism caused by acute and chronic complications [4, 5]. Some studies demonstrated low quality of life, changes in family lifestyle and symptoms of anxiety and depression among the parents of type 1 diabetes patients [6]. HRQoL refers to how people feel about their physical and mental health and can be accurately assessed by several validated tools, such as the Short Form-6 Dimensions (SF-6D) [7] and EuroQoL-5 Dimensions (EQ-5D) [8].

Brazil has a large multiethnic population as a result of centuries of miscegenation since Portuguese colonization in 1500. The population originated from African, European and Native American ancestral roots. Brazil had one of the largest slave trades in the Americas, receiving up to 4 million African slaves [9]. Several studies have not demonstrated an association between self-reported declaration of color and genetic ancestry [10, 11]. Despite the equivocal classification into races from a genetic point of view, self-reported color/race declaration has been widely used in demographic studies as well as in the development of public policies in Brazil [12]. A recent study in an aging cohort of a small city in Brazil showed that persons at higher levels of African and Native American genomic ancestry and those self-identified as nonwhite were more likely to report poor health than other groups, even after controlling for other confounding factors [13].

The aim of the present study was to evaluate the relationship between self-reported color/race and genomic ancestry with HRQoL of patients with type 1 diabetes.

Research design and methods

Study design and population

A nationwide multicenter cross-sectional study was performed by the Brazilian type 1 diabetes study group (BrazDiab1SG) on 1760 patients with type 1 diabetes. These subjects received health care from the National Brazilian Health Care System (NBHCS) from August 2011 to August 2014 at 14 public clinics; these clinics were of the secondary and tertiary care level and were located in 10 cities in all five Brazilian geographical regions (north, northeast, southeast, south and central-west). Patients were included in the study if they were diagnosed with type 1 diabetes by a physician (based on typical clinical presentation), needed continuous insulin use since their diagnosis and had more than 6 months of follow-up evaluations. Patients were excluded if they aged less than 13 years old, were pregnant or lactating, or had a history of acute infectious processes or diabetic ketoacidosis in the 3 months prior to assessment. Detailed methods have been described previously [14]. For the present study, we included 1698 patients because 62 (3.5%) participants had DNA extraction inadequate for analysis. The study was approved by each center's local ethics committee. All patients or their representatives signed a written informed consent for the study.

Health-related quality of life assessment

Information on HRQoL was obtained from self-completed questionnaires. We used two validated questionnaires to evaluate quality of life: SF-6D focused on productivity and activity impairment; EQ-5D focused on general self-rated health status. The first part of EQ-5D measures five health dimensions (mobility, self-care, usual activities, pain/discomfort and anxiety/depression), and the other part, EQ-VAS, comprises a visual scale of general health status ranging from 0 to 100. The SF-6D analyzes six quality of life domains such as physical function, role limitations, social function, pain, mental health and vitality.

Utility index scores were calculated for EQ-5D-3 L as well as for SF-6D data using validated algorithms developed by two Brazilian research groups, QALY Brazil [15] and Cruz et al. [16], respectively.

Genomic ancestry

The global and individuals genomic ancestry was inferred using a panel of 46 AIM-INDELs, as described in a protocol by Pereira et al. [17]. Genotyping of the AIM-INDELs was performed using the Qiagen Multiplex PCR Kit (QIAGEN Inc., Valencia, CA). Capillary electrophoresis on the automatic sequencer ABI 3500 (Applied Biosystems®) was used for detection of the polymorphisms and then analyzed by Gene Mapper software version 4.1 (Applied Biosystems®) and Structure software [18].

Self-reported color/race

Participants categorized themselves into one of the five self-reported color/race groups used for the Brazilian population censuses: white, brown ("pardo"), black, Asian (yellow-"amarela") and indigenous (indígena) as recommended by IBGE [19].

Demographic, clinical and laboratorial data measures

The following variables were assessed using a questionnaire during a clinical visit: gender, current age, years of study, age at diagnosis, diabetes duration, presence of

comorbidities, self-reported frequency of severe hypoglycemia in the last month (defined as blood glucose levels ≤ 70 mg/dl and the need of a third party to help overcome hypoglycemic symptoms), employment status and level of regular physical activity. Height (m) and weight (kg) were measured by a nurse or a physician during the clinical visit. BMI was determined by dividing an individual's weight (kg) by the square of their height (m^2). Overweight was defined as BMI between 25 and 29.9 kg/m^2, and obese as BMI ≥ 30 kg/m^2. HbA1c was measured using high-performance liquid chromatography (HPLC, Bio-Rad Laboratories, Hercules, California, USA). Patients with a diabetes duration greater than or equal to 5 years from diagnosis were screened for the following chronic diabetes-related complications: retinopathy, clinical nephropathy and foot pathologies.

Economic status was defined according to the Brazilian Economic Classification Criteria (ABEP), which is based on educational status, household income and possession of certain house appliances [20]. The following classes of economic status were considered for this analysis: very low, low, middle and high.

Statistical analysis

Categorical variables were presented as frequency (percentage). All normally distributed values were given as the mean ± standard deviation (SD), and all other values were given as the median (range). We used Chi-squared and Fisher's tests to compare categorical data; the Student *t* test and analysis of variance (ANOVA) were used for comparisons between groups with numeric variables when indicated. Genomic ancestry was stratified into quartiles for evaluation regarding demographics and clinical and HRQoL values. Simple linear regression was used to test linearity between EQ-VAS and HbA1c. In addition, we performed one regression model for each dependent variable (EQ-VAS, EQ-5D index and SF-6D index) using generalized linear models; each genomic ancestry was tested separately as a continuous variable adjusted for age, gender, years of study, HbA1c, practice of physical activity, duration of diabetes, current employment status, economic level, overweight/obesity status, occurrence of severe hypoglycemia in the last month and the presence of microvascular complications were tested as independent variables. Genomic ancestries were analyzed separately because proportions of African, European and Native American ancestries are complementary measures, whose sum equals 1. Residuals were inspected for each of the outcomes using graphs to consider how well the model performed. A two-sided $p < 0.05$ was considered statistically significant. All data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 20.0 (IBM, Chicago, IL, USA).

Results

Overview of the studied population

A total of 1698 (96.5%) patients with type 1 diabetes were included; 62 (3.5%) patients were excluded because the DNA extraction was inadequate for analysis. Table 1 shows clinical and demographic data of the study population.

Table 1 Clinical and demographic data of the studied population

Characteristics	
N	1.698
Proportion of genomic ancestry, median [IQR]	
African	0.16 [0.23]
European	0.68 [0.31]
Indigenous	0.11 [0.16]
Self-reported color/race, N (%)	
Caucasian	923 (54.40)
Black	132 (7.82)
Brown	610 (35.88)
Indigenous	15 (0.86)
Asians	18 (1.14)
Sociodemographic	
Gender, *N* (%)	
Male	749 (44.13)
Female	949 (55.87)
Age, y, mean (SD)	30.06 (11.93)
Economic status, N (%)	
High	52 (3.13)
Medium	774 (45.57)
Low	816 (48.06)
Very low	56 (3.34)
Years of study, y, mean (SD)	12.2 (3.77)
Unemployed, *N* (%)	871 (49.56)
Diabetes-related variables	
HbA1c (%), mean (SD)	9.0 (2.12)
HbA1c mmol/mol, mean (SD)	74.94 (23.21)
Duration of diabetes, y, mean (SD)	15.49 (9.26)
Presence of microvascular complication*, *N* (%)	1014 (59.78)
Episode of severe hypoglycemia last 30 days, N (%)	243 (14.35)
Practice of regular physical activity, *N* (%)	886 (52.24)
Overweight/obesity, *N* (%)	627 (36.97)
Health-related measures, mean (SD)	
EQ-VAS	71.46(17.26)
EQ-5D index	0.80 (0.16)
SF-6D index	0.85 (0.09)

y years, *SD* standard deviation

*Nephropathy, neuropathy, retinopathy

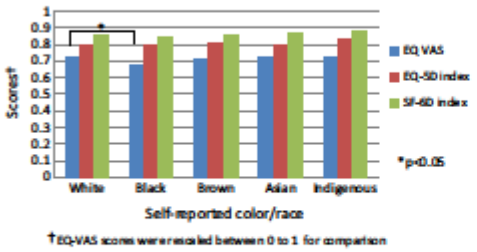

Fig. 1 HRQoL scores by self-reported color/race

Descriptive analysis of HRQoL by self-reported color/race and genomic ancestry

Patients who self-declared as black had lower health values for the EQ-VAS compared to those who self-declared as white (67.46 ± 18.45; 72.37 ± 16.44, respectively, $p = 0.02$). No differences were observed among self-declared ethnicities on the EQ-5D or SF-6D questionnaires (Fig. 1).

The simple linear regression model showed that African genomic ancestry significantly decreased EQ-VAS values by 9.51 for each increase of 1% in African genomic ancestry ($p < 0.001$). Table 2 shows distributions of demographic, clinical and HRQoL data stratified by quartiles of African genomic ancestry. EQ-VAS values were related to the levels of African genomic ancestry, especially those

Table 2 Clinical, demographic and laboratory data stratified by quartiles of African genomic ancestry

	Quartiles of African genomic ancestry				*p* value
	1st	2nd	3rd	4th	
N					
Health-related measures, mean (SD)					
EQ-VAS	71.82 (17.20)	73.67 (15.60)	71.88 (16.72)	68.47 (19.01)	< 0.001
EQ-5D index	0.79 (0.15)	0.81 (0.14)	0.81 (0.16)	0.79 (0.15)	0.15
SF-6D index	0.84 (0.09)	0.85 (0.09)	0.85 (0.09)	0.84 (0.09)	0.3
Self-reported color/race, N (%)					< 0.001
White	313 (33.41)	297 (32.24)	219 (23.69)	94 (10.16)	
Black	7 (5.27)	11 (8.29)	20 (15.23)	94 (71.21)	
Brown	104 (17.05)	109 (17.87)	173 (28.35)	224 (36.73)	
Indigenous	1 (6.72)	1 (6.74)	5 (33.26)	8 (53.78)	
Asian	2 (11.14)	5 (27.82)	8 (44.36)	3 (16.68)	
Sociodemographic					
Gender, male *N* (%)	195 (26.10)	183 (24.41)	196 (26.23)	175 (23.36)	0.47
Age, y, mean (SD)	31.19 (12.10)	31.05 (12.96)	28.38 (11.21)	26.72 (11.13)	0.01
Economic status, N (%)					< 0.001
High	18 (34.62)	13 (25.03)	9 (17.29)	12 (23.14)	
Medium	223 (28.78)	222 (28.71)	184 (23.78)	145 (18.66)	
Low	175 (21.41)	178 (21.76)	218 (26.71)	245 (30.09)	
Very low	11 (19.59)	10 (17.90)	14 (25.02)	21 (37.51)	
Years of study, y, mean (SD)	12.83 (4.39)	12.39 (3.57)	12.04 (3.51)	11.6 (3.44)	< 0.001
Unemployed, *N* (%)	189 (22.72)	213 (25.53)	214 (25.67)	218 (26.08)	0.13
Diabetes-related variables					
HbA1c (%), mean (SD)	8.85 (2.04)	8.78 (1.87)	9.01 (2.15)	9.39 (2.34)	< 0.001
HbA1c mmol/mol, mean (SD)	73.27 (22.39)	72.46 (20.49)	74.18 (23.56)	79.16 (25.67)	< 0.001
Duration of diabetes, y, mean (SD)	16.49 (9.72)	16.07 (9.95)	14.75 (8.78)	14.66 (8.41)	0.005
Presence of microvascular complication[a], *N* (%)	245 (26.57)	250 (24.68)	241 (23.82)	278 (27.43)	0.03
Episode of severe hypoglycemia last 30 days, *N* (%)	47 (19.31)	57 (23.48)	64 (26.29)	75 (30.92)	0.04
Practice of regular physical activity, *N* (%)	238 (26.92)	224 (25.26)	209 (23.66)	215 (24.30)	0.26
Overweight/obesity, *N* (%)	152 (24.21)	168 (26.78)	154 (24.60)	153 (24.41)	0.56

y years, *SD* standard deviation

p value is related to the comparison among all groups (ANOVA or X2)

[a]Nephropathy, neuropathy, retinopathy

in the 2nd versus 4th quartiles, with mean score varying from 73.67 (± 15.60) to 68.47 (± 19.01), respectively ($p < 0.001$). Patients at higher levels of African ancestry were younger and had lower duration of diabetes compared to those at the lowest level of African ancestry, respectively (26.72 ± 11.13; 31.19 ± 12.10 years, $p = 0.01$ and 14.66 ± 8.41; 16.49 ± 9.72 years, $p = 0.005$). A linear trend was observed between economic status and African ancestry, with more patients from high and medium classes in the lowest quartile of African ancestry in comparison with patients from low and very low classes (34.62%, 28.78%, 21.41%, 19.59%, respectively, $p < 0.001$). Years of school attendance were lower at higher levels of African ancestry (12.83 ± 4.39 at lowest versus 11.62 ± 3.44 at highest, $p < 0.001$).

In a linear simple regression model, HbA1c levels negatively correlated with EQ-VAS, with a decrease of 1.35 at EQ-VAS for each 1% increase in HbA1c.

There were no associations for the other two measurements of HRQoL used (EQ-5D index or SF-6D index) regarding self-reported color/race as well as genomic ancestry (data not shown).

When observing each domain of the EQ-5D, depression and anxiety were more prevalent than losses in mobility or daily self-care in our samples. As such, 57.42% of the patients reported some degree of depression or anxiety versus 15.02% reporting any losses in mobility or daily self-care activities, with a significant impact on EQ-VAS values for those with depression/anxiety compared to those without depression/anxiety (66.17 vs. 75.06, $p < 0.001$). These data were not associated with ancestry or self-reported color/race ($p = 0.06$).

Multivariate analysis with EQ-VAS as a dependent variable

A regression model with EQ-VAS as a dependent variable revealed, after adjustments, that African genomic ancestry had a negative correlation with EQ-VAS values. The independent variables associated with the reduction in EQ-VAS values were female gender, higher economic status, higher HbA1c, the presence of microvascular complications, sedentary lifestyle and overweight or obese status. Table 3 shows unadjusted and final adjusted model.

In the other three models with European or Native American genomic ancestries or self-reported color/race, none of these ancestry variables were associated with EQ-VAS scores. The variables that decreased EQ-VAS scores were as follows: female gender, higher economic status, higher HbA1c, the presence of microvascular complications, sedentary lifestyle and overweight or obese status, the same found with African ancestry described above (Supplemental Table S1).

Multivariate analysis with EQ-5D Index as a dependent variable

A regression model with EQ-5D index as a dependent variable revealed that none of the genomic ancestries or self-reported color/race was associated with EQ-5D utility score.

Table 3 Unadjusted and final adjusted regression for African genomic ancestry with EQ-VAS as dependent variable

Parameter	Unadjusted	Adjusted			
		95% confidence interval			
	OR	OR	Lower	Upper	Sig.
African genomic ancestry	0.87	0.92	0.86	0.99	0.03
Gender, female	0.95	0.97	0.95	0.99	0.02
Age, years	1.00	0.99	0.99	1.000	0.09
Economic status					
Very low	1.03	1.12	1.02	1.22	0.02
Low	0.98	1.02	0.95	1.09	0.52
Medium	1.03	1.05	0.98	1.12	0.17
High	0*	0*			
Years of study, years	1.01	1.00	1.00	1.01	0.15
Unemployment	0.98	0.99	0.97	1.02	0.52
HbA1c (%)	0.98	0.99	0.98	0.99	< 0.001
Duration of diabetes, years	1.00	1.00	1.00	1.002	0.94
Presence of microvascular complication	0.91	0.94	0.92	0.97	< 0.001
Episode of severe hypoglycemia last 30 days	0.98	0.98	0.95	1.01	0.28
Sedentary lifestyle	0.92	0.93	0.91	0.96	< 0.001
Overweight/obesity	0.97	0.97	0.95	0.99	0.02

*0 = reference group

For all three models (for each genomic ancestry), the variables significantly associated with lower utility values were as follows: female gender, sedentary lifestyle, unemployment, presence of microvascular complications, episode of severe hypoglycemia, younger age, fewer years of study, higher values of HbA1c and higher duration of disease (Supplemental Table S2).

Multivariate analysis with SF-6D index as a dependent variable

A regression model with SF-6D index as a dependent variable revealed that none of the genomic ancestries or self-reported color/race was associated with this utility score.

The variables negatively associated with SF-6D index were as follows: female gender, sedentary lifestyle, unemployment, presence of microvascular complications, episode of severe hypoglycemia and younger age (Supplemental Table S3).

Discussion

According to our knowledge, this was the first study to demonstrate that ancestry can influence HRQoL in patients with type 1 diabetes. Our findings suggest that type 1 diabetes Brazilian patients at higher levels of African genomic ancestry are more likely to report lower quality of life at the EQ-VAS scale, even after controlling for demographic, socioeconomic status and diabetes-related variables. Other variables responsible for a decrease in EQ-VAS values were: female gender, higher economic status, higher HbA1c, presence of microvascular complication, sedentary lifestyle and overweight/obesity status. However, there was no difference between the levels of genomic ancestry for the EQ-5D index or the SF-6D index.

These findings are in accordance with another study regarding ethnicity and health-related quality of life. The Bambui cohort study showed that elderly individuals who self-reported as nonwhite and those with higher levels of African genomic ancestry reported lower health status than those who self-reported as white and those who had higher levels of European ancestry [13]. Patients with higher degrees of African genomic ancestry might have lower scores on self-reported health status questionnaires, regardless of socioeconomic effects, due to the presence of implicit racial bias or perceived discrimination. Some studies have addressed the possibility of implicit racial bias and perceived discrimination in Brazil and others populations [21–24]. A large survey in Brazil showed that 9% of the participants reported discrimination and it was higher among those who self-reported as black versus white [25].

In all the three outcomes (EQ-VAS, EQ-5D index and SF-6D index), gender, physical activity and diabetes-related microvascular complications were statistically associated with HRQoL. Economic status and overweight/obesity were only associated with the EQ-VAS measure, while age, unemployment and severe hypoglycemia were only associated with the utilities values of EQ-5D and SF-6D.

Those differences can be expected due to inherited differences between questionnaires. EQ-VAS reveals a more general perception of health status, is a unique visual scale and is simpler to use, and it might have better acceptance in our population due to the low education level. SF-6D details levels for all six dimensions, takes longer to answer and requires more thoughtful choices, similar to EuroQol-5 Dimensions, which also requires higher levels of attention and comprehension to be answered when compared to the visual analogue scale. Other studies have been conducted specifically to analyze the differences between questionnaires and populations [26, 27]. Bharmal et al. observed that the EQ-VAS score among individuals reporting no limitations on EQ-5D was significantly lower when diabetes or other comorbidities were present [26].

Gender and age might influence HRQoL values. A US study showed that older individuals, as well as women, scored lower on the SF-6D and EQ-VAS [28]. It is important to highlight the relatively younger age of our population (mean age 30 years, 11–72 years) compared to that of other studies [3, 29], which may explain some of the differences observed in our population general values compared to others [27, 29]. These tests might not be the best tools for assessing HRQoL in young diabetic populations because the burden of a chronic disease in a young patient might have greater influence on predictors related to day-by-day management of disease or psychological factors [30]. Another option would be the diabetes quality of life (DQOL) [31] or its variation for younger populations, as the DQOLY has already been validated for the Brazilian population. However, because of their specificity, they do not allow comparisons with general population or cost utilities analysis.

We found that sedentary lifestyle had an important negative impact on HRQoL in our sample which is in accordance with previous data from our group [32]. Physical activity improves quality of life and diabetes management as shown in several studies [33, 34]. The Hvidoere study [35] observed that higher levels of physical activity were strongly associated with psychological well-being and therefore should be encouraged in this population.

The presence of microvascular complications was also significantly associated with lower HRQoL on all three measures. The 23rd year follow-up of DCCT/EDIC also showed that the presence of microvascular complications significantly decreased HRQoL on type 1 diabetes patients [36]. Diabetic retinopathy, although with few visual

symptoms in its early stages, can lead to significant vision loss with the progression of the disease. Similar pattern is observed in diabetic nephropathy, where patients might need dialysis or even renal transplant on its latest stages. Those conditions could bring losses on autonomy, self-care and quality of life [37, 38].

The prevalence of depression in the diabetic population varies; it has been reported at approximately 3.5% [39], 18% [40] and up to 31.1% [41] depending on the diagnostic criteria used. Depression was shown to have a significant decrease in HRQoL in type 1 diabetes patients [42] as well as an impact on clinical control [43]. In our study, 57.42% of the patients responded as feeling moderately/extremely anxious or depressed on EQ-5D. We also found a significantly decreased EQ-VAS score when comparing the groups with and without anxiety/depression (66.17 vs. 75.06, $p < 0.001$). It is important to highlight that the questionnaire used was not developed to diagnose depression or anxiety and does not distinguish between the two, which might lead to an overestimation in prevalence. However, it shows the importance of psychological assessment in daily clinical practice as a tool for the improvement on disease control and quality of life [44].

Overweight and obesity have been shown to decrease HRQoL in different studies and populations [45–47], even in young type 1 diabetes patients [46]. In our study, overweight and obese status was responsible for reducing HRQoL but only when assessed by EQ-VAS. Sach et al. [48] found obesity to be associated with a loss at HRQoL in all three measures, but their patients were from the UK general practice population (not a type 1 diabetes-specific population) and were older (age $\geq$ 45 years).

Our study consisted of a large, admixed, representative, population-based sample of Brazilian type 1 diabetes patients from the community setting of all geographical regions of the country. In addition, all participating centers followed a uniform and standardized protocol.

Lastly, as our findings are based on a cross-sectional study design, we cannot determine any causal effects. Another limitation is that we did not assess some variables that might influence HRQoL or the response to the questionnaires such as place of residence, cognitive function impairment, family support and environment. In addition, HbA1c was measured by HPLC method, but we were unable to standardize it according to the Diabetes Control and Complications Trial assay due to limited funding.

Conclusion

Our findings suggest that a higher level of African ancestry correlated with lower rates in quality of life even after adjustment for sociodemographic and diabetes-related data. The presence of implicit racial bias or perceived discrimination might be related to this finding. Gender, physical activity and diabetes-related microvascular complications were strongly associated with low HRQoL in all three questionnaires used. This fact highlights the importance of social aspects when assessing quality of life, as well as emphasizing the need for regular practice of physical activity and prevention of chronic complications to improve patients' quality of life.

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Compliance with ethical standards

Conflict of interest All authors declare no conflict of interests to disclose.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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5. DISCUSSÃO

Em nosso primeiro estudo descrito nos resultados, ao analisarmos o perfil HLA dos pacientes portadores de DM1 em um estudo multicêntrico no Brasil e sua distribuição de acordo com a cor autodeclarada, observamos que em todos os grupos étnicos, o haplótipo mais frequente foi HLA-DRB1*03:01~DQA1*05:01g~DQB1*02:01. Este haplótipo é o mais frequente em populações caucásicas e também o mais encontrado em estudos brasileiros prévios (84, 85). Isso demonstra que, apesar da população brasileira ser muito miscigenada, nos pacientes diabéticos tipo 1 há uma preponderância da influência genética caucásica.

Os dois principais alelos descritos na população de indivíduos com DM1 são do DRB1*03 e o DRB1*04, com uma frequência variando entre 20 a 30% (84). Suas maiores frequências são encontradas em populações caucásicas, mas ele também é descrito em Africano-americanos (18). No Brasil, a frequência destes alelos chega a 28% (85), similar ao que encontramos em nosso estudo (28,9%). Em nossa amostra, 63,3% dos pacientes apresentavam DRB1*03 e/ou DRB1*04 e destes, 39,2% possuíam ambos. No grupo controle (população proveniente do REDOME) apenas 5,1% apresentavam os dois alelos.

No nosso estudo, o haplótipo HLA-DRB1*07:01~DQA1*02:01~DQB1*02:02/03:03 apresentou frequência duas vezes maior no grupo controle quando comparado aos pacientes DM1. Estudos anteriores já demonstravam esse haplótipo protetor como muito prevalente, especialmente em populações caucásicas (3, 84). Porém este haplótipo parece estar relacionado à risco na população afro-Americana quando em associação com o HLA-DQA1*03:01 (18). O mesmo se observa com o alelo DRB1*13.

O genótipo mais fortemente associado a risco foi o DRB1*03/DRB1*04, com um OR de 12,1, seguido pela homozigose do DRB1*03 com uma razão de chances de 10,9 comparado com a população não diabética. O alelo DRB1*09 só apresentou risco quando associado ao DRB1*03 ou ao DRB1*04, diferente do que foi encontrado em estudos com populações africanas.

Estes achados podem estar relacionados ao fato da nossa população apresentar uma contribuição importante e preponderante da ancestralidade Europeia e uma baixa proporção de ancestralidade africana e ameríndia, como já descrito em estudo prévio do nosso grupo (55). É importante observar também que em nossa amostra, 51% dos pacientes se autodeclararam Brancos e apenas 9% Negros. Uma possível hipótese seria que, em

populações altamente miscigenadas, os alelos de risco para o DM1 sejam provenientes da herança ancestral Europeia/Caucasiana.

No segundo artigo apresentado como parte dos resultados desta tese, analisamos a relação dos alelos HLA com a RD em um estudo caso-controle aninhado a uma coorte de pacientes do estudo multicêntrico brasileiro (BrazDiab1SG). Neste estudo, não encontramos associação dos alelos e/ou haplótipos HLA com o desenvolvimento de RD severa. As associações de risco incialmente encontradas com o DRB1*13 e o haplótipo HLA-DRB1*13:02~DQA1*01:02~DQB1*06:04 e também as de proteção com o haplótipo HLA-DRB1*04:01~DQA1*03:01g~DQB1*03:02, perderam significância estatística após correção de Bonferroni para comparações múltiplas.

Apesar da evidente associação de alelos HLA com a predisposição ao desenvolvimento de DM1 (84, 86-88), seu papel em relação às complicações microvasculares ainda não está bem estabelecido. Alguns estudos encontraram associação do alelo DRB1*04 como um possível alelo de risco ao desenvolvimento de RD e o DRB1*03 conferindo proteção, porém outros estudos não encontram associação significativa (24, 26, 27, 89-93). É importante observar que há grande heterogeneidade entre esses estudos em relação ao desenho do estudo, à classificação da RD, definição do grupo controle e ajuste para fatores de riscos clássicos como duração do diabetes, tornando difícil a comparação dos resultados (38).

Uma possível explicação para ausência de associação dos alelos HLA com a RD pode estar relacionada à própria fisiopatologia da RD que está mais relacionada ao estresse oxidativo e não tão diretamente associada à resposta imune inata e de reconhecimento de antígenos. Com isso, os possíveis genes relacionados poderiam estar em regiões não-HLA ou próximas ao HLA. Diversos estudos de GWAS e genes candidatos foram realizados, porém os únicos genes que parecem estar mais fortemente associados são os polimorfismos da aldose redutase (AKR1B1) e do VEGF (38). Recentemente também se observou associação de modificações epigenéticas no DNA mitocondrial com a predisposição à RD (94). A continuidade dos estudos se faz necessária para estabelecer a predisposição genética da RD nas diferentes populações, pois a identificação precoce dos pacientes mais suscetíveis possibilita a prevenção mais eficaz desta debilitante complicação.

Nos outros dois artigos apresentados nesta tese, avaliamos o papel da ancestralidade genômica na RD e na qualidade de vida, respectivamente, dos pacientes portadores de DM1 na população brasileira também proveniente do estudo multicêntrico nacional (Brazdiab1SG). Ambos mostraram associações positivas. No primeiro deles encontramos associação positiva da ancestralidade africana com a presença de RD grave, mesmo após ajustes para fatores de

risco clássicos e socioeconômicos. No segundo, a ancestralidade africana se mostrou associada a uma menor qualidade de vida relacionada à saúde.

A associação de etnia com RD é controversa (95). Alguns estudos sugerem diferenças entre as taxas de prevalência da RD nas diferentes populações (96, 97) enquanto outros não encontram associação significativa após ajustes de fatores socioeconômicos (61, 98, 99). Estudo realizado em uma população Sul Africana mostrou maior prevalência de RD em Negros quando comparado com Indianos (98). Outro estudo mostrou que afro-americanos apresentavam maior prevalência de RD proliferativa comparada a Brancos, porém a etnia não se mostrou um fator de risco independente na análise multivariada (99). Uma análise realizada em pacientes provenientes de uma população Latina dos USA mostrou que a ancestralidade ameríndia estava associada a maior risco de RD (7). Porém, outro estudo americano, realizado em população Africano-Americana não mostrou associação da ancestralidade genômica com a RD após ajustes para fatores clínicos, demográficos e socioeconômicos (8). É importante ressaltar que apesar da ancestralidade ameríndia nos EUA ter a mesma origem que a apresentada no Brasil (100), a população Africano-Americana dos EUA apresenta maior proporção de ancestralidade africana comparada com a nossa (8), podendo justificar os resultados conflitantes encontrados. Em nossa amostra há maior miscigenação, evidenciada pela presença de apenas 5% dos pacientes apresentando ancestralidade genômica africana maior que 50%. Para ilustrar, nenhum paciente da nossa população apresentou percentual individual de ancestralidade genômica africana ou ameríndia maior que 90% e apenas 17,6% apresentaram ancestralidade genômica Europeia maior que 90%.

Fatores socioeconômicos e genéticos podem justificar as diferentes prevalências encontradas nas populações. Alguns estudos mostraram associação positiva entre baixo nível socioeconômico e pior controle metabólico (101, 102). Este fato também pode estar relacionado a outros fatores sócias e comportamentais incluindo inequidades no acesso à saúde (103). Em nosso estudo, o único determinante social relacionado à RD foi a ancestralidade genômica.

A ancestralidade genômica africana também esteve associada à pior qualidade de vida relacionada à saúde em nosso quarto artigo descrito nesta tese. Este achado está de acordo com o encontrado em outro estudo brasileiro que avaliou a relação da ancestralidade genômica e etnia com a qualidade de vida relacionada a saúde em uma coorte de idosos em Bambui (104). Nesta coorte, tanto a ancestralidade africana quanto a auto declaração de cor como não brancos foram estatisticamente associados a menor nota no estado de saúde. Em nossa análise apenas a ancestralidade genômica, e não a cor autodeclarada, se mostrou

associada a pior qualidade de vida após ajustes para fatores socioeconômicos. O fato de a ancestralidade africana estar relacionada a menores notas na percepção do estado de saúde mesmo após correção para fatores socioeconômicos pode ser explicado pela presença de preconceito racial implícito e discriminação percebida já descrita em diversas populações (75, 105-107). Neste contexto, o preconceito implícito significa tomar atitudes, fazer escolhas que de forma inconsciente são realizadas a partir da formação de estereótipos e que levam à discriminação e ao preconceito. Já a discriminação percebida é a percepção de preconceito relatada por um indivíduo como consequência de um ato, julgamento ou tratamento inadequado, sendo independente da verificação atual dos eventos e parece influenciar a saúde física e mental do indivíduo (108).

Além da ancestralidade africana, outros fatores também influenciaram a qualidade de vida em nosso estudo. Pacientes do gênero feminino, sedentários e com presença de pelo menos uma complicação microvascular apresentar redução no escore de qualidade de vida relacionada à saúde.

A associação de sedentarismo com redução da qualidade de vida já foi descrita em um estudo anterior do nosso grupo (70). A atividade física já se mostrou benéfica para melhorar a qualidade de vida e manejo clínico dos pacientes em diversos estudos (71, 72). O estudo Hvidoere mostrou que maiores níveis de atividade física representavam melhora no estado físico e mental e por isso devem sempre ser encorajadas nestes pacientes (73). A presença de complicações microvasculares também é responsável por redução na qualidade de vida, como foi demonstrado no estudo DCCT/EDIC. A RD e a nefropatia diabética podem evoluir para cegueira e doença renal terminal, respectivamente, levando a perdas de autonomia, autocuidado e consequentemente qualidade de vida.

Outro dado interessante encontrado neste estudo foi a presença de sintomas de depressão e ansiedade moderada a extrema em mais de 50% dos indivíduos. A prevalência de depressão na população de portadores de diabetes varia de 3,5% a 31,4% dependendo da população estudada e dos critérios diagnósticos de depressão. É importante ressaltar que o questionário aplicado em nossa análise não foi desenvolvido para realizar diagnostico de depressão e por isso os resultados podem estar sobrestimados. No entanto, mostra a importância da avaliação psicológica, multiprofissional no manejo clínico diário dos pacientes com diabetes.

Nosso estudo possui algumas limitações. Apesar de ser o primeiro estudo multicêntrico brasileiro de abrangência nacional com mais de 1500 pacientes, ele é um estudo transversal e, portanto, não permite inferir causalidade. Apesar desse fato não exercer grande impacto nas variáveis genéticas, estamos sujeitos a viés de classificação em relação ao desfecho da

retinopatia, por exemplo, onde o controle poderia se tornar um caso ao passar do tempo. Para minimizar esse possível viés, pareamos os pacientes pelo tempo de duração do diabetes em ambos os artigos sobre a RD. Outra limitação seria o fato de termos incluídos pacientes provenientes de serviços públicos de saúde de áreas urbanas, podendo gerar viés de seleção. Porém, no Brasil, a maioria dos pacientes portadores de DM1 é acompanhada em serviços públicos do SUS, restando uma minoria atendida exclusivamente pela rede privada de saúde, até mesmo devido ao acesso aos insumos disponibilizados aos pacientes pelo SUS. Em nosso estudo a classificação/diagnostico de DM1 não foi confirmada pela dosagem de auto anticorpos e o peptídeo C também não foi realizado, porém é importante ressaltar que mais de 96% dos pacientes incluídos neste estudo tiveram o diagnóstico de DM1 antes dos 30 anos de idade. Por fim, o diagnóstico de RD foi realizado por oftalmoscopia indireta e não por retinografia digital. Apesar das limitações inerentes a este método, estudo prévio do nosso grupo (109) mostrou alta concordância entre estes métodos e todos os oftalmologistas que realizaram o exame receberam treinamento prévio por um especialista em retina.

CONCLUSÃO

Com este estudo objetivamos estudar alguns marcadores genéticos e sua possível influência na RD e na qualidade de vida dos pacientes portadores de DM1 no Brasil. Conseguimos identificar que os principais alelos de risco (HLA-DRB1*03 e HLA-DRB1*04) e de proteção (HLA-DRB1*07 e HLA-DRB1*13) em nossa amostra se correlacionam com os alelos encontrados em populações caucásicas. Além disso, observamos que a ancestralidade genômica africana foi positivamente associada à presença de RD grave. Tal fato nos aponta para uma possível influência genética no desenvolvimento de RD. Porém, não encontramos associações fortes da RD com os genes do HLA de classe II em nossa análise. Provavelmente, devido ao caráter inflamatório da fisiopatologia da RD, especialmente associado ao estresse oxidativo e dano endotelial, outras regiões do nosso genoma devem estar mais fortemente associadas a esta importante complicação.

Por fim, observamos que a ancestralidade genômica africana foi associada a menor qualidade de vida relacionada à saúde, mostrando que também é um fator a ser considerado durante o acompanhamento dos nossos pacientes.

Novos estudos são necessários para melhor elucidar os mecanismos genéticos associados a esta importante doença e permitir detecção precoce e consequente melhoria em prevenção e controle.

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APÊNDICE –Self-reported color-race and genomic ancestry in an admixed population: A contribution of a nationwide survey in patients with type 1 diabetes in Brazil. (Artigo publicado, co-autoria)

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Self-reported color-race and genomic ancestry in an admixed population: A contribution of a nationwide survey in patients with type 1 diabetes in Brazil ☆

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ABSTRACT

Aims: The development of type 1 diabetes (T1D) and its chronic complications may have a genetic background. The primary objective of our study was to characterize the relationship between self-reported color-race and genomic ancestry (GA) in patients with T1D. As secondary objective, we aimed to characterize GA of patients with T1D from different urban geographical regions of Brazil, compared to healthy Brazilian controls from the same regions.
Methods: This was a cross-sectional, nationwide survey conducted in 14 public clinics from 10 Brazilian cities. Global and individual GA were inferred using a panel of 46 ancestry informative markers (AIMs) in 1698 T1D patients. Ancestry percentage was compared with published data of Brazilian healthy controls (n = 936) for the same AIMs.
Results: A higher median individual European ancestry was observed in T1D patients in comparison to controls 67.8 [31.2] vs. 56.3 [25.7]%, respectively (median [IQR]; $p < 0.001$). As for self-reported color-race in T1D group, 923 (54.3%) participants reported to be White, 610 (35.9%) Brown, 132 (7.8%) Black, 18 (1.1%) Asian and 15 (0.9%) Indigenous. European GA prevailed in those who self-reported as White (74.6%) and Brown (61.1%) and constituted 39.1% in Black self-reported patients.
Conclusions: Our study showed that T1D patients presented a higher percentage of European GA than the healthy population. Additionally, European GA was found in a considerable percentage of T1D patients who self-reported as non-White. Further studies are necessary to establish the influence of GA in the development of T1D as well its related chronic complications in admixed populations.

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1. Introduction

Type 1 diabetes (T1D) is an autoimmune chronic disease with an incidence that varies worldwide and is increasing in developed and in developing countries [1]. It is also a polygenic condition that has been related to an interaction among environmental and genetic factors [2]. Although almost 50% of its genetic predisposition is conferred by the major histocompatibility complex (MHC), up to 20% of the genetic predisposition to T1D has been linked to other genes out of the MHC complex [3,4]. The influence of genetic factors in the development of T1D and its related chronic complications is not completely understood due to differences in the frequencies of alleles that are related to the disease and can possibly reflect the variation of its incidence in different populations [2,5]. This variation can also be linked to differences in the ancestry background, migration and miscegenation rates among these populations [5]. This fact may limit the extrapolation of results obtained in one specific population to another. So far, most genetic studies in T1D have been performed in homogeneous populations, i.e., Caucasians with European ancestry [6,7]. In general, these studies have used self-reported color-race instead of genomic ancestry (GA) to stratify participants according to ethnicity [8]. However, this classification can result in biases for studies with allele frequencies, leading to false associations, mainly in admixed populations [9]. This fact could be related to distinct allelic frequencies in the original population resulting from different admixture degrees due to disproportionate ancestral heritage, even from one chromosome to another [8,10]. So, it is important to perform studies in admixed populations aiming to evaluate the relationship between self-reported color race, ancestry and allele frequencies [11,12].

Brazil has a heterogeneous population, formed basically by three ancestral roots that are highly admixed: Native Amerindians (NAM), Europeans (EUR) and Africans (AFR). The colonization started by the coast, with Europeans (particularly Portuguese) and Africans, spreading gradually to the internal part of the country that was originally populated by NAM. Afterwards, migrations and miscegenation have occurred, explaining the strong Brazilian's genetic variability. Since 1991, self-reported color-race has been used for Brazilian population censuses and is divided into five color-race groups: White, Black, Brown (*parda*), Asian (*amarela*) and Indigenous (*indígena*) [13].

Currently the risk of misclassification can be minimized by the availability of methodologies that allow the inference of GA, like AIMs. The use of this tool provides more knowledge on genes' structure and diversity in populations with a heterogeneous ancestry, such as the highly admixed Brazilian population [14], making possible a more efficient stratification of these populations [15].

We aimed to characterize the relationship between self-reported color-race and GA in T1D patients. As secondary objective, we aimed to characterize GA of patients with T1D from different urban geographical regions of Brazil, in comparison to a sample of healthy Brazilian controls from the same regions.

2. Subjects and methods

2.1. T1D participants

This was a cross-sectional, multicenter study conducted between August 2011 and August 2014 in 14 public clinics located in 10 Brazilian cities in five geographical regions (North, Northeast, Midwest, Southeast and South) with 1760 T1D participants. The methods have been described previously [16]. Briefly, T1D patients received health care from the SUS (National Brazilian Health Care System) and have been diagnosed by the presence of typical clinical presentation of T1D, including variable degrees of hyperglycemia, weight loss, polyuria, polydipsia, polyphagia and the need of continuous insulin use since the diagnosis. Included patients had at least 13 years of age, and were followed at each diabetes center for at least 6 months. Each clinic provided data from at least 50 patients with T1D who regularly attended the diabetes center.

This study was approved by the ethics committee of Pedro Ernesto University Hospital (Rio de Janeiro State University) and by the local ethics committee of each center. Written informed consent was obtained from all participants or their parents.

A standardized questionnaire was also applied during a clinical visit to evaluate clinical and demographic data such as gender, current age, birthplace, self-reported color-race, age at diagnosis and duration of diabetes.

2.2. DNA extraction and AIM-Indel genotyping

Genomic DNA was extracted from peripheral blood with the commercial kit SP QIA symphony by automation with QIA symphony equipment, following manufacturer's instructions (Qiagen, USA).

The global and individual GA was inferred using a panel of 46 AIM-INDEL, with a protocol described by Pereira et al. [17]. Genotyping was done by multiplex PCR followed by capillary electrophoresis with ABI 3500 sequencer. Allele naming was performed with the software Gene Mapper V.4.1 (Life Technologies, USA). We used the Structure V.2.3.3 software to estimate ancestry and the HGDP-CEHP diversity panel (Sub-Set H952) as reference date of ancestral populations.

The allele frequency of genotyped 46 AIM-INDELs and the T1D sample ancestry (n = 1698) were compared with published data of a Brazilian healthy population for the same markers, obtained from 936 unrelated healthy controls from the same geographic and metropolitan areas: Southeast – 509; Midwest – 84; North – 42; Northeast – 237 and South – 64 [18].

2.3. Statistical analysis

Estimates of individual and global ancestry were performed with the Structure software v2.3.3 [19]. Structure ran with 100,000 burning steps followed by 100,000 Markov Chain Monte Carlo (MCMC) interactions using the "Admixture MOD-

EL" default correlating allele frequencies and the number of populations (K = 3), designated as EUROPEAN (EUR), AFRICAN (AFR) and NATIVE AMERINDIAN (NAM). Kruskal-Wallis test was used to compare the GA percentage of patients relative to self-reported color-race, followed by multiple comparisons test with Action Software. The comparison of GA between T1D and controls was performed by Mann Whitney test. The comparison of EUR Ancestry among the five geographical regions was performed by ANOVA with Bonferroni correction or Kruskal-Wallis. Only patients who self-reported themselves as White, Black, and Brown were included in the analyses of GA and self-reported color race, due to the small number of patients that self-declared as Asians or Indigenous. Continuous data were expressed as median, interquartile range [IQR] or means (SD) when indicated. Categorical data were expressed as count or percentage. A two-sided p value less than 0.05 was considered significant.

Molecular diversity indices such as the observed allele frequency and expected heterozygosis, Hardy-Weinberg, Fixation Index (FST) values and p value between the genetic distances were calculated using Arlequin v.3.5 software [20]. The multidimensional scaling plot (MDS) of the pair wise FST matrix was used to represent the genetic distances between the assumed groups, using the software STATISTICA v7.0 (Statsoft, Tulsa, Oklahoma; http://www.statsoft.com/) and for the other analyses we used the Statistical Program for Social Sciences version 17.0 (SPSS, Inc., Chicago, Illinois).

3. Results

3.1. Overview of the studied population

For this study, 1698 (96.5%) T1D participants were enrolled; 62 (3.5%) patients were excluded because the DNA extraction was not adequate. Table 1 shows the demographic data of the enrolled population. The distribution by geographical region showed that in South region, control group had less individuals in comparison to patients with T1D ($p < 0.05$).

3.2. Overview of the studied population according to genetic ancestry

At intrapopulation level, GA heterogeneity was observed with a wide range of variation of ancestry proportions (Fig. 1): EUR (2.3–99.1%); AFR (0.2–88.7%) and NAM (0.3–69.5%). This fact was also noted in controls: EUR (4.7–92.9%), AFR (2.6–87.2%) and NAM (0.3–69.5%).

A higher median individual EUR ancestry proportion was observed in T1D patients in comparison to controls 67.8 [31.2] vs. 56.3 [25.7]%, respectively (median [IQR]; $p < 0.001$). T1D participants had lower percentage of NAM contribution in comparison to controls (10.5 [16.1] vs. 16.0 [14.2]%, $p < 0.001$) respectively, and a lower median individual proportion of AFR GA in comparison to controls (15.8 [23.3] vs 22.5 [22.7]%, $p < 0.001$), respectively. Only patients with T1D (N = 110, 6.3%) showed an EUR GA greater than 95%. These patients had a contribution of African and Native Amerindian GA of 1.55 [1.11] and 1.80 [1.12]%, respectively. No patients with T1D or controls had African or Native Amerindian GA greater than 95%.

Table 1 – Clinical and demographic data of the studied population.

Variable	T1D	Controls
N	1698	936
Female, n (%)	945 (55.7)	468 (50.0)
Age, y	30.06 ± 11.9	32.2 ± 8.8
Duration of diabetes, y	15.5 ± 9.3	
Self-reported color-race[#]		
White	923 (54.3)	
Black	132 (7.8)	
Brown[*]	610 (35.9)	
Asian[*]	18 (1.1)	
Indigenous	15 (0.9)	
Economic status		
High	52 (3.1)	
Medium	774 (45.6)	
Low	816 (48.1)	
Very low	56 (3.3)	
Geographic region n (%)		
Southeast	803 (47.3)	509 (54.4)
Northeast	465 (27.4)	237 (25.3)
South[#]	231 (13.6)	64 (6.8)
Midwest	154 (9.1)	84 (9.0)
North	45 (2.7)	42 (4.5)

y = year; data are presented as number (percentage) or mean ± standard deviation.
[*] IBGE classification: Asian – *Amarela*; Brown – *Parda*.
[#] The number of T1D participants and controls were not matched in the South region.

Brazil is divided in five geographical regions (North, Northeast, Midwest, Southeast and South). The estimates of GA of T1D patients and controls, by geographical region, can be seen in Fig. 2 and Table 2. A higher percentage of EUR GA contribution in T1D participants in comparison to controls was observed in all geographical regions. A lower percentage of AFR and NAM ancestry was noted in T1D in comparison to controls. A higher EUR GA was found in T1D group and controls from South region in comparison to all other regions ($p < 0.001$).

A significant decrease in EUR GA was observed from the South to the North region in control subjects ($p < 0.001$), which was not observed in T1D patients (Supplementary Fig. S1).

Pairwise genetic distances listed in supplementary Table S1 show significant differences between all T1D samples and the ancestral populations ($p < 0.0008$, after applying Bonferroni's correction for multiple tests). T1D groups were found to be genetically close to control groups, and all the inferred groups also showed greater proximity to the HGDP-CEPH European population. In order to illustrate the genetic distances a MDS plot based on pairwise FST matrix was created (Supplementary Fig. S2). In general, the Brazilian regional populations are all closer to the Europeans, although the positions in the MDS plot of the Northern populations indicate a significant NAM contribution. Genetic distances to NAM are lower for populations in the North and higher for populations in the South; the lowest genetic distances to EURs are noted in Southern populations. Finally, the genetic

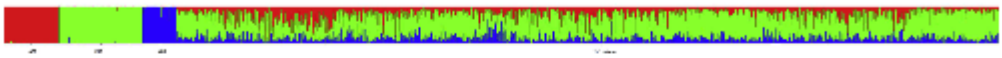

Fig. 1 – Individual ancestry estimates obtained for the HGDP-CEPH reference samples and T1D participants tested from Brazilian population using 46 AIM-INDELs. AFR: African; EUR: European; NAM: Native Amerindian; T1D Patients: participants with type 1 diabetes. Ancestry estimates were obtained using STRUCTURE, for the following options: k=3; 100,000 burning steps followed by 100,000 MCMC iterations; Admixture model ("Use population Information to test for migrants"); and allele frequencies were correlated and updated using only individuals with POPFLAG=1.

composition of the Northeast, Midwest and Southeast regions is very similar, with slightly lower genetic distances to AFR when compared to populations from the North or the South.

3.3. *Overview of the participants with type 1 diabetes according to genomic ancestry and self-reported color-race*

As for self-reported color-race, 923 (54.3%) T1D patients reported to be White, 610 (35.9%) Brown, 132 (7.8%) Black, 18 (1.1%) Asian and 15 (0.9%) Indigenous. Fig. 3 represents a box plot of the percentage of GA within the three major self-reported color-race groups considered in the analysis (White, Brown and Black). Regarding EUR GA, there were patients in all three self-reported categories within the range of 25.0 to 85.0% for ancestry proportion. On the other hand, for AFR GA, this overlap ranged from 5.0 to 45.0%, and for NAM GA the range was from 5.0 to 50.0%. EUR GA has predominated in all groups, except in patients who self-

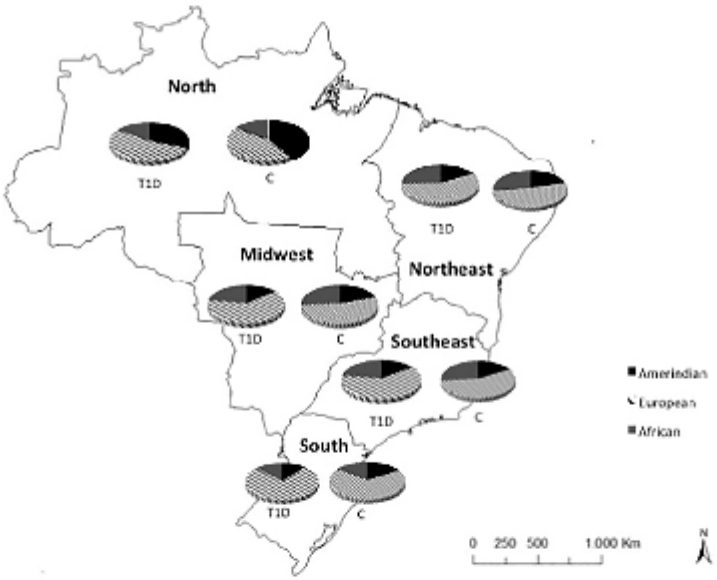

Fig. 2 – Geographical distribution of genomic ancestry in Brazil.

Table 2 – Comparison of Genomic ancestry between T1D and controls according to Brazilian geographical regions.

Geographical region	European		African		Native Amerindian	
	T1D	Control	T1D	Control	T1D	Control
Southeast	67.0 [27.7]	58.3 [26.4]*	16.5 [21.5]	22.5 [27.9]*	11.5 [15.8]	14.2 [12.3]
Midwest	70.6 [28.0]	56.6 [20.6]*	16.5 [23.7]	24.6 [13.9]*	7.0 [34.6]	18.5 [11.6]*
North	65.1 [30.3]	44.6 [27.7]*	12.0 [18.4]	14.3 [12.1]	16.2 [21.0]	36.5 [28.8]*
Northeast	62.0 [32.2]	51.7 [20.6]*	21.2 [25.2]	26.4 [18.0]*	11.9 [15.6]	19.3 [16.1]*
South	83.0 [22.7]	71.7 [19.0]*	5.5 [12.4]	11.3 [13.2]	6.7 [15.8]	14.8 [11.7]*

Data are presented as median individual proportion,% and [IQR]. T1D – participants with Type 1 diabetes.
* $p < 0.01$, T1D vs control.

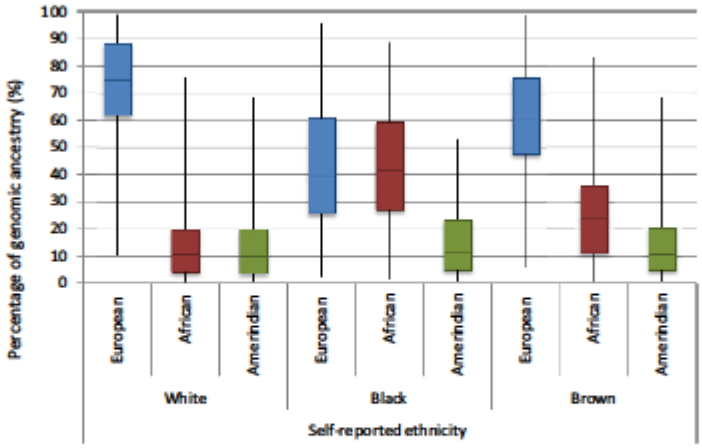

Fig. 3 – Boxplot representing genomic ancestry within self-reported color-race groups. European – European AIM ancestry; African – African AIM ancestry; Amerindian – Native American AIM ancestry. Median (solid line), First and Third Quartiles (box). Medians: White (74.6% European, 10.7% African, and 9.8% Native Amerindian), Black (39.1% European, 41.4% African, and 11.7% Native Amerindian), and Brown (61.1% European, 23.7% African, and 10.4% Native Amerindian).

reported as Black ($p < 0.0001$). A higher AFR GA was noted in patients who self-reported as Black in comparison to the other groups ($p < 0.001$). No significant difference in NAM GA was observed in those who self-reported as White, Black and Brown.

4. Discussion

Our study showed that T1D patients from different urban geographical regions of Brazil have a higher predominance of EUR ancestry and lower percentage of NAM and AFR ancestry than healthy Brazilian populations from similar geographical regions. It is important to emphasize that in both groups (T1D patients and healthy controls), we noted a higher proportion of EUR ancestry, followed by AFR ancestry. In both groups, the lowest observed ancestry was the NAM. Although EUR ancestry predominates in patients with T1D that self-reported color-race as White, a considerable proportion of this GA was also found in patients who self-reported as brown and black.

The Brazilian population is estimated to be of approximately 201 million, with almost 84.0% living in urban areas, and is considered to be one of the most admixed populations in the world. According to the last national census conducted by the Brazilian Institute of Geography and Statistics (IBGE) [13], self-reported color-race in Brazil was composed by White (47.5%), Black (7.5%), Brown (43.4%), Asian (1.1%) and Indigenous (0.9%) with a great variability among the geographical regions. The use of self-reported color-race for ethnic evaluation in admixed groups like the Brazilian population has been a matter of debate. For instance, Lima-Costa et al. [21] identified that the relationship between self-reported color-race and GA becomes more reliable when individuals are in the highest quartile of a specific GA. However, cases of misclassification were observed in all self-reported ethnic groups. It is possible that in our study some patients have been misclassified since EUR GA varied from 74.6% in patients who self-reported as White to 39.1% in patients who self-reported as Black and 61.1% in patients who self-reported as Brown. Patients who self-reported as Black or Brown could be considered highly admixed and represent a high percentage of the participants in this study (43.7%), according to the self-reported color race. Only patients with T1D (6.3%) showed an EUR GA greater than 95% with a very low contribution of African and Native Amerindian GA. These patients could probably be Non-Admixed as has recently been proposed [22].

According to the IBGE [23], color-race self-report is stepped (in order of relevance) taking in account characteristics such as: skin color (73.8%); family background (61.6%); physical traits (53.6%); culture/habits (24.9%); socioeconomic level (13.5%) and political option (2.9%). Santos et al. [24] have also reported that 20.0% of the individuals that were interviewed with four months interval visits, declared in each moment that they belonged to a different racial group. A study performed in the Brazilian population failed to point out a good correlation between GA and self-reported color-race in pairs of siblings [25]. Durso et al. [26] who also sought to associate polymorphisms related to skin pigmentation with GA and self-reported color-race in individuals from Rio de Janeiro and São Paulo, concluded that both (skin pigmentation and ancestral markers) were unable to predict the self-attributed skin color of these individuals.

Our study showed that in Brazil, T1D participants had a similar GA pattern observed in healthy subjects with a higher percentage of EUR ancestry followed by AFR and NAM ancestry [27]; however, their EUR Ancestry is significantly higher than that observed in controls from all the geographical

regions. Moreover, a great variability was observed across the country ranging from a median of 62.0 to 83.0% for EUR Ancestry (Northeast to South), 6.7% to 16.2% for NAM (South to North) and from 5.5 to 21.2% for AFR (South to Northeast). This regional variation observed in T1D patients agrees with previous reports from the general Brazilian population [18]. We could conclude that although most of our T1D patients have a predominant EUR ancestral contribution, they also present a genetic background from other ancestral groups, with different levels of admixture, making it difficult to extrapolate results obtained in studies performed in Caucasians to those to be performed with the Brazilian population.

This fact must be evaluated in genetic studies of major histocompatibility complex in admixed populations which remain so far poorly analyzed. A study conducted in the United States (US) with African-American T1D patients [28] showed a higher diversity of HLA haplotypes in African-American than in EUR populations. Some haplotypes that increase the risk for T1D were derived from EUR ancestry but others have not been reported in EUR populations with T1D. This fact was observed despite the lower prevalence of T1D in youths from all minorities, including African Americans compared to Caucasians [29]. In a study conducted by Gomes et al. [30] with T1D patients in São Paulo city showed that most HLA haplotypes that were associated with high risk for T1D were those found in EUR [6], African-American [28] and Asian [31] patients. However, it is important to note that although São Paulo is considered a cosmopolitan city, it probably does not reflect the GA of the whole Brazilian population as we have shown in our study. We attempted to evaluate, from different perspectives, the ancestral genetic composition of the Brazilian population with T1D, comparing our data to a previously published study conducted in healthy controls from similar geographical areas. Other studies analyze regions of the country with low demographic density, specific populations or residents of isolated villages that have remained without intense genetic admixture that frequently happens with populations living in urban cities [9].

Another point that must be evaluated in this context is the relationship between ethnicity and incidence of diabetes and it its chronic-related complications. In the US, the prevalence of T1D in youths is lower in all minorities compared to Caucasians [5] but its incidence is increasing mostly in Hispanics [32]. In Bauru, a southeastern city of Brazil an increasing incidence of T1D during the last three decades was found mainly in Caucasian children [33]. Regarding diabetes-related complications, most of the studies do not separate their data according to diabetes type and show conflicting results as has been described in a recent review [34] .The majority of studies conducted in the US, minorities showed an increased prevalence of retinopathy [35] which was not observed in patients with type 2 diabetes (T2D) in the United Kingdom (UK) after adjustment for retinopathy risk factors [36]. In the US the same was found for nephropathy (end-stage renal disease) [37]. However, in UK, Asians showed an increased prevalence of end-stage renal disease (ESRD)in comparison to Caucasians [38]. Few studies have been performed so far exclusively in patients with T1D. For instance, the Search Study showed a tendency for an increased prevalence of retinopathy in non-Caucasian youth with either T1D or T2D [39] which was not observed regarding albumin excretion rate. No data was described for ESRD [40]. A recent systematic review regarding South Asian patients with T1D did not show difference in the prevalence of retinopathy or nephropathy [41]. However, controversial data have been observed in studies included in this review, mainly because comparisons were done between South Asians and Caucasians. Moreover, in a prospective study with patients with T1D, self-reported ethnicity was not a risk factor for the development of retinopathy [42]. The above-mentioned data showed that not only the prevalence or incidence of T1D could be related to GA but also diabetes-related complications, although a consensus does not exist regarding this relationship. Otherwise, it is important to emphasize that so far the majority of the studies have used self-reported color-race and did not adjust their findings for socioeconomic status and quality of care which are important issues concerning this topic. The influence of GA in the natural history of other chronic diseases has been recently described for asthma [43] with a high clinical and laboratorial severity in Mexican-Americans with European ancestry.

Particular strengths of our study are the population-based ascertainment of diabetes cases in a large sample of Brazilian T1D patients from a wide range of ethnic groups diagnosed in the community setting, from all geographical regions of the country; also, all participating centers followed a uniform and standardized protocol. Similar to other population-based studies, we used a clinical definition of T1D assigned by healthcare providers that was applicable to all patients. Our study stands out because we consider the State of birth for the whole studied population and because we have evaluated only urban populations. We also consider self-reported color-race. To the best of our knowledge this is the first report that has performed a GA study in T1D patients from all geographical regions of Brazil. This geographic localization is affected by socioeconomic and cultural factors that reflect genomic distances and also gives subsidiary distinction between T1D and controls among these regions. Controversially, Midwest region was an exception as controls and T1D are not genetically distant, probably as a result of the recent and continuous migration to this region since Brasília's foundation as the federal capital of Brazil, in 1960, which attracted people from all other geographical regions.

One limitation of our study may be the characteristics of our studied sample. T1D participants had a public specialist care and lived in urban areas of the country. Although we could have missed some information about patients who received a primary attention care and lived in rural areas, they probably represent the minority of patients with T1D under treatment in Brazil. As aforementioned, we used the clinical definition of T1D, however autoantibodies and C-peptide levels were not measured. Although some patients with other types of diabetes might have been included, it is important to emphasize that 96.5% of them were diagnosed before 30 years of age, which reinforces the high probability that they most likely have T1D.

Recently it was shown, thorough cross-validation, experiment that the number of SNPs needed for ancestry inference can be successfully reduced to less than 0.1% of the original 650,000 while retaining close to 100.0% accuracy [44]. Although we used only 46 AIMs, this number has already

proved to be effective in the attribution of Continental GA in tri-hybrid populations such as the Brazilian, being considered efficient for what our study was designed for and also in genetic variability studies conducted with admixed populations, where the goal is usually to minimize the bias that ethnic particularities may bring.

In conclusion, our study showed that T1D patients from all geographical regions of Brazil, belonging to a highly admixed population, presented a higher percentage of EUR GA than the healthy population. Additionally, EUR GA was observed in considerable percentage of those patients with T1D who self-reported as Brown and Black. Further studies are necessary to establish the influence of GA in the development of T1D as well as in the development of diabetes-related chronic complications in admixed populations.

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Conflicts of interest

All authors declare they do not have conflict of interests.

Authors contributions

MBG, ABG, LCP, DAS wrote, analyzed and reviewed the paper. CAN wrote and reviewed the paper. DCS, MHP, BSVB and SAD reviewed the paper. MBG has full access to all study data and takes responsibility for the submission.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.diabres.2018.03.021.

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ANEXO - Aprovação do Comitê de Ética

HOSPITAL UNIVERSITÁRIO PEDRO ERNESTO/ UERJ

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Retinopatia diabética, HLA e ancestralidade no DM1: estudo multicêntrico BrazDiab1SG

Pesquisador: Deborah Conte Santos

Área Temática:

Versão: 1

CAAE: 52537915.9.0000.5259

Instituição Proponente: Hospital Universitário Pedro Ernesto/UERJ

Patrocinador Principal: FUN CARLOS CHAGAS F. DE AMPARO A PESQUISA DO ESTADO DO RIO DE JANEIRO - FAPERJ

DADOS DO PARECER

Número do Parecer: 1.401.697

Apresentação do Projeto:

Este estudo visa utilizar banco de dados de um estudo multicêntrico recém finalizado em pacientes diabéticos tipo 1, sob coordenação da Prof.ª. Marilia de Brito Gomes. Estudo corte transversal multicêntrico em pacientes DM1 (BrazDiab1SG) desenvolvido de 2008 a 2010 em 28 clinicas publicas de atenção secundária e terciária, localizadas em 20 cidades distribuídas nas 5 regiões geográficas brasileiras.

Objetivo da Pesquisa:

Determinar a associação entre ancestralidade e alelos do sistema de histocompatibilidade na prevalência da retinopatia diabética em uma coorte

nacional de pacientes DM1 atendidos em diversos serviços públicos de referencia para tratamento de diabetes no Brasil.

Objetivo Secundário:

Identificar os alelos de risco e classificar os pacientes de acordo com sua carga genética e grau de retinopatia.

Endereço: Avenida 28 de Setembro 77 - Térreo
Bairro: Vila Isabel **CEP:** 20.551-030
UF: RJ **Município:** RIO DE JANEIRO
Telefone: (21)2868-8253 **Fax:** (21)2264-0853 **E-mail:** cep-hupe@uerj.br

Avaliação dos Riscos e Benefícios:

Riscos:

O presente estudo não apresenta risco objetivo ao paciente tendo em vista que apenas utilizará banco de dados existente, sem necessidade de nova coleta de material.

Benefícios:

Possibilitará maior entendimento quanto a fisiopatologia e predisposição ao desenvolvimento de retinopatia diabética a partir da correlação com sua
contribuição genetica.

Comentários e Considerações sobre a Pesquisa:

Este estudo visa utilizar banco de dados de um estudo multicêntrico recém finalizado em pacientes diabéticos tipo 1, sob coordenação da Prof.ª.
Marilia de Brito Gomes /UERJ. A partir do banco de dados, objetivamos correlacionar a presença de retinopatia com dados da ancestralidade e
marcadores geneticos de cada individuo a fim de identificar os potenciais marcadores geneticos associados a esta complicação. Em anexo envio
aprovação prévia do estudo multicentrico pelo comitê de ética, descrição detalhada de coleta de dados realizada e modelo de termo de
consentimento que foi assinado por todos os participantes.

Considerações sobre os Termos de apresentação obrigatória:

Todos os documentos de apresentação obrigatória foram enviados a este Comitê, estando dentro das boas práticas e apresentando todas dados necessários para apreciação ética.

Recomendações:

Conclusões ou Pendências e Lista de Inadequações:

O trabalho pode ser realizado da fora como está apresentado. Diante do exposto e à luz da Resolução CNS nº466/2012, o projeto pode ser enquadrado na categoria – APROVADO. Para ter acesso ao PARECER CONSUBSTANCIADO: Clicar na "LUPA" (DETALHAR) - Ir em "DOCUMENTOS DO PROJETO DE PESQUISA ", clicar na opção da ramificação (pequeno triangulo no entrocamento do organograma) de pastas chamada – "Apreciação", e depois na Pasta chamada "Pareceres", o Parecer estará nesse local.

Considerações Finais a critério do CEP:

Tendo em vista a legislação vigente, o CEP recomenda ao Pesquisador: 1. Comunicar toda e qualquer alteração do projeto e termo de consentimento livre e esclarecido. Nestas circunstâncias a inclusão de pacientes deve ser temporariamente interrompida até a resposta do Comitê, após análise das mudanças propostas. 2. Os dados individuais de todas as etapas da pesquisa devem ser mantidos em local seguro por 5 anos para possível auditoria dos órgãos competentes. 3. O Comitê de Ética solicita a V. Sª., que encaminhe relatórios parciais e anuais referentes ao andamento da pesquisa ao término da pesquisa encaminhe a esta comissão um sumário dos resultados do projeto.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_641181.pdf	08/12/2015 18:20:57		Aceito
Outros	CEP_aprovacao.jpg	08/12/2015 18:19:28	Deborah Conte Santos	Aceito
Outros	PROJETO_CNPQ_Edital_Aprovado_CEP.doc	08/12/2015 18:14:39	Deborah Conte Santos	Aceito
Outros	DeclaracaoDeCiencia.jpg	08/12/2015 18:13:20	Deborah Conte Santos	Aceito
Projeto Detalhado / Brochura Investigador	ProjetoTeseDoutorado.docx	08/12/2015 18:11:21	Deborah Conte Santos	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE.docx	08/12/2015 18:10:57	Deborah Conte Santos	Aceito
Folha de Rosto	FolhaDeRosto.docx	08/12/2015 18:10:04	Deborah Conte Santos	Aceito

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

RIO DE JANEIRO, 03 de Fevereiro de 2016

Assinado por:
WILLE OIGMAN
(Coordenador)

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