NATHÁLIA LISBOA ROSA ALMEIDA GOMES

Novas perspectivas no diagnóstico etiológico dos distúrbios do desenvolvimento sexual 46,XY

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Orientadora: Profa. Dra. Berenice Bilharinho de Mendonça

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Normalização

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Abreviaturas dos títulos dos periódicos de acordo com *List of Journals Indexed in Index Medicus.*

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ABREVIATURAS

%	Percentual
5ARD2	5-α redutase tipo 2
ABraOM	Arquivo Brasileiro Online de Mutações (banco de dados populacional brasileiro)
ACMG	*American College of Medical Genetics*
AIS	*Androgen Insensitivity Syndrome* = Síndrome de Insensibilidade aos Andrógenos
AMH	Hormônio anti-Mulleriano
AS	Alelo selvagem
bp	pares de bases
CADD	*Combined Annotation Dependent Depletion* (ferramenta de predição *in silico*)
CAIS	*Complete Androgen Insensitivity Syndrome* = Síndrome de Insensibilidade Completa aos Andrógenos
CAPPesq	Comissão de Ética para Análise de Projetos de Pesquisa
ClinVar	banco de dados que possui informações de significado clínico de variantes descritas
cm	Centímetros
CNV	*Copy number variation* (variação do número de cópias)
dbSNP	*Single Nucleotide Polymorphism database*= banco de dados para avaliação de polimorfismos de nucleotídeo único
DDS	Distúrbios do desenvolvimento sexual
DGC	Disgenesia gonadal completa
DGP	Disgenesia gonadal parcial
DHT	Diidrotestosterona
DNA	Ácido desoxirribonucleico
dNTP	Desoxirribonucleotídeos Fosfatados
DO	Densidade óptica

DP	Desvio Padrão
EDTA	*Ethylenediamine tetraacetic acid* (ácido etilenodiamino tetracético)
ExAC	*Exome Aggregation Consortium* (banco de dados populacional)
FAPESP	Fundação de Amparo à Pesquisa do Estado de São Paulo
FATHMM	*Functional Analysis Through Hidden* (ferramenta de predição *in silico*)
FSH	Hormônio folículo estimulante
GAP	*GnRH-associated peptide*
GERP	*Genomic evolutionary rate profiling*
gnomAD	*Genome Aggregation Database (*banco de dados populacional)
HCFMUSP	Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo
hCG	*Human chorionic gonatropin* = Gonadotrofina coriônica humana
hg19 UCSC	*Human genome versão 19 do projeto genoma da Universidade da Califórnia de Santa Cruz*
HGMD	*Human Gene Mutation Database (*banco de dados que descreve variantes patogênicas e seus fenótipos*)*
IGV	*Integrative Genomics Viewer*
indels	Inserções e deleções
Kb	Kilobases
L	Litros
LH	Hormônio luteinizante
LIM42	Laboratório de Hormônios e Genética Molecular 42
Mb	Megabases
mL	Mililitros
NCBI	*National Center for Biotechnology and Information*
ND	Não descrito
ng	Nanogramas
nm	Nanômetro
nM	Nanomolar
ºC	Graus Celsius

PAIS	*Partial Androgen Insensitivity Syndrome* = Síndrome de Insensibilidade Parcial aos Andrógenos
PCR	Reação de cadeia polimerase
pH	Potencial hidrogeniônico
PM	Moderada evidência de patogenicidade, segundo critério ACMG
Polyphen2	*Polymorphism Phenotyping v2* (ferramenta de predição *in silico*)
PP	Evidência que suporta patogenicidade e uma variante, segundo critério ACMG
PREMiUM	Programa Rede de Equipamentos Multiusuários
PROVEAN	*Protein Variation Effect Analyzer (*ferramenta de predição *in silico*)
PSV	Evidência forte que suporta a patogenicidade de uma variante, segundo critério ACMG
SELA	Laboratório de Sequenciamento em Larga Escala
SIFT	*Sorting Intolerant from Tolerant Human Protein* (ferramenta de predição *in silico)*
SNP	*Single nucleotide polymorphism* = polimorfismo de um único nucleotídeo
SPLE	Sequenciamento paralelo em larga escala
SRTE	Síndrome de regressão testicular embrionária
T	Testosterona
T/A	Relação testosterona/ androstenediona
T/DHT	Relação testosterona/diidrotestosterona
TAE	tampão tris - acetato de sódio - HCl
vcf	*Variant call format = chamada de variantes*
XL	Herança Ligada ao X
Wg	Micrograma
WL	Microlitro
λ DNA	marcador de peso molecular lambda DNA

SÍMBOLOS DE GENES

AKR1C2	Aldo-Keto Reductase Family 1 Member C2
AKR1C3	Aldo-Keto Reductase Family 1 Member C3
AKR1C4	Aldo-Keto Reductase Family 1 Member C4
AMH	Anti-Mullerian Hormone
AMHR2	Anti-Mullerian Hormone Receptor Type 2
ARX	Aristaless Related Homeobox
ATRX	ATRX Chromatin Remodeler
AXIN1	Axin 1
BMP15	Bone Morphogenetic Protein 15
CBX2	Chromobox 2
CBX2.2	Isoforma 2 do gene Chromobox 2
CDH7	Cadherin 7
CITED2	Cbp/P300 Interacting Transactivator With Glu/Asp Rich Carboxy-Terminal Domain 2
CYP17A1	Cytochrome P450 Family 17 Subfamily A Member 1
CYP19A1	Cytochrome P450 Family 19 Subfamily A Member 1
DHCR7	7-Dehydrocholesterol Reductase
DHH	Desert Hedgehog Signaling Molecule
DHX37	DEAH-Box Helicase 37
DMRT1	Doublesex And Mab-3 Related Transcription Factor 1
DMRT2	Doublesex And Mab-3 Related Transcription Factor 2
ESR1	Estrogen Receptor 1
ESR2	Estrogen Receptor 2
FGF9	Fibroblast Growth Factor 9
FGFR2	Fibroblast Growth Factor Receptor 2
FOXL2	Forkhead Box L2
FSHR	Follicle Stimulating Hormone Receptor
GATA4	GATA Binding Protein 4
GDF9	Growth Differentiation Factor 9
GSK3β	Glycogen Synthase Kinase 3 Beta
HNF1B	HNF1 Homeobox B
HSD11B1	Hydroxysteroid 11-Beta Dehydrogenase 1
HSD17B3	Hydroxysteroid 17-Beta Dehydrogenase 3

HSD3B2	Hydroxy-Delta-5-Steroid Dehydrogenase, 3 Beta- And Steroid Delta-Isomerase 2
LHCGR	Luteinizing Hormone/Choriogonadotropin Receptor
LHX1	LIM Homeobox 1
LHX9	LIM Homeobox 9
MAMLD1	Mastermind Like Domain Containing 1
MAP3K1	Mitogen-Activated Protein Kinase Kinase Kinase 1
NANOS2	Nanos C2HC-Type Zinc Finger 2
NANOS3	Nanos C2HC-Type Zinc Finger 3
NR0B1	Nuclear Receptor Subfamily 0 Group B Member 1
NR5A1	Nuclear Receptor Subfamily 5 Group A Member 1
PAPPA	Pappalysin 1
PAX2	Paired Box 2
PBX1	PBX Homeobox 1
POR	Cytochrome P450 Oxidoreductase
PTDGS	Prostaglandin D2
RAC1	Rac Family Small GTPase 1
RSPO1	R-Spondin 1
RSPO2	R-Spondin 2
SOX 9	SRY-Box 9
SOX3	SRY-Box 3
SRD5A2	Steroid 5 Alpha-Reductase 2
SRY	Sex Determining Region Y
STAG3	Stromal Antigen 3
STAR	Steroidogenic Acute Regulatory Protein
STRA8	Stimulated By Retinoic Acid 8
TCF21	Transcription Factor 21
TBX2	T-Box Protein 2
TES	Testin LIM Domain Protein
WNT4	Wnt Family Member 4
WT1	WT1 Transcription Factor
WWOX	WW Domain Containing Oxidoreductase
ZFPM2	Zinc Finger Protein, FOG Family Member 2

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Resumo

Gomes NLRA. *Novas perspectivas no diagnóstico etiológico dos distúrbios do desenvolvimento sexual 46,XY* [tese]. São Paulo: Faculdade de Medicina, Universidade de São Paulo; 2019.

Os distúrbios do desenvolvimento sexual (DDS) 46,XY constituem um grupo de doenças com etiologia heterogênea, o que dificulta a análise molecular pela tradicional estratégia de estudo do gene candidato. O diagnóstico molecular é identificado em menos de 50% desses pacientes avaliados pelo sequenciamento por Sanger, especialmente aqueles com disgenesia gonadal (DG). As técnicas de sequenciamento paralelo em larga escala (SPLE), desenvolvidas nos últimos anos, permitem a análise simultânea de múltiplos genes, bem como a identificação de novos genes, como foi o caso do gene DEAH-box helicase 37 (*DHX37*). Este gene foi previamente identificado em nossa casuística como novo gene candidato à síndrome de regressão testicular embrionária (SRTE), considerada um espectro da DG. Neste contexto, os objetivos deste projeto foram: 1) determinar a etiologia molecular de uma coorte de pacientes com DDS 46,XY, identificando variantes em genes já associados à etiologia dos DDS e também em genes candidatos aos DDS; 2) identificar novos genes candidatos à etiologia dos DDS, utilizando SPLE por painel de genes-alvo e por sequenciamento exômico global, respectivamente. Para confecção do painel, foram incluídas as regiões exônicas de 63 genes já associados ou candidatos aos DDS na literatura, incluindo o *DHX37.* O sequenciamento foi realizado na plataforma Illumina. Os dados brutos foram analisados utilizando um *pipeline* para a identificação de variantes de ponto, *indels* e alteração de número de cópias. As variantes foram classificadas de acordo com os critérios do *American College of Medical Genetics*. A casuística estudada incluiu 100 casos esporádicos e 14 casos famílias de 7 famílias distintas, sendo 45 casos índices com DG (12 deles com SRTE), 54 casos classificados com DDS de etiologia indeterminada e 8 casos índices com DDS por provável defeito de síntese de andrógenos. Os casos esporádicos foram estudados por SPLE por painel, e os familiais foram estudados em sua maioria por sequenciamento exômico. O diagnóstico molecular foi elucidado em 20.5% dos pacientes, sendo identificadas variantes patogênicas ou possivelmente patogênicas em 6 dos 45 casos índices (13%) com DG, 10 dos 54 índices (18.5%) com DDS de etiologia indeterminada e em 6 dos 8 casos índices (75%) com DDS com suspeita diagnóstica, incluindo variantes no *NR5A1, DHX37, WT1, AMH, MAP3K1, SRD5A2 E HSD17B3*. Variantes de significado incerto foram identificadas em 12% da casuística, destacando-se as variantes no *GATA4, MAP3K1, ESR2, CBX2.2.* Em 5% dos pacientes foram identificadas duas variantes, sugerindo herança digênica. As variantes no *DXH37* (n=4) foram as mais frequentes sendo identificadas em quatro casos esporádicos e em três casos familiais de duas famílias distintas com fenótipos variados, de SRTE a DG parcial, a maioria deles sem derivados Mullerianos. As variantes estão localizadas em dois domínios conservados da proteína (helicase C-terminal e de ligação ao ATP) e sua frequência foi significativamente superior em pacientes com fenótipo de SRTE (44%) e DG (8%) em relação aos controles (p<0.001). Os achados desse trabalho

reforçam a relevância do SPLE na determinação etiológica dos DDS 46,XY e indicam que variantes no *DHX37* são causa frequente de DDS disgenético, em todo o seu espectro, especialmente de SRTE, sendo este gene importante não só na formação como na manutenção testicular.

Descritores: Transtornos do desenvolvimento sexual; Disgenesia gonadal 46 XY; Sequenciamento completo do exoma; Gene DHX37; Sequenciamento de nucleotídeos em larga escala; Gene NR5A1.

Abstract

Gomes NLRA. *Novel perspectives of the genetic etiology of the 46,XY disorders of sex development* [thesis]. São Paulo: “Faculdade de Medicina, Universidade de São Paulo”; 2019.

Since the genetic etiology of the 46,XY disorders of sex development (DSD) is highly heterogeneous, obtaining a definitive molecular diagnosis by single gene test is challenging. A molecular diagnosis is identified in less than 50% of these patients who are studied by Sanger sequencing, especially those with gonadal dysgenesis (GD). In the last years, massively parallel sequencing (MPS) technologies have been proposed as an efficient sequencing approach by analyzing multiple genes at the same time, as well as allowing the identification of novel genes, as the DEAH-box helicase 37 (*DHX37*). This gene was previously identified in our cohort as a candidate gene for the embryonic testicular regression syndrome (ETRS), which is considered part of the spectrum of gonadal dysgenesis (GD). In this context, the objectives of this project were: 1) to investigate the molecular etiology of a cohort of patients with 46,XY DSD, identifying variants in genes previously known to be associated with DSD and also in DSD candidate genes; 2) identify novel candidate genes for the DSD etiology, using MPS by panel sequencing and by whole exome sequencing (WES), respectively. We designed an amplicon-based capture panel of 63 genes for targeted sequencing including genes already associated and candidate genes for the DSD etiology, including the *DHX37.* Sequencing was performed in the Illumina platform. The data was analyzed by an in-house pipeline in order to identify the missense and indels variants and for copy number variations. The variants were classified according to the American College of Medical Genetics. We studied 100 sporadic cases and 14 familial cases from 7 different families, including 45 index-cases with GD (12 of them had ETRS), 54 cases classified as DSD of unknown etiology and 8 cases with androgen synthesis defect. The sporadic cases were studied by target MPS and most of the familial cases were studied by WES. A genetic diagnosis was established in 20.5% of the patients. Pathogenic or likely pathogenic variants were identified in 6 of 45 index-cases (18%) with GD, 10 of 55 index-cases (18.5%) with DSD of unknown etiology and 6 of 8 patients (75%) suspected of having a testosterone synthesis defect, including variants in *NR5A1, DHX37, WT1, AMH, MAP3K1, SRD5A2 E HSD17B3* genes. Variants of uncertain significance were identified in 12% of the cohort, highlighting variants in *GATA4, MAP3K1, ESR2, CBX2.2.* Two variants were identified in 5% of the patients, suggesting a digenic inheritance. The *DHX37* variants (n=4) were the most frequent and were identified in four sporadic cases and in three familial cases from two unrelated families with variable phenotypes, including ETRS and partial GD, most of them without Mullerian derivatives. The variants were identified in two conserved domains of the protein (helicase C-terminal and ATP binding domain) and their frequency was enriched among patients with ETRS (44%) and GD (9%) from the cohort in comparison to the frequency of deleterious variants among controls ($p<0.001$). The present findings reinforce the relevance of MPS in the 46,XY DSD investigation and indicate that *DHX37* variants are a frequent cause of

all GD spectrum of, especially for ETRS, being this gene important not only for the development but also for the maintenance of the testicular tissue.

Descriptors: Disorders of sex development; Gonadal dysgenesis 46 XY; Whole Exome Sequencing; DHX37 gene; High-throughput nucleotide sequencing; NR5A1 gene.

1 Introdução

1 INTRODUÇÃO

1.1 Mecanismos envolvidos no desenvolvimento sexual

O desenvolvimento sexual dos mamíferos é um processo complexo, determinado geneticamente e pode ser dividido em três etapas.

A primeira etapa consiste na determinação do sexo cromossômico. Após a fertilização do embrião, na dependência de qual cromossomo sexual foi recebido, a cascata do desenvolvimento sexual feminino ou masculino é deflagrada na presença do cromossoma X (determinando o cariótipo 46,XX) ou Y (determinando o cariótipo 46,XY), respectivamente [(1)]. As etapas que se seguem são as da determinação e da diferenciação sexual [(2)].

A fase da determinação sexual se refere aos processos envolvidos no desenvolvimento do sexo gonadal, ou seja, dos eventos que culminam com a transformação da crista urogenital, que é tecido embriológico indiferenciado, para o estado de gônada bipotencial e subsequente diferenciação em gônada feminina ou masculina [(3)].

A etapa da diferenciação sexual engloba os processos do desenvolvimento da genitália interna e externa que resultam no fenótipo feminino ou masculino [(3)].

Determinação sexual

A gônada bipotencial é constituída por duas populações celulares distintas: a germinativa e a somática, originárias do epitélio celômico e do mesênquima, respectivamente [(1)].

Em humanos, a fase de determinação sexual tem início por volta da sexta semana de vida intraembrionária e consiste no processo de

transformação do tecido somático gonadal bipotencial em gônada feminina ou masculina [1]. Trata-se de uma fase complexa envolvendo inúmeros genes e moléculas sinalizadoras que interagem entre si, culminando na formação do testículo ou do ovário [4] (**Figura 1**).

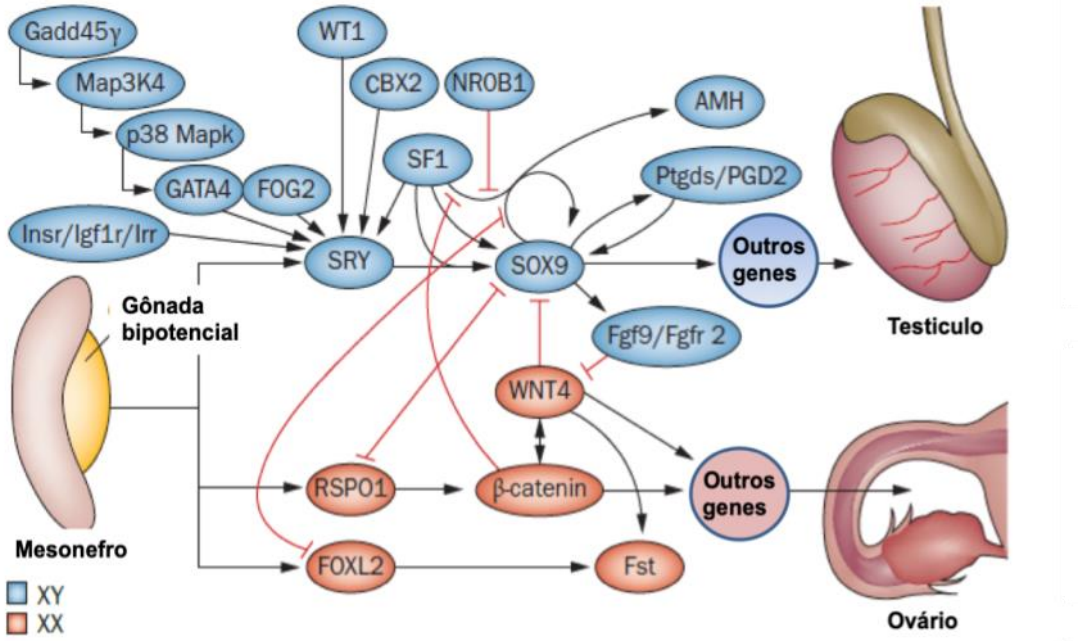
Em embriões XY, as células do epitélio celômico se diferenciam em células de Sertoli, que coalescem ao redor das células germinativas para formar os cordões sexuais dos testículos, futuros túbulos seminíferos(5). As células mesenquimais, esteroidogênicas, se diferenciam em células de Leydig, produtoras de testosterona [6].

De forma semelhante, nos embriões 46,XX, o epitélio celômico se diferencia nas células da granulosa e fornecem o suporte para as células da teca, de origem mesenquimal [1, 5]. Nas células da teca, a síntese de esteróides sexuais começa com a síntese de androstenediona, que é convertida em estrona e esta última em estradiol nas células de granulosa [6].

O controle genético do processo de determinação sexual é complexo e envolve uma rede de interação gênica, em que as vias da determinação testicular e ovariana são próprias e antagônicas [7]. Nos indivíduos 46,XY, o gene *SRY* desencadeia a cascata da diferenciação testicular [8, 9], regulado pelos genes *WT1* [10], *NR5A1* [11], *CBX2* [12], *GATA4* [13] e seu cofator *ZFPM2* [14] induzindo a expressão do gene *SOX9* [15]. Este tem um papel central, regulando a expressão de diversos genes da diferenciação sexual masculina, como *FGF9/FGFR2* [16], *PTGDS* [17], *AMH* [18], além de suprimir a expressão de genes indutores da diferenciação ovariana tais como o *RSPO1 e FOXL2* [7, 19]. Outros genes como o *DHH* [20, 21], *MAP3K1*[22, 23], *WWOX*[24] e *DMRT1* [25, 26] foram adicionados como participantes da via de determinação testicular após a identificação de alterações deletérias de ponto ou de número de cópias em associação com o fenótipo de distúrbios da determinação testicular em humanos e camundongos [19].

Nos indivíduos 46,XX, a *RSPO1* e o *FOXL2* ativam os genes *WNT4, β-catenina* e a *FST* [27-29] que, por sua vez, reprimem a expressão do *SOX9* [30]. O gene *NR0B1*, que está localizado no cromossomo X e que codifica a

proteína DAX1, pode atuar como co-repressor ou co-ativador do gene *NR5A1* dependendo do contexto. A duplicação deste gene está associada à alteração do desenvolvimento testicular, mostrando que em indivíduos 46,XY o seu papel é dose dependente [31].



Fonte: Adaptado de Ono e Harley [19]

Figura 1 - Rede de interação gênica responsável pela determinação gonadal. Genes conhecidos relatados aos DDS em humanos (letra maiúscula) ou somente em camundongo (letra minúscula). As setas mostram as vias de ativação e os conectores as vias de supressão

Diferenciação sexual

Este processo é dependente da ação dos hormônios secretados pelos testículos [3]. Por volta da sétima semana de gestação, os embriões dispõem de dois sistemas de dutos embrionários, os dutos paramesonéfricos (ou Mullerianos) que são precursores das trompas, útero, terço superior da vagina, e os dutos mesonéfricos (ou de Wolff) precursores dos vasos deferentes, epidídimo, vesículas seminais e duto ejaculatório. Assim, o desenvolvimento dos testículos leva à produção do hormônio antimulleriano,

secretado pelas células de Sertoli, responsável pela regressão dos dutos de Muller [3]. A testosterona, secretada pelas células de Leydig nos testículos fetais, age nos dutos de Wolff levando à formação das estruturas do sistema reprodutor masculino [7]. A testosterona é convertida perifericamente pela ação da enzima 5-α redutase tipo 2 em diidrotestosterona (DHT). Esta, por sua vez, age no tubérculo genital, que é o órgão precursor da genitália externa, originando a glande peniana e estimulando a fusão ventral das pregas uretrais, formando a uretra e o corpo peniano. As pregas labioescrotais unem-se medianamente, formando a bolsa escrotal, enquanto a uretra prostática se origina do seio urogenital. O processo de formação da genitália externa masculina se completa por volta da 12ª semana de gestação, enquanto o crescimento peniano ocorre principalmente no último trimestre da gestação [3].

Na ausência de secreção de testosterona e do hormônio antimulleriano, haverá regressão das estruturas de Wolff com manutenção de desenvolvimento das estruturas mullerianas, originando trompas uterinas, útero e vagina proximal, originando estruturas internas e externas femininas tanto nos indivíduos 46,XX quanto 46,XY com distúrbio da determinação sexual.

1.2 Distúrbios/ Diferenças do Desenvolvimento Sexual

Os distúrbios do desenvolvimento sexual (DDS) constituem um grupo de anomalias congênitas que afetam a diferenciação do sistema urogenital associadas a alterações em uma das três etapas do desenvolvimento, seja da determinação cromossômica, gonadal ou diferenciação sexual[32], resultando em um espectro fenotípico que abrange desde atipias genitais variadas, incongruência entre fenótipo hormonal e fenótipo sexual a distúrbios do desenvolvimento puberal e infertilidade [32]. Embora o diagnóstico dos DDS possa ser dado em qualquer idade, a apresentação mais comum é ao nascimento, quando se identifica a genitália atípica [33].

Cabe ressaltar que o termo “distúrbios do desenvolvimento sexual” veio em substituição ao antigo termo “intersexo” e foi proposto pelo Consenso de Chicago em 2006 [32, 33]. Nos últimos anos, vários especialistas têm o substituído por “diferenças” do desenvolvimento sexual [34, 35].

A causa mais frequente de DDS em indivíduos 46,XX é a forma clássica da hiperplasia adrenal congênita por deficiência da 21-hidroxilase, correspondendo a cerca de 90-95% de todos os casos [32, 36]. Em contraste, apenas 50% dos pacientes com DDS 46,XY têm um diagnóstico estabelecido, havendo diversas etiologias [32]. Podem ser decorrentes de disgenesia gonadal (em sua forma completa e parcial), de defeitos na produção de testosterona (incluindo a resistência ao hormônio luteinizante (LH) e defeitos na síntese de testosterona) ou ação da mesma, como na síndrome de insensibilidade aos andrógenos, nas formas parcial (PAIS) e completa (CAIS), ou defeitos na produção ou na ação do hormônio antimulleriano.

1.3 Determinação da etiologia dos DDS 46,XY

O diagnóstico dos DDS envolve alta complexidade, sendo necessária a combinação de achados fenotípicos, bioquímicos (hormonais) e os achados moleculares. O diagnóstico genético-molecular desses pacientes é importante, especialmente nos casos identificados logo ao nascimento, para auxiliar na determinação do sexo social, na predição da função endócrina gonadal, das possíveis condições de fertilidade e do desenvolvimento tumoral na vida adulta e fornecer um diagnóstico definivo [37].

Tradicionalmente, o método mais empregado para obtenção do diagnóstico molecular dos DDS 46,XY é o sequenciamento pelo método de Sanger [38], que se baseia na estratégia de avaliação do gene candidato. Assim, para a identificação de um possível gene candidato, primeiro é preciso ter um diagnóstico sindrômico (disgenesia gonadal, defeito de síntese de testosterona, defeito na ação da testosterona ou etiologia indeterminada).

Para tal, faz-se necessária a realização de exames de imagem, para verificar a presença de derivados mullerianos, e a dosagem de andrógenos, seja basal ou após estímulo com gonadotrofina coriônica humana (hCG) nas crianças em período fora da minipuberdade ou da puberdade. Contudo, há limitações desse tipo de abordagem.

Primeiramente, as dosagens hormonais devem ser preferencialmente obtidas após o estímulo com hCG, não havendo uniformidade nos protocolos usados para uso desse estímulo. Assim, pode ocorrer de o estímulo não ser suficiente para amplificar a produção de testosterona, especialmente na infância e pré-puberdade, devido à inatividade das células de Leydig neste período, de forma a permitir uma adequada avaliação dos esteroides desejados [39, 40].

Além disso, as dosagens dos esteroides possuem suas limitações metodológicas e em relação à padronização dos valores de referência [41]. O método mais empregado na rotina dos laboratórios é o imunoensaio (IE). Contudo, a especificidade de kits comerciais é reduzida, com frequente reatividade cruzada entre os diferentes tipos de esteroides, interferindo nas relações hormonais que são empregadas na avaliação dos defeitos de síntese de testosterona [41]. Dessa maneira, o ideal é acoplar o RIE à coluna de extração e/ou a cromatografia, o que não é a prática da maior parte dos laboratórios [41].

Mesmo em condições ideais, a sensibilidade das relações testosterona/androstenediona (T/A) e testosterona/ diidrotestosterona (T/DHT) para diagnosticar as deficiências de 17β- hidroxiesteroide desidrogenase tipo 3 (HSD17B3) e de 5-α redutase tipo 2 (5ARD2), respectivamente não é 100%, sendo também considerável o número de falsos positivos [39, 42].

A deficiência da 17β -hidroxiesteroide desidrogenase tipo 3 é sugerida por relação testosterona/ androstenediona (T/A) inferior a 0,8 [43]. Há relatos de pacientes com diagnóstico molecular confirmado de defeitos no gene *HSD17B3* com relação T/A superior a 0,8 em algumas coortes [43-45]. Além

disso, em uma coorte de 114 pacientes avaliados para essa relação (T/A), 5% (4/ 84) dos pacientes com diagnóstico de insensibilidade aos andrógenos (AIS) e 56% dos pacientes com disgenesia gonadal apresentavam uma relação alterada [39].

Em relação ao diagnóstico bioquímico de deficiência da 5-α redutase tipo 2, o ponto de corte da relação T/DHT é variável dentre as diferentes coortes, não havendo um consenso [46] [47]. Em crianças até 6 meses de idade há casos de relações T/DHT falsamente normais [48], e relações alteradas não são específicas da deficiência de 5-α redutase tipo 2 em qualquer idade, podendo estar presente em indivíduos com AIS [49, 50]. Assim, o diagnóstico definitivo, especialmente em crianças de até 6 meses, deve ser feito pela análise molecular.

Outro fato importante é que o volume de sangue necessário para a dosagem desses esteroides por IE é grande, o que pode ser complicado em se tratando de recém nascidos e crianças pequenas.

O emprego da espectrometria de massas, seja acoplada a cromatografia líquida ou gasosa, permite a adequada mensuração dos esteroides por identificar o analito específico de acordo com seu peso molecular. O seu emprego melhora a acurácia das dosagem dos andrógenos e, ainda, permite a dosagem de todos os esteroides em um único frasco, reduzindo o volume de sangue extraído [41]. Entretanto, considerando o alto custo, limitada disponibilidade do método e ainda ausência de valores de referencia padronizados, ele não é utilizado de rotina na prática clínica [41].

Ainda, a avaliação da presença de derivados Mullerianos por análise ultrassonográfica está associada a erros diagnósticos por ser examinador dependente [51], ficando o diagnóstico de certeza apenas após a avaliação laparoscópica, que é invasiva.

Por todos esses fatores expostos, classificar clinicamente um paciente com DDS 46,XY afim de se determinar um gene candidato é laborioso e sujeito a erros.

1.4 Diagnóstico molecular dos DDS 46,XY pelo sequenciamento pelo método de Sanger

Quando há evidência bioquímica inequívoca de que determinada via de produção hormonal está comprometida (como na deficiência de 17α-hidroxilase, de 3-β hidroxiesteroide desidrogenase tipo 2) ou nos casos com suspeita de CAIS ou PAIS, as chances de se identificar o diagnóstico genético pelo tradicional sequenciamento do gene candidato pelo método de Sanger são elevadas. Diferentemente, o grande número de genes envolvidos no processo da determinação testicular e da esteroidogênese, candidatos potenciais para a etiologia dos DDS disgenéticos ou de origem etiológica indeterminada, torna a abordagem gene a gene um trabalho árduo, demorado e com aplicação diagnóstica limitada na prática clínica.

Em nossa coorte de 173 casos índices com DDS 46,XY, dos 44 pacientes com defeitos da esteroidogênese (*CYP17A1, 3BHSD2, HSD17B3, SRD5A2)* e dos 29 pacientes com fenótipo de síndrome de insensibilidade aos andrógenos, o diagnóstico foi possível em 91.6% e 96.5% dos casos, respectivamente (dados não publicados). Entretanto, o diagnóstico molecular só foi elucidado em 21.5% (n=51) dos pacientes com disgenesia gonadal, após avaliação de 8 genes pelo método de Sanger (*NR5A1, SRY*, *MAP3K1, FGFR2, FGF9, GATA4, DMRT1, WT1*) e em 17% (n=36) dos pacientes com diagnostico indeterminado.

Assim, um número significativo de pacientes portadores de DDS 46,XY permanece sem diagnóstico etiológico, principalmente os pacientes com disgenesia gonadal e os indeterminados [32, 52]. Este baixo percentual indica que genes/vias ainda desconhecidos devam estar implicados na etiologia da determinação e desenvolvimento sexual.

Em conjunto, as dificuldades encontradas no diagnóstico dos DDS 46,XY vão desde o estabelecimento do diagnóstico sindrômico ao diagnóstico molecular, fazendo-se necessária a busca por métodos diagnósticos mais

sensíveis e acessíveis, bem como a identificação de novos genes envolvidos no processo.

1.5 Sequenciamento paralelo em larga escala (SPLE) para o diagnóstico molecular dos DDS 46,XY

Nos últimos anos, o sequenciamento paralelo em larga escala (SPLE) ocasionou grande evolução permitindo a genotipagem simultânea de diversas regiões do genoma [53, 54]. Pode ser realizado através do sequenciamento exômico por um painel de genes relacionados a um determinado fenótipo, da análise do exoma completo ou, ainda, do genoma integral de um paciente. A incorporação dessas técnicas na rotina diagnóstica de diversas doenças tem facilitado o diagnóstico molecular, com um menor custo [55-58], além de o sequenciamento exômico ou genômico permitirem a identificação de novos genes candidatos.

O primeiro estudo a utilizar a técnica de sequenciamento para avaliação da etiologia molecular de 10 pacientes com DDS 46,XY utilizou um painel de 35 genes relacionados aos DDS. Foi possível confirmar o diagnóstico molecular de 5 pacientes DDS 46,XY e identificar a etiologia de outros 2 de 5 pacientes sem diagnóstico [59]. A partir de então, outras coortes de pacientes com DDS 46,XY foram estudadas através de sequenciamento por painel com um percentual diagnóstico que varia de 29 a 47% **(Tabela 1)**.

Tabela 1 - Estudos que utilizaram SPLE por painel de genes associados aos DDS. A frequência do diagnóstico molecular inclui apenas variantes classificadas como patogênicas ou provavelmente patogênicas

Referência	Painel de genes	n	Frequência do diagnóstico molecular (%)
Arboleda, VA *et al*. 2013 [59]	35	14	28,6
Baxter, RM *et al*. 2015 [60]	64	40	34,5
Dong *et al.* 2016 [61]	219	21	38,1
Eggers *et al.* 2016 [62]	64	278	43
Kim, JH *et al.* 2017 [63]	67	37	29,6
Fan,Y *et al.* 2017 [64]	80	32	28.1
Wang, H *et al.* 2018 [65]	33	70	42.9
Hughes, LA *et al.* 2019 [66]	30	80	34
Xu, Y *et al.* 2019 [67]	2.742	96	46.9

Além disso, diversos casos de DDS tiveram o diagnóstico molecular elucidado a partir do sequenciamento exômico global, ampliando os espectros fenotípicos. Como exemplo, a variante p.Arg92Trp no *NR5A1,* gene associado apenas ao DDS 46,XY, foi identificada em pacientes com DDS 46,XX ovotesticular e testicular [68, 69].

Novos genes candidatos à etiologia dos DDS 46,XY têm sido identificados através dessa técnica, merecendo destaque o gene DEAH-box helicase 37 (*DHX37*), descrito pela primeira vez em nossa casuística [70].

1.6 DEAH-box helicase 37 (*DHX37*): novo gene candidato à etiologia do DDS 46,XY disgenético

O *DHX37* tornou-se um gene candidato à etiologia do DDS após a identificação da variante em heterozigose c.G923A; p.Arg308Gln por sequenciamento exômico de 4 casos familiares de micropênis e síndrome de regressão testicular embrionária (SRTE) de 2 famílias distintas e 2 casos esporádicos com mesmo fenótipo, de famílias não relacionadas, incluindo um caso esporádico chinês americano, sem que haja um efeito fundador dessa variante [70, 71]. A segregação da variante nessas famílias mostrou padrão de herança variável: materna, *de novo* e paterna, em uma das família, sendo o pai carreador assintomático [70, 71].

A SRTE é considerada um espectro da disgenesia gonadal parcial e é caracterizada pela ausência de tecido gonadal, associada a atipia genital ou a micropênis isolado [72, 73]. Relatos prévios da ocorrência de SRTE em irmãos sugere uma possível base genética, até então desconhecida [72].

O *DHX37*, está localizado no 12q24.31, pertence à família das RNA helicases, que utilizam ATP para se ligar ou remodelar ácidos nucleicos, complexos ácido nucléico-proteína ou ambos. A proteína codificada possui uma região muito conservada formada por quatro aminoácidos (DEAH - Asparato-Glutamato-Alanina- Histidina). Membros da família das helicases participam de vários processos celulares envolvidos na alteração da estrutura secundária do RNA, como a iniciação da tradução, *splicing* alternativo mitocondrial ou nuclear e biogênese ribossômica [74]. Este gene

tem expressão testicular, como mostrado pelo *the Human Protein Atlas* [75], porém seu papel no desenvolvimento gonadal ainda é desconhecido.

Há um caso descrito de paciente portador de DDS 46,XY sindrômico, com micropênis e criptorquidia, em que foi verificada deleção no cromossomo 12q24.31-33, contendo o gene *DHX37* [76].

Até o presente momento, os pacientes com SRTE haviam sido pouco estudados nas diversas coortes de pacientes com DDS 46,XY, permanecendo sem um diagnóstico etiológico, especialmente os casos familiais. Apenas o gene *NR5A1* foi previamente associado ao fenótipo de SRTE, descrito apenas em um caso isolado [77].

2 Justificativa

2 JUSTIFICATIVA

Dispomos de uma grande coorte de pacientes com DDS 46,XY de etiologia genética indeterminada, a maioria já estudada pelo tradicional método de Sanger, incluindo um número considerável de pacientes com SRTE.

Considerando que as novas técnicas de SPLE têm ampliado o diagnóstico molecular de pacientes em diversas estudos, esperamos elucidar a etiologia dos pacientes com DDS 46,XY da nossa coorte, utilizando tais técnicas.

Ainda, levantamos a hipótese de que variantes deletérias no gene *DHX37* possam ser causa não só de SRTE, como identificado até o momento, mas também da disgenesia gonadal, em todo o seu espectro e pretendemos ampliar as evidências genéticas de sua associação com este fenótipo.

A identificação da causa genética dos DDS 46,XY permite o aconselhamento genético e o seguimento adequado desses pacientes. A identificação de novos genes candidatos amplia a possibilidade de conhecer novas vias do desenvolvimento sexual.

3 Objetivos

3 OBJETIVOS

3.1 Objetivo geral

Determinar a etiologia molecular de casos familiais e esporádicos de uma coorte de pacientes com DDS 46,XY sem etiologia molecular definida por técnica de sequenciamento paralelo em larga escala.

3.2 Objetivos específicos

3.2.1 Avaliar a presença de variantes alélicas patogênicas em genes já associados aos DDS

3.2.2 Verificar a presença de variantes no recém identificado *DHX37* potencialmente causadoras de DDS 46,XY

3.2.3 Identificar novos genes candidatos aos DDS

4 Pacientes e Métodos

4 PACIENTES E MÉTODOS

O projeto foi aprovado pela Comissão de Ética para Análise de Projetos de Pesquisa (CAPPesq) do HCFMUSP (número: 1.968.070). O consentimento informado formal foi concedido pelos pacientes ou responsáveis legais (**Anexo 1**).

A confidencialidade dos resultados obtidos foi garantida a todos os pacientes. Os achados de defeitos genéticos relacionados às doenças em investigação serão informados aos pacientes e/ou seus responsáveis legais.

4.1 Pacientes

Os pacientes foram convidados a participar do estudo por serem portadores de DDS 46,XY sem etiologia molecular definida.

Todos os pacientes têm cariótipo 46,XY em pelo menos 30 células analisadas obtidas de sangue periférico, sem outros estigmas que pudessem os classificar como sindrômicos.

Os pacientes foram selecionados, em sua maioria, no Ambulatório de Endocrinologia do Desenvolvimento do Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo (HC-FMUSP), além de alguns casos provenientes de outros serviços (Hospital das Clínicas de Porto Alegre/ RS e Hospital Nacional Prof. Dr. A. Posadas, Buenos Aires/ Argentina).

Dos 173 casos índices com DDS 46,XY que já haviam sido previamente avaliados para variantes em pelo menos um gene candidato associado aos DDS pela técnica de Sanger, foram selecionados 76 casos sem diagnóstico molecular prévio além de outros 31 casos que não haviam

sido estudos previamente. A **Figura 2** representa o fluxograma de seleção dos pacientes da casuística.

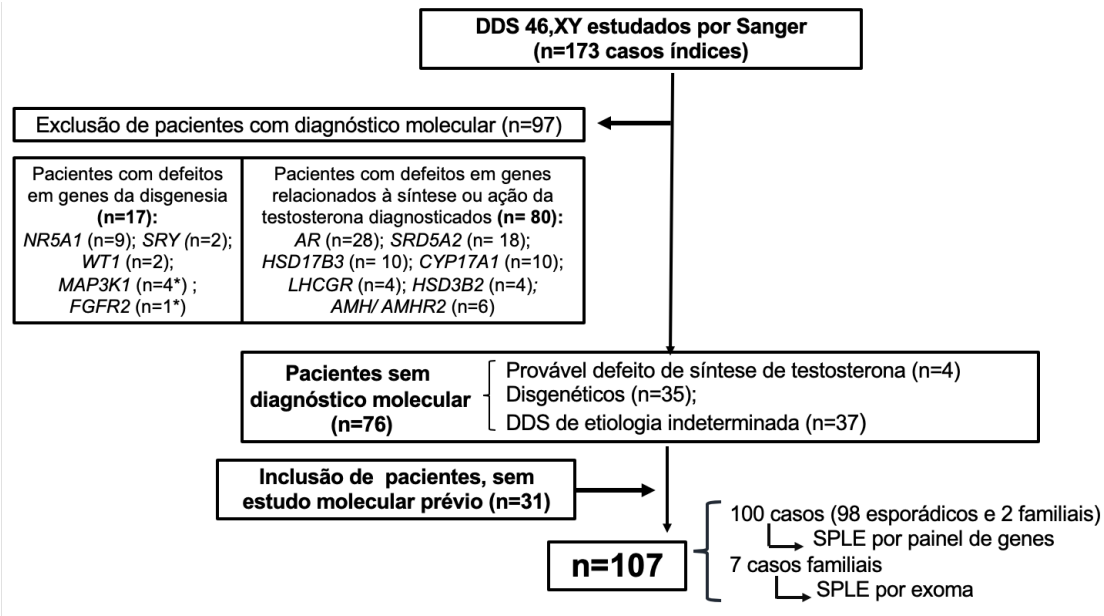


Figura 2 - Fluxograma representando a seleção dos pacientes da casuística do estudo. O asterisco marca que um dos casos índices possui variantes no gene da *MAP3K1* em associação ao *FGFR2* [78]

Os pacientes foram classificados em três grupos de acordo com o fenótipo:

1) DDS 46,XY por disgenesia gonadal: inclui pacientes com derivados mullerianos e/ou pelo menos uma das gônadas disgenéticas histologicamente ou ausentes, nas formas completa e parcial.

2) DDS 46,XY de etiologia indeterminada: inclui pacientes a princípio não disgenéticos, em que estudo hormonal (basal e/ou após estímulo com hCG) e/ou por imagem não encontrou a etiologia do DDS ou aqueles em que a avaliação hormonal não foi possível,

seja por gonadectomia prévia ou pela indisponibilidade de exames hormonais;

3) DDS 46,XY não disgenético com provável defeito na produção de andrógenos sem diagnóstico molecular definido

Foram incluídos 100 casos para sequenciamento em painel de genes e 14 casos familiais de 7 famílias para sequenciamento exômico, totalizando 114 casos.

Dos 100 casos selecionados para painel de genes, 98 são esporádicos e dois casos são familiais. Estes dois casos familiais foram estudados por painel devido à indisponibilidade do DNA dos familiares na ocasião (**Tabela 2**). Há histórico de consanguinidade em 13 casos esporádicos e em três casos familiais.

O detalhamento dos dados clínicos, laboratoriais que permitiram a classificação de cada paciente em um dos três grupos supracitados bem como os genes que foram previamente estudados por Sanger em cada um deles pode ser visto nos **Anexos 1, 2 e 3**. O heredograma bem como o fenótipo de cada uma das famílias estudadas está na **Figura 3**.

No grupo de pacientes com disgenesia gonadal, 30% deles tiveram os genes *SRY* e *NR5A1* sequenciados e 25% dos indeterminados foram sequenciados para variantes no *AR* e *NR5A1*, considerando a alta prevalência de variantes nestes genes nestes subgrupos.

Tabela 2 - Casuística selecionada de pacientes com DDS 46,XY para estudo por SPLE

DDS 46,XY		**Sequenciados por painel**		**Sequenciados por exoma**	**Total**
Grupo	**Subgrupo**	Casos Esporádicos (n)	Casos Familiais (n)	Casos Familiais (n de casos índices)	
Disgenesia gonadal	Forma completa	14	0	2	16
	Forma Parcial	17	0	0	17
	SRTE	10	1	1	12
Etiologia Indeterminada		52	0	2	54
Defeito de síntese de andrógenos		5	1	2	8
Total		98	2	7	**107**

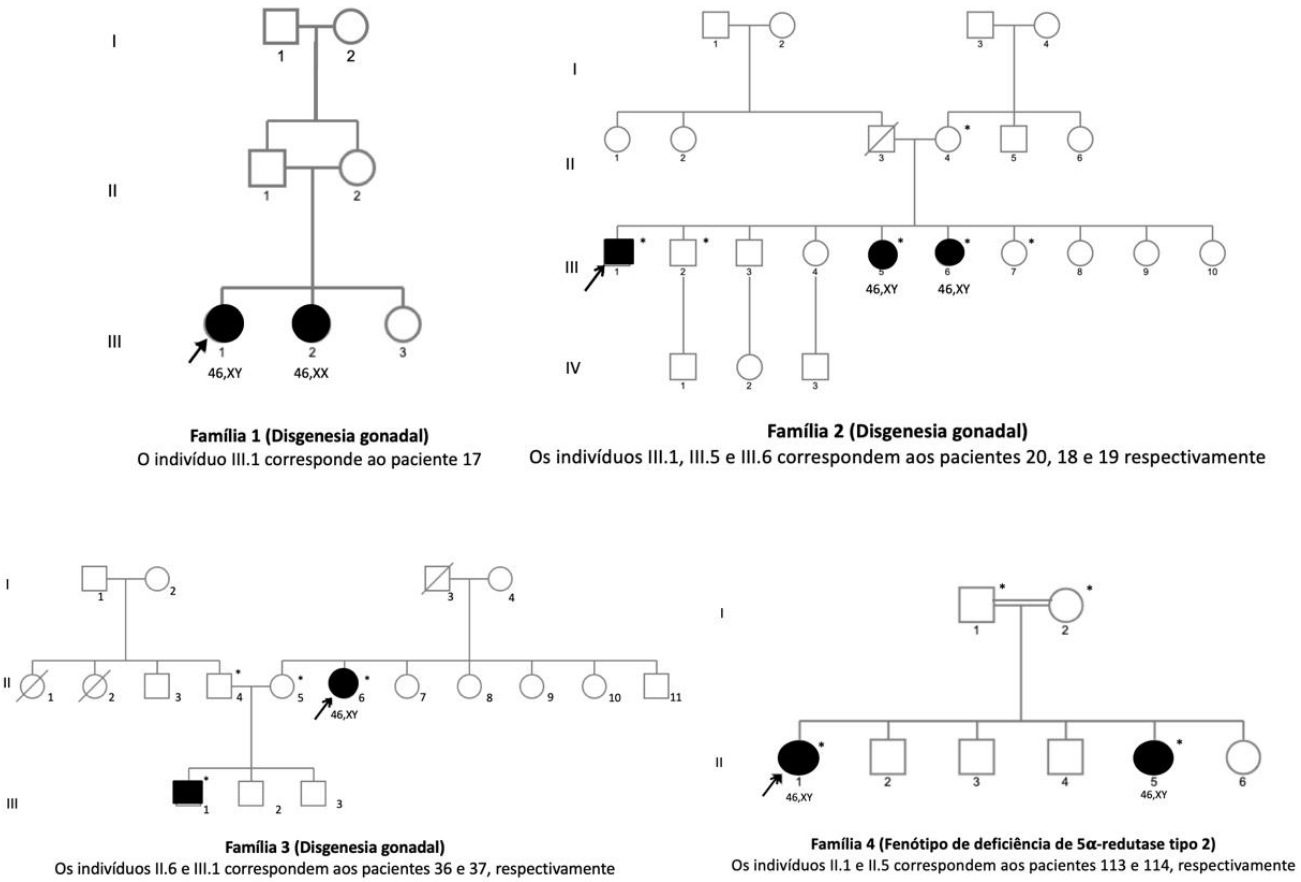


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Figura 3 - Heredogramas das famílias de pacientes com DDS 46,XY estudadas por sequenciamento exômico. Os quadrados representam os indivíduos do sexo social masculino e os círculos aqueles com sexo social feminino. As figuras preenchidas na cor preta representam os indivíduos afetados. A linha diagonal representa os indivíduos falecidos. O asterisco marca aqueles que foram sequenciados.

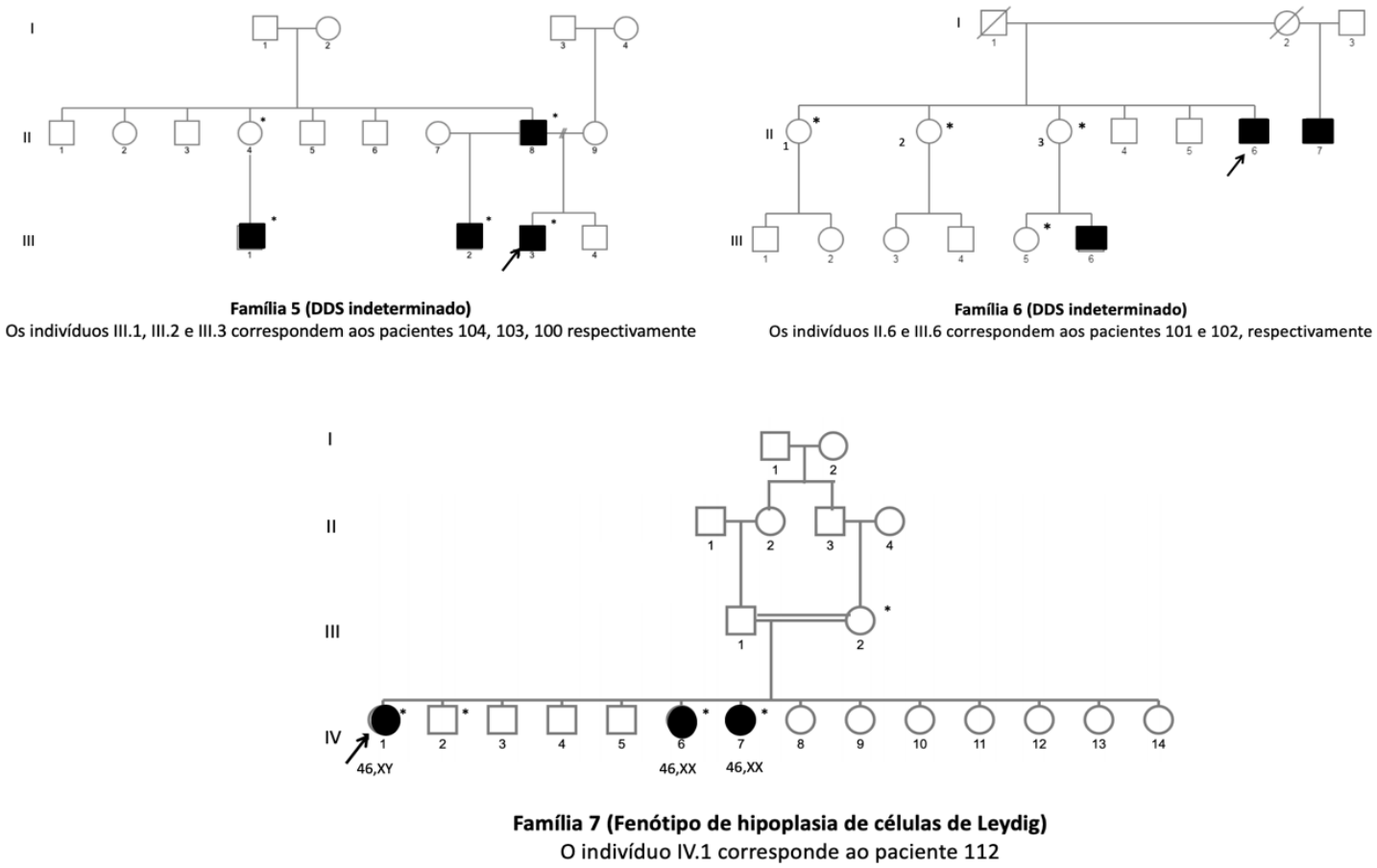

Figura 3 - Heredogramas das famílias de pacientes com DDS 46,XY estudadas por sequenciamento exômico. Os quadrados representam os indivíduos do sexo social masculino e os círculos aqueles com sexo social feminino. As figuras preenchidas na cor preta representam os indivíduos afetados. A linha diagonal representa os indivíduos falecidos. O asterisco marca aqueles que foram sequenciados.

4.2 Métodos

4.2.1 Extração e armazenamento do DNA Genômico

As amostras de DNA dos pacientes e seus familiares foram obtidas a partir de leucócitos de sangue periférico ou de células extraídas por *swab* da mucosa oral. O DNA foi extraído e armazenado de acordo com procedimento padronizado no Laboratório de Hormônios e Genética Molecular/LIM42 da Unidade de Endocrinologia do Desenvolvimento, Faculdade de Medicina da Universidade de São Paulo, baseado no método de Miller e colaboradores [(79)].

A concentração do DNA foi estimada a partir da leitura da densidade óptica por espectrofotometria (*Biophotometer, Eppendorf,* Hamburgo, Alemanha); no comprimento de onda de 260nm (1 unidade DO 260 = 50 µg/mL). O grau de pureza foi avaliado pela relação A260/280, sendo superior a 1,75. A integridade do material foi verificada com eletroforese em gel de agarose (*Thermo Fisher Scientific,* Waltham, MA, EUA) a 1% em TAE (Tris 0,004 M; Ácido Acético Glacial; EDTA 0,001 M pH 8,0) contendo *SYBR Safe DNA gel stain* (*Invitrogen, Thermo Fisher Scientific*, Waltham, MA, EUA) na concentração de 0,1 µL/mL de gel com posterior visualização por transiluminação com luz ultravioleta para verificar sua integridade. Foram utilizados 500 ng do marcador de peso molecular *λ DNA-HindIII digest* (250 ng/WL) e 20 ng do *λ* DNA (10 ng/WL) (ambos *Thermo Fisher Scientific,* Waltham, MA, EUA) como padrão de massa. Em seguida, as amostras foram mantidas congeladas a menos 20º C até seu uso.

4.2.2 Sequenciamento Paralelo em Larga Escala

O sequenciamento paralelo em larga escala envolve três etapas fundamentais: (1) o preparo das bibliotecas, (2) a amplificação clonal, (3) o sequenciamento propriamente, seguindo-se pelo processamento por bioinformática para análise dos dados.

4.2.2.1 Preparo das bibliotecas

O preparo das bibliotecas dos painéis e dos exomas foi realizado de acordo com o protocolo *SureSelectXT Target Enrichment System for Illumina Paired-End Sequencing Library (Agilent Technologies, Santa Clara, CA, EUA).* A diferença entre o preparo destas bibliotecas está no *kit* de sondas utilizados para captura das áreas de interesse: para as bibliotecas do painel foi utilizado um *kit* customizado e para os exomas um comercial.

4.2.2.1.1 Preparo das bibliotecas do painel

O painel customizado de genes-alvo associados ao DDS foi elaborado a partir da ferramenta *Agilent SureDesign 2.0 (Agilent Technologies,* Santa Clara, CA, EUA). Essa ferramenta desenha sondas específicas para seleção e captura das regiões genômicas de interesse, com base na metodologia *SureSelectXT (Agilent Technologies,* Santa Clara, CA, EUA).

Para o desenho do painel, foram incluídas as regiões exônicas de 63 genes relacionados aos DDS, incluindo uma sequência de até 50 bp antes e após cada éxon. Foram incluídos 42 genes que participam do processo de determinação gonadal e da diferenciação sexual, englobando genes envolvidos na esteroidogênese, que codificam receptores hormonais, e outros cujos defeitos moleculares fossem responsáveis por DDS associados a malformações múltiplas (**Tabela 3),** bem como 21 genes candidatos à etiologia dos DDS, extraídos da literatura além do gene *DHX37* (**Tabela 4**).

A região alvo total do painel é de aproximadamente 485kb.

Tabela 3 - Lista dos genes já associados aos DDS incluídos no painel de genes para o sequenciamento paralelo em larga escala

Gene	Fenótipo reportado no OMIM (número do OMIM) ou relato na literatura (L) de associação com DDS em humanos (n=41)	Herança
	Determinação gonadal	
BMP15	Disgenesia ovariana 2 (300510); Falência ovariana prematura 4 (300510)	LX
CBX2	Disgenesia gonadal completa 46,XY (613080) [80]	AR
DHH	Disgenesia gonadal parcial 46XY, com neuropatia minifascicular (607080); Disgenesia gonadal completa 46XY 7 (233420)	AR
DMRT1	Disgenesia testicular e ovariana (L)[2];	AD
DMRT2	Haploinsuficiência da região 9p ocasionando disgenesia gonadal 46,XY (L)[2]	NA
FGFR2	Craniossinostose com disgenesia gonadal completa 46,XY (L)[2]	AD, AR
FSHR	Disgenesia ovariana 1 (233300)	AR
FOXL2	Insuficiência ovariana prematura 3 (608996); Blefarofimose, epicanto inverso, e ptose, tipo 1 e 2 (110100)	AD
GATA4	Anomalias testiculares com ou sem cardiopatia (615542)	AD
MAP3K1	Disgenesia gonadal completa 46XY 6 (613762)	AD
NR0B1	Disgenesia gonadal completa 46XY 2 (300018);	LX
NR5A1	Disgenesia gonadal completa 46, XX 4 (617480); Disgenesia gonadal parcial 46XY 3 (612965); Insuficiência ovariana prematura 7 (612964)	AD
RSPO1	Cetatose palmoplantar e hermafroditismo verdadeiro (610644); (610644)	AR
SOX 9	Displasia campomélica com reversão sexual dominante (114290)	AD
SOX3	DDS testicular 3 (300833); DDS ovotesticular 46,XX (L) [81];	XL
SRY	Hermafrodistismo verdadeiro 46XX 1 (400045); Disgenesia gonadal 46XY 1 (400044)	Ligada ao Y
STAG3	Insuficiência ovariana prematura 8 (615723)	AR
WNT4	Aplasia dos ductos de Muller (158330)	AD
WT1	Síndrome de Denys-Drash (194080); Síndrome de Frasier (136680)	AD
WWOX	Disgenesia gonadal 46,XY (L)[2]	Ligada ao Y
ZFPM2	Disgenesia gonadal completa 46XY 9 (616067);	AD

continua

Tabela 3 - Lista dos genes já associados aos DDS incluídos no painel de genes para o sequenciamento paralelo em larga escala (conclusão)

Gene	Fenótipo reportado no OMIM (número do OMIM) ou relato na literatura (L) de associação com DDS em humanos (n=41)	Herança
	Diferenciação sexual	
AKR1C2	Disgenesia gonadal completa 46XY 8 (614279)	AR
AKR1C4	Disgenesia gonadal completa 46XY 8, modificado (614279)	AR
AMH	Síndrome de persistência dos ductos de Muller, tipo I (261550)	AR
AMHR2	Síndrome de persistência dos ductos de Muller, tipo 2 (261550)	AR
AR	Síndrome de insensibilidade aos andrógenos (300068)	LX
CYP17A1	Deficiência de 17,20-lyase, isolada (202110)	AR
CYP19A1	Deficiência de aromatase (613546); Síndrome de deficiência de aromatase (139300)	AD
DHCR7	Síndrome de Smith-Lemli-Opitz (270400)	AR
HSD17B3	Pseudohermafroditismo masculino com ginecomastia (264300)	AD
HSD3B2	Deficiência de 3ß- hidroxiesteróide desidrogenase tipo 2 (201810)	AD
LHCGR	Hipoplasia de células de Leydig com pseudohermafroditismo (238320); Hipoplasia de células de Leydig com hipogonadismo hipergonadotrófico (238320)	AR
POR	Síndrome de Antley-Bixler com anomalias genitais e distrúrbios da esteroidogênese (201750); Distúrbio da esteroidogênse por defeito do citocromo P450 oxiforedutase (613571)	AR
SRD5A2	Pseudohermafroditismo com hipospádia (264600)	AR
STAR	Hiperplasia adrenal lipóide (201710)	AR
Outros (DDS sindrômico, síndrome de Rokitansky, hipospádia isolada)		
ARX	Hidranencenfalia com genitália normal (300215)	LX
ATRX	Síndrome ATRX com anomalias genitais (301040)	LX (AD e AR)
CDH7	Síndrome CHARGE (214800); Hipogonadismo hipogonadotrófico com ou sem anosmia (612370)	AD
HNF1B	Variantes neste gene foram identificadas em pacientes com Rokitansky (L) [82]	AD
LHX1	Variantes neste gene foram identificadas em pacientes com Rokitansky (L) [82]	AD
MAMLD1	Hipospadia 2, Ligada ao X (300758)	LX

Tabela 4 - Lista dos genes candidatos incluídos no painel de genes para o sequenciamento paralelo em larga escala

Gene	Genes candidatos na literatura (L) (n=21)	Herança
AKR1C3	Produção de testosterona na reticular da suprarrenal (L) [83]	NA
AXIN1	Sinalizador da via Wnt-beta-catenina (L) [84]	NA
CITED2	Regulador do gene *NR5A1* (L)[2]	NA
DHX37	**Gene candidato à etiologia do DDS disgenético**	**AD**
ESR1	Reversão sexual em ratos machos *knockout* para ESRα /ESRß (O)	NA
ESR2	Disgenesia ovariana (618187);	AR;AD
FGF9	Ratos *knockout* XY têm reversão sexual (L) [7, 30]	NA
GDF9	Participa do desenvolvimento ovariano (L) [85]	NA
GSK3β	Participa da sinalização da Wnt-beta-catenina (L) [86]	NA
HSD11B1	Deficiência de cortisona tipo 1 (614662)	AD
LHX9	Participa da formação gonadal (L) [7]	NA
NANOS2	Expresso em testículos desde a via intraembrionárias (L) [87]	NA
NANOS3	Ratos *knockout* do Nanos3 têm espermatogênese reduzida (L) [88]	NA
PAPPA	Expresso nos folículos ovarianos e vesículas seminais (L) [89]	NA
PAX2	Participa da via do *WT1* (L)(90) e da via da diferenciação gonadal (L) [91]	NA
PBX1	Müllerian development in the mouse (L)[13]	NA
PTDGS	Necessário para a formação testicular (L) [7]	NA
RAC1	Essencial para o desenvolvimento do folículo primordial em ratos (L) [14]	NA
RSPO2	Essencial para o desenvolvimento do folículo primordial (L) [92]	NA
STRA8	Replicação pré-meiótica do DNA (L) [93]	NA
TCF21	Participante da via do *SRY* (L) [94]	NA
TES	Aumenta a ativação do *Sox9* (L) [7]	NA

4.2.2.1.2 Preparo das bibliotecas do exoma

As bibliotecas para o sequenciamento exômico dos pacientes foram preparadas com os *kits* comerciais disponíveis, o *SureSelectXT Human All Exon V5 ou V6 (Agilent Technologies,* Santa Clara, CA, EUA).

4.2.2.2 Protocolo de preparo das bibliotecas

Para preparo das bibliotecas, três microgramas ou 200 ng de DNA genomico foram fragmentados por ação mecânica, por sonicação, no equipamento *Covaris* (*Covaris Inc*, Woburn, MA, EUA), gerando fragmentos de tamanho entre 150 e 550 pb.

A seguir, adaptadores, que são sequências complementares às sequências de oligonucleotídeos presentes na *flow-cell*, foram adicionados às extremidades dos fragmentos de DNA. Em sequência, foram adicionados os *barcodes* (códigos de identificação específicos para o DNA de cada paciente). Os fragmentos ligados aos adaptadores foram amplificados em uma reação em cadeia da polimerase (PCR).

Na etapa subsequente, as bibliotecas foram enriquecidas através da adição de sondas biotiniladas que hibridizam ao DNA fragmentado apenas nas regiões de interesse, sendo específicas para os genes alvos desenhados no painel ou genes do exoma. O enriquecimento de *beads* magnéticos acoplados a estreptavidina às sondas biotiniladas permitiram a captura das regiões de interesse, que foram purificadas, antes de serem amplificadas (**Figura 4)**.

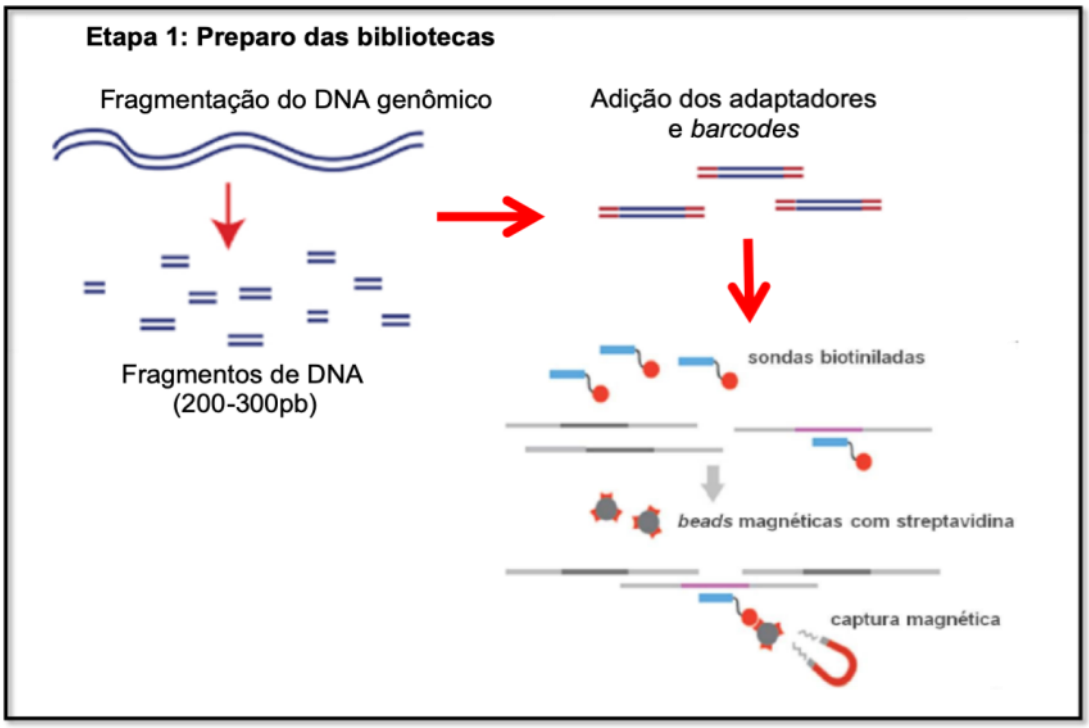

Figura 4 - Esquema da preparação das bibliotecas para SPLE

4.2.2.3 Amplificação clonal

As bibliotecas, com seus fragmentos de DNA ligados aos adaptadores, foram depositadas em uma lâmina especial, denominada *flowcell*, cuja superfície estão fixados, de maneira paralela, duas espécies de oligonucleotídeos. Após a ligação dos adaptadores aos seus oligonucleotídeos complementares presentes na superfície da *flowcell*, acontece a clusterização, onde cada fragmento é amplificado várias vezes, através de uma reação conhecida como PCR em ponte. Ao final desta etapa, são gerados *clusters* (clones) de moléculas de DNA idênticas à molécula original, ligadas covalentemente à superfície da *flowcell.*

4.2.2.4 Sequenciamento

Após a amplificação clonal, as moléculas *antisense* foram removidas enzimaticamente e o processo de sequenciamento foi iniciado com o acoplamento de um oligonucleotídeo iniciador especial. Em seguida,

nucleotídeos modificados com terminadores marcados com fluoróforos específicos foram adicionados ao meio, dando início ao sequenciamento por síntese. A cada ciclo de incorporação, foram geradas imagens de toda a superfície da *flowcell* através de um escâner de fluorescência em cada um dos comprimentos de onda específico para cada fluoróforo. Os *clusters* presentes na superfície da *flowcell* foram então identificados e mapeados. A sobreposição das imagens produzidas a cada ciclo de incorporação de cada *cluster* propiciou a identificação da sequência de bases nucleotídicas que o originou.

Na metade dos ciclos, ao final do sequenciamento do primeiro *read* (ou seja, da sequência *sense)*, a inversão dos fragmentos ligados na *flowcell* foi seguida pela reclusterizacao de forma a propiciar o sequenciamento do mesmo fragmento no sentido reverso (do segundo *read*, a sequência *antissense*), chamado sequenciamento em *paired end* do SPLE.

As etapas de amplificação clonal e do sequenciamento propriamente, tanto do painel customizado de genes quanto dos exomas, foram realizados em sequenciadores da plataforma Illumina (*Illumina, Inc, San Diego*, CA, EUA) do Laboratório de Sequenciamento em Larga Escala (SELA) da Rede PREMiUM, Multiusuário FAPESP, localizado na Faculdade de Medicina da Universidade de São Paulo.

A maior parte dos painéis customizados foi sequenciada no equipamento *NextSeq 500*, com o *kit NextSeq MidOutput V2* 2x150 ciclos e 8 amostras foram sequenciadas no equipamento *MiSeq 4000,* com o *kit V2 2x150 ciclos* (ambos *Illumina, Inc, San Diego*, CA, EUA).

Os exomas foram sequenciados no *HiSeq 2500*, com o *kits HiSeq SBS Kit V3 2x100 ciclos* e *V4 2x 125 ciclos* (ambos *Illumina, Inc, San Diego*, CA, EUA).

A **Figura 5** representa, de forma esquemática, as etapas de amplificação clonal e sequenciamento do SPLE.

A amplificação clonal forma os *clusters* através das PCR em ponte. O sequenciamento é feito pela incorporação de nucleotídeos que emitem sinais que são captados e escaneados, gerando o arquivo *fastq.

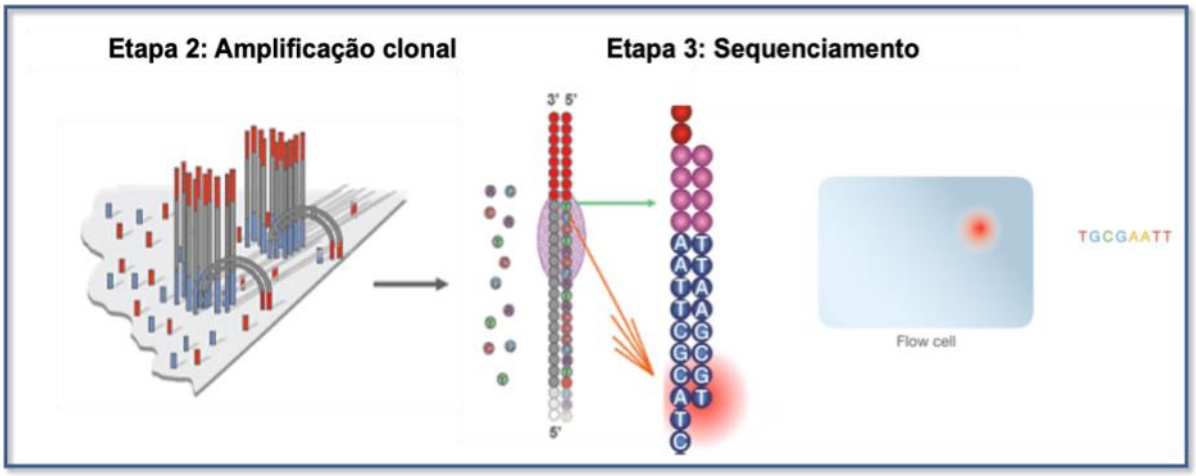

Figura 5 - Esquema das etapas de amplificação clonal e sequenciamento por síntese. Ambas as etapas ocorrem na superfície da *flow cell*

4.2.2.5 Análise por Bioinformática

Para realização da análise por bioinformática, os dados encontrados foram processados e transferidos para uma planilha de dados para a análise.

As sequências lidas foram separadas entre si de acordo com seus *barcodes,* sendo então pareadas as sequências *sense* e *antisense*, criando sequências contíguas. Estas foram alinhadas para comparação com a sequência de DNA referência, de forma a permitir a comparação entre o sequenciamento obtido e a sequência referência. A sequência referência do genoma humano utilizada foi *hg19* UCSC (ou b37 GRC/NCBI) e o alinhamento foi realizado pelo *software BWA (www.bio-bwa.sourceforge.net),* sendo gerado o arquivo *.bam.

A seguir, foi feita a chamada das variantes, realizada pelos software *Platyplus* e *Freebays*, que identificam as variantes de um único nucleotídeo

(SNVs) e pequenas deleções e inserções *(indels*), gerando o arquivo em formato *.vcf (*variant call format)*.

A última etapa antes da análise consistiu na anotação das variantes, realizada pelo *software Annovar*, sendo gerada a planilha para análise de dados, com as variantes identificadas bem como com dados de suas frequências nos bancos públicos de dados populacionais e análise de predição *in silico.*

4.2.2.6 Seleção e classificação das variantes candidatas de ponto ou pequenas *indels*

Com o grande volume de dados gerados pelo sequenciamento paralelo em larga escala, as alterações genéticas de interesse devem ser filtradas em meio a centenas ou milhares de polimorfismos, não relacionados ao fenótipo. Nesta etapa, foi aplicada uma sequência de filtros de forma a gerar uma lista de variantes potencialmente relacionadas ao fenótipo de cada paciente para, se possível, identificar a variante associada à doença.

Foram utilizados os seguintes critérios para priorização das variantes candidatas:

- Seleção de variantes presentes nos pacientes
  - No caso dos exomas, foi realizada também a filtragem das variantes presentes nos familiares, de acordo com o tipo de herança cabível para cada caso
- Seleção das variantes com frequência inferior a 1% nos principais bancos populacionais: 1000 Genomes Project (http://www.1000genomes.org), o Exome Aggregation Consortium – ExAC (http://exac.broadinstutite.org), gnomAD (www.gnomad.broadinstitute.org), que é uma extensão do ExAC e inclui dados de 123.136 exomas e 15.496 genomas sequenciados de indivíduos com diversas comorbidades e como parte de

pesquisa genética populacional, além do banco brasileiro *ABraOM* (www.abraom.ib.usp.br)

- Para os exomas, a filtragem inicial incluiu variantes com frequência inferior a 0.1% em um primeiro momento, ampliando para frequência de 1% em filtragens subsequentes, nos casos sem diagnóstico na primeira filtragem

- Seleção das variantes com frequência alélica inferior a 1% (*minor allele frequency*, *MAF*) no nosso próprio banco de dados do Laboratório de Sequenciamento em Larga Escala- SELA (http://intranet.fm.usp.br/sela), que até o momento conta com cerca de 760 indivíduos sequenciados por exoma (entre casos índices e familiares de diversas casuísticas, incluindo DDS, baixa estatura, falência ovariana prematura, doenças osteometabólicas e hiperplasia macronodular da adrenal), dados não publicados.
- Seleção de variantes localizadas em éxons e sítios de *splicing;*
- Avaliação das variantes raras codificadoras ou em sítio de *splicing* em relação ao efeito sobre a proteína:
  - Seleção das variantes alélicas já descritas como patogênicas pelo ClinVar;
  - Seleção daquelas variantes que causam perda de função da proteína (*stopgain, stoploss, frameshift)*;
  - Seleção das variantes não sinônimas preditas como deletérias em pelo menos 3 dentre os seguinte algoritmos de predição *in silico*: SIFT [(95)], PolyPhen2 , Mutation Taster [(96)], Mutation Acessor [(97)], CADD (phred) [(98)] para mutações do tipo *missense*, além do escore de conservação (GERP++) [(99)];
  - Seleção de variantes com predição de alteração de *splicing* pelo *dbscsnv* [(100)] *(ADA ou RF SCORE >0,6),* incluindo as variantes sinônimas. No **Anexo 4** estão especificados os

critérios que cada um desses algoritmos utiliza bem como a classificação utilizada.

- No caso dos exomas, dado o grande número de variantes candidatas encontradas em genes com função desconhecida, foram utilizadas ferramentas de priorização de genes candidatos- VarElect (www.ve.genecards.org) e GeneDistiller [101] assim como a função e expressão tecidual de cada gene foi verificada manualmente no GeneCards (https://www.genecards.org) e no HumanProtein Atlas [75], respectivamente, além de publicações relacionadas no PubMed (https://www.ncbi.nlm.nih.gov/pubmed).

Após a seleção das variantes, foi verificada a plausibilidade patológica, bem como a pesquisa de relatos prévios de sua ocorrência no Ensembl (www.ensembl.org), HGMD (hwww.hgmd.cf.ac.uk/ac/index.php), ClinVar (www.ncbi.nlm.nih.gov/clinvar).

As variantes relevantes foram visualmente confirmadas no programa *Integrative Genomics Viewer (IGV)* que permite a visualização do sequenciamento a partir do arquivo *.bam, de forma a verificar a cobertura do sequenciamento, bem como permitir a identificação de variantes chamadas incorretamente.

Por fim, as variantes candidatas a serem causadoras do fenótipo foram segregadas na família pelo sequenciamento pelo método de Sanger [38].

4.2.2.7 Confirmação das variantes alélicas pela técnica de Sanger

Para a realização do sequenciamento por Sanger, foram sintetizados oligonucleotídeos iniciadores específicos para amplificar por PCR as regiões que flanqueiam as variantes. As amplificações foram acompanhadas de um controle negativo e os produtos amplificados foram submetidos a uma purificação enzimática. O sequenciamento foi realizado com o *kit ABI*

PrismTM Big Dye Terminator Cycle Sequencing Ready Reaction Kit 3.1 (*Applied Biosystems*, *Thermo Fisher Scientific*, Waltham, Massachusetts, EUA) e os produtos desta reação foram submetidos a uma eletroforese capilar no sequenciador automático *ABI Prism 3130xl Genetic Analyzer* (*Applied Biosystems, Thermo Fisher Scientific*, Waltham, Massachusetts, EUA). A leitura dos eletroferogramas foi realizada através do programa *Sequencher 4.10.1* (*GeneCodes Corporation,* Ann Arbor*,* MI, EUA), sempre em comparação à sequência referência do gene e a um eletroferograma de um indivíduo normal.

4.2.2.8 Identificação de alterações de número de cópias (CNVs)

Para identificação das CNVs foi utilizado o *software* CONTRA (COpy Number Analysis for Targeted Resequencing) [102], que é capaz de identificar perdas ou ganhos através de uma relação logarítmica em relação a uma linha de base que foi gerada a partir dos dados de todos os pacientes nas corridas dos painéis, que funcionam como controle. Para isso, ele fragmenta todas as regiões sequenciadas em outras menores e libera o resultado de cada região em *log-ratio* juntamente com uma análise de significância (valores de *p*). *Log-ratios* próximos de -1,0, com valores de *p* ajustados inferiores a 0,05, indicam a presença de uma deleção. Não é possível utilizar este *software* para análise de CNVs em exomas.

As alterações de CNVs encontradas foram confirmadas por MPLA. De forma sucinta, todas as reações são realizadas de acordo com as instruções do protocolo dos *kits SALSA MLPA probemix P334-A2 Gonadal Development Disorder (MRC Holland, Amsterdam, Holanda),* que avalia CNVs nos genes *DMRT1, CYP17A1, SRD5A2 e HSD17B3* e *SALSA MLPA KIT P185-B1 Intersex,* que avalia os genes *SRY, WNT4, DAX1, SOX9 e NR5A1*. As amostras processadas com o *kit* foram submetidas a uma eletroforese capilar no sequenciador automático *ABI Prism 3130XL Genetic Analyzer* e analisadas pelo programa *GeneMapper 5.0* (ambos *Applied Biosystems, Thermo Fisher Scientific*, Waltham, Massachusetts, EUA). Os

dados obtidos foram analisados pelo programa *MRC Coffalyzer* (*MRC Holland*, Amsterdam, Holanda), que determina a relação das áreas dos picos de interesse com as áreas dos picos das regiões controle. Considerou-se como normal a razão entre essas áreas entre 0,7 e 1,3; considerou-se como deleção quando a mesma for inferior a 0,7 e como duplicação quando for superior a 1,3.

4.2.2.9 Classificação das variantes

Todas as variantes de interesse foram classificadas de acordo com os critérios do *American College of Medical Genetics and Genomics* [(103)], publicados em 2015. Esta classificação leva em consideração os critérios de frequência em bancos de dados populacionais, predição *in silico,* dados de estudos funcionais, de segregação familiar e publicações prévias na literatura. De acordo com o peso de cada critério, as variantes são classificadas como: (1) patogênica, (2) provavelmente patogênica, (3) variante de significado incerto, (4) provavelmente benigna ou (5) benigna. A descrição pormenorizada de cada critério assim como os critérios que permitem classificar as variantes estão descritos nos **Anexos 5 e 6.**

4.3 Análise estatística

Afim de verificar se havia associação entre a frequência de variantes deletérias identificadas no *DHX37* na população de pacientes com DDS 46,XY em relação à da população geral, a frequência alélica das variantes raras e possivelmente deletérias identificadas nestes dois grupos foi comparada, utilizando um teste x^2 , sendo considerada significância estatística para $p<0,05$. Para calcular a frequência das variantes, foram incluídos os seis casos índices (três casos esporádicos e seis casos familiais de três famílias) com SRTE da casuística da Unidade de Endocrinologia da FM-USP que foram previamente estudados na tese de doutorado da

Thatiana Evelyn da Silva apresentada à Faculdade de Medicina [70]. Para análise estatística foi utilizado o software SIGMAstat statistical software package (Windows version 3.5; SPSS Inc., San Rafael, CA).

5 Resultados

5 RESULTADOS

5.1 Avaliação dos painéis

Em relação às métricas das corridas, a cobertura média foi de 494X e desvio padrão de 244. Mais que 99% das regiões alvo foi coberta mais de 20 vezes. Assim, a cobertura foi considerada adequada.

5.1.1 Variantes alélicas candidatas identificadas na avaliação dos painéis

O sequenciamento dos 100 pacientes, gerou um total de 16.217 variantes, com uma média de 665 variantes por paciente (variando de 448 a 931 variantes), restando em média 10 variantes com *MAF* < 1% em regiões codificadoras (1 a 23 variantes). Após a exclusão das variantes sinônimas (sem predição de alteração do *splicing*), restaram em média 6 variantes por pacientes (1 a 14 variantes).

Por fim, após a aplicação de todos os critérios de seleção de variantes, foram identificadas 38 variantes candidatas de ponto distintas em 31 pacientes. Da totalidade, 23 estão em heterozigose, 10 em heterozigose composta e 5 em homozigose. A maioria é do tipo *missense,* 4 estão localizadas em sítio de *splicing e* uma variante determina um códon de parada. Ainda, foi identificada uma alteração de número de cópias, identificada pela *CONTRA*.

A caracterização das variantes candidatas identificadas por SPLE por painel (predição de patogenicidade pelo *SIFT, POLYPHEN e CADD*, escore de conservação do nucleotídeo pelo GERP, presença e frequência em bancos de dados populacionais, a segregação na família), classificação

pelos critérios ACMG bem como a identificação do paciente em que foram encontradas estão apresentadas nas **Tabelas 5 e 6.**

Foram identificadas 14 (37%) variantes novas**.** Trinta e uma (81%) variantes foram identificadas em genes classicamente relacionados ao DDS 46,XY e 7 variantes foram identificadas em dois genes candidatos aos DDS (6 variantes no gene *DHX37* e uma no *ESR2*). Cinco pacientes apresentam variantes candidatas em 2 genes (**Tabela 7**).

Tabela 5 - Caracterização e classificação das variantes identificadas por SPLE com painel de genes já associados aos DDS

Paciente	Classificação fenotípica do DDS	Gene (transcrito)	Variante	Alteração proteica	Estado da variante	Descrição prévia	Segregação
64	Indeterminado (gonadectomia)	*AMH (*NM_000479)	c.T902A	p.Leu301Gln	HMZ	Nova	ND
1	DG completa	*CHD7* (NM_017780)	c.C2066T	p.Thr689Met	HTZ	Sim (rs373706363)	ND
106	Defeito de síntese de andrógenos (Def. da 17-hidroxilase)	*CY17A1* (NM_000102)	Dup exon 1, 2 c.T1216C	 p.Trp406Arg	HTZ composta	Nova Sim (CM033603)	Irmã afetada tem o mesmo genótipo
23	DG parcial	*CBX2.2* (NM_032647.3)	c.460delT	p.Cys154Alafs*62	HTZ	Sim (rs552892809)	ND
114	Indeterminado (gonadectomia)	*DHH* (NM_021044)	c.T1004C	p.Leu335Pro	HMZ	Nova	ND
25	GD (parcial)	*GATA4 (*NM_002052)	c.C1220G	p.Pro407Arg	HTZ	Sim (rs115099192)	Mãe: c.C1220G
62	Indeterminado	*GATA4 (*NM_002052)	c.C1220G	p.Pro407Arg	HTZ	Sim (rs115099192)	Mãe: c.C1220G
105	Indeterminado	*HSD17B3* (NM_000197)	c.C242T c.277+4A>T	p.Thr81Met	HTZ composta	Sim (rs746859258) Sim (CS002140)	Pai: c.277+4A>T Mãe: c.C242T
111	Defeito de síntese de andrógenos (Def. da 17HSD2)	*HSD17B3* (NM_000197)	c.201+1G>T c.277+4A>T		HTZ composta	Sim (rs746001525) Sim (CS002140)	Pai: c.201+1G>T Mãe: c.277+4A>T
22	DG parcial	*NR5A1* (NM_004959)	c.C86G	p.Thr29Arg	HTZ	Sim (CM118936)	ND
6	DG completa	*NR5A1* (NM_004959)	c.C1019T	p.Ala340Val	HTZ	Sim (rs780199277)	ND
56	Indeterminado (gonadectomia)	*NR5A1* (NM_004959)	c.C72A	p.Hys24Gln	HTZ	Nova	2 irmãos e 3 irmãs: AS
80	Indeterminado	*NR5A1* (NM_004959)	c.C937T	p.Arg313Cys	HTZ	Sim (CM118686)	Mãe: AS; pai: ND

continua

Tabela 5 - Caracterização e classificação das variantes identificadas por SPLE com painel de genes já associados aos DDS (continuação)

Paciente	Gene (transcrito)	Variante	Alteração proteica	*MAF*	Predição *in silico* PolyPhen	SIFT	CADD_phred	GERP	dbscSNV	Estudo funcional	Critérios ACMG	Classificação ACMG
64	*AMH (*NM_000479)	c.T902A	p.Leu301Gln	0	D	D	26.7	3.11		Não	PM1, PM2	VUS
1	*CHD7* (NM_017780)	c.C2066T	p.Thr689Met	gAD:0.00003	D	T	25.9	5.5		Não	PP3,PB1	VUS
106	*CY17A1* (NM_000102)	Dup exon 1, 2		0	0	0	0	0		Não	PVS1,PS2,PS3	Patogênica
		c.T1216C	p.Trp406Arg		D	D	25.3	3.88		Sim [104]	PS3,PM2,PP3,PP4, PP5	Provavelmente patogênica
23	*CBX2.2 (*NM_032647.3)	c.460delT	p.Cys154Alafs*62	gAD: 0.001418	D	D	ND	-3.5		Sim	PP3,PM2,PP5	VUS
114	*DHH* (NM_021044)	c.T1004C	p.Leu335Pro	0		D	31	4.77		Não	PM1,PM2,PP2,PP3	Provavelmente patogênica
25	*GATA4 (*NM_002052)	c.C1220G	p.Pro407Arg	gAD (0.00006); Abr (0.0008)	P	D	25.8	4.78		Não	PM2,PP3,PP5	VUS
62	*GATA4 (*NM_002052)	c.C1220G	p.Pro407Arg	gAD (0.00006); Abr (0.0008)	P	D	25.8	4.78		Não	PM2,PP3,PP5	VUS
105	*HSD17B3* (NM_000197)	c.C242T	p.Thr81Met	gAD(0.0004)	D	D	25.9	4.79		Não	PM1,PM2,PM3,PP2,PP3	Provavelmente patogênica
		c.277+4A>T		gAD:0.0003			.		0.99	Não	PVS1,PM2, PM3, PP3	Patogênica
111	*HSD17B3* (NM_000197)	c.201+1G>T		gAD:0.000008					1	Não	PVS1,PM2,PP3	Provavelmente patogênica
		c.277+4A>T		gAD:0.0003					0.99	Não	PVS1,PM1, PM2, PP3	Patogênica
22	*NR5A1* (NM_004959)	c.C86G	p.Thr29Arg	0	D	D	17.8	4.0		Não	PM1,PM2,PP2,PP3	Provavelmente patogênica
6	*NR5A1* (NM_004959)	c.C1019T	p.Ala340Val	gAD (0.00002)	D	D	27	5.1		Não	PM1,PM2,PP3	VUS
56	*NR5A1* (NM_004959)	c.C72A	p.Hys24Gln	0	D	D	25.5	5.12		Não	PM1,PM2,PP3,PM5	Provavelmente patogênica
80	*NR5A1* (NM_004959)	c.C937T	p.Arg313Cys	0	D	D	25.5	5.12		Não	PM1, PM2, PP3, PP5	Provavelmente patogênica

Tabela 5 - Caracterização e classificação das variantes identificadas por SPLE com painel de genes já associados aos DDS (continuação)

Paciente	Classificação fenotípica do DDS	Gene (transcrito)	Variante	Alteração proteica	Estado da variante	Descrição prévia	Segregação
68	Indeterminado (gonadectomia)	*SRD5A2* (NM_000348)	c.G547A	p.Gly183Ser	HMZ	Sim (rs121434247/CM920637)	ND
108	Defeito de síntese de andrógenos (Def. da 5ARD2)	*SRD5A2* (NM_000348)	c.C736T	p.Arg246Trp	HMZ	Sim (rs121434244/CM920645)	ND
59	Indeterminado (gonadectomia)	*SRD5A2* (NM_000348)	c.T558G c.A337G	p.Phe186Leu p.Gln126Arg	HTZ composta	Sim (CM994668) Sim (rs368386747/CM920634)	Pai: c.T558G Mãe: c.A337G
110	Defeito de síntese de andrógenos (Def. da 5ARD2)	*SRD5A2* (NM_000348)	c.G604A c.T394C	p.Gly203Ser p.Cys133Arg	HTZ composta	Sim (rs9332961/ CM971419) Nova	ND
109	Defeito de síntese de andrógenos (Def. da 5ARD2)	*SRD5A2* (NM_000348)	c.A337G c.G596C	p.Gln126Arg p.Gly190Ala	HTZ composta	Sim (rs368386747/CM920634) Nova	Mãe:c.A337G Pai: c.G596C
70	Indeterminado	*WT1* (NM_024426)	c.1432+4C>T		HTZ	Sim (CS971933)	*De novo* (Mae e pai AS)
39	DG (SRTE)	*ZFPM2* (NM_012082)	c.A2501G	p.Lys834Arg	HMZ	Sim (rs113289249)	ND

continua

Tabela 5- Caracterização e classificação das variantes identificadas por SPLE com painel de genes já associados aos DDS (continuação)

					Predição *in silico*							
Paciente	**Gene (transcrito)**	**Variante**	**Alteração proteica**	***MAF***	**PolyPhen**	**SIFT**	**CADD_phred**	**GERP**	**dbscSNV**	**Estudo funcional**	**Critérios ACMG**	**Classificação ACMG**
68	*SRD5A2* (NM_000348)	c.G547A	p.Gly183Ser	gAD (0,0002)	D	D	34	3.45		Sim [105]	PS3,PM2,PP3,PP5	Provavelmente patogênica
108	*SRD5A2* (NM_000348)	c.C736T	p.Arg246Trp	0	B	D	24.0	4.1		Não	PM2,PP3,PP5,PS3	Provavelmente patogênica
59	*SRD5A2* (NM_000348)	c.T558G	p.Phe186Leu	0	D	P	17.43	5.55		Sim [106]	PM2,PM3,PP3,PP2	Provavelmente patogênica
		c.A337G	p.Gln126Arg	gAD (0.00008)	D	.	16.57	2.79		Não	PS3,PM2,PP3,PP5	Provavelmente patogênica
110	*SRD5A2* (NM_000348)	c.G604A	p.Gly203Ser	gAD (0.00008)	D	.	13.45	-3.5		Sim [107]	PS3,PM2,PP3	Provavelmente patogênica
		c.T394C	p.Cys133Arg	0	D	.	15.99	5.27		Não	PM2,PM3,PP3,PP4	Provavelmente patogênica
109	*SRD5A2* (NM_000348)	c.A337G	p.Gln126Arg	gAD (0.00008)	D	.	15.2	5.66		Não	PS3,PM2,PP3,PP5	Provavelmente patogênica
		c.G596C	p.Gly190Ala	0	D	.	15.34	5.27		Não	PM2,PM3,PP3,PP2	Provavelmente patogênica
70	*WT1* (NM_024426)	c.1432+4C>T		0						Sim [108]	PVS1,PS2,PS3	Patogênica
39	*ZFPM2* (NM_012082)	c.A2501G	p.Lys834Arg	gAD (0.0006); Abr (0.007)	D	D	24	5.8		Não	BS1, PP3	VUS

Abreviações: Def.; Deficiência, HTZ, Heterozigoto; HMZ, Homozigoto; ND, não descrito; gAD, gnomAD; ABr, ABRaOM; AS, alelo selvagem

Tabela 6 - Caracterização e classificação das variantes alélicas identificadas por SPLE por painel em genes candidatos aos DDS, incluindo o *DHX37*

									Predição *in silico*							
Paciente	**Classificação fenotípica do DDS**	**Gene (transcrito)**	**Variante**	**Alteração proteica**	**Estado da variante**	**Descrição prévia**	**Segregação**	***MAF***	**PolyPhen**	**SIFT**	**CADD_phred**	**GERP**	**dbscSNV**	**Estudo funcional**	**Classificação ACMG**	**Critérios ACMG**
3	DG (completa)	*ESR2* (NM_001291712)	c.T1277G	p.Leu426Arg	HTZ	Nova	ND	0	D	D	26.9	5.7		Sim[109]	VUS	PM1,PM2,PP3
53	Indeterminado (gonadectomia)	*DHX37* (NM_032656)	c.C911T	p.Thr304Met	HTZ	Nova	Mãe e 4 irmãos: AS	0	D	D	29.8	5.3		Não	Provavelmente patogênica	PM1,PM2,PP2,PP3
57	Indeterminado	*DHX37* (NM_032656)	c.C1399G	p.Leu467Val	HTZ	Sim (rs149331610)	ND	gAD (0.0002); Abr (0,0008)	D	D	24	0.19		Não	VUS	PM2,PP3
21	DG (parcial)	*DHX37* (NM_032656)	c.G923A	p.Arg308Gln	HTZ	Sim [70]	Mãe e pai: AS	gAD (0.00006677)	D	D	35	5.3		Não	Patogênica	PM2, PP2,PP3,P2, PM1
44	DG (SRTE)	*DHX37* (NM_032656)	c.C2020T	p.Arg674Trp	HTZ	Sim [70]	ND	0	D	D	33	4.2		Não	Provavelmente patogênica	PM2,PP2,PP3,PM1
41	DG (SRTE)	*DHX37* (NM_032656)	c.C2020T	p.Arg674Trp	HTZ	Sim [70]	Mãe: c.C2020T	0	D	D	33	4.2		Não	Provavelmente patogênica	PM2,PP2,PP3,PM1

Abreviações: HTZ, Heterozigoto; HMZ, Homozigoto; ND, não descrito; gAD, gnomAD; ABr, ABRaOM; AS, alelo selvagem

Tabela 7 - Variantes alélicas possivelmente digênicas identificadas por SPLE por painel, em genes já associados aos DDS 46,XY e no gene candidato *DHX37*

									Predição *in silico*							
Paciente	**Classificação fenotípica do DDS**	**Gene (transcrito)**	**Variante**	**Proteína**	**Estado da variante**	**Descrição prévia**	**Segregação**	**MAF**	**PolyPhen**	**SIFT**	**CADD_phred**	**GERP**	**dbscSNV**	**Estudo funcional**	**Classificação ACMG**	**Critérios ACMG**
107	Defeito de síntese de andrógenos	*MAP3K1* (NM_005921)	c.834+1G>T		HTZ	Nova	Mãe e pai: AS				22,9	5,72	0.9	Não	Patogênica	PVS1, PS2, PM2
	(Def da 5ARD2)	*SRD5A2* (NM_000348)	c.337C>G	p.Leu113Val	HTZ	Sim (rs28383048)		0.002	B	B	1.3	(-3.79)		Não	Provavelmente benigna	PP2, BP2, BP6
28	DG (parcial)	*GATA4* (NM_002052)	c.C1103G	p.Pro368Arg	HTZ	Sim (rs775379687)	Pai: c.C1103G	gAD(0.00004) Abr (0.0008)	D	D	27.8	5.8			VUS	PM2,PP3
		DHX37 (NM_032656)	c.C2020T	p.Arg674Trp	HTZ	Sim[70]	Mãe:c.C2020T	0	D	D	33	4.2		Não	Provavelmente patogênica	PS4,PM2, PP3
71	Indeterminado	*WWOX* (NM_016373)	c.T253G	p.Tyr85Asp	HTZ	Sim (rs781063964)	ND		D	T	23.9	5.41		Não	VUS	PM1,PM2, PP3
		DHX37 (NM_032656)	c.G2209A	p.Ala737Thr	HTZ	Sim (rs199752789)	ND	gAD:0.00001	D	D				Não	VUS	
77	Indeterminado	*NR5A1* (NM_004959)	c.C77T	p.Gly26Val	HTZ	Nova	ND	0	D	D	29.7	4.01		Não	Provavelmente patogênica	PM1,PM2, PP3,PP5
		DHX37 (NM_032656)	c.G1474C	p.Ala494Pro	HTZ	Nova	ND	0						Não	VUS	
78	Indeterminado	*MAP3K1* (NM_005921)	c.A1628G	p.His543Arg	HTZ	Sim (rs1054518497)	Mãe: AS	gAD:0.000004	D	D	23.1	5.97		Não	VUS	PM2
		GATA4 (NM_002052)	c.G644T	p.Arg215Ile	HTZ	Novel	Mãe: c.G644T	0	D	D	32	4.72		Não	VUS	PM1,PM2, PP3

Abreviações: HTZ, Heterozigoto; HMZ, Homozigoto; ND, não descrito; gAD, gnomAD; ABr, ABRaOM; AS, alelo selvagem

5.2 Alteração de número de cópias identificadas pelo *CONTRA*

Foi identificada uma alteração de número de cópias na paciente número 106 com fenótipo da deficiência da enzima 17-α hidroxilase, porém com genótipo incompleto.

Previamente, foi identificada pelo método de Sanger a variante alélica c.T1216C;p.Trp406Arg no gene *CYP17A1*, também encontrada na análise do painel, em heterozigose. Trata-se de variante ausente em bancos de dados populacionais, já conhecida e associada ao fenótipo (CM033603) em diversas coortes [110] e com estudo funcional publicado [104].

O *CONTRA* identificou uma duplicação dos éxons 1 e 2 da *CYP17A1,* que foi confirmado por MLPA (**Figura 6**). Trata-se de alteração nova, ausente em bancos de dados populacionais, classificada como provavelmente patogênica.

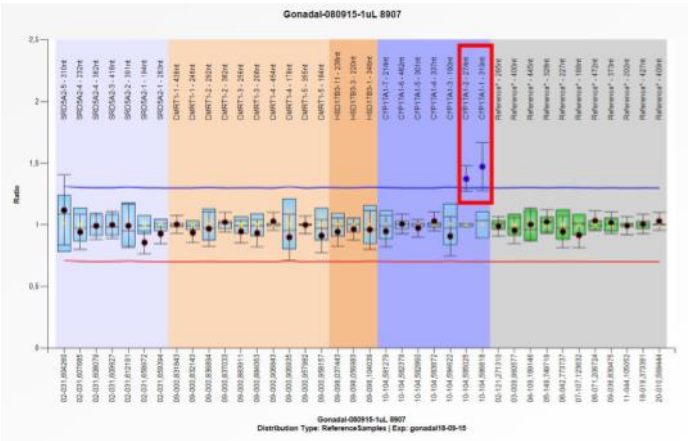

Figura 6 - Análise do resultado no MLPA da paciente 20, com fenótipo de deficiência da 17α-hidroxilase. A região destacada pelo retângulo vermelho realça a duplicação dos éxons 1 e 2 do *CYP17A1.* A razão entre pico da área de interesse e controle foi de 1,38 e 1,47, para estes éxons, respectivamente

5.3 Avaliação do sequenciamento exômico global

5.3.1 Métrica das corridas

Em relação às métricas das corridas dos exomas avaliados, média de 125X e desvio padrão de 83. Mais que 96% das regiões alvo foi coberta mais de 10 vezes (mínimo de 91%) e mais de 93,5% foi coberta mais de 20 vezes em média (mínimo de 84%).

5.3.2 Variantes encontradas no sequenciamento exômico global

Foram identificadas 2 variantes candidatas distintas em 4 pacientes de 2 das 7 famílias avaliadas (famílias 6 e 3). Ambas as variantes são inéditas e do tipo mutação de ponto, sendo a variante da família 6 localizada em sítio de *splicing* do gene *NR5A1* e, portanto, do tipo perda de função (*loss of function*). A variante da família 3 foi identificada no gene candidato *DHX37.*

A caracterização das variantes candidatas identificadas (predição de patogenicidade pelo SIFT, POLYPHEN e CAAD, escore de conservação do nucleotídeo pelo GERP, presença e frequência em bancos de dados populacionais, tipo de herança – de acordo com a segregação realizada) e classificação pelos critérios ACMG estão apresentados na **Tabela 8.**

Tabela 8 - Variantes alélicas identificadas por SPLE por exoma em duas famílias de pacientes com DDS 46,XY

Paciente (Família)	Classificação fenotípica do DDS	Gene (transcrito)	Variante	Alteração proteica	Estado da variante	Descrição prévia	Segregação	*MAF*	PolyPhen	SIFT	CADD_phred	GERP	dbscSNV	Estudo funcional	Classificação ACMG	Critérios ACMG
36 e 37 (F3)	DG (parcial e SRTE)	*DHX37* (NM_032656)	c.C1784T	p.Ser595Phe	HTZ	Nova	AD (materna)	0	D	B	24.3	4.13		Não	Provavelmente patogênica	PP1,PM2,PP3
101 e 102 (F6)	Indeterminado	*NR5A1* (NM_004959)	c.C1139-3C>A		HTZ	Nova	AD (materna)	0	D	D	25.2	2.09	0.99	Não	Patogênica	PM2,PSV1, PP3

Abreviações: HTZ, Heterozigoto; HMZ, Homozigoto; ND, não descrito; gAD, gnomAD; ABr, ABRaOM; AS, alelo selvagem

5.3.2.1 Variante c.1139-3C>A no *NR5A1* identificada por sequenciamento exômico

A variante c.1139-3C>A, localizada em sítio de *splicing*, foi identificada por sequenciamento exômico da família 6 em dois pacientes com DDS 46,XY de etiologia indeterminada (o caso índice e seu sobrinho, representados no heredograma como casos II.6 e III.6, respectivamente (**Figura 7).**

Foram selecionados para sequenciamento exômico os dois indivíduos afetados e a irmã do caso índice (representada no heredograma como caso II.3). A filtragem inicial foi feita para padrão de herança autossômica dominante e ligada ao X. Do total de 1045557 presentes nos 3 indivíduos, restaram 21 variantes candidatas, todas em genes não relacionados às vias da determinação/ diferenciação sexual, exceto a variante c.1139-3C>A no *NR5A1*, como representado de forma esquemática na **Figura 8.**

A variante em questão está ausente em bancos de dados populacionais e no banco do SELA. Ela é predita como deletéria pelo *dbscSNV* (ADA escore 0,99) e, segundo o *Human Splice Finder,* ela modifica o sítio de *splicing,* sem que um novo seja criado. O gene *NR5A1* não tolera variantes com perda de função (*loss of function*), conforme previsão no ExAC (http://exac.broadinstitute.org). Houve segregação familiar com o fenótipo nos indivíduos 46,XY estudados. Os dados em questão permitem classificá-la como patogênica.

Após a identificação da variante, outros indivíduos da família foram estudados por Sanger e a segregação confirmou herança autossômica dominante de origem materna. Os indivíduos 46,XX II.1, II.3 e III.5 são, a princípio, portadores assintomáticos da variante, sendo que as duas primeiras mantiveram ciclos menstruais regulares após os 40 anos de idade e a terceira tem 17 anos, com ciclos menstruais regulares. Em nenhum dos casos há relato de insuficiência da suprarrenal.

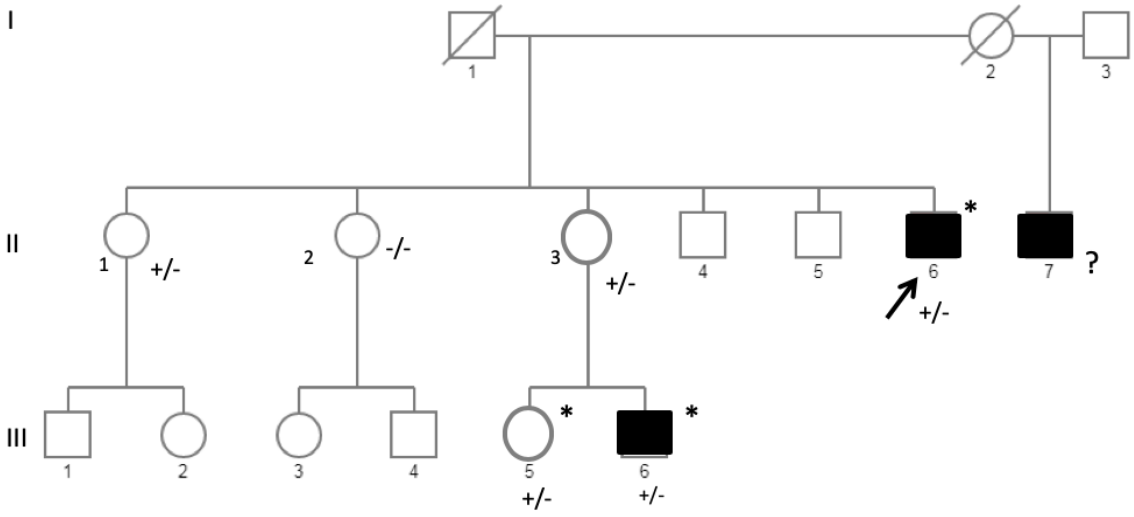

Figura 7 - Heredograma da Família 6. Os quadrados representam os indivíduos de sexo social masculino e os círculos aqueles com sexo social feminino. As figuras preenchidas por preto representam os indivíduos com DDS 46,XY. O asterisco marca aqueles estudados por sequenciamento exômico. O genótipo do *NR5A1* está representado para aqueles indivíduos que foram estudados; +/- representa heterozigoto e -/- representa homozigose para o alelo selvagem

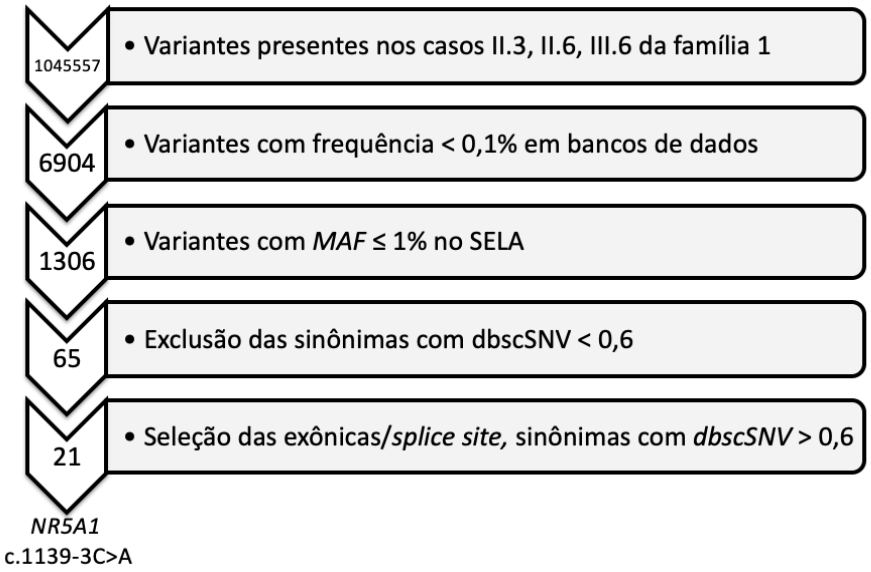

Figura 8 - Fluxograma da análise das variantes obtidas por sequenciamento exômico na família 6

5.3.2.2.2 Variante c.C1784T; p.Ser595Phe no *DHX37* identificada por sequenciamento exômico

A variante em questão foi identificada por sequenciamento exômico da família 3 em dois pacientes classificados como disgenesia gonadal (o caso índice, paciente 36, com a forma parcial, e seu sobrinho, paciente 37, com SRTE, casos II.6, III.1 do heredograma da **Figura 9**) e na irmã do caso índice (indivíduo II.5).

Foram incluídos na avaliação por exoma o caso índice, seu sobrinho, a mãe e pai deste. A filtragem inicial foi feita para padrão de herança autossômica dominante materna. Do total de 150455 variantes, após a devida filtragem, restaram 27 variantes candidatas em genes não relacionados às vias da determinação/ diferenciação sexual, exceto pela variante em questão no *DHX37,* como mostra o fluxograma da **Figura 10.** Ainda, a variante foi segregada pelo método de Sanger nos indivíduos II.4, II.7, II.8 e II.9, não sendo identificada nos mesmos.

A variante em questão é inédita, é predita como deletéria pelos algoritmos *in silico* (**Tabela 8**), está ausente em todos os bancos de dados populacionais e no banco do SELA e o nucleotídeo em questão é evolutivamente conservado. A segregação mostrou padrão de herança autossômico dominante de origem materna, sendo a mulher (indivíduo II.5) carreadora assintomática da variante.

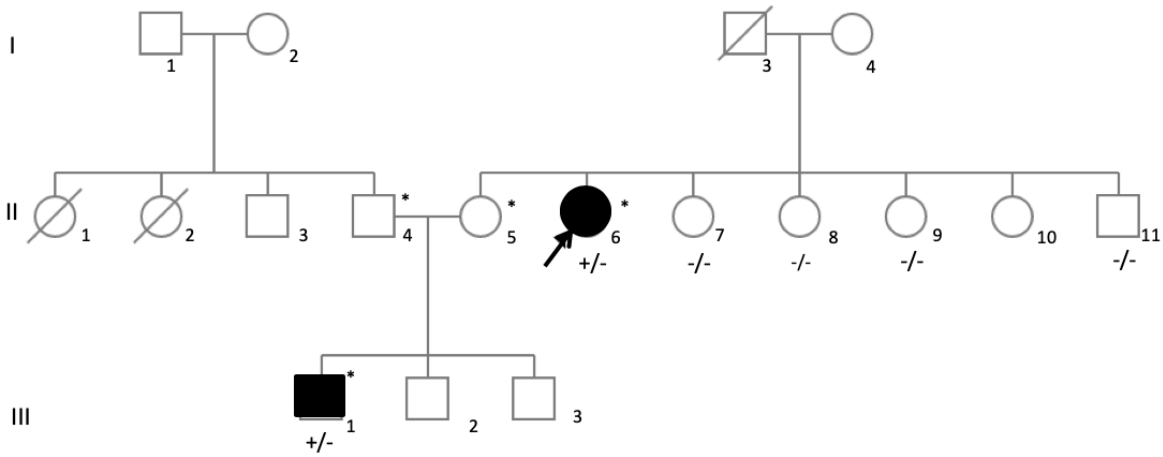

Figura 9 - Heredograma da Família 3. Os quadrados representam os indivíduos de sexo social masculino e os círculos aqueles com sexo social feminino. As figuras preenchidas por preto representam os indivíduos com DDS 46,XY. O asterisco marca aqueles estudados por sequenciamento exômico. O genótipo do *DHX37* está representado para aqueles indivíduos que foram estudados; +/- representa heterozigoto e -/- representa homozigose para o alelo selvagem.

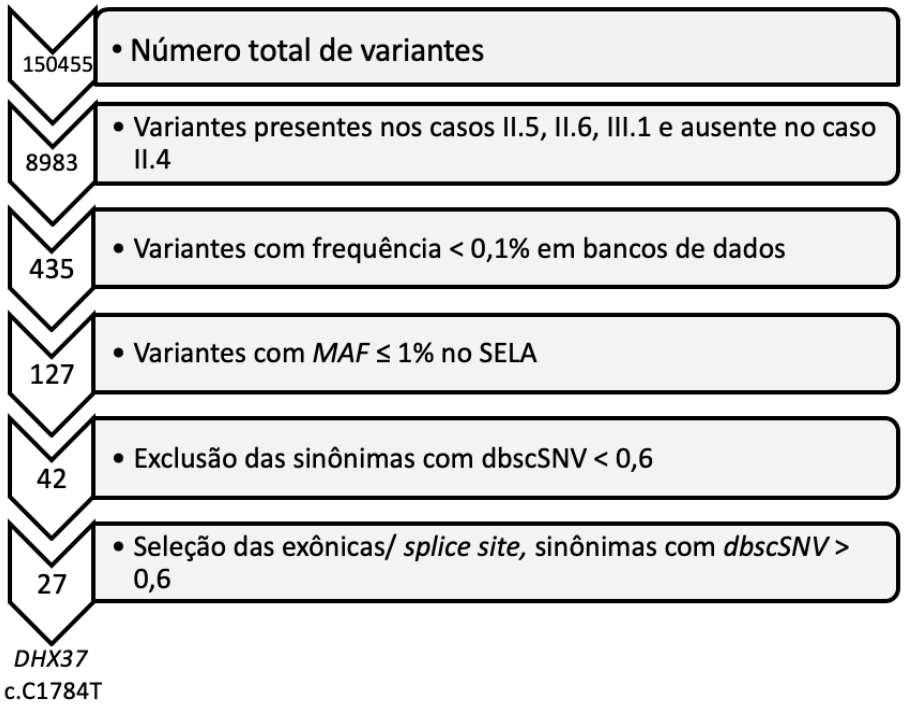

Figura 10 - Fluxograma da análise das variantes obtidas por sequenciamento exômico na família 3

5.4 Fenótipo dos pacientes e descrição das variantes identificadas em genes já associados à etiologia dos DDS

Sete variantes distintas classificadas como patogênicas ou possivelmente patogênicas foram identificadas em genes previamente relacionados à formação testicular (*NR5A1, WT1, MAP3K1)* em seis casos esporádicos e nos dois casos familiais da família 6, todas em heterozigose, sendo as variantes no gene *NRA51* as mais frequentes (5 variantes). Apenas um destes pacientes foi inicialmente classificado como disgenético (paciente 22), sendo a maioria classificada como DDS de etiologia indeterminada por gonadectomia (pacientes 56) e por perfil hormonal inconclusivo (pacientes 70, 77, 80, casos familiais 101 e 102), além de uma paciente classificada como tendo deficiência da 5ARD2 (paciente 107). Quatro entre os estes cinco pacientes classificados como DDS de etiologia indeterminada têm variantes no *NR5A1*. Nenhum deles possui derivados Mullerianos.

O paciente 70, no qual foi identificada a variante no *WT1* c.1432+4C>T, evoluiu com proteinúria nefrótica aos 5 anos de idade e foi encaminhado para nefrologia pediátrica, sendo submetido a biópsia renal que confirmou diagnóstico de gromeruloesclerose segmentar focal (GESF).

No paciente 107, com perfil clínico sugestivo de deficiência da 5ARD2 (histórico de virilização na puberdade e relação T/DHT= 44), foi identificada a variante patogênica c.1432+4C>T na *MAP3K1* em associação à variante em heterozigose p.Leu113Val no gene *SRD5A2* (rs28383048), classificada como provavelmente benigna. Não foram identificadas alterações de número de cópias neste gene. Ainda, a paciente apresenta a variante polimórfica p.Val89Leu (rs523349) em homozigose.

Outras oito variantes classificadas como de significado incerto foram identificadas no genes *GATA4, CBX2.2, MAP3K1, DHH, WWOX* e *ZFPM2* em sete pacientes (pacientes 23, 25, 28, 39, 62, 71, 78, 114), sendo quatro delas identificadas em associação a variantes em outros genes relacionados

à disgenesia. Estes pacientes possuem fenótipos variados incluindo aqueles com DDS de etiologia indeterminada por perfil hormonal inconclusivo (n= 4) ou por gonadectomia (n=1), DG parcial (n=1) e SRTE (n=1).

Doze variantes de ponto distintas e uma alteração de número de cópias foram identificadas em genes da diferenciação sexual (*CYP17A1, HSD17B3, SRD5A2*) em sete casos esporádicos (casos 105, 111, 68, 108, 59, 110, 109) e no caso familial 106 , todas com herança autossômica recessiva. As variantes em genes relacionados à esteroidogênese foram identificadas em cinco pacientes classificados como DDS por provável defeito de síntese de andrógenos (casos 106, 108, 109, 110, 111), dois pacientes com DDS de etiologia indeterminada por gonadectomia prévia (pacientes 59 e 68) e um paciente com perfil hormonal inconclusivo (caso 105).

O paciente 105 apresentou virilização na puberdade associada a disforia de gênero (criado no sexo social feminino com identidade de gênero masculina), é filho de pais consanguíneos (primos de 1º grau) e apresentava clitoromegalia importante (clitóris 6 cm), orifício perineal único e gônadas inguinais palpáveis bilateralmente, sem derivados Mullerianos. Avaliação laboratorial mostrou testosterona adequada para o sexo masculino (626 ng/dL). Ao teste de estímulo com hCG verificou-se: testosterona sérica 756 ng/dL, DHT 38 ng/dL, androstenediona 800 ng/dL, permitindo cálculo das relações T/DHT=19,6 (aumentada), T/A=0,94 (normal). Embora a relação T/A estivesse normal, neste caso foram identificadas as variantes c.277+4A > T e c.C242T no *HSD17B3*, em heterozigose composta, confirmadas no programa IGV (**Figura 11**).

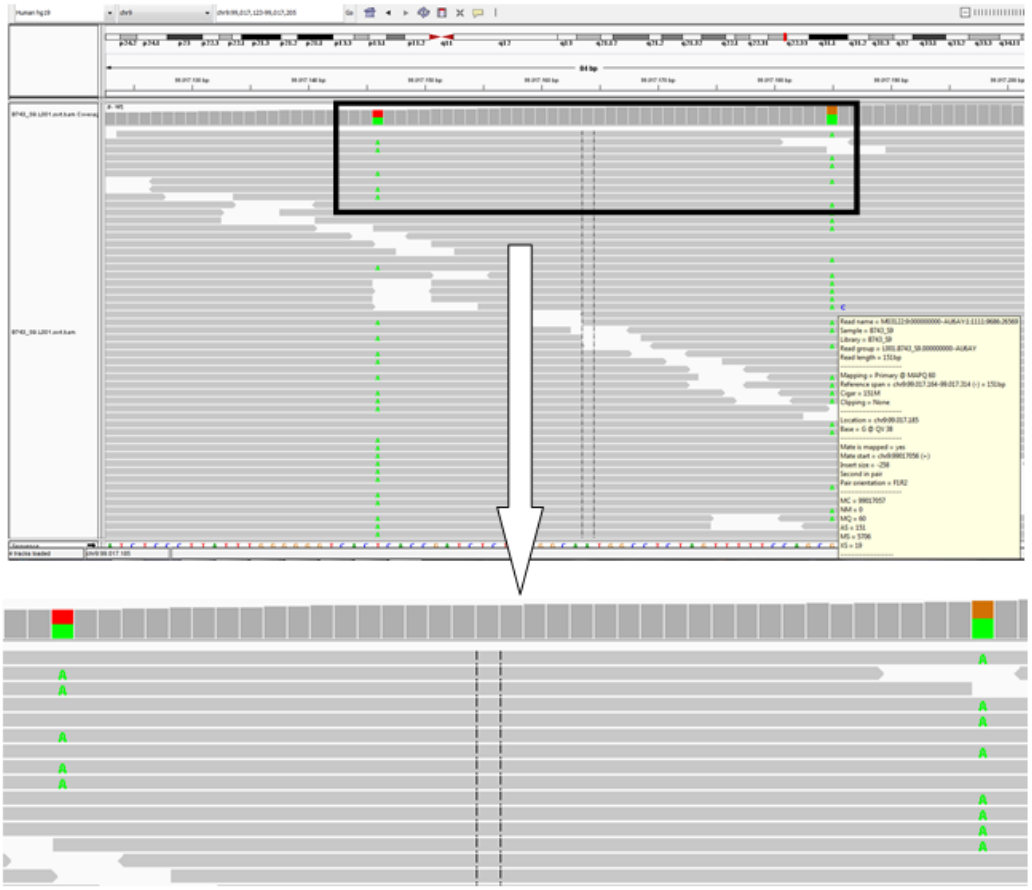

Figura 11 - IGV das variantes c.C242T e c.277+4A>T no *HSD17B3*, em heterozigose composta, identificadas no paciente 105

5.5 Fenótipo dos pacientes e descrição das variantes identificadas em novos genes candidatos à etiologia dos DDS

5.5.1 Gene *ESR2*

A variante c.T1277G; p.Leu426Arg no *ESR2* foi identificada em heterozigose na paciente 3, filha de pais consanguíneos, com fenótipo de disgenesia gonadal completa.

Trata-se de variante alélica nova, não encontrada em bancos de dados populacionais ou no banco do SELA, predita como deletéria, que altera um nucleotídeo evolutivamente conservado. Não dispomos de familiares para a segregação.

O gene ESR2 é expresso nos testículos desde 8 semanas de vida embrionária [111, 112]. Foi realizado estudo funcional da variante em colaboração com o grupo da Universidade de Ghent, na Bélgica, coordenado pela Profa. Elfride De Baere que mostrou, por ensaio de luciferase, que houve aumento significativo da ativação do receptor, independente do ligante específico da unidade β do receptor estrogênico [109].

5.5.2 Gene *DHX37*

Ao todo, variantes patogênicas ou possivelmente patogênicas foram identificadas em quatro casos esporádicos e em três casos familiais de duas famílias distintas (a família 3, estudada por sequenciamento exômico e a família do paciente 41, cujo caso índice foi estudado por painel, sendo a variante posteriormente segregada no demais dos familiares) [113].

A variante p.Arg308Gln, anteriormente identificada em pacientes com fenótipo de SRTE [70], foi identificada uma paciente com disgenesia gonadal parcial (paciente 21), caracterizada por genitália externa feminina, derivados Mullerianos ausentes e gônadas disgenéticas.

A variante p.Arg674Trp que previamente identificada em três indivíduos de uma família chilena com SRTE [70], foi identificada não só em mais um caso esporádico (paciente 44) e um caso familial de origem argentina (paciente 41) com este fenótipo, como também em uma paciente com disgenesia gonadal parcial (paciente 28), caracterizada por atipia genital verificada ao nascimento, pela presença de derivados mullerianos e gônadas disgenéticas. Nesta última paciente, foi também identificada a variante no *GATA4,* p.Pro368Arg, classificada como de significado incerto, de herança paterna.

Ainda, outras duas novas variantes, a p.Ser595Phe e a p.Thr304Met foram identificadas em uma família com dois indivíduos afetados (paciente 36 com disgenesia gonadal parcial e o paciente 37 com SRTE) e o caso 53, criado no sexo feminino, gonadectomizado na infância e, portanto, classificado inicialmente como DDS de etiologia indeterminada.

Todas as variantes têm baixa frequência (< 0,001) ou estão ausentes em bancos de dados populacionais, são preditas como deletérias pelos algoritmos de *in silico* (**Tabelas 6 e 7**) e alteram nucleotídeos conservados da proteína. A herança em todos os casos foi autossômica dominante, de origem materna (dois casos) e *de novo* (caso 21), como mostram os heredogramas da **Figura 12.**

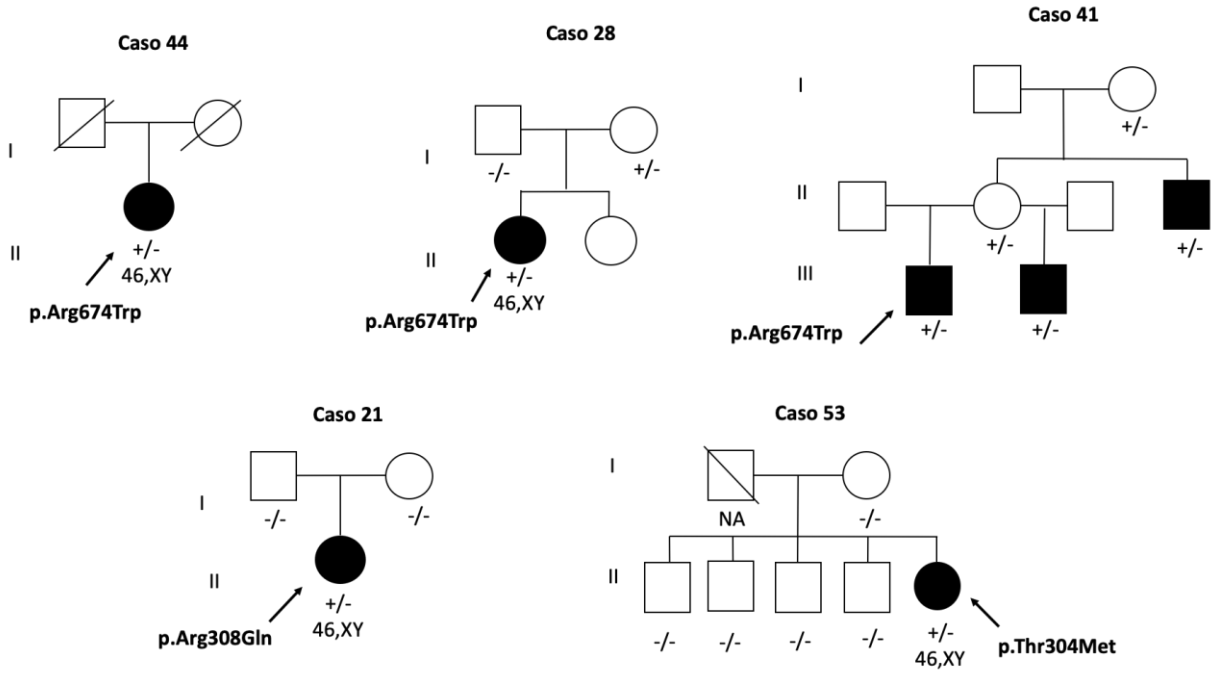

Figura 12 - Heredogramas dos casos com variantes deletérias no *DHX37* identificadas por sequenciamento por painel de genes. Os quadrados representam os indivíduos de sexo social masculino e os círculos aqueles com sexo social feminino. As figuras preenchidas na cor preta representam os indivíduos afetados. A linha diagonal representa os indivíduos falecidos. O genótipo do *DHX37* está representado para aqueles indivíduos que foram estudados; +/- representa heterozigoto e -/- representa homozigose para o alelo selvagem.

Seguindo os critérios ACMG, as variantes foram classificadas como patogênicas (p.Arg308Gln) ou possivelmente patogênicas (p.Arg674Trp, p.Ser595Phe e p.Thr304Met).

A **Tabela 9** descreve os fenótipos relacionados às variantes, na totalidade da casuística da Unidade de Endocrinologia da FM-USP.

Tabela 9 - Frequência das variantes patogênicas/provavelmente patogênicas do *DHX37* em toda a coorte da Unidade de Endocrinologia da FMUSP, de acordo com o fenótipo

	Número de pacientes afetados de acordo com a classificação do DDS 46,XY			
			SRTE	
	Indeterminado	Disgenesia gonadal	Estudo Prévio [70]	Estudo Atual
Variantes no *DHX37*				
p.Arg308Gln	0	1	4	0
p.Arg674Trp	0	1	1	2
p.Ser595Phe	0	1*	0	1*
p.Thr304Met	1	0	0	0
% /n de pacientes afetados	1.8 (1/54)	8 (3/37)	44 (8/18)	

Legenda: o asterisco marca indivíduos de uma mesma família com fenótipos diferentes

As quatro variantes estão localizados nos domínios conservados da proteína na região *helicase core* que contém o domínio helicase de ligação ao ATP (posições dos aminoácidos 262-429) e o domínio helicase c-terminal (posição dos aminoácidos 585-674) (**Figura 12).**

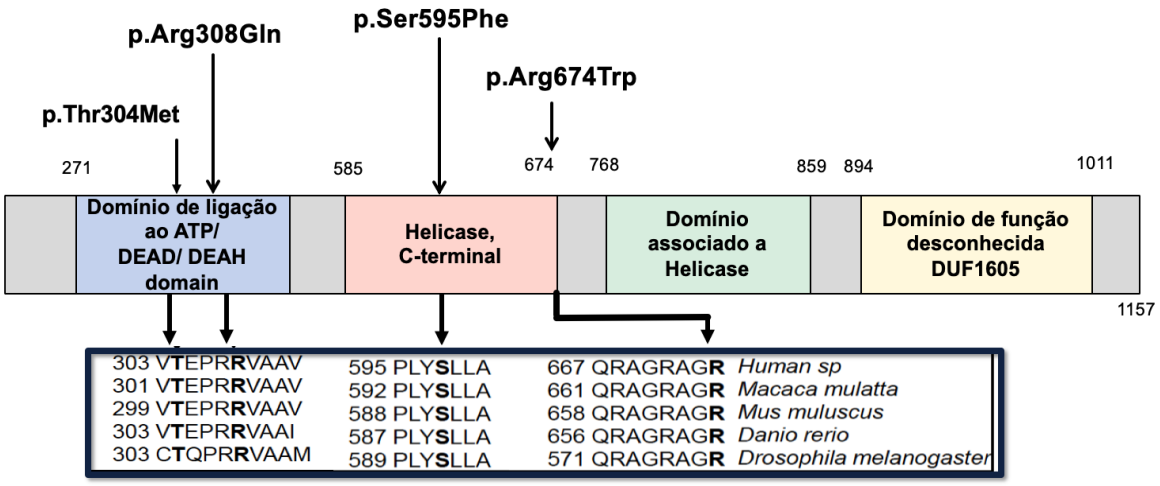

Figura 13 - Desenho esquemático da proteína do DHX37 com a identificação dos domínios e a localização das variantes identificadas. O quadro abaixo mostra que as variantes envolvem aminoácidos muito conservados entre as diferentes espécies

5.6 Comparação da frequência alélica de variantes deletérias do *DHX37* na coorte de pacientes com DDS 46,XY e na população

Com o objetivo de avaliar se há evidência genética entre a associação de variantes alélicas deletérias no *DHX37* com o fenótipo de disgenesia gonadal [114], foram selecionadas dos bancos de dados populacionais (gnomAD e ABraOM) variantes neste gene com características similares àquelas identificadas nos pacientes com DDS 46,XY, de forma a permitir a comparação da frequência dessas variantes com as da presente coorte. Assim, foram selecionadas variantes não sinônimas com frequência alélica (MAF) de até 0.01, localizadas nos domínios conservados de ligação ao ATP e Helicase C-terminal, que fossem preditas como patogênicas por pelo menos quatro das ferramentas de predição *in silico* SIFT [95], PolyPhen2 [115], Mutation Taster [96], Mutation Acessor [97], CADD [98]. Todas as variantes identificadas estão em heterozigose.

A frequência dessas variantes, tanto na coorte de pacientes com DDS 46,XY quanto na de DDS disgenético, mas principalmente nos pacientes com SRTE, está significativamente aumentada em relação aos controles dos bancos de dados populacionais (**Tabela 10).**

Tabela 10 - Comparação entre a frequência das variantes raras e possivelmente deletérias localizadas nos domínios conservados entre os pacientes da presente coorte e dos bancos de dados populacionais

Parâmetro	Número de indivíduos com variantes potencialmente deletérias	Número total de indivíduos	Frequência das variantes	*p*
gnomAD	568	141456	0.4%	
ABraOM	1	609	0.2%	
DDS 46,XY	11	109	10%	<0.001
Disgenéticos	3	37	8%	<0.001
SRTE	8	18	44%	<0.001

5.7 Resumo dos achados moleculares

A análise total dos resultados moleculares obtidos pelo sequenciamento está resumida na **Tabela 11**. O diagnóstico molecular foi considerado elucidado nos pacientes nos quais foram identificadas variantes patogênicas ou possivelmente patogênicas**.**

Em relação aos resultados obtidos por sequenciamento por exoma, foram identificadas variantes candidatas e diagnósticas em 5 dos 42 casos índices (11.9%) com disgenesia gonadal, 9 dos 52 índices (17.2%) com DDS de etiologia indeterminada e em todos os 6 casos índices (100%) com DDS por provável defeito de síntese de andrógenos.

Em relação aos resultados do sequenciamento por exoma, foram identificadas variantes candidatas e diagnósticas em 2 das 7 famílias (28.5%), sendo uma das famílias com DDS disgenético e a outra como DDS de etiologia indeterminada.

Nas duas famílias classificadas como DDS por provável defeito de síntese de andrógenos (família 4, pacientes 113 e 114 e família 7, paciente 112, por provável deficiência de 5ARD2 e hipoplasia de células de Leydig, respectivamente) não foram identificadas variantes candidatas. Permaneceram ainda sem diagnóstico molecular as famílias 1 e 2 (com fenótipo de disgenesia gonadal) e a família 5 (classificada como DDS de etiologia indeterminada).

Dessa forma, totalizando a casuística, foram identificadas variantes patogênicas ou possivelmente patogênicas em 6 dos 45 casos índices (13%) com disgenesia gonadal, 10 dos 54 índices (18.5%) com DDS de etiologia indeterminada e 6 dos 8 casos índices (75%) com DDS por provável defeito de síntese de andrógenos.

Tabela 11 - Resumo dos achados moleculares obtidos na casuística de pacientes com DDS 46,XY

DDS 46,XY	Pacientes com variantes alélicas		Pacientes sem variantes
	Número e (%) de variantes P e PP	**Número e (%) de pacientes com VUS**	**Número e (%)**
Disgenesia gonadal (n=45)	6 (13.3)	6 (13.3)	34 (75.5)
Forma completa (n=16)	0	3 (18.5)	11 (78.5)
Forma parcial (n=17)	3 (17.6)	3 (11.7)	12 (70.5)
SRTE (n=12)	3 (25)	1 (8.3)	8 (60)
Indeterminado (n=54)	10 (18.5)	6 (11)	39 (72)
DDS por provável defeito de síntese de andrógenos (n=8)	6 (75)	0	0
Total (n=107)	22 (20.5)	13 (12.1)	73 (68.2)

6 Discussão

6 DISCUSSÃO

Os pacientes com DDS eram tradicionalmente diagnosticados através de uma combinação de avaliação fenotípica e laboratorial endocrinológica, de forma a guiar o diagnóstico molecular pela estratégia de gene candidato. Trata-se de um processo demorado, custoso e muitas vezes sem sucesso no diagnóstico molecular final, especialmente nos casos de DDS 46,XY, particularmente os disgenéticos.

Neste contexto, as técnicas de sequenciamento paralelo em larga escala emergiram na última década como promissoras, propiciando a determinação da etiologia genética precoce nos DDS [60-62]. Além disso, o sequenciamento por exoma propicia a identificação de novos genes candidatos aos DDS, como foi o caso do gene *DHX37* [70].

De forma inédita no Brasil, o presente estudo avaliou a frequência de diagnóstico molecular em uma coorte de 100 pacientes sequenciados por painel de genes relacionados aos DDS. Variantes patogênicas ou provavelmente patogênicas foram encontradas em 20% dos casos.

Esta é a segunda maior coorte de DDS até então avaliada por SPLE por painel. Comparando os resultados obtidos com o dos estudos prévios, esta é a que obteve um dos menores percentuais de esclarecimento diagnóstico, em relação apenas às variantes identificadas em genes já associados aos DDS, especialmente em relação aos pacientes com disgenesia gonadal [59-63]. Neste ponto, cabem algumas ponderações em relação aos nossos resultados. Esta é uma coorte que já foi amplamente estudada: 71% dos pacientes já foi sequenciada para alterações em pelo menos um gene associado aos DDS, enquanto nas demais coortes esse percentual fica entre 30% [62]- 50% [60] dos casos.

Outro aspecto relevante é em relação à classificação das variantes dos estudos prévios. Embora os autores coloquem que a classificação foi

feita de acordo com os critérios do ACMG, variantes novas em genes associados aos DDS e preditas como deletérias pelos algoritmos *in silico,* foram consideradas provavelmente patogênicas nestes estudos, muitas vezes independente da segregação ter sido ou não realizada e na ausência de estudos funcionais [(60)-(62)]. Se seguíssemos estes critérios, sete variantes que foram identificadas em nossa coorte e que foram rigorosamente classificadas com VUS, seriam consideradas diagnósticas.

Entretanto, tal conduta pode levar a falsos diagnósticos moleculares. Como exemplo, a variante no *GATA4* p.Pro407Gln (c.C1220A) foi previamente classificada como deletéria devido à sua associação com cardiopatia congênita, sendo considerada como causadora de DDS por outros autores [(62)]. Contudo, esta variante foi identificada em dois pacientes da nossa coorte, ambos sem cardiopatia, como também em outro paciente sem alterações genitais e com puberdade espontânea do banco de dados do SELA.

Até o momento, apenas quatro variantes comprovadamente patogênicas por estudo funcional no *GATA4* (p.Gly221Arg, p.Cys238Arg, p.Trp228Cys e p.Pro226Leu), todas localizadas no domínio de ligação ao DNA N-terminal (em dedo de zinco), foram identificadas em indivíduos com atipia genital, com ou sem cardiopatia, com grande variabilidade fenotípica [(116, 117)]. As variantes alélicas localizadas fora do domínio de ligação ao DNA do *GATA4* são causas recorrentes de cardiopatia e parecem alterar a migração da proteína em direção ao núcleo em cardiomiócitos, justificando os fenótipos encontrados nestes casos [(118)]. Entretanto, em relação à cascata de determinação sexual, não é possível dizer o mesmo, haja visto que variantes alélicas que causam fenótipo de doença cardíaca em ratos parecem não ocasionar fenótipo em gônadas, pois a redução da transcrição dos diversos promotores do *GATA4* pode ser compensada pela interação preservada com o *NR5A1*[(119)].

Assim, tanto a variante p.Pro407Gln como outras variantes no *GATA4* identificadas fora do domínio de ligação do DNA não devem ser consideradas como deletérias, sem que haja outras evidências favoráveis.

Apenas 14% dos pacientes com disgenesia gonadal estudados por painel tiveram seu diagnóstico molecular elucidado no presente estudo, sendo que a maior parte das variantes foi identificada no *DHX37*. Entretanto, das sete variantes identificadas em genes previamente associados à formação testicular, seis foram identificadas em pacientes classificados como de etiologia indeterminada, nos quais os derivados Mullerianos não foram encontrados, e apenas uma delas foi encontrada em um paciente classificado como disgenético. As variantes no *NR5A1* foram as mais frequentes nesse grupo de pacientes, previamente classificados como de etiologia indeterminada.

Variantes no *NR5A1* são uma das causas mais frequentes de DDS 46,XY, com espectro fenotípico variável, correspondendo a cerca de 10-15% dos pacientes em diversas coortes, a grande maioria sem insuficiência adrenal [120, 121]. Em nossa coorte, variantes alélicas neste gene estiveram associadas a fenótipos variados, desde disgenesia gonadal completa a hipospádia perineal isolada, a maioria (5 dos 6 pacientes) sem derivados Mullerianos[122]. Dessa maneira, o diagnóstico molecular propiciou a classificação adequada dos pacientes com disgenesia, orientando quanto ao seguimento adequado, especialmente em relação ao risco de desenvolvimento de tumor gonadal e o risco elevado de infertilidade futura.

Variantes classificadas como patogênicas ou possivelmente patogênicas no *DHX37* foram as mais frequentes nesta casuística, estando presentes em cinco casos esporádicos e em uma família, sendo a principal causa de disgenesia gonadal, especialmente de SRTE. Quatro dos seis casos esporádicos e uma entre as duas famílias de pacientes disgenéticos com diagnóstico molecular possuem uma variante diagnóstica no *DHX37*. Além disso, variantes neste gene, antes associadas apenas ao fenótipo de SRTE, foram também identificadas em pacientes com disgenesia gonadal

parcial, havendo variabilidade fenotípica inclusive em indivíduos de uma mesma família. Tais dados ratificam que a SRTE faz parte do espectro de apresentação de apresentação da disgenesia gonadal [113].

A grande parte dos pacientes com variantes no *DHX37* não possui derivados Mullerianos, independente do grau de virilização da genitália externa, sugerindo função residual das células de Sertoli durante o início do desenvolvimento testicular. A associação dessas variantes com indivíduos com genitália externa masculina e micropênis mostra também função preservada das células de Leydig até pelo menos 12 semanas de vida intraembrionária nestes indivíduos. Estes dados sugerem que o *DHX37* tem um papel importante não apenas na determinação, como também na manutenção testicular durante as fases precoces do desenvolvimento gonadal.

Embora o *DHX37* seja um gene que codifica uma proteína composta por 1.157 aminoácidos, todas as variantes identificadas estão localizadas em dois domínios muito conservados entre as diferentes espécies (o domínio de ligação ao ATP e o domínio helicase C-terminal). Todas as trocas de aminoácidos nestes domínios foram preditas como deletérias por diversas ferramentas de predição *in silico.*

Assim, o presente estudo fornece diversas linhas de evidências genéticas que indicam que variantes no *DHX37* estão associadas ao espectro da disgenesia gonadal 46,XY [114]. Primeiramente, as variantes segregam com o fenótipo com padrão de herança autossômico dominante na maior parte das famílias e duas variantes *de novo* (caso 21 deste estudo e o caso chinês-americano previamente reportado pelo grupo [70]). Ainda, fornecemos evidência estatística do tipo caso-controle ao verificar a frequência de variantes deletérias nos domínios conservados do *DHX37* foi significativamente maior entre os pacientes com disgenesia gonadal, especialmente no indivíduos com SRTE de toda a casuística em relação à dos bancos de dados populacionais (44% *versus* 0.4% e 0.2% nos bancos do gnomAD e ABraOM, respectivamente).

O *DHX37* emerge como um importante gene na etiologia disgenesia gonadal, sendo suas variantes deletérias responsáveis por uma frequência que segue à do *NR5A1* em toda a casuística da Unidade de Endocrinologia do Desenvolvimento da USP [121, 123] (12% e 10% dos pacientes com disgenesia gonadal, para o *NR5A1* e o *DHX37* respectivamente).

Nossos dados foram corroborados pelos achados de uma coorte de pacientes europeus, na qual foram identificadas as mesmas variantes p.Thr304Met (n=2), p.Arg308Gln (n=5), além de outra variante envolvendo troca de diferente aminoácido na mesma posição Arg674 (Arg674Gln; n=2) e mais outras quatro variantes distintas no *DHX37,* todas nestes mesmos domínios [124], em associação com fenótipo de disgenesia gonadal (11% da casuística de pacientes disgenéticos) e de SRTE (25% destes pacientes).

O gene *ESR2* se tornou candidato à etiologia dos DDS após a identificação por sequenciamento exômico global por Baere *et al* de duas variantes neste gene, a p.Asn181del em homozigose e a p.Gly84Val em heterozigose, em um paciente sindrômico com genitália externa feminina, sem derivados Mullerianos e em outro paciente com disgenesia gonadal parcial 46,XY, respectivamente [109]. Além disso, variantes alélicas polimórficas foram associadas à ocorrência de hipospádia [125-128]. A identificação de uma terceira variante no estudo atual, a p.Leu426Arg, motivou um estudo em colaboração com este grupo para avaliação funcional. Estudos prévios mostraram que há expressão do *ESR2* em gônadas embrionárias [111]. As variantes em questão estão presentes em baixa frequência ou ausentes em bancos de dados populacionais, estão localizadas em domínios importantes da proteína (domínio altamente conservado do ER-β de ligação ao DNA, domínio N terminal e domínio de ligação ao ligante, respectivamente) e são preditas como deletérias pelos algoritmos de predição *in silico.* A análise estrutural das variantes p.Asn181del e p.Leu426Arg demonstra haver impacto na conformação da proteína [129]. A avaliação do impacto na atividade de transcrição por ensaio da luciferase mostrou que houve aumento significativo da ativação, dependente do ligante, no caso da variante p.Asn181del, e independente do

ligante, para a variante p.Leu426Arg. Postula-se que o *ESR2* interaja com a via *MAPK,* que está envolvida na via da determinação sexual, através de via não genômica. Os receptores estrogênicos, como receptores nucleares, possuem uma via alternativa de ativação, independente de ligante, que envolve a interação com outros fatores de transcrição. Já foi mostrado que o ER-β pode ativar a via *MAPK* em células humanas do câncer colo retal. Assim, as variantes levariam a hiperativação da via alternativa do ER-β com consequente aumento da via da *MAPK,* alterando o balanço entre *SOX9* e β-catenina, resultado em hipoexpressão do *SOX9* e falência do desenvolvimento testicular, de forma similar ao que ocorre com as variantes ativadoras da *MAP3K1* que ocasionam fenótipo de disgenesia gonadal [129]. O *ESR2* emerge como gene associado a etiologia do DDS 46,XY. Nossa variante permanece como de significado incerto, por não ter sido possível realizar a segregação familiar.

O gene *CBX2* já está sabidamente associado à disgenesia gonadal e possui seus alvos e suas funções dentro da cascata da determinação testicular já estão estabelecidos [12, 80, 130]. No presente estudo, identificamos uma variante em heterozigose na isoforma 2 (*CBX2.2*), p.Cys154Alafs*62 em um paciente com disgenesia gonadal parcial. Outra variante nesta mesma isoforma p.Cys132Arg havia sido previamente identificada durante estudo de tese de doutorado da aluna Camila Gomes [131] em outro paciente com disgenesia gonadal parcial. A identificação dessas variantes motivou o estudo em colaboração com Biason-Lauber. Foram avaliados os possíveis alvos dessa isoforma através de ferramentas de avaliação de análise de ontologia gênica, e realizado o estudo funcional para avaliar o impacto dessas variantes nestes alvos, quantificando a expressão destes alvos por PCR em tempo real (RT-qPCR). Assim, as variantes falham na ativação dos alvos EMX2, HOXA13 e MAK, apresentando comportamento similar aos controles negativos e muito abaixo do controle com alelo selvagem, indicando o possível papel do *CBX2.2* na cascata da determinação sexual [132].

O sequenciamento paralelo em larga escala não só permitiu a identificação de novos genes como também de mais de uma variante em genes associados ao DDS 46,XY sugerindo que heranças oligogênicas poderiam ser a base de variações fenotípicas como as observadas no hipogonadismo hipogonadotrófico congênito [60, 62, 133] e na insuficiência ovariana prematura [85, 134].

Em cerca de 4% dos pacientes com DDS 46,XY da maior coorte previamente avaliada, foram identificadas variantes oligogênicas, sendo todos eles classificados como DDS de etiologia indeterminada (o que inclui aqueles pacientes com hipospádia isolada) [62]. Variantes no gene *AR* foram as mais comuns nesta coorte, em associação a defeitos em outros genes.

Na presente coorte, foram identificadas 10 variantes em cinco pacientes (5% dos pacientes) envolvendo mais de um gene relacionado ao DDS. Nossa casuística não incluiu pacientes com defeitos no gene *AR,* uma vez que estes foram previamente estudados pelo sequenciamento por Sanger. A maior parte dos defeitos digênicos foi identificada em genes associados à formação testicular.

Outros estudos também reportaram seus achados oligogênicos, embora em um número menor de pacientes, de forma a buscar variantes que justificassem os diferentes fenótipos em pacientes com variantes no *NR5A1* [135] e *GATA4* [117]. Postula-se que a baixa correlação genótipo-fenótipo relacionada às variantes do *NR5A1* e aos domínios em que se localizam possa se dever a variantes em outros genes moduladores do fenótipo. Mazen *et al* identificaram a variante c.C937T; p.Arg313Cys no *NR5A1*, *de novo,* em um paciente com disgenesia gonadal, com subvirilização importante da genitália. Esta mesma variante havia sido descrita em um paciente com hipospádia isolada [133]. A identificação de uma variante na *MAP3K1,* de origem materna, foi considerada como possível modulador do fenótipo, a despeito de ser predita como benigna pelo PolyPhen e não haver estudo funcional. Werner *et al* identificaram a variante p.Tyr211Thrfs*83 no *NR5A1* em um paciente com disgenesia gonadal

completa e em seu irmão com disgenesia gonadal parcial. A variabilidade fenotípica foi atribuída a uma variante no gene candidato *TBX2,* encontrada apenas no primeiro, de herança materna [136].

O paciente 77 desta coorte é portador da mutação p.Gly26Val no *NR5A1*, e da variante p.Ala492Pro de significado incerto do *DHX37*. A variante p.Gly26Val no *NR5A1* é nova, porém variantes localizadas no mesmo domínio da proteína, de ligação ao DNA, estão comumente associadas a quadro de subvirilização importante da genitália [137, 138].

Outro exemplo, todos os pacientes de nossa coorte que são carreadores da variante p.Arg674Trp no *DHX37* têm um fenótipo de SRTE, exceto o paciente 28 com disgenesia gonadal parcial e genitália externa atípica pouco virilizada, que também tem uma variante de significado incerto em heterozigose no *GATA4* .

Embora seja difícil provar o papel dessas diferentes variantes no fenótipo do DDS [117, 135], estudos em animais transgênicos mutantes para as variantes são necessários para comprovar uma relação de causa e efeito.

Embora na maior parte (75%) dos pacientes com DDS por provável defeito de síntese de andrógenos fossem identificadas variantes deletérias nos genes relacionados ao diagnóstico em questão, em uma paciente com fenótipo sugestivo de deficiência da 5ARD2 foi identificada uma variante patogênica no gene da *MAP3K1* associada a uma variante classificada como provavelmente benigna em heterozigose na *SDR5A2,* além do polimorfismo p.Val30Leu em homozigose neste mesmo gene. Este polimorfismo está associado ao decréscimo da atividade enzimática da 5-alfa redutase em 30% [139] e é provável que, por estar em homozigose, tenha contribuído para o fenótipo de deficiência da 5α- redutase tipo 2 .

As alterações de número de cópias (*copy number variations*, CNVs) são responsáveis por menos de 10% da etiologia genética dos pacientes não sindrômicos com DSD 46, XY e por um percentual maior de pacientes sindrômicos que foram estudados por hibridização genômica comparativa de matriz (aCGH) [25]. Sabe-se que o sequenciamento exômico global tem o

potencial de detectar CNVs nas regiões de codificação, mas um número limitado de métodos foi desenvolvido para a análise de CNVs em dados de sequenciamento paralelo em larga escala por painel de genes [60]. A aplicação do software CONTRA nos dados do painel e identificou uma duplicação no *CYP17A1* em um paciente com deficiência de 17α-hidroxilase, com genótipo prévio incompleto. Nenhuma outra alteração de número de cópias foi identificada nos demais pacientes da casuística.

O grande número de pacientes que ainda permanece sem um diagnóstico molecular, incluindo as famílias com fenótipo de hipoplasia das células de Leydig e de deficiência de 5 α- redutase tipo 2, indicam que mecanismos ou vias que não são avaliadas ou não foram suficientemente avaliadas por estas técnicas, tais como variantes não exônicas, variantes em genes ainda desconhecidos, além de fatores epigenéticos e ambientais, podem ser as causadoras dos fenótipos em questão.

7 Conclusões

7 CONCLUSÕES

1. O presente estudo representa a primeira coorte brasileira de pacientes com DDS 46,XY avaliados por SPLE. Foram encontradas variantes alélicas patogênicas ou possivelmente patogênicas em 20.5% dos pacientes.

2. Pacientes com DDS 46,XY não disgenético, classificados como DDS por provável defeito de síntese de andrógenos, tiveram os melhores resultados diagnósticos (75%), permanecendo a maior parte dos pacientes com DDS disgenético sem etiologia molecular, o que indica a presença de mecanismos ou vias que não foram avaliadas ou desconhecidas.

3. Os achados reforçam que variantes no *DHX37* são uma nova e frequente causa de DDS 46,XY disgenético, especialmente da SRTE e indicam que este gene participa não só da diferenciação como também na manutenção testicular.

8 Anexos

Anexo 1

HOSPITAL DAS CLÍNICAS DA FACULDADE DE MEDICINA DA UNIVERSIDADE DE SÃO PAULO-HCFMUSP

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

DADOS DE IDENTIFICAÇÃO DO SUJEITO DA PESQUISA OU RESPONSÁVEL LEGAL

1 NOME: .:.....

NOME DO PAI: .....
NOME DA MÃE: .....
DOCUMENTO DE IDENTIDADE No : ..... SEXO : .M □ F □
DATA NASCIMENTO: ...../...../.....
ENDEREÇO.....No.....APTO:.....
BAIRRO.....CIDADE.....
CEP:.....TELEFONE (.....) .....

2.RESPONSÁVEL LEGAL.....

NATUREZA (grau de parentesco, tutor,curador etc.).....
DOCUMENTO DE IDENTIDADE :.....SEXO: M □ F □
DATA NASCIMENTO.: ...../...../.....
ENDEREÇO: .....No ..... APTO: .....
BAIRRO:.....CIDADE:.....
CEP:.....TELEFONE:(.....).....

DADOS SOBRE A PESQUISA

1. TÍTULO DO PROTOCOLO DE PESQUISA

Estudo dos genes relacionados ao distúrbio do desenvolvimento sexual

PESQUISADOR : Berenie Bilharinho de Mendonça

CARGO/FUNÇÃO: Professora titular

INSCRIÇÃO CONSELHO REGIONAL No 20305

UNIDADE DO HCFMUSP: Endocrinologia

3. AVALIAÇÃO DO RISCO DA PESQUISA:

RISCO MÍNIMO ☐ RISCO MÉDIO ☐

RISCO BAIXO ☐ RISCO MAIOR ☐

4.DURAÇÃO DA PESQUISA : 2 anos

Rubrica do sujeito de pesquisa ou responsável_____ Rubrica do pesquisador_____

HOSPITAL DAS CLÍNICAS DA FACULDADE DE MEDICINA DA UNIVERSIDADE DE SÃO PAULO-HCFMUSP

Em qualquer etapa do estudo, você terá acesso aos profissionais responsáveis pela pesquisa para esclarecimento de eventuais dúvidas no endereço: LIM 42 do Hospital das Clinicas da Universidade de Sao Paulo, localicado na rua Eneas de Carvalho Aguiar, 255 Sao Paulo/SP.Telefone(s) 11 26617512. Se você tiver alguma consideração ou dúvida sobre a ética da pesquisa, entre em contato com o Comitê de Ética em Pesquisa (CEP) – Rua Ovídio Pires de Campos, 225 – 5o andar – tel: 2661-6442 ramais 16, 17, 18 – e-mail: cappesq@hcnet.usp.br.
É garantida a liberdade da retirada de consentimento a qualquer momento e deixar de participar do estudo, sem qualquer prejuízo à continuidade de seu tratamento na Instituição.
As informações obtidas serão analisadas em conjunto com outros pacientes, não sendo divulgado a identificação de nenhum paciente.

Rubrica do sujeito de pesquisa ou responsável_____ Rubrica do pesquisador_____

HOSPITAL DAS CLÍNICAS DA FACULDADE DE MEDICINA DA UNIVERSIDADE DE SÃO PAULO-HCFMUSP

Acredito ter sido suficientemente informado a respeito das informações que li ou que foram lidas para mim, descrevendo o estudo" Eu discuti com o Dr. Berenice B Mendonça sobre a minha decisão em participar nesse estudo. Ficaram claros para mim quais são os propósitos do estudo, os procedimentos a serem realizados, seus desconfortos e riscos, as garantias de confidencialidade e de esclarecimentos permanentes. Ficou claro também que minha participação é isenta de despesas e que tenho garantia do acesso a tratamento hospitalar quando necessário. Concordo voluntariamente em participar deste estudo e poderei retirar o meu consentimento a qualquer momento, antes ou durante o mesmo, sem penalidades ou prejuízo ou perda de qualquer benefício que eu possa ter adquirido, ou no meu atendimento neste Serviço.

Assinatura do paciente/representante legal Data / /

Assinatura da testemunha Data / /

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para casos de pacientes menores de 18 anos, analfabetos, semi-analfabetos ou portadores de deficiência auditiva ou visual.

Declaro que obtive de forma apropriada e voluntária o Consentimento Livre e Esclarecido deste paciente ou representante legal para a participação neste estudo.

Assinatura do responsável pelo estudo Data / /

Anexo 2 – Fenótipo dos pacientes com DDS 46,XY disgenético

Paciente	Sexo	IC avaliação	Genitália externa	Falo/ Clitoris (cm) DP	Orifícios perineais	Uretra	Localização das gônadas	Genitália Interna	Histologia das gônadas	T basal (ng/dL)	T pós hCG (ng/dL)	LH (U/L)	FSH (U/L)	Caso familial	Consanguinidade	Genes avaliados por Sanger
1	F	17	Feminina	Normal	2	Feminina	Abdominais	Feminina Completa	Disgenéticas	25*	ND	13	51	Não	Não	*DMRT1,FGF9, FGFR2*
2	F	22	Feminina	Normal	2	Feminina	Abdominais	Feminina Completa	Disgenéticas	<14*	ND	17	74	Não	Não	*SRY,NR5A1,DMRT1 GATA4,FGF9,FGFR2*
3	F	24	Feminina	Normal	2	Feminina	Abdominais	Feminina Completa	Disgenéticas	<14*	ND	9	41	Não	Sim	*SRY, DMRT1 GATA4,FGF9,FGFR2*
4	F	23	Feminina	Normal	2	Feminina	Abdominais	Feminina Completa	Disgenéticas	35*	ND	21	76	Não	Não	*SRY, DMRT1 GATA4,FGF9,FGFR2*
5	F	19	Feminina	Normal	2	Feminina	Abdominais	Feminina Completa	Disgenéticas	14	ND	14	75	Não	Não	*SRY,NR5A1,DHH, DMRT1 GATA4,FGF9,FGFR2*
6	F	21	Feminina	Normal	2	Feminina	Abdominais	Feminina Completa	Dsgenéticas	<14*	ND	11	38	Sim	Sim	*SRY*
7	F	45	Feminina	2,5cm	2	Feminina	Abdominais	Feminina Completa	Disgenéticas	<14*	14	14	57	Não	Não	*SRY,NR5A1,DMRT1 GATA4,FGF9,FGFR2*
8	F	13	Feminina	Normal	2	Feminina	Abdominais	Feminina Completa	Disgenéticas	38	ND	34	107	Não	Não	*SRY*
9	F	ND	Feminina	Normal	2	Feminina	Abdominais	Feminina Completa	Ausentes	ND	ND	11	42	Não	Não	*DMRT1 GATA4,FGF9,FGFR2*

continua

Anexo 2 – Fenótipo dos pacientes com DDS 46,XY disgenético (continuação)

Paciente	Sexo	IC avaliação	Genitália externa	Falo/ Clitoris (cm) DP	Orifícios perineais	Uretra	Localização das gônadas	Genitália Interna	Histologia das gônadas	T basal (ng/dL)	T pós hCG (ng/dL)	LH (U/L)	FSH (U/L)	Caso familial	Consanguinidade	Genes avaliados por Sanger
10	F	23	Feminina	Normal	2	Feminina	Abdominais	Feminina Completa	Disgenéticas	22	ND	22	64	Não	Não	*SRY,NR5A1,DHH, DMRT1 GATA4,FGF9,FGFR2*
11	F	15,5	Feminina	Normal	2	Feminina	Abdominais	Feminina Completa	Disgenéticas	<11	ND	25	103	Não	Não	-
12	F	25	Feminina	Normal	2	Feminina	Abdominais	Feminina Completa	Disgenéticas	55	ND	37	57	Não	Não	*SRY, FGF9, FGFR2,GATA4*
13	F	10,6	Feminina	Normal	2	Feminina	Abdominais	Ausente	Ausentes	22	ND	0,8	6,4	Não	Não	-
14	F	22	Feminina	Normal	2	Feminina	Abdominais	Feminina completa	Disgenéticas	<14*	ND	17	74	Não	Não	*DMRT1 GATA4, FGF9,FGFR2*
17	F	25	Feminina	Normal	2	Feminina	Ausentes	Feminina completa	Ausentes	<14*	ND	43	97	Sim (F1)	Sim	*SRY, NR5A1*
18	F	18	Feminina	Normal	2	Feminina	Abdominais	Feminina completa	ND	ND	ND	ND	ND	Sim (F2)	Não	*SRY, NR5A1*
19	F	56	Feminina	Normal	2	Feminina	Abdominais	Feminina completa	ND	ND	ND	ND	ND	Sim (F2)	Não	-
20	M	47	Atípica	8,5 cm (-3,0)	2	Perineal	Ausentes	Útero	Ausentes	25	ND	15	56	Sim (F2)	Não	SRY

continua

Anexo 2 – Fenótipo dos pacientes com DDS 46,XY disgenético (continuação)

Paciente	Sexo	IC avaliação	Genitália externa	Falo/ Clitoris (cm) DP	Orifícios perineais	Uretra	Localização das gônadas	Genitália Interna	Histologia das gônadas	T basal (ng/dL)	T pós hCG (ng/dL)	LH (U/L)	FSH (U/L)	Caso familial	Consanguinidade	Genes avaliados por Sanger
21	F	7,7	Feminina	Normal	2		Abdominais	Ausentes	Disgenéticos	16	ND	<0,1	4,9	Não	Não	-
22	F	1	Atípica	2cm	1	Perineal	Grandes lábios	Útero	Testículos infantis	25	ND	30	88	Não	Não	*SRD5A2*
24	M	23	Atípica	8cm (-3,3)	1	Perineal	Inguinais	Útero	Disgenéticos Tumor de cel. Germ	420	ND	34	72	Não	Não	*NR5A1, SRY, DMRT1, GATA4, FGF9, FGFR2*
23	M	34	Atípica	5cm (-5,2)	1	Perineal	Abdominais	Útero hipoplásico	Ausentes	75	120	10	78	Não	Sim	*NR5A1, SRY, DMRT1, GATA4, FGF9, FGFR2*
24	F	43	Atípica	Cirurgia prévia	2	-	Abdominais	Útero	Disgenéticos	324	ND	32	52	Não	Não	*GATA4, FGF9, FGFR2 SRY, NR0B1, DMRT1,*
25	M	4	Atípica	Cirurgia prévia	1	-	Tópico (E) Ausente (D)	Útero	Disgenéticos	42	493	ND	ND	Não	Não	*SRY, DMRT1,GATA4 FGF9, FGFR2*
26	M	1,7	Atípica	2cm (-3,3)	1	Tópica	Inguinal (D) Ausente (E)	Ausentes	Ausentes	<14	211	ND	ND	Não	Não	*AR*
27	F>M (17anos)	27	Atípica	Cirurgia prévia	Cirurgia prévia	Cirurgia prévia	Cirurgia prévia	Útero	Testículos infantis (aos 2 anos)	ND	ND	ND	ND	Não	Não	*AR*

continua

Anexo 2 – Fenótipo dos pacientes com DDS 46,XY disgenético (continuação)

Paciente	Sexo	IC avaliação	Genitália externa	Falo/ Clitoris (cm) DP	Orifícios perineais	Uretra	Localização das gônadas	Genitália Interna	Histologia das gônadas	T basal (ng/dL)	T pós hCG (ng/dL)	LH (U/L)	FSH (U/L)	Caso familial	Consanguinidade	Genes avaliados por Sanger
28	F	3,7	Atípica	3cm	2	Perineal	Abdominais	Útero	Disgenéticos	25	337	ND	ND	Não	Não	*GATA4, FGF9, FGFR2 NR5A1, SRY, DMRT1*
29	M	7,4	Atípica	4,5cm (-1,7)	1	Perineal	Abdominais	Ausentes	Disgenéticos	<14*	<14*	ND	ND	Não	Não	*NR5A1, SRY, NR0B1, DMRT1 GATA4, FGF9, FGFR2*
30	M	16	Atípica	Cirurgia prévia	Cirurgia prévia	Cirurgia prévia	Cirurgia prévia	Útero	ND	ND	ND	ND	ND	Não	Não	*NR5A1, SRY, DMRT1, GATA4, FGF9, FGFR2*
31	F	1,8	Atípica	3,5cm	1	Perineal	Abdominais	Útero	ND	14*	107	<0,6	2,7	Não	Não	*GATA4, FGF9, FGFR2 NR5A1, SRY, NR0B1, DMRT1*
32	M	7	Masculina	3cm (-3,2)	1	Tópica	Ausentes	Ausentes	Disgenéticos	ND	ND	ND	ND	Sim	Não	-
33	M	5	Atípica	4,2cm (-2,0)	1	Perienal	Inguinal (E) Tópica (D)	Útero	ND	<14	509	<0,6	<1,0	Não	Não	*SRY, DMRT1, GATA4, FGF9, FGFR2*
34	M	4,3	Atípica	2,3cm (-3,5)	1	Perineal	Abdominais	Útero	Disgenéticos	<14	189	<0,6	3	Não	Não	*SRY*

continua

Anexo 2 – Fenótipo dos pacientes com DDS 46,XY disgenético (continuação)

Paciente	Sexo	IC avaliação	Genitália externa	Falo/ Clitoris (cm) DP	Orifícios perineais	Uretra	Localização das gônadas	Genitália Interna	Histologia das gônadas	T basal (ng/dL)	T pós hCG (ng/dL)	LH (U/L)	FSH (U/L)	Caso familial	Consanguinidade	Genes avaliados por Sanger
35	M	0,1	Atípica	2cm (-3,75)	1	Perineal	Abdominais	ND	Ausentes	310	ND	ND	ND	Não	Não	*SRY, DMRT1, GATA4, FGF9, FGFR2*
36	F	18.9	Atípica	Cirurgia prévia	1	Cirurgia prévia	Abdominais	Ausentes	Disgenético (D) Ausente (E)	ND	ND	ND	ND	Sim (F3)	Não	*DMRT1, GATA4, FGF9, FGFR2*
37	M	9	Masculina	3,4 cm (-2,9)	1	Tópica	Ausentes	Ausentes	Ausente	<14	ND	19	43	Sim (F3)	Não	*SRY, DMRT1, GATA4, FGF9, FGFR2*
38	M	35	Masculina	12cm** (-0,8)	1	Tópica	Ausentes	Ausentes	Ausente	219 **	ND	18	43	Não	Não	-
39	M	25	Masculina	6,2cm (-4,4)	1	Tópica	Ausentes	Ausentes	Ausente	14*	17	18	44	Não	Não	-
40	M	15,7	Masculina	5cm (-5,2)	1	Tópica	Ausentes	Ausentes	Ausente	10	ND	46	90	Não	Não	-
41	M	0.1	Masculina		1	Tópica	Ausentes	Ausentes	Ausente	38	40	<0.5	NA	Sim	Não	-
42	M	4	Masculina	4,5cm (-2,0)	1	Tópica	Ausentes	Ausentes	Ausente	<10	ND	10	104	Não	Não	*SRY*

continua

Anexo 2 – Fenótipo dos pacientes com DDS 46,XY disgenético (conclusão)

Paciente	Sexo	IC avaliação	Genitália externa	Falo/ Clitoris (cm) DP	Orifícios perineais	Uretra	Localização das gônadas	Genitália Interna	Histologia das gônadas	T basal (ng/dL)	T pós hCG (ng/dL)	LH (U/L)	FSH (U/L)	Caso familial	Consanguinidade	Genes avaliados por Sanger
43	M	5	Masculina	ND	1	Tópica	Ausentes	Ausentes	Ausentes	ND	ND	ND	ND	ND	ND	*NR5A1, NR0B1, GATA4, FGF9, FGFR2*
44	M>F (17 anos)	30	Masculina	5,5cm (-4,8)	1	Tópica	Ausentes	Ausentes	Ausente	29	ND	10	40	Não	Não	*AR*
45	M	37	Masculina	8cm (-3,3)	1	Tópica	Ausentes	Ausentes	Ausente	ND	ND	ND	ND	ND	ND	*SRY*
46	M	20	Masculina	6,5cm (-4,25)	1	Tópica	Ausentes	Ausentes	Ausente	24	ND	21	45	Não	Não	-
47	M	7	Masculina	3cm (-3,2)	1	Tópica	Ausentes	Ausentes	Ausente	<14*	ND	<0,6	<1,0	Não	Não	-
48	M	21	Masculina	3,9cm (-5,8)	1	Tópica	Ausentes	Ausentes	Ausente	<14	ND	15	46	Não	Não	-

Legenda: Idade cronológica, IC; Feminino, F; Masculino, M; Não disponível, ND.

Anexo 3 - Fenótipo dos pacientes com DDS 46,XY de etiologia indeterminada

Paciente	Sexo	IC da avaliação	Clitoris/ Falo (DP)	Uretra	Localização das gônadas	T basal (ng/dL)	LH (U/L)	FSH (U/L)	T pós hCG (ng/dL)	T/DHT	T/A	Caso familial (número da família)	Consanguinidade	Genes avaliados previamente
49	M	4	5cm (-0,5)	Perineal	Inguinais	<11	0,3	2,6	ND	ND	ND	Não	Não	*SRY*
50	M	1,3	4 (-0,87)	Perineal	Inguinais	<11	<0,1	<1,0	342	5,5	6	Não	Sim	*AR*
51	M	1,9	3,3 (-1,0)	Perineal	Inguinais	<14	<0,6	<1,0	315	21	3,7	Não	Não	*AR, NR5A1*
52	M	**13**	4,2 (-5,6)	Cirurgia prévia	Inguinais	101	0,8	1,8	ND	ND	1,7	Não	Não	*AR, NR5A1*
53	F	30	Cirurgia prévia	Cirurgia prévia	Cirurgia prévia	ND	ND	ND	ND	ND	ND	Não	Não	*AR, NR5A1*
54	M	3,1	3,3cm (-2,6)	Glandar	Retráteis	48*	<0,6	<1,0	336	8,4	5,6	Não	Não	*AR, NR5A1*
55	F	0,2	1,5	Perineal	Grandes lábios	<10	3	12	183	1,66	9,2	Não	Não	*AR, NR5A1, MAMDL1, NR0B1*
56	F>M (32 anos)	32	Cirurgia prévia	Cirurgia prévia	Cirurgia prévia	ND	ND	ND	ND	ND	ND	Não	Não	*AR, NR5A1*
57	M	23	6cm (-4,2)	Perineal	Cirurgia prévia	20	52	43	ND	ND	ND	Não	Sim	*LHR*
58	F	0,2	1 cm	Tópica	Grandes lábios	37	ND	ND	86	ND	ND	Não	Não	*AR, NR5A1, SRD5A2, SRY, HSD17B3,*
59	F>M (40 anos)	5	Cirurgia prévia	Cirurgia prévia	Cirurgia prévia	ND	ND	ND	ND	ND	ND	Não	Não	*AR*

continua

Anexo 3 - Fenótipo dos pacientes com DDS 46,XY de etiologia indeterminada (continuação)

Paciente	Sexo	IC da avaliação	Clitoris/ Falo (DP)	Uretra	Localização das gônadas	T basal (ng/dL)	LH (U/L)	FSH (U/L)	T pós hCG (ng/dL)	T/DHT	T/A	Caso Familial	Consanguinidade	Genes avaliados previamente
60	M	22	9,5cm (-2,5)	Perineal	Tópicas	674	5,5	4,2	ND	12	ND	Não	Não	*AR*
61	M	1	ND	Perineal	ND	ND	ND	ND	ND	ND	ND	Não	Não	*NR5A1, AR*
62	M	2,3	4,5cm (-0,7)	Perineal	Retratéis	<11	<0,6	<1,0	174	ND	4,4	Não	Não	*AR*
63	M	2	5cm (-0,1)	Perineal	Tópicas	ND	ND	ND	ND	ND	ND	Não	Não	*SRY*
64	M	12	5cm (-1,27)	Tópica	Cirurgia prévia	ND	ND	ND	ND	ND	ND	Não	Sim	-
65	F	18	Cirurgia prévia	Cirurgia prévia	Cirurgia prévia	ND	ND	ND	ND	ND	ND	Não	Sim	*NR5A1, AR, HSD17B3*
66	M	0,1 2,2	2,2cm (-3,25)	Perineal	Inguinais	250	0,3	6	ND	29	8	Não	Não	-
67	F	25	2cm	Perineal	Inguinais	13	6,6	<2,0	146	17	ND	Não	Não	*AR, NR5A1*
68	F	25	Cirurgia prévia	Cirurgia prévia	Cirurgia prévia	ND	ND	ND	ND	ND	ND	Não	Sim	*AR, NR5A1*
69	M	7	5,5cm (-0,7)	Perineal	Inguinais	<11	<0,6	<1,0	193	11	19,3	Não	Não	-
70	M	1,8	4,5cm (-0,25)	Perineal	Inguinal (D) Tópica (E)	<11	1	4,4	496	2,4	12,4	Não	Não	*AR*
71	M	12,9	6cm (-0,45)	Perineal	Criptorquidia bilateral	619	3,3	7	ND	ND	ND	Não	Não	*AR*
72	M	12	7cm (-3,9)	Corpo peniano	Tópicas	<14	<0,6	<1,0	747	4,1	6,8	Não	Não	-

continua

Anexo 3 - Fenótipo dos pacientes com DDS 46,XY de etiologia indeterminada (continuação)

ID	sexo	IC da avaliação	Clitoris/ Falo (DP)	Uretra	Localização das gônadas	T basal (ng/dL)	LH (U/L)	FSH (U/L)	T pós hCG (ng/dL)	T/DHT	T/A	Caso familial	Consanguinidade	Genes avaliados previamente
73	M	23	7cm (-4,7)	Perineal	Criptorquidia bilateral	880	14,6	5,1	ND	ND	ND	Não	Não	*AR*
74	M	1	1,5cm (-3,5)	Perineal	Tópicas	<11	0,2	1,2	202	11,9	4	Não	Sim	*AR, SRY*
75	M	0,2	3cm (-3,5)	Perineal	Tópicas	ND	ND	ND	ND	ND	ND	Não	Não	-
76	M	0,2	3cm (-1,12)	Perineal	Tópicas	319	3,8	2,3	ND	4	6,3	Não	Não	*AR, SRY*
77	M	0,3	3cm (-1,2)	Perineal	Tópica (E) Inguinal (D)	350	ND	ND	ND	12,5	ND	Não	Não	-
78	M	6	3,5 (-2,0)	Tópica	Inguinais	<12	0,1	2,5	ND	ND	ND	Não	Não	-
79	M	11	3cm (-3,0)	Perineal	Tópicas	<12	<0,1	0,9	ND	ND	ND	Não	Sim	-
80	M	0,1	2,6cm (-1,6)	Perineal	Tópicas	36	4,6	10,3	ND	ND	ND	Não	Não	-
81	M	0,4	3cm (-1,1)	Perineal	Tópicas	<12	1,4	1,7	ND	ND	ND	Não	Não	-
82	M	0,8	3,5cm (-1,0)	Corpo peniano	Tópicas	<12	ND	ND	ND	ND	ND	Não	Não	-
83	M	0,1	3,5cm 0	Perineal	Tópicas	157	2	7,6	ND	ND	3,1	Não	Não	-

continua

Anexo 3 - Fenótipo dos pacientes com DDS 46,XY de etiologia indeterminada (continuação)

ID	sexo	IC da avaliação	Clitoris/ Falo (DP)	Uretra	Localização das gônadas	T basal (ng/dL)	LH (U/L)	FSH (U/L)	T pós hCG (ng/dL)	T/DHT	T/A	Caso familial	Consanguinidade	Genes avaliados previamente
84	M	0,7	3,0cm (-1,6)	Perineal	Tópicas	<12	0,4	<0,6	ND	ND	ND	Não	Não	-
85	M	14	4,5cm (-1,72)	Perineal	Tópicas	31	1,3	3,9	ND	ND	ND	Não	Não	-
86	M	0,3	3,2cm (-1,87)	Perineal	Tópicos	394	ND	ND	ND	ND		Não	Não	*CYP21A2*
87	M	0,1	2,8cm (-1,3)	Perineal	Inguinal (D) Tópico (E)	510	ND	ND	785	5,3	7,2	Não	Não	*NR5A1, AR*
88	M	5,9	3cm (-3,3)	Perineal	Tópicos	<11	ND	ND	512	7,5	51	Não	Não	*NR5A1, AR*
89	M	3,9	4,8cm (-0,7)	Perineal	Inguinais	<11	2,8	3,7	352	14	ND	Não	Não	*NR5A1, AR*
90	M	6,7	2cm (-4,5)	Perineal	Tópicas	<11	1	1,7	248	4,13	ND	Não	Não	*NR5A1, AR*
91	M	1,5	2cm (-3,3)	Perineal	Tópicas	<14	<1,0	<0,6	676	ND	9,6	Não	Não	*NR5A1, AR*
92	M	3,7	2cm (-3,8)	Perineal	Tópicas	<14	ND	ND	415	11,8	2	Não	Não	*NR5A1, AR*
93	M			Perineal	Tópicas	ND	ND	ND	ND	ND	ND	Não	Não	-
94	M	6,4	4,2cm (-2,0)	Perineal	Tópicas	<14	ND	ND	168	5,7	3,4	Não	Não	-
95	M	7,4	3,5cm (-2,7)	Perineal	Tópicas	418	3,3	6,4	ND	2,24	4,6	Não	Não	*AR*

continua

Anexo 3 - Fenótipo dos pacientes com DDS 46,XY de etiologia indeterminada (Conclusão)

ID	sexo	IC da avaliação	Clitoris/ Falo (DP)	Uretra	Localização das gônadas	T basal (ng/dL)	LH (U/L)	FSH (U/L)	T POS HCG (ng/dL)	T/DHT	T/A	Caso familial (número da família)	Consanguinidade	Genes avaliados previamente
96	M	14,9	8,5cm (-3,2)	Corpo peniano	Tópicos	<14	<0,6	<1,0	92	ND	ND	Não	Não	-
97	M	0,6	2cm (-2,8)	Perineal	Tópicos	<12	0,2	<1,0	637	13	2,1	Não	Não	*AR, NR5A1*
98	M	1	3,5cm (-4,1)	Perineal	Tópicos	<14	<0,6	<1,0	366	1,9	7,3	Não	Não	*AR*
99	F	28	ND	Feminina	Abdominais	ND	ND	ND	ND	ND	ND	Não	Sim	NR5A1, AR
100	M	0,1	1,5cm (-5,0)	Perineal	Inguinal (D) Tópca (E)	38	<0,6	4,7	ND	ND	ND	Sim (F5)	Não	*AR, LH*
101	F>M (17 anos)	17	4,7cm (-5,3)	Perineal	Criptorquidia bilateral	148	35	48	137	4,7	1,34	Sim (F6)	Não	*AR*
102	M	1,3	3,8cm (-1,1)	Perineal	Inguinais	<11	<1,0	<0,1	125	ND	ND	Sim (F6)	Não	-
103	M	0,3	3,5cm (-0,5)	Perineal	Tópicos	162	2,4	2,4	1031	5	20	Sim (F5)	Não	-
104	M	9	5,5cm (-0,8)	Perineal	Inguinais	79	<1,0	<0,1	83	ND	ND	Sim (F5)	Não	-
105	F>M (15 anos)	15	6cm	Perineal	Inguinais	626	7,4	13	756	19	0,94	Não	Sim	*SRD5A2, AR, NR5A1*

Legenda: Feminino, F; Masculino, M; Não disponível, ND; Testosterona, T; Diidrostestosterona, DHT; Androstenediona, A; Direito, D; Esquerdo, E

Anexo 4 - Fenótipo dos pacientes com DDS 46,XY com provável defeito de síntese de andrógenos

Paciente	Sexo	Idade cronológica	Clitóris/ Falo (DP)	Orifícios perineais	Uretra	Localização das gônadas	T basal (ng/dL)	LH (U/L)	FSH (U/L)	T POS HCG (ng/dL)	T/DHT	T/A	Caso familial (número da família)	Consanguinidade	Genes avaliados previamente
106	F	17	Feminina	2	-	Abdominais	<11	19	27,4	ND	ND	ND	Sim	Não	*CYP17A1*
107	F	12	4cm	1	Perineal	Abdominais	378	17	41	ND	44,4	1,68	Não	Não	*SRD5A2*
108	F	17,7	3,8	2	-	Inguinais	451	7,8	23	647	21	10,6	Não	Não	-
109	M	0,1	2cm (-3,75)	1	Perineal	Tópicos	317	0,4	1,3	ND	28,8	ND	Não	Não	-
110	F	0,1	1,5cm	1	Perineal	Inguinais	406	7,4	ND	ND	21	0,72	Não	Não	-
111	F	18	7cm	1	Perineal	Inguinais	272	17,5	29,6	ND	3,83	0,27	Não	Não	-
112	F	24	<1.8cm	1	Perineal	Inguinais	<14	50	48	NA	NA	NA	Sim (F7)	Sim	*LHCGR*
113	F	0,1	1cm	1	Perineal	Abdominais	ND	ND	ND	ND	ND	ND	Sim (F4)	Sim	*SRD5A2*
114	F	1	Clitoris hipertrofiado	1	Perineal	Inguinais	<14	ND	ND	224	56	4,5	Sim (F4)	Sim	*SRD5A2*

Legenda: Feminino, F; Masculino, M; Não disponível, ND; Testosterona, T; Diidrostestosterona, DHT; Androstenediona, A;

Anexo 5

Sites de predição utilizados neste estudo

Ferramentas	**Critérios avaliados**	**Classificação**	**Critérios patogênicos**
dbscSNV_ADA_SCORE dbscSNV_RF_SCORE	Avalição de *splicing*	Entre 0 e 1	≥0,6
SIFT	Baseada no grau de conservação da sequência proteica	T: tolerado; D: deletério	D
CADD	Avalia conservação, dados funcionais e análise de outras ferramentas *in silico* (como SIFT e PolyPHEN0	C-scores ≥ representa variantes entre as 10% mais deletérias; ≥20 representa entre as 1% mais deletérias; critérios sugerido ≥15	≥15
Mutation Acessor	Baseada no grau de conservação da sequência proteica	L: efeito leve; M: moderado; H:alto	M e H
Mutation Taster	Combina informações de banco de dados bioquímicos e utiliza teorema de Bayes	N e P: não deletério/ polimorfismo A e D: deletério	A e D
Polyphen2	Efeito das variantes sobre sequências e estruturas proteicas	B: benigno; P: possivelmente patogênico; D: deletério	P e D
GERP	Avalia conservação do aminoácido envolvido	Numérica, variando de -12.3 a 6.17	≥ 2

Fonte: Adaptado de Richards e colaboradores, 2015(103).

Anexo 6

Tabela com os critérios utilizados para qualificar as variantes alélicas segundo o *American College of Medical Genetics* (ACMG)

Critério muito forte para patogenicidade	
PVS1	Variante *nonsense, frameshift*, em sítios de *splice* canônicos ±1 ou 2 códons de iniciação ou deleção de 1 ou vários exons (*null variant*) em um gene no qual variantes com perda de função (LoF) são mecanismos conhecidos de doença
Critérios fortes para patogenicidade	
PS1	Troca de aminoácido já estabelecida como patogênica, independente da troca do nucleotídeo
PS2	Variante de novo em um paciente com doença sem história familiar (com paternidade confirmada)
PS3	Presença de estudos funcionais (*in vitro* ou *in vivo*) bem estabelecidos que suportem o efeito prejudicial no gene ou sua prote.na
PS4	A prevalência da variante nos indivíduos afetados é significativamente maior quando comparada com a prevalência em controles
Critérios moderados para patogenicidade	
PM1	Variante localizada em uma região *hotspot* para mutações e/ou região importante já estabelecida com um domínio funcional da proteína, sem variantes descritas como benignas
PM2	Variante ausente nos bancos de dados populacionais (1000Genomes, ExAC), ou presente em frequência extremamente baixa, para os casos recessivos
PM3	Variante detectada em *trans* com uma variante patogênica para as doenças recessivas
PM4	Modificação no tamanho da proteína causada por deleções ou inserções *in-frame* em região não repetitiva ou por variantes que perdem o códon de parada (tipo *stop-loss*)
PM5	Nova variante *missense* em um aminoácido em que uma variante *missense* diferente já foi estabelecida como patogênica
PM6	Variante considerada *de novo*, sem confirmação da paternidade
Critérios fracos para patogenicidade	
PP1	Segregação da variante (em um gene conhecido como causador de doença) com a doença em uma família com vários membros afetados
PP2	Variante *missense* em um gene com uma baixa taxa de mutações *missense* benignas e no qual esse tipo de variante. considerado um mecanismo comum de doença
PP3	Várias ferramentas computacionais suportam a evidência de efeito deletério no gene ou proteína (conservação evolutiva, impacto no s*plicing* etc)
PP4	O fenótipo do paciente ou a história da família são bastante especíicos de uma doença monogênica
PP5	Variante recém-publicada como patogênica, porém o laboratório não apresenta condições de fazer um estudo funcional independente
Critérios fracos para benignidade	
BP1	Variante *missense* em um gene no qual variantes com perda de função (*LoF*) são mecanismos conhecidos de doença
BP2	Variante detectada em *trans* com uma variante patogênica dominante com penetrância completa ou observada em *cis* com uma variante patogênica em qualquer tipo de herança
BP3	Deleções ou inserções *in-frame* em região repetitiva sem função conhecida
BP4	Várias ferramentas computacionais sugerem ausência de efeito deletério no gene ou proteína (conservação evolutiva, impacto no *splicing* etc)
BP5	Variante encontrada em um caso com uma base molecular alternativa para a doença
BP6	Variante recém publicada como benigna, porém o laboratório não apresenta condições de fazer um estudo funcional independente
BP7	Variante sinônima cujas ferramentas de predição de *splicing* não predizem impacto na sequência consenso de *splice* nem a criação de um novo sítio, e o nucleotídeo não conservado
Critérios fortes para benignidade	
BS1	A frequência do alelo é maior do que a esperada para a doença
BS2	Variante para doença recessiva (em homozigose), dominante (em heterozigose) ou ligada ao X (hemizigose), com penetrância completa e de início precoce, observada em indivíduo adulto saudável
BS3	Presença de estudos funcionais (*in vitro* ou *in vivo*) bem estabelecidos mostrando ausência de efeito prejudicial para a proteína ou *splicing*
BS4	Ausência de segregação nos indivíduos afetados da família
Critério que definem benignidade por si só	
BA1	Frequência alélica maior que 5% nos bancos de dados populacionais (1000Genomes, ExAC)

Fonte: Adaptado de Richards e colaboradores, 2015(103). PVS: critério muito forte para patogenicidade; PS: critério forte para patogenicidade; PM: critério moderado para patogenicidade; PP: critério fraco para patogenicidade; BP: critério fraco para benignidade; BS: critério forte para benignidade; BA: critério benigno por si só. *Lof*: perda de função (de *loss of function*); 1000Genomes:1*000Genomes*; Project; ExAC: *Exome Aggregation Consortium*.

Anexo 7

Tabela com a classificação das variantes alélicas de de acordo com a combinação dos critérios do ACMG

Classificação de acordo com a combinação dos critérios ACMG
Variante patogênica (I) 1 critério muito forte para patogenicidade (PVS1) e (a) ≥ 1 critérios fortes para patogenicidade (PS1-PS4) ou (b) ≥ 2 critérios moderados para patogenicidade (PM1-PM6) ou (c) 1 critério moderado para patogenicidade (PM1-PM6) e 1 crit.rio fraco para patogenicidade (PP1-PP5) ou (d) ≥ 2 critérios fracos para patogenicidade (PP1-PP5) ou (II) ≥ 2 critérios fortes para patogenicidade (PS1-PS4) ou (III) 1 critério forte para patogenicidade (PS1-PS4) e (a) ≥ 3 critérios moderados para patogenicidade (PM1-PM6) ou (b) 2 critérios moderados para patogenicidade (PM1-PM6) e ≥ 2 critérios fracos para patogenicidade (PP1-PP5) ou (c) 1 critérios moderados para patogenicidade (PM1-PM6) e ≥ 4 critérios fracos para patogenicidade (PP1-PP5)
Variante provavelmente patogênica I) 1 critério muito forte para patogenicidade (PVS1) e 1 critério moderado para patogenicidade (PM1-PM6) ou (II) 1 critério forte para patogenicidade (PS1-PS4) e 1 ou 2 critérios moderado para patogenicidade (PM1-PM6) ou (III) 1 critério forte para patogenicidade (PS1-PS4) e ≥ 2 critérios fracos para patogenicidade (PP1-PP5) ou (IV) ≥ 3 critérios moderados para patogenicidade (PM1-PM6) ou (V) 2 critérios moderados para patogenicidade (PM1-PM6) e ≥ 2 crit.rios fracos para patogenicidade (PP1-PP5) ou (VI) 1 critério moderado para patogenicidade (PM1-PM6) e ≥ 4 crit.rios fracos para patogenicidade (PP1-PP5)
Variante de significado incerto (I) outra combinação de critérios não mencionada acima ou (II) critérios para benignidade e patogenicidade contraditórios
Variante provavelmente benigna (I) 1 critério forte para benignidade (BS1-BS4) e 1 critério fraco para benignidade (BP1-BP7) ou (II) ≥ 2 critérios fracos para benignidade (BP1-BP7)
Variante benigna (I) 1 critério benigno por si só (BA1) ou (II) ≥ 2 critérios fortes para benignidade (BS1-BS4)

Fonte: Adaptado de Richards e colaboradores, 2015 [103]. PVS: critério muito forte para patogenicidade; PS: critério forte para patogenicidade; PM: critério moderado para patogenicidade; PP: critério fraco para patogenicidade; BP: critério fraco para benignidade; BS: critério forte para benignidade; BA: critério benigno por si só.

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10 Apêndices

PUBLICAÇÕES

Apêndice 1

Evilen da Silva T, **Gomes NL,** Lerário AM, Keegan CE, Nishi MY, Carvalho FM, et al. Genetic evidence of the association of DEAH-box helicase 37 defects with 46,XY gonadal dysgenesis spectrum. J Clin Endocrinol Metab. 2019.

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Genetic evidence of the association of DEAH-box helicase 37 defects with 46,XY gonadal dysgenesis spectrum

Thatiana Evilen da Silva PhD, Nathalia Lisboa Gomes MD, Antonio Marcondes Lerário MD, Catherine Elizabeth Keegan MD, Mirian Yumi Nishi PhD, Filomena Marino Carvalho MD, Eric Vilain MD, Hayk Barseghyanm PhD, Alejandro Martinez-Aguayo MD, María Verónica Forclaz MD, Regina Papazian MD, Leila Cristina Pedroso de Paula MD, Eduardo Corrêa Costa MD, Luciani Renata Carvalho MD, Alexander A Jorge MD, Felipe Elias MS, Rod Mitchell, MBChB, PhD, Elaine Maria Frade Costa MD, Berenice Bilharinho Mendonca MD, Sorahia Domenice MD

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DHX37 defects in 46,XY Gonadal Dysgenesis

Genetic evidence of the association of DEAH-box helicase 37 defects with 46,XY gonadal dysgenesis spectrum

Thatiana Evilen da Silva PhD[1*], Nathalia Lisboa Gomes MD[1*], Antonio Marcondes Lerário MD,[2,3] Catherine Elizabeth Keegan MD,[4,5] Mirian Yumi Nishi PhD,[1] Filomena Marino Carvalho MD,[6] Eric Vilain MD,[7] Hayk Barseghyanm PhD,[7] Alejandro Martinez-Aguayo MD,[8] María Verónica Forclaz MD,[9] Regina Papazian MD[9], Leila Cristina Pedroso de Paula MD[10], Eduardo Corrêa Costa MD,[10] Luciani Renata Carvalho MD,[1] Alexander A Jorge MD[1], Felipe Elias MS[1], Rod Mitchell, MBChB, PhD[11], Elaine Maria Frade Costa MD,[1] Berenice Bilharinho Mendonca MD,[1,2*] Sorahia Domenice MD,[1*]

[1]*Unidade de Endocrinologia do Desenvolvimento, Laboratório de Hormônios e Genética Molecular (LIM/42) da Disciplina de Endocrinologia e Metabologia do Hospital das Clínicas da Faculdade de Medicina, Universidade de São Paulo, São Paulo, Brasil*

[2]*Laboratório de Sequenciamento em Larga Escala (SELA), Faculdade de Medicina da Universidade de São Paulo, São Paulo, Brasil*

[3]*Division of Metabolism, Endocrinology and Diabetes, Department of Internal Medicine, University of Michigan, Ann Arbor, MI, USA*

[4]*Department of Pediatric Genetics, University of Michigan Medical School, Ann Arbor, MI, USA*

[5]*Department of Human Genetics, University of Michigan Medical School, Ann Arbor, MI, USA*

[6]*Departamento de Patologia, Faculdade de Medicina, Universidade de São Paulo, São Paulo, SP, Brasil*

[7]*Center for Genetic Medicine Research, Children's National Health System, Children's Research Institute, Children's National Medical Center, Washington, DC, USA*

[8]*Division de Pediatria, Escuela de Medicina, Pontificia Universidad Catolica de Chile, Santiago, Chile*

[9]*Servicio de Pediatría, Hospital Nacional Prof. Dr. A. Posadas, Buenos Aires, Argentina*

[10]*Hospital de Clínicas de Porto Alegre, Programa de Atendimento às Desordens do Desenvolvimento Sexual (PADS), Brasil, UFRGS*

[11]*MRC Centre for Reproductive Health, Queens Medical Research Institute, Edinburgh, UK*

ORCiD numbers:

0000-0002-1072-7903

Gomes

Nathalia Lisboa

0000-0001-7071-0225

Evilen da Silva

Thatiana

0000-0002-8336-6432

Lerário

Antonio Marcondes

0000-0002-7780-6542

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Keegan

Catherine Elizabeth

0000-0001-6603-3188

Nishi

Mirian Yumi

0000-0002-5838-3636

Carvalho

Filomena Marino

0000-0002-5557-3709

Vilain

Eric

0000-0002-1677-3513

Martinez-Agueyo

Alejandro

0000-0002-7313-2130

Carvalho

Luciani Renata

0000-0003-3739-4755

Elias

Felipe

0000-0003-4650-3765

Mitchell

Rod

0000-0003-2567-7360

Jorge

Alexander Augusto de Lima

0000-0003-1762-1084

Mendonca

Berenice B

0000-0002-4883-7630

Domenice

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*First authors da Silva, TE and Gomes, NL and last authors Mendonca, BB and Domenice, S contributed equally to this article.

Context:46,XY gonadal dysgenesis (GD) is a heterogeneous group of disorders with a wide phenotypic spectrum, including embryonic testicular regression syndrome (ETRS). Objective: To report a novel gene for 46,XY GD etiology, especially for ETRS. Design: Screening of familial cases of 46,XY GD using WES and sporadic cases by target gene panel sequencing. Setting: Tertiary referral center for Differences/Disorders of sex Development (DSD). Patients and methods: We selected 87 patients with 46,XY DSD (17 familial cases from eight unrelated families and 70 sporadic cases); 55 patients had GD (among them, ten patients from five families and eight sporadic cases had ETRS) and 32 patients had 46,XY DSD of unknown etiology. Results: We identified four heterozygous missense rare variants classified as pathogenic or likely pathogenic in DEAH-box helicase 37 (DHX37) gene in five families (n=11 patients) and in six sporadic cases. Two variants were recurrent: the p.Arg308Gln (in two families and in three sporadic cases) and the p.Arg674Trp (in two families and in two sporadic cases). The variants were specifically associated with ETRS (7/14 index cases; 50%). The frequency of rare, predicted to be deleterious DHX37 variants in this cohort (0.14) is significantly higher than that observed in gnomAD database (0.004; $p<0.001$). Immunohistochemistry analysis in human testis showed that DHX37 is mainly expressed in germ cells, at different stages of testis maturation, in Leydig cells and rarely in Sertoli cells. Conclusion: This strong genetic evidence identifies DHX37 as a new player in the complex cascade of male gonadal differentiation and maintenance.

Descrition of a novel gene, DHX37 involved in gonadal dysgenesis etiology, especially in embrionyc testicular regression syndrome.

Introduction

46,XY gonadal dysgenesis (GD) represents a heterogeneous group of disorders/differences of sex development (DSD) characterized by abnormal gonadal development leading to a wide phenotypic spectrum. Variable degrees of external genitalia undervirilisation are observed, ranging from micropenis to female-like genitalia and partially- or fully-developed Mullerian derivatives.The gonads from these patients display a wide spectrum of histological abnormalities, ranging from ovarian-like stroma with disorganized seminiferous tubules to complete absence of gonadal tissue (1). Embryonic testicular regression syndrome (ETRS) is considered a part of the clinical spectrum of 46,XY gonadal dysgenesis (2). Most individuals with ETRS present with micropenis or atypical genitalia and lack of gonadal tissue on one or both sides (2).

Partial or complete Mullerian duct regression associated with micropenis suggests an intrinsically functional testis in the first months of fetal life subsequent loss of testicular function before the last trimester of gestation, when the increase in penile length occurs.

Numerous genes are known to be involved in the process of gonadal determination (3). However, a genetic diagnosis is identified in less than 40% of the patients with 46,XY GD (4). Moreover, few patients with ETRS were included in large cohorts of 46,XY DSD previously studied (4). However, the fact that some familial cases of ETRS were reported indicates a genetic etiology (5,6).

In the present work, high throughput parallel sequencing methods, including whole-exome sequencing (WES) and targeted DSD-gene panels, were used to investigate the

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underlying genetic etiology in a large cohort of 46,XY patients with GD and 46,XY DSD patients with unknown etiological cause.

We identified recurrent rare variants in DEAH (Asp-Glu-Ala-His) box polypeptide 37 (*DHX37*) in several affected individuals from distinct families, establishing a novel genetic cause for 46,XY gonadal dysgenesis spectrum, including ETRS.

Ethics

This study was approved by the Ethics Committee of the Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo, the Institutional Review Board of the University of Michigan Medical School, the Hospital de Garrahan Escuela de Medicina, Pontificia Universidad Católica de Chile, and the Hospital Nacional Prof. Dr. A. Posadas, Buenos Aires, Argentina. Written informed consent was obtained from all patients, their parents or legal guardians.

Subjects and Methods

We studied eighty-seven 46,XY DSD patients without previous molecular diagnosis, including 17 familial cases of 46,XY GD from 8 non-consanguineous families and 70 sporadic cases (38 with GD and 32 with 46,XY DSD of unknown etiology). Out of the 55 patients with GD, 10 patients from 5 families and 8 sporadic cases had an ETRS phenotype. The patients had different nationalities: Brazilian (81 patients), Argentinian (three siblings), Chilean (two siblings) and Chinese-American (one patient).

All patients have a normal GTG-banded metaphases 46,XY karyotype.

The 46,XY DSD patients were classified as having complete GD (CGD) if they had female external genitalia, Mullerian derivatives and streak gonads; as partial GD (PGD) if they had atypical external genitalia, Mullerian derivatives and at least one gonad with histopathological features of dysgenetic testis; as ETRS if they had micropenis, partially developed Mullerian derivatives and no gonadal tissue or small area of gonadal stroma; and as 46,XY DSD of unknown etiology if hormonal profile was not conclusive or not available due to previous gonadectomy. In this latter group, molecular defects of LHCG and androgen receptors, *CYP17A1*, *HSD17B3*, *HSD3B2*, and *5ARD2* genes were ruled out by DNA sequencing.

Genomic DNA

For molecular diagnosis, genomic DNA was extracted from peripheral blood leukocytes by the proteinase K-SDS salting-out method (7).

Genetic study

Whole exome sequencing (WES) was performed in 14 familial cases from 7 families. In all but one family, the probands and their first-degree relatives and other affected family members were studied.

Sixty-eight sporadic cases were studied by targeted massively parallel sequencing. *DHX37* was studied by Sanger sequencing in two sporadic cases and in three patients from Family 2 (Figure 1) (8).

Enrichment for massively parallel sequencing was performed with Nextera Exome Enrichment Kit (Illumina, San Diego, CA), followed by paired-end sequencing on the Illumina HiSeq 2500 System (Illumina, San Diego, CA)

For target sequencing, we designed an amplicon-based capture panel against exonic regions of 63 genes, including 43 genes known to be associated with human DSDs and 20 candidate genes, including *DHX37* (3,9). Target sequences were captured using a custom Sure Select Target Enrichment System Kit (Agilent Technologies, Santa Clara, CA, USA) and sequencing was performed on the Illumina MiSeq platform (San Diego, CA, USA).

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Sanger sequencing was used to confirm the potentially pathogenic variants identified by massively parallel sequencing and for segregation analysis. Sequencing was performed on the ABI 3730XL DNA Analyzer (Applied Biosystems) using the BigDye (Applied Biosystems), followed by data analysis using a Genetic Analyzer (ThermoFisher Scientific).

The identified variants were classified according to American College of Medical Genetics (ACMG) criteria (10).

Data analysis

The exome and the targeted panel sequencing data were screened for rare variants (minor allele frequency < 0.1% in the public databases: Genome Aggregation Database (gnomAD) (11), 1000 Genomes (12), and in the Brazilian population database (ABraOM) (13), located in exonic and consensus splice site regions. Subsequently, the filtration pipeline prioritized potentially pathogenic candidate variants (loss of function variants and variants classified as pathogenic by multiple *in silico* programs). For variants identified by WES, we selected variants that fitted an autosomal-dominant model.

The sequencing reads carrying candidate variants were visually confirmed using the Integrative Genomics Viewer (Broad Institute, Cambridge, MA). Candidate variants were segregated in the family members by Sanger method.

The filtering of the variants is provided in Supplementary data (8).

Histological analysis

Immunohistochemical staining

Eight formalin-fixed paraffin-embedded testicular tissue samples from 46,XY individuals with different chronological ages (27 and 33 weeks gestational age, 1, 53, and 180 days of age, 13, 23 and 53 years of age) were collected during autopsy and used for DHX37 expression analysis by immunohistochemistry. All samples were sliced into 3-µm-thick sections using an automatic Leica RM2255 microtome (Leica Biosystems, Nussloch, Germany). The sections were briefly stretched in xylol at 600°C for 20 min, cooled in xylol, and dried in an incubator (Fanem Orion 515, São Paulo, Brazil) at 600°C. Sections were subjected to hematoxylin-eosin (HE) staining for histological analysis. For immunohistochemical study, slides were deparaffinized with xylene, hydrated in ethanol, washed in phosphate-buffered saline (0.01 M/pH 7.4), and blocked using methanol and hydrogen peroxide. Epitope exposure was carried out by placing the slide in boiling 10 mM citric acid (pH 6) or 100 mM EDTA (pH 9), followed by blocking non-specific protein. Rabbit polyclonal anti-DHX37 antibody (NB110-40581; Novus Biologicals, USA) was added at a dilution factor of 1:50. Dilution was standardized after testing on ovarian and skin tissues where protein expression was identified in cytoplasm of oocytes and nuclear membranes of ovarian stromal and squamous cells. The samples were incubated with universal secondary antibodies using the Novo Link Detection Systems kit (Leica Biosystems, USA) according to the manufacturer's instruction.

Statistical analysis

To test the genetic evidence for the association between *DHX37* and GD phenotype, we performed aggregate variant analyses comparing allele frequencies among our 46,XY DSD cohort and public databases [gnomAD and ABraOM].

Variants with similar characteristics of the *DHX37* variants observed in our cohort (rare nonsynonymous variants with a minor allele frequency of 0.01 and located in the two highly conserved protein (ATP-binding and Helicase C-terminal domains) that are predicted to be pathogenic by at least four *in silico* tools (Mutation Taster, SIFT, PolyPhen-2, Mutation Assessor and PROVEAN) were selected. Allele frequency differences between groups were analyzed by X^2 test, and statistical significance was set at $p<0.05$. Statistical analyses were

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performed using SIGMAstat statistical software package (Windows version 3.5; SPSS Inc., San Rafael, CA).

Results

Patient phenotype and *DHX37* variants

Firstly, WES identified the same *DHX37* variant p.Arg308Gln (c.923G>A) (GenBank: NM_032656.3) in heterozygous state in two unrelated Brazilian families with ETRS (Families 1 and 2). All the affected individuals have the same phenotype (micropenis and absence or bilateral rudimentary gonadal tissue) (Figure 1, Table 1). A founder effect for p.Arg308Gln variant was ruled out in Families 1 and 2.

The p.Arg308Gln variant was also identified by WES in a Chinese-American sporadic case of ETRS from Michigan University performed in Eric Vilain's laboratory (sporadic case F6:II-1, Figure 1, Table 2).

As a novel candidate gene for 46,XY DSD, *DHX37* was included in our target DSD-panel. The same p.Arg308Gln variant was identified in another two sporadic cases: one had ETRS (sporadic case F7:II-1) and the other had PGD (sporadic case F8:II-1) (Figure 1; Table 2).

A further three different heterozygous *DHX37* missense variants (the p.Arg674Trp, p.Ser595Phe and p.Thr304Met) were identified in seven affected members from three families and in three sporadic cases (Figure 2).

All of these four variants are predicted to be pathogenic by at least four *in-silico* prediction tools (Table 3) and are absent in genomic population databases, except for the p.Arg308Gln, which has a very low allele frequency (0.00003) in the gnomAD database (Tables 4-5).

The p.Arg674Trp (c.2020C>T) variant was identified in the two Chilean brothers, both with ETRS (cases F3:II-1 and F3:II-2, Family 3), and also in the three Argentinian affected members (two brothers with ETRS and their uncle with PGD; cases F4:III-1, F4:III-2 and F4:II-4, respectively, Family 4) (Figure 1, Table 1). In addition, the p.Arg674Trp variant was also identified in another two Brazilian sporadic cases, one patient with ETRS (sporadic case F10:II-1) and the other with PGD (sporadic case F11:II-1) (Figure 1, Table 2)

The p.Ser595Phe (c.1784C>T) variant was identified in two affected individuals from the same Brazilian family (Family 5). The proband had PGD and her nephew had ETRS (F5:II-6 and F5:III-1, respectively) (Figure 1,Table 1).

The p.Thr304Met (c.911C>T) was identified in a Brazilian female (sporadic case F9: II-5), who had previously undergone bilateral gonadectomy and genitoplasty (Figure 1,Table 2).

The p.Arg308Gln variant is classified as pathogenic and the other three variants, p.Arg674Trp, p.Ser595Phe and p.Thr304Met, are classified as likely pathogenic accordingly the ACMG criteria (Tables 4-5).

Segregation analysis of *DHX37* variants

Segregation analysis of the *DHX37* variants in eight families displayed a sex-limited autosomal dominant pattern, maternally inherited in five families (F2, F3, F4, F5, F11). In the Family 1, the presence of the p.Arg308Gln variant in the asymptomatic father suggests an autosomal dominant pattern of inheritance with incomplete penetrance (Figure 1). In two sporadic cases (F6. II-1 and F8.II-1), the confirmed paternity displayed a *de novo* status of the p.Arg308Gln *DHX37* variant.

DHX37 gene and its protein structure

DHX37 is located in the 12q24.31 region. It is a member of the large DEAH family of proteins and encodes an RNA helicase (14). The DHX37 protein (NP_116045) comprises 1157 amino acids and four main domains. The conserved motifs of the helicase core region

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contain the Helicase ATP-binding domain (position 262-429) and the Helicase superfamily c-terminal domain (position 585-674); the two other domains are the helicase associated domain (position 768-859) and the oligonucleotide/oligosaccharide-binding-fold domain (position 894-1011) (Figure 2). All the identified variants are located in the helicase core region (Figure 2).

DHX37 protein was identified in different testicular cells

DHX37 expression was characterized in testes from newborns, children and adults using immunohistochemistry. DHX37 was expressed in fibroblasts, endothelial cells and epithelial cells of epididymis. These cells were used as internal positive controls for immunohistochemistry. We found DHX37 expression in Leydig cell cytoplasm and in germ cells at different stages of maturation. Our analysis indicates that DHX37 expression in spermatogonia is characterized by a regular perinuclear halo pattern in both newborns (five samples) and adults (three samples). This pattern of staining differs from that seen in Leydig cells (granular cytoplasmatic) and during other stages of maturation of germ cells. A progressive condensation of protein around the nucleus was observed as cells differentiate from spermatocytes to spermatids, generating a localized paranuclear dot-like pattern. There was no staining in spermatozoa. Rare Sertoli cells displayed a weak and focal cytoplasmatic stain (Figure 3).

Frequency of the *DHX37* variants in our 46,XY DSD cohort

The allele frequency of rare and predicted to be deleterious *DHX37* variants identified in our cohort of 46,XY DSD patients [11/78 index cases (0.14)] was markedly higher than that observed in individuals from gnomAD [568 /141456 individuals (0.004; $p<0.001$)] and from a Brazilian cohort [1/609 individuals (0.002); $p<0.001$)].

Discussion

The present study analyzed a large cohort of 46,XY DSD patients without a molecular diagnosis, most of whom had a GD phenotype, including a large number of familial and sporadic cases with ETRS.

Pathogenic or likely pathogenic allelic variants in the *DHX37* were identified in 11 familial cases from 5 unrelated families and in six sporadic cases. Deleterious variants are recurrent in familial and sporadic cases of 46,XY GD in patients of different nationalities.

The *DHX37* gene has never been directly associated with gonadal development, but deletions or rearrangements of the 12q24 chromosomal region, which contains *DHX37* gene, have been associated with atypical genital development (15). Four syndromic patients with micropenis or perineal hypospadias, and/or hypergonadotropic hypogonadism are reported to have deletions or rearrangements involving the 12q24 region (15-17).

The *DHX37* gene encodes a RNA helicase protein which is involved in RNA-related processes, including transcription, splicing, ribosome biogenesis (18), translation and degradation (14,19). *DHX37* is required for maturation of the small ribosomal subunit in human cells, through its catalytic activity, required for dissociation of the U3 snoRNA from pre-ribosomal complexes (20). Disturbance of human ribosome production is associated with cancer and genetic diseases known as ribosomopathies (21).

Disease-causing variants in the DExH-box helicase 30 (DHX30), were previously described in syndromic patients with global developmental delay, intellectual disability, severe speech impairment and gait abnormalities. Functional studies of allelic variants in *DHX30* demonstrated that they affect protein folding or stability interfering with the RNA binding (mutations located in Motif Ia) or with ATPase activity (mutations located in Motif II and VI) (19,20). Two DHX37 allelic variants found in the present study are located in the same motifs.

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Despite lack of experimental evidence to formally demonstrate the deleterious effects of the four variants identified in the present study, they are located in the highly conserved helicase core region of the DHX37 protein.

The spontaneous p.Leu489Pro Dhx37 pathogenic variant was identified in Zebrafish in association with behavior scape defects (22). This study demonstrated that Dhx37 is involved in pre-mRNA splicing reinforcing the role of Dhx37 in RNA-related processes.

Although there is no direct evidence of *DHX37* being involved in mRNA processing during gonadal development, DExD/H-box RNA helicase genes are differentially expressed between males and females during the critical period of male sex differentiation in channel catfish (23).

Further, we show population evidence that the *DHX37* variants are enriched among the 46,XY GD patients in comparison with the population database. The statistical analysis confirmed that the predicted deleterious *DHX37* variants located in the helicase core region are more frequently identified in our 46,XY DSD cohort than in the public databases, emphasizing that this finding was not by chance ($p<0.01$).

Therefore, *in vitro* and *in vivo* studies on DHX37 mechanism have demonstrated a role of DHX37 in ribosome biogenesis (23). Based on this new knowledge, 46,XY gonadal dysgenesis could be classified as a ribosomopathy, expanding the etiological mechanisms of dysgenetic 46,XY DSD spectrum.

Since the discovery of the sex-determining region Y (*SRY*) variants in patients with GD in 1990 (24), several genes have been associated with the molecular etiology of this disorder. The nuclear receptor subfamily 5 group A member 1 (*NR5A1*) and Mitogen-Activated Kinase Kinase Kinase 1 (*MAP3K1*) variants are the most frequent causes of 46,XY gonadal dysgenesis identified to date (25-28).

In this study we found pathogenic/ likely pathogenic variants in *DHX37* in patients with 46,XY GD at a frequency of 14%, which is slightly higher than the frequency of *NR5A1* defects (11%) in our whole cohort (26,29). Considering only the ETRS phenotype (micropenis and absence of uni or bilateral testicular tissue) this frequency increases to 50% (7/14 families).

In the literature, different inheritance patterns have already been described in 46,XY gonadal dysgenesis kindreds (30), including the description of asymptomatic male carriers of proven pathogenic variants of genes involved in testicular determination, such as *SRY* and *NR5A1* genes (31,32). Uncertain mechanisms might prevent the appearance of the phenotype in asymptomatic 46,XY carriers.

Maternal inheritance was observed in all familial cases with pathogenic/ likely pathogenic variants in *DHX37* with the exception of family 1, where the variant was inherited from a seemingly unaffected father carrier.

In adult humans, the DHX37 protein is expressed in the ovarian stroma and in the cells within seminiferous tubules (Human Protein Atlas database) (33-35). In our study, the immunohistochemistry analysis of normal testicular tissue from newborn, pubertal and adult males revealed that DHX37 is expressed during specific stages of germ cell maturation, in Leydig cells and rarely in Sertoli cells.

An elaborate paracrine cell-cell network transporting signaling molecules between germ cells and Sertoli cells has been described (36). Indeed, *in vitro* studies have shown that there is a bidirectional trafficking between Sertoli and germ cells, and that each cell type regulates the function of the other (37-40). In addition, RNA expression profiles of DHX37 in human testicular cancer cells are higher than in other tissues (The Human Protein Atlas – Pathology), suggesting that DHX37 may be involved in the regulatory process of the cell proliferation in the testis (33-35).

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The present study provides several lines of genetic evidence to indicate that defects in *DHX37* are associated with 46,XY GD spectrum, mainly with ETRS. First, we observed that the variants segregate with the DSD phenotype in a dominant inheritance pattern in most of the families and that two *de novo* variants were identified. Second, we provide statistical evidence that rare *DHX37* variants are enriched in the analyzed 46,XY DSD cohort in comparison with public databases involving a large number of individuals not selected by this phenotype.

In conclusion, our findings indicate that *DHX37* is a new player in the complex cascade of male gonadal differentiation and maintenance, thus establishing a novel and frequent molecular etiology for 46,XY gonadal dysgenesis spectrum, which includes a high proportion of individuals with embryonic testicular regression syndrome.

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Corresponding author: Berenice B Mendonca , Hospital das Clínicas, Faculdade de Medicina da Universidade de São Paulo, Disciplina de Endocrinologia e Metabologia. Av. Dr. Enéas de Carvalho Aguiar, 155, 2° andar, bloco 6, CEP: 05403-900, São Paulo, Brasil., Email: beremen@usp; sorahiad@gmail.com, Phone number: 55 11 2661-7564, 55 11 2661-7512

All authors have nothing to disclose.

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Figure 1. Pedigrees of the eleven families with potential disease-causing *DHX37*variants. Filled symbols represent affected individuals. The affected males (46,XY males) are indicated by filled squares and the affected individuals raised as females (46,XY females) are shown by large dark dots within the squares. Symbols with a diagonal line represent deceased individuals. The *DHX37* genotype is shown for the individuals whose DNA sample was available; +/- indicates a heterozygous state and -/- indicates a homozygous state for wild-type allele. NA- DNA not available. Paternity and maternity was confirmed in families 6 and 8.

Figure 2. The identified variants are localized within conserved helicase domains of *DHX37*. Top: Schematic protein structure of DHX37 showing conserved motifs of the helicase core region, the helicase associated domain (HA2) and the oligonucleotide/oligosaccharide-binding-fold. Middle: Nucleotide-interacting motifs (I, II, and VI), nucleic acid-binding motifs (Ia, Ib, and IV), motif V, which binds nucleic acid and interacts with nucleotides, and motif III, which couples ATP hydrolysis to RNA unwinding (N- N terminus; C- C terminus). Bottom: Amino acids within conserved motifs of the helicase core region. The position of the first and last amino acid within each motif is denoted below left and right, respectively. The position of the allelic variants identified in this study are indicated with vertical arrows and shown in bold in the different species sequence.

Figure 3. Immunoexpression patterns of DHX37 in testis tissues. **A-** Newborn testis showing strongly positive staining in occasional spermatogonia (arrow) among numerous Sertoli cells, some of which show weak cytoplasmic staining (original magnification 100X). **B-** Seminiferous tubules of a 13 year old boy demonstrating tubules with predominance of Sertoli cells, all of them negative for DHX37. Note some positive stromal cells (arrow) (original magnification 100X). **C-** Adult testis of a 54 year old man showing positive Leydig (arrow) and germ cell staining. **D-** Detail of (**C**) showing the different pattern of stain in different stages of germ cells. Note strong perinuclear halo in spermatogonia, progressive paranuclear condensation in spermatocytes and spermatids, and absence of DHX37 expression in spermatozoa (original magnification 20x).

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Table 1. Phenotype of 46,XY DSD patients with familial embryonic testicular regression syndrome with rare and predicted pathogenic or likely pathogenic *DHX37* variants

Nationality	Brazilian		Brazilian		Chilean	Argentinian				Brazilian	
Variables											
Patient	F1:III-2	F1:III-3	F2:II-4	F2:III-1	F3:II-1	F3:II-2	F4:III-1	F4:III-2	F4:II-4	F5:II-6	F5:III-1
Sex of rearing	Male	Male	Male	Female	Male	Male	Male	Male	Male	Female	Male
Age at presentation (yrs)	2.2	1.8	14.0	1.8	0.6	24 days	Newborn	4	2	18	10
Diagnosis	ETRS	ETRS	ETRS	ETRS	ETRS	ETRS	ETRS	ETRS	PGD	PGD	ETRS
External genitalia	Micropenis	Micropenis	Micropenis	Micropenis	Micropenis	Micropenis	Micropenis	Micropenis	Micropenis	Atypical	Micropenis
Gonads	Non-palpable	Non-palpable	Non-palpable	Non-palpable	Non-palpable	Non-palpable	Non-palpable	Non-palpable	Left testis - scrotum	Previous gonadectomy	Non-palpable
Histologic analysis	Small bilateral dysgenetic gonads	Left gonad not found. Right dysgenetic gonad	No gonadal tissue found	Left gonad not found. Small right dysgenetic gonad.	Small bilateral dysgenetic gonads	Small bilateral dysgenetic gonads	No gonadal Tissue	No gonadal tissue	Right gonad not found Left dysgenetic testis with GCNIS *	Bilateral dysgenetic gonads'	No gonadal tissue
Wolffian derivatives	Present	Present	Present	Present	Present	Present	Present	Present	Present	Present	Present
Müllerian Derivatives: Tubes	Present ¥	Absent	Present ¥	Present ¥	Absent	Present	Present ¥	Present ¥	Absent	Absent	Absent
Müllerian Derivatives: Uterus	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
LH (IU/L)	14.5	12	3.5	1.9	<0.5	<0.5	<0.5	<0.5	26	NA	19
FSH (IU/L)	117	133	87	56	10.9	9.5	NA	9	112	NA	43
Basal Testosterone (ng/dL)	<10	<10	<10	NA	16	<10	38	27	16	NA	21
Testosterone after hCG test (ng/dL)	<10	NA	29	26	18	<10	40	29	NA	NA	NA
Allelic variant	p.Arg308Gln	p.Arg308Gln	p.Arg308Gln	p.Arg308Gln	p.Arg674Trp	p.Arg674Trp	p.Arg674Trp	p.Arg674Trp	p.Arg674Trp	p.Ser595Phe	p.Ser595Phe
Variant state	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous

NA- not available; PGD- partial gonadal dysgenesis; GCNIS - germ cell neoplasia in-situ; * testicular biopsy; ¥ - Rudimentary Fallopian tubes;
Conversion factors to SI units: T, ng/dL to nmol/L, multiply by 0.0347

Table 2. Phenotype of 46,XY DSD patients with sporadic gonadal dysgenesis spectrum and heterozygous rare pathogenic or likely pathogenic *DHX37* variants

Nationality	Chinese-American	Brazilian				
Variables						
Patient	F6:II-1	F7:II-1	F8:II-1	F9:II-5	F10:II-1	F11:II-1
Social sex	Male	Male to Female	Female	Female	Male to female	Female
Age at presentation (yrs)	0.18	30	7.7	35	19	3.7
Diagnosis	ETRS	ETRS	PGD	Previous gonadectomy	ETRS	PGD
External genitalia	Micropenis	Micropenis	Female	Previous genitoplasty	Micropenis	Atypical
Gonads	Non-palpable	Non-palpable	Non-palpable	NA	Non-palpable	Non-palpable
Histological analysis	No gonadal tissue	No gonadal tissue	Bilateral dysgenetic gonads	NA	No gonadal tissue	Bilateral dysgenetic gonads

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Wolff derivatives		Present	Present	Present	NA	NA	Present
Mullerian derivatives	Tubes	Absent	Present	Absent	NA	Absent	Present
	Uterus	Absent	Absent	Absent	Absent	Absent	Absent
LH (IU/L)		0.1	10	0.1	NA	23	NA
FSH (IU/L)		0.4	40	4.9	NA	62	NA
Basal Testosterone (ng/dL)		<10	NA	<10	NA	21	25
Testosterone after hCG test (ng/dL)		<10	<10	NA	NA	NA	33
Allelic variant		p.Arg308Gln	p.Arg308Gln	p.Arg308Gln	p.Thr304Met	p.Arg674Trp	p.Arg674Trp
Variant state		Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous

NA: not available; PGD- Partial gonadal dysgenesis; Conversion factors to SI units: T, ng/dL to nmol/L, multiply by 0.0347.

Table 3. *In silico* prediction analysis of *DHX37* allelic variants identified in 46,XY DSD patients

Families	Nucleotide changed	AA changed	Functional domain	*In silico* prediction tools						
				Mutation Taster	Mutation Assessor	SIFT	Polyphen-2	PROVEAN	CADD	GERP
F1, F2, F6, F7, F8	c.923G>A	p.Arg308Gln	Helicase ATP-binding	Disease Cause (score: 0.999)	High functional impact (score: 4.38)	Protein function affected (score 0.001)	Probably damaging (score 1.000)	Deleterious (score -3.93)	35	5.3
F3,F4,F10, F11	c.2020C>T	p.Arg674Trp	Helicase superfamily C-terminal domain	Disease Cause (score: 1.000)	Middle functional impact (score: 4.83)	Protein function affected (score 0.001)	Probably damaging (score 1.000)	Deleterious (score -7.42)	33	4.2
F5	c.1784C>T	p.Ser595Phe	Helicase superfamily C-terminal domain	Disease Cause (score: 1.000)	High functional impact (score: 4.26)	Protein function affected (score 0.001)	Benign (score 0.24)	Deleterious (score -5.57)	24.4	4.13
F9	c.911C>T	p.Thr304Met	Helicase ATP-binding	Disease Cause (score 1.000)	High functional impact (score: 4.45)	Protein function affected (score 0.001)	Probably damaging (score 1.000)	Deleterious (score -5.89)	29.8	5.3

Table 4. *DHX37* missense allelic variants identified in 46,XY DSD patients and their frequency in population databases

Families	cDNA position	AA change	Phylogenetic Conservation	State	dbSNP	MAFs in population databases				
						1000 Genomes	ExAC	gnomAD	ABraOm	ESP6500
F1, F2, F6, F7, F8	c.923 G>A	p.Arg308Gln	Highly conserved	Heterozygous	Not available	Absent	Absent	0.0000667 Non-Finnish European	Absent	Absent
F3, F4, F10, F11	c.2020C>T	p.Arg674Trp	Highly conserved	Heterozygous	Not available	Absent	Absent	Absent	Absent	Absent
F5	c.1784C>T	p.Ser595Phe	Highly conserved	Heterozygous	Not available	Absent South Asian	Absent	Absent	Absent	Absent
F9	c.911C>T	p.Thr304Met	Highly conserved	Heterozygous	Not available	Absent	Absent	Absent	Absent	Absent

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Table 5. Pathogenicity classification of DHX37 variants according to the American College of Medical Genetics and Genomics guidelines

Familiss	Nucleotide changed	AA changed	Population Data	Computational and prediction data	De novo data	Other data	Classification
F1, F2, F6, F7, F8	c.923G>A	p.Arg308Gln	PM2 [a]	PP2 [b]	PS2 [d]	PM1 [e]	Pathogenic
				PP3 [c]			
F3, F4, F10,F11	c.2020C>T	p.Arg674Trp	PM2 [a]	PP2 [b]		PM1 [e]	Likely
				PP3 [c]			Pathogenic
F5	c.1784C>T	p.Ser595Phe	PM2 [a]	PP2 [b]		PM1 [e]	Likely
				PP3 [c]			Pathogenic
F9	c.911C>T	p.Thr304Met	PM2 [a]	PP2 [b]		PM1 [e]	Likely
				PP3 [c]			Pathogenic

PM2: moderate piece of evidence for pathogenicity; PP3: supporting evidence for pathogenicity by computational (in silico) data;

PS2: strong support for pathogenicity when the variants are de novo; PP4: supporting evidence using phenotype; PM1: pathogenic moderate;

VUS: Variant of Uncertain Significance.

a Absent from controls (or at extremely low frequency if recessive) in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium

b Missense variant in a gene that has a low rate of benign missense variation and in which missense variants are a common mechanism of disease

c Multiple lines of computational evidence support a deleterious effect on the gene or gene product (conservation, evolutionary, splicing impact, etc.)

d De novo (both maternity and paternity confirmed) in a patient with the disease and no family history.

e Located in a mutational hot spot and/or critical and well-established functional domain without benign variation.

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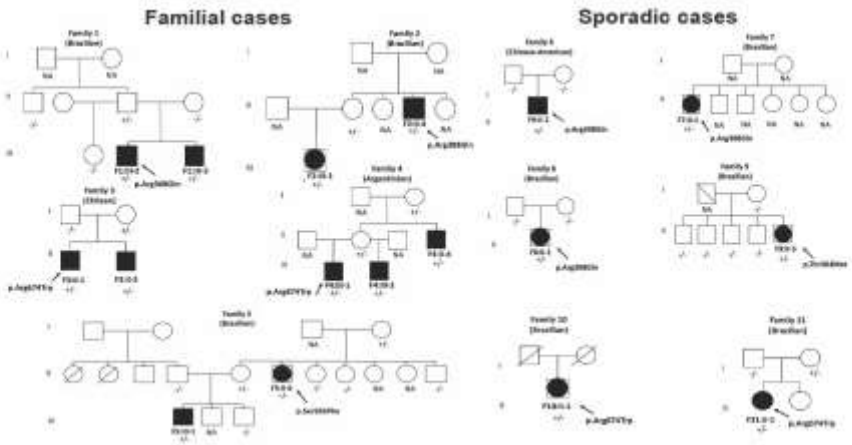

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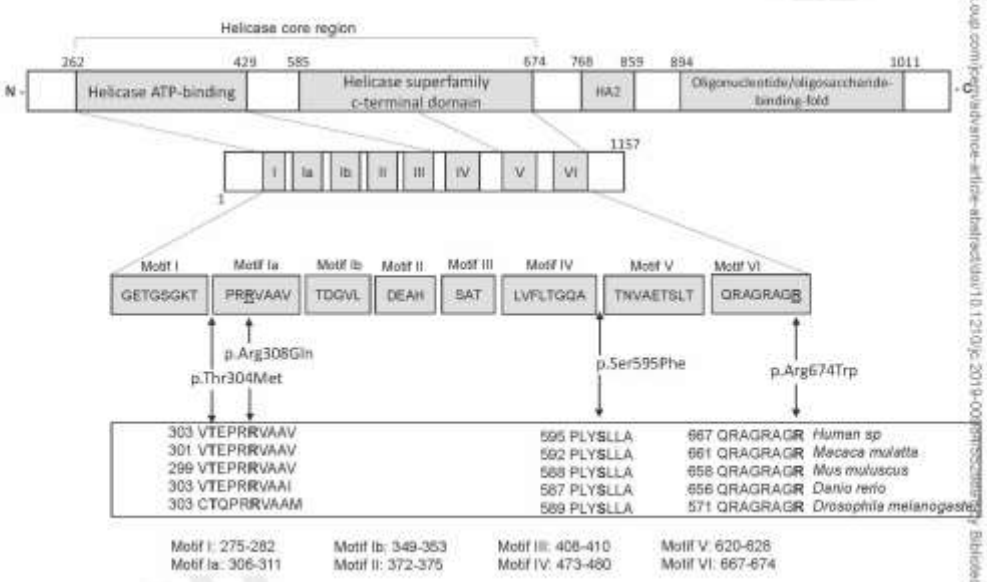

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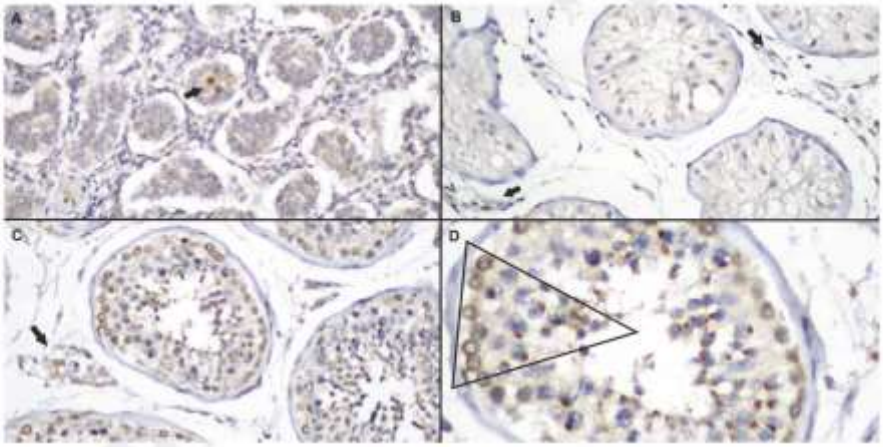

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Apêndice 2 -

Baetens D, Gürian T, Mendonça BB, **Gomes NL**, De Gauwer L, Peelman F, Verdin H, Vuylsteke M, Van der Linden M, ESR2 Study Grou, Stoop H, Looijenga LH, De Bosscher K, Cools M, De Baere E. Biallelic and monoallelic *ESR2* variants associated with 46,XY disorders of sex development. Genetics in Medicine. 2017.

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Biallelic and monoallelic *ESR2* variants associated with 46,XY disorders of sex development

Dorien Baetens, PhD[1], Tülay Güran, MD[2], Berenice B. Mendonca, MD, PhD[3], Nathalia L. Gomes, MD[3], Lode De Cauwer, BSc[4,5], Frank Peelman, PhD[4,5], Hannah Verdin, PhD[1], Marnik Vuylsteke, PhD[6], Malaïka Van der Linden, MSc[7], *ESR2* Study Group[8], Hans Stoop, PhD[9], Leendert H. Looijenga, PhD[9], Karolien De Bosscher, PhD[4,5], Martine Cools, MD, PhD[10] and Elfride De Baere, MD, PhD[1]

Purpose: Disorders or differences of sex development (DSDs) are rare congenital conditions characterized by atypical sex development. Despite advances in genomic technologies, the molecular cause remains unknown in 50% of cases.

Methods: Homozygosity mapping and whole-exome sequencing revealed an *ESR2* variant in an individual with syndromic 46,XY DSD. Additional cases with 46,XY DSD underwent whole-exome sequencing and targeted next-generation sequencing of *ESR2*. Functional characterization of the identified variants included luciferase assays and protein structure analysis. Gonadal *ESR2* expression was assessed in human embryonic data sets and immunostaining of estrogen receptor-β (ER-β) was performed in an 8-week-old human male embryo.

Results: We identified a homozygous *ESR2* variant, c.541_543del p.(Asn181del), located in the highly conserved DNA-binding domain of ER-β, in an individual with syndromic 46,XY DSD. Two additional heterozygous missense variants, c.251G > T p.(Gly84Val) and c.1277T > G p.(Leu426Arg), located in the N-terminus and the ligand-binding domain of ER-β, were found in unrelated, nonsyndromic 46,XY DSD cases. Significantly increased transcriptional activation and an impact on protein conformation were shown for the p.(Asn181del) and p.(Leu426Arg) variants. Testicular ESR2 expression was previously documented and ER-β immunostaining was positive in the developing intestine and eyes.

Conclusion: Our study supports a role for *ESR2* as a novel candidate gene for 46,XY DSD.

Genet Med advance online publication 26 October 2017

Key Words: 46,XY DSD; disorders of sex development; *ESR2* variants; novel candidate gene; whole-exome sequencing

INTRODUCTION

In humans, gonadal fate is determined by the presence or absence of sex-specific gene expression. In the bipotential stage of development, transcription factors such as Wilms' tumor 1, nuclear receptor subfamily 5 group A member 1 (NR5A1) and GATA-binding protein 4 (GATA4) lead to upregulation of the sex-determining region Y gene (*SRY*) in the male embryo, thereby initiating the male developmental signaling cascade, with the expression of other male-specific genes such as *SRY-box 9* (*SOX9*).[1] In addition, several reports indicate an important role for the mitogen-activated protein kinase (MAPK) pathway in the early phases of (male) sex determination.[2–4] In female embryos lacking the Y chromosome, female-specific genes like *R-spondin 1* and *forkhead box L2* (*FOXL2*) are expressed among others.[1,5] Animal studies have revealed multiple and long-lasting interactions between these male and female pathways; for instance, the loss of Foxl2 or Dmrt1 in murine adult granulosa and Sertoli cells, respectively, results in trans-differentiation to an opposite cell fate.[6,7]

Subtle imbalances of this strictly regulated sex-specific gene network can lead to disorders or differences of sex development (DSD).[1] Currently, mutations in genes known to be involved in sex development explain the molecular pathogenesis in only a minority of 46,XY DSD cases.[1,7] Next-generation-sequencing-based approaches such as whole-exome sequencing improve the identification of causal genetic defects, and the discovery of new genes and signaling pathways implicated in sex development.[8,9] Elucidating the underlying causes of DSD can facilitate sex assignment and

[1]Center for Medical Genetics, Ghent University and Ghent University Hospital, Ghent, Belgium; [2]Zeynep Kamil Maternity and Children's Diseases Training and Research Hospital, Division of Pediatric Endocrinology and Diabetes, Istanbul, Turkey; [3]Disciplina de Endocrinologia, Laboratório de Hormônios e Genética Molecular LIM/42, Unidade de Adrenal, Disc. de Endocrinologia e Metabologia, Hospital das Clínicas, Faculdade de Medicina da Universidade de São Paulo, São Paulo, Brazil; [4]Receptor Research Laboratories, Nuclear Receptor Lab, Department of Medical Protein Research, VIB, Ghent, Belgium; [5]Department of Biochemistry, Ghent University, Ghent, Belgium; [6]GENOMDX ltd, Statistics for Genomics, Melle, Belgium; [7]Department of Medical and Forensic Pathology, Ghent University and Ghent University Hospital, Ghent, Belgium; [8]Department of Pathology, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands; [9]Department of Pediatrics, Division of Pediatric Endocrinology, Ghent University and Ghent University Hospital, Ghent, Belgium. Correspondence: Elfride De Baere (Elfride.DeBaere@ugent.be)
[10]The members of the *ESR2* Study Group are listed after Acknowledgments.
The last two authors contributed equally to this work.

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lead to improved management, a better prognosis and a more accurate evaluation of gonadal function and gonadal germ cell cancer risk.[10]

So far, mutations in genes encoding nuclear receptors, such as *NR5A1* (also known as the steroidogenic factor 1 gene, or *SF1*)[11] and the androgen receptor *NR3C4* gene[12] have been implicated in DSD pathogenesis as well as the duplication of the nuclear receptor subfamily 0 group B member 1 gene (*NR0B1*, *DAX1*).[13] Here, we identified biallelic and monoallelic estrogen receptor 2 (*ESR2*) variants in one syndromic and two nonsyndromic 46,XY DSD cases, respectively, putting forward *ESR2* as a novel disease gene for 46,XY DSD and expanding the spectrum of nuclear receptors implicated in 46,XY DSD etiology.

MATERIALS AND METHODS

Study subjects

Laboratory and clinical investigations of cases 1–3 are summarized in **Table 1**. Cases and/or parents provided written informed consent for the study. Detailed case reports can be found in **Supplementary Data S1–S3** online.

Genetic studies

Homozygosity mapping was performed by genome-wide single-nucleotide polymorphism (SNP) genotyping in case 1 and in two unaffected siblings using the HumanCytoSNP-12 BeadChip platform (Illumina, San Diego, CA). Plink software, integrated in ViVar, identified homozygous regions of > 1 Mb, which were ranked according to their length and the number of SNPs, as described.[14] Only homozygous regions unique to the index case were selected. The presence of relevant known DSD genes was excluded using BioMart filtering. Next, different gene prioritization tools (Endeavour, GeneDistiller, GeneWanderer, PosMed, SUSPECTS, and ToppGene) were used to identify candidate genes in the selected regions.[15] For this purpose, known DSD genes were used as training genes (**Supplementary Data and Supplementary Table S1** online).

Sanger sequencing and targeted next-generation sequencing

Primers were developed for polymerase chain reaction amplification and sequencing of all coding exons, intron–exon boundaries and untranslated regions of *ESR2* (**Supplementary Data and Supplementary Table S2** online). Sanger sequencing (cases 1 and 2) was performed using an ABI 3730XL DNA Analyzer (Applied Biosystems, Carlshad, CA) with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) followed by data analysis with SeqScape Software v2.5 (Life Technologies, Europe, Ghent, Belgium). Targeted next-generation sequencing of 63 DSD disease and candidate genes including *ESR2* was performed (case 3) with a MiSeq System instrument. Details of the target enrichment, variant annotation and filtering can be found in **Supplementary Data S4** online.

Segregation analysis for the *ESR2* variant found in case 1 was performed in the available family members. Apart from the mother, no family members were available for further analysis in case 2. For case 3, no parental DNA was available for segregation analysis. Variant classification was performed following the American College of Medical Genetics and Genomics (ACMG) guidelines of 2015.[16]

Whole-exome sequencing was performed to exclude the presence of other likely pathogenic variants. Exome enrichment was performed with the Nextera Rapid Capture Exome kit (Illumina), followed by paired-end sequencing on HiSeq 2000 (2 × 100 cycles) (Illumina). Reads were mapped against the human reference genome sequence (National Center for Biotechnology Information, GRCh37/hg19) with the CLC Genomics Workbench v6.4 (Qiagen, Venlo, The Netherlands) followed by postmapping duplicate read removal, coverage analysis, and quality-based variant calling. More thorough variant annotation was executed using Alamut-HT v1.1.5 software (Interactive Biosoftware, Rouen, France) and a complementary literature search after extensive screening (**Supplementary Table S3–S5** online).

Targeted NR5A1 testing

In cases 1 and 2, targeted next-generation sequencing of the coding region of *NR5A1* was performed using a previously described workflow,[17] and copy-number variant analysis was performed using multiplex ligation-dependent probe amplification (SALSA MLPA P185 Intersex probemix; MRC-Holland, Amsterdam, The Netherlands). In case 3 *NR5A1* sequence variants and copy-number variants were excluded by the aforementioned targeted next-generation sequencing panel analysis.

Structural analysis of the estrogen receptor-β (ER-β) variants

Models for the wild-type ER-β DNA-binding domain (DBD) were built using the standard homology modeling procedure in YASARA Structure (http://yasara.org/products). We used the structure of the ER-β bound to a DNA response element (pdb code 1HCQ) as a template.[18] We built models for dimers of the wild-type DBD with and without DNA. A model for the p.(Asn181del) (N181del) mutation (found in case 1) was built in YASARA Structure based on the wild-type model, by deleting residue N181 and introducing a new peptide bond between residues 180 and 182, followed by energy minimization (the em_runclean protocol in YASARA Structure). The homodimer models for the wild-type and N181del mutant DBDs were further subjected to 500 ps of molecular dynamics with the YASARA2 forcefield, followed by energy minimization using the md_refine procedure.[19]

Using FoldX and the estrogen-bound ER-β ligand-binding domain (LBD) structure with pdb code 3OLL, we calculated an in silico estimate of the Gibbs energy of protein folding for the mutant and wild-type LBD using the RepairPDB and BuildModel commands.[20,21] For each mutant, we report the

Table 1 Laboratory and clinical investigations in cases 1–3

	Case 1 (15 y)*		Case 2 (12.5 y)*		Case 3 (24 y)*	
Clinical phenotype						
General	Height: 165 cm		Height: 158.6 cm		Height: 168 cm	
	Parental height: mother 158 cm; father 171 cm		Parental height: not available		Parental height: not available	
	Weight: 49 kg		Weight: 44.9 kg		Weight: 58.8 kg	
	Breast and pubic hair development: Tanner stages 1 and 3				Breast and pubic hair development: Tanner stages 1 and 4	
Urogenital system	Female external genitalia		Atypical genitalia (clitoromegaly, urogenital sinus)		Female external genitalia	
	Absence of uterus, fallopian tubes, gonads, and vagina		Absence of a uterus		Prepubertal uterus	
	Anal atresia, rectovestibular fistula					
Ocular system	Blepharophimosis, ptosis, nystagmus, severe degenerative myopia, left esotropia, and retinal detachment		No eye abnormalities		No eye abnormalities	
Dysmorphism	Flat-triangular face, prognatism, broad-depressed nasal bridge, dysplastic ears, and high-arched and narrow palate. Bilateral syndactyly (toes **II/III**).		Absent		Absent	
Hormonal investigations						
		Local reference range		Local reference range		Local reference range
LH (mIU/ml)	23.1	M (1.7–8.6)	12.6	6.1–21	9	0.95–9.3 UI/l
		F (2.4–12.6)				
FSH (mIU/ml)	78.6	M (1.42–15.4)	25.6	15–42	41	1.7–9.3 UI/l
		F (1.37–9.9)				
Testosterone (ng/ml)	0.01	M (0.28–11.1)	Not available	Not available	<14	F (<14 a 98 ng/dl)
		F (0.06–0.82)				
AMH (ng/ml)	<0.1	M (3.8–159.8)	Not available	Not available	Not available	Not available
		F (<8.9)				
Inh B (pg/ml)	<5	M (15–405)	Not available	Not available	Not available	Not available
		F (<186)				
Imaging						
Ultrasound	Absence of gonads but presence of a large cystic lesion of 70 mm × 42 mm × 37 mm at the right adrenal region instead. Prostate volume was estimated to be 3 cm^3		Absence of uterus		No visible gonads	

Table 1 Continued

	Case 1 (15 y)[a]	Case 2 (12.5 y)[a]	Case 3 (24 y)[a]
	Hepatomegaly (154 mm in long axis; Riedel lobe)		Prepubertal uterus: 6.3 cm[3]
	Splenomegaly (126 mm in long axis)		
Pelvic MRI	Confirmed ultrasound findings	Not available	Not available
Bone age (according to Greulich and Pyle)	11 years; 12.5 (female)	11.5 years	Not available
Genetic investigations			
Karyotype	46,XY	46,XY	46,XY
FISH *SRY*	Positive	Positive	Positive
FOXL2	Negative	Not relevant	Not relevant
NR5A1	Negative (no SNV and CNV)	Negative (no SNV and CNV)	Negative (no SNV and CNV)
AR	Negative	Negative	Negative
ESR2	c.541_543del p.(Asn181del) (Hom)	c.251C > T p.(Gly84Val) (Het)	c.1277T > G p.(Leu426Arg) (Het)

AMH, anti-Müllerian hormone; CNV, copy-number variation; F, female; FSH, follicle-stimulating hormone; Het, heterozygous; Hom, homozygous; inh α, inhibin α; LH, luteinizing hormone; M, male; MRI, magnetic resonance imaging; SNV, single-nucleotide variation.
[a]Assessment at start of estrogen supplementation.

average ΔΔG for the folding of mutant versus wild-type of 20 BuildModel calculations. The 3OLL protein structure was visualized in UCSF Chimera.[22] The effect of the p.(Leu426Arg) (L426R) mutation (found in case 3) was visualized by replacing L426 with the best-fitting arginine rotamer in the Dunbrack backbone-dependent rotamer library.

ESR2 expression analysis in human embryonic tissue

Assessment of human embryonic expression data sets

ESR2 expression in the testis was assessed in RNA sequencing data generated by Gerrard et al.[23] in human embryos of Carnegie stages 18 and 22 and in array expression data generated by del Valle et al.[24] in embryonic gonadal and adrenal tissues.

Immunohistochemistry

Tissue slides (4 µm) were prepared from a formalin-fixed, paraffin-embedded 8-week-old male fetus obtained after spontaneous abortion. Before immunohistochemistry, a heat-induced antigen retrieval step (Tris-EGTA, pH 9.00) was included. We used a standard ABC protocol for the detection of ER-β. After blocking endogenous peroxidase and biotin (Avidin/Biotin Blocking Kit SP-2001) and overnight incubation with mouse anti-ER-β (dilution 1/50; Dako M7292), detection was performed with a biotinylated rabbit antimouse antibody (dilution 1/150, 30 min; Dako E413) followed by 30 min of incubation with Avidin–Biotin–HRP-labeled complex (VECTASTAIN Elite ABC HRP Kit PK--6100). Visualization was done with DAB/H_2O_2 and hematoxylin counterstaining.

Luciferase assays

Plasmid construction

Plasmids containing the *ESR2* variants were constructed with site-directed mutagenesis starting from a pcDNA–flag–ER-β plasmid (35562; Addgene, Cambridge, MA). Mutagenesis primers were designed using the NEBaseChanger software (http://nebasechanger.neb.com/). After amplification, the plasmids were digested with *Dpn*I to remove nonmutated DNA. After verifying that mutations had correctly been inserted via sequence analysis, mutated plasmids were transformed in chemically competent *Escherichia coli* (DH10b cells) and subsequently isolated with the NucleoBond Xtra Midi/Maxi kit (Macherey-Nagel, Düren, Germany).

Cell culture and luciferase assays

HEK293T cells were cultured in Dulbecco's minimal essential medium without phenol red, supplemented with 10% charcoal-stripped fetal bovine serum (Life Technologies, Europe, Ghent, Belgium) and 10 µg/ml gentamycin at 37 °C and 8% CO_2. A total of 300,000 cells per well were plated in 6-well plates. Cells were transfected with a total of 2 µg of DNA using a standard calcium phosphate transfection method. After 24 h, cells were washed with phosphate buffered saline and subsequently stimulated with

2,3-bis(4-hydroxyphenyl)propionitrile (DPN) to a final concentration of 10 nM. Following lysis with 1× cell culture lysis reagent (25 mM Tris-phosphate pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-cyclohexylenedinitrilotetraacetic acid, 10% glycerol, and 1% Triton X-100), luciferase activity was measured 24 h after stimulation on a TopCount luminometer (Canberra–Packard, Meriden, CT). To normalize for differences in transfection efficiency, a plasmid constitutively expressing β-galactosidase was cotransfected in all experiments (Tropix Galacto-Star system; Applied Biosystems, Foster City, CA). Each transfection was replicated in at least three independent experiments. Differences in expression between wild-type and mutated ER-β were demonstrated for variants 1 and 2 using a control western blot (data not shown).

RESULTS

Identification of a homozygous *ESR2* variant in case 1 with syndromic 46,XY DSD

Given the possible parental consanguinity, we performed homozygosity mapping to identify runs of homozygosity containing the putative underlying genetic cause of the syndromic 46,XY DSD phenotype in case 1. Twenty-one homozygous regions larger than 1 Mb were identified (**Supplementary Data and Supplementary Table S6** online). The largest one—with a length of 20 Mb—is located on chromosome 14 and contains 465 genes, none of which are known DSD genes. The gene encoding 5α-reductase, *SRD5A2*, was present in another small homozygous region (3.6 Mb); however, no potentially pathogenic variants were found in its coding region. Variant prioritization ranked *ESR2* as the top candidate gene (**Supplementary Data and Supplementary Table S7** online). Sequencing of *ESR2* revealed a homozygous 3-bp deletion in exon 9, c.541_543del, leading to an in-frame deletion, p.(Asn181del) (**Figure 1a,b**). This deletion affects a highly conserved amino acid located in the DBD (**Figure 1a, b**). The deletion was found to be absent in 92 tested ethnically matched control chromosomes and 320 other tested control chromosomes. This variant is reported at very low frequencies in publicly available databases (the Exome Sequencing Project, the Exome Aggregation Consortium 0.3.1, and gnomAD); however, no homozygotes have previously been identified (variant details in **Supplementary Data and Supplementary Table S8** online). Segregation analysis showed that both parents (**Figure 1b**; I:1 and I:2) and one healthy 46,XX sibling (**Figure 1b**; II:2) are heterozygous carriers of the deletion, while another 46,XX sibling (**Figure 1b**; II:3) is homozygous for the wild-type allele. Variant classification following the ACMG guidelines categorizes p.(Asn181del) as likely pathogenic (class 4) (**Table 2**). Whole-exome sequencing in the index case and the parents made the presence of other potentially pathogenic coding variants very unlikely. An overview of the variant filtering, performed as previously described,[14] is given in **Supplementary Tables S3** and **S4** online.

Identification of two heterozygous *ESR2* missense variants in case 2 and 3 with nonsyndromic 46,XY DSD

Targeted resequencing of the *ESR2* coding region in a Belgian 46,XY DSD cohort ($n = 73$) and a Brazilian 46,XY DSD cohort ($n = 40$) revealed two heterozygous missense variants in two unrelated cases with nonsyndromic 46,XY DSD. In case 2, a heterozygous *ESR2* variant c.251G > T was found in exon 2, leading to a missense change p.(Gly84Val) affecting a highly conserved amino acid (up to fruit fly) located in the N-terminal domain, which contains the first activation function of the receptor (**Figure 1a**). The physicochemical difference between Gly and Val is moderate (a Grantham distance of 109). Several prediction programs (SIFT, PolyPhen-2, and MutationTaster) suggest a deleterious effect on protein function. The variant is absent in 412 tested control chromosomes and in genomic databases such as dbSNP, the Exome Sequencing Project, and the Exome Aggregation Consortium. In gnomAD, it occurs in 0.000396% (no homozygotes) (**Table 2**). Following the ACMG guidelines, p.(Gly84Val) could be scored as likely pathogenic (class 4) (**Table 2**). Whole-exome sequencing did not reveal any other potentially pathogenic variants in case 2. An overview of the different filtering steps and variants is given in **Supplementary Tables S3** and **S5** online. In case 3, a heterozygous variant—c.1277T > G in exon 8 of *ESR2*—was found, leading to the missense change p.(Leu426Arg) in the LBD and affecting a highly conserved amino acid (up to *Tetraodon*). The Grantham distance between Leu and Arg is moderate (i.e., 102) and several prediction programs show a potential deleterious effect. This variant is not present in the consulted genomic databases (dbSNP, the Exome Sequencing Project, the Exome Aggregation Consortium, and gnomAD), nor in 214 Brazilian exomes (**Table 2**). Following the ACMG guidelines, p.(Gly84Val) could be classified as a variant of unknown significance (class 3) (**Table 2**). Whole-exome sequencing did not reveal any other potentially pathogenic variants in case 3. **Figure 1** and **Table 2** summarize the location and characteristics of the identified *ESR2* variants.

Structural analysis of the ER-β variants

The ER-β DBD binds to DNA as a homodimer. We built homology models for the wild-type and p.(Asn181del) mutant homodimer. Residue Asn181 is part of a longer extended loop that connects the main DNA-binding helix to a zinc coordination site, which is part of the homodimer interface. The Asn181 deletion shortens this connecting loop and causes a 15° tilt of the main DNA-binding helix (**Figure 2a,b**). Molecular dynamics simulation, followed by energy minimization, suggests that this slightly affects the orientation between the two DBDs in the homodimer, with an increased number of contacts between the two domains (**Figure 2a,b**). It is therefore very likely that the Asn181 deletion affects homodimerization and/or DNA binding of the ER-β.

There was no template available for remodeling of the p.(Gly84Val) variant, and no homology models could be

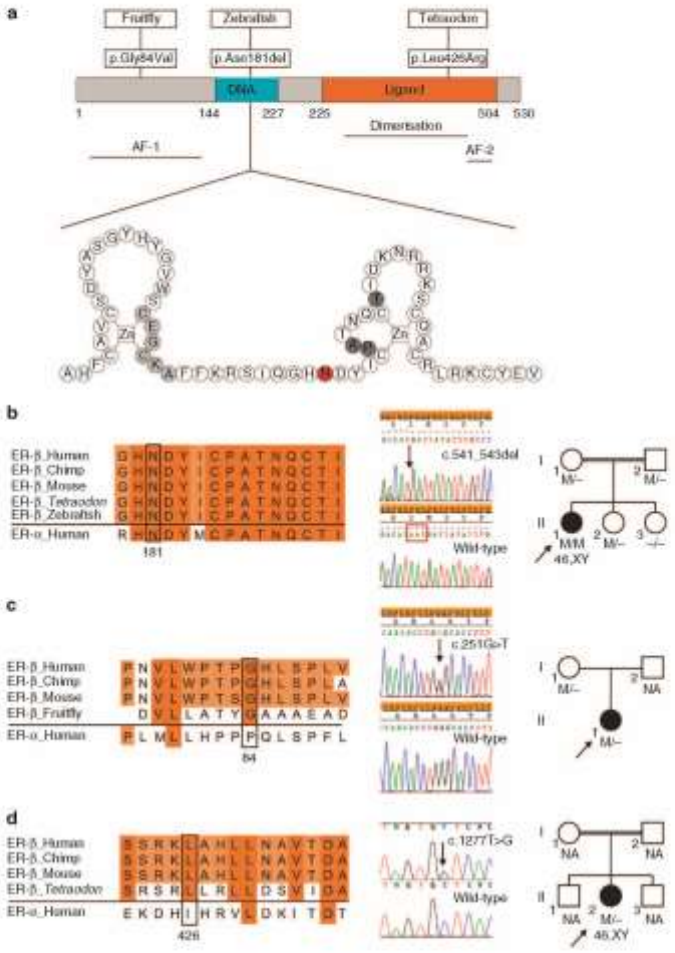

Figure 1 Identification of three *ESR2* variants. (**a**) Schematic overview of the ER-β protein with detailed representation of the DNA-binding domain. The light gray circles represent P-box amino acids, the dark gray circles represent the dimerization interface and the red circle represents the deleted amino acid in case 1. (**b–d**) Left: protein alignment; middle: electropherogram; and right: pedigree, for the deleted amino acid Asn181 (N181) found in case 1 conserved up to *Tetraodon* and in human ER-α (**b**); the affected amino acid in case 2, Gly84 (G84) conserved up to the fruit fly (**c**); and the affected amino acid in case 3, Leu426 (L426) conserved up to *Tetraodon* (**d**). In (**b–d**), the affected individual is represented by a black symbol. M, mutation; NA, not available.

obtained. To assess the possible influence of the p.(Leu426Arg) missense variant on the ER-β protein structure, we made *in silico* calculations of ΔΔG with FoldX and tried to model the best rotamer position for each variant with UCSF Chimera. The p.(Leu426Arg) variant is located in the LBD, which was previously characterized by X-ray crystallography. Leu426 is a buried residue of helix 9 (**Figure 2a**). FoldX energy calculations using the BuildModel command indicate that the p.(Leu426Arg) mutation affects the folding energy of the protein and has a destabilizing effect (*in silico*

Table 2 Variant classification of the identified *ESR2* variants

Variant	cDNA	Protein	Nucleotide conservation	Amino acid conservation	SIFT	PolyPhen2	MutationTaster	Grantham distance	Public databases	ACMG classification
1	c.541_543del (Hom)	p.(Asn181del)	/	Highly conserved: zebrafish	/	/	/	/	dbSNP: rs750091675 ESP: 0.04% ExAC: 0.041%, no homozygous cases reported gnomAD: 0.0368%, no homozygous cases reported	Class 4 (likely pathogenic) Criteria: ≥ 3 moderate pathogenic arguments (PM2, PM4, PM1, and PM3)[19]
2	c.251G > T (Het)	p.(Gly84Val)	Moderately conserved: phyloP: 4.22	Highly conserved: fruit fly	Tolerated	Probably damaging	Disease-causing	109	Absent in dbSNP, ESP, and ExAC gnomAD: 0.000396% (singleton)	Class 4 (likely pathogenic) Criteria: ≥ 3 moderate pathogenic arguments (PM2, PM1, and PM6)[19]
3	c.1277T > G (Het)	p.(Leu426Arg)	Highly conserved: phyloP: 8.708N	Highly conserved: Tetraodon	Deleterious	Probably damaging	Disease-causing	102	Absent in dbSNP, ESP, ExAC, and gnomAD	Class 3 (uncertain significance) Criteria: classification criteria for classification as benign or pathogenic are not met (PM2, PM1, and PP3)[19]

ACMG, American College of Medical Genetics and Genomics; dbSNP, Single Nucleotide Polymorphism database; ESP, Exome Sequencing Project; ExAC, Exome Aggregation Consortium; Het, heterozygous; Hom, homozygous; PM, moderate pathogenic criterion; PP, supporting pathogenic criterion

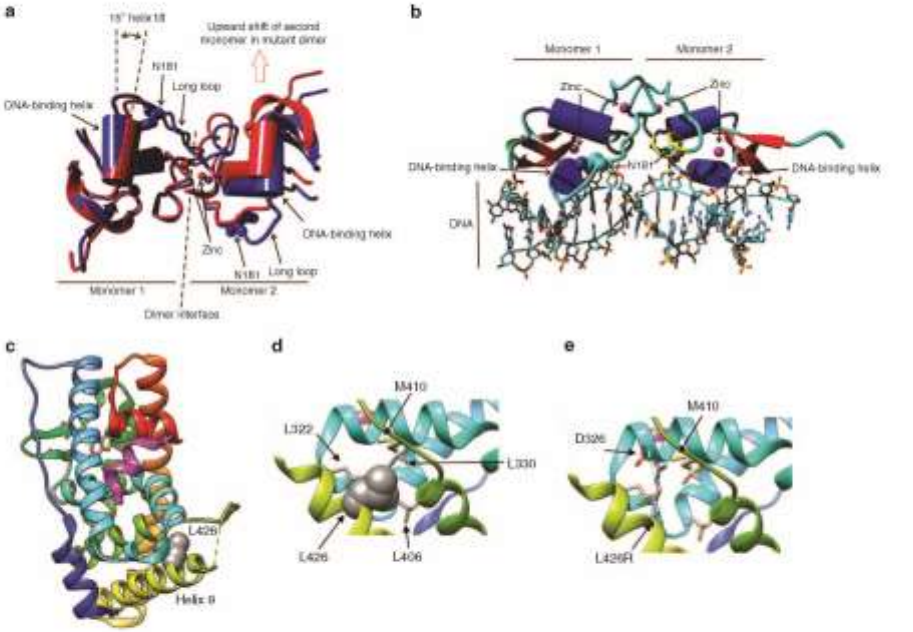

Figure 2. Structural analysis of mutated and wild-type ER-β. (**a**) Effect of the Asn181 deletion on a dimer of the ER-β DNA-binding domain. A model for a dimer of both the wild-type (blue) and Asn181del mutant (red) DNA-binding domain is shown. Monomer 1 of both dimers is superposed. The Asn181 (N181) deletion shortens a long loop between the DNA-binding helix and the zinc-binding site at the dimer interface. As a result, the DNA-binding helix tilts towards the dimer interface. The red arrow indicates an upward shift of the second monomer versus the first monomer in the mutant dimer. The molecules are rotated 90° compared with (**b**) and the DNA-binding helices are directed towards the viewer. (**b**) Model of the wild-type ER-β DNA-binding domain bound to DNA. The position of Asn181 is indicated. (**c**) Position of the Leu270 (L270) and Leu426 (L426) residues in the ER-β ligand-binding domain structure. Atoms of L270 and L426 are shown as gray spheres. (**d**) L426 is buried in a hydrophobic environment in the ligand-binding domain structure. (**e**) When L426 is mutated to the best-fitting Arg rotamer in UCSF Chimera, the Arg side chain clashes with residues D326 and M410 (red lines).

$\Delta\Delta G = 2.3$ kcal/mol). Leu426 fits in a hydrophobic pocket and engages in interactions with Leu322, Leu330, Leu406, and Met410 (**Figure 2b**). Modeling the best-fitting Arg rotamer at the position of Leu426 in UCSF Chimera shows that this mutation abolishes most of these hydrophobic interactions, while even the best-fitting rotamer of the Arg side chain introduces strong steric clashes in the absence of drastic energy minimization (green lines) (**Figure 2c**). The p.(Leu426Arg) variant is likely to affect the stability or folding of the LBD domain. Folding of the p.(Leu426Arg) variant estrogen-bound LBD domain into a wild type-like structure would at least require drastic structural changes in the environment of residue 426, which could hamper LBD dimerization, as helix 9 and the loop between helix 8 and 9 (which interacts with Leu426 via Met410) are both part of the symmetrical LBD dimer interface.

Expression studies in human embryos

In RNA sequencing data generated by Gerrard et al.[23] in embryos of Carnegie stages 18 and 22 (7 and 8 weeks; i.e., around the time of sex determination), *ESR2* expression could be observed in testicular tissues. In array expression data generated by del Valle et al.[24] in a genomic atlas of human adrenal and gonadal development, early expression of *ESR2* could be confirmed. In a human male embryo with a gestational age of 8 weeks, ER-β immunostaining was positive in the developing eye, especially in the corneal epithelium and endothelium, primitive lens neurons, primitive retinal cells and retinal pigment epithelium, mucosa of the developing intestine, and neuronal system (specifically the primitive neurons of the germinal matrix). ER-β staining was negative in the developing testis at this stage of development (**Supplementary Data and Supplementary Figure S1** online).

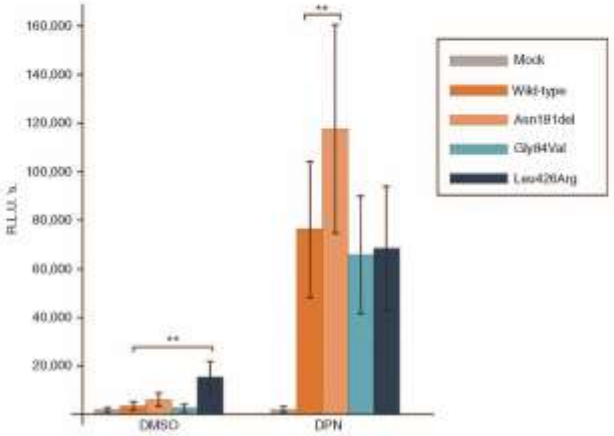

Figure 3 Transcriptional activation assays. Results are shown as the mean ± SEM for relative luciferase intensity units (R.L.U.). The analysis involved the fitting of a hierarchical generalized linear model to the R.L.U. data, as implemented in Genstat v18, with transfection and stimulation as fixed terms (Poisson distribution; log link) and replicates ($n = 4$ or higher) nested within experimenter as additional random terms (gamma distribution; log link). $^{**}P < 0.01$. The difference between WT and Asn181del is significant at the 0.001 level. For variants 1 and 2, the experiment was repeated 11 times respectively with three to six technical replicates each time; for variant 3, the experiment was repeated seven times with six replicates each time. DMSO, dimethylsulfoxide; DPN, 2,3-bis(4-hydroxyphenyl)propionitrile.

Transcriptional activation assays

The impact of the identified variants on transcriptional activation was assessed using luciferase assays with TK-3xERE-promoter constructs. The p.(Asn181del) variant resulted in significantly increased transcriptional activation after stimulation with the ER-β-specific ligand, DPN ($P < 0.001$). For the two missense variants, p.(Gly84Val) and p.(Leu426Arg), no significant alterations of ligand-dependent transcriptional activation were observed (**Figure 3**). Interestingly, the p.(Leu426Arg) variant shows a higher transcriptional activation compared with wild-type ER-β when the cells are not stimulated with DPN ($P = 0.0014$).

DISCUSSION

The identification of likely pathogenic variants in *ESR2* in unrelated individuals with 46,XY gonadal dysgenesis suggests an unanticipated role for ER-β in early gonadal development. Previously, polymorphic *ESR2* variants have been associated with milder variations in sexual development such as hypospadias and micropenis,[25–29] and a heterozygous *ESR2* variant c.1295C > A leading to a missense change p.(Ala432Asp) in the LBD of ER-β was recently reported in a girl with primary amenorrhea.[30]

Here, we identified a biallelic in-frame deletion in a girl with syndromic 46,XY DSD and two monoallelic missense variants in two unrelated cases with nonsyndromic 46,XY DSD. The data of case 1 are suggestive of absent gonadal development, while the data of cases 2 and 3 are compatible with partial and complete gonadal dysgenesis. A combination of different parameters including population frequencies and in silico predictions support a likely pathogenic classification for two of the three variants found (**Table 2**). Structural analyses support a deleterious effect of the p.(Asn181del) and p.(Leu426Arg) variants on protein conformation, but are not available for the p.(Gly84Val) variant due to the absence of a suitable model for this region. A significant increase in transcriptional activation was shown for the p.(Asn181del) and p.(Leu426Arg) variants and resulted in increased activity in the absence of an ER-β-specific ligand.

In recently published human embryonic gonadal expression databases, *ESR2* expression was found in early stages of gonadal development. In an 8-week-old male human embryo, ER-β immunostaining was shown in the developing intestine, eye, and neuronal systems, potentially reflecting the extra-gonadal involvement in case 1. However, no testicular immunostaining was observed at this developmental time point. Given the severity of the observed gonadal phenotypes, it cannot be excluded that ER-β plays a role even earlier during development.

An interesting finding in this respect is the fact that nonclassical estrogen signaling has been shown previously to interact with the MAPK pathway[31]—a signaling pathway involved in the earliest stages of gonadal development—although the operating mechanisms are different in humans and mice. In humans, MAPK signaling is essential for tilting the gene expression balance towards *SOX9* expression; gain-

of-function mutations of *MAP3K1* lead to higher phosphorylation of downstream targets, causing decreased expression of *SOX9* and increased expression of female-specific genes like *β-catenin*.[1,31,32] In mice, the Mapk pathway acts through direct upregulation of *Sry*; homozygous loss-of-function variants of *Map3k4* are reflected in a male-to-female sex reversal phenotype.[3,33]

Nuclear receptor family members, to which the ERs belong, are known to exert their functions through different action mechanisms. The classical genomic pathway involves ligand binding and receptor dimerization, followed by nuclear translocation and activation or repression of target gene expression.[34,35] Estrogen binds on both ER-α and ER-β receptors; however, binding results in partially overlapping but also differential transcriptional effects.[36,37] Alternative action mechanisms involve interactions with other transcription factors, ligand-independent receptor activation, and activation of different signaling pathways. For instance, it has been shown that ER-β can activate specific *MAPK* pathways in human colon cancer cell lines. The scarce experimental data regarding these alternative pathways indicate that these processes are highly cell specific, making it hard to investigate them in a development gonadal context as the currently available cell lines do not mimic the embryonic cellular environment. We hypothesize that the *ESR2* variants found here could lead to increased activation of the MAPK pathway via a nongenomic working mechanism, thereby shifting the expression balance from SOX9 to β-catenin signaling, and resulting in decreased SOX9 activation and failure of testicular development (**Supplementary Data and Supplementary Figure S2** online). Experimental validation of this hypothesis is challenging given the cell-type specificity of these alternative pathways and the fact that no relevant cell line resembling the undifferentiated gonad is available. Furthermore, it remains complex to acquire an experimental setup that resembles the earliest phases of sex development as all available gonadal cell lines are differentiated.

A differential action mode of MAPK signaling in mice and humans can explain the absence of a sex reversal phenotype in ER-β knockout mouse models. Over the years, several ER-β KO mice have been generated. A first mouse knockout model was characterized by female sub- or infertility and normal reproductive capacity in males.[38] This mouse was generated through insertion of a neomycin resistance gene into exon 3 of the *Esr2* gene by homologous recombination in embryonic stem cells. Several transcripts were still being processed, however, leading to an incomplete knockout. Moreover, ER-β knockout mice originating from the same initial ER-β mutant but raised and examined in different laboratories presented with a range of different phenotypes. A new ER-β knockout mouse used Cre/LoxP-mediated excision of the third exon of *Esr2*, resulting in complete absence of any *Esr2* transcript. These mice show infertility in both sexes, but no sex reversal. A similar situation has been reported for another nuclear receptor, *NR0B1* (*DAX1*), for which duplications lead to male-to-female sex reversal in humans, but delayed testis development in mice.[39]

Differences in zygosity have been shown for the *ESR2* variants found here, and may relate to the nonsyndromic phenotype in the two monoallelic cases compared with the syndromic phenotype in the biallelic case. Moreover, a different working mechanism cannot be excluded for the monoallelic missense variants found, although they may all act via the same dysregulated *MAPK* pathway. Differences in zygosity and mutation-dependent action mechanisms have been described for other nuclear receptors, such as *NR2E3*, implicated in both autosomal dominant and recessive retinal dystrophies with clinically distinct phenotypes.[40]

CONCLUSION

We identified biallelic and monoallelic variants in *ESR2* in patients with syndromic and nonsyndromic 46,XY DSD, respectively, putting forward *ESR2* as a novel candidate gene for 46,XY DSD etiology. It is interesting to speculate about an unanticipated role for ER-β in early gonadal development; i.e., the development and stabilization of the bipotential gonad and/or its differentiation into a functional testis.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/gim

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ESR2 STUDY GROUP

Zeynep Atay (Pediatric Endocrinology and Diabetes, Marmara University, Turkey); Abdullah Bereket (Pediatric Endocrinology and Diabetes, Marmara University, Turkey); Ronald R de Krijger (Reinier de Graaf Hospital, Delft, The Netherlands); Katleen de Preter (Center for Medical Genetics, Ghent University and Ghent University Hospital, Belgium); Sorahia Domenice (Disciplina de Endocrinologia, Laboratorio de Hormonios e Genetica Molecular LIM/42, Unidade de Adrenal, Disciplina de Endocrinologia e Metabologia, Hospital das Clinicas, Faculdade de Medicina da Universidade de São Paulo, São Paulo, Brazil); Serap Turan (Pediatric Endocrinology and Diabetes, Marmara University, Turkey).

DISCLOSURE

The authors declare no conflict of interest.

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Apêndice 3

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ORIGINAL ARTICLE

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Assembling the jigsaw puzzle: CBX2 isoform 2 and its targets in disorders/differences of sex development

Patrick Sproll[1†] | Wassim Eid[1,2†] | Camila R. Gomes[3] | Berenice B. Mendonca[3] | Nathalia L. Gomes[3] | Elaine M.-F. Costa[3] | Anna Biason-Lauber[1]

[1]Division of Endocrinology, University of Fribourg, Fribourg, Switzerland
[2]Department of Biochemistry, Medical Research Institute, University of Alexandria, Alexandria, Egypt
[3]Medical School, University of Sao Paulo, Sao Paulo, Brazil

Correspondence
Anna Biason-Lauber, Division of Endocrinology, University of Fribourg, Fribourg 1700, Switzerland.
Email: anna.lauber@unifr.ch

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Abstract

Background: One of the defining moments of human life occurs early during embryonic development, when individuals sexually differentiate into either male or female. Perturbation of this process can lead to disorders/differences of sex development (DSD). Chromobox protein homolog 2 (*CBX2*) has two distinct isoforms, CBX2.1 and CBX2.2: the role of CBX2.1 in DSD has been previously established, yet to date the function of the smaller isoform CBX2.2 remains unknown.

Methods: The genomic DNA of two 46,XY DSD patients was analysed using whole exome sequencing. Furthermore, protein/DNA interaction studies were performed using DNA adenine methyltransferase identification (DamID) to identify putative binding partners of CBX2. Finally, in vitro functional studies were used to elucidate the effect of wild-type and variant CBX2.2 on selected downstream targets.

Results: Here, we describe two patients with features of DSD i.e. atypical external genitalia, perineal hypospadias and no palpable gonads, each patient carrying a distinct CBX2.2 variant, p.Cys132Arg (c.394T>C) and p.Cys154fs (c.460delT). We show that both CBX2.2 variants fail to regulate the expression of genes essential for sexual development, leading to a severe 46,XY DSD defect, likely because of a defective expression of EMX2 in the developing gonad.

Conclusion: Our study indicates a distinct function of the shorter form of CBX2 and by identifying several of its unique targets, can advance our understanding of DSD pathogenesis and ultimately DSD diagnosis and management.

KEYWORDS
CBX2, DamID, DSD, EMX2, gonad development

1 | INTRODUCTION

Disorder of sex development (DSD) combines a broad spectrum of different phenotypes, defined as congenital conditions in which the development of the gonad and/or anatomical sex is atypical. Current data indicate that approximately 1 in 4,500 birth exhibit genital anomalies, for which in only around 50% of cases a causal gene variant can be found (Hughes et al., 2006). One of the genes implicated in DSD is *CBX2/M33*, a member of the Polycomb group (PcG) proteins, that are conserved regulatory factors initially discovered in *Drosophila*, best known for

†These authors contributed equally to this work.

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their role in maintaining silent expression states of Hox genes during development by regulating chromatin structure and chromosome architecture at their target loci.

Knocking out M33 in mice affected the development of the genital ridge in both XX and XY embryos, suggesting that it functions upstream of Sry. The majority of XY mice showed sex reversal, whereas ovarian development was impaired in XX animals (Katoh-Fukui et al., 1998). Cbx2 is able to activate Sf1 expression during spleen and adrenal development, suggesting that Cbx2 in mice has a role as transactivator, distinct from its chromatin modifier function (Katoh-Fukui et al., 2006). Additionally, it has been shown that forced expression of Sry or Sox9 in Cbx2 KO mice could rescue to sex reversal in XY mice, although they presented with smaller gonads compared to WT mice (Katoh-Fukui et al., 2012). Katoh-Fukui et al. concluded that Cbx2 may regulate testis determination by regulating, directly or indirectly, Sry and additionally might influence gonadal size through the regulation of other genes. Our group made the discovery of a loss-of-function double heterozygote CBX2.1 variant in a 46, XY girl with ovarian-like tissue at histology, normal uterus and external female genitalia, accidentally diagnosed because of a discrepancy between prenatal karyotype and phenotype at birth. Functional studies demonstrated that the CBX2.1 variant does not properly bind to and does not adequately regulate the expression of target genes essential for sex development such as *SF1/NR5A1*. Our data identified CBX2.1 as essential for normal human male gonadal development, suggested that it lies upstream of SRY in the human sex development cascade and identified a novel autosomal recessive cause of DSD. From a more mechanistic point of view, we demonstrated that CBX2.1 might have a role as transactivator, distinct from its known function as chromatin-modifier (Biason-Lauber, Konrad, Meyer, DeBeaufort, & Schoenle, 2009).

The function of the shorter isoform CBX2.2 is not yet well-explored. To study this isoform, we investigated the c.394T>C (p.Cys132Arg) and the c.460delT (p.Cys154fs) CBX2.2 variants, from two individuals with 46, XY DSD, characterized by atypical external genitalia and dysgenetic testis.

2 | MATERIALS AND METHODS

2.1 | Subjects

The study was approved by our institutional ethics committee and written informed consent was given.

2.1.1 | Patient one

The first patient, a white 9 months-old girl born at term and small for gestational age (2.300 g), was referred at the outpatient clinic of Hospital das Clínicas of University of Sao Paulo, with atypical external genitalia noticed at birth, characterized by microphallus (2.5 cm), perineal hypospadias and absence of palpable gonads. The karyotype was 46, XY. No Müllerian derivatives were found at pelvic ultrasonography and retrograde uretrocistography showed a blind vagina. At 2 years of age, a human chorionic gonadotropin (hCG) stimulation test was performed (two doses of 2,000 U) and no testosterone increase and steroid precursor accumulation was found. Since childhood, the patient showed a male behavior and after psychological evaluation, changed to male social sex at 5 years of age. He had a normal mental development. At 10 years of age he was submitted to exploratory laparotomy which disclosed bilateral atrophic testis that were removed. Anatomopathological data showed dysgenetic testes characterized by immature tubules with Sertoli cells only and a few atypical spermatogonias. No Leydig cells were identified in the interstitium. By 17 years of age, he started androgen replacement with testosterone esters. At this time his penile size was 12 × 3 cm, serum LH level was 16 U/L, FSH level was 54 U/L, and testosterone level was 230 ng/dl 14 days after exogenous testosterone (NV LH: 1,4–9,2 UI/L; FSH: 1,0–12 UI/L. Total testosterone: 271–965 ng/dL).

2.1.2 | Patient two

The second patient was a white 34 years of age male at the time of referral to the outpatient clinic of Hospital das Clínicas of University of Sao Paulo. He presented with atypical genitalia, characterized by microphallus (5.0 cm), bilateral cryptorchidism, perineal hypospadias and scrotum biphidus. 46, XY karyotype was confirmed by chromosome analysis and laboratory evaluation showed hypergonadotropic hypogonadism with LH levels of 10 U/L, FSH levels of 78 U/L, and testosterone levels of 75 ng/dL. A gonadectomy was performed, which revealed no gonadal tissue on histology evaluation and furthermore, a hypoplastic uterus was found. Genitoplasty, followed by testicular prosthesis implant was performed. In the meantime, testosterone replacement was started by using testosterone cypionate. The final penile length was 10 cm.

2.2 | Whole exome sequencing

Genomic DNA samples from both patients were analysed by whole exome sequencing (WES) on a SureSelect Human All Exon V.6.0 capture kit (Agilent, Santa Clara, California, USA) and a HiSeq 4000 Sequencer (Illumina, San Diego, California, USA). The reads were screened with FastQ Screen (Babraham Bioinformatics, Cambridge, UK) for possible contamination and a quality control has been performed with FastQC (Babraham Bioinformatics). Read

alignment has been performed with Bowtie 2 (Johns Hopkins University) against a human reference sequence (GRCh37.p13/hg19). GATK HaplotypeCaller (Broad Institute) was used for calling SNPs and indels, and the aligned data was visualized with the Integrative Genomics Viewer (Broad Institute). Genes with known variants causing similar phenotypes were screened first (e.g. *SRY*, *NR5A1/SF1*, *DMRT1*, *TCF21*, *GATA4*, *DHH*, *FGF9*, *FGFR2*, and *PGD2*). Then, a list of 85 genes known to be involved in 46,XY and 46;XX DSD in general, was used to further screen the WES data (Supporting information Table S1) (Baetens et al., 2017). Finally, additional potential candidate variants were selected based on occurrence in the general population (MAF < 0.5% in ExAC) and on the predicted influence on the protein level (SIFT, PolyPhen, Meta-SNP, PhD-SNP and SNAP).

2.3 | Genome-wide analysis of CBX2.2 binding sites

In order to study the functional consequences of the variants, we first had to identify potential targets of CBX2.2 in NT2/D1 (ATCC CRL-1973) Sertoli-like cells. Even though they are pluripotent cells, these cells express most of the important Sertoli-cell markers (including SOX9, SF1 etc.) (Knower et al., 2007). To analyse protein/DNA interaction we used the DamID-seq method that couples next-generation sequencing to DamID (DNA adenine methyltransferase identification) assay (Eid, Opitz, & Biason-Lauber, 2015). DamID is a methodology independent of antibodies, fixation, chromatin, shearing and other technically challenging features of Chromatin Immuno Precipitation (ChIP), and is a powerful tool for the mapping of genomic binding sites of chromatin binding proteins (Vogel et al., 2006). Briefly, human CBX2.2 cDNA (Origene, Rockville, Maryland, USA) was amplified and cloned into a pENTR11 plasmid (Invitrogen, Carlsbad, California, USA), then recombined into the destination vector pLgw-V5-EcoDam-RFC1 to generate pLgw-V5-EcoDam-CBX2, using the Gateway LR Clonase II enzyme mix according to the manufacturer's directions (Invitrogen). Lentiviral packaging plasmids, pRSV-Rev (Addgene, plasmid 12253), pCMV-dR8.2 (Addgene, plasmid 8455) and pMD2.G (Addgene plasmid 12259) were obtained from Addgene. DamID was performed using a lentiviral transduction protocol as previously described (Vogel, Peric-Hupkes, & van Steensel, 2007), using pLgw-EcoDam-V5-CBX2 (EcoDam-CBX2) or pLgw-V5-EcoDam (Dam-only). EcoDam-CBX2 or Dam-only were transfected into HEK239T cells (ATCC CRL-11268) together with the packaging plasmids (pRSV-Rev, pCMV-dR8.2, pMD2.G) using Metafectene (Biontex Laboratories, Munich, Germany). 48 hr later, the supernatant containing the lentivirus was harvested and added to NT-2D1 cells with 1 mg/ml polybrene (Santa Cruz Biotechnology, Dallas, Texas, USA), in two consecutive rounds of transduction. Two days after the first infection, genomic DNA was isolated using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany). The isolated genomic DNA was subject to DpnI digestion, ligation of DamID adaptors, and DpnII digestion. Samples without addition of DpnI or ligation adaptors served as negative controls. DpnII-digested fragments were used as templates to amplify methylated genomic fragments. DNA libraries were prepared using the TruSeq DNA LT Sample Prep Kit (Illumina) and the libraries were sequenced on a HiSeq2000 sequencer (Illumina). Cells expressing free Dam protein served as a control to correct for the unspecific DNA accessibility by Dam alone and for sequencing biases.

2.4 | Immunofluorescence

Immunofluorescence was performed as previously reported (Eid et al., 2010). Briefly, cells grown on cover slips were pre-extracted for 5 min on ice using 25 mM HEPES (Sigma-Aldrich, St. Louis, Missouri, USA) pH 7.4, 50 mM NaCl (Acros Organics, Geel, Belgium), 1 mM EDTA (Acros Organics), 3 mM $MgCL_2$ (Sigma-Aldrich), 300 mM sucrose (Sigma-Aldrich), and 0.5% Triton X-100 (Sigma-Aldrich) before fixation in 4% (w/v) formaldehyde (Sigma-Aldrich) in PBS (Thermo Fisher Scientific, Waltham, Massachusetts, USA) for 15 min at room temperature (RT). Cover slips were incubated overnight at 4°C with primary antibodies anti-V5 antibody (Santa Cruz Biotechnology) and Alexa-conjugated secondary antibodies (Invitrogen) for 1 hr at RT. The cover slips were mounted with Vectrashield (Vector Laboratories, Burlingame, California, USA) containing DAPI. Images were acquired using a Nikon eclipse Ni-E microscope system.

2.5 | Functional analysis of the CBX2.2 mutants

We generated the p.Cys132Arg and p.Cys154fs variant CBX2.2s by site directed mutagenesis of wild-type cDNA (Origene), according to the manufacture's manual (Quickchange II Site-Directed Mutagenesis Kit; Agilent).

The influence of WT and CBX2.2 variants on the expression of selected candidate genes was studied by means of quantitative real-time PCR, performed with ABI StepOnePlus Real-Time PCR system, PCR products were quantified fluorometrically using the KAPA SYBR FAST master mix (KAPA Biosystems, Wilmington, Massachusetts, USA). The reference mRNA from cyclophilin was used for data normalization. Primers sequence and qRT-PCR conditions for CBX2 isoforms and putative targets are available upon request. All samples were run in triplicates

and finally the normalized relative expression values ($2^{-\Delta\Delta C_t}$) of multiple independent experiments were plotted against the relative expression values of the empty vector which was set as 1. Unpaired *t*-test was performed using GraphPad Prism version 6.07 for Windows (GraphPad Software, La Jolla, California, USA).

2.6 | Gene-ontology enrichment analysis

ToppCluster was used for GO-enrichment analysis of CBX2.2 Dam-ID target genes (Kaimal, Bardes, Tabar, Jegga, & Aronow, 2010). GO-enrichment permits to analyse functional features of gene sets, clustering them by their involvement in pathways related to Molecular Function, Biological Process and/or Cellular Component. GO-terms with *p*-values ≤0.05 and more than five target genes associated to the corresponding GO-term were defined as significant. CBX2.2 Dam-ID target genes were clustered depending on GO-terms and visualized using spring-embed layout with Cytoscape v3.3.0 (Shannon et al., 2003).

3 | RESULTS

3.1 | Identification of CBX2.2 mutations

No variants in the coding sequence of genes potentially causing a similar 46, XY DSD phenotype were found in the WES data of both patients including *SRY*, *TCF21*, *GATA4*, *DHH*, and *PGD2*. Patient one carries a *DMRT1* variant (c.133T>A, p.Ser45Thr), a synonymous FGF9 variant (c.1284A>G, p.Ser149Ser) and a synonymous FGFR2 variant (c.1343A>G, p.Val232Val), all common in the general population and predicted to be tolerable (Table 1). The second patient carries the same *DMRT1*, *FGF9*, and *FGFR2* variants as patient one and two *SF1/NR5A1* variants (c.437G>C, p.Gly146Ala, and c.499C>T, p.Pro125-Pro), which are also common in the general population and predicted to be tolerable (Table 1).

Screening the 85 genes implicated in 46, XY and 46XX DSD, revealed a c.394T>C variant in *CBX2.2*, leading to a p.Cys132Arg missense exchange in patient one and a c.460delT deletion, leading to a p.Cys154fs frameshift in patient two (Figure 1). The corresponding refSNP cluster ID numbers are rs782325368 and rs552892809 respectively. Both variants are rare, with a MAF on ExAC of 0.00001647 (p.Cys132Arg) and 0.001418 (p.Cys154fs) and are predicted to be disease causing. *CBX2.1* of patient one carries two known allelic variants (rs71368052 and rs3751956; Figure 1). ExAC predicts for *CBX2* a pLI (probability of LOF intolerance) of 0.95 (0 = gene tolerant to LOF; 1 = gene completely intolerant to LOF).

The WES data were further analysed to find other rare variants potentially causing the observed phenotype. Besides CBX2.2, there are 27 and 30 other gene variants in both patients, respectively, which were predicted to have a high to moderate impact (missense-variants, frameshift-variants, stop-gained or splice acceptor/donor-variants) and are rare (MAF < 0.5%) (Supporting information Tables S2 and S3). The unavailability of the parents DNAs prevented us from further filtering the variants. To analyze the putative relevance of these variants for the clinical phenotype, with the help of Pathway Studio 11 (Elsevier), we searched for connection between these genes with rare variants and female gonad development, male gonad development, gonad development, sex development, and sex determination. According to the literature, the only candidate gene implicated in these processes is CBX2, allowing us to conclude that the CBX2.2 variants are the most likely candidates for the observed phenotypes of the patients and prompted us to further analyze these variants.

3.2 | Identification of CBX2.2 genomic targets

To analyze the pathophysiological consequences of the CBX2.2 variants, we had to face the challenge of not knowing any targets for CBX2.2 and the consequent lack of functional assays. To gain insights into the function of CBX2.2 we applied the DamID-seq method to identify its direct targets. We prepared two replicates for CBX2.1 and CBX2.2 fusion proteins or free Dam and sequenced the DNA libraries separately. Immunofluorescence analysis showed that the CBX2.2-Dam construct correctly localized to the nucleus (Figure 2). All pairs of replicates had highly correlated read densities along the genome (Pearson correlation coefficients >0.90, *p*-value <2e-16) and thus were combined for the follow-up analyses. Approximately 30% of CBX2.2 binding peaks were found in the promoter region, defined as 5Kb upstream of transcription start sites (Supporting information Figure S1). After normalizing the data to the controls, we identified 1901 enriched binding sequences of CBX2.2 within the promoter region of various genes, with a *p*-value <1e-5, of which 451 were shared with CBX2.1 and were excluded from further analysis. *SOX9* is one of the common targets and has also been excluded since it can be assumed that CBX2.1 might compensate for CBX2.2 deficiency. In silico bioinformatic analysis using Pathway Studio 11 (Elsevier) identified six potential CBX2.2 unique targets. Target selection was based on their reported putative involvement in sexual differentiation, their role in human disease and animal models or their specific expression in issues involved in sex development (gonads, sex organs, hypothalamus, and pituitary), as previously reported (Eid et al., 2015). Empty spiracles homeobox 2 (*EMX2*) (Simeone et al., 1992), Male germ-cell associated kinase (*MAK*) which is exclusively

TABLE 1 Variants found by whole exome sequencing in genes known to be related in 46, XX and 46, XY DSD for both patients

	Genes	Zygosity	AA. Sub.	SIFT	PhD-SNP	SNAP	Meta-SNP	PolyPhen	ExAC (MAF)	rs ID
Common mutations	LEPR	Hom	Q223R	0.28	0.363	0.665	0.15	0.047	0.5103	rs1137101
	LHX4	Het	N328S	1	0.063	0.45	0.068	0.004	0.485	rs7536561
	KISS1	Het	P81R	0.28	0.016	0.61	0.075	0.972	0.3413	rs4889
	AKR1C4	Hom	Q250R	1	0.324	0.39	0.437	0.001	0.2055	rs17306779
	WWOX	Hom	A179T	0.39	0.221	0.385	0.38	0.139	0.4507	rs11545029
	AMH	Hom	V515A	0.77	0.044	0.345	0.044	0	0.9799	rs10417628
	FBLN2	Het	T854A	0.3	0.102	0.465	0.266	0.012	0.7364	rs9843344
	SRD5A1	Het	A39G	—	0.091	0.255	0.044	0.001	0.6079	rs248793
	MAP3K1	Hom	D806N	0.19	0.396	0.555	0.112	0.092	0.5984	rs702689
	MAP3K1	Hom	V906I	0.76	0.137	0.51	0.059	0	0.7648	rs832582
	PROP1	Het	A142T	0.66	0.087	0.345	0.104	0.001	0.5113	rs4072924
	ZFPM2	Het	A403G	1	0.016	0.37	0.1	0	0.1146	rs11993776
	ZFPM2	Het	A1055V	0.16	0.093	0.535	0.17	0.046	0.01771	rs16873741
	DMRT1	Het	S45T	0.12	0.078	0.48	0.058	0.024	0.2678	rs3739583
	HSD17B3	Het	G289S	1	0.448	0.495	0.239	0	0.073306	rs2066479
	FGFR2	Hom	V232V	—	—	—	—	—	0.7797	rs1047100
	FGF9	Hom	S149S	—	—	—	—	—	0.8398	rs9509841
Patient one	PBX1	Hom	G21S	0.12	0.106	0.356	—	0.049	0.2977	rs2275558
	AMH	Hom	S49I	0	0.206	0.625	0.137	0.068	0.7876	rs10407022
	FSHR	Het	S654N	0.77	0.164	0.49	0.127	0	0.5727	rs6166
	FSHR	Het	A281T	0.5	0.113	0.47	0.187	0.005	0.5446	rs6165
	HHAT	Het	S182N	0.13	0.78	0.725	0.71	0.986	0.1439	rs2294851
	INSL3	Hom	T60A	1	0.051	0.115	0.05	0	0.6451	rs6523
	CYP21A2	Het	R103K	1	0.153	0.62	0.313	0.005	0.3341	rs75730897
	POR	Het	A503V	0.37	0.164	0.435	0.215	0	0.3639	rs1057868
	CYP11B1	Het	R43Q	1	0.219	0.48	0.27	0	0.08037	rs4534
	MAMLD1	Hom	P334S	0.02	0.206	0.565	0.178	0.22	0.08321	rs41313406
	MAMLD1	Hom	N662S	0.19	0.202	0.635	0.213	0.007	0.1089	rs2073043
Patient two	FBLN2	Hom	N839K	0.74	0.299	0.28	0.166	0.627	0.001426	rs61731214
	RXFP2	Het	I580V	0.27	0.122	0.53	0.225	0.02	0.1487	rs17076657
	NOBOX	Het	K664R	0.63	0.059	0.48	0.048	0.003	0.02763	rs77802098
	ZFPM2	Het	S657G	0.28	0.023	0.680	0.102	0.031	0.01258	rs28374544
	NR5A1	Het	G146A	0.5	0.187	0.43	0.246	0.001	0.1183	rs1110061
	NR5A1	Het	P125P	—	—	—	—	—	0.02645	rs1110062
	BMP15	Hom	S5R	0.01	0.736	0.23	0.475	0.235	0.01587	rs113099187

Variants are sorted into common between both patients, variants specific for patient one and variants specific for patient two. The effects of the variants on the protein level were predicted by SIFT, PhD-SNP, SNAP, Meta-SNP, and PolyPhen. Scores predicting a damaging effect of the variant are colored in red. In order to determine the occurrence in the general population, ExAC was used (rare: MAF < 0.005).

expressed in testis and potentially important for spermatogenesis (Matsushime, Jinno, Takagi, & Shibuya, 1990), Homeobox A13 (*HOXA13*), a transcription factor which has been linked to syndromes affecting genitourinary development (Scott, Morgan, & Stadler, 2005), WD repeat-containing protein 77 (*WDR77*) also known as androgen receptor-associated protein, a 44-kd protein interacting with Polycomb and a component of a methyltransferase complex involved in testis development and tumor formation (Liang et al., 2007), Twist Family BHLH Transcription Factor 1 (*TWIST1*), a HAT binding protein that also interacts with Polycomb proteins, was found to be related to androgen

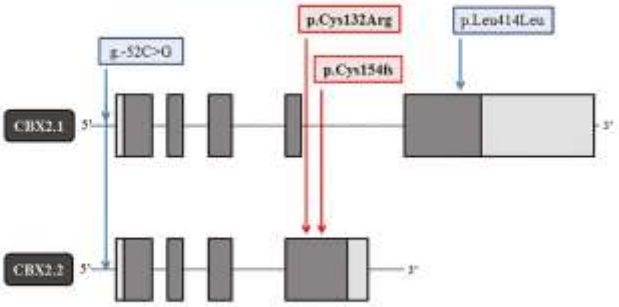

FIGURE 1 Schematic representation of the gene organization of both CBX2 isoforms. The CBX2.2 variants are depicted in red (Patient one: p.Cys132Arg; Patient two: p.Cys154fs). The two known SNPs found in CBX2.1, g.-52C>G (rs71368052) and p.Leu414Leu (rs3751956), are depicted in blue

receptor expression (Shiota et al., 2010) and Basonuclein 2 (*BNC2*), which has been linked to hypospadias and distal urethral development (Bhoj et al., 2011). The analysis of the expression of these genes was used as an assay for WT versus mutant CBX2.2 function (see below).

3.3 | Gene ontology analysis of CBX2.2 targets

In order to retrieve a functional profile of the high-throughput gene sets and therefore better understand the underlying biological processes, we performed an unbiased Gene Ontology enrichment analysis of the DamID targets. CBX2.2 targets showed an over-representation of Gene Ontology terms related to development and morphogenesis (Figure 3), mainly in generic developmental terms like embryo development, heart development, and neuron development. Of particular interest for us, the analysis showed that the CBX2.2 targets are also overrepresented for the Gene Ontology term urogenital system development, additionally demonstrating the involvement of CBX2.2 in human sex development.

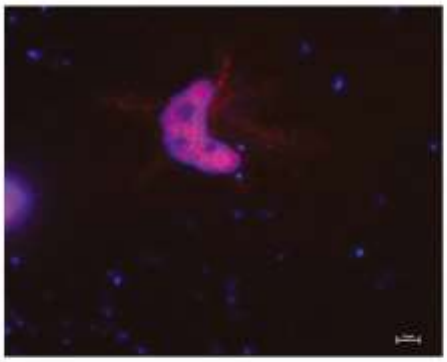

FIGURE 2 Dam-CBX2.2 localization in NT2-D1 cells. NT2-D1 cells were transfected with the pLgw-EcoDam-V5-CBX2 (Dam-CBX2) and 48 hr later cells were detergent-extracted and fixed. Dam-CBX2 was visualized by indirect immunofluorescence using a V5 antibody, nuclei were visualized by DAPI. Bar 10 μm

3.4 | Functional characterization of CBX2.2 variants

To independently validate our findings, we performed functional studies using quantitative Real-time PCR (RT-qPCR) for the six selected targets. For the first variant (p.Cys132Arg), WT CBX2.2 increased the expression of EMX2, MAK, HOXA13, WDR77, and BNC in NT2-D1 cells significantly ($p < 0.05$) by 3.2, 1.9, 2.0, 1.5, and 1.6, respectively, compared to the EV transfection (Figures 4 and 5). Using the CBX2.2 variant, the effect on EMX2, MAK, and HOXA13 significantly ($p < 0.05$) diminishes to expression values of 1.3, 1.0, and 0.8, with the expression of the target genes behaving essentially like the control, indicating that the effect is CBX2.2-specific (Figure 4). Overexpression of the first CBX2.2 variant (p.Cys132Arg) increased the expression values for WDR77, BNC2, and TWIST1, compared to the EV, to 1.1, 1.3, and 1.0 respectively (Figure 5). However, they show no significant difference in expression under either WT or CBX2.2 variant expression. Similarly, by transfecting of the CBX2.2 variant found in the second patient (p.Cys154fs), the effect on the expression of EMX2, MAK, and HOXA13 also diminished significantly to expression values of 1.0, 0.8, and 0.7, again behaving essentially like the control (Figure 4). The expression values for WDR77, BNC2, and TWIST1 changed to 3.5, 3.5, and 1.3, respectively, when overexpressing the second CBX2.2 variant (p.Cys154fs) (Figure 5). Similar to the first variant, there is no difference in expression under either WT or the second CBX2.2 variant. The CBX2.2 variant mRNAs were expressed at a level similar to that of the wild-type mRNA, suggesting that the variants do not influence mRNA stability. In order to ensure that the effect of CBX2.2 on EMX2 is isoform

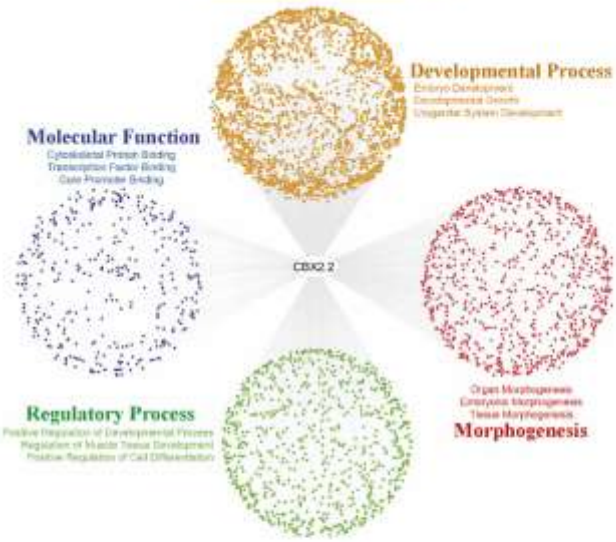

FIGURE 3 Representation of Gene Ontology (GO) enrichment analysis by Cytoscape. Every dot represents a gene related to the enriched GO-terms, which have been clustered depending on their function. In orange are the GO-terms represented which are involved in developmental processes, in red all GO-terms related to morphogenesis, in green regulatory processes and in blue are the GO-terms involved in molecular function

specific, we performed overexpression studies of CBX2.1 in NT2-D1 cells. Overexpression of CBX2.1 showed no effect on EMX2 expression (Supporting information Figure S2).

4 | DISCUSSION

Our patients represent the first two known variants in CBX2.2, the less known isoform of CBX2, associated to 46, XY DSD. In mice and men, loss-of-function of CBX2/M33, causes male-to-female and 46, XY DSD (MIM 613080) respectively. CBX2 isoform 1 was shown to be a transcription factor aside from its role as chromatin modifier and we could established that CBX2 isoform 1 regulates sex development by stimulating the male-specific (e.g. *SF1* and *SOX9*) and inhibiting the female-specific factors (such as *FOXL2*) (Biason-Lauber et al., 2009; Eid et al., 2015). As for isoform 2, no function was known, mainly because of the lack of animal models, as there is no evidence of a CBX2.2 homologue in any other mammalian, including other primates, and the absence of human patients with CBX2.2 specific defects. The identification of the first such 46, XY DSD patients allowed us to investigate the function of CBX2.2.

Analysis of WES from the patients genomic DNA, showed no rare (MAF < 0.005) and potentially damaging variants related to 46, XY DSD, besides *CBX2* (Table 1). Furthermore, none of the 26 genes carrying rare variants (MAF < 0.005) in both patients and are predicted to have a high to moderate pathogenic impact, had any reported link to sex development or had previously been mentioned in DSD (Supporting information Tables S2, and S3) as analyzed using Pathway studio. This allows us to state that the CBX2.2 variants are the most likely cause for the observed phenotype of the patients.

For the identification of potential CBX2.2 binding targets and subsequent functional analysis of CBX2.2 variant function, we used the Sertoli-like NT2/D1 cells, currently still the best available human cell model to study Sertoli-cell development.

Using whole genome protein/DNA interaction and next generation sequencing approaches, we identified a large number of direct and unique potential targets of CBX2.2. Of all the CBX2.2 specific targets, the most relevant for sex development seems to be *EMX2*. *EMX2* is a paired-like homeobox transcription factor, homologue of the Drosophila empty spiracle gene (Simeone et al., 1992). In the mouse, *Emx2* is essential for the development of several organs/tissues, such as the kidney and the urogenital system (Miyamoto, Yoshida, Kuratani, Matsuo, & Aizawa, 1997) and the central nervous system (CNS) (Galli et al., 2002; Gangemi et al., 2001; Heins et al., 2001; O'Leary, Chou, & Sahara, 2007). The absence of neurological impairment usually associated to *EMX2* variants (Granata et al., 1997) or *EMX2* haploinsufficiency (Piard et al., 2014) in our patients, might suggest that CBX2.2 regulates EMX2 expression specifically in the developing gonad and

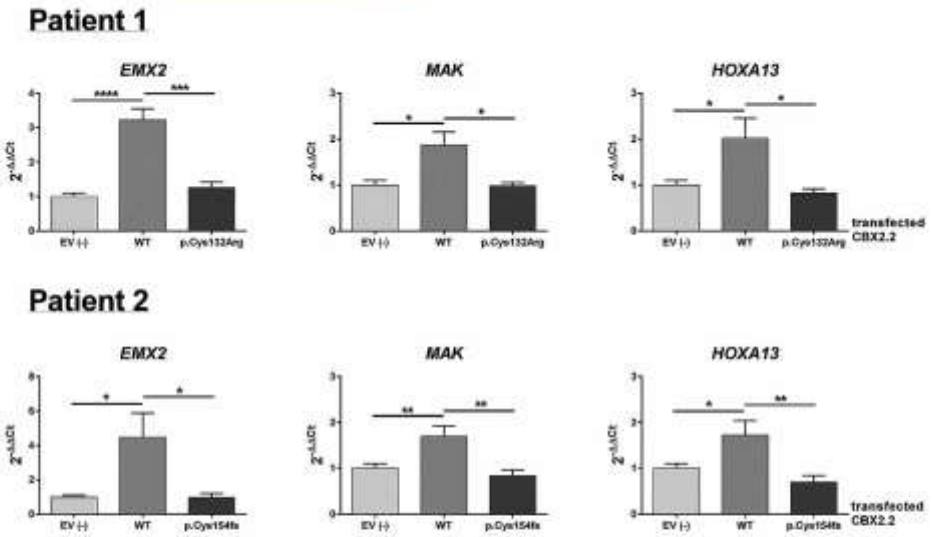

FIGURE 4 qRT-PCR quantification of mRNA levels for the putative CBX2.2 targets EMX2, HOXA13, and MAK. NT2-D1 cells were transfected with empty vector (EV), wild-type (WT) CBX2.2 or CBX2.2 variants (p.Cys132Arg or p.Cys154fs) from patient number one or patient number two. Relative expression levels ($2^{-\Delta\Delta Ct}$) of target genes were determined by qRT-PCR after normalization to cyclophilin as an endogenous control. Following WT CBX2 overexpression, the relative expression of EMX2, HOXA13 and MAK, was upregulated compared to cells transfected with empty vector (EV). Introducing either variant of CBX2.2 (p.Cys132Arg or p.Cys154fs) into the cells showed an effect comparable to negative control (i.e. no effect) on the expression of EMX2, HOXA13, and MAK, suggesting that failed regulation of these targets might contribute to the pathophysiology of the disease. The data in all graphs are the average of three independent experiments, error bars represent standard deviation from the mean (SEM) and values are expressed as relative to control = 1. (****) $p < 0.0001$; (***) $p < 0.001$; (**) $p < 0.01$; (*) $p < 0.05$

not the CNS. *Emx2* XY KO mice have gonadal agenesis (Miyamoto et al., 1997) due to proliferative defects of the coelomic epithelium, which gives rise to Sertoli cells. This implicated Emx2 in polarity of epithelial cells and their transition to mesenchymal cells, most likely through EGFR downregulation (Kusaka et al., 2010). Emx2 expression is stimulated by Wnt and Bmp signalling in nervous system development (Theil, Aydin, Koch, Grotewold, & Ruther, 2002) and it is repressed by HOXA10 during development of the endometrium of mice and humans (Daftary & Taylor, 2004; Troy, Daftary, Bagot, & Taylor, 2003) but very little is known about the control of *EMX2* in the developing gonad. In patients, deletions encompassing *EMX2* cause 46,XY DSD ranging from hypospadias to gonadal dysgenesis (Piard et al., 2014), confirming its role in human gonadal development (MIM 269160). In human development, EMX2 and CBX2 are simultaneously expressed in the gonadal anlage at 7 weeks of gestation, i.e. prior to the expression of SRY and testis determination, suggesting a role in the formation of the early gonad (Ostrer, Huang, Masch, & Shapiro, 2007). Subsequently, CBX2 is highly expressed in both sexes at 8 GW (but higher in female than male) then slightly decreases in male until basal, but still detectable expression level at 18 GW (E. Lecluze, F. Chalmel, INSERM-Rennes, personal communication). In agreement with our data, Emx2 was also found downregulated in Cbx2-deficient mouse gonads (Katoh-Fukui et al., 2012). Based on our results, it is intriguing to hypothesize that CBX2.2 variants that impair expression of EMX2 might lead to severe defects of gonadal development in 46, XY individuals, similar to those individuals with *EMX2* haploinsufficiency (Piard et al., 2014) and might explain the severe testicular dysgenesis phenotype of the present CBX2.2 deficient patients, different from the ovarian-like gonadal phenotype found in the 46,XY DSD CBX2.1 deficient patient (Biason-Lauber et al., 2009).

Another target of CBX2.2 is HOXA13, a homeobox protein whose variants lead to and hand-foot-genital syndrome (also known as hand-foot-uterinesyndrome) or the related Guttmacher syndrome, dominantly inherited

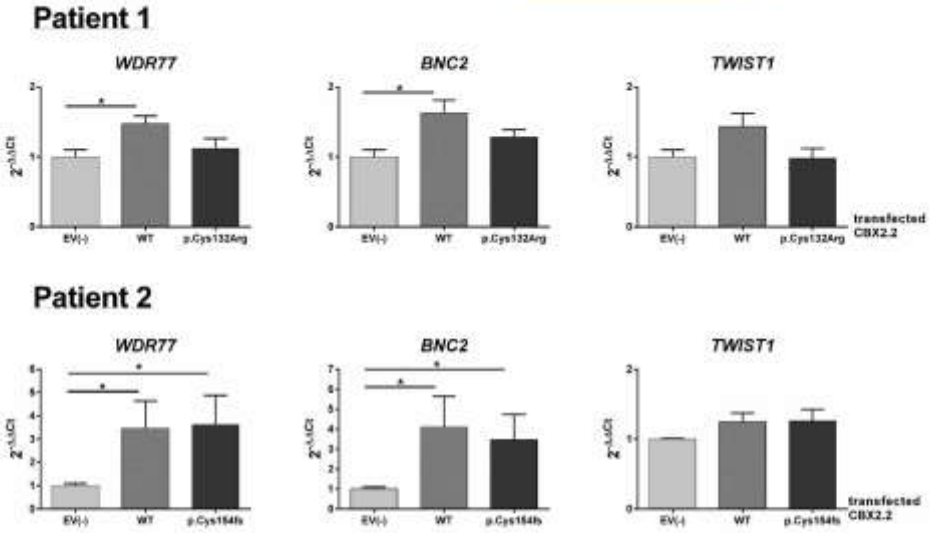

FIGURE 5 qRT-PCR quantification of mRNA levels for the putative CBX2.2 targets WDR77, BNC2, and TWIST1. NT2-D1 cells transfected with empty vector (EV), wild-type (WT) CBX2.2 or CBX2.2 variants (p.Cys132Arg or p.Cys154fs) from patient number one or patient number two. Relative expression levels ($2^{-\Delta\Delta C_t}$) of target genes were determined by qRT-PCR after normalization to cyclophilin as an endogenous control. Following WT CBX2 overexpression, the relative expression of WDR77 and BNC2 was up-regulated compared to cells transfected with empty vector (EV). Both CBX2.2 variants had stimulatory effects similar to those of WT CBX2.2 on the expression level of WDR77, BNC2, and TWIST1, likely excluding them from the mechanism of disease. The data in all graphs are the average of three independent experiments, error bars represent standard deviation from the mean (SEM) and values are expressed as relative to control = 1. (*) $p < 0.05$

conditions characterized by Müllerian duct fusion defects of varying degree in females and hypospadias in males (Mortlock & Innis, 1997; Scott et al., 2005) (MIM 140000). *Hoxa13* mutant mice show loss of Fgf8 and Bmp7 expression and a decrease of the androgen receptor in male genital organs (Morgan, Nguyen, Scott, & Stadler, 2003) implicating *Hoxa13* in male sex differentiation and might partly explain the patients' phenotype. This suggests that the mechanism of sex disorder in these CBX2.2-deficient patients might affect the sex developmental processes primarily through impaired sex/gonadal developement due to failure to regulate *EMX2*.

Our data suggest that both CBX2 isoforms are important for human sex development but they apparently have different functions, as shown by the difference in gonadal phenotype between loss-of-function of CBX2.1, which leads to residual gonadal development (Biason-Lauber et al., 2009) and loss-of-function of CBX2.2, which leads to the here described 46, XY gonadal dysgenesis. DamID-Seq analysis underlines the difference in function, since CBX2.1 and CBX2.2 only share about 25% of direct binding sequences.

There are other examples of the involvement of multiple isoforms in gonad development, the most prominent one being Wilms Tumor 1 (WT1). *WT1* codes for different isoforms, most important for gonad development are WT1−KTS and WT1+KTS, which differ only by the three amino acids Lysine (K), Threonine (T) and Serine (S). It has been shown that WT−KTS is required for gonad primordium survival, while the +KTS form seems mainly involved in the determination of the male pathway (Wilhelm & Englert, 2002).

The same appears to be the case for *CBX2*, as each isoform presents with distinct functions during gonad development. While CBX2.2 seems to be important for early bipotential gonad development, CBX2.1 seems to be mainly involved in testis determination. Nothing is known about the transcriptional control of *CBX2*, which could further help to elucidate the reason behind the different function of the isoforms and their spatiotemporal expression. However, alternative splicing and the existence of multiple isoforms with different functions during sex development and development in general, appear to be an important mechanism to control gene/protein cascades and

differentiation pathways. Functional studies and analysis of spatiotemporal expression-differences for isoforms could expand our current knowledge about sex development and further characterization of how *CBX2* and its targets fit into an ever expanding sex developmental network can impact our understanding of DSD pathogenesis and ultimately DSD diagnosis and management.

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CONFLICT OF INTEREST

No conflict of interest declared.

ORCID

Patrick Sproll http://orcid.org/0000-0001-5968-2307

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Apêndice 4

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ORIGINAL ARTICLE

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Long-term outcomes and molecular analysis of a large cohort of patients with 46,XY disorder of sex development due to partial gonadal dysgenesis

Nathalia L. Gomes[1] | Antônio Marcondes Lerário[1,2] | Aline Zamboni Machado[1] | Daniela Rodrigues de Moraes[1] | Thatiana Evilen da Silva[1] | Ivo J. P. Arnhold[1] | Rafael Loch Batista[1] | José Antônio Diniz Faria Júnior[1] | Elaine F. Costa[1] | Mirian Y. Nishi[1] | Marlene Inacio[1] | Sorahia Domenice[1] | Berenice B. Mendonca[1]

[1]Unidade de Endocrinologia do Desenvolvimento, Laboratório de Hormônios e Genética Molecular/LIM42, Hospital das Clínicas, Disciplina de Endocrinologia, Faculdade de Medicina da Universidade de São Paulo, São Paulo, Brazil

[2]Department of Internal Medicine, Division of Metabolism, Endocrinology and Diabetes, University of Michigan, Ann Arbor, MI, USA

Correspondence
Berenice B. Mendonca, Divisão de Endocrinologia, Hospital das Clínicas, FMUSP, São Paulo, Brazil.
Email: berenen@usp.br

Nathalia L. Gomes, Divisão de Endocrinologia, Hospital das Clínicas, FMUSP, São Paulo, Brazil
Email: nathalialisboa.endocrino@gmail.com

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Summary

Background: Follow-up data on patients with 46,XY partial gonadal dysgenesis (PGD) until adulthood are scarce, making information on prognosis difficult.

Objective: To analyse the long-term outcomes of patients with 46,XY PGD regarding testosterone production, germ cell tumour risk, genotype and psychosexual adaptation.

Methods: A retrospective longitudinal study of 33 patients (20 assigned male and 13 patients assigned female at birth). Molecular diagnosis was performed by Sanger sequencing or by targeted massively parallel sequencing of 63 genes related to disorders of sex development (DSDs).

Results: Age at first and last visit ranged from 0.1 to 43 and from 17 to 53 years, respectively. Spontaneous puberty was observed in 57% of the patients. During follow-up, six of them had a gonadectomy (four due to female gender, and two because of a gonadal tumour). At last evaluation, five of six patients had adult male testosterone levels (median 16.7 nmol/L, range 15.3-21.7 nmol/L) and elevated LH and FSH levels. Germ cell tumours were found in two postpubertal patients (one with an abdominal gonad and one patient with Frasier syndrome). Molecular diagnosis was possible in 11 patients (33%). *NR5A1* variants were the most prevalent molecular defects (n = 6), and four of five patients harbouring them developed spontaneous puberty. Gender change was observed in four patients, two from each sex assignment group; all patients reported satisfaction with their gender at final evaluation. Sexual intercourse was reported by 81% of both gender and 82% of them reported satisfaction with their sexual lives.

Conclusion: Spontaneous puberty was observed in 57% of the patients with 46,XY PGD, being *NR5A1* defects the most prevalent ones among all the patients and in those with spontaneous puberty. Gender change due to gender dysphoria was reported by 12% of the patients. All the patients reported satisfaction with their final gender, and most of them with their sexual life.

KEYWORDS
atypical genitalia, disorder of sex development, gonadal dysgenesis, puberty

1 | INTRODUCTION

The 46,XY disorder of sex development (DSD) due to gonadal dysgenesis is a congenital disorder caused by alterations in the complex process of gonadal determination.[1] There is a wide phenotype spectrum ranging from a partial form, characterised by variable degrees of external genitalia undervirilisation, development of Mullerian derivatives and testosterone production, to a complete form with female external and internal genitalia.

There are scarce data on long-term follow-up of 46,XY partial gonadal dysgenesis (PGD) patients, regarding spontaneous puberty,[2] risk of a gonadal tumour development[3] and gender adjustment, making it difficult to provide comprehensive information to parents.

Our aim was to describe the phenotype, genotype and long-term outcomes of a large cohort of patients with 46,XY PGD followed until adulthood.

2 | SUBJECTS AND METHODS

Thirty-three patients with 46,XY PGD were included in this retrospective longitudinal study conducted at Hospital das Clínicas of São Paulo (HCFMUSP). Twenty-six patients were initially evaluated at our service, and 7 had already had a previous genitoplasty and/or gonadectomy elsewhere. Written informed consent was obtained from all the patients. The clinical and molecular data from 8 patients were previously reported.[4-7]

Inclusion criteria were as follows: ≥17 years of age at last evaluation, a 46,XY karyotype in a G-banded karyotyping analysis of at least 30 peripheral blood lymphocytes, atypical genitalia associated with the presence of Mullerian derivatives and/or at least one gonad with histopathological features compatible with testicular dysgenesis. Data regarding sex assignment, age at first and last evaluation, external genitalia appearance, the hormonal profile throughout the follow-up and at last visit, pubertal development and gonadal tumour incidence were collected from medical records. Patients were assumed to be at prepubertal age if they were younger than 9 years, at pubertal age if they were 9.1-16 years old, and at adult age if they were older than 17 years. Micropenis is defined as a normally structured penis which in its fully stretched length is 2.5 standard deviations (SDs) below the mean for age[8] and microphallus is defined as a micropenis associated with hypospadias.

The external masculinisation score (EMS) was calculated as previously described.[9] To determine the hormonal profile, luteinising hormone (LH), follicle-stimulating hormone (FSH) and testosterone were measured by immunoradiometric or immunofluorimetric assays at the first and at each semi-annual follow-up visit. Spontaneous puberty was assumed if virilisation was observed in pubertal patients or was reported by the patients that came in adulthood in conjunction with the presence of pubertal signs (the presence of secondary sex characteristics, such as increased penile length and testis diameter > 2.5 cm, when they were palpable) and with male serum testosterone levels without the use of exogenous testosterone.

For prepubertal patients, a human chorionic gonadotropin (hCG) test was performed by means of 4 intramuscular injections at 50-100 IU/kg each, with a 4-day interval between the injections. A single dose of 5000 IU of hCG was administered to adult patients. Serum levels of testosterone were measured before and 72 hours after the last hCG injection.

Data on a gonadal histology were collected from medical records.

Physical evaluation of the palpable scrotal testes of male patients was performed at every medical visit (each semester or annually), and testicular ultrasonography was performed once every 2 years. If a suspicious nodule was identified, tumour markers (β-HCG, α-fetoprotein and carcinoma embryonic antigen) were tested, and a gonadectomy was indicated if needed.

Continuous variables were described as median and range. Differences in the categorical variables among the groups were analysed by the Chi-square test or Fisher's exact test, when appropriate. The Mann-Whitney *U* test served as a nonparametric test, and data with $P < .05$ were considered statistically significant. All analyses were performed in SPSS Statistics 24.0 software (Chicago, IL).

Evaluation of psychosexual characteristics was performed on 21 patients by a psychologist specializing in DSDs. Self-reported gender identity, the self-reported gender role in childhood, the desire to change gender and satisfaction with gender and with their sexual life were analysed via a questionnaire (see Appendix 1).

For molecular diagnosis, genomic DNA was obtained from peripheral blood leucocytes by the proteinase K–SDS salting-out method.[10] Six genes involved in testicular dysgenesis (*SRY*, *NR5A1*, *CBX2*, *MAPK3*, *FGF9* and *FGFR2*) were previously sequenced by the Sanger method in patients 1, 3, 10, 14, 26 and 30. Patients 15 and 31 had only *WT1* variants screened, considering their phenotypic features, as previously reported in great detail.[5,6] The entire coding region and the exon-intron boundary areas of each gene were PCR-amplified with specific primers. The PCR products were sequenced according to the protocol of the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Life Technologies Corporation, CA, USA) on an ABI Prism Genetic Analyzer 3130XL (Life Technologies Corporation, CA, USA).

Twenty-seven patients were analysed during the 2010s by targeted massively parallel sequencing. An amplicon-based capture panel was designed against exonic regions of 63 genes, including 43 genes already associated with human DSDs and 20 candidate genes involved in gonadal determination pathways or with a DSD phenotype in rats[1,11-18] (see Appendix 2). Capture of the target sequences was performed using a custom Sure Select Target Enrichment System Kit (Agilent). Sequencing was performed on the Illumina MiSeq platform. Paired-end reads (2 × 300) were aligned to the hg19 assembly of the human genome with BWA-MEM.[19] The aligned reads were sorted and converted to the BAM format using the bamsort tool from the biobambam2 suite (https://launchpad.net/biobambam2). Mean coverage was over 95× for all the samples, and more than 96% of the RefSeq gene coding regions was covered at 20× or greater. Single-nucleotide

variants and small insertions or deletions (indels) were simultaneously called in all samples in the Freebayes software (https://github.com/ekg/freebayes). Annotation of the variants was performed in ANNOVAR.[20] For prioritizing the most likely pathogenic variants, we filtered out those with a minor allele frequency >0.5% in available population databases Genome Aggregation Database (gnomAD),[21] 1000 Genomes,[22] and in the Brazilian population database ABraOM.[23] To assess the possible impact of the novel nonsynonymous variants on protein structure and function, we employed in silico algorithms (SIFT, PolyPhen2, Mutation Assessor, and CADD) and conservation scores (GERP++, PhyloP). These variants were considered deleterious when predicted as pathogenic by at least three algorithms. The variants were classified according to the American College of Medical Genetics and Genomics guidelines (ACMG).[24]

3 | RESULTS

The patients' age at first visit ranged from 10 days to 43 years (median 13 years), and at last visit, from 17 to 53 years (median 26.5 years). Follow-up ranged from 3 to 26 years (median 13.2 years). Nineteen patients had Mullerian derivatives (57%). Histological analysis identified testicular dysgenesis in 18 patients (67%) and the absence of gonadal tissue in 9 (33%).

3.1 | Sex assignment and the EMS

Thirteen patients were assigned female and 20 were assigned male at birth. Twenty-six patients were assigned before the year 1990 (16 patients to male and 10 patients to female), and seven patients were assigned between 1990 and 1999 (4 patients to male and 3 patients to female). The median EMS at first evaluation in patients without a previous genitoplasty was 3.5 (1.0-5.5) for the patients assigned female and 6.0 (3.0-7.5) for the patients assigned male at birth ($P = .002$). This difference in the EMS between the two sexes was observed in the patients assigned before and after the year 1990.

Four patients changed their gender in adulthood, two from male to female and two from female to male. These four patients visited our hospital at an adult age.

The patients were grouped on the basis of their gender and not on their sex assignment.

3.2 | Female gender group (n = 13)

Two patients came at prepubertal age (patients 1 and 2), four at pubertal (patients 3-6) and seven after pubertal age (patients 7-13). Three patients had already had a gonadectomy and genitoplasty (patients 6, 7 and 9). Amongst the 10 patients without previous genital surgery, external genitalia ranged from normal female (patient 12), female with clitoromegaly (patients 1-5, 8 and 11) to micropenis (patient 11). Six patients had two perineal openings (patients 1, 3, 4, 5, 6 and 13), and five patients had a single perineal opening (patients 2, 8, 10, 11 and 12). All the patients had bilateral cryptorchidism. These patients had elevated serum gonadotropin levels with predominance of FSH levels (range from 38 to 77 IU/L) over LH levels (range from 5 to 32 IU/L) Table 1.

All female patients had undergone feminizing genitoplasty and bilateral gonadectomy. Oestrogen replacement was started at a median age of 14 (10-31 years) with normal breast development.

3.3 | Male gender group (n = 20)

Twelve patients were evaluated at prepubertal age (patients 14-18, 24-30), three at pubertal age (patients 19, 20 and 31) and five after puberty (patients 21-23, 32 and 33). At first evaluation, three patients had a previous genitoplasty (patients 20, 21 and 29), two had undergone bilateral gonadectomy (patients 20, 21) and one unilateral gonadectomy (patient 29) Tables 2,3.

Regarding the 17 remaining patients, 12 had microphallus and proximal hypospadias (71%), 14 had bilateral cryptorchidism (82%), had unilateral cryptorchidism and one patient had both testes lying inside the scrotum.

The patients with low basal and/or hCG-stimulated testosterone levels had undergone bilateral gonadectomy. All of them received testosterone replacement at a median age of 15 (11.7-48 years). Their median phallus size at first visit was −3.4 SD (−6.1 to −1.7 SD), and in adulthood, after testosterone replacement, the phallus size reached a median of 9 cm (range 6.7-12 cm), corresponding to −2.7 SD (range −4.1 to −0.9 SD).

Patients with preserved testosterone secretion with one atrophic cryptorchid testis had had unilateral gonadectomy. The median phallus size of these patients at first visit was −3.3 SD (range −4.3 to −0.3 SD). In adulthood, phallus size reached a median of 8 cm (6.5-9.2 cm), corresponding to −3.3 SD (−4.3 to −2.6 SD).

There was no statistically significant difference in phallus size SDs between the patients with preserved testosterone secretion and those who received testosterone replacement and also at their first and last evaluation.

3.4 | Testosterone production in patients with 46,XY PGD

At first evaluation, 28 patients who did not undergo bilateral gonadectomy in childhood were evaluated regarding testosterone production (Figure 1). Fourteen of them were at prepubertal age (patients 1, 2, 14-18, and 24-30). Eight of them had normal hCG-stimulated testosterone levels (patients 1, 2, and 25-30), median of 13.8 nmol/L (6.2-22.1 nmol/L) and one patient at minipuberty had normal basal testosterone levels (patient 24). The other five individuals (patients 14-18) showed very low hCG-stimulated testosterone levels (undetectable to 2.9 nmol/L). All the male patients with impaired testosterone secretion and two female patients (patients 1 and 2) had had bilateral gonadectomy.

Among the 21 patients without bilateral gonadectomy, 12 went through spontaneous puberty (patients 3, 4, 5, 13, 25-29, and 31-33), including four female patients (patients 3, 4, 5, and 13; Figure 1). These female patients and the two male patients who developed a gonadal tumour (patients 31 and 32) underwent bilateral gonadectomy. Patient 29 progressively lost testosterone secretion and started testosterone replacement at the age of 34 (Figure 1).

At last evaluation, five male patients maintained normal male adult testosterone levels (median 16.7 nmol/L). Four patients had high LH (median 11 IU/L) and FSH levels (median 24 IU/L), and one subject had normal gonadotropin levels (patient 25) at 17 years of age Table 4.

Altogether, twelve patients (57%) had gone through spontaneous puberty. At last evaluation, five of eight patients with male social sex who had gone through spontaneous puberty still had normal testosterone production.

3.5 | Psychosexual follow-up according to gender

Ten patients assigned female and 11 patients assigned male at birth were evaluated. Gender dysphoria and gender change were observed in four patients, two from each gender group. None of the patients reported nonbinary or gender-fluid feelings.

Patients 8 and 11 were assigned male at birth. The former had atypical genitalia, and the latter had severe micropenis and bilateral cryptorchidism. They clearly displayed female behaviour, preferring female activities and clothes since childhood. They received proper medical and psychological assistance at the ages of 19 and 30, respectively. At the time, their hormonal profile showed hypergonadotropic hypogonadism without pubertal signs. Their psychological analysis revealed female gender identity and gender dysphoria. They changed their gender to female, had feminizing genital surgery and were treated with conjugated oestrogens.

Patients 21 and 33 had atypical genitalia and were first assigned female at birth. They had manifested male behaviour since childhood, preferring boys' hobbies and clothes.

Patient 21 had a feminizing genitoplasty and gonadectomy at 1.6 years of age elsewhere and had no psychological evaluation and follow-up. At age 19, he changed his gender to male, and testosterone replacement was started. At 27 years of age, he came to our institution looking for neophallus surgery.

Patient 33 never had medical assistance. He had had male gender identity since he was 9 years old. Virilisation due to spontaneous puberty was noticed when he was 15 years of age. At the time, he changed his gender to male. He was first seen at our service at age 26, when masculinizing genitoplasty was performed.

At final evaluation, all the 21 patients had gender identity concordant with their self-reported gender role in childhood and were satisfied with their gender. Four females (40%) and eight males (73%) had a steady partner. Penetrative sexual intercourse was reported by eight females (80%) and by nine males (81%), among whom six females (75%) and eight males (81%) reported satisfaction with sexual life and orgasm.

None of the patients from both genders have offspring or adopted children.

3.6 | Testosterone production and gender

There was no relation between postnatal testosterone levels and gender considering the highest serum testosterone level at baseline condition or after the hCG stimulation test observed at the follow-up ($P = .9$).

3.7 | Incidence of gonadal tumours

During follow-up, patients underwent bilateral gonadectomy due to female gender, for impaired testosterone secretion, for an atrophic cryptorchid testis, or because of a gonadal tumour. Thirteen patients had bilateral or unilateral gonadectomy at prepubertal age at a median age of 4 years (1.2-8.8), and no evidence of germ cell neoplasia was found. Fifteen patients had a gonadectomy at pubertal age or in adulthood at a median age of 21 (9.9-47.9), and a testicular tumour was found in two subjects (patients 31 and 32).

Patient 31 had bilateral gonadoblastoma at ages 18 and 20 and an in situ germ cell neoplasia in the right testis, despite the scrotal position of both testes, due to a *WT1* (Wilms' tumour 1) mutation, as previously reported.[6] Patient 32 had a mixed germ cell tumour (80% embryonal carcinoma, 15% yolk sac tumour, 5% choriocarcinoma) with a gonadoblastoma in the left abdominal gonad at 23 years of age associated with very high levels of hCG (536 IU/L; reference level <3 IU/L). He underwent bilateral gonadectomy and chemotherapy with a good response.

3.8 | Molecular diagnosis

Pathogenic or likely pathogenic variants were found in nine sporadic cases and in two familial cases, eight identified by Sanger sequencing and three by targeted massively parallel sequencing (Table 5). All the identified variants are heterozygous and located in genes previously associated with gonadal dysgenesis phenotypes (*NR5A1*, *SRY*, *WT1*, *MAP3K1* and *FGFR2*). Nine variants had already been described,[4-7,23] and two variants are novel (in *MAP3K1* and *FGFR2*, the familial cases). None of the variants was found in population databases, including the Brazilian ABraOM.[23] *NR5A1* defects were the most common, being responsible for 18% of the cases, and in silico and in vivo studies corroborated the deleteriousness of *NR5A1* variants, as previously reported by our group.[4,7] None of these patients had adrenal failure. Segregation analysis by Sanger sequencing was possible in eight of ten families and confirmed segregation with the phenotype in five families and de novo status of the two *WT1* variants.

4 | DISCUSSION

The DSD due to 46,XY PGD is a rare disorder. It represents 19.6% in our cohort of 250 patients with a 46,XY DSD. The current study

TABLE 1 Phenotypes at first evaluation of 46,XY PGD female gender patients

Patient	1	2	3	4	5	6[a]	7[a]	8[b]	9[a]	10	11[b]	12	13[c]
Age (y)	1.2	3.7	9.4	11	12.3	16	18.9	19	27	31	30	38	43
EM score	2	4	1	4	5	NA	NA	6	NA	3	6	3	NA
Clitoris/ phallus size (cm)	2.5 × 1.0	3.0 × 0.9	3.0 × 0.9	6.0 × 2.0	3.0 × 1.0	Previous surgery	Previous surgery	5.5 × 1.7	Previous surgery	4.1 × 1.0	Micropenis	0.8 × 1.0	Previous surgery
Perineal openings	2	1	2	2	2	2	1	1	2	1	1	1	2
Gonads location	RG: labial LG: ABD	ABD	ABD	RG:ING LG:ABD	ING	LG:ING	ABD	Not found	NA	Not found	Not found	Not found	ABD
Mullerian derivatives	Present	Present	Present	Absent	Present	Present	Absent	Absent	NA	Absent	Present	Absent	Present
Age at gonadectomy (y)	1.4	3.9	9.9	12	15.2	14	5	9	14	31	30	38	43
Gonadal histology	Dysgenetic	Dysgenetic	Dysgenetic	Dysgenetic	Dysgenetic	NA	RG:Dysgenetic LG:NGT	NGT	NGT	NGT	NGT	NGT	RG: NGT LG:Dysgenetic
Basal T (nmol/L)	2.6[d]	0.9[d]	4.0[d]	14.4[e]	10.3[e]	NA	NA	<0.9[d]	NA	1.2[d]	1.0[e]	<0.9[d]	11.2[e]
T after hCG (nmol/L)	19.9[e]	11.7[e]	NA	NA	NA	NA	NA	NA	NA	1.3	NA	NA	NA
LH (IU/L)	<1.0	NA	5.1	13	24	NA	NA	23	NA	18	10	11	32
FSH (IU/L)	<0.6	NA	50	77	69	NA	NA	62	NA	57	40	38	52
Affected genes	MAP3K1/ FGFR2	-	MAP3K1/ FGFR2	NR5A1	NR5A1	NR5A1	-	-	-	NR5A1	-	-	-

ABD, abdominal; EM score, external masculinization score; ING, inguinal; LG, left gonad; NA, not available; NGT, no gonadal tissue; PGD, partial gonadal dysgenesis; RG, right gonad.

[a]Previous gonadectomy and genitoplasty in another hospital; incomplete data;

[b]Patients that changed from male to female social sex and underwent previous testosterone replacement;

[c]Previous genitoplasty in another hospital;

[d]Radioimmunoassay (RIA), normal male value: prepubertal age <0.9 nmol/L; adults: 8.7-35.7 nmol/L elmmunofluorometric assay (IFMA), normal male value: prepubertal age <0.65 nmol/L; adults 9.4-33.5 nmol/L.

TABLE 2 Phenotypes at first evaluation of 46,XY PGD male gender patients and impaired testosterone secretion

Patient	14	15	16	17	18	19	20[a]	21[a,b]	22	23
Age (y)	1.9	2.0	7	7.4	9	14.9	16	27	34	47.9
EM score	4	7	6	7	6	6	NA	NA	3	3
Phallus size (cm)	2.0 × 1.0	3.0 × 1.0	3.0 × 1.0	4.5 × 2.0	3.4 × 1.4	3.6 × 1.2	Previous surgery	Previous surgery	5.0 × 1.2	8.5 × 3.0
Phallus size (SD)	−3.4	−2.3	−3.4	−1.7	−2.9	−6.1	NA	NA	−5.2	−3.0
Urethra location	Perineal	Perineal	Topic	Perineal	Topic	Topic	NA	NA	Perineal	Perineal
Gonad location	ABD	ABD	Not found	ABD	Not found	Not found	Cryptorchidism	NA	Not found	Not found
Mullerian derivatives	Absent	Absent	Absent	Absent	Absent	Present	Present	Present	Present	Present
Age at gonadectomy (y)	9.9 and 10.5	2.5 and 4.7	15	4 and 11	9.0	15	NA	1.7	34	48
Gonadal hystology	Dysgenetic	Dysgenetic	Dysgenetic	Dysgenetic	NGT	NGT	NA	Infantile testes	NGT	NGT
Basal T (nmol/L)	0.9[e]	<0.9[e]	NA	<0.9[e]	<0.9[e]	<0.9[e]	NA	NA	2.6[d]	0.9[d]
T after hCG (nmol/L)	0.9[e]	2.9[e]	NA	<0.9[e]	<0.9[e]	NA	NA	NA	4.1[d]	NA
LH (IU/L)	NA	NA	NA	NA	NA	20	NA	NA	10	16
FSH (IU/L)	NA	NA	NA	NA	NA	41	NA	NA	78	57
Affected genes	-	*WT1*	-	-	-	-	-	-	-	-

ABD, abdominal; EM score, external masculinization score; ING, inguinal; NA, not available; NGT, no gonadal tissue; PGD, partial gonadal dysgenesis.

[a]Previous gonadectomy and genitoplasty in another hospital; incomplete data; [b]Patient that changed from female to male social sex; [c]Radioimmunoassay (RIA), normal male value: prepubertal age <0.9 nmol/L; adults: 8.7-35.7 nmol/L; [d]Immunofluorometric assay (IFMA), normal male value: prepubertal age <0.65 nmol/L; adults 9.4-33.5 nmol/L

TABLE 3 Phenotypes at first evaluation of 46,XY PGD male gender patients and preserved testosterone secretion

Patient	24	25	26	27	28	29[a]	30	31	32	33[b]
Age (y)	Newborn	0.5	0.6	0.9	1.7	4.0	4.3	13	23	26
EM score	6	6	7.5	7.5	6	NA	4	5	4.5	5.5
Phallus size (cm)	2.0 × 1.0	3.0 × 0.9	2.5 × 1.0	3.5 × 1.2	2.0 × 1.0	Previous surgery	2.5 × 1.0	8.7 × 4.2	8.0 × 2.7	6.5 × 2.5
Phallus size (SD)	−3.75	−1.12	−2.25	−0.34	−3.3	NA	− 3.5	−2.8	−3.3	−4.3
Urethra location	Penile	Perineal	Perineal	Perineal	Topic	NA	Perineal	Perineal	Perineal	Perineal
Gonad location	RG: ABD LG: topic	Topic	RG: ING LG: ABD	RG: ABD LG: ING	RG: ING LG: not found	RG: NA LG: topic	ABD	ING	RG: ING LG: ABD	RG: ING LG: topic
Mullerian derivatives	Present	Absent	Present	Present	Absent	Present	Present	Absent	Present	Present
Age at gonadectomy (y)	8	NA	1.1	1.2	2	4	4 and 6	19 and 21	23	NA
Gonadal histology	NA	NA	LG: dysgenetic	RG: dysgenetic	LG: dysgenetic	RG: dysgenetic	Dysgenetic	Bilateral gonadoblastoma RG: in situ GCT	RG: Dysgenetic LG: mixed GCT	NA
Basal T (nmol/L)	10.7[c]	<0.9[c]	<0.9[c]	NA	2.6[c]	1.5[c]	<2.9[c]	18.5[d]	14.6[d]	13.7[d]
T afterhCG (nmol/L)	NA	15.9[c]	6.2[c]	22.1[c]	7.1[c]	17.1[c]	6.6[c]	21.4[d]	NA	17.3[d]
LH (IU/L)	NA	NA	<0.6	<0.6	NA	NA	<1.0	13.3	34	10.8
FSH (IU/L)	NA	NA	<1.0	<1.0	NA	NA	3.0	39.6	75.5	20.7
Affected genes	-	NR5A1	SRY	-	-	-	-	WT1	-	NR5A1

ABD, abdominal; EMS, external masculinization score; GCT, germ cell tumour; NA, not available; NGT, no gonadal tissue; PGD, partial gonadal dysgenesis.

[a]Previous unilateral gonadectomy and genitoplasty perfomed in another hospital; [b]Patient that changed from female to male social sex; [c]Radioimmunoassay (RIA), normal male value: prepubertal age <0.9 nmol/L; adults: 8.7-35.7 nmol/L dImmunofluorometric assay (IFMA), normal male value: prepubertal age <0.65 nmol/L; adults 9.4-33.5 nmol/L;

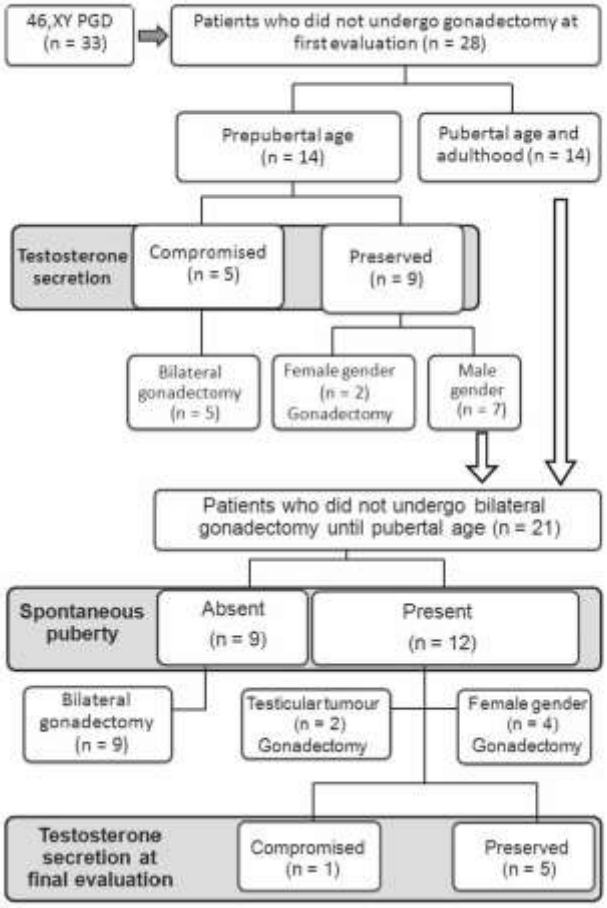

FIGURE 1 Flow chart of patients' follow-up regarding testosterone secretion and gonadal tumour incidence

is the largest 46,XY PGD cohort showing clinical outcomes and molecular analysis.

Sex assignment is the most controversial issue of DSD management. In our cohort, most patients were assigned before the 1990s, and male sex assignment was significantly more frequent in patients with a higher EMS, in a ratio of 1.5 males to 1.0 female. The International Disorder of Sex Development (I-DSD) Registry reported an increase in the male-to-female sex assignment ratio in 46,XY dysgenetic DSD babies with time, starting from a ratio of 0.4 before the 1990s to a ratio of 1.5 in children born after 1999, regardless of the EMS.[26]

Despite this increasing trend on male sex assignment, there are scarce data on pubertal development of those patients. In our cohort, spontaneous puberty was observed in 57% of the patients who did not undergo bilateral gonadectomy in childhood. In adulthood, all the male patients maintained testosterone secretion, except for the oldest, who showed a decrease in testosterone secretion at the age of 34. They all had high LH levels (>10 IU/L), with the exception of the youngest, indicating partial Leydig cell dysfunction. Regarding reproductive function, high FSH levels (>20 IU/L) were found in most patients manifesting compromised spermatogenesis although the sperm count was not performed.

In one retrospective study on pubertal development of 46,XY PGD patients, 9 of 10 patients had gone through spontaneous puberty with high FSH levels and progressive elevation of LH.[2] These

TABLE 4 Phenotypes of 46,XY PGD male gender patients who had preserved testosterone secretion at final evaluation

Patient	25	26	27	28	33
Age (y)	17	17	17.6	21	28
Penile length (cm)	8.0 × 3.0	8.0 × 3.0	7.5 × 2.5	9.2 × 2.5	6.5 × 2.5
Z phallus (SD)	−3.3	−3.3	−3.6	−2.6	−4.3
Testis final size (cm)	4.5 × 2.5	6.7 × 2.1	4.0 × 2.5	4.3 × 2.0	6.5 × 2.5
Basal T[a] (nmol/L)	441	561	625	482	396
LH (IU/L)	5.6	14	11	11	10.8
FSH (IU/L)	3.7	26	25	24	20.7

PGD, Partial gonadal dysgenesis.
[a]Immunofluorometric assay (IFMA), normal male value: prepubertal age <0.65 nmol/L; adults 9.4-33.5 nmol/L.

patients had a mild-gonadal-dysgenesis phenotype as 60% had a penile urethra opening and all of them were assigned male at birth.[2]

In humans, the process of gonadal determination is quite complex[1] and a molecular defect was identified in 20% and 40% of the 46,XY gonadal dysgenesis patients who were studied by Sanger[27] and target massively parallel sequencing, respectively.[28] *NR5A1* and *MAP3K1* allelic variants were the most frequent molecular diagnosis.[28]

In our study, likely pathogenic or pathogenic allelic variants were identified in 33% of the patients, in one of the following genes: *NR5A1*, *SRY*, *WT1*, *MAP3K1* and *FGFR2*. *NR5A1* defects (n = 6) were the most frequent in the whole cohort and also among the 12 patients who developed spontaneous puberty (n = 4). Moreover, three of these patients were assigned female at birth owing to their severely undervirilised genitalia. This finding is in agreement with other reports.[29-35] In those cases, the severe undervirilisation of external genitalia could not predict virilisation in adulthood because testosterone secretion recovered during puberty for unknown reasons.[29-34]

SRY defects have been mostly associated with complete gonadal dysgenesis and rarely with partial gonadal dysgenesis.[25,37-39] The *SRY* p.Arg30Ile pathogenic allelic variant was identified in one of our patients with spontaneous puberty. This same variant was also found in another Brazilian family, including affected members with various phenotypes, ranging from complete to partial gonadal dysgenesis.[25] In vitro studies proved the deleteriousness of the variant.[25] None of the reported patients with PGD due to *SRY* variants had preserved testosterone secretion.[25,37-39]

Missense defects in Wilms' tumour suppressor gene 1 (*WT1*) cause Frasier and Denys-Drash syndromes.[40,41] One of our patients with normal size testes and spontaneous puberty harboured the most common allelic variant in intron 9 of *WT1*, which is associated with Frasier syndrome.[6] This syndrome is generally characterised by bilateral gonadal dysgenesis, female external genitalia, renal failure in the second decade of life and high risk of testicular gonadoblastoma. Instead, our patient had a predominantly male phenotype, with normal penile length and perineal hypospadias resembling the Denys-Drash phenotype. Even though there are five other cases of Frasier syndrome with a male phenotype,[42-44] including one with a normal male phenotype,[45] there are no reports of patients with spontaneous puberty.

The novel heterozygous variants *MAP3K1*p.Leu639Pro and *FGFR2* p.Ser453Leu were found simultaneously in two 46,XY sisters within our cohort, both inherited from their unaffected mother. Both had severe undervirilised genitalia at first evaluation, although normal male testosterone levels were reached after hCG stimulation in the 1.2-year-old child, and her 9.4-year-old sister had pubertal male testosterone levels.

MAP3K1 was first associated with 46,XY DSDs by Pearlman et al.[46] Targeted massively parallel sequencing has revealed previously reported and novel *MAP3K1* variants not only in patients with complete gonadal dysgenesis but also in patients with PGD. There is no information on hormone profile and pubertal development of those patients.[28,47,48]

FGFR2 variants most commonly cause craniosynostosis syndromes without any gonadal phenotype. Although there is one report of a heterozygous *FGFR2* p.Cys342Tyr variant that was associated with complete gonadal dysgenesis and no report of patients with PGD.[49] The *FGFR2* p.Ser453Leu allelic variant found in one of our families is located in the hotspot region for pathogenic variants responsible for craniosynostosis phenotypes; however, our patients and their mother did not have any skull problems.

The mechanism by which the gonadal FGF signal is transduced intracellularly remains unclear, but *FGFR2* and *MAP3K1* are members of the RAS/RAF/MEK/ERK signalling pathway, and these patients may have a digenic inheritance cause of gonadal dysgenesis.

The prevalence of germ cell tumours in PGD is variable. Reported rates range between 16% and 30%[3] and for Denys-Drash and Frasier syndromes is as high as 40%-60%.[50] In our cohort, two patients (7%) had a germ cell tumour: one had an invasive seminoma, and the other had bilateral gonadoblastoma associated with in situ germ cell neoplasia. Both patients had additional factors for germ cell tumour development: one had an abdominal gonad, and the other had both testicles lying within the scrotum but carried a *WT1* mutation.

TABLE 5 Deleterious variants' description and testosterone production at puberty in patients with 46,XY partial gonadal dysgenesis

Patient	T secretion at puberty	Gene	Transcript ID	Allelic variant[a]	Protein alteration	Previous published/reference	Variant segregation in family members	ACMG classification
4	Preserved	*NR5A1*	NM_004959	c.T1073C	p.Leu358Pro	Yes/2	M: C with POI	Likely pathogenic
5	Preserved	*NR5A1*	NM_004959	c.C633G	p.Tyr211*	Yes/2	M: AC; F:NA	Pathogenic
6	Previous gonadectomy	*NR5A1*	NM_004959	c.G77A	p.Gly26Glu	Yes/2	NA	Likely pathogenic
10	Absent	*NR5A1*	NM_004959	c.1058_1065del	p.Glu353Ala fs*31	Yes/5	NA	Pathogenic
25	Preserved	*NR5A1*	NM_004959	c.1183_1185del	p.Glu395del	Yes/2	NA	Pathogenic
33	Preserved	*NR5A1*	NM_004959	c.C741A	p.Cys247*	Yes/2	NA	Pathogenic
26	Preserved	*SRY*	NM_003140	c.G89T	p.Arg30Ile	Yes/23	F:AC	Likely pathogenic
15	Previous gonadectomy	*WT1*	NM_024426	c. A742T	p.Lys248*	Yes/3	De novo	Pathogenic
31	Preserved	*WT1*	NM_024426	IVS 9 + 4C > T	Splice site change	Yes/4	De novo	Pathogenic
1 and 3 (sisters)	Previous gonadectomy[b]	*MAP3K1* and	NM_005921	c.T1916C	p.Leu639Pro	Novel	M:AC; P:WT	Likely pathogenic
1 and 3 (sisters)	Previous gonadectomy[b]	*FGFR2*	NM_000141	c.C1358T	p.Ser453Leu	Novel	M:AC; P:WT	Likely pathogenic

AC, asymptomatic carrier; C, carrier; F, father; M, mother; NA, not available; POI, premature ovarian failure; WT, wild type.
The references of the previously reported variants were included. The novel variants are highlighted in bold. [a]All the variants are in heterozygous state and are absent in population database. [b]These female social sex patients had preserved testosterone secretion at childhood and underwent bilateral gonadectomy.

Pre- and postnatal androgen exposure seems to contribute to male gender identity in patients with the 46,XY DSD due to 5alpha-reductase type 2 and 17beta-hydroxysteroid dehydrogenase type 3 deficiencies. In those patients, despite the severe undervirilisation and female rearing, a high rate of gender change to male is observed, ranging from 50% to 63% and from 39% to 64%, respectively.[51] In 46,XY PGD, testosterone production is quite variable during foetal and adult life. In our cohort, postnatal testosterone levels were not related to the gender. In addition, among the 5 patients assigned female at birth who virilised at puberty, 3 harboured *NR5A1* variants, and gender change to male was observed in one patient, who reported gender dysphoria since childhood.

Gender dysphoria had rarely been observed in patients with PGD. To our knowledge, only one case of female-to-male gender change has been reported.[34] This patient harboured a *NR5A1* variant, was assigned female at birth, presented with virilisation at puberty and changed his gender at 18 years of age.[34] Nonetheless, no gender change was observed among another six PGD patients with *NR5A1* defects already described, who had gone through spontaneous virilisation.[29-31,33,35,36] Together with the already published cases of *NR5A1* defects, among the 10 patients assigned female at birth with virilisation at puberty, two patients changed their gender to male. This data does not suggests that testosterone production at puberty is a determinant of gender change, but it should play a role in 46,XY PGD patients' gender, although the small sample size does not allow us to make any conclusion.

The psychosexual evaluation in adulthood revealed that the patients were satisfied with their final social sex, and ~80% of the patients reported satisfaction with their sexual life.

5 | CONCLUSION

The present study represents the largest 46,XY PGD cohort showing clinical outcomes and molecular analysis. Spontaneous puberty was observed in 57% of the patients with 46,XY PGD, being *NR5A1* defects the most prevalent among all patients and among those with spontaneous puberty. A germ cell tumour was detected only after puberty in 7% of the patients. Gender change due to gender dysphoria was reported by 12% of the patients. All the patients reported satisfaction with their gender and most of them with their sexual life.

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CONFLICT OF INTEREST

The authors report no conflict of interest in this work.

ORCID

Nathalia L. Gomes http://orcid.org/0000-0002-1072-7903
Rafael Loch Batista http://orcid.org/0000-0002-3819-7377
Elaine F. Costa http://orcid.org/0000-0001-8677-8515
Mirian Y. Nishi http://orcid.org/0000-0001-6603-3188
Sorahia Domenice http://orcid.org/0000-0002-4883-7630
Berenice B. Mendonca http://orcid.org/0000-0003-1762-1084

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APPENDIX 1

Questionnaire applied to 46,XY disorder of sex development (DSD) patients due to partial gonadal dysgenesis regarding gender identification and sexual life quality

Social sex and gender identity

- At birth, your assigned social sex was: 1 - female 2 - male 3 - undefined
- Have you ever wished to change your gender? 1 - yes 2 - no
- Have you changed your gender?: 1 - yes 2 - no
- How old were you when you started thinking about to change your gender?
- How old were you when you changed your gender?
- Define your gender identification: 1 - female 2 - male 3 - both 4 - none

Self- reported gender role at childhood

- At childhood, you used to behave like you were: 1 - a girl 2 - a boy 3 - both
- At childhood, how you used to feel about your gender?
  - If assigned female at birth, you used to feel like a girl:
    1 Never
    2 Almost never
    3 Sometimes
    4 Often
    5 Always
  - If assigned male at birth, you used to feel like a boy:
    1 Never
    2 Almost never
    3 Sometimes
    4 Often
    5 Always

APPENDIX 1 (Continued)

- Which were your preferable toys at childhood

Classification of the preferable toys at childhood according gender:

Typically female toys	Typically boys toys	Neutral boys
Dolls, make-ups, drawing, reading, costumes, board games	Cars and trucks, building games (like Lego)	Plays with ball, running, group activities

Sexual life aspects

- Do you have regular sexual intercourses? 1 - yes 2 - no
- Do you have a steady partner? 1 - yes 2 - no
- Do you have penetrative sexual intercourses? 1 - yes 2 - no
- Are you satisfied with your sexual life? 1 - yes 2 - no
- Do you have orgasm during intercourse? 1 - yes 2 - no

APPENDIX 2

List of genes related to disorders of sex development (DSDs) included in the panel for targeted massively parallel sequencing

Gene	Associated phenotype already related to human DSDs reported in OMIM (OMIM number) or in the literature (L)[1] (n = 43)	Inheritance
Gonadal development genes		
BMP15	Ovarian dysgenesis 2 (300510); Premature ovarian failure 4 (300510)	XL
CBX2	46,XY sex reversal 5 (613080); 46,XY complete gonadal dysgenesis (L)	AR
DHH	46XY partial gonadal dysgenesis, with minifascicular neuropathy (607080); 46XY sex reversal 7 (233420)	AR
DMRT1	Dysgenetic testis or ovotestis (L)	AD
DMRT2	Haploinsufficiency 9p sex-determining gene leads to gonadal dysgenesis (L)	NA
FGFR2	46,XY sex reversal with craniosynostosis (L)	AD, AR
FOXL2	Premature ovarian failure 3 (608996); blepharophimosis, epicanthus inversus, and ptosis, type 1 and 2 (110100)	AD
GATA4	Testicular anomalies with or without congenital heart disease (615542)	AD
MAP3K1	46XY sex reversal 6 (613762)	AD
NR0B1	46XY sex reversal 2, dosage-sensitive (300018)	XL
NR5A1	46, XX sex reversal 4 (617480); 46XY sex reversal 3 (612965); premature ovarian failure 7 (612964)	AD
RSPO1	Palmoplantar hyperkeratosis and true hermaphroditism (610644); palmoplantar hyperkeratosis with squamous cell carcinoma of skin and sex reversal (610644)	AR
SOX 9	Campomelic dysplasia with autosomal sex reversal (114290)	AD
SOX3	46,XX testicular or ovotesticular DSD (L); 46,XX Sex Reversal 3 (300833)	XL
SRY	46XX sex reversal 1 (400045); 46XY sex reversal 1 (400044)	Y-linked
STAG3	Premature ovarian failure 8 (615723)	AR
WNT4	Mullerian aplasia and hyperandrogenism (158330)	AD
WT1	Denys-Drash syndrome (194080); Frasier syndrome(136680)	AD
WWOX	46,XY gonadal dysgenesis (L)	Y-linked
ZFPM2	46XY sex reversal 9 (616067)	AD
Sexual differentiation genes		
AKR1C2	46XY sex reversal 8 (614279)	AR
AKR1C4	46XY sex reversal 8, modifier (614279)	AR
AMH	Persistent Mullerian duct syndrome, type I (261550)	AR
AMHR2	Persistent Mullerian duct syndrome, type I (261550)	AR
AR	Androgen insensitivity (300068); Hypospadias 1, X-linked (300633)	X-L
CYP17A1	17,20-lyase deficiency, isolated (202110)	AR
CYP19A1	Aromatase deficiency (613546); aromatase excess syndrome (139300)	AD
CYP21A2	Adrenal hyperplasia, congenital, due to 21-hydroxylase deficiency (201910)	AR
DHCR7	Smith-Lemli-Opitz syndrome (270400)	AR
FSHR	Ovarian dysgenesis 1 (233300)	AR

APPENDIX 2 (Continued)

Gene	Associated phenotype already related to human DSDs reported in OMIM (OMIM number) or in the literature (L)[5] (n = 43)	Inheritance
HSD11B1	Cortisone reductase deficiency 2 (614662)	AD
HSD17B3	Pseudohermaphroditism, male, with gynecomastia (264300)	AD
HSD3B2	3-beta-hydroxysteroid dehydrogenase, type II, deficiency (201810)	AD
LHCGR	Leydig cell hypoplasia with pseudohermaphroditism (238320); Leydig cell hypoplasia with hypergonadotropic hypogonadism (238320)	AR
POR	Antley-Bixler syndrome with genital anomalies and disordered steroidogenesis (201750); Disordered steroidogenesis due to cytochrome P450 oxidoreductase (613571)	AR
SRD5A2	Pseudovaginalperineoscrotal hypospadias (264600)	AR
STAR	Lipoid adrenal hyperplasia (201710)	AR
Other (syndromic DSD, isolated hypospadias)		
ARX	Hydranencephaly with abnormal genitalia (300215)	X-L
ATRX	ATR-X syndrome with gonadal abnormalities (301040)	X-LD/X-LR
CDH7	CHARGE syndrome (214800); hypogonadotropic hypogonadism with or without anosmia (612370)	AD
HNF1B	Mayer-Rokitansky-Kuster-Hauser syndrome (L)	AD
LHX1	Mayer-Rokitansky-Kuster-Hauser syndrome (L)	AD
MAMLD1	Hypospadias, X-linked (300758)	XL

Gene	Candidate genes associated with human DSDs selected from the literature (L) and in OMIM (O) (n = 20)	Inheritance
AKR1C3	Testosterone production in the adrenal reticularis[18]	NA
AXIN1	Wnt-beta-catenin signaling (O)	NA
CITED2	An upstream regulator of *NR5A1* (L)[1]	NA
ESR1	Sex reversal in ESRA/ESRB knockout males (O)	NA
ESR2	46,XY DSD candidate gene (L)[11]	AR;AD
FGF9	XY mice KO results in male-to-female sex reversal (L)[12]	NA
GDF9	Ovarian development (L)[13]	NA
GSK3β	Wnt-beta-catenin signalling (O)	NA
LHX9	Gonadal formation in mouse model (L)[1]	NA
NANOS2	Expressed in adult and foetal testis (O)	NA
NANOS3	Nanos3-null mice present reduced spermatogenesis (O)	NA
PAPPA	Expressed in ovarian follicles and in the seminal vesicles and fluid (O)	NA
PAX2	WT1 pathway (L)[15]	NA
PBX1	Müllerian development in the mouse (L)[15]	NA
PTDGS	Required for testis formation (L)[1]	NA
RAC1	Formation of primordial follicles in mouse (L)[16]	NA
RSPO2	Essential for primary follicle development (L)[19]	NA
STRA8	Premeiotic DNA replication (L)[14]	NA
TCF21	SRY pathway (L)[17]	NA
TES	Testis-specific enhancer of *Sox9* (L)[1]	NA

Apêndice 5

Domenice S, Zamboni Machado A, Moraes Ferreira F, Ferraz-de-Souza B, Marcondes Lerario A, Lin L, Nishi NY, **Gomes NL** et al. Wide spectrum of NR5A1-related phenotypes in 46,XY and 46,XX individuals. Birth Defects Res C Embryo Today. 2016;108(4):309-20.

Wide Spectrum of NR5A1-Related Phenotypes in 46,XY and 46,XX Individuals

Sorahia Domenice[1], Aline Zamboni Machado[1], Frederico Moraes Ferreira[2], Bruno Ferraz-de-Souza[1], Antonio Marcondes Lerario[1], Lin Lin[3], Mirian Yumie Nishi[1], Nathalia Lisboa Gomes[1], Thatiana Evelin da Silva[1], Rosana Barbosa Silva[1], Rafaela Vieira Correa[4], Luciana Ribeiro Montenegro[1], Amanda Narciso[1], Elaine Maria Frade Costa[1], John C Achermann[3], and Berenice Bilharinho Mendonca*[1]

Steroidogenic factor 1 (NR5A1, SF-1, Ad4BP) is a transcriptional regulator of genes involved in adrenal and gonadal development and function. Mutations in *NR5A1* have been among the most frequently identified genetic causes of gonadal development disorders and are associated with a wide phenotypic spectrum. In 46,XY individuals, NR5A1-related phenotypes may range from disorders of sex development (DSD) to oligoazoospermia, and in 46,XX individuals, from 46,XX testicular and testicular DSD to primary ovarian insufficiency (POI). The most common 46,XY phenotype is atypical or female external genitalia with chromosomally, intracelle gonads, and absence of Müllerian derivatives. Notably, an underdeveloped external genitalia is frequently seen at birth, while spontaneous virilization may occur later, at puberty. In 46,XX individuals, *NR5A1* mutations are a rare genetic cause of POI, manifesting as primary or secondary amenorrhea, infertility, hypoestrogenism, and elevated gonadotropin levels. Mothers and sisters of 46,XY DSD patients carrying heterozygous *NR5A1* mutations may develop POI, and therefore require appropriate counselling. Moreover, the recurrent heterozygous p.Arg92Trp NR5A1 mutation is associated with variable degrees of testis development in 46,XX patients. A clear genotype-phenotype correlation is not seen in patients bearing *NR5A1* mutations, suggesting that genetic modifiers, such as pathogenic variants in other testis/ovarian-determining genes, may contribute to the phenotypic expression. Here, we review the published literature on NR5A1-related disease, and discuss our findings at a single tertiary center in Brazil, including ten novel *NR5A1* mutations identified in 46,XY DSD patients. The ever-expanding phenotypic range associated with *NR5A1* variants in XY and XX individuals confirms its pivotal role in reproductive biology, and should alert clinicians to the possibility of NR5A1 defects in a variety of phenotypes presenting with gonadal dysfunction.

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Key words: disorders of sex development; NR5A1 gene; gonadal dysgenesis; primary ovarian failure; ovarioitis; adrenal insufficiency

Introduction

NR5A1 (nuclear receptor subfamily 5 group A, member 1), previously known as *SF1* (Steroidogenic Factor-1) and *Ad4BP* (Adrenal 4-Binding Protein), was first cloned by Parker and colleagues in 1992, in an attempt to identify a protein that could activate the promoters of steroid hydroxylase enzymes (Lala et al., 1992).

NR5A1 is expressed in steroidogenic tissues (Honda et al., 1993; Ikeda et al., 1993; Morohashi et al., 1994; Parker and Schimmer, 1997; Ramayya et al., 1997; Morohashi,

Additional Supporting Information may be found in the online version of this article.

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The authors declare that they have no competing financial interests.

[1]Sorahia Domenice, Aline Zamboni Machado, Bruno Ferraz-de-Souza, Antonio Marcondes Lerario, Mirian Yumie Nishi, Nathalia Lisboa Gomes, Thatiana Evelin da Silva, Rosana Barbosa Silva, Luciana R. Montenegro, Amanda Narciso, Elaine Maria Frade Costa, and Berenice Bilharinho Mendonca are from the Laboratório de Hormônios e Genética Molecular (LIM42), Unidade de Endocrinologia do Desenvolvimento, Disciplina de Endocrinologia e Metabologia do Hospital das Clínicas, Faculdade de Medicina, Universidade de São Paulo, São Paulo, Brasil

[2]Frederico Moraes Ferreira is from the Ciencias da Saúde, Universidade Santo Amaro, São Paulo, Brasil and Laboratorio de Imunologia, Instituto do Coração, Faculdade de Medicina, Universidade de São Paulo, São Paulo, Brasil

[3]Lin Lin and John C. Achermann are form the Genetics & Genomic Medicine, University College London (UCL) Great Ormond Street Institute of Child Health, University College London, London, United Kingdom

[4]Rafaela V. Correa is from the Núcleo de Atenção Médica Integrada (NAMI), Universidade de Fortaleza, Ceará, Brasil

Key words: disorders of sex development; NR5A1 gene; gonadal dysgenesis; primary ovarian failure; ovarioitis; adrenal insufficiency

*Correspondence to: Berenice Bilharinho Mendonca, Laboratório de Hormônios e Genética Molecular (LIM42), Unidade de Endocrinologia do Desenvolvimento, Disciplina de Endocrinologia e Metabologia do Hospital das Clínicas, Faculdade de Medicina, Universidade de São Paulo, Av. Dr. Enéas de Carvalho Aguiar, 155, 7 andar, São Paulo, SP CEP 05403-900, Brasil. E-mail berenmen@usp.br

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1999), pituitary gonadotrophs (Barnhart and Mellon, 1994; Ingraham et al., 1994; Ngan et al., 1999), and neurons located at the dorsomedial portion of the ventromedial hypothalamus (VMH) (Ramayya et al., 1997; Morohashi et al., 1999). In developing embryos, *Nr5a1* is expressed in the urogenital ridge, representing the first marker of gonadal and adrenal differentiation (Ikeda et al., 1994; Morohashi et al., 1995, 1999; Ramayya et al., 1997; Hanley et al., 1999, 2001). Throughout human development, NR5A1 is expressed in the steroid-secreting adrenal cortex, in Leydig and Sertoli cells, as well as in granulosa and theca cells.

NR5A1 stimulates the expression of several genes required for the development and maintenance of the male differentiation cascade. It regulates the expression of *LHCGR* and the steroidogenic enzymes *STAR*, *CYP11A1*, and *CYP17A1* in Leydig cells, required for testosterone biosynthesis. NR5A1 also increases the expression of insulin-like polypeptide 3 (INSL3), which regulates testicular descent and is a survival factor for male germ cells in adults (Zimmermann et al., 1998; Tremblay and Robert, 2005). Anti-Mullerian hormone (AMH) and its receptor, AMHR2, essential factors for male reproductive tract development, are also regulated by NR5A1. In Sertoli cells, NR5A1 regulates the expression of the testis-determining genes *SRY* and *SOX9* (Jeyasuria et al., 2004).

Mutations in *NR5A1* are emerging as a frequent genetic cause of human 46,XY disorders of sex development (DSD), having been identified throughout the globe. In South America, familial and sporadic DSD patients bearing NR5A1 defects have been described in Brazil and Argentina (Lourenço et al., 2009; Ciaccio et al., 2012; Gabriel Ribeiro de Andrade et al., 2014; Fabbri et al., 2016). Here, we review the phenotype associated with ten novel and one previously described *NR5A1* allelic variants identified in a Brazilian cohort of 46,XY and 46,XX DSD patients followed at a single tertiary center. Our findings are discussed in light of previously published reports, and several aspects of the phenotypic spectrum associated with NR5A1 mutations in humans are reviewed.

Patients

This is a retrospective study approved by the Ethics Committee of Hospital das Clinicas, University of Sao Paulo Medical School. Written informed consents were obtained from all patients or their parents/guardians.

The full cohort of DSD patients followed at the Developmental Endocrinology Unit at Hospital das Clinicas consisted of 83 patients with 46,XY DSD (48 patients with 46,XY gonadal dysgenesis and 35 patients with 46,XY DSD of unknown cause) and 90 patients with 46,XX DSD (70 patients with primary ovarian failure (44 patients had primary amenorrhea and 26 patients had secondary amenorrhea) and 20 patients with 46,XX SRY negative disorders of ovary development (16 with 46,XX ovotesticular DSD (OTDSD) and 4 with 46,XX testicular DSD (TDSD)).

Molecular Analysis

Mutational analysis of *NR5A1* (Ensembl transcript ESNT00000373588) was performed by Sanger sequencing and by targeted massively parallel sequencing (TMPS), as detailed in Supporting Information.

Identified allelic variants were analyzed according to the joint American College of Medical Genetics and Genomics Association for Molecular Pathology Guidelines (Richards et al., 2015) (see Supporting Information)

IN SILICO ANALYSIS

Alignment of wild-type and mutated *NR5A1* sequences was performed using MUSCLE (Edgar, 2004) and the NCBI's Reference Sequence (RefSeq) database. To evaluate the impact of NR5A1 nonsynonymous mutations at the tertiary protein structure, several structural models were built for wild-type and mutated molecules in the YASARA suite (Krieger et al., 2004), using as templates the crystal structures of the human liver receptor homologue 1 (LRH-1, NR5A2) DNA-binding domain (DBD) in complex with the *hCYP7A1* promoter (PDB accession code 2A66), and the human NR5A1 ligand-binding domain (LBD) in complex with di-pamitoyl-3-SN-phosphatidylethanolamine (PDB accession code 1ZDT). Stereo chemical quality of generated structural models was accessed with MOLPROBITY (Davis et al., 2007). Two molecular mechanics simulations were carried in explicit solvent for each model. Trajectories were calculated with the YAMBER3 force field. Ten thousand simulation steps of 2.5 fs were recorded for each wild type and its corresponding mutated molecules. The final confirmation of the lowest energy models was confirmed for each pair of simulations. The volumes of the cavities of the models were calculated using KVFINDER (Oliveira et al., 2014). Molecular models were drawn using PyMOL (www.pymol.org).

IN VITRO FUNCTIONAL STUDIES

Transient gene expression assays were performed using human embryonic kidney TSA-201 cells to analyze transcriptional activation of murine *Cyp11a1* and rat *Cyp19* promoters by wild-type or mutant NR5A1 (Lin et al., 2007) (see Supporting Information).

Results

We identified 11 pathogenic *NR5A1* variants, including 10 novel variants in 46,XY DSD patients and one previously reported variant in a 46,XX testicular DSD patient.

CLINICAL AND HORMONAL CHARACTERISTICS OF 46,XY DSD PATIENTS BEARING PATHOGENIC NR5A1 VARIANTS

Nine of ten 46,XY DSD patients presented with atypical genitalia at birth and one had female external genitalia. Nine patients were assigned as female at birth, one of

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TABLE 1. *Clinical Phenotype of Ten 46,XY DSD Patients and One 46,XX Testicular DSD Patient in Whom NR5A1 Mutations Were Identified*

Patient	Karyotype	Age at first evaluation (years)	External genitalia	Mullerian structures	Age at surgery	Sex of rearing	Family
1	46,XY	0.5	Atypical Phallus 3 cm, perineal hypospadias, palpable testes	A	Genitoplasty at 1.6 years	Male	NA
2[a]	46,XY	0.7	Atypical Clitoromegaly, palpable gonads	A	Gonadectomy at 9 m[a]; Genitoplasty at 2 years	Female	NA
3	46,XY	4.9	Atypical Single perineal opening, palpable gonads	A	Gonadectomy and Genitoplasty at 4.9 years	Female	M – WT, F – NA
4[a]	46,XY	12	Atypical Clitoromegaly (2.0 cm),single perineal opening, palpable gonads	A	Gonadectomy at 3 years	Female	NA
5	46,XY	12.3	Atypical Clitoromegaly (4.0 cm), two perineal openings, no palpable gonads. Virilization at puberty	A	Gonadectomy and Genitoplasty at 12.5 years	Female	Mother is a carrier of the mutation and presented with POI
6	46,XY	13	Atypical Clitoromegaly (3cm), labial fusion, no palpable gonads	P	Gonadectomy and Genitoplasty at 15.5 years	Female	Mother is a carrier of the mutation, F – NA
7[a]	46,XY	16.7	Atypical Clitoromegaly, left gonad palpable	P	Gonadectomy at 14 years, Genitoplasty at 15 years	Female	NA
8	46,XY	21	Female genitalia	P	Not performed	Female	NA
9[a]	46,XY	26	Atypical Phallus 6.5 cm, perineal hypospadias, palpable testes	A	Genitoplasty at 26 years	Female to Male (9 years)	NA
10[a]	46,XY	39	Atypical, clitoromegaly (3cm), two perineal opening	A	Gonadectomy at 14 years, Genitoplasty at 39 years	Female	NA
11*	46,XX	39	Atypical, no palpable gonads	A	Genitoplasty in childhood	Male	NA

[a]Surgery performed previously to the evaluation in our center.
P, present; A, absent; F, father; M, mother; NA, not available; years, years of age; POI, primary ovarian insufficiency.

whom reassigned to male at 9 years of age (patient 9, Table 1); only one patient was assigned as male at birth (patient 1, Table 1). The initial assessment at our specialist unit ranged from 0.75 to 39 years of age. Only three patients were seen in childhood, and the remaining after puberty; four of these patients had previously undergone bilateral gonadectomy. The patient with normal-appearing female external genitalia was referred at 21 years of age due to primary amenorrhea (patient 8, Table 1).

Basal hormonal evaluation was available for seven patients. Gonadotropin levels were available in three prepubertal and in four postpubertal patients. All postpubertal patients had elevated gonadotropin levels, mainly FSH. Among four postpubertal patients, basal testosterone levels were normal for age in three (360, 155, and 414 ng/dl, respectively) and low in the single patient with normal female external genitalia (patient 8). A stimulation test with hCG was performed in four patients (two prepubertal and two pubertal) and in all of them testosterone levels increased adequately (Table 2).

TABLE 2. *Hormonal Profiles of Brazilian 46,XY DSD and 46,XX Testicular DSD Patients in Whom NR5A1 Mutations Were Identified*

Patient	Age (years)	FSH (IU/l)	LH (IU/l)	Testosterone (ng/dl)	
				Basal	After hCG
1	0.5	NA	NA	11	458
	13	6	6	NA	508
2*	0.7	2	4	NA	12
3	4.9	6	<0.6	14	169
4*	12	NA	NA	NA	NA
5	12.3	77	13	41.4	NA
6*	13	NA	NA	NA	277
7	13	69	24	155	295
8	21	60	16	14	NA
11*	26	21	14	360	499
9*	39	NA	NA	NA	NA
10*	59	45	28	178	NA

Chronological age corresponding to the hormonal evaluation.
Conversion factors to SI units: T, ng/dl to nmol/l, multiply by 0.0347
*Surgery performed previously to the evaluation in our Center
NA, not available.

Seven patients underwent bilateral gonadectomy; age at gonadectomy ranged from 9 months to 15.5 years. The two patients who were raised as males had inguinal testes and underwent bilateral orchiopexy. Gonadectomy was not performed in one patient who discontinued follow-up care. In three patients, a uterus was identified by pelvic ultrasound or MRI (Table 1).

None of the patients had symptoms or signs of adrenal insufficiency, and normal basal adrenal function was documented in six patients.

A family history of DSD was presented in three cases. Patient 6 had two paternal cousins with atypical genitalia. Patient 7 reported two second-degree cousins with primary amenorrhea. Patient 5's mother, who also carried the *NR5A1* variant, developed ovarian insufficiency at 39 years.

CLINICAL AND HORMONAL CHARACTERISTICS OF THE 46,XX DSD PATIENT BEARING A PATHOGENIC *NR5A1* VARIANT

Although mutations in *NR5A1* have been reported in a small proportion of women with primary ovarian insufficiency (POI), *NR5A1* mutations were not identified in 70 patients with POI in our cohort. However, the previously described p.Arg92Trp NR5A1 mutation was found in heterozygous state in one 46,XX patient with testicular DSD. This patient (Patient 11, Table 1) was first seen in our Unit when he was 59 years old. He was born with atypical genitalia and had undergone masculinizing genitoplasty during childhood. Spontaneous secondary sexual characteristics such as phallic enlargement were absent and testosterone replacement was started at 15 years of age. Bilateral mastectomy was performed at 18 years of age.

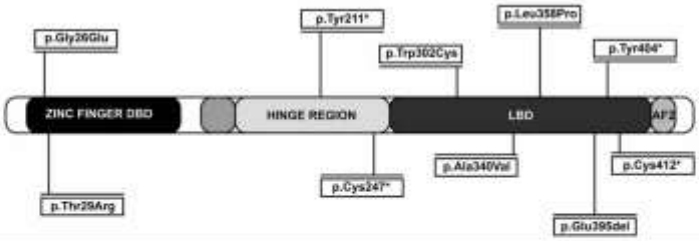

FIGURE 1. *The ten novel mutations identified in 46,XY DSD patients and their localization on the NR5A1 protein. DBD, DNA-binding domain; LBD, ligand-binding domain; AF2, activation function domain.*

TABLE 3. *Molecular Characterization of NR5A1 Defects Identified in Heterozygous State in Ten 46,XY and in One 46,XX Testicular DSD Patients*

Patient	Mutation (DNA)	Mutation (protein)	Domain	Functional impact	Bind energy
8	c.77G>A	p.Gly26Glu	DBD (1st zinc finger)	In silico prediction: damaging	↓
9	c.86C>G	p.Thr29Arg	DBD	In silico prediction:damaging	NA
5	c.663C>G	p.Tyr211*	Hinge	Truncated protein	NA
2	c.741C>A	p.Cys247*	Hinge	Truncated protein	NA
7	c.906G>C +c.437G>C	p.Trp302Cys +p.Gly146Ala	LBD Hinge	In silico prediction: damaging	↑
1	c. 1019C>T	p.Ala340Val	LBD	In silico prediction: damaging	NA
3	c.1073T>C	p.Leu358Pro	LBD	Impaired transactivation[a] ↑	↑
6	c.1183_1185del	p.Glu395del	LBD	In silico prediction: damaging	↓
10	c.1212C>G +c.437G>C	p.Tyr404* +p.Gly146Ala	LBD Hinge	Truncated protein	↑
11	c.1236C>A	p.Cys412*	LBD	Truncated protein	NA
4	c.274C>T	p.Arg92Trp	DBD (A box)	Ref	NA

The alignment of the rows is according to mutation location in the NR5A1protein. Binding energy to the [a]ligand di-pamitoyl-3-SN-phosphatidylethanolamine of the mutated complex compared to the non-mutated complex.

Functional studies of mutated NR5A1 activity shown a reduction in the transcriptional activity of Cyp11a1 and Cyp19 promoters.

↓, reduced bind energy; ↑, increased bind energy; DBD, DNA-binding domain; LBD, ligand-binding domain; NA, not available.

He did not have symptoms or signs of adrenal insufficiency, and basal adrenal profiling was normal. Peripheral blood leukocyte DNA was PCR-analyzed for Y-specific genes such as *SRY, TSPY, AMGY, DYZ3, DYS280*, and *DYS1*, all of which were not amplified and therefore considered absent. Pelvic MRI performed at 59 years of age revealed small bilateral testes in the inguinal regions; a uterus was not identified.

MOLECULAR CHARACTERISTICS OF NOVEL NR5A1 DEFECTS

Ten novel heterozygous *NR5A1* mutations were identified in 46,XY DSD patients (Fig. 1), including five nonsynonymous variants (p.Gly26Glu, p.Thr29Arg, p.Trp302Cys, p.Ala340Val, p.Leu358Pro), four stop-gain variants (p.Tyr211*, p.Cys247*, p.Tyr404*, p.Cys412*), and one frameshift variant (p.Glu395-del) (Table 3; Fig. 1). In three cases DNA samples were obtained from patients' mothers; two of them carried *NR5A1* mutations (Table 3).

The previously described common nonsynonymous p.Gly146Ala variant (rs1110061) was identified in two patients in association with p.Trp302Cys and p.Tyr404* *NR5A1* mutations, respectively.

IN SILICO STUDIES OF THE NR5A1 VARIANTS IDENTIFIED IN 46,XY PATIENTS

Primary sequence analyses showed that all NR5A1 mutated amino acids are extremely conserved among non-redundant homologous and relative sequences. Regarding potential and binding energies studies, native and mutated energy distributions of the p.Gly26Glu, p.Trp302Cys, p.Leu358Pro, p.Tyr404*, and p.Glu395del variants were compared. The LBD domain mutations p.Trp302Cys and p.Tyr404* both led to a higher energy state and therefore to a less stable molecule in comparison with the wild-type protein (Fig. 2).

The p.Gly26Glu variant, located in the DBD, establishes a polar contact not found in the original molecule, increasing its affinity for DNA binding and making the NR5A1 dissociation from the DNA helix difficult, thus potentially impairing transcriptional regulation. Interestingly, the p.Gly26Glu variant seems to stabilize the DBD, leading to a smaller energy compared to that of the native domain (Fig. 2). As a consequence, an opposite behavior was observed for binding energies. While all LBD mutated molecules studied increased the bind energy to the ligand di-pamitoyl-3-SN-phosphatidylethanolamine, p.Gly26Glu provided smaller binding energy to the DNA helix. Potential and binding energy means, variations, and overall conformations between mutated and native molecules are shown in Table 4.

Figure 3 presents the lowest energy molecular models of all mutated domains superposed to their respective native versions. Replacement of Gly by Glu at position 26 creates an additional contact not present in the original

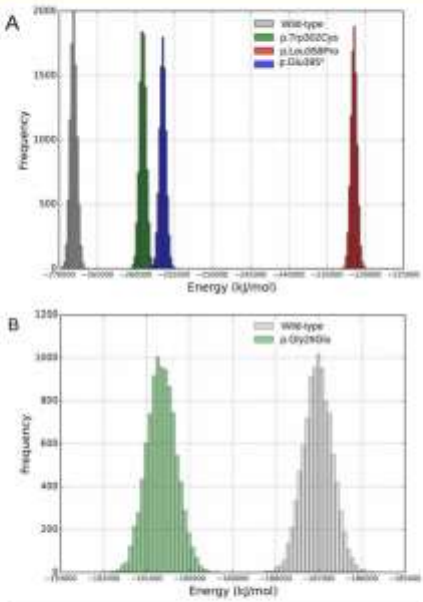

FIGURE 2. (**A**) *Energy distributions of the native NR5A1 LBD domain and mutated molecules. A higher energy is associated with a less stable molecule compared to the native due loss of amino acid contacts.* (**B**) *Energy distributions of the native NR5A1 DBD domain and its p.Gly26Glu mutated molecule. A smaller energy is associated to a more stable molecule, compared to the native due additional amino acids contacts.*

DBD domain. The negatively charged Glu26 Oε2 forms a salting bridge with the Arg79 Nε, which makes the β-hairpin 21-20 less flexible and closer to the ligand DNA. In the native LBD domain, the Try302imidazolic ring contributes to form a hydrophobic environment of the active site bottom cleft required to accommodate fatty ligands. Removal of such a bulky amino acid at that position induces a whole structural reorganization so that the central cavity of the protein shrinks, decreasing the occluded volume from 885 Å3 to 724 Å3. In turn, the p.Leu358Pro mutation is located in a hydrophobic core surrounded by four α-helices. In the original molecule, Leu358 establishes several contacts with a network of hydrophobic residues. Although proline is a non-polar amino acid, its side chain is too small compared to leucine and cannot effectively reach the surrounded hydrophobic residues. Therefore, many hydrophobic contacts were lost, which may explain

TABLE 4. *Variation of the Potential and Binding Energies Means Overall 5,000 Conformations Between Mutated and Native Molecules.*

Mutation/domain	ΔE (kJ/mol)	ΔE_{bind} (kJ/mol)
p.Gly26Glu/DBD	3633	−1,746
p.Tyr302Cys/LBD	9053	36
p.Leu358Pro/LBD	36,639	36
p.Glu385 del/LBD	11,686	69

All samples distributions passed Shapiro-Wilk normality test, being means compared with Welch's t-test.

All resultant p values were less than 2.2×10^{-16}.

the potential energy increasing. This change may be responsible for the reduction of transcriptional activity observed in the functional studies described below.

The variants p.Tyr211* and p.Cys247* are located in hinge region of NR5A1 that is important for stabilizing the LBD and interaction with other proteins that control NR5A1 transcriptional activity.

Finally, the Glu385 deletion is located within LDB helix-9, which is in the opposite direction of the ligand cleft. Together with the N-terminal helix-1, these two external helices tightly embrace the entire domain. In addition, the removal of the Glu385 slightly rotates the helix-9 axial axis; weakening several lateral contacts and making the helix unroll half a turn. All these modifications are predicted to be transmitted throughout the whole domain decreasing its affinity to the ligand.

The results of the molecular simulation analyses strongly support the hypothesis that all these novel mutations potentially interfere with the activity of the NR5A1 protein. Its structural stability and its ability to bind the di-palmitoyl-3-SN-phosphatidylethanolamine and probably other fat can be affected by the presence of the mutations. The function of such fat acid has not been cleared; however, it certainly provides additional contacts and stability to the whole molecule to perform its activities. Altogether, the simulation results strengthen the hypothesis that the studied mutations are very likely deleterious.

IN VITRO FUNCTIONAL STUDIES OF THE P.LEU358PRO NR5A1 VARIANT

In vitro studies showed a markedly impaired transcriptional activity of the p.Leu358Pro mutant NR5A1, with a reduction of more than 70% and 80% in the transactivation of the *Cyp11a1* and *Cyp19* promoters, respectively (Fig. 4).

Discussion

NR5A1 MUTATIONS IN 46,XY INDIVIDUALS

Since the first 46,XY DSD patient with the p.Gly35Glu *NR5A1* mutation described by Achermann et al. (1999), the spectrum of phenotypes associated with *NR5A1* mutations has greatly expanded. This initial patient bearing the

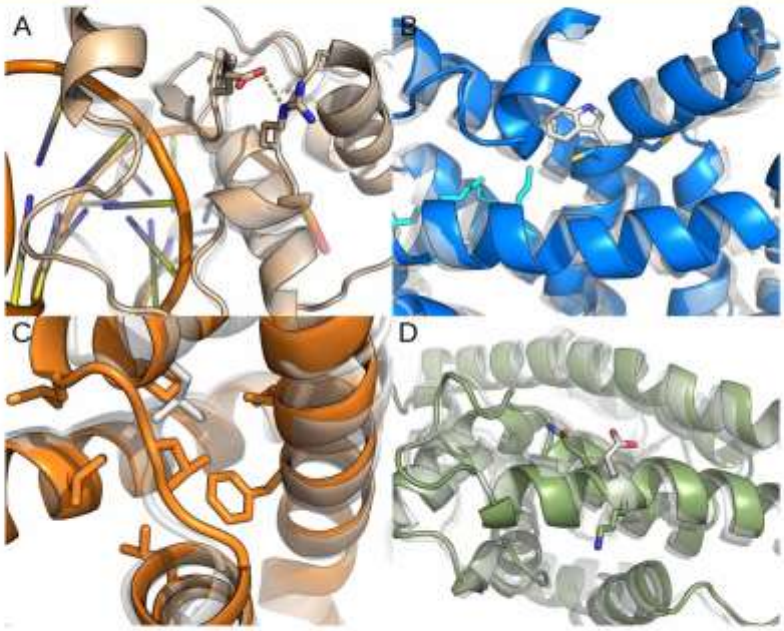

FIGURE 3. *Superimposed Models. Superimposition of the native (white) and mutated smallest energy NR5A1 models (colored). (**A**) DBD domain showing Glu26 and Arg79 salt bridge, (**B**) LBD domain showing Trp403Cys, (**C**) LBD hydrophobic network with Leu358Pro in the center, and (**D**) LBD helix 9 rotated and unwound half a turn with the Glu385 deletion.*

heterozygous p.Gly35Glu mutation presented with adrenal failure and gonadal dysgenesis with persistent Mullerian derivatives (Woo et al., 2015), a phenotype that closely resembled the phenotype observed in murine *Nr5a1* knockout models (Luo et al., 1994). Gonadal dysgenesis and adrenal insufficiency were also present in the second reported NR5A1-related 46,XY DSD, in a patient bearing the homozygous p.Arg92Gln mutation (Achermann et al., 2002).

The expected phenotype associated with *NR5A1* mutations was shifted when a heterozygous 8-bp microdeletion was found in a 46,XY DSD patient who presented with clitoromegaly, absence of uterus and gonads but normal adrenal function (Correa et al., 2004). After this first report, the study of several cohorts of individuals with 46,XY DSD has shown that adrenal insufficiency is a rare finding in patients with NR5A1 defects (Lin et al., 2006; Guran et al., 2016).

Reported heterozygous *NR5A1* mutations support the model that partial NR5A1 dysfunction can result in several degrees of impaired Leydig cell function and androgen biosynthesis, leading to predominantly abnormal gonadal phenotypes, which can range from complete testicular dysgenesis with Mullerian structures, through mild clitoromegaly or atypical genitalia without Mullerian derivatives, to proximal hypospadias associated with undescended testis (Köhler et al., 2009), or even micropenis with absent gonads (Philibert et al., 2007).

Currently, *NR5A1* mutations represent one of the most frequent defects associated with 46,XY gonadal dysgenesis, accounting for up to 20% of cases (Suntharalingham et al., 2015).

More than 80 different *NR5A1* variants, distributed across the full length of the protein, have been described and the majority are nonsynonymous mutations (Pedace et al., 2014; Tantawy et al., 2014; Woo et al., 2015; Fabbri et al., 2016). Most of these mutations are located in the DBD and are in a heterozygous state or compound heterozygous state with the p.Gly146Ala (rs1110061) variant,

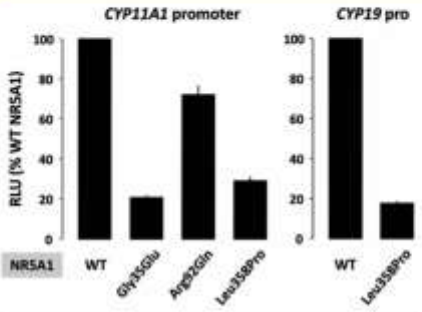

FIGURE 4. *Transcriptional activation of target gene promoters by wild-type (WT) and mutant NR5A1 in human embryonic kidney TSA 201 cells. In vitro activation of CYP11A1 and CYP19 gene promoters by the novel NR5A1 mutant p.Leu358Pro was reduced, in comparison to WT NR5A1 (29% and 18% of WT, respectively). Notably, the degree of transcriptional activity impairment of the CYP11A1 promoter by p.Leu358Pro NR5A1 was comparable to that of the well known NR5A1 mutant p.Gly35Glu (Lin et al., 2007) and more severe than p.Arg92Gln (Achermann et al., 2002). Results represent the mean (SEM) for three assays, each performed in triplicate, and shown relative to wild-type transactivation. RLU, relative luciferase units.*

with the exception of two mild mutations described in homozygous state (Achermann et al., 2002; Soardi et al., 2010). These findings reinforce the concept that NR5A1 dosage is critical to normal gonadal development. However, a clear correlation between the location of a mutation, its in vitro functional performance, and the associated phenotype is not observed. Indeed, family members bearing the same *NR5A1* mutation may present with variable phenotypes (Warman et al., 2011).

There are some hypotheses to explain the phenotypic variation associated with a similar *NR5A1* mutation. First, interallelic association with the known p.Gly146Ala polymorphism (rs1110061) may further reduce NR5A1 activity and contribute to more severe phenotypes (WuQiang et al., 2003; Hasegawa et al., 2004; Wada et al., 2006; Reuter et al., 2007; Köhler et al., 2008; Lourenço et al., 2009; Bashamboo et al., 2010a; Paris et al., 2011; Camats et al., 2012). However, two patients in our cohort also bore the p.Gly146Ala variant besides the pathogenic NR5A1 variants, p.Trp302Cys and p.Tyr404*, and their phenotype was not strikingly distinct or more severe.

The contribution of other genetic modifiers has also been suggested to explain phenotypic variability. Exome sequencing analyses of DSD patients have identified pathogenic variants or variants of uncertain significance in several genes involved in sexual development (Bashamboo et al., 2010b; Bashamboo et al., 2014). In a 46,XY patient with atypical external genitalia, palpable inguinal gonads, absent uterus in pelvic ultrasonography, and poor testosterone response to hCG stimulation, Mazen and colleagues (2016) identified, by exome sequencing, the previously described p.Arg313Cys *NR5A1* mutation in compound heterozygous state with a p.Gln237Arg *MAP3K1* variant. This *NR5A1* mutation was previously reported in association with mild hypospadias (Allali et al., 2011), and a possible digenic inheritance was proposed to explain the phenotypic heterogeneity (Mazen et al., 2016).

Progressive androgen production and virilization in adolescence has been observed in several XY patients with *NR5A1* mutations, in contrast to the severe undervirilized external genitalia found in most patients (Cools et al., 2012; Gabriel Ribeiro de Andrade et al., 2014; Tantawy et al., 2014; Fabbri et al., 2016). The almost normal testosterone levels after hCG stimulation or at pubertal age suggest that NR5A1 action may be less implicated in pubertal steroidogenesis than during fetal life. NR5A1 is expressed in the both fetal and adult Leydig cells, but differences in its actions at both stages are not completely known. The study of a hypomorphic mouse model of *Nr5a1* indicated differential impairment of fetal and adult Leydig cell development, whereby *Nr5a1* may regulate the differentiation of fetal Leydig cells, whereas in the adult it may regulate progenitor cell formation and/or survival (Karpova et al., 2015). Different actions of NR5A1 in fetal and postnatal Leydig cells populations might contribute to the switch from birth to pubertal phenotypes.

In contrast, fetal Sertoli cell function seems to be preserved in the most patients with heterozygous *NR5A1* mutations, based on the common observation of absent Müllerian derivatives and primitive seminiferous tubules on histology. In our cohort, three of 10 patients had Müllerian derivatives although in one of them the uterine volume remained infantile after three years of estrogen replacement.

The reviewed data of seventy-two 46,XY DSD patients with *NR5A1* mutations reported in the literature, for whom information on presence or absence of Müllerian derivatives was available, suggested that Müllerian derivatives are present in about 24% of the cases (Pedace et al., 2014; Tantawy et al., 2014; Woo et al., 2015; Fabbri et al., 2016). However, persistently elevated FSH levels after puberty found in all patients studied suggest an impairment of Sertoli cells function in post pubertal age (Pedace et al., 2014).

Male infertility has been also related to the presence of *NR5A1* defects (Bashamboo et al., 2010a; Ferlin et al., 2015). Patients with moderate/severe oligospermia or azoospermia and *NR5A1* mutations may have normal testosterone and normal low or low inhibin B levels, but they are at potential risk of deterioration of testicular hormonal secretion with age and may need counseling regarding

preservation of sperm and regular monitoring of endocrine function (Bashamboo et al., 2010a).

The presence of ovotestis was described in a 46,XY girl, with the 9q33.3-q34.1 deletion encompassing *NR5A1* and *LMX1B* genes causing genitopatellar syndrome associated with female external genitalia and clitoromegaly (Schlaubitz et al., 2007).

NR5A1 MUTATIONS IN 46,XX SUBJECTS

The first reported 46,XX patient with a *NR5A1* mutation (p.Arg255Leu) presented with isolated adrenal insufficiency, but a follow-up description of ovarian function at postpubertal age is lacking (Biason-Lauber and Schoenle, 2000). Subsequently, it was shown that *NR5A1* mutations are also a genetic cause of POI, with phenotypes ranging from primary to secondary amenorrhea, associated with infertility, hypoestrogenism, and elevated gonadotropin levels (Lourenço et al., 2009; Camats et al., 2012) Sisters and mothers of 46,XY DSD patients carrying heterozygous *NR5A1* mutations may also develop premature ovarian failure (Lourenço et al., 2009; Camats et al., 2012; Fabbri et al., 2016).

Several genes required for ovarian steroidogenesis, and follicle growth and maturation are regulated by *NR5A1*, explaining why mutant *NR5A1* may lead to progressive loss of ovarian function and female reproductive capacity (Lourenço et al., 2009; Camats et al., 2012; Fabbri et al., 2016). Women bearing *NR5A1* defects should receive appropriate counseling and fertility guidance, enabling potential oocyte cryopreservation, due to the risk of developing ovarian insufficiency later in life.

In contrast to its prominent pathogenic role in 46,XY DSD, NR5A1 defects are a rare cause of sporadic POI of unknown etiology in women (1.4–1.6%). (Suntharalingham et al., 2015) Sixteen different *NR5A1* variants distributed across the full length of the protein have been described in patients with premature ovarian failure, and the majority of them are nonsynonymous mutations (Suntharalingham et al., 2015)

The spectrum of disorders associated with *NR5A1* variants in 46,XX individuals was further expanded by the demonstration of the specific recurrent heterozygous p.Arg92Trp NR5A1 variant, in association with variable degrees of testis development in 46,XX patients from unrelated families (Baetens et al., 2016; Bashamboo et al., 2016; Igarashi et al., 2017). The p.Arg92Trp *NR5A1* mutation was described in ten *SRY* negative 46,XX DSD patients, six with testicular DSD and four with ovotesticular DSD. This mutation was found in two familial cases: the first one comprising two 46,XX ovotesticular DSD sisters and the second one involving one 46,XX testicular DSD patient and his 46,XY sister with partial gonadal dysgenesis. In these two families a maternal inheritance was identified. Atypical genitalia was the most frequent presentation (six patients), but a phenotypic spectrum ranging from female external genitalia with clitoromegaly (one patient), to male external genitalia with micropenis (two patients) or penoscrotal hypospadias (one patient) was observed. Three of eight patients had a uterus, and two had a hemiuterus. Gonads were palpable in seven patients and the other three 46,XX ovotesticular patients had bilateral abdominal ovotestis.

The p.Arg92Gln NR5A1 mutation, located at the same residue, was previously described in homozygous state in a 46,XY phenotypic female with adrenal insufficiency and in homozygous state in a 46,XX girl with early-onset primary adrenal insufficiency. This variant was also present, in heterozygous state, in her phenotypically normal sister, mother, and father. (Achermann et al., 2002; Guran et al., 2016) Differences in the transactivating activity of the p.Arg92Gln and the p.Arg92Trp mutants may be responsible for the significant phenotypic differences observed (Igarashi et al., 2017) Nevertheless, the contribution of modifying allelic variants in other genes involved in gonadal development may not be completely excluded.

In vitro assays demonstrated that the p.Arg92Trp mutant lost its binding capacity to a consensus NR5A1 response element and reduced activation of some minimal promoters (*Amh*, *Cyp11a1*) (Bashamboo et al., 2016). This mutated protein was also less sensitive to NR0B1-induced suppression on the *SOX9* TESCO element, leading to testis formation (Igarashi et al., 2017). These findings suggest a dysregulation of normal female gonadal development by p.Arg92Trp NR5A1, tipping the balance toward testis development in 46,XX individuals.

Conclusion

In a single-center Brazilian cohort, we have identified 10 novel *NR5A1* mutations in 46,XY DSD patients and the previously described p.Arg92Trp mutation in one 46,XX testicular DSD patient. Additionally, no mutations were found in 70 patients with POI. Our findings reinforce those of previous reports and contribute to expanding our understanding of NR5A1-related phenotypes in humans.

NR5A1 mutations are associated with a wide spectrum of disorders of gonadal development, ranging from DSD to oligo/azoospermia in 46,XY individuals and 46,XX ovotesticular and testicular phenotypes to primary ovarian failure in 46,XX individuals. The most frequent phenotype in 46,XY patients is atypical or female external genitalia with clitoromegaly, palpable gonads, and absence of Müllerian derivatives. Postnatally, Leydig cells function seems to be preserved in several patients with *NR5A1* mutation, while Sertoli and germ cells are more profoundly affected. The severely undervirilized external genitalia frequently seen at birth, contrasts with spontaneous virilization at puberty in several patients. Phenotypic variability observed in patients with similar NR5A1 defects may be related to the association with genetic modifiers or pathogenic variants in other testis/ovarian-determining genes.

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