UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

"TWIN PEAKS": INVESTIGANDO MISTÉRIOS SOBRE A GEMELARIDADE NO BRASIL

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Sumário

Lista de abreviaturas, símbolos e unidades

‰	Por mil
ANVISA	Agência Nacional de Vigilância Sanitária
CG	Cândido Godói
DZ	Dizigótico
ExAC	*Exome Aggregation Consortium* – Consórcio de Agregação de Exomas (Acesso em: http://exac.broadinstitute.org/)
FIV	Fertilização *in vitro*
FSH	*Follicule Stimulating Hormone* – Hormônio Folículo Estimulante
FSHB	*Follicule Stimulating Hormone Beta Subunit* – Subunidade Beta do Hormônio Folículo Estimulante
FSHR	*Follicule Stimulating Hormone Receptor* – Receptor do Hormônio Folículo Estimulante
GWAS	*Genome Wide-Association Study* – Estudo de Associação Genômica Ampla
IBGE	Instituto Brasileiro de Geografia e Estatística
IDH	Índice de Desenvolvimento Humano
INaGeMP	Instituto Nacional de Genética Médica Populacional
km	Quilômetro
LD	*Linkage Disequilibrium* – Desequilíbrio de Ligação
LH	*Luteinizant Hormone* – Hormônio Luteinizante
LSP	Linha São Pedro
MIM	*Mendelian Inheritance in Man* – Herança Mendeliana no Homem (Acesso em: https://www.omim.org/)
mRNA	*Messenger RNA* – RNA Mensageiro
MTHFR	*Methylenetetrahydrofolate Reductase* – Metilenotetrahidrofolato Redutase
MZ	Monozigótico
RMA	Reprodução Medicamente Assistida
RN	Recém-nascido
RS	Rio Grande do Sul

SAM	S-adenosil metionina
SIM	Sistemas de Informações sobre Mortalidade
SINASC	Sistema de Informações sobre Nascidos Vivos
SMAD3	*SMAD Family Member 3* – Membro 3 da Família SMAD
SNPs	*Single Nucleotide Polymorphisms* – Polimorfismos de Nucleotídeo Único
TG	Taxa Gemelar
TNF	*Tumor Necrosis Factor* – Fator de Necrose Tumoral
TP53	*Tumor Protein P53* – Proteína Tumoral 53
VEGFA	*Vascular Endothelial Growth Factor A* – Fator de Crescimento Endotelial Vascular A

Resumo

O nascimento de gêmeos na espécie humana é rodeado por muitos mistérios e, há bastante tempo, desperta a curiosidade tanto leiga quanto científica. Os estudos com gêmeos vêm contribuindo para o entendimento de diversas áreas da biologia humana; porém, aspectos epidemiológicos e etiológicos relacionados aos nascimentos gemelares em si permanecem pouco elucidados. Para além de mera curiosidade, pesquisas nessa área podem ajudar a compreender mecanismos relacionados à reprodução em geral, além de serem importantes em um contexto de saúde pública, já que a gestação gemelar representa riscos adicionais à mãe e à prole. Em países industrializados observa-se um aumento acentuado nas taxas gemelares (TGs) que parece estar relacionado ao aumento da idade materna na primeira gravidez e tecnologias de Reprodução Medicamente Assistida (RMA). No Brasil, os aspectos geográficos e sociodemográficos dos nascimentos gemelares ainda não foram profundamente estudados. Assim, este estudo objetivou responder a três questões principais relacionadas aos nascimentos de gêmeos: "Quantos?" "Onde? e "Como?", isto é, considerando aspectos epidemiológicos e etiológicos. Para o estudo epidemiológico, desenhamos um estudo de base de dados populacional e investigamos as TGs em duas dimensões: espacial e temporal. Para isso, utilizamos dados provenientes do Sistema de Informações sobre Nascidos Vivos (SINASC) e analisamos mais de 41 milhões de nascimentos que ocorreram em todos os 5.565 municípios brasileiros entre 2001 e 2014. Encontramos uma TG nacional média de 9,41 por 1.000 nascimentos e o modelo de análise de séries temporais revelou tendência de aumento global ao longo do período estudo, mas com notáveis diferenças regionais. De fato, resultados da "Análise de Cluster e Outlier" (*Anselin Local Moran's I*) revelaram concentração de municípios com altas TGs em uma área que vai do sul do Nordeste brasileiro até o Rio Grande do Sul (Global Moran Index = 0.062, $P < 0.001$). Além disso, encontramos correlação positiva entre o Índice de Desenvolvimento Humano (IDH) local e as TGs em diferentes cenários, sugerindo que o IDH pode ser um importante indicador de RMA no Brasil. Nossas análises também revelaram aumento de 26.42% na TG entre as mulheres com mais de 44 anos durante o período estudado. A tendência de aumento temporal encontrada para algumas regiões do país está de acordo com o que é observado em outros países industrializados, enquanto que a análise geográfica revelou duas situações bem distintas dentro do Brasil. Por sua vez, os

estudos acerca da etiologia dos nascimentos de gêmeos foram concentrados em investigar polimorfismos de nucleotídeo único (SNPs) em mães de Cândido Godói (CG), uma pequena cidade do Sul do Brasil conhecida como a "Cidade dos Gêmeos". Este título é devido à alta TG observada no município e à recorrência do traço nas famílias locais, com forte descendência europeia. Nós desenhamos um estudo caso-controle e genotipamos sete SNPs relacionados à foliculogênese (rs6166:C>T em *FSHR*, rs11031006:G>A próximo a *FSHB*, e rs17293443:T>C in *SMAD3*) ou a gestações de sucesso (rs1801131:T>G e rs1801133:G>A em *MTHFR*, rs2010963:C>G em *VEGFA*, e rs1800629:G>A em *TNF*) em 44 mães de gêmeos (casos) e 102 mães de filhos únicos (controles), todas residentes de CG. Para todos os SNPs, a distribuição das frequências alélicas e genotípicas foi similar entre casos e controles. Diferentes combinações de alelos de risco e análises haplotípicas também foram homogeneamente distribuídas entre ambos os grupos. Assim, estes resultados sugerem uma ausência de associação entre os nascimentos gemelares em CG e sete SNPs relacionados à foliculogênese ou a gestações de sucesso, mas é possível que outras variantes genéticas ligadas a ambos os processos possam estar envolvidas neste fenômeno que possui uma base genética subjacente.

Palavras-chave: gêmeos; taxas gemelares; polimorfismos genéticos; foliculogênese; gravidez de sucesso.

Abstract

The birth of twins in human species is surrounded by many mysteries and it has long aroused both lay and scientific curiosity. Studies with twins have contributed to the understanding of several areas of human biology; however, the epidemiological and etiological aspects related to twin births by themselves remain unclear. Beyond mere curiosity, research in this field may help us to understand the mechanisms related to reproduction in general, and it is important in a public health context since a twin pregnancy represents additional risks to the mother and offspring. Some countries have reported a striking increase in twinning rates (TRs), which seems to be related mainly to delayed childbearing and to Medically-Assisted Reproduction (MAR) technologies. In Brazil, the epidemiological scenario of twin births has not been studied while considering its territorial and sociodemographic magnitude. Therefore, this study aimed to answer three main questions related to twin births: "How many?", "Where" and "How?", that is, we investigated twinning in light of epidemiology and etiology. For the epidemiological study, we carried out a population-based study, investigating twin births in two dimensions: spatial and temporal. For that, we used data from Brazil's Live Birth Information System (SINASC), and we analyzed over 41 million births that occurred in all 5,565 Brazilian municipalities between 2001 and 2014. We found an average TR of 9.41 per 1,000, and a first-order autoregressive model of time-series analysis revealed a global upward trend over time, but with important regional differences. In fact, a Cluster and Outlier Analysis (Anselin Local Moran's I) was performed and identified clusters of high TR in an area stretching from the south of Brazil's Northeast Region to the South Region (Global Moran Index = 0.062, $P < 0.001$). Furthermore, we found a positive correlation between the local Human Development Index (HDI) and TRs in different scenarios, suggesting that the HDI may be an important proxy indicator of MAR in Brazil. We also found a sharp increase (26.42%) in TR in women aged over 44 years. The upward temporal trend in TRs is in line with recent observations from other countries, whereas the spatial analysis revealed two very different realities within our country. In turn, studies on the etiology of twin births were focused on single nucleotide polymorphisms (SNPs) in mothers from Cândido Godói (CG), a small southern Brazilian city known as the "Twin's Town". This title is due to the high TR attributed to the municipality and to the recurrence of the twin trait through the

local families, who have a strong European descent. We performed a case-control study and genotyped seven SNPs related to folliculogenesis (rs6166:C>T in *FSHR*, rs11031006:G>A near *FSHB*, and rs17293443:T>C in *SMAD3*) and to a successful pregnancy (rs1801131:T>G and rs1801133:G>A in *MTHFR*, rs2010963:C>G in *VEGFA*, and rs1800629:G>A in *TNF*) in 44 mothers of twins (cases) and 102 mothers of singletons (controls), all of them from CG. For all SNPs, the distributions of the genotypic and allelic frequencies were similar between the cases and controls. Different combinations of risk alleles and haplotypic analyses were also homogeneously distributed between both groups. Thus, these results suggest a lack of association between twin births in CG and seven SNPs related to folliculogenesis or successful pregnancies, but it is possible that other genetic variants linked to both processes may be involved in this phenomenon, which has an underlying genetic basis.

Keywords: twins; twinning rates; genetic polymorphisms; folliculogenesis; successful pregnancy.

CAPÍTULO I

INTRODUÇÃO

1. INTRODUÇÃO

1.1. Sobre o título

"Twin Peaks" é uma famosa série estadunidense de televisão criada na década de 1990 por David Lynch e Mark Frost. À época, esta série revolucionou a linguagem televisiva por conta de, dentre outros, seus personagens idiossincráticos, pela fuga de lugares-comuns presentes em outras obras da época e, sobretudo, pelas altas doses de mistério características do seu roteiro (Lavery 1995; Poniewozik 2007).

Decidimos batizar este trabalho de "Twin Peaks" porque estudamos um fenômeno que é rodeado por semelhante clima de mistério: o nascimento dos gêmeos na espécie humana. Ainda, além de possuir "twin" no nome (do inglês, *gêmeo*), a trama da série desdobra-se seguindo a investigação do fictício agente de investigações Dale Cooper. Em termos gerais, o ofício do cientista em um laboratório pode ser comparado ao do detetive investigativo, e o eminente cientista do século XX, Albert Einstein, fez comparação equivalente em seu livro *The evolution of Physics* (Einstein and Infeld 1938).

Por fim, acreditamos que um trabalho científico pode ser mais atrativo quando situado em contextos além dos jargões e que, quando possível, a Ciência deve ser misturada às outras áreas de produção do conhecimento, incluindo a Cultura Popular. Afinal, antes de serem separadas por pastas acadêmicas, todas elas são derivadas da comum e eterna tentativa humana de entender-se a si mesma e ao mundo.

1.2. A Gemelaridade

A simbologia do zodíaco astrológico, o mito da fundação de Roma, a tradicional rejeição em algumas tribos indígenas, o nascimento de duas crianças dividindo o mesmo corpo e as experimentações nazistas em uma pequena cidade do sul do Brasil. Esta miscelânea de fatos históricos e ficcionais reflete o fascínio dos seres humanos pela gemelaridade ao longo dos séculos. Além de despertar curiosidade, os gêmeos fornecem um caminho para o entendimento de eventos ligados à embriogênese humana e a diversos processos patológicos (Hall 2003).

Nas últimas décadas, tem havido um aumento significativo nas taxas de gemelaridade principalmente nos países desenvolvidos e a busca por explicações para este

fenômeno nunca fora tão latente, sobretudo porque gestações gemelares aumentam o risco de complicações para a gestante e a prole, além de acarretarem custos hospitalares adicionais (Delbaere et al. 2008; Laskov et al. 2013; Dawson et al. 2016). Todavia, dados da epidemiologia dos nascimentos gemelares são pouco estudados em países em desenvolvimento, e no Brasil o cenário é semelhante (Geraldo et al. 2008; Smits and Monden 2011; Otta et al. 2016).

Apesar dos esforços tanto leigos quanto científicos, pouco é conhecido sobre os processos etiológicos ligados ao nascimento de gêmeos. Na literatura especializada, acumulam-se associações entre variáveis genéticas, epigenéticas e ambientais ao fenômeno característico de caráter multifatorial (McNamara et al. 2016). Mesmo assim, nem sempre os resultados são concordantes e é notório que ainda há muito a esclarecer.

1.3.   Tipos de Gêmeos

De acordo com sua origem, os gêmeos podem ser classificados como monozigóticos (MZ), que surgem a partir da divisão de um óvulo fecundado, ou dizigóticos (DZ), que ocorrem a partir da liberação de mais de um óvulo e subsequente fecundação por diferentes espermatozoides. Embora tal dicotomia clássica tenha sido questionada na literatura científica, a divisão em MZ e DZ continua sendo elucidativa em estudos especializados (Shur 2009; Herranz 2015).

Em linhas gerais, a gemelaridade MZ (*Mendelian Inherintance in Man* – MIM: 276410) é considerada um evento pós-fertilização resultante da divisão do precoce embrião ao longo das duas primeiras semanas de desenvolvimento. O período no qual tal fracionamento ocorre estabelece a corionicidade e a amniocidade das gestações (Benirschke 2013; Herranz 2015). Outrora considerados "idênticos", atualmente acumulam-se evidências de que o DNA dos gêmeos MZ apresenta uma miríade de diferenças no tocante a mutações somáticas e mitocondriais, diferenças no controle epigenético, anomalias cromossômicas, dentre outros (Hall 2003; Shur 2009; Benirschke 2013).

Por sua vez, a gemelaridade DZ (MIM: 276400) está relacionada à seleção dos folículos em desenvolvimento contidos no ovário, onde, ao invés de um, dois folículos são maturados e ambos os ovócitos são liberados prontos para a fecundação por diferentes espermatozoides. Logo, os gêmeos DZ apresentam o mesmo nível de identidade genética

que irmãos de gestação única, isto é, compartilham entre si aproximadamente 50% dos seus genes e, evidentemente, podem ser do mesmo sexo ou de sexos opostos (Hoekstra et al. 2007).

1.4.   Epidemiologia

A taxa de gemelaridade (TG) é definida como o número de gestações gemelares por 1000 (‰) nascimentos em um determinado ano. Notavelmente, esta taxa varia de acordo com espaço e tempo observados, a qual, sobretudo em países desenvolvidos, apresentou um acentuado declínio por volta de 1900 e, em contraste, passou a aumentar constantemente a partir de 1970 (Hoekstra et al. 2007; Smits and Monden 2011).

Nos Estados Unidos, por exemplo, a TG passou de 9,5‰ em 1975 para 16,9‰ em 2011, representando um aumento de aproximadamente 75%. Durante o mesmo período, também aumentou em outros países industrializados como Inglaterra e País de Gales (de 9,9 a 16,1‰), Alemanha (de 9,2 a 17,2‰), Dinamarca (de 9,6 a 21,2‰) e Coreia do Sul (5,0 a 14,6‰) (Burt and Klump 2012; Pison et al. 2015; Dawson et al. 2016).

O aumento da idade materna na época do nascimento e o uso de técnicas ligadas à Reprodução Medicamente Assistida (RMA) são tidos como possíveis responsáveis dos modernos aumentos na taxa de nascimentos gemelares (Shur 2009; Boothroyd 2016; Dawson et al. 2016). Pison et al. (2015) ressaltaram que a prática da RMA ocuparia um protagonismo no aumento da TG, levantando uma importante questão de saúde, visto que nascimentos gemelares estão ligados a complicações tanto para a mãe quanto para os bebês.

O continente africano e, em especial, a África Central representa uma vasta zona onde são encontradas as maiores taxas de nascimentos gemelares, com países como Guiné Equatorial e República Democrática do Congo apresentando taxas acima de 18,0‰. O maior índice mundial foi observado em Benin, com 27,9 nascimentos gemelares a cada 1.000 nascimentos (Nylander 1969; Smits and Monden 2011). Nestas situações reforça-se a importância de outras causas que a não a RMA nos nascimentos gemelares, como predisposição genética ou fatores ambientais particulares. Em geral, países da Ásia e América do Sul apresentam índices menores (de abaixo de 9,0 a 12,0‰) (Aisien et al.; Smits and Monden 2011).

O trabalho de Smits and Mondem (2011) refere uma TG para o Brasil de 8,8‰. Embora muito informativo, o estudo é baseado em dados de 1996 e não considera a diversidade territorial e sociodemográfica do Brasil. Outros poucos estudos têm focado neste tema e os dados disponíveis são geralmente restritos a estados como São Paulo e Rio Grande do Sul, com resultados discordantes e prevalências maiores em São Paulo (Colletto and Beiguelman 2001; Colletto 2003; Geraldo et al. 2008; Otta et al. 2016).

Até pouco tempo, considerava-se que as diferenças na TG entre os países e ao longo do tempo fossem devidas à variação na gemelaridade DZ, pois a epidemiologia de gêmeos MZ mantinha-se constante ao redor do mundo (aproximadamente 4,0‰) (Hall 2003; Hoekstra et al. 2007). Todavia, observou-se que a prática da RMA também pode influenciar os índices de gemelaridade MZ (Parazzini et al. 2016; Sobek et al. 2016; Dawson et al. 2016).

1.5. Etiologia da Gemelaridade

Não há, na literatura especializada, uma causa *sui generis* para a gemelaridade. Atualmente, conhece-se um conjunto de fatores genéticos e ambientais que, embora não consensuais, se somam e podem tornar determinada pessoa (em especial mulheres), famílias ou ainda populações inteiras mais suscetíveis aos nascimentos gemelares. A distribuição destes fatores não é homogênea entre gêmeos MZ e DZ, e a maior parte das explicações pode ser aplicada apenas à gemelaridade DZ.

1.5.1. Fatores Ambientais

Como já mencionado, existe uma forte correlação entre procedimentos ligados à RMA e a TG em países desenvolvidos. Tal associação é devida às estratégias relacionadas ao próprio método, como indução da ovulação e implantação de múltiplos embriões, potencializando as taxas de gemelaridade DZ e MZ, de modo que o nascimento de gêmeos é considerado o evento adverso mais comum relacionado à RMA (Nakasuji et al. 2014; Boudjenah et al. 2014; Sobek et al. 2016).

Outro fator que parece influenciar a gemelaridade (sobretudo DZ) é a idade materna avançada (Lambalk et al. 1998; Dawson et al. 2016). A notável diminuição da TG no início do século XX reflete, dentre outros fatores, a diminuição da idade materna média,

enquanto que seu aumento a partir de 1970 também tem sido associado a uma idade materna elevada ao nascimento (Hall 2003; Helle 2008). O mecanismo subjacente proposto seria a presença de distúrbios em decorrência da idade avançada no mecanismo de retroalimentação responsável pela liberação do FSH, mas o assunto ainda permanece alvo de debate (Simoni and Casarini 2014).

Demais fatores maternos associados à gemelaridade DZ seriam maior paridade, etnia, status socieconômico, taxa de ovulação, composição corporal, variação sazonal, tabagismo e influência dietética (Hankins and Saade 2005; Steinman 2006; Morales-Suárez-Varela et al. 2007; Hoekstra et al. 2007; Shur 2009; Steinman 2009; Dawson et al. 2016). Uma possível associação com o ácido fólico permanece controversa (Haggarty et al. 2006; Muggli and Halliday 2007; Enciso et al. 2016).

Entretanto, fatores ambientais não são suficientes para explicar por completo a ocorrência de gemelaridade, sobretudo porque têm sido relatadas comunidades livres da influência dos fatores ambientais mais comumente associados (como metodologias ligadas à RMA), além de que, em alguns casos, o fenótipo "ter gêmeos" é claramente prevalente em determinados núcleos familiares (Matte et al. 1996; Hoekstra et al. 2008; Shur 2009).

1.5.2. Fatores Genéticos

A ligação entre caracteres genéticos e nascimentos gemelares tem sido explorada pela comunidade científica há tempos, em grande parte através de estudos familiares onde foi possível identificar forte predisposição intrafamiliar ao nascimento de gêmeos DZ (Hall 2003). Outrora considerada um traço passado exclusivamente pela mãe, a gemelaridade DZ hoje em dia é compreendida como sendo transmitida tanto via materna quanto paterna, com expressão limitada ao sexo feminino (Hoekstra et al. 2007; Shur 2009).

Em linhas gerais, a gemelaridade DZ parece ser influenciada por muitos genes e não demonstra ser um simples fenótipo dominante ou recessivo (Derom et al. 2006; Hoekstra et al. 2007). Além disso, é possível que fatores epigenéticos também possam estar relacionados (Burt and Klump 2012; McNamara et al. 2016). Por sua vez, a genética da gemelaridade MZ permanece ainda mais enigmática e não existem componentes definitivamente implicados, muito embora a participação de um componente genético seja considerada "indubitável" (Shur 2009; Herranz 2015; Mbarek et al. 2016).

Tem sido possível a caracterização de genes ou variantes genéticas associadas ao nascimento de gêmeos DZ, seja por abordagem gene-cadidato (Al-Hendy et al. 2000; Palmer et al. 2006; Tagliani-Ribeiro et al. 2011; Sirugo et al. 2012; Huang et al. 2015) ou por estudos livres de hipótese (Derom et al. 2006; Palmer et al. 2006; Painter et al. 2010; Mbarek et al. 2016). Porém, os resultados nem sempre são concordantes e não tem sido possível a identificação de genes maiores de predisposição.

Extensamente relacionada ao mecanismo de poliovulação, a genética da gemelaridade DZ tem sido implicada, embora não exclusivamente, à rota do hormônio folículo estimulante (FSH) (Al-Hendy et al. 2000; Hoekstra et al. 2007; Simoni and Casarini 2014; Mbarek et al. 2016). Em consonância, a incidência da gemelaridade DZ espontânea tem sido também proposta como um potencial marcador para fecundidade (Derom et al. 2011).

Além da rota do FSH, alguns outros candidatos foram implicados nos nascimentos gemelares, como o *PPARG* (Busjahn et al. 2000), *PTX3* (Sirugo et al. 2012), *GDF9* (Palmer et al. 2006) e *FIGLA* (Palmer et al. 2006), cuja investigação destes genes não foi incluída neste trabalho. Ao invés disso, decidimos investigar variantes genéticas relacionadas a uma gestação de sucesso (tanto em *MTHFR*, quanto em *VEGFA* e *TNF)*, pois, independentemente dos fatores precoces ligados à fecundação em si, é preciso que haja um organismo preparado para manter uma gestação gemelar.

1.5.2.1.   A rota do FSH

A múltipla ovulação tem sido considerada o fenômeno biológico fundamental da gemelaridade, porém, é flagrante que suas bases genética e molecular permanecem pobremente compreendidas (Sirugo et al. 2012). De fato, a gemelaridade DZ espontânea tem-se mostrado associada a um aumento na concentração de FSH na mãe, dada a sua relevante função de estimular o crescimento e a maturação do folículo ovariano (Lambalk et al. 1998; Hoekstra et al. 2007; Bernard et al. 2010).

O FSH é uma gonadotrofina sintetizada e liberada pela glândula pituitária anterior após estímulo hipotalâmico que induz o crescimento folicular e a maturação dos ovários, bem como a síntese de estradiol. O hormônio consiste de uma subunidade α e subunidades β (FSHB), e se liga não-covalentemente a um receptor presente nas células gonadais (FSHR) (Bernard et al. 2010).

O metabolismo do FSH está contido em um contexto de retroalimentação que envolve outra importante gonadotrofina de origem pituitária, o hormônio luteinizante (LH) (Bernard et al. 2010; Simoni and Casarini 2014). Falhas neste mecanismo de *feedback* decorrentes da idade avançada podem levar ao desenvolvimento folicular múltiplo e, na presença de dois ovócitos de qualidade, uma gestação gemelar pode ocorrer (Beemsterboer et al. 2006; Van der Stroom et al. 2013).

Pesquisas nas duas últimas décadas revelaram o papel de variantes nos genes que codificam FSHR e FSHB na determinação dos níveis hormonais séricos e na resposta a órgãos-alvo (Simoni and Casarini 2014). Tão logo, variantes em *FSHR* e *FSHB* associadas à gemelaridade DZ foram descritas. Como exemplo, Al-Hendy et al. (2000) identificaram uma associação entre a gemelaridade e duas variantes em desequilíbrio de ligação (LD) no éxon 10 de *FSHR*, rs6166:C>T e rs6165:C>T, sugerindo que a homozigosidade para ambas estaria associada com gemelaridade DZ repetida, por meio da produção de um FSHR mais sensível. Todavia, tais resultados foram recebidos com certo criticismo (Derom et al. 2001; Gromoll and Simoni 2001; Lambalk 2001).

Mbarek et al. 2016, por meio de um robusto estudo de associação genômica (GWAS – *genome wide association study*) em mães de gêmeos DZ e mulheres-controle de ancestralidade europeia, identificaram dois polimorfismos genéticos (rs11031006:G>A, próximo a *FSHB* e rs17293443:T>C, em *SMAD3*) como variantes de risco para a gemelaridade, ambos relacionados a diversos aspectos da capacidade reprodutiva e saúde. Ainda no mesmo estudo, os achados foram replicados em uma grande coorte na Islândia e foram, inclusive, associados a demais aspectos relacionados à fertilidade feminina.

Foi sugerido que mulheres carreando o alelo rs11031006-G, próximo a FSHB, teriam maior depleção do *pool* folicular ovariano. Este polimorfismo mostrou-se importante para diversos aspectos reprodutivos femininos, como idade mais precoce no desenvolvimento das mamas, menarca, menopausa, dentre outros. Por sua vez, *SMAD3* codifica um membro de uma família de proteínas transdutoras de sinais e moduladores transcricionais, sendo altamente expresso no ovário humano, onde participa no mecanismo de resposta ao FSH, e o alelo rs7293443-C parece aumentar as chances de gemelaridade DZ (Mbarek et al. 2016)

1.5.2.2.   MTHFR e a rota do folato

O metabolismo do folato está intimamente relacionado à síntese e ao reparo do DNA, controle da expressão gênica e muitos outros processos biológicos de fundamental importância para a divisão celular e o desenvolvimento do embrião (Enciso et al. 2016). Variantes em genes envolvidos na rota do folato e no ciclo da homocisteína/metionina têm sido implicados não só em malformações e patologias no curso gestacional, mas também em diversos aspectos da reprodução, incluindo a gemelaridade, muito embora os resultados não sejam absolutamente concordantes (Haggarty et al. 2006; Hoekstra et al. 2007; Huang et al. 2015).

Em termos de prevalência e impacto, variações no *MTHFR*, o qual codifica a enzima 5,10-metilenotetrahidrofolato redutase, estão entre as mais importantes biologicamente. Dentre elas, destacam-se os SNPs nas posições 677 (rs1801133:G>A) e 1298 (1801131:T>G) do mRNA, cujos alelos A e G, respectivamente, diminuem a atividade da enzima MTHFR e, consequentemente, influenciam nos níveis séricos de homocisteína e demais metabólitos envolvidos (Forges et al. 2007).

Há uma série de evidências revisadas por Forges et al. (2007) que mostram que a atividade reduzida de MTHFR e a subsequente redução de SAM (S-adenosilmetionina, doador universal do grupamento metil para reações de metilação) podem influenciar em diversos aspectos da reprodução humana, inclusive no nascimento de gêmeos. Tal associação parece ser mantida tanto em um cenário de RMA, quanto no caso de gestações naturalmente concebidas (Hasbargen et al. 2000; Haggarty et al. 2006; Huang et al. 2015). Adicionalmente, foi mostrado recentemente que ambos os polimorfismos anteriormente citados podem influenciar no sucesso reprodutivo em níveis distintos, desde a implantação do embrião até a sua viabilidade (Enciso et al. 2016).

1.5.2.3 *Manutenção de uma gestação de sucesso*

O processo de implantação (ou nidação) requer a interação direta entre o blastocisto e o endométrio materno, o qual precisa ser receptivo. A receptividade do endométrio é um processo ativo, limitado no tempo e que envolve a regulação da expressão de centenas de genes, onde alguns componentes maternos representam uma ameaça para o feto, enquanto outros são tão úteis quanto necessários (Hoozemans et al. 2004).

Certas citocinas inflamatórias são fundamentais na relação materno-fetal e, consequentemente, importantes para o processo de implantação. Especificamente, o TNF

(fator de necrose tumoral) é importante durante os períodos de pré e peri-implantação e consideráveis desbalanços nos níveis de TNF estão associados com perdas gestacionais (Hoozemans et al. 2004; Chaouat et al. 2007). Sabe-se que polimorfismos genéticos podem impactar nos níveis de TNF e influenciar na taxa de implantação do embrião e de gestações múltiplas (Vialard et al. 2013).

Além disso, o desenvolvimento saudável do embrião não seria possível sem um adequado suprimento sanguíneo materno. O VEGFA (fator de crescimento endotelial vascular) é expresso durante a pré e peri-implantação no estroma uterino, possuindo o papel crítico de regulador da angigogênese, a qual, por sua vez, é importante para o crescimento folicular, qualidade do ovócito, vascularização endometrial e taxas de implantação (Chaouat et al. 2007; Boudjenah et al. 2014).

De fato, em um cenário de RMA, Boudjenah et al. 2014 investigaram 13 SNPs possivelmente associados com o risco de falha de implantação recorrente em mulheres sem fatores de infertilidade, dos quais dois SNPs individuais nos genes rs2010963:C>G em *VEGFA* e rs1800629:G>A *TNF* mostraram-se significativamente associados com a taxa de implantação e/ou as taxas de gestação, além de mostrarem que a combinação de ambos pode modificar o potencial de implantação.

1.6. Cândido Godói: a "Cidade dos Gêmeos"

O município de Cândido Godói (CG, 27°57'07''S; 54°45'07''O) está localizado na região noroeste do estado brasileiro do Rio Grande do Sul, a 524 km da capital Porto Alegre (Figura 1). A cidade possui mais de 6500 habitantes, a maioria pertencendo à zona rural (IBGE 2010) e cuja população é majoritariamente descendente de alemães e poloneses (Matte et al. 1996).

Dividida em 24 seções ou distritos chamados "linhas", cuja intercomunicação geralmente é complicada, CG é conhecida como "a cidade dos gêmeos", por apresentar uma TG duas vezes superior à nacional (estimada em 1%), sendo que em uma das seções da cidade, Linha São Pedro, a TG varia entre 7% e 10% (Matte et al. 1996; Tagliani-Ribeiro et al. 2011). Ao que nos consta, são poucos os relatos na literatura que descrevem uma alta prevalência de gemelaridade natural a uma região geográfica restrita livre da influência das metodologias de RMA (Nylander 1969; Hamamy et al. 2004; Sahab Khan and Skandhan 2011; Sunde et al. 2013).

Os estudos da gemelaridade em CG datam a partir de 1996, quando Matte et al. confirmam a alta TG na cidade, especialmente na LSP, atribuindo a principal causa a fatores genéticos devido, sobretudo, à ausência dos principais fatores ambientais comumente relacionados aos nascimentos gemelares (como metodologias ligadas à RMA) e à alta recorrência familial, além das características intrínsecas à formação da cidade. Assim, foi sugerido que teria ocorrido uma concentração de genes que predispunham à gemelaridade em uma população fundadora, a qual teria permanecido relativamente isolada com um alto grau de endocruzamento (Matte et al. 1996).
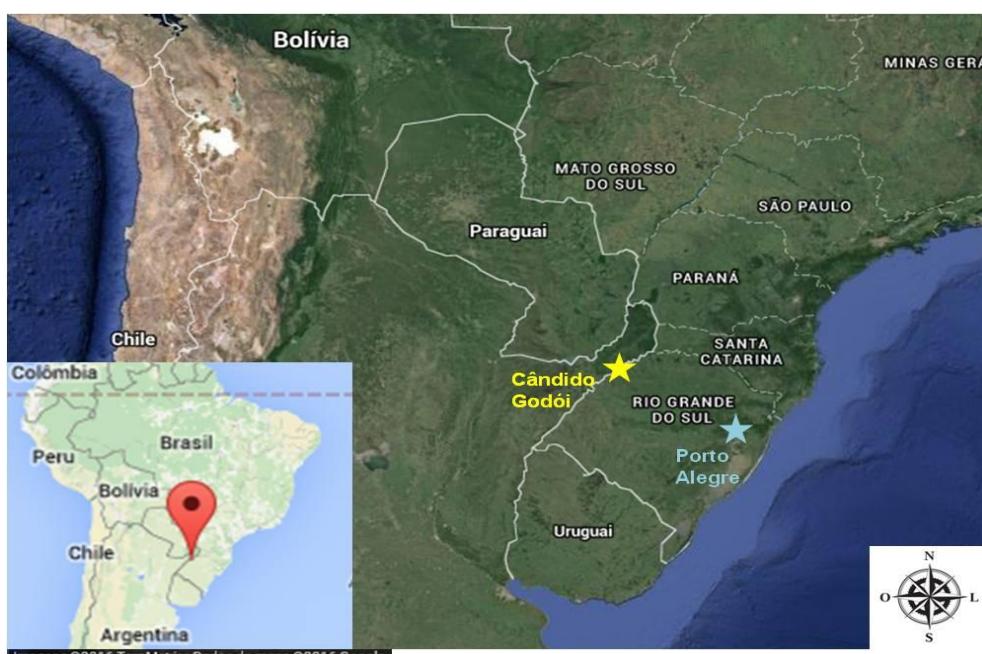
Figura 1: Localização geográfica do município de Cândido Godói. **Fonte:** Adapatado de *Google Maps* 2018 (https://maps.google.com)

De fato, mais de 10 anos depois dos estudos iniciais, a hipótese do efeito genético do fundador é confirmada por meio da análise de registros de batismo e de isonímia, em contraste a hipóteses alternativas que também buscavam explicar o fenômeno da gemelaridade em CG, como experimentações nazistas e demais mitos locais (Tagliani-Ribeiro et al. 2011; De Oliveira et al. 2013).

Além disso, Tagliani-Ribeiro et al. (2012) realizaram um estudo caso-controle em mães de gêmeos DZ (casos) e mãe de filhos únicos (controles) originárias do município. Considerando que a via p53 desempenha um importante papel nas gestações através de

implantação do blastocisto e da sobrevivência do embrião, os autores analisaram cinco SNPs e identificaram o alelo P72 de *TP53* (rs1042522) como um forte fator de risco para a gemelaridade local.

Embora estes resultados contribuam para o esclarecimento do fenômeno, é notável o caráter multigênico da gemelaridade e é bem provável que outras variantes genéticas estejam ligadas à alta recorrência gemelar (Palmer et al. 2006; Hoekstra et al. 2007; Huang et al. 2015). Além disso, novos estudos fornecem novas pistas para o entendimento das bases biológicas subjacentes aos nascimentos de gêmeos em Cândido Godói, um município onde a taxa de gemelaridade natural ainda pode ser observada, isto é, onde a alta incidência gemelar não demonstra ser alterada pela influência de RMA.

CAPÍTULO II

JUSTIFICATIVA

2. JUSTIFICATIVA

A despeito de despertar a curiosidade na espécie humana há séculos, a gemelaridade é uma característica multifatorial ainda pouco compreendida. O estudo com gêmeos tem contribuído para o entendimento de diversas áreas da biologia humana, mas principalmente aspectos etiológicos e epidemiológicos relacionados aos nascimentos gemelares permanecem pouco conhecidos (Hall 2003; McNamara et al. 2016).

Comparado ao nascimento de filhos únicos, o nascimento de gêmeos está associado a diversas complicações para a mãe e para a prole, acarretando cuidados e custos hospitalares adicionais (Corsello and Piro 2010; Dawson et al. 2016). Ultimamente, diversos países têm relatado tendência ascendente na incidência de nascimentos de gêmeos, o que tem gerado preocupações principalmente no campo da RMA, onde a gemelaridade é considerada o principal fator iatrogênico associado (Pison et al. 2015; Fellman 2016; Dawson et al. 2016). No Brasil, o cenário epidemiológico dos nascimentos gemelares ainda não foi estudado considerando a sua magnitude territorial e sociodemográfica.

Do ponto de vista etiológico, alguns estudos apontam variantes genéticas específicas aumentando a chance de uma mulher portadora ter filhos gêmeos (Al-Hendy et al. 2000; Hasbargen et al. 2000; Palmer et al. 2006; Painter et al. 2010; Huang et al. 2015; Mbarek et al. 2016). Porém, além dos resultados nem sempre concordarem, não parece haver uma única via biológica que leve ao nascimento de gêmeos. Pesquisas nessa área podem, além de aprofundar o entendimento sobre a reprodução humana, contribuir para o desenvolvimento de estratégias que tratem pessoas com infertilidade (Lambalk, 2001).

Resultados importantes sobre a etiologia dos nascimentos gemelares foram obtidos a partir de estudos em alguns poucos locais conhecidos como "cidades dos gêmeos", como a pequena cidade de CG localizada no Sul do Brasil. Nesta cidade, a gemelaridade ocorre a uma taxa naturalmente alta e o traço gemelar corre entra as famílias locais, o que tem levado à elaboração de teorias com significado nem sempre comprovadas pelo método científico (Tagliani-Ribeiro et al. 2011). Assim, estudos em CG podem contribuir não apenas para uma melhor compreensão sobre uma questão local, mas também para o estudo de questões ainda mais profundas sobre a reprodução humana.

CAPÍTULO III

OBJETIVOS

3. OBJETIVOS

3.1.   OBJETIVO GERAL

Estudar aspectos relacionados à epidemiologia da gemelaridade no Brasil e investigar a associação de variantes genéticas relacionadas à foliculogênese e à manutenção da gestação na população de Cândido Godói (RS).

3.2   OBJETIVOS ESPECÍFICOS

Estudo epidemiológico:

- Caracterizar as taxas gemelares brasileiras de acordo com geografia (país, regiões, estados e municípios) e tempo (2001 a 2014);
- Entender possíveis tendências e variações, além de identificar fatores epidemiológicos potencialmente implicados;
- Contextualizar os resultados encontrados no Brasil com outros países.

Estudo molecular:

- Estudar a associação dos SNPs rs11031006:G>A próximo a *FSHB*, rs17293443:T>C em *SMAD3*, rs1801131:T>G em *MTHFR*, rs2010963:C>G em *VEGFA*, e rs1800629:G>A em *TNF* com os nascimentos gemelares em CG;
- Integrar os resultados encontrados com as genotipagens de outros dois SNPs (rs6166:C>T em *FSHR*, e rs1801133:G>A em *MTHFR*) já realizados no laboratório (Tagliani-Ribeiro 2011);
- Comparar as frequências encontradas com outras populações já estudadas e relacionar estes achados com os nascimentos gemelares na população em questão.

CAPÍTULO IV

ARTIGO I

Twin Peaks: a spatial and temporal study of twinning rates in Brazil

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ARTIGO I – *Twin Peaks: a spatial and temporal study of twinning rates in Brazil*

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ABSTRACT

Twin births are an important public health issue due to health complications for both mother and children. While it is known that contemporary factors have drastically changed the epidemiology of twins in certain developed countries, in Brazil, relevant data are still scarce. Thus, we carried out a population-based study of live births in spatial and temporal dimensions using data from Brazil's Live Birth Information System, which covers the entire country. Over 41 million births registered between 2001 and 2014 were classified as singleton, twin or multiple. Twinning rates (TR) averaged 9.41 per 1,000 for the study period and a first-order autoregressive model of time-series analysis revealed a global upward trend over time; however, there were important regional differences. In fact, a Cluster and Outlier Analysis (Anselin Local Moran's I) was performed and identified clusters of high TR in an area stretching from the South of Brazil's Northeast Region to the South Region (Global Moran Index = 0.062, $P < 0.001$). Spearman's correlation coefficient and a Wilcoxon matched pairs test revealed a positive association between Human Development Index (HDI) and TRs in different scenarios, suggesting that the HDI might be an important indicator of Medically Assisted Reproduction (MAR) in Brazil. Furthermore, there was a sharp increase of 26.42% in TR in women aged 45 and over during study period. The upward temporal trend in TRs is in line with recent observations from other countries, while the spatial analysis has revealed two very different realities within the same country. Our approach to TR using HDI as a proxy of MAR can be applied to other developing countries with regional inequalities resembling those found in Brazil.

Keywords: twin births; assisted reproduction; maternal age; developing world; human development index; delayed childbearing

INTRODUCTION

Twin Peaks is an award-winning American TV series of the 90s created by Mark Frost and David Lynch, whose plot famously boasts high levels of mystery (Frost and Lynch, 1990). A similar mystery surrounds the "twinning peaks" of developing countries: while the incidence of twinning is well known for high-income countries, equivalent information is scarce for the developing world, mainly due to a lack of representative data (D'addato, 2007; Pison et al., 2015).

Twinning is an important public health issue. It has been shown that morbidity and mortality are both higher in twin babies than singletons (Hall, 2003; Corsello and Piro, 2010). Health complications associated with twinning include reduced birth weight, preterm births and congenital defects (Corsello and Piro, 2010; Jauniaux et al., 2013; Dawson et al., 2016). In addition, mothers of twins are at an increased risk of diabetes, preeclampsia, postpartum depression and maternal mortality (Delbaere et al., 2008; Ananth and Chauhan, 2012; Laskov et al., 2013).

The twinning rate (TR) is defined as the number of twin births per 1,000 (‰) deliveries. In industrialized countries, rising maternal age and the increased use of assisted reproductive techniques (ARTs) have sent TRs on a dramatic upward trajectory (Zegers-Hochschild et al., 2009; Pison et al., 2015; Malamitsi-Puchner et al., 2017). TRs are thus a useful proxy for the use of ARTs, and where reliable statistics on ART use are scarce, comprehensive information on TRs can be valuable (Pison et al., 2015).

The epidemiology of twins in the Brazilian population remains understudied. Currently available data, derived mainly from the two states of São Paulo and Rio Grande do Sul, are highly discordant (Colletto and Beiguelman, 2001; Colletto, 2003; Geraldo et al., 2008; Otta et al., 2016). To the best of our knowledge, the only study to report a TR for the country as a whole, although very informative, is based on data from 1996 and addressed twinning across 75 low- and middle-income countries. As such, it did not consider Brazil's territorial and socio-demographic diversity (Smits and Monden, 2011).

Here, we present data on twin births from 41 million live births registered in 5,565 municipalities across Brazil between 2001 and 2014. An important aim of the present paper is to characterize Brazilian TRs across the two dimensions of space and time. In addition, we aim to understand trends and variation in twinning data, and to identify factors relevant to the epidemiology of twins.

MATERIAL AND METHODS

Brazil is the largest country in South America, with a population over 200 million. It is divided into five geographical regions: North, Northeast, Midwest, Southeast and South (Fig 1a). The political and administrative organization of the country comprises a federal district (seat of the federal government) and 26 states (the highest administrative unit), with a total of 5,565 municipalities (the lowest administrative unit) (IBGE 2010; IBGE 2017).

Fig 1. Brazilian geographical features. Brazilian geographical regions and the states mentioned in the paper.

Data Sources

Information on all live births registered in Brazil between 2001 and 2014 was obtained from the Brazilian Health Department's public Live Birth Information System (SINASC). This system covers all Brazilian regions, states and municipalities (Brasil, 2017) and has been shown to be a reliable tool for the measurement of population health indicators (Frias et al., 2014). While SINASC provides information on singleton, twin and multiple births, it does not include stillbirths. Because twin births are associated with higher mortality than singletons (Hall, 2003; Corsello and Piro, 2010), SINASC data may therefore underestimate true TRs.

TRs were calculated as follows: TR = [(individual twin births/2)/ total of deliveries) x 1,000]. Multiple birth rates were calculated using the same formula, but dividing individual multiple births by three (assuming that the majority of multiple births were triplets). We were able to analyze 41,013,511 deliveries that occurred across Brazil between January 1st, 2001 and December 31st, 2014, inclusive. Of these, 385,477 were of twins (0.94%) and 9,502 of multiples (0.02%). A total of 73,547 deliveries with an unknown type of birth were not included in our analyses.

SINASC data includes information on maternal factors linked to twinning, such as marital status, educational level and age (Delbaere et al., 2008; Ananth and Chauhan, 2012). Information on the sex composition of twin births, however, is not publicly available. It

was therefore not possible to estimate the frequency of monozygotic (MZT) and dizygotic (DZT) twins according to Weinberg's method (Fellman and Eriksson, 2006). Thus, like in other similar studies, the TRs reported here represent combined rates of MZT and DZT (Smits and Monden, 2011; Ananth and Chauhan, 2012; Pison et al., 2015; Fellman, 2016; Malamitsi-Puchner et al., 2017).

The use of ART-related procedures is a major factor associated with twinning peaks across the world (Hall, 2003; Ananth and Chauhan, 2012; Pison et al., 2015; Sobek et al., 2016); however, information on ART is not recorded in the SINASC database. In Brazil, access to reproductive technologies is linked to socioeconomic factors (Makuch and Bahamondes, 2012; Bahamondes and Makuch, 2014; Corrêa and Loyola, 2015; Tavares et al., 2016). As a proxy for the use of ARTs, we therefore decided to include the Human Development Index (HDI) in our analyses. The HDI is a well-established summary measure of human development, which factors in health, education, and income (Brazilian Institute of Geography and Statistics, 2000, 2013). It varies between 0 and 1; the closer it is to 1, the greater the human development (United Nations Development Programme, 2013). HDI values were obtained from the Brazilian censuses of 2000 and 2010.

Statistical Methods

To examine temporal trends for each type of birth in the country as a whole, we plotted singleton, twin and multiple rates over time. In addition, TRs were described separately for the five geographic regions, and for seven categories of maternal age at birth: ≤19, 20−24, 25−29, 30−34, 35−39, 40−44 and ≥45 years.

In our time series analysis, we used a first-order autoregressive model to identify temporal trends of TRs from 2001 to 2014. Because women over 30 are more prone to seek fertility treatment (Pison et al., 2015; Tavares et al., 2016), we included a maternal age of 30 or above as a co-variable in our model. Statistically significant parameter estimates ($P < 0.05$) support the relevance of time and/or maternal age to explain the observed TRs. Time-series analysis was performed using R version 3.2.3 software (R Core Team, 2016).

Spatial analyses were conducted in ArcGis version 10.3 software using the IBGE cartographic database, which is publicly available on the IBGE website (Brazilian Institute

of Geography and Statistics, 2015). The threshold for statistical significance was set to P < 0.05. First, we calculated the TR for each municipality in the first (2001) and last year (2014) of our analysis, and the average TR for the analyzed time span. Second, we calculated the Global Moran Index (GMI) to test the global spatial dependence of TRs (Anselin, 2010). Third, we performed a Cluster and Outlier Analysis (Anselin Local Moran's Index) to identify groups of municipalities with similar TRs or HDI (clusters). This analysis generated a map indicating statistically significant hot spots of municipalities with high TRs surrounded by other municipalities with high TRs (high-high areas), as well cold spots (low-low areas) and spatial outliers (high-low and low-high) (ESRI, 2017). The identification of outliers is especially useful in the case of municipalities with a high TR, which are surrounded by municipalities with low TRs. These outliers can be due to the occasional, random, occurrence of twin births against the very low background live birth rate of a small city, thus causing a substantial variation in gross TRs. Data from recently established municipalities were joined with those from the municipality from which they had emerged, in order to avoid mismatches in the analysis (Brazilian Institute of Geography and Statistics, 2014). A similar cluster map was generated from the 2010 HDI data.

SPSS v18.0 software was used to obtain Spearman's rank correlation coefficient (rs) for each individual state's TR and HDI data. Since TR and HDI data were not consistently available for the same years, the coefficient was calculated for three different scenarios: TR data from 2001 and HDI data from 2000; TR and HDI data from 2010; average TR for the 2001-2014 period and HDI data from 2010. For the last scenario at the municipality level, we also performed a graphic Cluster and Outlier Analysis using normalized data and applying a spatial correlation index with maximum (max) and minimum (min) values of both indicators using the formula:

$$\sqrt{\frac{(TR - TRmax)}{(TRmax - TRmin)} \times \frac{(HDI - HDImax)}{(HDImax - HDImin)}}$$

Finally, we divided the municipalities into three categories according to 2010 HDI figures: low (≤ 0.599; n = 1399), medium ($0.600-0.699$; n = 2233) and high (≥ 0.700; n = 1933). For each category, we compared TRs from 2001 and 2014 using the Wilcoxon matched pairs test.

Ethical Approval

Ethical approval was not required for this study because it was based on fully anonymous data publicly available.

RESULTS

For the analyzed time period, the global average rate of twin births in Brazil was 9.41‰ or one twin birth in 106.3 live births. Over time, the average rate increased by 17.34‰, from 8.65‰ in 2001 to 10.15‰ in 2014. The global average rates of singleton and multiple births were 990.37‰ and 0.23‰, respectively (Fig 2). Fig 3 presents the distribution of TR in Brazil for the different classes of maternal age over the 14 years of the study. The increase in twinning seems to be more pronounced for women 30 years or older, and a particularly sharp increase of 26.42% was observed in women aged 45 and over.

Fig 2. Temporal distribution. Singleton, twin and multiple birth rates per 1,000 births in Brazil, 2001-2014.

Fig 3. Maternal age and twinning rates. Twinning rates among different classes of maternal age in Brazil, 2001-2014.

On average, mothers of twins or multiples had completed more years of education than mothers of singletons. From 2001 to 2014, 19.77% of mothers of twins or multiples had completed 12 or more years of education (values for individual years went up from 13.79% in 2001 to 25.35% in 2014), compared to 14.86% of mothers of singletons (11.75% in 2001 to 17.36% in 2014). Similarly, the proportion of married mothers was greater among mothers of twins and multiples than among those of singletons. From 2001 to 2014, 41.84% of mothers of twins and multiples were married (45.93% in 2001 to 40.63% in 2014), compared to 35.91% of mothers of singleton (41.15% in 2001 to 32.74% in 2014).

In the autoregressive time-series model, the temporal parameter estimate was statistically significant, revealing an upward trend of TRs, both for the country as a whole and for all states of the Southeast, South and Midwest regions, as well as Bahia and Paraiba in the Northeast (Table 1). However, the temporal trend was no longer statistically significant for the whole of Brazil, as well as for most individual states, when factoring in a maternal age

above 30. Instead, a positive relationship between maternal age and TR emerged, both for Brazil as a whole and for all individual states, with the exception of Roraima and Tocantins in the North, and Maranhão and Piauí in the Northeast.

Table 1: Twinning rates (years 2001 and 2014 and average) and its percentage variation, as well the parameters estimated in the autoregressive models.

Location	Twinning rate (‰)			Variation (%)	AR (temporal parameter)[a]	AR (temporal parameter)[b]	Co-variable maternal age ≥ 30[b]
	2001	2014	Average				
BRAZIL	8.65	10.15	9.41	17.34%	0.97*	0.47	0.86*
NORTH	6.92	7.54	7.31	8.88%	0.51	-0.35	0.69*
Roraima	7.68	8.47	8.17	10.22%	-0.17	-0.53*	0.54*
Acre	7.28	9.12	7.54	25.20%	0.29	0.56	1.38*
Amazonas	6.42	7.17	7.06	11.73%	0.11	-0.36	0.72*
Roraima	7.51	6.67	6.88	-11.12%	-0.35	-0.21	0.69
Pará	6.81	7.41	7.19	8.86%	0.06	-0.21	0.62*
Amapá	7.32	6.35	7.48	-13.25%	0.02	0.16	0.59
Tocantins	7.42	8.53	7.76	14.92%	0.01	-0.57*	0.66*
NORTHEAST	8.37	9.07	8.71	8.35%	0.65*	0.04	0.55*
Maranhão	8.56	7.60	8.02	-11.17%	0.47	0.02	-1.15
Piauí	8.58	8.92	8.45	3.97%	-0.20	-0.06	0.33
Ceará	8.42	9.29	8.46	10.31%	-0.46	-0.40	0.44*
Rio Grande do Norte	8.07	9.00	8.34	11.45%	0.47	0.34	0.73*
Paraíba	8.89	9.79	8.99	10.16%	0.54*	0.03	0.83*
Pernambuco	8.37	9.11	8.83	8.84%	0.44	-0.40	0.71*
Alagoas	7.34	8.56	8.22	16.74%	0.05	-0.11	1.39*
Sergipe	8.42	9.36	9.08	11.19%	0.09	-0.02	0.81*
Bahia	8.43	9.69	9.31	14.91%	0.72*	-0.11	0.84*
SOUTHEAST	9.20	11.33	10.34	23.16%	0.96*	0.42	0.92*
Minas Gerais	9.27	10.83	10.13	16.74%	0.90*	0.45*	0.82*
Espírito Santo	8.47	10.20	9.28	20.47%	0.87*	-0.25	0.90*
Rio de Janeiro	8.88	10.43	9.91	17.45%	0.84*	0.41	0.97*
São Paulo	9.35	11.98	10.69	28.12%	0.94*	0.21	0.96*
SOUTH	9.09	11.02	10.07	21.19%	0.93*	0.05	1.17*
Paraná	9.01	10.58	9.86	17.36%	0.87*	-0.51*	1.21*
Santa Catarina	8.52	10.72	9.74	25.75%	0.81*	0.20	1.18*
Rio Grande do Sul	9.48	11.70	10.51	23.41%	0.78*	-0.18	1.12*
MIDWEST	8.31	10.17	9.07	22.39%	0.92*	-0.27	0.81*
Mato Grosso do Sul	7.96	9.93	8.73	24.82%	0.80*	0.19	1.03*
Mato Grosso	7.73	10.30	8.70	33.25%	0.81*	-0.46*	0.98*
Goiás	8.61	9.57	8.91	11.12%	0.65*	-0.72*	0.76*

| Distrito Federal | 8.60 | 11.59 | 10.12 | 34.69% | 0.66* | 0.48 | 0.83* |

AR: Autoregressive
[a]Model considering only the time period
[b]Model considering time and the co-variable maternal age ≥ 30
*Stastitically significant at 0.05 level

While an upward trend for TRs was found in all regions, there were substantial differences between regions (S1 Fig). The highest average TR, and the highest increase in twinning over time, was observed in the Southeast Region (10.39‰ average TR; 23.16% increase between 2001 and 2014). The lowest TR was found in the North Region (7.32‰ average TR, varying from 6.92‰ in 2001 to 7.54‰ in 2014). Finally, the lowest percentage increase was found in the Northeast (8.35%), which also had the second lowest average TR (8.71‰).

We found a clear pattern in the spatial distribution of TRs. The GMI was 0.004 for 2001; 0.013 for 2014; and 0.062 for the average TR (all $P < 0.001$), indicating that twinning rates are spatially dependent in all three scenarios. From 2001 to 2014, TRs increased mainly in municipalities of the Southeast and South (S2 Fig). A cluster of high average TRs was also observed in Paraiba, in the east of the Northeast Region; however, the highest average rates were spatially concentrated in a large strip following the coastline from Bahia in the south of the Northeast Region to Rio Grande do Sul in the South (Fig 4A).

Fig 4. Cluster and Outlier Analysis. (A) Distribution of average Twinning Rates (TRs) across Brazilian municipalities. (B) Distribution of Human Development Index (HDI) figures from the 2000 census across Brazilian municipalities. (C) Spatial correlation index from normalized average TR and 2010's HDI across Brazilian municipalities.

The correlation analysis between TRs and HDI for each state revealed a statistically significant association in the three different scenarios studied: TRs from 2001 and HDI figures from the 2000 census ($r_s = 0.496$; $P = 0.008$); TRs and HDI figures from 2010 ($r_s = 0.719$; $P < 0.001$); and average TRs and HDI figures from 2010 ($r_s = 0.617$; $P < 0.001$). Considering this last scenario at the municipality level, the spatial correlation revealed an overlap involving both indicators (Fig 4A and 4B), with a statistically significant spatial concentration (GMI = 0.399; $P < 0.001$) (Fig 4C).

The highest TRs were observed in municipalities with a high HDI, and the lowest TRs were found in municipalities with a low HDI (S3 Fig). In municipalities with a high HDI, TRs were statistically higher in 2014 than 2001 (mean difference = 1.92; $P < 0.001$). In contrast, no statistically significant differences in TRs between 2014 and 2001 were found in municipalities with a low (mean difference = -0.74; $P = 0.091$) and medium (mean difference = 0.27; $P = 0.331$) HDI.

DISCUSSION

Although twin births can have important consequences for both mother and children and are considered an important public health issue, our knowledge of the epidemiology of twinning in the developing world is limited (D'addato, 2007; Smits and Monden, 2011). Here, we report an average TR of 9.41 per 1,000 deliveries for the Brazilian population between 2001 and 2014, with a peak at 10.15‰ in 2014.

The average rate reported here is slightly higher than 8.8‰, the only previously available figure for the country as a whole (Smits and Monden, 2011). However, our analysis is based on a larger and more recent sample (over 41 million versus 11,099; 2001−2014 versus 1996). In addition, we present a regionally differentiated analysis, which also takes into account other factors potentially associated with twinning. Other studies have reported TRs for specific localities of the country, but the data underlying these reports are outdated and/or limited to specific regions (Colletto and Beiguelman, 2001; Colletto, 2003; Otta et al., 2016).

The average rate found here for Brazil is close to that of other South American and some Asian countries, where TRs are below 9‰, and considerably lower than that of Central Africa, which has the highest incidence of twinning worldwide (Bulmer, 1970; Hall, 2003; Hoekstra et al., 2007; Smits and Monden, 2011). While the average rate of twinning in the Brazilian population is lower than in a number of developed countries, including the United States, France, England and Wales, and Norway, among others, the global upward trend in twinning seen in Brazil mirrors the recent trends seen in some of these countries, where increased twinning is mainly associated with two factors: delayed childbearing and/or ART (Pison et al., 2015; Dawson et al., 2016; Fellman, 2016).

Reproductive procedures, such as ovarian stimulation in hypofertile women, in vitro fertilization (IVF), assisted hatching and blastocyst culture, are associated with a large increase in the chance of multiple births, the vast majority of which are twin births (Aston et al., 2008; Ananth and Chauhan, 2012; Jauniaux et al., 2013). The common practice of transferring several embryos to enhance the probability of a successful pregnancy directly increases the chances of a twin birth, despite a recent tendency to transfer fewer embryos (Corsello and Piro, 2010; Jauniaux et al., 2013). However, other

factors, such as the type of ovarian stimulation and the medium used in embryo culture, may also increase the incidence of MZT (Vaughan et al., 2016).

In Brazil, the high cost of ART represents a significant economic barrier to the use of reproductive technologies (Makuch and Bahamondes, 2012; Bahamondes and Makuch, 2014; Corrêa and Loyola, 2015; Tavares et al., 2016), and access to these procedures is thus generally limited to those who can afford the hefty price tag associated with them. Our results indicate that, in the absence of accurate information on ART in the Brazilian population, the positive correlation between HDI and TRs in different scenarios can help us understand the spatial and temporal distribution of twinning.

There is marked heterogeneity among the five geographical regions of Brazil in terms of demography, socioeconomics, culture, and healthcare. These differences are further accentuated by widespread internal inequalities (Paim et al., 2011). Despite significant recent social advances, some areas in Brazil, particularly in the North and Northeast, are still characterized by a HDI typical of very poor societies. Meanwhile, the HDI of other regions, notably the Southeast and South, falls into the range of developed countries (Brazilian Institute of Geography and Statistics, 2013; United Nations Development Programme, 2013).

In a recent study, Otta et al. (2016) reported an average TR of 11.96‰ for São Paulo, the capital of the homonymous state. In our study, the highest average TRs were seen in the state of São Paulo (10.69‰) and the geographic region it belongs to, the Southeast (10.34‰), both characterized by a high HDI. Interestingly, but perhaps not surprisingly, official figures from 2014 show that the vast majority of assisted reproduction centers are located in the Southeast (Anvisa, 2014). In stark contrast with this, in the North region, where the HDI is lower, some states have average TRs below 7‰, and no significant upward trend can be observed over the study period.

While relatively short, the 14-year period considered has been a time of enormous social and economic change across the country, as reflected by the important upward change of the national HDI from 0,612 in 2000 to 0,727 in 2010. While the indices rose more in some parts of the country than in others, they increased in all states of the country (Brazilian Institute of Geography and Statistics, 2000, 2013; United Nations Development Programme, 2013). These and other changes have affected the health and health behavior of Brazilians, in addition to causing positive educational changes (Paim et al., 2011;

Madalozzo, 2012; Tejada et al., 2017). Taken together, such changes can result in delayed childbearing, even though some parts of the country may be affected more than others (Madalozzo, 2012; United Nations Development Programme, 2013). We found mothers of twins and multiples to have a higher educational level than singleton mothers, and increased twinning was particularly evident among older women. Especially in those aged 45 years and over, there has been a significant increase in twinning in recent years, which is likely due to the use of ART caused by difficulties in falling pregnant at an advanced maternal age.

We thus believe that the increasing TRs across Brazil, seen mainly in women over 30, as well the particularly sharp rise of twinning in women aged 45 and over, are due to a combination of five major factors: first, delayed childbearing, particularly among higher-income women (Makuch et al., 2011; Bahamondes and Makuch, 2014; Tejada et al., 2017). Second, infertility increases with maternal age (Leridon, 2004) and can reach high rates in developing countries (Ombelet, 2009; Makuch et al., 2011). Third, a recent (if unevenly spread) improvement in human development across Brazil (Madalozzo, 2012; United Nations Development Programme, 2013). Fourth, an increase in the availability of ART services (Makuch and Bahamondes, 2012; Anvisa, 2014). Fifth, as recently emphasized by Corrêa & Loyola (2015), current ethical norms suggesting the transfer of no more than two embryos in fertility procedures amount to a recommendation, rather than an obligation or law. As a result, low standards in ART services across the country may imply an increased risk of multiple pregnancies. In addition to these major factors, an increased likelihood of spontaneous twins in older women has been recognized (Ananth and Chauhan, 2012).

The democratization of access to ART in Brazil is currently a hot debate involving the scientific community, federal and state governments and the general population (Ombelet, 2009; Makuch et al., 2011; Makuch and Bahamondes, 2012; Corrêa and Loyola, 2015; Tavares et al., 2016), and the universal access to reproductive health is one of the UN Millennium Development Goals (United Nations, 2015). The impact of infertility on health and well-being is undeniable, and since available treatments are currently beyond the reach of most Brazilian women (Makuch et al., 2011; Tavares et al., 2016), access to ART services needs to be widened. In this context, we emphasize, however, that the implementation of this public health goal requires special caution, due to the enhanced

probability of twin births, which is considered to be the main iatrogenic effect of assisted medicine (Jauniaux et al., 2013). In some countries, TRs have declined after reaching a peak, a development which has mainly been attributed to changes in ART procedures, such as a reduction in the number of embryos transferred to the uterus (D'addato, 2007; Pison et al., 2015).

While TRs are internationally regarded as a useful indicator of the use of medical reproductive services (Pison et al., 2015), further studies are needed to clarify the effects of ART in Brazil. The inclusion of information about ART services involved in a birth in SINASC, or even the creation of an independent database system would be very useful for such future studies.

Other factors besides maternal age and HDI as a proxy for ART may be involved in the mystery behind increased twinning, and the difficulty of analyzing them represents a limitation of this study. For example, parity has been related to twinning, and greater parity has been associated with a higher risk of twin pregnancies (Hall, 2003; Hoekstra et al., 2007). However, Brazil has achieved an intermediary stage of the demographic transition with decreasing fertility levels, making this explanation less likely (Madalozzo, 2012; Tejada et al., 2017). Another factor that has been related to twin births is maternal diet, but the reports on this are conflicting (Bulmer, 1970; Hall, 2003; Fellman, 2016).

The Brazilian population is one of the most ethnically admixed in the world. It has formed by extensive admixture between Europeans, Africans and Native Americans (Pimenta et al., 2006). Geographically, the highest levels of European ancestry are observed in the South, while African ancestry is highest in the East, and Native American ancestry in the North (Ruiz-Linares et al., 2014). Twin births are known to be linked to ethnic background and genetic predisposition (Bulmer, 1970; Hall, 2003; Tagliani-Ribeiro et al., 2012; Dawson et al., 2016). African ancestry in particular is associated with increased twinning rates (Bulmer, 1970; Hall, 2003; Smits and Monden, 2011). It might thus be tempting to explain our results as a reflection of genetic ancestry. However, the observed TRs were highest in the South and Southeast regions, where European ancestry is most prominent, rather than in the East, where African ancestry is particularly common. Moreover, genetic ancestry would not have changed enough over the study period to explain the upward trends in TRs observed here (Pison et al., 2015).

Our results thus indicate that the temporal and spatial distribution of TRs across Brazil are mainly linked to the effects of maternal age and ART, with local HDI figures serving as an important proxy for the socioeconomic changes underlying this scenario. Given the importance and urgency of the topic and given the lack of reliable statistics on ART across the developing world, we believe that our approach can be applied to other countries with territorial and socioeconomic profiles similar to those of Brazil, and in particular the other major emerging economies of the BRICS association.

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FUNDING STATEMENT

SUPPORTING INFORMATION

S1 Fig. Twinning rates across geographical regions. Rates of twin births in Brazil and its geographical regions, 2001-2014.

S2 Fig. Cluster and Outlier Analysis showing the distribution of Twinning Rates across Brazilian municipalities in the years 2001 (A) and 2014 (B).

S3 Fig. Twinning rates and HDI. Twinning Rates across Brazilian municipalities grouped according to HDI categories.

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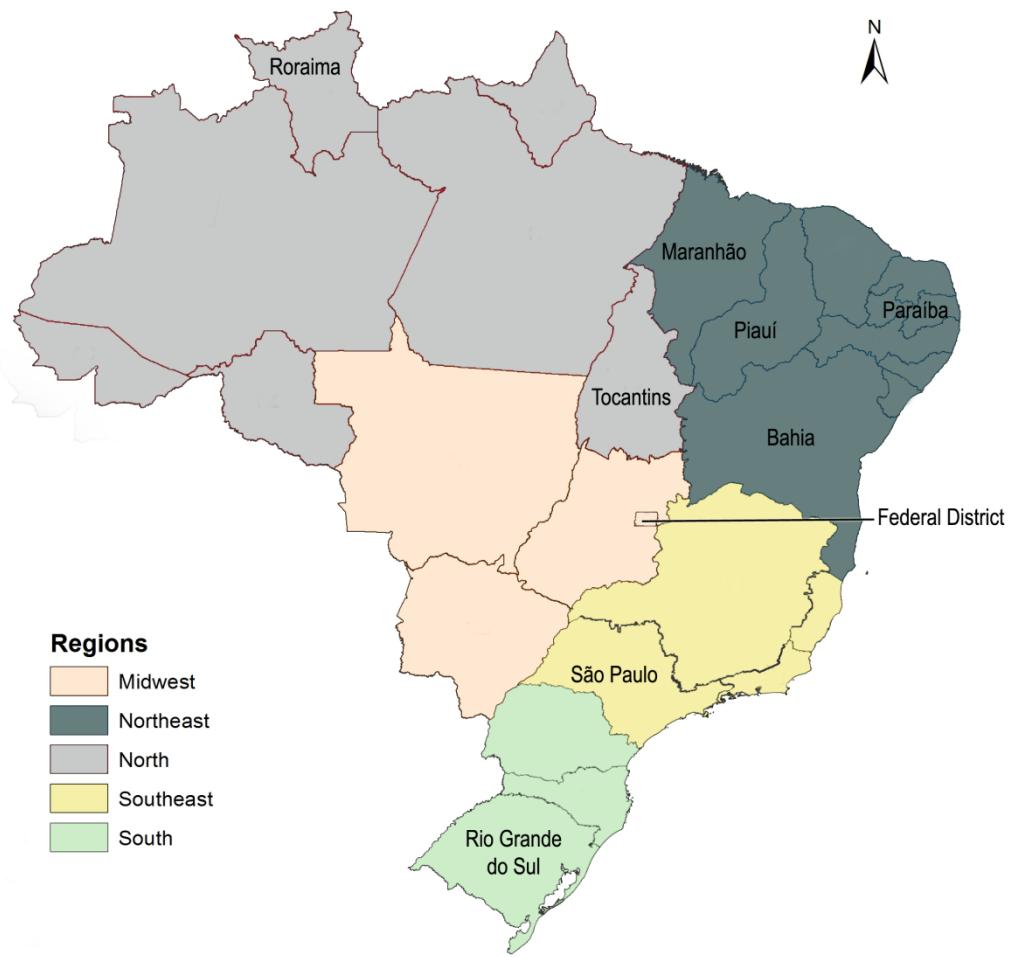
Fig 1. Brazilian geographical features. Brazilian geographical regions and the states mentioned in the paper.
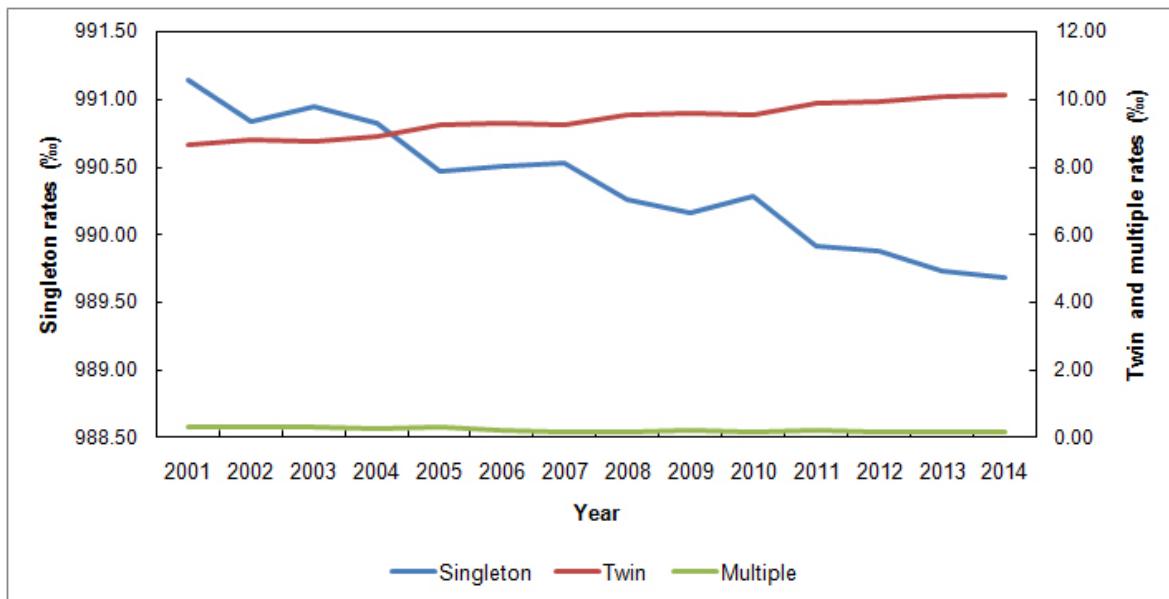
Fig 2. Temporal distribution. Singleton, twin and multiple birth rates per 1,000 births in Brazil, 2001-2014.
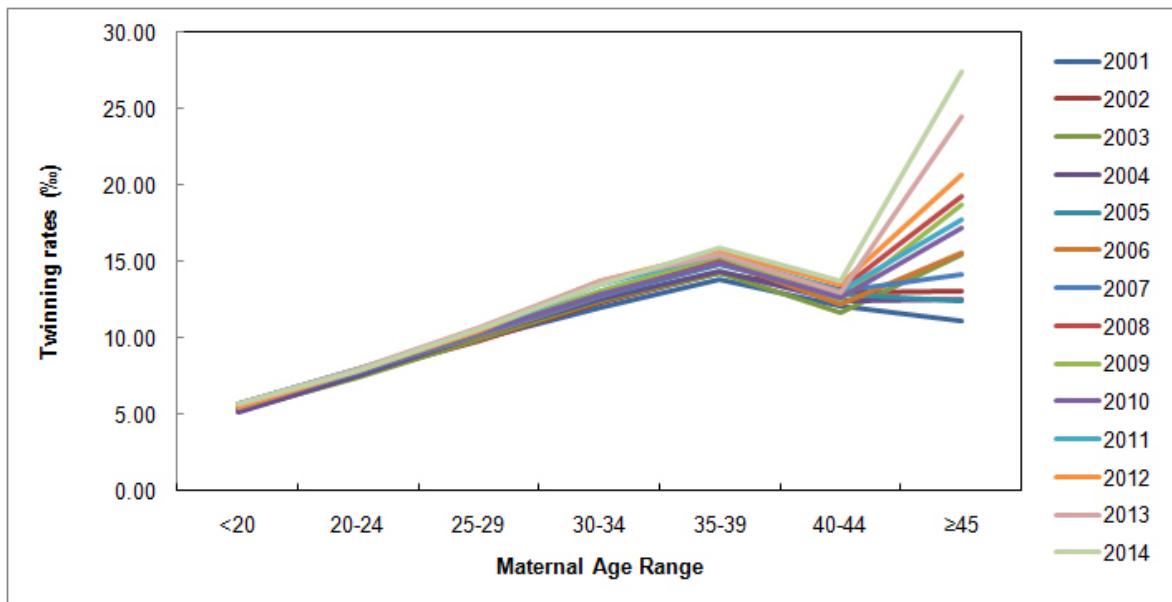
Fig 3. Maternal age and twinning rates. Twinning rates among different classes of maternal age in Brazil, 2001-2014.
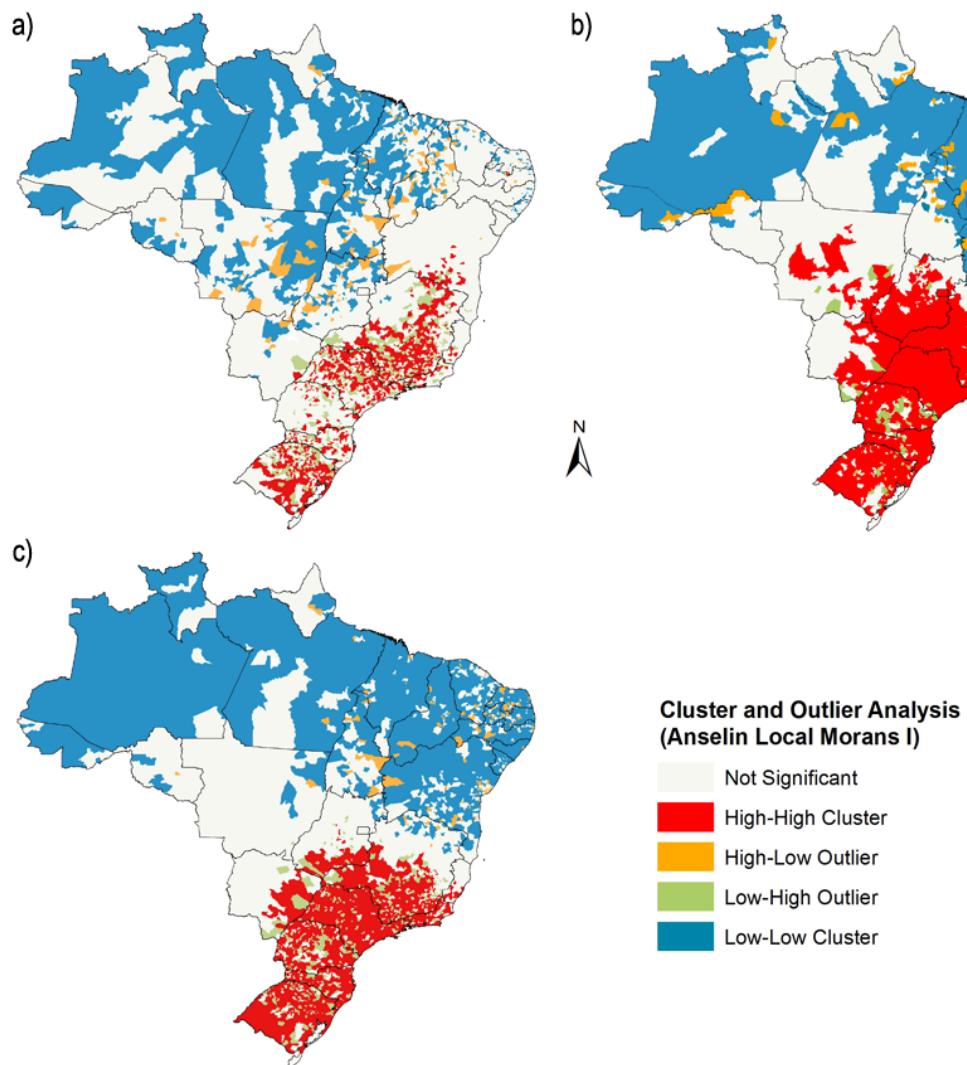
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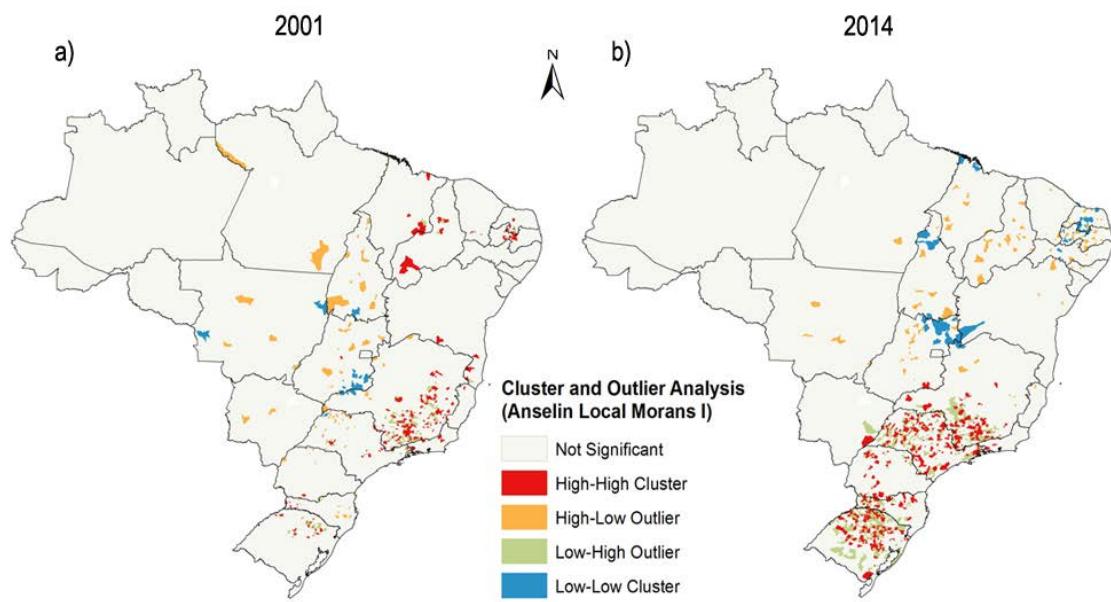
S2 Fig. Cluster and Outlier Analysis showing the distribution of Twinning Rates across Brazilian municipalities in the years 2001 (A) and 2014 (B).
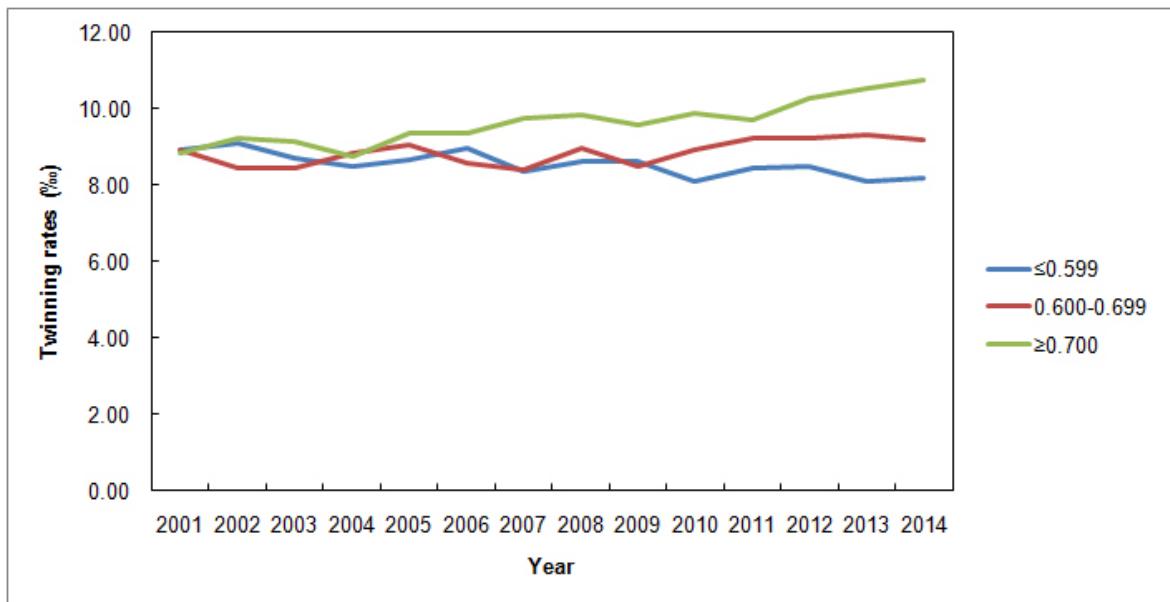
S3 Fig. Twinning rates and HDI. Twinning Rates across Brazilian municipalities grouped according to HDI categories.

CAPÍTULO V

ARTIGO II

Genetic variants linked to folliculogenesis and successful pregnancy in mothers from a Twin's Town

Artigo em preparação para posterior submissão na revista *Reproduction*

ARTIGO II – *Genetic variants linked to folliculogenesis and successful pregnancy in mothers from a Twin's Town*

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Short Title: Genetic polymorphisms and twin births

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Keywords: twin birth; folliculogenesis; successful pregnancy; genetic polymorphism; founder effect.

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ABSTRACT

Cândido Godói (CG) is a small city in South Brazil in which natural twin births (both monozygotic and dizygotic) occur at an unusually high rate and the twin trait runs through the local families, which are mostly European descendants. The aim of this study was to investigate this phenomenon from a genetic point of view, evaluating single nucleotide polymorphisms (SNPs) related to folliculogenesis (rs6166:C>T in *FSHR*, rs11031006:G>A near *FSHB*, and rs17293443:T>C in *SMAD3*) and successful pregnancies (rs2010963:C>G in *VEGFA*, rs1800629:G>A in *TNF*, rs1801131:T>G and rs1801133:G>A in *MTHFR*,) in 44 mothers of twins (cases) and 102 mothers of singletons (controls), all of them from CG. Genotypes were determined by real-time PCR (TaqMan SNP Genotyping Assay). For all SNPs, the distributions of the genotypic and allelic frequencies were similar between cases and controls. Interestingly, a deviation from the Hardy-Weinberg equilibrium was detected for SNP rs11031006:G>A near *FSHB* in the control population. Different combinations of risk alleles and haplotypic analyses were homogeneously distributed between cases and controls. Therefore, these results suggest a lack of association between the seven studied SNPs and twin births in CG; however, we hypothesized that other genetic variants related to folliculogenesis or successful pregnancies may be involved in this phenomenon, which has an underlying genetic basis. Identifying such genetic components may be important not only for the Brazilian "Twin's Town," but also for a better understanding of twinning in general.

INTRODUCTION

There are a few places in the world where twin births are recorded at unusually high rates and the twin trait runs through the local families (Nylander, 1969; Matte et al., 1996; Hamamy et al., 2004; Sahab Khan & Skandhan, 2011; Sunde et al., 2013). These cities are known as "Twin's Towns" and, besides being surrounded by much mystery and speculation, they represent a unique opportunity to study a fascinating and puzzling natural phenomenon: human twinning.

Cândido Godói (CG) is a small city, located in the northwest region of Rio Grande do Sul (South Brazil), where a high frequency of natural twin births has been observed, especially in a small district of the city (Matte et al., 1996). Our research group has contributed to the understanding of this noteworthy phenomenon, which was once associated with Nazi experiences (Tagliani-Ribeiro et al., 2011). We have argued that a genetic founder effect must have occurred during the settlement of CG and that genetic factors may help to explain the familial aggregation of twinning in that city (Tagliani-Ribeiro et al., 2012; De Oliveira et al., 2013).

The genetic component of human twinning has been investigated for some time. Some genes and, more specifically, some single nucleotide polymorphisms (SNPs) have been associated with maternal susceptibility for twin births (Al-Hendy et al., 2000; Hasbargen et al., 2000; Tagliani-Ribeiro et al., 2012; Huang et al., 2015; Mardini et al., 2017) ; however, the results are not always concordant, and more importantly, there does not seem to be only one pathway that leads to the "having twins" phenotype in humans. In addition, most efforts have focused on identifying variants that lead to the birth of dizygotic (DZ, or fraternal) twins, whereas monozygotic ones (MZ, or identical) remain even more enigmatic (Derom et al., 2006; Palmer et al., 2006; Painter et al., 2010; Sirugo et al., 2012; Mbarek et al., 2016). In CG, both type of twin births run through local families (Matte et al., 1996; Tagliani-Ribeiro et al., 2011).

Classically, DZ twinning has been related to the mechanism of polyovulation (although not the only one), in which multiple oocytes from separate follicles are fertilized by two different sperms (for a review, see Hoekstra et al. 2007). Folliculogenesis is closely related to the follicle-stimulating hormone (FSH) and specific variants in genes from this pathway seem to influence the likelihood of DZ twin births, especially SNPs in *FSHR*

(OMIM: 136435), *FSHB* (OMIM: 136530), and *SMAD3* (OMIM: 603109) (Al-Hendy et al., 2000; Mbarek et al., 2016).

However, regardless of the early mechanism that leads to MZ or DZ twinning, there must be a prepared organism to sustain a successful pregnancy, i.e., it is necessary that post-fecundation events ensure the possibility that a twin pregnancy will succeed to term, which can be modulated by specific genetic variants (Tagliani-Ribeiro et al., 2012; Boudjenah et al., 2014; Huang et al., 2015). Thus, events such as embryo implantation and *in utero* embryo survival may contribute to the maintenance of a twin pregnancy, and SNPs in the genes *MTHFR* (OMIM: 607093), *TNF* (OMIM: 191160), and *VEGFA* (OMIM: 192240) have been listed as good candidates, although the results do not always agree (Hasbargen et al., 2000; Haggarty et al., 2006; Vialard et al., 2013; Boudjenah et al., 2014).

In fact, due to their important roles in implantation and in utero survival, we studied some SNPs in the p53 pathway, through a case-control study, in mothers of twins and mothers of singletons from CG (Tagliani-Ribeiro et al., 2012). The rs1042522-C allele in the *TP53* gene was a strong risk factor for twinning in that city, but it is probably only part of the genetic repertoire that mediates this intriguing phenomenon.

Therefore, in this work we decided to test seven SNPs related to folliculogenesis (which would affect DZ twin births) and to successful pregnancies (which would affect both kinds of twin births) in the population from CG, a Twin's Town in South Brazil.

MATERIAL AND METHODS

Subjects

We performed a case-control study in which cases were represented by mothers of twins and controls by mothers of singletons; all the mothers were from CG. A detailed characterization of this sample was given by Tagliani-Ribeiro et al. (2012). Briefly, the case group was composed of 44 women who gave birth to twins, which were divided into 21 mothers of DZ twins and 10 mothers of MZ twins (besides one woman who had triplets, two of them MZ, the remaining 12 women were mothers of same-sex twins who were not investigated for zygosity). The case samples represented 40% of all mothers of twins born in CG after 1955. The control group was comprised of 102 women who only gave birth to singletons and had no first-degree relative who was a twin. All the women signed written consent forms before the collection of information and blood samples. This research project was approved by the Hospital de Clínicas de Porto Alegre Ethics Research Committee under protocol number 09-359.

Molecular analysis

DNA was extracted according to the method of Lahiri and Nurnberger (1991) and quantified using a NanoDrop 2000 (Thermo Scientific, USA). We studied a total of seven SNPs (Table 1) in genes related to folliculogenesis (rs6166:C>T in *FSHR*, rs11031006:G>A near *FSHB*, and rs17293443:T>C in *SMAD3*) and to successful pregnancies (rs2010963:C>G in *VEGFA*, rs1800629:G>A in *TNF*, and rs1801131:T>G and rs1801133:G>A in *MTHFR*). Genotypes were determined by allelic discrimination using a TaqMan SNP Genotyping Assay (Applied Biosystems, USA); the demand assay numbers are listed in Table 1. Not all samples were genotyped for all polymorphisms because some samples were exhausted. The real-time PCR reactions were performed in 48-well plates and each reaction contained: 2 ng of genomic DNA, 2x TaqMan Genotyping Master Mix (Applied Biosystems), probes specific for each polymorphism (40x), and enough water to reach 10 μl. For quality control, a subsample of 10% of all samples was genotyped in duplicate for each polymorphism. The reactions were conducted in a StepOne[TM] PCR Real-Time System and the genotyping results were analyzed in StepOne Software v2.2.2 (Applied Biosystems, USA). For SNPs in the same chromosome, linkage disequilibrium (LD) analysis was performed using Haploview 4.2 (Barrett et al., 2005), and PHASE v.2.1 (Stephens et al., 2001) was used for Bayesian haplotype estimation.

Statistical analysis

Genotypic and allelic frequencies were calculated through simple counting. The Hardy-Weinberg equilibrium (HWE) was tested for cases and controls using the chi-squared test (χ^2). Differences in the distributions of the genotypic and allelic frequencies in the case and control groups were tested through Pearson's chi-squared and the two-tailed Fisher's exact test, respectively. Mothers of DZ twins (n = 21) were compared separately with control samples. Due to the remarkable European ancestry present in CG (Matte et al., 1996; Tagliani-Ribeiro et al., 2011), we decided to compare the frequencies of the case group with the allelic frequencies attributed to the European population according to the 1,000 Genomes Project Phase 3 (Auton et al., 2015) and the Exome Aggregation Consortium – ExAC (Lek et al., 2016). We also performed allelic combinations by considering a gene-gene additive effect and according to a dominant model in which either one or two copies of the risk allele are required for an increasing likelihood of the phenotype, and Pearson's chi-squared test was used to test any difference in the frequency between the cases and controls. Statistical analysis was done in SPSS 18.0 for Windows (BM SPSS, IBM Corp, Armonk, NY, USA). The threshold for statistical significance was set at P < 0.05.

RESULTS

A total of 44 mothers of twins (case group) and 102 mothers of singletons (control group) were studied, all of them from CG. Table 2 shows the distributions of the genotypic and allelic frequencies of the studied SNPs in the mothers of both groups. All studied polymorphisms were in HWE for the case and control groups (all $P > 0.05$), except for rs11031006:G>A near *FSHB* in the control group ($\chi^2 = 8.39$; $P = 0.0038$). Due to the deviation from the HWE, rs11031006:G>A near FSHB was not included in later analyses.

No significant differences were found in the genotype and allelic distributions of the seven studied SNPs ($P > 0.05$) between cases and controls. The absence of statistical significance was maintained when we compared only the mothers of DZ twins with the control group (Table 2). Furthermore, the distribution of the rs1800629-A allele in *TNF* was statistically significantly different between cases (23.3%) and the healthy European population (13.4%; $P = 0.019$) according to the 1,000 Genomes Project Phase 3, but this difference was not maintained when comparing cases with the European (Non-Finnish) population recorded in the ExAC (22.1%; $P = 0.794$).

Different combinations of risk alleles for twin births were statistically homogeneous across the cases and controls for all polymorphisms (Table 3). Both polymorphisms in *MTHFR* were in linkage disequilibrium in this population (D' = 0.95; LOD = 10.33), unlike SNPs in *VEGFA* and *TNF* (D' = 0.067; LOD < 2.0). According to haplotype analysis in *MTHFR*, we found three haplotypes in the case group and four in the control group (Table 4). Although the risk haplotype was overrepresented in the case group, no statistically significant difference was found between the groups ($P = 0.623$).

DISCUSSION

Although evident, the genetic component for human twinning is still poorly understood. In this work, we hypothesized that SNPs in genes related to folliculogenesis or to successful pregnancies could be involved in the high natural rate and intrafamilial transmission of twin births in CG, a Twin's Town located in South Brazil.

SNPs in the FSH pathway have been considered in twinning due to the involvement of this hormone with folliculogenesis, a key event related to DZ twinning (for a review, see Hoekstra et al. 2007). A polymorphism in the gene encoding the FSH receptor (*FSHR* rs6166:C>T) was associated with DZ twin births (Al-Hendy et al., 2000), but we did not find any association between this SNP and twin births (overall or only DZ) in CG. This result is in agreement with others who did not replicate this association (Liao et al., 2001; Montgomery et al., 2001; Derom et al., 2006).

We also decided to evaluate two other polymorphisms related to folliculogenesis: rs11031006:G>A near *FSHB*, which encodes one of the two subunits (β-subunit) of FSH (for a review see, Simoni and Casarini 2014); and rs17293443:T>C in *SMAD3*, which encodes a transduction factor that regulates the synthesis of FSH (Li et al., 2017). Both polymorphisms were recently associated with DZ twinning through a genome-wide association study (GWAS) in mothers of DZ twins and a subsequent replication in a large cohort from Iceland (Mbarek et al., 2016). We did not replicate the association between rs17293443:T>C in *SMAD3* and twin births in CG, and we found a deviation from the HWE for the SNP rs11031006:G>A near *FSHB* in the CG control population.

Deviations in HWE can be caused by genotyping errors, but we have excluded this possibility because the samples were randomly tested more than once and the genotyping accuracy rate was greater than 99%. Sample size is another factor that may be responsible for random errors in allelic frequencies, and our sample number is limited, although representative of a naturally small population. In addition, Hardy-Weinberg disequilibrium may be related to heterozygous advantage, population admixture, copy number variants, or inbreeding (Li & Leal, 2009). Although this deviation was only observed for rs11031006:G>A near *FSHB*, a high degree of inbreeding in the CG population has already been demonstrated (Tagliani-Ribeiro et al., 2011; De Oliveira et al., 2013). In addition to DZ twinning, rs11031006:G>A near FSHB was strongly associated with polycystic ovary syndrome (PCOS) in women of European ancestry (Hayes et al., 2015). Therefore, we are

led to believe that inbreeding, together with the limited sample size observed in our study, may underlie the observed deviation in a SNP that has been strongly related to human reproduction. In addition, the deviation from HWE is also found in some healthy American populations according to the 1,000 Genomes Project Phase 3 (P < 0.05) and, when considering only women of European ancestry, the P-value of the HWE is borderline (P = 0.060) (Auton et al., 2015).

Considering that the frequency of both kinds of twins (MZ and DZ) is increased in CG, we decided also to test genetic variants related to successful pregnancies. Firstly, embryo implantation is a multifactorial event that depends on the interplay between the embryo and maternal endometrium, which needs to be receptive (for a review, see Hoozemans et al. 2004). VEGFA and TNF are important factors in this process, although the biological basis of such involvement is far from fully understood (for a review, see Hoozemans et al. 2004; Boudjenah et al. 2014). Beyond implantation, genetic variants in the genes encoding VEGFA and TNF could be associated with multiple pregnancies due to their potential role in crucial processes in the course of a successful pregnancy, such as implantation, decidualization, angiogenesis, and embryo survival (Vialard et al., 2013; Boudjenah et al., 2014).

In fact, the rs1800629-A allele in *TNF* was associated with high implantation and multiple pregnancy rates after IVF in women without known infertility factors (Vialard et al., 2013). Also, Boudjenah et al. (2014) identified associations between multiple pregnancies and rs1800629:G>A in *TNF* and rs2010963:G>C in *VEGF* (alone or in combination) in a sample within a medically-assisted reproduction context. However, our results did not confirm the association of both polymorphisms alone or in combination with twin births in CG. Although the rs1800629-A allele in *TNF* was more frequent in the case group than in the healthy European population (Auton et al., 2015), this association was not maintained for the more robust data provided by ExAC for the non-Finnish European population (Lek et al., 2016).

In addition, folate deficiency, caused either by dietary folate intake or by genetic polymorphism, affects ovarian function, implantation, embryo development, and the chance of multiple births (for a review, see Thaler 2014). In twin pregnancies, demands for folic acid are considerably increased and some studies have linked the maternal folic acid status with the chance of twin births, although that association has not always been

replicated (Czeizel et al., 1994; Källén, 2004; Haggarty et al., 2006; Muggli & Halliday, 2007). Two common missense variants (rs1801131:T>G and rs1801133:G>A) of the *MTHFR* gene decrease the enzyme's catalytic activity, resulting in lower levels of serum folic acid, and these missense variants affect different contexts of human reproduction, including variations in embryo survival and twin births, especially in women with low folic acid concentrations (Hasbargen et al., 2000; Montgomery et al., 2003; Haggarty et al., 2006; Enciso et al., 2016). Hasbargen et al. (2000) found a lower frequency of the A-allele in *MTHFR* rs1801133:G>A in dichorionic twin mothers when compared to singleton mothers, and, more recently, it was shown that the rs1801131-G allele in *MTHFR* was overrepresented amongst women who had undergone several unsuccessful assisted reproductive treatments (Enciso et al., 2016). In a context of medically assisted reproduction, it was showed that folic acid intake can influence twin births, but the genotypes of both polymorphisms were statically homogenous between the twin and singleton mothers (Haggarty et al., 2006).

In this study, although both the risk allele and the risk haplotype in *MTHFR* were found in greater numbers in the cases than in the controls, there was no statistical support for a consistent association. It has also been considered whether only DZ twinning would be affected by changes in folic acid levels (Källén, 2004), but in our study, the frequency of polymorphisms was statistically similar between the cases (overall or DZ alone) and controls. Our results are in agreement with those of Montgomery et al. (2003), who studied both polymorphisms in mothers of DZ twins and did not find any association between *MTHFR* genotype and twinning. Beyond genotypes, the association between the likelihood of twin pregnancy and maternal folic acid levels not always had statistical support, and it has been questioned due to possible confounding factors. The question is still a matter of debate (Vollset et al., 2005; Muggli & Halliday, 2007).

This study had some limitations. It would be interesting to evaluate data on FSH, ovarian reserve, or folic acid levels in our sample; however, this is a population-based study and many women included in the case or control groups are no longer at reproductive age. Therefore, we have considered that these data, at the present time, would not be informative. We could be missing a minor effect due to the small sample size, but the cases sampled in this study represented 40% of all mothers of twins born in CG after 1955.

To sum up, the present study suggests a lack of association between twin births in CG and seven SNPs related to folliculogenesis and successful pregnancies. Finally, it is important to consider that this city has a very particular founding history and it is possible that other genetic variants linked to both processes may explain the high twinning rate found in the region.

DECLARATION OF INTEREST

The authors have no competing interests to declare.

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Table 1: General characteristics of the seven SNPs studied in this work.

Gene	SNP	HGVS name	Common name	Locus	Probe	Annotation	Risk Allele for Twin Births	RAF[a]	RAF[b]
FSHR	rs6166:C>T	NC_000002.12:g.48962782C>T	FSHR Asn680Ser	2p16.3	C___2676874_10	missense	T[c]	0.53	0.55
near *FSHB*	rs11031006:G>A	NC_000011.10:g.30204981G>A	-	11p14.1	C__32036787_10	intergenic	G[d]	0.87	-
SMAD3	rs17293443 :T>C	NC_000015.10:g.67145525T>C	-	15q22.33	C__33991338_10	intron	C[d]	0.25	-
VEGFA	rs2010963:C>G	NC_000006.12:g.43770613C>G	VEGF +405 G/C	6p21.1	C___8311614_10	5' UTR	G[g]	0.67	-
TNF	rs1800629:G>A	NC_000006.11:g.31543031G>A	TNFα -308	6p21.33	C___7514879_10	upstream	A[g]	0.12	0.22
MTHFR	rs1801131:T>G	NC_000001.11:g.11794419T>G	MTHFR A1298C	1p36.22	C____850486_20	missense	T[e]	0.69	0.68
MTHFR	rs1801133:G>A	NC_000001.11:g.11796321G>A	MTHFR C677T	1p36.22	C___1202883_20	missense	G[f]	0.65	0.66

Abbreviations: SNP, single nucleotide polymorphism; RAF, risk allele frequency for twin births. [a]Frequency to European women according to 1,000 Genomes Project Phase 3 (Auton *et al.*, 2015); [b]Frequency to European (Non-Finnish) according to ExAC (Lek *et al.*, 2016); [c]Based on Al-Hendy et al. (2000); [d]Based on Mbarek et al. (2016); [e]Based on Hasbargen et al. (2000); [f]Based on Enciso et al. (2016); [g]Based on Boudjenah et al. (2014).

Table 2: Genotypic and allelic frequencies of the studied SNPs.

SNP	Cases % (n)	Controls % (n)	P	Cases DZ % (n)	P
FSHR rs6166:C>T	**n = 42**	**n = 101**		**n = 20**	
TT	26.2 (11)	23.8 (24)	0.798[a]	10.0 (2)	0.323[a]
TC	52.4 (22)	49.5 (50)		65.0 (13)	
CC	21.4 (9)	26.7 (27)		25.0 (5)	
T	52.4 (44)	48.5 (98)	0.604[b]	42.5 (17)	0.495[b]
HWE (_P_)	0.746	0.928			
FSHB rs11031006:G>A	**n = 43**	**n = 102**		**n = 21**	
GG	83.7 (36)	82.4 (84)	-	90.5 (19)	-
GA	16.3 (7)	13.7 (14)		9.5 (2)	
AA	0 (0)	3.9 (4)		0 (0)	
G	91.9 (79)	89.2 (182)	-	95.2 (40)	-
HWE (_P_)	0.561	0.004			
SMAD3 rs17293443:T>C	**n = 43**	**n = 102**		**n = 21**	
CC	11.6 (5)	3.9 (4)	0.193[a]	9.5 (2)	0.553[a]
CT	27.9 (12)	34.3 (35)		33.3 (7)	
TT	60.5 (26)	61.8 (63)		57.1 (12)	
C	25.6 (22)	21.1 (43)	0.442[b]	26.2 (11)	0.539[b]
HWE (_P_)	0.080	0.752			
MTHFR rs1801131:T>G	**n = 43**	**n = 102**		**n = 21**	
TT	48.8 (21)	42.6 (43)	0.741[a]	47.6 (10)	0.795[a]
TG	39.5 (17)	45.5 (47)		38.1 (8)	
GG	11.6 (5)	11.9 (12)		14.3 (3)	
T	68.6 (59)	65.4 (133)	0.590[b]	66.7 (28)	1.000[b]
HWE (_P_)	0.590	0.877			
MTHFR rs1801133:G>A	**n = 42**	**n = 101**		**n = 20**	
GG	50.0 (21)	43.6 (44)	0.689[a]	45.0 (9)	0.691[a]
GA	42.9 (18)	45.5 (46)		45.0 (9)	
AA	7.1 (3)	10.9 (11)		10.0 (2)	
G	71.4 (60)	66.3 (134)	0.487[b]	67.5 (27)	0.597[b]
HWE (_P_)	0.746	0.843			
VEGFA rs2010963:C>G	**n = 43**	**n = 102**		**n = 21**	
GG	39.5 (17)	43.1 (44)	0.813[a]	33.3 (7)	0.894[a]
GC	48.8 (21)	43.1 (44)		52.4 (11)	
CC	11.6 (5)	13.7 (14)		14.3 (3)	
G	63.9 (55)	64.7 (132)	0.894[b]	59.5 (25)	1.000[b]
HWE (_P_)	0.698	0.575			
TNF rs1800629:G>A	**n = 43**	**n = 102**		**n = 21**	
AA	2.3 (1)	1.0 (1)	0.642[a]	0 (0)	0.731[a]
AG	41.9 (18)	36.3 (37)		38.1 (8)	
GG	55.8 (24)	62.7 (64)		61.9 (13)	
A	23.3 (20)	19.1 (39)	0.428[b]	19.1 (8)	1.000[b]
HWE (_P_)	0.257	0.081			

Abbreviations: SNP, Single Nucleotide Polymorphism; Cases DZ, only mothers of DZ twins.
[a]Pearson's chi-square; [b]Two-tailed Fisher's exact test. [c]Pearson's chi-square with Yates' correction.

Table 3: Different combinations of risk alleles for twin births in mothers of twins (cases) and of singleton (controls) in CG.

Alleles[a]	Cases % (n)	Controls % (n)	P[b]
FSHR rs6166-T + *SMAD3* rs17293443-C	29.3 (12)	26.7 (27)	0.638
MTHFR rs1801131-T + *MTHFR* rs1801133-G	80.5 (33)	77.2 (78)	0.670
VEGFA rs2010963-G + *TNF* rs1800629-A	34.9 (15)	33.3 (34)	0.296
MTHFR rs1801131-T + *MTHFR* rs1801133-G + *VEGFA* rs2010963-G + *TNF* rs1800629-A	26.8 (11)	26.7 (27)	0.383

[a]Includes the presence of the allele in homozygosis or heterozygosis; [b]Pearson's chi-square.

Table 4: Haplotype analysis of rs1801131:T>G and rs1801133:G>A in mothers of twins (cases) and of singleton (controls) in CG.

Gene	Haplotype[b]	Total % (n)	Cases % (n)	Controls (%)	P[a]
MTHFR	T-G	33.9 (99)	38.6 (34)	31.9 (65)	0.623
	T-A	31.9 (93)	28.4 (25)	33.3 (68)	
	G-G	33.9 (99)	33.0 (29)	34.3 (70)	
	G-A	0.3 (1)	0 (0)	0.5 (1)	

[a]Pearson's chi-square; [b]rs1801131:T>G and rs1801133:G>A, respectively.

CAPÍTULO VI

DISCUSSÃO

6. DISCUSSÃO

Além de despertar fascínio e de ser fonte de especulação tanto leiga quanto científica ao longo dos anos, a gemelaridade tem fornecido um caminho para o entendimento da nossa própria biologia. A Ciência e, em especial, a Genética vem utilizando este experimento biológico natural para investigar o comportamento, a fisiologia, o desenvolvimento e outras peculiaridades dos seres humanos. Entretanto, ainda há muito a ser esclarecido no tocante à gemelaridade *per se*.

"Quantos gêmeos nascem?" e "Como os gêmeos nascem?" são duas questões ainda em aberto. Respondê-las não se trata meramente de resolver curiosidades científicas, mas também de uma importante questão de saúde, visto que uma gestação gemelar representa riscos adicionais à mãe e à prole (Corsello and Piro 2010; Caserta et al. 2014). Ainda, o nascimento de gêmeos é atualmente considerado o principal efeito iatrogênico relacionado RMA, o qual, juntamente com outros componentes ambientais como o atraso à maternidade, tem contribuído para o aumento contemporâneo global no número de gêmeos (muito embora este dado seja escasso em países em desenvolvimento) (Ananth and Chauhan 2012; Pison et al. 2015; Dawson et al. 2016).

Neste trabalho, investigamos os nascimentos gemelares conforme duas perspectivas principais: epidemiológica e etiológica. Do ponto de vista epidemiológico, investigamos as taxas gemelares no Brasil entre 2001 e 2014 em duas dimensões – espacial e temporal. Para os estudos etiológicos, testamos algumas variantes genéticas relacionadas à foliculogênese e a gestações de sucesso em mães de Cândido Godói, uma pequena cidade localizada no sul do Brasil e popularmente conhecida como a "Cidade dos Gêmeos".

Epidemiologicamente, a TG é um indicador de saúde definido como o número de nascimentos gemelares por 1.000 (‰) nascimentos em uma determinada localidade. No Brasil, estudos nessa área são concentrados principalmente nos estados do Rio Grande do Sul e de São Paulo, cujos dados são altamente discordantes (Colletto and Beiguelman 2001; Colletto 2003; Geraldo et al. 2008; Otta et al. 2016). Ao que nos consta, existe apenas um estudo que atribui uma TG para o Brasil como um todo e, embora seja muito informativo, ele não considera a diversidade territorial e sociodemográfica do Brasil, além de apresentar dados de 1996 que podem não refletir a realidade de maneira fidedigna (Smits and Monden 2011).

Nós estudamos as TGs no Brasil a partir de dados do Sistema de Informações sobre Nascidos Vivos (SINASC), braço do Ministério da Saúde que cobre todo o território nacional. De acordo com nossos resultados, a TG brasileira aumentou de 8.65‰ em 2001 para 10.15‰ em 2014 e este aumento foi ainda mais acentuado em mulheres acima de 45 anos. Embora seja similar à TG antes reportada para todo o Brasil (8.8‰; Smits and Monden 2011), nós detectamos, a partir da nossa análise de séries temporais, uma tendência ascendente para o país como um todo e apenas para estados das regiões Sudeste, Sul e Centro-Oeste, além de Bahia e Paraíba. Ao introduzirmos a idade acima de 30 anos como covariável no modelo de séries temporais, detectamos uma relação positiva entre idade materna e TG para o Brasil e para todos os estados, exceto Piauí e Maranhão.

A TG brasileira encontrada neste estudo está próxima das TGs atribuídas para outros países da América do Sul e alguns países asiáticos (9,0 a 12,0‰), e é consideravelmente menor que aquelas atribuídas para países da África Central, onde são registradas as maiores incidências de gemelaridade ao redor do mundo (que atinge até 27,9 nascimentos gemelares a cada 1000 nascimentos) (Bulmer 1970; Hoekstra et al. 2007; Smits and Monden 2011). Altas taxas de nascimentos gemelares também são recentemente encontradas em países industrializados, como Estados Unidos, França, Inglaterra e Noruega, porém, nesses países a tendência de aumento é associada a dois fatores principais: maternidade atrasada e/ou RMA (Pison et al. 2015; Fellman 2016; Dawson et al. 2016).

A partir dos resultados das nossas análises espaciais, nós somos levados a acreditar que algo semelhante deve estar acontecendo no Brasil, embora de maneira heterogênea. Isto porque um claro padrão de distribuição espacial também foi detectado, com as maiores TGs concentradas na região Sudeste e Sul, e as menores localizadas nas regiões Norte e Nordeste, o que foi corroborado pela análise de Cluster and Outlier (*Anselin Local Moran's Index*). Com dados fornecidos pelo Instituto Brasileiro de Geografia e Estatística (IBGE), nós concluímos que altas TGs são significativamente concentradas em municípios com alto Índice de Desenvolvimento Humano (IDH) e as mais baixas TGs em municípios com um baixo IDH.

No Brasil, o alto custo associado à RMA e o acesso limitado a estes procedimentos, tanto dentro da rede de saúde pública quanto de planos privados de saúde, representam uma importante barreira econômica para o uso de tecnologias reprodutivas (Makuch and

Bahamondes 2012; Corrêa and Loyola 2015; Tavares et al. 2016). Embora o período de 14 anos seja curto, muitas mudanças econômicas e sociais ocorreram neste país, e tais mudanças afetam a vida dos brasileiros de muitas maneiras, inclusive na vida reprodutiva (Madalozzo 2012; United Nations Development Programme 2013; Tejada et al. 2017).

Assim, nós hipotetizamos que a tendência de aumento nas TGs dentro do Brasil vista principalmente em mulheres com idade mais avançada é devida a uma combinação de cinco fatores principais: 1) atraso na maternidade, que tem ocorrido principalmente entre as mulheres de alta renda (Makuch and Bahamondes 2012; Tejada et al. 2017); 2) o fato da infertilidade aumentar com a idade materna e atingir altas taxas em países em desenvolvimento (Ombelet 2009; Makuch et al. 2011); 3) o recente e desigual melhoramento no desenvolvimento humano na população brasileira (Madalozzo 2012; United Nations Development Programme 2013); 4) o aumento na disponibilidade de serviços de RMA em alguns lugares do país (Makuch and Bahamondes 2012; Anvisa 2014), e 5) fraca fiscalização dos procedimentos ligados à MAR (como o fato da transferência de não mais que dois embriões nestes procedimentos ser apenas uma recomendação, ao invés de uma lei ou de uma obrigação estrita) (Corrêa and Loyola 2015).

Para o estudo acerca da etiologia dos nascimentos gemelares, nós revisitamos as pesquisas em Cândido Godói, uma pequena cidade localizada no Noroeste do Rio Grande do Sul, no sul do Brasil. Nós desenhamos um estudo caso-controle e genotipamos sete SNPs relacionados à foliculogênese (rs6166:C>T em *FSHR*, rs11031006:G>A próximo a *FSHB*, e rs17293443:T>C in *SMAD3*) ou a uma gestação de sucesso (rs1801131:T>G e rs1801133:G>A em *MTHFR*, rs2010963:C>G em *VEGFA*, e rs1800629:G>A em *TNF*). Para cada SNP, os alelos de risco para a gemelaridade foram apontados tendo como base estudos anteriores que avaliaram a influência dos mesmos com diferentes contextos da reprodução humana (Al-Hendy et al. 2000; Hasbargen et al. 2000; Boudjenah et al. 2014; Mbarek et al. 2016; Enciso et al. 2016).

Entre casos e controles, nenhuma diferença estatisticamente significativa foi encontrada entre as distribuições alélicas e genotípicas para os sete SNPs estudados. Embora a frequência do alelo rs1800629-A em *TNF* tenha sido estatisticamente maior nos casos que na população europeia saudável de acordo com o Projeto 1.000 Genomas Fase 3 (*1,000 Genomes Project Phase 3*; Auton et al. 2015), tal diferença não foi mantida quando

a frequência nos casos foi comparada a dados mais robustos obtidos no ExAC (*Exome Aggregation* Consortium) para a população europeia não-finlandesa (Lek et al. 2016).

O alelo rs1800629-A em *TNF* já foi associado com alta taxa de implantação e gestações múltiplas após fertilização *in vitro* em mulheres sem algum fator conhecido de fertilidade (Vialard et al. 2013). Além disso, Boudjenah et al. (2014), em uma amostra dentro do contexto de RMA, identificaram uma associação entre gestações múltiplas e rs1800629:G>A em *TNF* e rs2010963:G>C em *VEGF* (sozinhos ou em combinação), mas nossos resultados não confirmam o envolvimento de ambos os polimorfismos com os nascimentos gemelares em CG.

Os outros dois SNPs possivelmente associados a uma gravidez de sucesso (rs1801131:T>G e rs1801133:G>A em *MTHFR*) também não foram estatisticamente associados à gemelaridade neste estudo, muito embora tanto o alelo quanto o haplótipo de risco tenham sido majoritariamente representado no grupo de casos quando comparado aos controles. Embora ambos os polimorfismos diminuam a atividade catalítica de MTHFR e resultem em níveis mais baixos de ácido fólico circulante, a associação de ambos com os nascimentos gemelares permanece controversa (Hasbargen et al. 2000; Haggarty et al. 2006; Montgomery et al. 2014; Thaler 2014; Enciso et al. 2016). Ao contrário do encontrado por Hasbargen et al. (2000) e Enciso et al. (2016), este resultado está de acordo com o relatado por Montgomery et al. (2001), o qual estudou ambos os polimorfismos em mães de gêmeos DZ e não encontrou nenhuma associação entre os genótipos em *MTHFR* e a gemelaridade. Para além dos genótipos, a associação entre os níveis de ácido fólico materno e os nascimentos gemelares foi questionada devido a possíveis fatores de confusão e a questão permanece matéria de debate (Vollset et al. 2005; Muggli and Halliday 2007).

Além disso, nossos resultados não fornecem suporte para uma associação entre os SNPs relacionados à foliculogênese com a gemelaridade (total ou apenas DZ) em CG. Em consonância, outros estudos também não têm replicado essa hipótese (Liao et al. 2001; Montgomery et al. 2001; Derom et al. 2006). Muito embora a rota do FSH seja uma via classicamente relacionada à gemelaridade DZ humana (Lambalk 2001; Hoekstra et al. 2007), é importante considerar que o nascimento de gêmeos é um fenótipo complexo e não é razoável considerar que exista apenas uma via pela qual este fenômeno ocorre em nossa espécie.

Este estudo possuiu algumas limitações. Por exemplo, seria interessante avaliar dosagens de FSH, reserva ovariana e/ou os níveis de ácido fólico em nossa amostra. Todavia, este é um estudo de base populacional e muitas participantes já não se encontravam em sua idade reprodutiva e, assim, nós consideramos que este dado no momento presente não seria muito informativo. Além disso, o tamanho amostral pode ter sido outra limitação, principalmente no que tange a variantes de pequeno efeito, mas os casos amostrados neste estudo representam 40% de todas as mães de gêmeos nascidos em CG após 1995.

Em conclusão, este estudo sugere uma ausência de associação entre os nascimentos gemelares em CG e sete SNPs relacionados à foliculogênese e a gestações de sucesso. Finalmente, é importante considerar que CG tem uma história de fundação muito particular e é possível que outras variantes genéticas ligadas a ambos os processos relacionados à reprodução ajudem a explicar a alta TG encontra na região.

CAPÍTULO VII

CONCLUSÕES E PERSPECTIVAS

7. CONCLUSÕES E PERSPECTIVAS

Na primeira parte deste trabalho, nós retratamos um desenho detalhado sobre a epidemiologia dos nascimentos gemelares no Brasil e encontramos que a TG brasileira se distribuiu de maneira heterogênea ao longo do país, com alta incidência e tendência ascendente em regiões com um alto IDH. Altas taxas de nascimentos gemelares foram encontradas principalmente em mulheres com idade mais avançada. Em conjunto, este cenário pode ser explicado por uma cadeia de fatores que iniciam no atraso da maternidade, passam pela desigualdade no acesso à RMA e terminam na fraca legislação no tocante às tecnologias reprodutivas. Nossos resultados podem fomentar o atual debate sobre a democratização do acesso à RMA no Brasil, e acreditamos que nossa abordagem de utilizar o IDH como "proxy" para RMA pode ser utilizado em demais países com características sociodemográficas semelhantes às do Brasil.

Ainda utilizando o próprio SINASC, pretendemos aplicar uma metologia semelhante à utilizada neste trabalho para elucidar diferenças sociais e epidemiológicas relacionadas aos nascimentos gemelares, utilizando variáveis como Cor/raça do RN, Apgar, Peso e Anomalia congênita. Além disso, podemos utilizar outro banco de dados público, o Sistema de Informação de Mortalidade (SIM), para traçar um perfil epidemiológico no Brasil sobre as causas de morte em gêmeos *versus* em únicos.

Na segunda parte deste trabalho, nós focamos na etiologia da gemelaridade, estudando alguns SNPs relacionados à foliculogênese ou a uma gestação de sucesso na população de CG, a "Cidade dos Gêmeos". As variantes foram escolhidas a partir de estudos hipótese-dependente ou de varredura genômica e nossos resultados sugerem uma ausência de associação entre as sete variantes genéticas e o fenótipo de estudo. Pesquisas adicionais sobre o papel destas variantes no contexto reprodutivo, bem como a sua investigação em demais populações com características similares a CG podem ajudar a elucidar a etiologia dos nascimentos gemelares na espécie humana.

CAPÍTULO VIII

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8. REFERÊNCIAS BIBLIOGRÁFICAS

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CAPÍTULO IX

Anexo

9. **ARTIGO ANEXO –** *Lack of Association between Genetic Polymorphisms in IGF1 and IGFBP3 with Twin Births in a Brazilian Population (Cândido Godói, Rio Grande do Sul)*

Artigo aceito para publicação na revista *Genetics and Molecular Biology*

Neste trabalho, nós hipotetizamos que o fator de crescimento semelhante à insulina do tipo 1 (IGF-1), que é classicamente envolvido na reprodução e desenvolvimento de mamíferos, pode estar associado aos nascimentos gemelares em CG. Assim, nós investigamos dois polimorfismos genéticos relacionados ao metabolismo do IGF-1 (*IGF1* (CA)n and *IGFBP3* rs2854744) em 39 mães de gêmeos e 214 mães de não-gêmeos (97 delas morando em CG e 117 morando em Porto Alegre). Nós não identificamos diferenças estatisticamente significativas entre ambos os polimorfismos e os nascimentos gemelares em CG. As limitações foram as mesmas deste presente trabalho.

Lack of Association between Genetic Polymorphisms in *IGF1* and *IGFBP3* with Twin Births in a Brazilian Population (Cândido Godói, Rio Grande do Sul)

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Runnig Head: Twin births and genetic variants

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ABSTRACT

Insulin-like growth factor (IGF-1) is an important peptide hormone involved in the reproduction and fetal development of mammals, and it is suggested that it may influence the human twinning rate. Thus, this study aimed to test such possible association, investigating the genetic polymorphisms *IGF1* (CA)n and *IGFBP3* rs2854744 in the population from Candido Godoi (CG), a small city located in the South of Brazil where is found a high prevalence of twin births. A case-control study was performed comprising a total of 39 cases (representing about 40% of the mothers of twins who were born in CG after 1995) and 214 controls (mothers of non-twin children) — 97 of whom were living in CG while 117 were living in Porto Alegre. DNA was extracted from blood leucocytes and genotyping was performed. According to the statistical analyses, there was no statistically significance difference in the frequencies of both studied genetic polymorphisms when comparing case group with control groups. Thus, our results pointed to a lack of association between polymorphisms *IGF1* (CA)n and *IGFBP3* rs2854744 and the twin births in CG, but further investigations in other populations with different characteristics must be performed to confirm the role of IGF-I in human twinning.

Although genetic factors related to twin births in the human species have been investigated by different approaches (Montgomery et al. 2001; Painter et al. 2010; Tagliani-Ribeiro et al. 2012; Mbarek et al. 2016), there is still a confusing scenario of which genes or alleles may be related to etiology of twinning, overall because it is a complex phenomenon and different causes may be involved in different situations (Lambalk et al. 1998; Huang et al. 2015; Mardini et al. 2017).

The small city in the South of Brazil called Cândido Godói (CG, latitude $27^o45'07''$, longitude $54^o45'07''$) has attracted the attention of researchers and curious observers, due to high rates of natural monozygotic (MZ) and dizygotic (DZ) twin births found there and because this trait run among local families (Matte et al. 1996; Tagliani-Ribeiro et al. 2011). Studies previously performed in this population have suggested that the founder effect hypothesis linked to geographical isolation may explain such peculiarity, associated to the finding of a single nucleotide polymorphism (SNP) of *TP53* as a strong risk factor for twinning, possibly due to its important role in blastocyst implantation and *intra utero* embryo survival (Tagliani-Ribeiro et al. 2012). However, it is expected that other factors, including other genetic polymorphisms, may be contributing with the high twinning rates found in CG.

Besides the p53 pathway, other factors possibly related to twin births have been studied in different contexts, among which, factors related to individual endocrine profiles stand out (Montgomery et al. 2001; Rickard et al. 2012). Insulin-like growth factor (IGF-1), for example, has previously been proposed to be associated with twin births in humans (Steinman 2009), and biochemical and genetic studies has already related such factor to twinning in cattle (Echternkamp et al. 1990; Echternkamp et al. 2004; Kim et al. 2008).

IGF-1 is an important peptide hormone for cellular differentiation and growth, and it is also involved with mechanisms related to reproduction and fetal development of mammals (Adam et al. 2000; Echternkamp et al. 2004; Thomas et al. 2016). In human reproduction, IGF-1 acts as a pituitary regulator of follicular growth and potentiates the action of both gonadotrophins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Ohlsson et al. 2009). In the ovary, it regulates the differentiation of granulosa cells and the development of the follicle, and may influence the twinning rate (Lambalk et al. 1998; Erickson and Shimasaki 2001). Furthermore, traits associated with IGF-1, such as

ethnicity and body mass index covaries with twinning ratio (Steinman 2009; Rickard et al. 2012).

Genetic factors are known to influence IGF-1 levels and its individual variation (Rosen et al. 1998; Kwasniewski et al. 2016). The *IGF1* gene has a polymorphic microsatellite of cytosine and adenine nucleotides, (CA)n, which range in sizes from 10 to 24 CA repeats, located at the *IGF1* promoter region, approximately 1 kb upstream from the transcription start site (GenBank accession number AB133839.1) (Jernström et al. 2001). In the Caucasian population, the most common allele contains 19 CA repeats (NG_011713.1:g.4248CA[19]), commonly known as (CA)19, which corresponds to fragments of 192bp after amplification (Costalonga et al. 2012). Interestingly, an exception can be found in the results by Kato et al. (2003), in which a Caucasian population presented the allele with 18 CA repeats as the most frequent, and the authors listed the small sample number and the population admixture as possible explanations for such discrepancy. The length of CA repeats has been associated with serum IGF-1 levels and to different human phenotypes, as well as breast and endometrial cancer (Wagner et al. 2005; Kwasniewski et al. 2016), bone disorders (Kim et al. 2002) and others (Costalonga et al. 2012; Kaczmarek et al. 2015).

In turn, the *IGFBP3* gene (which synthesizes insulin-like growth factor binding protein 3) is responsible for transporting about 90% of the circulating IGF-1 and is capable of deregulating the levels of IGF-1 present in the plasma (Al-Zahrani et al. 2005; Ohlsson et al. 2009). There is evidence that the polymorphism rs2854744: C>A (NG_011508.1:g.4797C>A) in *IGFBP3* is a genetic factor influencing the levels of IGFBP-3 and IGF-1, and it has been shown that women homozygous to the allele A have higher levels of circulating IGFBP-3, and consequently, higher levels of circulating IGF-1 (Ali et al. 2003; Al-Zahrani et al. 2005; Costalonga et al. 2009; Ohlsson et al. 2009).

Taking into consideration the role of *IGF1* (CA)n and *IGFBP3* rs2854744 polymorphisms in the metabolism of IGF-1 and in the reproductive context, the aim of this study was to investigate whether such genetic polymorphisms can help to explain the high rates of twin births found in the city of Cândido Godói, in the state of Rio Grande do Sul, Brazil.

We designed a population-based case-control study, with mothers of twins in case group and mothers of singletons in two control groups. The study was approved by the

ethics committee of the Hospital de Clínicas of Porto Alegre (HCPA) under protocol #09-359. The case group was formed by 39 women, who were mothers of twins and resided in the municipality of CG. This sample number represents about 40% of the mothers of twins who were born in CG after 1995 (Tagliani-Ribeiro et al. 2011). To compare with case group, we studied a control group composed by 97 mothers of non-twin children and who were born in the municipality of CG. The characteristics of both case and control groups from CG were already described in Tagliani-Ribeiro *et al.*, 2012.

In addition, considering the common ancestral origin of CG population, we decided to include an external control group to determine the frequency of the investigated alleles in the state of Rio Grande do Sul. Thus, we included a second control group consisting of 117 mothers of non-twin children living in Porto Alegre (PA), the capital city of state. The control group from PA showed a mean age of 22.6 years and a mean number of pregnancies of 2.5, similar values to control group from CG (Tagliani-Ribeiro et al. 2012).

Taking into account that both MZ and DZ twins have increased prevalence in CG (Matte et al. 1996; Tagliani-Ribeiro et al. 2011) and that our biological hypothesis is focused in aspects related not only to ovulation, but also to fertilization and embryo development, we decided to analyze births of MZ and DZ twins combined.

Genomic DNA of the blood samples was extracted in accordance with that described by Lahiri and Nurnberger 1991. For the *IGF1* (CA)n polymorphism, polymerase reaction chain (PCR) was performed as described by Rosen et al. 1998, using primers sequences 5' 5`GCTAGCCAGCTGGTGTTATT3' and 5'ACCACTCTGGGAGAAGGGTA3'. The analysis of fragments was then performed by capillary electrophoresis in an Applied Biosystems® 3500 Genetic Analyzer sequencer. In order to test the veracity of the results, one sample from each homozygote pair was sequenced for the following alleles: *IGF1* (CA)19, (CA)20, (CA)21 and (CA)22. The samples were purified with the EXO I and SAP enzymes and then subjected to Sanger sequencing. In turn, the rs2854744 of the *IGFBP3* gene was determined via the TaqMan SNP Genotyping Assay, through allelic discrimination using the C___1842665_10 assay in accordance with the manufacturer's instructions (Applied Biosystems, USA). The reactions were conducted in the StepOnePlus™ PCR Real-Time System, and the reaction products were analyzed in the StepOne v. 2.2.2 software.

The Hardy-Weinberg equilibrium was calculated using version 3.11 of the Arlequin program. Simple comparisons of *IGF1* allelic frequencies in the cases and controls were done using the *G*-Test (Likelihood ratio *chi-square*), with a 95% confidence interval, in version 11.15 of the WinPEPI program. For analysis of the *IGFBP3* gene, the Two-tailed Fisher's exact test and Person's *chi-square* were used to compare the allelic and genotypic frequencies between case and control groups using IBM SPSS v.18.0 software (IBMCorp., Armonk, NY).

The samples of case and control groups were in Hardy-Weinberg equilibrium for both of the polymorphisms tested (*p* > 0.05 in cases and control groups for both polymorphisms). The analyses of the *IGF1* (CA)n polymorphism revealed eight different alleles in CG, varying of 11 to 22 CA repeats (Table 1). In turn, the alleles with 23 and 24 CA repeats (with 200 and 202 pb respectively) were found in Porto Alegre, even that at a low frequency. In all studied populations, the most frequent allele was that with 19 CA repeats, with 192 pb. Although the allele with 22 CA repeats (with 198 pb) have occurred more frequently in the mothers of twins (cases, 7.7%) than in the both control groups, there was no statistical significance in the distribution of *IGF1* (CA)n between cases and control groups from CG (p = 0.182) and from PA (p = 0.065). Similarly, for the *IGFBP3* rs2854744 polymorphism, there was no statistically significant difference between cases and controls groups, both for allelic and genotypic frequencies (Table 2).

Although IGF-1 has been considered an important candidate to understanding human twinning (Steinman 2009), we did not find statistical association between two genetic polymorphisms commonly related to its metabolism in a population with a very peculiar foundation history and that shows increased rates of twin births (Matte et al. 1996; Tagliani-Ribeiro et al. 2011).

Interestingly, upon analyzing the results obtained in studies of the *IGF1* (CA)n polymorphism in various continents (Table 3), it could be seen that the frequency of the allele with 22 repeats is low — it ranged from 1.5% in European populations (Vaessen et al. 2001; Rietveld 2009) to 3.4% in a study conducted in China (Xie et al. 2010). In the case group of the present study, this allele occurred at a frequency of 7.7%, while in the controls, from both CG and PA, these frequencies were similar to those observed in studies conducted in Europe and North America (Vaessen et al. 2001; Kato et al. 2003; Wen et al. 2005; Cleveland et al. 2006; Rietveld 2009).

In conclusion, our results pointed to a lack of association between twin births in CG and polymorphisms *IGF1* (CA)n and *IGFBP3* rs2854744. Unfortunately, we could not access the serum IGF-1 levels and comparing it with the investigated genetic polymorphisms. However, genetic variants related to such factor have been independently associated with a plenty of different phenotypes (Wagner et al. 2005; Cleveland et al. 2006; Kaczmarek et al. 2015). Furthermore, to the best of our knowledge, this is the first study to investigate the *IGF1* (CA)n and *IGFBP3* rs2854744 polymorphisms in human twinning and further investigations in other populations with different characteristics must be performed to confirm the role of IGF-1 in human twin births.

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Conflict of Interest

None.

Ethical Standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

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Table 1. Frequency of *IGF1* (CA)n polymorphism in cases and controls.

Number of CA[a] repeats	Cases (n = 39)		CG[b] controls (n = 97)			PA[c] controls (n = 117)		
	N	%	N	%	p[d]	N	%	p[d]
11	-	-	1	0.5	0.55	1	0.4	0.55
16	-	-	2	1.0	0.40	2	0.9	0.40
17	1	1.3	3	1.5	0.66	3	1.3	0.97
18	4	5.1	15	7.8	0.37	21	9.0	0.25
19	50	64.1	112	57.8	0.31	148	63.2	0.75
20	13	16.7	47	24.2	0.13	29	12.4	0.38
21	4	5.1	11	5.7	0.86	26	11.1	0.10
22	6	7.7	3	1.5	0.01	2	0.9	<0.001
23	-	-	-	-	-	1	0.4	0.55
24	-	-	-	-	-	1	0.4	0.55
Total	**78**	**100**	**194**	**100**	**0.18**	**234**	**100**	**0.07**

[a]CA: cytosine-adenosine; [b]CG: Cândido Godói; [c]PA: Porto Alegre; [d]*G-test*

Table 2. Frequency of *IGFBP3* rs2854744 gene polymorphism in cases and controls.

Allele	Cases (n = 39)		CG[a] controls (n = 97)		p[c]	PA[b] controls (n = 117)		p[c]
	N	%	N	%		N	%	
C	44	56.4	104	53.6	0.689	112	47.9	0.239
A	34	43.6	90	46.4		122	52.1	
Genotype	N	%	N	%	p[d]	N	%	p[d]
CC	14	35.9	32	33.0		32	27.4	
CA	16	41.0	40	41.2	0.927	48	41.0	0.484
AA	9	23.1	25	25.8		37	31.6	

[a]CG: Cândido Godói; [b]PA: Porto Alegre; [c]Two-tailed Fisher's exact test; [d]Person's *chi-square* test

Table 3: Percentage of *IGF1* (CA)n polymorphism in some general and non-diseased populations around the world.

REGION	BRAZIL	NORTH AMERICA			EUROPE		CHINA	
Number of CA[a] repeats	This study[b] N = 214	Cleveland et al. (2006) N = 736	Kato et al. (2003) White N = 112	Kato et al. (2003) Black N = 114	Rietveld (2009) N = 5386	Vaessen et al. (2001) N = 1080	Wen et al. (2005) N = 2172	Xie et al. (2010) N = 446
10	-	-	0.9	-	-	-	-	-
11	0.5	0.1	-	-	-	-	0.1	-
13	-	-	0.9	0.9	-	-	-	-
15	-	-	0.9	9.7	-	0.2	0.2	-
16	0.9	0.3	2.7	3.5	-	0.3	0.1	0.2
17	1.4	1.4	7.1	18.4	1.9	1.9	10.5	7.6
18	8.4	6.0	36.6	25.4	4.6	4.1	16.7	15.9
19	60.7	64.3	30.4	18.4	65.3	65.9	35.1	38.8
20	17.8	18.7	16.1	14.9	19.4	18.7	7.7	6.3
21	8.6	7.5	4.5	6.1	6.9	7.4	26.3	27.1
22	1.2	1.6	-	1.8	1.5	1.5	3.1	3.4
23	0.2	0.1	-	0.9	-	-	0.1	0.5
24	0.2	-	-	-	-	-	-	0.2
Others	-	-	-	-	0.4	-	-	-

[a]CA: cytosine-adenosine; [b]Frequencies of the control samples from Cândido Godói and Porto Alegre