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Desirée Deconte

Doenças monogênicas raras: caracterização molecular e computacional de variantes genéticas

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Resumo

Introdução: As doenças monogênicas representam um grupo extenso de alterações fenotípicas que afetam a população mundial: existem, hoje, cerca de 7.000 doenças monogênicas conhecidas. Apesar de extensas investigações para elucidar a etiologia molecular específica de doenças raras, a identificação de genes relacionados a elas são, ainda, escassos. Com a etiologia desconhecida, o diagnóstico para a maioria dos pacientes com suspeita de alguma condição monogênica rara continua imprecisa. Sendo assim, a necessidade de buscar o diagnóstico e a caracterização molecular para esses pacientes é essencial para estabelecer correlações entre o genótipo e o fenótipo, melhorar o manejo e o tratamento destes pacientes e para possibilitar a identificação de fatores etiológicos e prognósticos para um aconselhamento familiar mais preciso. **Objetivos:** Realizar a avaliação molecular de pacientes com suspeita clínica de diferentes alterações monogênicas raras associadas aos genes *CHST3, EDNRB, FGFR2, FOXC2, KIAA0586, NFIX* e *SOX9,* bem como realizar a predição *in silico* das variantes encontradas. **Material e Métodos:** O DNA de todos os pacientes e seus respectivos progenitores foi isolado pelo método de *salting-out* e amplificado pelo método de reação em cadeia da polimerase (PCR) convencional com o primers desenhados para cobrir todas as regiões codificantes dos genes. O sequenciamento das amostras foi realizado através do sequenciador automático ABI-PRISM 3130 Genetic Analyzer e os eletroferogramas gerados analisados com o auxílio do programa Chromas Lite. Para as análises *in silico* serão usadas à nível gênico as 25 ferramentas recomendadas pelas Diretrizes para Interpretação de Variantes de Sequências da ACMG quanto às análises preditivas para troca de

sentido, alteração de sítios de splicing e conservação de nucleotídeos. No nível protéico, os quatro níveis hierárquicos de sua estrutura serão avaliados Além disso, algoritmos de predição estipulados pela *American College of Medical Genetics and Genomics* e a *Association for Molecular Pathology* serão utilizados para realizar a avaliação dos critérios de patogenicidade das variantes encontradas a nível populacional, de estrutura proteica e conservação evolutiva. **Resultados:** Foram encontradas variações de sequência nos genes *CHST3, EDNRB, FGFR2, KIAA0586* e *NFIX.* No paciente diagnosticado com displasia esquelética foi encontrada uma nova variante missense em homozigose no gene *CHST3* por NGS, confirmada, posteriormente, por sequenciamento de Sanger. Alterações no gene *FGFR2* foram encontradas tanto para o indivíduo diagnosticado com síndrome de Pfeiffer, quanto para o paciente com síndrome de Apert. Na síndrome de Pfeiffer, uma variante missense *de novo* foi encontrada na região de sítio aceptor de splicing entre os exons 8 e 9. Para a síndrome de Apert, outra variante missense *de novo* em heterozigose foi encontrada. Ainda, uma nova variante também foi identificada no indivíduo com síndrome de Marshall-Smith: duplicação de sete nucleotídeos no éxon 8 do gene *NFIX* . Para o paciente com diagnóstico de síndrome de Shah-Waardenburg foi encontrada uma variante missense em homozigose no gene *EDNRB*. A análise do painel de genes para ciliopatias identificou uma variante em *KIAA0586* herdada do pai no paciente diagnosticado com síndrome de Hydrolethalus. Ainda, uma análise por rtPCR encontrou uma possível variante intrônica também no gene *KIAA0586* na amostra materna, possivelmente herdada pela filha. Por fim, para os genes *FOXC2* e *SOX9* não foi encontrada nenhuma mutação no sequenciamento dos

respectivos pacientes. **Conclusão:** Neste trabalho foi avaliado um único paciente com suspeita de doença monogênica rara para cada gene associado ao respectivo fenótipo. Apesar de nossa análise molecular cobrir toda a região codificante dos genes, para dois destes pacientes não foi observada nenhuma mutação. Para os demais casos, foram observadas duas mutações previamente descritas para pacientes com craniossinostoses (rs1057519041 e rs77543610) e quatro variantes novas. Para as mutações novas, análises *in silico* foram realizadas para auxiliar na elucidação da probabilidade de dano ao genoma, bem como na predição da proteína alterada e, ainda, na compreensão do funcionamento do gene investigado. Estes resultados reforçam a existência de uma heterogeneidade genética entre casos de mesmo diagnóstico clínico, bem como a importância do diagnóstico molecular para cada paciente de forma a fornecer um diagnóstico completo mais efetivo e preciso e um aconselhamento genético mais personalizado.

Palavras-chave: sequenciamento, doença monogênica, análise *in silico*, gene, variante.

Abstract

Introduction: Monogenic conditions represent a large group of phenotype alterations that affect the population worldwide. Nowadays there are on average 7.000 known monogenic diseases. Despite extensive investigations to diagnose molecular causes for rare phenotype conditions, the identification for related genes are still very low. With an unknown etiology, for most patients with a rare disease the diagnosis remains inaccurate. Therefore the need for a precise molecular characterization and an optimum diagnosis is fundamental to correlate genotype and phenotype, to give a better treatment for this group of patients and to be able to identify etiologic factors to perform a better genetic counseling. **Aim of study:** To perform a molecular analysis of patients with a suspicious diagnosis of rare monogenic diseases associated with the following genes: *CHST3, EDNRB, FGFR2, FOXC2, KIAA0586, NFIX* e *SOX9*. Also, for new identified variants, to predict its function using *in silico* tools. **Materials and methods:** DNA from both patient and parents blood samples were isolated by salting-out method and amplified by conventional polymerase chain reaction (PCR) with primers designed to cover all gene coding regions. Sample's sequencing was performed using the automatic sequencer ABI-PRISM 3130 Genetic Analyzer and electropherograms were analyzed with the Chromas Lite program. For *in silico* assessments, 25 computational tools recommended by the ACMG Guidelines for Interpreting Sequence Variants will be used to analyze the variant at gene level. At protein level, all four structures levels will be properly evaluated. Moreover, prediction algorithms stipulated by the American College of Medical Genetics and Genomics and the Association for Molecular Pathology will be used to evaluate the new variants' pathogenicity

criteria at a population level, as well as protein structure and evolutionary conservation. **Results:** We found genetic alterations in *CHST3, EDNRB, FGFR2, KIAA0586* and *NFIX* genes. In the individual diagnosed with skeletal dysplasia we found a new recessive homozygous missense variant at gene *CHST3* by NGS, later confirmed by Sanger sequencing. Gene alterations at *FGFR2* were found for both patients diagnosed with Pfeiffer syndrome and Apert syndrome. In the individual with Pfeiffer syndrome we found a *de novo* mutation at an acceptor splicing site in intron 8 – exon 9 boundary at *FGFR2*. For the patient with Apert syndrome another *de novo* heterozygous missense variant was found on the same gene. A new variant was also identified in the individual diagnosed with Marshall-Smith syndrome: a duplication of seven nucleotides was found on *NFIX* gene's exon 8. For the patient with Shah-Waardenburg syndrome, we found a new homozygous missense variant at *EDNRB*. An exome analysis of genes associated with ciliopathies identified a variant on *KIAA0586* on the patient with Hydrolethalus syndrome inherited from the father. Moreover, rtPCR analysis showed an intronic variant at *KIAA0586* on the mother's genome, which may be also inherited by the child. No mutation was found in the patients' sequence associated with *FOXC2* e *SOX9* genes. **Conclusion:** In this study we investigated one patient for each gene associated with a rare monogenic disease diagnosis. Although all coding region sequencing analysis was performed, two patients did not have any variant found in their sequence. For the other six cases, we found two previously reported variants in probands with craniosynostosis (rs1057519041 e rs77543610) and four brand new variants. For the new molecular findings, we performed *in silico* assessments in order to elucidate variants probability on

genome damage, as well as protein structure prediction. Moreover, computational tools aid us to better understand the investigated gene function and roles in the human genome. Our results reinforce the existence of a genetic heterogeneity between cases of same clinical diagnosis, as well as the importance of molecular diagnosis for each patient in order to provide a more effective and precise diagnosis and a personalized genetic counseling.

Keywords: sequencing, monogenic disease, *in silico* assessment, gene, variant.

Lista de abreviaturas

DNA: *deoxyribonucleic acid*

dNTPs: desoxinucleotídeos

ddNTPs: didesoxinucleotídeos

HMIPV: Hospital Materno Infantil Presidente Vargas

MLPA: *Multiplex Ligation-dependent Probe Amplification*

MRSHSS: síndrome de Marshall-Smith

NFAT: fator nuclear das células T ativas

NFI: Fator Nuclear I

NGS: sequenciamento de próxima geração

NMD: *non-mediated RNA decay*

OMIM: *Online Mendelian Inheritance in Man*

PCR: *Polimerase Chain Reaction*

SRY: região Y de determinação de sexo

UFCSPA: Universidade Federal de Ciências da Saúde de Porto Alegre

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1. REFERENCIAL TEÓRICO

As doenças monogênicas, também conhecidas como mendelianas, são condições genéticas em que a alteração de um único gene é suficiente para acarretar no aparecimento de determinadas manifestações clínicas.[1,2] Atualmente, existem aproximadamente 7.000 doenças monogênicas conhecidas ao redor do mundo, sendo que apenas metade dos genes responsáveis por estas patologias foram identificados.[3] Enquanto a prevalência para a maioria das doenças causadas por variações em um único gene é baixa e definida, então, com raras, as doenças mendelianas juntas acometem aproximadamente 10 indivíduos a cada 1.000 nascimentos de acordo com dados fornecidos pela Organização Mundial da Saúde.[4] Além disso, os fenótipos apresentados por indivíduos diagnosticados com síndromes monogênicas frequentemente envolvem morte, incapacidade ou malformação congênita, implicando em uma série de problemas tanto para as famílias afetadas como para o sistema de saúde público e privado.[2,5]

Avanços recentes em técnicas de biologia molecular, como análises cromossômicas em *microarray*, painéis genéticos e sequenciamento de genoma ou exoma, tem motivado novas abordagens para o mapeamento de genes associados a doenças e estimulou a rápida descoberta de genes relacionados doenças monogênicas.[6,7,8] De fato, uma nova visão para os transtornos monogênicos está surgindo, uma vez que pacientes com sintomas antes não explicados estão sendo diagnosticados com variantes patogênicas causais específicas.[8,9] Entretanto, apesar do benefício claro do advento de tecnologias mais avançadas, alguns conceitos previamente consolidados foram questionados: a aparição significativa de variabilidade fenotípica entre indivíduos

com o mesmo achado molecular e, da mesma forma, a heterogeneidade de variantes genéticas dentro de uma mesma condição clínica instigaram a necessidade de reavaliar o diagnóstico molecular de doenças monogênicas.[1,6,9,10] Ainda, a identificação de variantes novas, ainda não descritas na literatura, alertou a comunidade científica para a necessidade de criação de uma padronização da nomenclatura, bem como a criação de ferramentas que elucidassem a probabilidade da variante descoberta ser causativa do fenótipo clínico observado.[8,11]

A *American College of Medical Genetics and Genomics* e a *Association for Molecular Pathology* estipulou normas e orientações para a interpretação de variantes genéticas de forma a aprimorar a investigação e a elucidação da potencial relação genótipo-fenótipo nas doenças raras. Isso se deve ao fato de que cada paciente pode ser único no mundo, sendo a variação identificada como possível causadora do fenótipo analisado ainda não descrita (“nova mutação”) e não caracterizada em termos de funcionalidade. Algoritmos de predição e critérios de patogenicidade devem ser considerados na análise a fim de providenciar uma avaliação mais fidedigna do nível de importância patológica de variantes na população. Sabe-se que polimorfismos existem em diferentes populações e que essas variantes não necessariamente indicam risco de patogenicidade para o indivíduo portador.[11]

Concomitantemente aos algoritmos de predição, análises de bioinformática permitem, também, a investigação a nível protéico das alterações ocasionadas por variantes, possibilitando a elucidação de causas moleculares e a realização da associação entre genótipo-fenótipo.[12,13] Estratégias combinadas de genética médica, biologia molecular e bioinformática têm sido bem-sucedidas ao auxiliar

na compreensão de diferentes doenças com etiologia genética, como fibrose cística e deficiência de frutose-1,6-bifosfatase.[14,15] A análise da desordem proteica é um exemplo de análise *in silico* que pode ser realizada para avaliar o tipo de alteração que a variante encontrada causa na estrutura e na conformação da proteína.[16,17] Dessa forma, a análise molecular aliada à análise computacional proporciona uma investigação mais completa de novas variantes genéticas, auxiliando na elucidação de causas moleculares e na associação entre genótipo-fenótipo, bem como na relevância da mesma a nível populacional.

A genética médica refere-se a toda aplicação prática da genética à medicina. Ela abrange o estudo da herança das doenças nas famílias, a identificação de genes causadores de doenças, a análise dos mecanismos moleculares através dos quais os genes causam doenças, o diagnóstico e a possibilidade de tratamento e prevenção de doenças genéticas.[18] Ela abrange também o aconselhamento genético, que consiste na informação quanto aos riscos, prognóstico e tratamento de apoio dispensado aos pacientes e suas famílias.[18,19] Atualmente as análises de sequenciamento de exoma e genoma (WES e WGS) permitem a identificação de muitas variações genéticas novas e ferramentas computacionais auxiliam na elucidação das consequências fenotípicas destas variações.[7] Sendo assim, cada descoberta bem-sucedida é uma futura contribuição para possíveis diagnósticos, prevenção e oportunidades terapêuticas para as doenças correspondentes e, ainda, um auxílio para a elucidação dos mecanismos biológicos e patológicos destes fenótipos.[20,21]

Sendo assim, o estudo molecular de doenças monogênicas permite não apenas o ganho científico na associação cada vez mais precisa de causa e consequência entre genótipo e fenótipo, mas, também, viabiliza o compartilhamento dessas

informações com a comunidade externa através do aconselhamento genético. A elucidação da etiologia do gene, do mecanismo molecular e da manifestação clínica de doenças mendelianas permite a educação da família a respeito da herdabilidade genética, além de proporcionar um melhor prognóstico e apoio a longo prazo para os pacientes e seus familiares.

1.1. Fenótipos clínicos associados a mutações em *CHST3*

Variantes patogênicas encontradas ao longo do gene *CHST3* (OMIM #603799) estão geralmente associadas a deficiências esqueléticas, coletivamente chamadas de displasias esqueléticas ou osteocondrodisplasias. A prevalência desse grupo de malformações na população é de 1 para 5.000 nascidos vivos e o grau de severidade pode variar entre apresentações leves à eventuais fenótipos mais graves.[22,23]

A malformação esquelética congênita é frequentemente observada ao nascimento com neonatos apresentando baixa estatura e múltiplos deslocamentos e/ou subluxações de articulações.[24] A priori, as mutações encontradas no gene *CHST3* eram associadas diretamente a condição denominada displasia espondiloepifisária congênita (OMIM #143095), uma malformação genética de herança autossômica recessiva regularmente descrita em famílias com eventual grau de consanguinidade.[25,26,27] Entretanto, com a ampliação dos achados clínicos e variedade significativa de severidade entre os casos, o gene supracitado é hoje referenciado como causador do grupo de alterações conhecidas como displasias esqueléticas.[23,25] As características clínicas relatadas com mais frequência e que convergem entre os casos já

descritos incluem grandes contraturas articulares, cifose progressiva, pé torto e luxação da articulação do quadril.[23-28,]

O gene *CHST3* está localizado no cromossomo 10(10q22.1) e codifica a proteína carboidrato sulfotransferase 3 (C6ST) responsável por catalisar a sulfatação de condroitina, um proteoglicano encontrado na matriz extracelular da maioria das células envolvidas na migração e diferenciação celular.[29] A condroitina, por sua vez, é um dos componentes que compõem os glicosaminoglicanos, estruturas ligadas a proteínas centrais e macromoléculas amplamente distribuídas pelo corpo. A sulfatação do glicosaminoglicano cria uma carga negativa em sua superfície crucial para a sua função biológica, sendo os genes que codificam carboidrato sulfotransferases imprescindíveis para a sulfatação correta das cadeias desses polissacarídeos.[30,31]

O gene *CHST3* é expresso principalmente no tecido cardíaco, nos músculos esqueléticos, na placenta e no timo. Estudos de associação de genoma completo indicam que variantes patogênicas encontradas nesse gene resultam em defeitos congênitos estruturais como displasias esqueléticas. Em sua maioria, fenótipos que contemplam displasia espondiloepifisária com deslocação articular, baixa estatura e escoliose estão entre os mais descrito na prática clínica.[25,28,31] Atualmente, mais de 30 variantes associadas à condições genéticas foram relatadas na literatura.[23] Os achados moleculares são heterogêneos e variam de mutações pontuais à duplicações no gene *CHST3* com padrões de herança variando entre autossômicos dominantes e recessivos.[23,25,28,32]

1.2. Fenótipos clínicos associados a mutações em *EDNRB*

A síndrome de Waardenburg, tipo 4 (também conhecida como síndrome de Shah-Waardenburg) (OMIM #277580) é a condição genética associada com maior frequência à variantes patogênicas encontradas no gene *EDNRB* (OMIM #131244). Entre as características fenotípicas mais comuns da patologia estão a perda de cabelo, anormalidades de pigmentação, deficiência auditiva neurossensorial e doença de Hirschsprung.[33] A prevalência estimada da síndrome na população mundial é de 1 a cada 42.000 nascidos vivos.[34] Apesar das características clássicas e bem definidas da condição genética, a manifestação clínica da doença tem uma variabilidade expressiva entre os indivíduos afetados com o padrão de herança variando entre autossômico dominante e recessivo.[35]

As variantes encontradas no gene *EDNRB* são normalmente apresentadas em homozigose, com raras descrições de achados moleculares em indivíduos heterozigotos.[35,36] Entretanto, quando identificadas, as alterações genéticas em apenas uma cópia do gene são normalmente associadas diretamente ao fenótipo de doença de Hirschsprung (OMIM #600155). Por outro lado, as alterações apresentadas em homozigose estão relacionadas à manifestação da síndrome de Shah-Waardenburg propriamente dita, a qual incorpora, inclusive, o distúrbio gastrointestinal caracterizado pela doença de Hirschsprung.[35,36,37] Ainda, estudos apontam para a possibilidade do envolvimento do gene no desenvolvimento do melanoma, uma vez que o mesmo está relacionado à produção de melanócitos - células produtoras de melanina, proteínas responsáveis pela pigmentação da pele. Entretanto, a associação do

gene ao desenvolvimento do câncer de pele ainda não está bem elucidada na literatura.[38]

O gene *EDNRB* está localizado no cromossomo 13 (13q22.3) e codifica a proteína denominada receptor endotelial tipo B, responsável por atuar no mecanismo de sinalização celular, transmitindo informações extracelulares para o ambiente intracelular.[39] O receptor codificado pelo gene interage com proteínas endoteliais no intuito de regular processos biológicos críticos como o desenvolvimento e funcionamento de vasos sanguíneos, proliferação e divisão celular e produção de hormônios específicos.[39,40,41] A endotelina 3 é a proteína com maior destaque na interação com o receptor uma vez que a atuação conjunta dos mesmos possui um papel importante no desenvolvimento das células da crista neural.[41,42] A formação de nervos entéricos e produção especializada de melanócitos também foram associadas à expressão de endotelina 3 e do receptor codificado pelo gene *EDNRB*.[38,40] Atualmente, as variantes encontradas nesse gene foram associadas a fenótipos de alteração de pigmento e obstruções intestinais, bem como atuação no desenvolvimento de melanomas.[38,40,42]

1.3. Fenótipos clínicos associados a mutações em *FGFR2*

Variantes genéticas patogênicas encontradas nos receptores 1 e 2 do fator de crescimento fibroblástico (*FGFR1* e *FGFR2*) estão relacionadas ao desenvolvimento de craniossinostoses com padrão de herança autossômico dominante como a síndrome de Pfeiffer (OMIM #101600), de Apert (OMIM #101200), de Crouzon (OMIM #123500) e de Jackson-Weiss (OMIM #123150). As mutações em *FGFR1* (OMIM #136350) produzem um fenótipo clássico de

malformações de membros inferiores e superiores, presença variável de craniossinostose e características craniofaciais mais leves, enquanto variantes em *FGFR2* (OMIM #176943) são associadas a um quadro clínico mais severo com presença de anormalidades de fusão dos ossos cranianos, hipertelorismo associado a uma proptose extrema, anomalias variáveis de mãos e pés, anomalias vertebrais e óbito no período neonatal.[43]

Entre as variantes patogênicas descritas, mais de 94% das mutações encontradas em indivíduos diagnosticados com síndrome de Pfeiffer ocorrem nos éxons 8 e 10 do gene *FGFR2*. Além do fenótipo para Pfeiffer, alterações genéticas nesses dois éxons também estão associadas a outras craniossinostoses.[44,45] Apesar do conjunto de síndromes de anormalidades cranianas convergirem para achados genéticos de sobreposição, para a síndrome de Apert a maioria expressiva das mutações descritas na literatura estão alocadas no éxon 7.[46] Embora a maioria das variantes patogênicas descritas no gene *FGFR2* sejam encontradas em éxons que codificam o domínio extracelular IgIII, alterações ao longo de toda região codificante do gene já foram identificadas e associadas a craniossinostoses.[47,48]

O gene *FGFR2* está localizado no cromossomo 10 (10q26.1) e codifica uma proteína transmembrana chamada receptor 2 do fator de crescimento fibroblástico, a qual possui um papel significante em alguns processos importantes do desenvolvimento embrionário como a divisão celular, a proliferação celular e a regulação do crescimento e da maturação da célula de acordo com o tecido alvo. A família FGF é composta de pelo menos 22 ligantes conhecidos que se ligam a cinco receptores de tirosina quinase. Essa junção resulta na ativação de domínios quinase e fosforilação do receptor.[49,50] Os

receptores transmembrana codificados pela família *FGFR*, com exceção do gene *FGFR5*, possuem uma região extracelular composta por três domínios de imunoglobulinas: IgI, IgII e IgII. Esses três domínios afetam diretamente a expressão dos éxons 8, 9 e 10 do gene *FGFR2*, aonde encontram-se a maioria das variantes patogênicas em indivíduos com alterações do gene.[51,52,53] Variantes neste gene são normalmente associadas a craniossinostoses e resultam na sinalização desregulada do gene e no fechamento prematuro das suturas cranianas.[54] A expressão de *FGFR2* é encontrada em abundância na cartilagem basal do crânio, sendo altamente expressa nas suturas para diferenciação de osteoblastos e de células osteoprogenitoras com posterior apoptose.[55,56,57] A maioria das variantes patogênicas em *FGFR2* são mutações pontuais missenses, porém com relatos de deleções, inserções e alterações em sítios de *splicing* já descritos na literatura.[58]

1.4. Fenótipos clínicos associados a mutações em *FOXC2*

Variantes patogênicas identificadas no gene *FOXC2* (OMIM #602402) estão relacionadas a síndrome de linfedema-distiquíase (OMIM #153400), condição rara, autossômica dominante, caracterizada por distúrbios do desenvolvimento do sistema linfático e distiquíase - anomalia congênita que compõe o surgimento de fileiras extras de cílios oculares junto à glândula Meibomiana ou na pálpebra superior.[59] O primeiro relato de linfedema foi descrito por Meige em 1898,[60] sendo o fenótipo identificado na época homenageado com o nome do mesmo. Acredita-se que muito casos de síndrome de linfedema-distiquíase tenham sido rotulados erroneamente como doença de Meige, uma vez que muitos dos indivíduos diagnosticados não eram examinados

e nem questionados a respeito da anomalia ocular.[59] O linfedema normalmente surge durante a puberdade, podendo ser desenvolvido em idades mais avançadas. Por outro lado, a distiquíase surge precocemente, normalmente sendo diagnosticada no nascimento.[59] Atualmente aproximadamente 70 variantes diferentes foram descritas no gene *FOXC2*, sendo todas associadas à síndrome de linfedema-distiquíase.[61]

O gene *FOXC2* está localizado no cromossomo 16 (16q24.1) e codifica um fator de transcrição essencial para a formação de diversos tecidos e órgãos, visto que a proteína traduzida atua no controle e regulação de outros diversos genes. Estudos indicam que o gene possui um papel importante na formação de vasos sanguíneos, bem como no desenvolvimento pulmonar, ocular, renal, cardíaco e do trato urinário, além de participar, também, do sistema de transporte das células do sistema imunológico (vasos linfáticos).[62,63]

A maioria das alterações genéticas descritas no gene *FOXC2* são pequenas inserções ou deleções e variantes nonsense, que geram um códon de parada prematuro e, consequentemente, proteínas truncadas.[59,64] Apesar de incomum, variantes missenses também foram reportadas.[65] As alterações encontradas no gene estão diretamente associadas a fenótipos relacionados ao mal desenvolvimento do sistema linfático, seja em cenários de hiperplasia ou hipoplasia.[61] Além disso, o envolvimento do gene também foi relacionado à prevenção da obesidade e à resistência à insulina induzida pela dieta.[66]

1.5. Fenótipos clínicos associados a mutações em *KIAA0586*

As variantes patogênicas identificadas no gene *KIAA0586* (OMIM #610178) foram associadas a diversas ciliopatias como a síndrome de Joubert

(OMIM #616490), a síndrome de displasia torácica de Short-Rib (OMIM #616546) e, recentemente, a síndrome de Hydrolethalus (OMIM #236680 e #614120), todas caracterizadas pelo padrão de herança autossômico recessivo.[67,68,69] As ciliopatias são um conjunto de condições genéticas raras caracterizadas por alterações da cilia celular que acarretam em fenótipos complexos que incluem a presença de doenças fibrocísticas dos rins e do fígado, degeneração da retina, defeitos estruturais e funcionais do sistema nervoso central e dos olhos, crescimento ósseo anormal, lateralidade anormal dos órgãos internos e polidactilia.[70]

Assim como a sobreposição fenotípica, as ciliopatias também apresentam uma heterogeneidade expressiva de variantes moleculares descritas: síndromes diferentes associadas a um mesmo gene e diferentes genes associadas a uma mesma síndrome. As alterações patogênicas identificadas no gene *KIAA0586*, entretanto, estão normalmente relacionadas a apresentações clínicas mais leves de indivíduos diagnosticados com síndrome de Joubert e de Short-Rib.[67,71,72] Apesar de menos frequente, fenótipos mais severos como os manifestados por indivíduos portadores da síndrome de Hydrolethalus são relacionadas a variantes de mudança de fase de leitura com formação de proteínas truncadas.[68]

O gene *KIAA0586* está localizado no cromossomo 14 (14q23.1) e codifica uma proteína centrossomal conservada necessária para a funcionalidade correta da via de sinalização do *hedgehog*, para o desenvolvimento dos vertebrados e para a ciliogênese.[68,73] Os cílios são um componente essencial da transdução de sinal durante o desenvolvimento embrionário e as variantes patogênicas no gene são frequentemente associadas a um amplo grupo de condições genéticas denominadas de ciliopatias.[67,69] As principais características clínicas observadas

as indivíduos com variantes no gene englobam a degeneração da retina, doenças renais, malformações esqueléticas, anomalias cerebrais e diversos graus de comprometimento neurológico.[67,74]

1.6. Fenótipos clínicos associados a mutações em *NFIX*

A síndrome de Marshall-Smith (OMIM #602535) é uma condição rara, autossômica dominante, descrita pela primeira vez em 1971 por Marshall et al., (1971)[75] sendo caracterizada por traços dismórficos, atraso de desenvolvimento e maturação óssea anormal.[76] Poucos indivíduos ao redor do mundo foram diagnosticados com esta condição, entretanto, a mesma parece estar presente em todas as populações, afetando homens e mulheres igualmente, e, geralmente, decorrentes de variantes *de novo* localizadas no gene *NFIX* (19p13.13) (OMIM #164005).[76,77] Entretanto, variantes patogênicas neste gene também podem acarretar em outro fenótipo clínico conhecido como síndrome de Sotos-like, ou síndrome de Malan (OMIM #614753).[78]

A síndrome de Malan é caracterizada por supercrescimento leve a moderado, macrocefalia com fronte proeminente e atraso moderado do desenvolvimento. Normalmente deleções completas do gene e variantes nonsense e/ou missense que afetam o domínio de ligação do DNA são associadas a essa condição, sendo frequentemente localizadas nos éxons 2 e 3 do gene. Por outro lado, a síndrome de Marshall-Smith costuma estar associada a mutações de mudança de fase de leitura ou a variantes em sítios de splicing que evitam a degradação de RNA mensageiro mediada por mutação sem sentido, sendo agrupadas entre os exons 6 e 10. Ainda, devido à grande variação de transcritos alternativos do

gene *NFIX*, é possível que as diferentes variantes formadas acarretem em diferentes efeitos fenotípicos.[78,79]

A família Fator Nuclear I (NFI) consiste em quatro genes intimamente relacionados (A, B, C e X), cada um possuindo múltiplas variantes de splicing e padrões de expressão distintos, mas que se sobrepõem. Esses genes codificam proteínas com o domínio de ligação de DNA N-terminal e o domínio C-terminal conservados que se ligam como homo ou heterodímeros em sequências específicas do DNA e desempenham funções importantes na regulação da expressão gênica em diversos tecidos.[80,81] A família NFI é amplamente expressa durante o desenvolvimento embrionário e na idade adulta em mamíferos. Entretanto, o foco das pesquisas a respeito da função dessas proteínas tem sido, principalmente, em relação a sua participação no desenvolvimento cerebral, em que essas proteínas aparecem intimamente associadas à função das células da glia.[82,83] As proteínas NFI conseguem se ligar diretamente ao promotor e regular a atividade de transcrição da proteína ácida fibrilar glial (GFAP), um marcador de células gliais.[84]

Ainda, a família NFI de fatores de transcrição, principalmente o gene *NFIX*, tem sido amplamente descrita no desenvolvimento cerebral e implicada em múltiplos transtornos do desenvolvimento neurológico,[76,85] músculo esquelético[85,86] e sistema hematopoiético.[87]

1.7. Fenótipos clínicos associados a mutações em *SOX9*

A displasia campomélica (OMIM #114290) é uma displasia esquelética rara, autossômica dominante, caracterizada por dismorfias faciais distintivas, sequência de Pierre Robin com fenda palatina, encurtamento e curvatura dos

ossos longos e pés tortos. Outros achados incluem laringotraqueomalácia com compromisso respiratório e reversão de sexo em indivíduos do sexo masculino. A maioria dos indivíduos afetados acabam indo a óbito ainda no período neonatal devido a insuficiência respiratória.[88,89] Mais de 70 variantes patogênicas foram descritas no gene *SOX9* (OMIM #608160) e relacionados a displasia campomélica. A maioria das alterações dentro do gene impactam diretamente no funcionamento adequado da proteína traduzida e, consequentemente, impedem que o gene atue na regulação de outros genes essenciais para o desenvolvimento embrionário. Indivíduos com manifestações mais leves da displasia campomélica normalmente apresentam variantes em genes próximos ao *SOX9*.[90,91,92]

O gene *SOX9* está localizado no cromossomo 17 (17q24.3) e codifica um fator de transcrição essencial para o desenvolvimento embrionário. A proteína SOX9 se liga a regiões específicas do DNA para regular a atividade de outros genes, principalmente dos que atuam no controle do desenvolvimento esquelético e na determinação do sexo antes do nascimento, um vez que a proteina codificada pertence a região Y de determinação de sexo (SRY).[90,93,94] Ainda, durante a condrogênese, os alvos do SOX9 incluem genes codificadores de colágeno *Col2a1* e *Col11a2*, justificando o surgimento de fenótipos relacionados a displasias esqueléticas quando na presença de variantes patogênicas.[95] As alterações genéticas identificadas no gene *SOX9* variam de deleções e inserções de nucleotídeos a variantes sem sentido, a maioria implicando em mudanças na fase de leitura e tradução de uma proteína truncada e disfuncional. Em suma, os fenótipos associados as variantes descritas permeiam entre displasias esqueléticas e reversão de sexo.[93,96,97,98]

As doenças genéticas representam um importante problema de saúde pública, impactando diretamente no número elevado de internações pediátricas e óbitos infantis. Apesar de todos os avanços ocorridos nas últimas décadas, a etiologia de grande parte das malformações e síndromes descritas na literatura ainda não são conhecidas. Entretanto, estratégias de caracterização molecular de pacientes com doenças monogênicas tem como objetivo estabelecer correlações genótipo-fenótipo, auxiliar na identificação de fatores etiológicos e visar um aconselhamento familiar mais preciso. Sendo assim, a união entre a genética médica, biologia molecular e bioinformática têm sido bem-sucedida na busca pela elucidação e compreensão de diferentes condições genéticas.

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3. OBJETIVOS

GERAL:

Realizar a avaliação molecular de pacientes com diagnóstico clínico suspeito de diferentes alterações monogênicas raras associadas aos genes investigados neste estudo e diagnosticadas no Serviço de Genética Clínica da Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA).

ESPECÍFICOS:

a) Realizar a descrição clínica do relato de caso para cada paciente analisado.

b) Realizar a análise computacional através de algoritmos de predição para a estrutura e função proteica, bem como a conservação evolutiva das variantes encontradas.

c) Avaliar as variantes encontradas para critérios de patogenicidade através das normas e orientações estipuladas pelo *American College of Medical Genetics and Genomics* e a *Association for Molecular Pathology* para a interpretação de variantes.

4. ARTIGOS CIENTÍFICOS REDIGIDOS EM INGLÊS

4.1. Artigo gene *NFIX*

“Unusual features in a child with Marshall-Smith syndrome due to a novel NFIX variant: evidence for an abnormal protein function”

Desirée Deconte, Catarine Benta Lopes dos Santos, Camila Ohomoto de Morais, Tatiane Mayumi Yonamine, Letícia Thaís Nogueira, Maria Angélica Tosi Ferreira, Vinícius Bonetti Franceschi, André Luís Soares Longhi, Rolando André Rios Villacis, Silvia Regina Rogatto, Rodrigo Ligabue-Braun, Paulo Ricardo Gazzola Zen, Rafael Fabiano Machado da Rosa, Marilu Fiegenbaum

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Unusual features in a child with Marshall-Smith syndrome due to a novel *NFIX* variant: Evidence for an abnormal protein function

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ABSTRACT

Marshall-Smith syndrome (MSS) is a rare genetic condition characterized by different clinical features, such as accelerated skeletal maturation, growth retardation, neuropsychomotor delay, and characteristic facial abnormalities. This study describes a 2-year-old boy presenting longiline appearance, macrocephalus with trigonocephaly, prominent eyes with bluish sclera, middle face hypoplasia, micrognathia, dysplastic ears, hirsutism, narrow and asymmetrical thorax, hypoplastic scrotal sac, cryptorchidism, joint laxity, hypoplastic toenails, and large sacral dimple with prominent coccyx. Abdominal ultrasonography showed an enlarged left ectopic kidney and skull computed tomography revealed signs of early closure of the metopic suture. High-resolution GTG banding karyotype and high-density CytoScan HD array did not identify abnormalities associated to the patient clinical findings. *NFIX* sequencing revealed a *de novo* variant consisted of a seven nucleotides duplication in exon 8. We suggest that some clinical findings, as glaucoma, trigonocephaly and ectopic kidney are underestimated and should be considered within the clinical spectrum of the syndrome. In addition, the *NFIX* variant identified in our patient is unusual among patients with MSS and has not yet been described in the literature. We present some mechanisms that could be involved in the impairment of proper NFIX protein function.

1. Introduction

Marshall-Smith syndrome (MSS) is a rare genetic condition characterized by accelerated skeletal maturation, growth retardation, neuropsychomotor delay, breathing difficulties and characteristic facial abnormalities, including prominent forehead, shallow orbits, bluish sclera, low nasal bridge and micrognathia (Adam et al., 2005; Travan et al., 2008; van Balkom et al., 2011; Wang, 2002). However, since the description by Marshall et al. (1971), more than 100 different clinical findings have been described. To date, about 70 cases have been reported in the literature (van Balkom et al., 2011). Most of them occur sporadically and without description of family recurrence. More recently, Malan et al. (2010) identified variants in the *NFIX* gene (nuclear transcription factor I), mapped on 19p13.3, in patients with MSS.

We report a patient with MSS syndrome presenting unusual clinical features as trigonocephaly, congenital glaucoma and ectopic kidney, and a novel *NFIX* variant. We also present some mechanisms that could be involved in the impairment of proper NFIX protein function.

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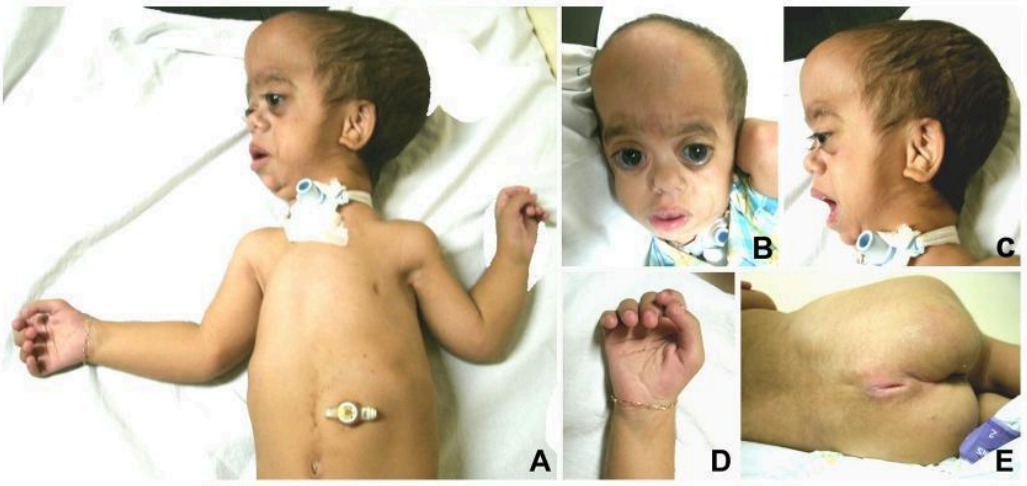

Fig. 1. Appearance of the patient at 2 years old showing high and prominent forehead, hypoplasia of the middle face, macrocephalus with trigonocephaly, prominent eyes with bluish sclera, low nasal bridge, microretrognathia (A–C), dysplastic ears with horizontal overfold of the helices (A and C), hypertricosis (E), narrow and asymmetrical thorax (A), right hand (D) and large sacral dimple (E).

2. Material and methods

High-resolution GTG banding karyotype analysis was performed from lymphocyte cell cultures. DNA was isolated from peripheral blood lymphocytes using the Gentra Puregene Blood Kit (Qiagen, Valencia, Ca, USA). Germline copy number variations (CNVs) were investigated using the high-density CytoScan HD Array platform (Affymetrix, Santa Clara, CA, USA). The data were analyzed by the Chromosome Analysis Suite software v.3.1 (Affymetrix, NetAffx Annotation Files version 33.1 - hg19). Gains and losses were identified considering at least 50 and 25 markers, respectively. The identified CNVs were compared with the DGV (Database of Genomic Variants, http://dgv.tcag.ca/dgv/app/home, updated in May 2016) and the Affymetrix Database of Variants (aDGV). Only rare alterations, described in a frequency <0.5% in the aDGV and <0.05% in the DGV were considered as significant.

Whole-Exome Sequencing (WES) was captured by SureSelect Human All Exon Kit (Agilent Technologies, Inc., Santa Clara, CA, USA) and sequenced on an Illumina NovaSeq 6000 instrument (Illumina, Inc., San Diego, CA, USA). Bioinformatics analysis were conducted as follows: (i) raw reads quality was accessed with FASTQC (v0.11.5) (Andrews, 2010); (ii) human reference mapping was performed against hg19/UCSC genome using Burrows-Wheeler Aligner (BWA v0.7.17) (Li and Durbin, 2009); (iii) alignment file (SAM) was converted and sorted in BAM format, and PCR duplicates were removed using SAMtools (v1.7) (Li, 2011); (iv) variant calling was performed using BCFtools[9] (v1.7) and FreeBayes (v1.0.0) (Garrison and Marth, 2012), and only variants with quality score $\geq$ 20 and read depth $\geq$ 100 were retained; (v) Variant Call Format (VCF) files generated by both callers were intersected using BEDtools (v2.26.0) (Quinlan and Hall, 2010), resulting in 539,789 variants (25,136 deletions, 23,630 insertions and 491,023 SNPs); (vi) genetic variants were functionally annotated using ANNOVAR (v2019Oct24) (Wang et al., 2010) and the results were filtered with custom scripts, in order to include gene information, clinically relevant variants and mutation scores (e. g. SIFT and PolyPhen). A variant was considered to be detrimental if it was predicted to be damaging in at least two of the *in silico* tools. Missense variants were automated in InterVar (http://wintervar.wglab.org/) and divided into five categories: pathogenic, likely pathogenic, of uncertain significance, likely benign, and benign.

Additionally, *NFIX* gene was amplified by conventional polymerase chain reaction (PCR) using primers designed to investigate the entire coding region. All eleven primers pairs were designed according to the NCBI reference sequence (NG_032925.2) through Primer3 program (http://frodo.wi.mit.edu/primer3/). Amplification reactions had a final volume of 25 μL (0.4 mmol/L of dNTPs, 1.5 mmol/L of $MgCl_2$, 0.4 μmol/L of each primer, 1 U of Taq DNA polymerase, and 100 ng of genomic DNA). PCR conditions were as follows: an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30s, 52–57 °C for 30s, 72 °C for 30s, and a final extension at 72 °C for 5 min. ExoSAP-IT protocol was performed to purified all PCR products through an incubation of 5 min (4 min at 37 °C and 1 min at 80 °C). DNA sequencing was performed using ABI-PRISM 3130 Genetic Analyzer and all electropherograms analyzed with Chromas Lite program. Sequence conservation and phylogeny of NFIX were assessed with ConSurf and PhylomeDB, respectively (Ashkenazy et al., 2016; Huerta-Cepas et al., 2014). Physical chemical properties for the predicted proteins were inspected with ProtParam combined with data for disordered proteins and had their disorder propensity evaluated with PrDOS (Gasteiger et al., 2005; Lieutaud et al., 2016; Ishida and Kinoshita, 2007). Multiple sequence alignment of NFIX isoforms (Q14938, Q14938–2 to Q14938–6) and their respective alterated forms was performed through Clustal-Omega (https://www.uniprot.org/align/).

3. Results

3.1. Clinical findings

The patient is the first child of non-consanguineous parents. The mother was 31 years old and the father was 26 years old. There was no description of similar cases in the family. His gestation presented polyhydramnios, identified in the seventh month. The mother denies smoking, alcohol intake, or use of illicit drugs. The child was born by cesarean section, with 38 weeks of gestation, weighing 3320 g, measuring 55 cm, with a head circumference of 39 cm and Apgar scores of 3 in the first minute and 4 in the fifth. After birth, he stayed for three months in the neonatal intensive care unit due to high respiratory obstruction by micrognathia/glossoptosis. He underwent tracheostomy at 18 days and gastrostomy at 2 months. He had frequent episodes of pneumonia. A congenital glaucoma was also identified, and the surgery was performed at 5 months of age.

On physical examination, at 2 years of age, he measured 84 cm (percentile 10th–25th) and weighed 8370 g (above 3rd percentile). His head had a circumference of 49 cm (percentile 50th) and a longiline appearance. He had a high and prominent forehead, hypoplasia of the middle face, macrocephalus with trigonocephaly, prominent eyes with bluish sclera, low nasal bridge, irregular dentition, microretrognathia,

Table 1
Literature review of Marshall-Smith syndrome clinical findings in comparison with our patient.

Findings	% patients ($N = 68$)	Our Patient
Respiratory problems	73.5	+
Growth retardation	19.1	
Failure to thrive	29.4	+
Hypotonia	22.1	+
Hypertricosis	51.5	+
High forehead	39.7	+
Prominent forehead	44.1	+
Proptosis	75	+
Blue sclerae	61.8	+
Midface hypoplasia	47.1	+
Prominent premaxilla	32.4	
Low nasal bridge	35.3	+
Anteverted nares	47.1	
Small nose	36.8	
Short philtrum	22.1	
Everted lips	14.7	
Gum hypertrophy	22.1	
Narrow high-arched palate	14.7	
Irregular dentition	16.2	+
Micrognathia	48.5	+
Retrognathia	20.1	+
Low-set ears	29.4	
Kyphoscoliosis	42.6	+
Umbilical hernia	17.6	
Large hands	16.2	
Bone fractures	17.6	
Neurological		
Dilated ventricles	17.6	+
Agenesis/hypoplasia corpus callosum	17.6	
Cervical cord compression	4.4	
Spine stenosis	4.4	
Pachygyria	4.4	
Hypoplastic cerebellum	4.4	
Brain hypoplasia	4.4	+
Agenesis of septum pellucidum	1.5	
Tethered spinal cord	1.5	
Ophthalmologic		
Myopia	22.1	
Optic nerve atrophy	14.7	
Glaucoma	11.8	+
Megalocornea	4.4	
Corneal erosion	1.5	
Audiometric		
Hearing loss	33.8	+
Cardiac		
Congenital heart defect	16.2	
Respiratory		
Choanal stenosis/atresia	14.7	
Laryngomalacia	7.4	
Pulmonary hypertension	5.9	
Laryngostenosys	2.9	
Abdominal		
Hydronephrosis	4.4	
Pyloric stenosis	2.9	
Wilms tumor	1.5	
Ectopic kidney	–	+
Skeletal		
Abnormal bone maturation	88.2	+
Thin long bones	23.5	
Wide phalanges	19.1	+
Small distal phalanges	10.3	
Osteopenia	8.8	
Bowing of long bones	7.4	
Thin/long ribs	4.4	
Hypersegmented sacrum-coccyx	4.4	
Trigonocephaly	4.4	+
Hypersegmented sternum	2.9	
Immunological		
IgA deficiency	2.9	
T-cell immunodeficiency	1.5	

abnormal ears with horizontal overfold of the helices. Hypertricosis, narrow and asymmetrical thorax, hypoplastic scrotal sac, cryptorchidism, elongated and tapered toes, joint laxity, hypoplastic toenails and large sacral dimple with prominent coccyx were also featured (Fig. 1 and Table 1).

The child presented significant delay in neuropsychomotor development (he was hypotonic, and at 2 years of age he still did not speak and had difficulty to pick up objects and to support his head), as well as a single episode of seizure that lasted a few seconds. Electroencephalogram revealed basic disorganized activity with no symmetry, nor epileptiform activity. From this point onwards, it was prescribed phenobarbital. Head magnetic resonance imaging showed subarachnoid cisterns, enlarged telencephalic grooves, mainly in the frontal lobes, in addition to moderate triventricular dilatation (Figs. 2B and C).

Skull computed tomography with 3D reconstruction revealed signs of early closure of the metopic suture (trigonocephaly), as well as prominence of frontotemporal cortical sulci and absence of the pellucid septum. However, assessment of neurosurgery at that time did not indicate surgical treatment for trigonocephaly (Table 2).

Radiographies of hands and wrists, performed at 2 years and 8 months of life, showed acceleration in bone maturation and marked enlargement of the phalanges (bone age was 6 years and standard deviation was 5.97 months). Skull radiograph showed macrocrania with anteroposterior diameter accentuation (Fig. 2A). Feet radiograph revealed forefoot adduction. Spine radiograph showed levoconvex lumbar scoliosis, and levorotatory rotation of vertebral bodies from T8 to L4 segments (Table 2).

Echocardiography was normal. Abdominal ultrasonography showed an enlarged left ectopic kidney. Renal scintigraphy with dimercaptosuccinic acid (DMSA) showed a larger left kidney than the right kidney, but without capture defects (Table 2).

The patient also had recurrent episodes of pneumonia requiring hospitalization. He needed prolonged use of oxygen therapy. Videofluoroscopy of swallowing revealed oropharyngeal dysphagia with an important risk of bronchoaspiration. He underwent motor and respiratory physiotherapy and was also accompanied by a speech language pathologist. Otoacoustic emission revealed hearing impairment in both ears (Table 2).

3.2. Molecular findings

The patient presented a normal karyotype (46,XY). Using the high-density CytoScan HD Array platform, two rare CNVs were detected: loss of 4q22.1 (91557928-91799021, hg19) [which encompasses two exons, of two isoforms, of the gene *CCSER1* (*FAM190A*)] and loss of 9p23 (10545858-10587679, hg19) (which involves only the intron 1 of the *PTPRD* gene). Whole-exome sequencing identified 82 missense variants considered to be damaging by both *in silico* tools. However, further analysis by InterVar revealed that none of the variants would be pathogenic or likely pathogenic as shown in Suppl. Table 1. Similar analyses in his parents were not performed.

Sanger sequencing analysis of the *NFIX* gene also revealed a *de novo* variant: a duplication of seven nucleotides in exon 8 (NG_032925.2:g.90963_90969dupCCAGCAG; NC_000019.10:g.13081732_13081738dupCCAGCAG NM_002501.3:c.1131_1137dupCCAGCAG; NM_001271044.2:c.1107_1113dupCCAGCAG; NM_001271043.2:c.1155_1161dupCCAGCAG) (Fig. 3). The uneven number of base pairs added in the patient's DNA leads to a reading frame shift, which modifies the entire reading frame of all six isoforms (Suppl. Fig. 1). The duplication in exon 8 was also confirmed by Whole-Exome Sequencing (WES) through the alignment of reads against human reference genome (hg19) using Burrows-Wheeler Aligner (BWA v0.7.17) (Li and Durbin, 2009) and visualization with the Integrative Genomics Viewer (IGV v2.8.0) (Thorvaldsdóttir et al., 2013).

The sequence conservation analysis revealed that the N-terminus (dimerization and DNA binding region) of the protein is much more

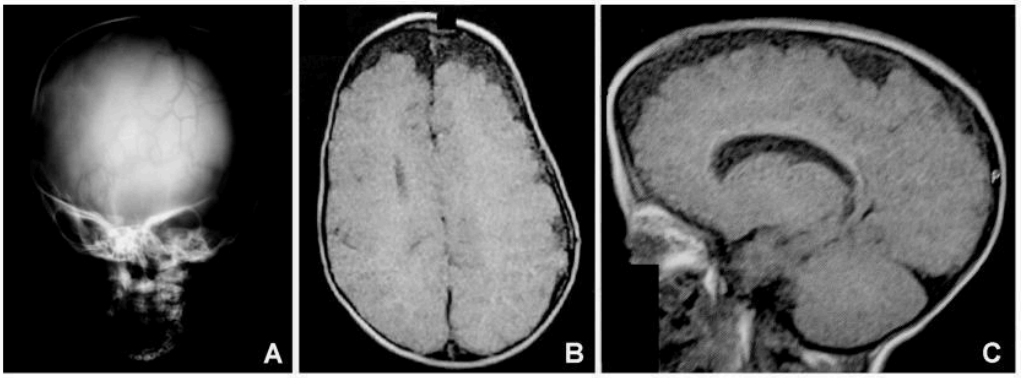

Fig. 2. Skull radiograph revealing macrocrania with anteroposterior diameter accentuation (A). Magnetic resonance imaging of the head showed subarachnoid cisterns, enlarged telencephalic grooves, mainly in the frontal lobes, in addition to moderate triventricular dilatation (B and C).

Table 2
Literature review of the clinical findings of Marshall-Smith syndrome patients using complementary exams compared with our patient.

Findings	% patients (N = 68)	Our Patient
Neurological		
Dilated ventricles	17.6	+
Agenesis/hypoplasia corpus callosum	17.6	
Cervical cord compression	4.4	
Spine stenosis	4.4	
Pachygyria	4.4	
Hypoplastic cerebellum	4.4	
Brain hypoplasia	4.4	+
Agenesis of septum pellucidum	1.5	
Tethered spinal cord	1.5	
Ophthalmologic		
Myopia	22.1	
Optic nerve atrophy	14.7	
Glaucoma	11.8	+
Megalocornea	4.4	
Corneal erosion	1.5	
Audiometric		
Hearing loss	33.8	+
Cardiac		
Congenital heart defect	16.2	
Respiratory		
Choanal stenosis/atresia	14.7	
Laryngomalacia	7.4	
Pulmonary hypertension	5.9	
Laryngostenosys	2.9	
Abdominal		
Hydronephrosis	4.4	
Pyloric stenosis	2.9	
Wilms tumor	1.5	
Ectopic kidney	–	+
Skeletal		
Abnormal bone maturation	88.2	+
Thin long bones	23.5	
Wide phalanges	19.1	+
Small distal phalanges	10.3	
Osteopenia	8.8	
Bowing of long bones	7.4	
Thin/long ribs	4.4	
Hypersegmented sacrum-coccyx	4.4	
Trigonocephaly	4.4	+
Hypersegmented sternum	2.9	
Immunological		
IgA deficiency	2.9	
T-cell immunodeficiency	1.5	

conserved than the C-terminus (transactivation and repression region) of the protein (Suppl. Fig. 2). This pattern is also shown in the phylogenetic reconstruction of NFIX evolutionary history (Suppl. Fig. 3).

Despite being marginal, the detected alteration could be able to change the normal folding tendencies of the protein. This is especially observed for the final segment of their C-termini, that in some cases present a disordered to ordered transition, as revealed by disorder profiling (Fig. 4). This transition is much rarer than the opposite (ordered to disordered transition), that is also observed for some isoforms, making these NFIX mutations even more unique in terms of genotype to phenotype association.

4. Discussion

The clinical findings of our patient were compatible with the diagnosis of MSS. In addition, molecular analysis through sequencing showed a variant in the *NFIX* gene, which confirmed this diagnosis. The clinical profile of MSS is usually characterized by a multisystemic involvement, with more than 100 different findings already described. However, the presence of some features is noteworthy in our patient. These consist of early closure of the metopic suture (trigonocephaly), glaucoma (in our case, congenital) and ectopic kidney. In principle, these findings are not considered part of the clinical spectrum of the syndrome.

Trigonocephaly has the appearance of a triangle-shaped skull. It occurs by a premature fusion of the metopic suture and has been reported in about 0.67:10,000 births, representing the third most common type of craniosynostosis (around 10–20% of the patients). Trigonocephaly has been described in several syndromes, as 9p monosomy (OMIM #158170) and valproic acid embryopathy (Stevenson and Hall, 2006). MSS is usually associated with several bone abnormalities. These include very distinct craniofacial findings, such as macrocephaly, bitemporal narrowing, prominent forehead, occipital flattening, and hypoplasia of the middle face (Adam et al., 2005; Travan et al., 2008; van Balkom et al., 2011; Wang, 2002; Butler, 2004; Diab et al., 2003; Passalacqua et al., 2011; Shaw et al., 2010; Sumiya et al., 2002). To our knowledge, no patients with MSS syndrome presenting trigonocephaly have been reported. However, a detailed analysis of the case reports revealed that some patients had description of trigonocephaly. Three of 68 patients (4.4%) described in the literature presented this finding (Shaw et al., 2010; Sumiya et al., 2002). These results denote that trigonocephaly may be an underreported feature that could be part of the clinical spectrum of MSS. Neither the case report made by Sumiya et al. (2002) nor the study developed by Shaw et al. (2010), who also had trigonocephaly, underwent molecular analysis of the *NFIX* gene.

Glaucoma is an optic neuropathy characterized by optic disc damage and visual field loss due to increased intraocular pressure. This feature is a significant global health problem that affects 67 million people, being the second cause of blindness worldwide (Mantravadi and Vadhar, 2015). Glaucoma can be congenital and has a strong genetic base, which can be part of the clinical spectrum of some genetic diseases as Axenfeld-Rieger syndrome (OMIM: #180500) and Aniridia (OMIM: #106210) (Lewis et al., 2017). In our review, we found that 11.8% of the patients

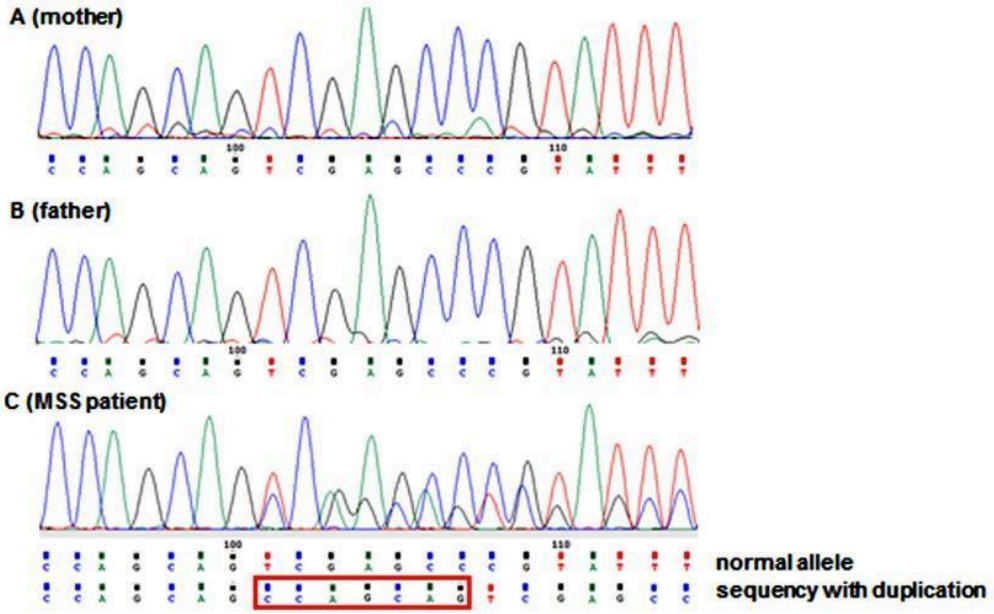

Fig. 3. A) Mother's electropherogram. B) Father's electropherogram. C) Patient's electropherogram with the duplicated sequence labeled in a red rectangle. The comparison between wild and mutated allele is illustrated by the nucleotide sequence to clarify the duplication. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

with MSS had glaucoma, a finding also underestimated (van Balkom et al., 2011; Shaw et al., 2010; Hou, 2004). In the literature, only one of eight patients with MSS and glaucoma had congenital glaucoma, as observed in our patient (Hou, 2004). Shaw et al. (2010) pointed that glaucoma in MSS happens due to congenital anomalies of morphology of the anterior chamber or trabecular meshwork, suggesting that glaucoma in MSS has a congenital origin.

Renal abnormalities have been described in patients with MSS such as hydronephrosis and Wilms tumor (Ng et al., 2007). However, ectopic kidney, as presented in our patient, has not been described in the literature. Apparently, the rarity of the description of these findings (in this case, trigonocephaly, glaucoma and ectopic kidney) may be directly related to an undetailed investigation (Diab et al., 2003; Shaw et al., 2010).

Most patients have a survivability that does not exceed the neonatal or early childhood period (Seidahmed et al., 1999). This mainly happens due to respiratory distress by obstruction of the upper airways and chronic and recurrent lung infections (Travan et al., 2008; Wang, 2002; Seidahmed et al., 1999). However, long-term survival has been described especially in cases where respiratory problems were predicted and aggressively managed (Adam et al., 2005; Travan et al., 2008). The increased survival also raises the need of strategies to evaluate and treat other abnormalities present in these patients, as possibly trigonocephaly, glaucoma and ectopic kidney.

MSS has been previously associated with *NFIX* variants (Malan et al., 2010; Passalacqua et al., 2011). Variants in this gene can lead to two different phenotypes: Sotos-like syndrome and MSS. The first one is usually associated with whole gene deletions and nonsense and/or missense variants affecting the DNA-binding domain clustered in exons 2 and 3. Frameshift and splice-site variants that avoid nonsense-mediated RNA decay, on the other hand, are related with MSS and are distributed along exons 6 to 10 (Klaassens et al., 2015; Martinez et al., 2015) (Table 3). Most of the *NFIX* variants associated with MSS are *de novo* and located in more than 50–55 nucleotides upstream in exon-exon junction (Malan et al., 2010; Passalacqua et al., 2011).

The exact mechanism that explains the genotype-phenotype correlation on the variant found in our patient is still unknown. However, some hypotheses can be raised. Recently a nine-amino-acid transactivation domain (9aaTAD) was identified in *NFIX*. *trans*-Activating domains (TADs) are transcriptional factors that contain binding sites, which enable other proteins to bind and perform molecular interactions. A 9aaTAD motif may be required for the function of the transactivation domain of NFIX and its related transcription factors (Piskacek et al., 2020). The variant described in this work could inactivate this motif, which would allow the mutant allele to codify an abnormal protein. The formation of an abnormal protein could cause a dominant negative effect on the wild-type protein and, consequently, implicate in the observed phenotype (Malan et al., 2010; Martinez et al., 2015; Piskacek et al., 2020). On the other hand, although some variants associated with MSS are known to escape NMD, the hypothesis that a protein is not translated from a mutant allele cannot be cut off. Therefore, our frameshift variant could set off NMD, avoid an abnormal protein codification and lead to a milder phenotype due to haploinsufficiency.

Regarding protein structure, NFIX has a conserved N-terminal domain, involved in protein dimerization and binding to DNA, and a variable C-terminal domain, called transactivation and repression region (Fig. 4) (Mason et al., 2009). While the former recognizes and binds a palindromic sequence present in viral and cellular promoters, the latter is proposed to be involved in the multiple interactions described for this protein. NFIX interacts with at least 14 different proteins, including multiple RNA polymerase subunits, zinc finger protein 614, and glutamine-rich protein 1 (Rolland et al., 2014; Gurrieri et al., 2015). The protein presents major intrinsically disordered regions allowing its classification as an IDP (intrinsically disordered protein) (Uversky, 2013).

IDPs take part in multiple physiopathological processes, being involved for instance in normal and disturbed cell signaling and senescence, neurodegenerative disorders and different types of cancer (Uversky et al., 2008; Uversky, 2014; Martinelli et al., 2019). In the case of NFIX, conformational disorder seems to be essential for its native functions (as revealed by its disorder profile). Some disordered regions can undergo disordered-to-ordered transitions when performing (each of) their functions, including interactions with nucleic acids, especially during transcription (Shammas, 2017; Munshi et al., 2018). However, multiple functions of disordered regions involve highly dynamic states

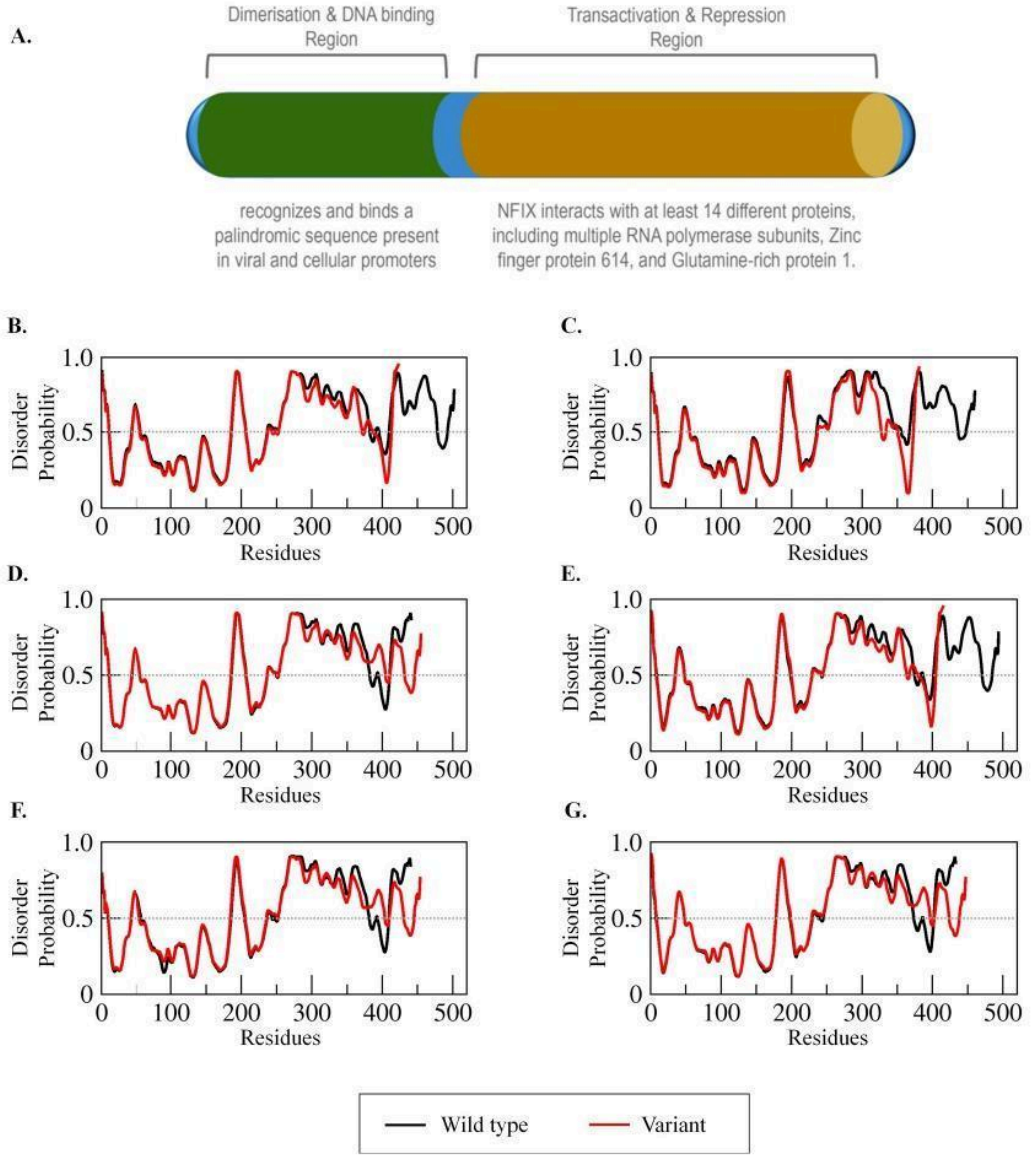

Fig. 4. NFIX structure and disorder prediction. Schematic representation of NFIX protein regions (A); Disorder probability plots for six NFIX isoforms and their variants. (B). Isoform 1; (C). Isoform 2; (D). Isoform 3; (E). Isoform 4; (F) Isoform 5; (G) Isoform 6.

with minimal change in order and some involve ordered-to-disordered transitions (Uversky, 2017; Hahn, 2018). The physical chemical analyses of all NFIX variants explored in this work (new, pathogenic and non-pathogenic) did not reveal clear alterations that could further explain the differences observed in MSS-related variants.

NFIX is involved in the mechanism of cell proliferation and differentiation, including chondrocytes and osteoblasts (Malan et al., 2010; Passalacqua et al., 2011). This could explain the relation with the bone finding of cranioestenosis (trigonocephaly) observed in our patient and previously described in some cases (Shaw et al., 2010; Sumiya et al., 2002). Campbell et al. (2008) observed defects not only in brain development but also in the opening of the eye. This finding may have some relation with the finding of glaucoma verified in patients with MSS. Although the uncommon description of renal anomalies in patients with MSS, an association between *NFIX* and ectopic kidney has not been reported.

5. Conclusions

In conclusion, we believe that the clinical findings found in this work (trigonocephaly, glaucoma and ectopic kidney) may be underestimated and should be considered within the clinical spectrum of the syndrome. In addition, the *NFIX* gene variant identified in our patient has not been described in the literature. A protein change caused by this variant, such as others described in MSS, could be explained by different molecular mechanisms. Therefore without experimental validation, any potential impact of the variant remains speculative. Thus, further studies are necessary to evaluate the functional effects of this specific variant and its respective protein function, as well as genotype-phenotype correlations.

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Supplementary data to this article can be found online at https://doi.

Table 3
NFIX mutations described in the literature and the novel mutation identified in our patient (highlighted in bold).

Phenotype	HGVS c.	Exon
Sotos-like	c.203 T > C	2
not provided	c.327delC	2
Sotos-like	c.362_366dup	2
Inborn genetic diseases	c.385C > T	2
Sotos-like	c.386G > C	2
not provided	c.583 + 1G > A	2
Sotos-like	c.592C > T	3
not provided	c.661C > T	4
not provided	c.690G > A	4
Sotos-like	c.740C > G	5
not provided	c.929delC	6
not provided	c.974_978delATGCA	6
Marshall-Smith	c.979 + 1G > A	6
Marshall-Smith	c.983_984insC	7
Marshall-Smith	c.994_995delAA	7
Marshall-Smith	c.1018_1019insT	7
Marshall-Smith	c.1032_1036delCTCTC	7
Marshall-Smith	c.1035_1036delTC	7
Marshall-Smith	c.1061_1062insT	7
Marshall-Smith	c.1072_1073insC	7
Marshall-Smith	c.1104_1120del17	8
not provided	c.1111_1112insC	8
not provided	c.1140_1141insC	8
Marshall-Smith (our patient)	c.1208_1214dupCCAGCAG	8
Marshall-Smith	c.1267delG	8

Positions were based on NCBI reference sequence NM_001271043.2. HGVS: Human Genome Variation Society; c. represents the coding sequence position according to RefSeq (http://varnomen.hgvs.org/).

org/10.1016/j.genrep.2020.100991.

CRediT authorship contribution statement

Desirée Deconte: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft. **Catarine Benta Lopes dos Santos:** Investigation, Writing – original draft. **Camila Ohomoto de Morais:** Investigation, Writing – original draft. **Tatiane Mayumi Yonamine:** Investigation, Writing – original draft. **Letícia Thaís Nogueira:** Investigation, Writing – original draft. **Maria Angélica Tosi Ferreira:** Investigation, Writing – original draft. **Vinícius Bonetti Franceschi:** Software, Formal analysis, Investigation. **André Luís Soares Longhi:** Investigation. **Rolando André Rios Villacis:** Investigation. **Silvia Regina Rogatto:** Investigation. **Rodrigo Ligabue-Braun:** Software, Validation, Formal analysis, Investigation, Writing – original draft. **Paulo Ricardo Gazzola Zen:** Conceptualization, Methodology, Supervision. **Rafael Fabiano Machado Rosa:** Conceptualization, Methodology, Investigation, Writing – original draft, Supervision. **Marilu Fiegenbaum:** Conceptualization, Methodology, Investigation, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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4.2. Artigo gene *KIAA0586*

“*Expanding the phenotypic spectrum of pathogenic KIAA0586 variants: from Joubert Syndrome to Hydrolethalus Syndrome*”

Desirée Deconte, Bruna L. Diniz, Jéssica K. Hartmann, Laira F. Zottis, Mateus A. de Souza, Paula G. Zen, Rafael F. M. Rosa, Marilu Fiegenbaum

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Expanding the Phenotypic Spectrum of Pathogenic *KIAA0586* Variants: From Joubert Syndrome to Hydrolethalus Syndrome

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Abstract: *KIAA0586* variants have been associated with a wide range of ciliopathies, mainly Joubert syndrome (JS, OMIM #616490) and short-rib thoracic dysplasia syndrome (SRTD, OMIM #616546). However, the hypothesis that this gene is involved with hydrolethalus syndrome (HSL, OMIM #614120) and orofaciodigital syndrome IV (OMIM #258860) has already been raised. Ciliopathies' clinical features are often overlapped despite differing in phenotype severity. Besides *KIAA0586*, *HYLS1* and *KIF7* are also known for being causative of ciliopathies, indicating that all three genes may have similar or converging genomic pathways. Overall, the genotypic and phenotypic spectrum of ciliopathies becomes wider and conflicting while more and more new variants are added to this group of disorders' molecular pot. In this case report we discuss the first Brazilian individual clinically diagnosed with hydrolethalus syndrome and molecular findings that demonstrate the role of *KIAA0586* as a causative gene of a group of genetic disorders. Also, recent reports on individuals with intronic and exonic variants combined leading to ciliopathies support our patient's molecular diagnosis. At the same time, we discuss variable expressivity and overlapping features in ciliopathies.

Keywords: ciliopathies; hydrolethalus syndrome; *KIAA0586*; pathogenic variant; inheritance; variable expressivity

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1. Introduction

The cilia are an essential component of signal transduction during embryonic development involved in cellular detection and the management of external signals. Dysfunctional cilia are often associated with ciliopathies, a heterogeneous group of genetic diseases that manifest during childhood and adolescence [1–3]. The majority of syndromes related to genes involved in ciliary function are monogenic and have an autosomal recessive trait. Although the phenotypic spectrum is known to be wide, the main clinical features observed in ciliopathies encompass retinal degeneration, renal disease, skeletal malformations, brain anomalies, and neurological impairment [1–6].

Genes related to ciliopathies are called *ciliary genes*, and over 800 genes have already been identified as involved in the ciliary–centrosome function [7]. Among the ciliary genes, *KIAA0586* (OMIM #610178, 14q23.1), the human ortholog of mouse and chicken *TALPID3*, encodes a conserved centrosomal protein required for sonic hedgehog signaling (Shh), vertebrate development, and ciliogenesis [8–10]. Previous animal model studies showed that the *KIAA0586* knockdown phenotype is associated with severe ciliogenesis

impairment and Shh disruption [11,12]. Primary cilia have an essential role in sonic hedgehog signaling in mammals, and their function is essential for correct embryonic formation. As a result, variants in ciliary genes like *KIAA0586* are associated with severe clinical consequences [11,12].

Pathogenic variants in *KIAA0586* are often associated with a wide range of ciliopathies, such as Joubert syndrome (JS, OMIM #616490) and short-rib thoracic dysplasia syndrome (SRTD, OMIM #616546), and the hypothesis of this gene being involved with hydrolethalus syndrome (HSL, OMIM #614120) and orofaciodigital syndrome IV (OMIM #258860) has been raised in the literature [4–6,8,13]. Although the severity of ciliopathies may differ, a variety of clinical manifestations are frequently overlapped. Moreover, these diseases have molecular heterogeneity, with several genes being associated with one or more ciliopathies [4,14]. Therefore, the etiology of these syndromes as well as the role of the involved genes are yet to be elucidated to understand the genotype–phenotype correlation behind ciliopathies disorders [14].

Here we report on the first Brazilian individual clinically diagnosed with hydrolethalus syndrome and molecular findings that instigate the discussion on the role of *KIAA0586* as a causative gene of ciliopathies. Also, we provided an overview of the wide phenotype range in the ciliopathies spectrum as well as the overlapping genes between them by gathering previous cases reported in the literature and proposing whether gene expression variability among ciliopathies is a possibility.

2. Results

2.1. Clinical Findings

The proband was the first child of young and non-consanguineous parents with no similar cases in the family, although the father's sister was described as being born with clubfoot. Both parents have German ancestry. A previous history of pregnancy loss was denied.

The mother had two episodes of bleeding in the third month of pregnancy. She used piperidolate hydrochloride and progesterone but denied smoking or any drug use during gestation. In the second trimester, ultrasound and magnetic resonance imaging (MRI) identified Dandy–Walker malformation, corpus callosum agenesis, microphthalmia, reduced chest diameter, short limbs, increased amount of amniotic fluid (polyhydramnios), and a retrovesical cystic mass, which, postnatally, was found to be a didelphic uterus with hydrometrocolpos; the fetal echocardiography was normal. The parents chose not to perform fetal karyotyping by amniocentesis.

The proband, female, was born prematurely at 35 weeks and 2 days by cesarean section, weighing 2649 g (percentile 70) and measuring 46 cm (percentile 53), with a head circumference of 33 cm (percentile 83) and Apgar score of three at the first minute and five at the fifth minute. She presented respiratory distress and needed to be placed on mechanical ventilation. During the physical examination, a large anterior fontanelle, deep-set eyes with an impression of microphthalmia, low and broad nasal root, median cleft lip, cleft palate, micrognathia, hypoplastic and malformed tongue, dysplastic, small and low-set ears with a preauricular pit on the left, short neck with redundant skin, narrow thorax, postaxial polydactyly of hands, wrists arthrogryposis; camptodactyly of fingers, labia majora hypoplasia, anteriorly placed anus, and broad/bifid hallux (preaxial polydactyly) were noted. A brain ultrasound confirmed the findings observed during the prenatal period and identified the presence of dilated lateral ventricles. Abdominal/pelvic ultrasound showed pielocalicial and ureteral dilatation, as well as a didelphic uterus with hydrometrocolpos. The ophthalmologic assessment confirmed the apparent bilateral microphthalmia. A radiographic evaluation showed an elongated skull with a large posterior fossa, a small jaw, a narrow thorax with little expanded lungs and short ribs, wide iliac bones with a small sciatic notch, underdeveloped vertebrae, and humerus shortening. The child died within 25 days of life due to sudden respiratory dysfunction. A postnatal MRI could not be performed.

2.2. Genetic Analysis Results

Chromosomal microarray analysis revealed a deletion at chromosome 7q (71,869,384–76,820,289), a deletion at chromosome 14q (38,465,567–38,584,405), and two deletions at chromosome Xq (62,036,670–67,171,633; 71,632,324–78,216,024). Gene content for these deletions can be found in Supplementary Table S1. In addition, genes in the deleted regions were crossed with all ciliary genes described in the KEGG database to identify potential involvement in cilia function (Supplementary Table S2). At chromosome 7, two ciliary genes—*CLDN3* and *CLDN4*—were found to be deleted; however, neither of them justifies the clinical features presented in our case. Moreover, none of the alterations identified by CMA were associated with the proband phenotype. A pathogenic variant in *KIAA0586* was identified by NGS (NM_001244189.1:c.4018del, p.Glu1340ArgfsX10) in both the father's and proband's samples. This variant was not described in the literature. The mother sequence was normal. However, rtPCR analysis showed a probable intronic mutation at *KIAA0586* in the mother's sample, which led to an aberrant transcript. This result suggests that the second causative variant may be a mutation not identified by NGS inherited from the mother. Although CMA results showed a deletion on chromosome 14, the deleted region does not comprise the *KIAA0586* nor any known gene (Supplementary Table S1).

Unfortunately, proband rtPCR could not be performed and the inheritance of the mother's intronic variant was not confirmed. Hydrolethalus is an autosomal recessive syndrome, which implies that a variant in both alleles is to be expected to justify the lethal phenotype. Future research should consider intronic analysis before rejecting any autosomal recessive disease hypothesis or suspicion.

3. Discussion

Hydrolethalus syndrome (HLS) is a lethal genetic syndrome with an autosomal recessive trait characterized by the association of postaxial polydactyly and preaxial polydactyly of hands and feet, micrognathia, and hydrocephaly or anencephaly with a key-hole defect of the occipital bone [15]. Researchers have associated *HYLS1* and *KIF7* as key genes in this disorder [16,17]. The *HYLS1* gene showed alternative splicing, where the transcript was found to be present in multiple tissues during fetal development [18]. Also, an experiment with mice harboring the *HYLS1* variant showed that the animals die shortly after birth and exhibit developmental defects that resemble several manifestations of the human syndrome [19]. The *KIF7* gene, on the other hand, encodes a cilia-associated protein that regulates the hedgehog signaling pathway. In vivo genetic interaction studies indicated that the knockdown of *KIF7* could exacerbate the ciliopathy transcripts since KIF7 expression in cell lines causes defects in cilia formation and induces abnormal centrosome duplication [17,20]. However, the case reported in this paper differs from those already described, since the molecular finding on an HLS individual was on the *KIAA0586* gene.

Although *KIAA0586* is not the first candidate gene for HLS, it is associated with ciliopathies [6,8,21]. Ciliopathies have overlapping features, including renal and liver disorders, retinal degeneration, central nervous system defects, abnormal bone growth, and polydactyly [2,3]. Joubert syndrome was one of the first ciliopathy disorders recognized after the identification of the subcellular localizations and functions in the defective proteins [2,3,22]. JS phenotype is based on neurological features, abnormal eye movements, ataxia, cognitive impairment, and agenesis of the cerebellar vermis [23]. To date, several genes have been identified as causing the syndrome. However, many patients remain undiagnosed, suggesting that genetic heterogeneity may justify the reason why molecular diagnosis can be challenging [21]. Whole exome sequencing (WES) is preferable, owing to ciliopathies' high diagnostic yield. In 2017, Vilboux et al. [24] suggested that if WES is not possible, then updated Joubert syndrome gene panels are preferable in most cases. In addition, in the absence of mainly phenotypic clues, prioritization of the most prevalent JS genes, including *KIAA0586*, may be reasonable. The mutation of known genes accounts for approximately half of the cases. Similar to our study, in 2015, Stephen et al. [25] identified a novel locus with a homozygous splice site mutation in *KIAA0586*, a known lethal ciliopathy

locus in model organisms, in patients clinically diagnosed with JS. The truncating *KIAA0586* mutations observed may result in loss of gene function and consequently abnormal tissue polarity, centrosome length and orientation, and centriolar satellites.

The phenotype of KIAA0586-affected subjects may range from a hydrolethalus phenotype to SRTDS. In 2015, Alby et al. [4] identified homozygous nonsense and splicing *KIAA0586* mutations as responsible for lethal ciliopathies in patients with similar phenotypes to that of HLS. All the fetuses and individuals reported by the authors showed lethal phenotypes consistent with a defect in hedgehog signaling and were similar to the phenotype described in the animal models of *KIAA0586*. SRTD with or without polydactyly refers to a group of skeletal ciliopathies with an autosomal recessive trait that is characterized by a constricted thoracic cage, short ribs, shortened tubular bones, polydactyly, and a 'trident' appearance of the acetabular roof [26]. Some forms of SRTD are lethal in the neonatal period due to respiratory insufficiency secondary to a severely restricted thoracic cage, whereas others are compatible with life [27]. Looking at all clinical descriptions, our case showed features shared in all three syndromes (Figure 1).

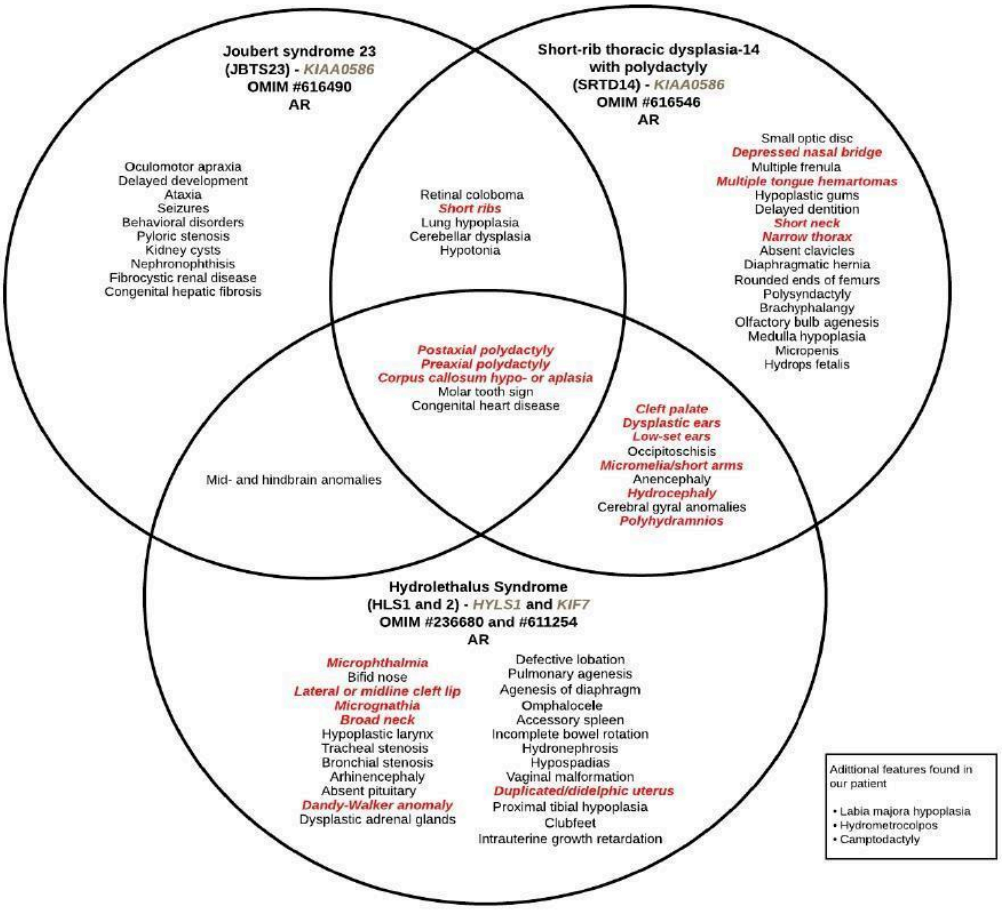

Figure 1. Venn diagram showing described overlapping clinical phenotypes between ciliopathies, as well as single syndrome features. Features written in red correspond to the ones found in our patient. AR: autosomal recessive.

Overlapping features in genetic syndromes are increasing as we deepen our research on molecular diagnosis. At the same time, the number of new genetic disorders is also rising since new technologies are available to map and explore known genes that cause specific clinical phenotypes [28]. Although clinical and molecular diagnosis is crucial to

elucidating an individual rare condition, addressing a possible variable expressivity of phenotypes with too many different names can be confusing and hinder the process more than aid. There are several explanations for variable expressivity and clinical heterogeneity in genomic disorders: genetic locus, variant type, number of genes involved, multiple allelism, protein isoforms, and modifier genes are some of them. In addition, a new theory of "true oligogenic" was also raised to understand such complexity: authors have proposed that the actions of two or more recessive genes with heterozygous mutations may result in a phenotype only when the mutations coexist (which may be our index case) [3,28,29].

Our patient showed a paternal inherited variant on *KIAA0586*, however, after an exome panel investigation for ciliopathies, we did not succeed in completely unraveling the molecular disarray. As the majority of ciliopathies are autosomal recessive inheritance, a heterozygous mutation would not fulfill the requirements to express the syndrome. Curiously, the proband mother had an intronic truncated variant on the same gene that could provide the missing "other half". Recent studies reported cases of individuals diagnosed with ciliopathies that have both a heterozygous variant on an exon and a heterozygous variant on an intron, which justified the phenotype [30–32]. In these cases, the intronic variants led to a cryptic exon that somehow disrupted the encoded protein. Therefore, since exonic and intronic variants can be causative of ciliopathies, we believe that despite the known exon inherited variant, the aberrant intronic variant may have been inherited from the mother, leading to the clinical hydrolethalus phenotype. The abnormal intronic region would, then, replace the expected second exonic mutation. Although the intronic variant hypothesis could be a plausible response, we cannot assess the proband molecular diagnosis. Furthermore, the involvement of other genes or variants cannot be discarded.

4. Materials and Methods

Molecular Analysis

High-resolution GTG banding karyotype analysis was performed from lymphocyte cell cultures (46, XX). DNA was isolated from peripheral blood lymphocytes using the Gentra Puregene Blood Kit (Qiagen, Valencia, CA, USA). The germline copy number variations (CNVs) were investigated using a high-density CytoScan HD Array platform (Affymetrix, Santa Clara, CA, USA) and the final data were analyzed by the Chromosome Analysis Suite software v.3.1 (Affymetrix, NetAffx Annotation Files version 33.1—hg19). A new generations sequencing (NGS) panel for ciliopathies (Cildag V1 panel) was performed in both the patient and parent's samples using the SureSelect 174 genes kit (Agilent, Santa Clara, CA, USA) and the NextSeq500 sequencer (Illumina, San Diego, CA, USA). Gene coordinates cited throughout the manuscript were based on the genome build GRCh38/hg38.

The medical ethics committee of Universidade Federal de Ciências da Saúde de Porto Alegre and Hospital Santa Casa de Misericórdia de Porto Alegre approved the study (69178217.7.0000.5345). The study was conducted in accordance with the Helsinki declaration.

5. Conclusions

To sum up, as the number of ciliopathy-associated genes increases and the range of overlapping features between ciliopathies becomes more common, the need to better understand the genotype–phenotype correlation becomes clearer. In addition, elucidating the variable expressivity is essential to comprehending how many different syndromes we have. To achieve that, NGS panels may have to start including screening of intronic regions since the current scenario involves mostly molecular analysis focused on exploring exons. Overall, phenotype severity and molecular diagnosis should be investigated together to better propose a possible degree of ciliopathies disorders.

Supplementary Materials: The supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms25147900/s1.

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Informed Consent Statement: Informed consent was obtained from the parents of the index patient.

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5. CONCLUSÕES

Para esta tese, diferentes condições monogênicas foram abordadas a fim de realizar a análise molecular de indivíduos afetados, e, também, discutir o cenário atual do diagnóstico molecular para doenças genéticas raras em geral. As estratégias de investigação utilizadas contemplaram sequenciamentos convencionais de Sanger, painel gênico por sequenciamento de nova geração e exoma. Além das técnicas moleculares, algoritmos de predição foram utilizados como ferramentas complementares após a identificação de variantes patogênicas ainda não descritas na literatura.

Ao total, oito indivíduos foram analisados para a presença de alterações genéticas, sendo cada paciente diagnosticado clinicamente para uma doença monogênica rara distinta (Tabela 1).

Tabela 1. Descrição do diagnóstico clínico das doenças monogênicas raras apresentadas nesta tese.

Suspeita Clínica	Displasia Esquelética	Síndrome de Waardenburg	Síndrome de Apert	Síndrome de Pfeiffer	Doença de Meige	Síndrome de Hydrolethalus	Síndrome de Marshall-Smith	Displasia Campomélica
Padrão de Herança	AR	AD, AR	AD	AD	AD	AR	AD	AD
OMIM	#143095	#277580	#101200	#101600	#153400	#236680	#602535	#114290
Abordagem Molecular	Painel NGS; Sanger	Sanger	Sanger	Sanger	Sanger	Painel NGS; Sanger	Sanger, Exoma	Sanger
Gene	*CHST3*	*EDNRB*	*FGFR2*	*FGFR2*	*FOXC2*	*KIAA0586*	*NFIX*	*SOX9*
Variante Detectada	c.673A>G	c.888G>C	c.758C>G	c.940-2A>G	N/A	c.4018del	c.1168_1174dupCCAGCAG	N/A
Etiologia	Herdada	Desconhecida	*De novo*	*De novo*	N/A	Herdada	*De novo*	N/A
Descrita na Literatura	Não	Não	Sim	Sim	N/A	Não	Não	N/A
Patogenicidade	Patogênica	Patogênica	Patogênica	Patogênica	N/A	N/A	Patogênica	N/A

AR: autossômica recessiva; AD: autossômica dominante

Entre os sete genes analisados, quatro indivíduos apresentaram variantes patogênicas novas, ainda não descritas na literatura e/ou depositadas em banco de dados. Para dois pacientes foram identificadas variantes previamente discutidas na literatura já associadas a indivíduos portadores do mesmo fenótipo (*FGFR2*: rs1057519041 e rs77543610). Apesar de nossa análise molecular cobrir toda a região codificante dos genes, para dois destes pacientes não foi possível identificar alterações em suas sequências de DNA. Ainda, verificamos alterações *de novo* em três pacientes e variantes herdadas em dois indivíduos.

Variações monogênicas estão associadas a um extenso grupo de patologias que, em 1997, ganharam um banco de dados online para catalogar e notificar fenótipos de doenças monogênicas e seus genes associados. A plataforma OMIM, *Online Mendelian Inheritance in Man*, é regularmente atualizada e focada em doenças genéticas com padrões de herança mendelianos em humanos. Em 8 de abril de 2024, o OMIM descreveu 7.514 fenótipos com base molecular conhecida e cerca de 4.900 genes com variantes relacionadas a doenças monogênicas. Entretanto, a plataforma também registrou outros 1.501 fenótipos que ainda não possuem base molecular conhecida. Além disso, do total aproximado de 5 mil genes com variantes descritas, apenas 3.433 possuem um único fenótipo associado; 891 são associadas a dois fenótipos, 323 a três manifestações clínicas e 253 a mais de quatro diagnósticos clínicos (OMIM. Gene Map Statistics. 2024. Disponível em: <https://omim.org/statistics/geneMap>. Acesso em: 04 de Maio de 2024).

Além do significativo número de fenótipos monogênicos sem elucidação molecular, há um escasso número de casos registrados para cada doença

monogênica, tornando os achados moleculares pontuais para um limitado número de indivíduos e desmotivando pesquisadores a investirem em linhas de pesquisa para essas doenças.[99] Hoje, sabe-se que o conceito de doença monogênica não é simples e que, ao contrário do que se pensava, esse grupo de patologias apresenta uma alta heterogeneidade alélica e de loco que dificulta a construção de painéis genéticos aplicáveis a pacientes com o mesmo fenótipo. Entretanto, esses achados atípicos do conceito Mendeliano encontrados em doenças monogênicas acabaram contribuindo para um grande conhecimento das formas poligênicas da doença humana: os diferentes tipos de variantes patogênicas, suas consequências e a complexidade fenotípica para doenças relacionadas ao mesmo gene foram descobertos no curso da busca contínua por loco de doença mendeliana.[100,101,102]

As mutações *de novo* são justificadas pela ausência da alteração genômica nos pais de um indivíduo portador. Dentre os sequenciamentos analisados foram encontradas mutações *de novo* em três indivíduos, sendo as alterações encontradas no gene *FGFR2* já descritas na literatura. A variante encontrada no paciente diagnosticado com síndrome de Pfeiffer está localizada no sítio aceptor de *splicing*, uma região crucial do DNA, cuja função é delimitar o início do éxon para posterior formação do RNA mensageiro maduro. A troca de nucleotídeo nessa região do gene *FGFR2* pode fazer com que o íntron adjacente permanecesse ou que o éxon subsequente fosse excluído no momento do splicing, acarretando na formação de uma proteína anormal e/ou não-funcional.[103] Essa mutação já foi descrita anteriormente e associada a síndromes de craniossinostose como Pfeiffer e Apert.[44,104] A alteração genética encontrada no indivíduo com síndrome de Apert, por outro lado, não implica

diretamente em alterações de regiões imprescindíveis para a formação proteica. Entretanto, a variante Pro253Arg do gene *FGFR2* é considerada patogênica e já foi consolidada na literatura como associada a diversas craniossinostoses.[105,106]

Para o paciente com síndrome de Marshall-Smith foi identificada uma variante *de novo* até então não descrita na literatura. Essa alteração no genoma consiste na duplicação de sete nucleotídeos, cuja adição na sequência de DNA altera as três isoformas do gene *NFIX*. A mudança na fase de leitura do RNA mensageiro é verificada em todas as isoformas do gene, ocorrendo em duas o prolongamento da cadeia peptídica acarretado e em uma a formação de um *stop códon* prematuro. Normalmente variantes que implicam na mudança da fase de leitura são degradadas por um sistema de vigilância chamado de degradação de RNA mensageiro mediada por mutação sem sentido (do inglês, *nonsense-mediated RNA decay*, NMD), ou seja, um mecanismo que reconhece RNA mensageiros alterados e impede que esses codifiquem proteínas não funcionais.[76] Entretanto, portadores de variantes no gene *NFIX* associados ao fenótipo de Marshall-Smith parecem gerar proteínas que são capazes de exercer um efeito dominante negativo sobre o alelo selvagem e escapar do mecanismo de vigilância NMD.[76]

Além dos achados de etiologia *de novo*, para a família dos indivíduos diagnosticados com displasia esquelética e síndrome de Hydrolethalus foi observada a herança de alelos patogênicos. Para o paciente com displasia esquelética, ambos os progenitores eram portadores heterozigotos para a variante encontrada no gene *CHST3*. A alteração encontrada ainda não foi descrita na literatura, entretanto variantes identificadas no gene *CHST3* estão

diretamente associadas ao fenótipo de displasia esquelética.[23,24,28] Ainda, algoritmos de predição como o PolyPhen indicam que esta alteração *missense* apresenta um alto risco de dano ao genoma do indivíduo portador (score 0.9999/1), corroborando com a hipótese de a variante justificar a clínica apresentada.

Por outro lado, o indivíduo com síndrome de Hydrolethalus herdou uma mutação do pai e a segunda não foi detectada através do painel de sequenciamento exônico para ciliopatias. Apesar de ter herdado um alelo paterno mutado, o padrão de herança autossômica recessiva da condição genética não justifica a manifestação clínica apresentada. Entretanto, suspeita-se que o paciente tenha herdado, também, uma alteração intrônica materna. Ambas variantes são encontradas no gene *KIAA0586*. A alteração genética paterna configura-se em uma deleção que acarreta na mudança da fase de leitura e, consequentemente, na formação de uma proteína anormal. Estudos recentes com indivíduos portadores destas condições já evidenciaram a presença concomitante de variantes exônicas somadas a variantes intrônicas, auxiliando na validação da hipótese gerada para o nosso paciente.[107,108,109]

Eventualmente, a origem da variante patogênica do indivíduo portador pode não ser identificada, uma vez que dependemos diretamente da disponibilidade do DNA dos pais. Para o paciente diagnosticado com síndrome de Shah-Waardenburg, foi encontrada uma alteração em homozigose no gene *EDNRB*, ainda não descrita na literatura. Entretanto, como o DNA dos progenitores estava indisponível, não foi possível confirmar se a variante identificada pelo sequenciamento de Sanger é *de novo*, ou o resultado da

herança de alelos de pais heterozigotos. Apesar da variante encontrada em nosso paciente ainda não ser associada como causadora do fenótipo de Shah-Waardenburg, alterações no gene *EDNRB* são frequentemente relacionadas à síndrome. Ainda, o algoritmo de predição PolyPhen indica que esta alteração na sequência de DNA apresenta um alto risco de dano ao genoma do indivíduo portador (score 0.928/1).

As variantes genéticas identificadas como novas devem ser inseridas em banco de dados internacionais por seus descobridores como uma forma de alimentar informações relacionadas ao diagnóstico molecular de síndromes genéticas. Por muitas condições serem raras, o compartilhamento de achados é imprescindível para as associações entre genótipo e fenótipo serem cada vez melhor elucidadas. Entretanto, os bancos de dados genéticos dependem diretamente de cientistas e pesquisadores para serem atualizados. Sendo assim, hoje, não é possível afirmar que os dados contidos nestes registros são fidedignos e atuais. Portanto, além da investigação da etiologia molecular e clínica, é necessário refletir como podemos garantir que a informação genética está sendo disseminada em tempo real.

Outro desafio enfrentado pela comunidade científica relacionado a variantes genéticas - principalmente mutações novas - é a padronização de sua nomenclatura e, principalmente, da patogenicidade da mesma frente ao tipo de alteração encontrada. A *American College of Medical Genetics and Genomics* e a *Association for Molecular Pathology* estipulou normas e orientações para a interpretação de variantes genéticas de forma a aprimorar a investigação e a elucidação da potencial relação genótipo-fenótipo nas doenças raras. Algoritmos de predição e critérios de patogenicidade devem ser considerados

na análise a fim de providenciar uma avaliação mais fidedigna do nível de importância patológica de variantes na população. Sabe-se que polimorfismos existem em diferentes populações e que essas variantes não necessariamente indicam risco de patogenicidade para o indivíduo portador.[11]

O diagnóstico molecular, por outro lado, vem avançando exponencialmente nas últimas décadas. Atualmente, diversas tecnologias podem ser utilizadas de forma complementar para auxiliar na elucidação de doenças genéticas, bem como na etiologia dos genes. Técnicas que revolucionaram a ciência como a Reação em Cadeia da Polimerase (PCR) e o sequenciamento de Sanger foram cruciais para o desenvolvimento e aprimoramento de metodologias que levaram a criação de painéis genéticos, técnicas com base no RNA, varreduras genômicas e sequenciamento de íntrons, éxons e do genoma completo.[110]

Apesar das vantagens das tecnologias mais recentes, onde a análise de variantes genéticas é em grande escala, o sequenciamento de Sanger, por exemplo, permanece com um lugar essencial na genômica clínica. Um dos motivos para sua permanência é devido ao seu papel essencial para confirmar variantes de sequências identificadas por sequenciamentos de nova geração (NGS). Devido ao grande número de primers, reações e interpretações necessárias para analisar cada variante, a confirmação de todas as alterações genômicas encontradas pelo NGS seria inviável. Entretanto, em casos em que uma variante específica seja questionada quanto ser ou não relevante ou causadora de um fenótipo, o sequenciamento de Sanger é o método mais fácil e eficaz para resolver essas incertezas.[111] Sendo assim, de forma a alcançar um maior nível de evidência científica para os achados moleculares se torna

necessário entender que as metodologias moleculares antigas são complementares às novas e vice-versa.

Além das técnicas moleculares, as ferramentas da análise *in silico* mostraram ser uma grande aliada na busca pelo diagnóstico molecular preciso. Com o intuito de auxiliar na elucidação da função dos genes, bem como no esclarecimento da alteração funcional das diversas variantes patogênicas ainda sem significado clínico, a bioinformática se tornou imprescindível na busca pela consolidação da correlação genótipo-fenótipo das diversas condições genéticas raras. Algoritmos de predição, como o SIFT e o PolyPhen, proporcionaram a avaliação fidedigna de dano ao genótipo de variantes ainda não conhecidas, enquanto as análises de bioinformática permitiram a investigação das variantes à nível protéico - auxiliando ainda mais no esclarecimento da associação genótipo-fenótipo de diversas patologias.[11,12,13] O conceito de desordem proteica abordado no artigo do paciente diagnosticado com síndrome de Marshall-Smith, por exemplo, ilustra a importância das análises *in silico* para não só entender, como também justificar o porquê de a proteína traduzida pelo alelo anormal ser suficiente para gerar determinado fenótipo clínico.

Em suma, a busca pelo diagnóstico molecular é extensiva, mas, hoje, contamos com uma diversidade de ferramentas para auxiliar na jornada até a correlação precisa entre genótipo e fenótipo. A clínica médica fornece o primeiro norte na busca pela causa de um determinado fenótipo, enquanto as metodologias moleculares permitem a consolidação do diagnóstico genético. Ainda, a bioinformática fornece informações complementares que auxiliam na compreensão e na elucidação do porquê a alteração molecular encontrada causar as características clínicas presentes no indivíduo. As diversas áreas de

conhecimento juntas se mostram promissoras como aliadas no objetivo comum de proporcionar a montagem final do quebra-cabeça complexo que são as doenças genéticas, principalmente as consideradas monogênicas e raras.

6. CONSIDERAÇÕES FINAIS

O presente estudo faz parte de um projeto maior intitulado “Estudo de aspectos etiológicos e clínicos de pacientes com doenças genéticas ou síndromes malformativas atendidos no Serviço de Genética Clínica**”,** aprovado pelos Comitês de Ética em Pesquisa (CEP) da UFCSPA (Parecer N° 2.230.086). Deste projeto maior estão sendo desenvolvidos diversos outros trabalhos e projetos, incluindo trabalhos de conclusão de cursos, dissertações e teses.

O Serviço de Genética Clínica da Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA) é parceria direta do Complexo Hospitalar Santa Casa de Porto Alegre (CHSCPA). A colaboração entre as instituições visa descrever as características clínicas, laboratoriais e prognósticas de pacientes portadores de doenças genéticas ou síndromes malformativas a fim de contribuir com o diagnóstico molecular muitas vezes inexistente para esses pacientes e familiares.

O aprimoramento da investigação molecular aliada à prática clínica e a ferramentas de bioinformática permite uma investigação mais completa de novas variantes genéticas, auxiliando na elucidação de causas moleculares e na associação entre genótipo-fenótipo. Ainda, a complementação entre áreas afins reflete no prognóstico clínico mais preciso, bem como no aconselhamento genético de famílias com indivíduos acometidos por síndromes genéticas.

Esta tese de doutorado gerou dois artigos publicados e um manuscrito submetido a periódicos científicos. Os resultados moleculares identificados para os pacientes diagnosticados clinicamente com displasia esquelética, síndrome de Shah-Waardenburg e síndrome de Apert estão descritos no

apêndice deste trabalho e os dados encontrados encontram-se em elaboração para futura submissão.

7. APÊNDICES

7.1. Resultado do gene *CHST3*

Para o paciente clinicamente diagnosticado com displasia esquelética, um painel de NGS direcionado para essa condição genética foi realizado a fim de identificar a alteração molecular. A varredura de éxons de todos os genes contemplados no painel identificou uma nova variante em homozigose no éxon 3 do gene *CHST3* (NG_012635.1:g.48343A>G, NM_004273.5:c.673A>G, NP_004264.2:p.Ser225Gly), ainda não descrita na literatura.

Com o intuito de verificar a etiologia da alteração genética e, também, confirmar o resultado encontrado, o sequenciamento convencional por Sanger foi realizado para o paciente e seus progenitores. Ao analisar os eletroferogramas da família, foi identificado que tanto a mãe quanto o pai do paciente apresentavam a variante em heterozigose e que, portanto, o filho havia herdado ambos os alelos mutados dos pais (Figura 1).

A probabilidade de dano ao genoma da nova variante foi analisada através do algoritmo de predição PolyPhen. A alteração *missense* foi considerada como altamente provável de causar prejuízo à funcionalidade da proteína traduzida e, consequentemente, corroborar para a expressão das características clínicas apresentadas pelo paciente (Figura 3).

Para os demais éxons, nenhuma alteração na sequência de DNA foi identificada.

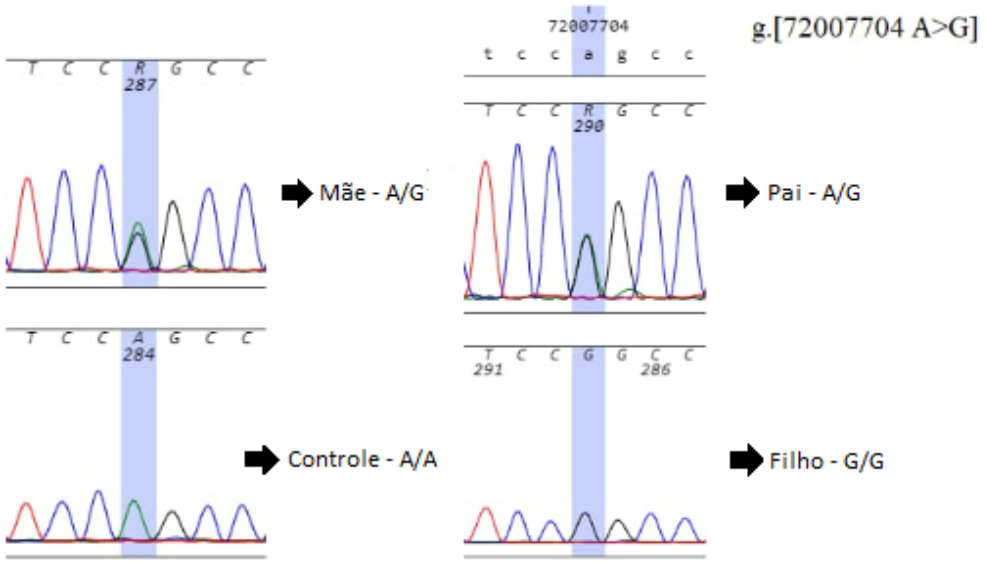

Figura 1. Eletroferogramas ilustrando a variante encontrada em heterozigose nos pais, em homozigose no filho e ausente na amostra controle.

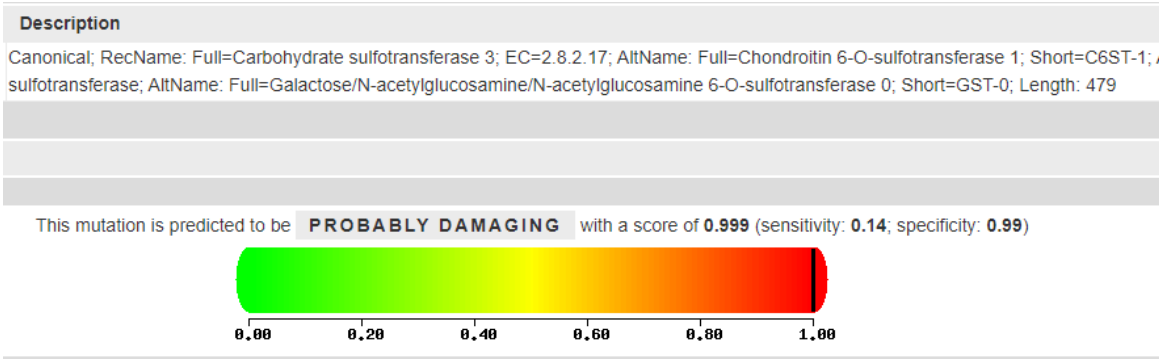

Figura 2. Resultado da predição do algoritmo PolyPhen para a variante S225G.

7.2. Resultado do gene *EDNRB*

Para a análise do gene *EDNRB*, pares de primers foram desenhados para todos os nove éxons que compõem a sua região codificante. Como estratégia, optamos por sequenciar inicialmente os éxons com variantes já descritas no banco de dados do NCBI e associadas ao fenótipo clínico da

síndrome de Shah-Waardenburg. Dessa forma, uma nova variante *missense* no éxon 4 do gene foi identificada (Figura 3) (NG_011630.3:g.78603G>C, NM_000115.5:c.888G>C, NP_000106.1:p.Met296Ile).

Uma vez que a alteração encontrada no sequenciamento do paciente ainda não foi descrita na literatura, o algoritmo de predição PolyPhen foi utilizado para verificar a probabilidade de dano ao genoma. A troca de aminoácidos gerada pela variante indica uma forte probabilidade de prejuízo à proteína traduzida pelo gene, podendo, dessa forma, justificar o fenótipo apresentado pelo paciente (Figura 4).

Infelizmente, o DNA dos pais do paciente estava indisponível para análise e, portanto, não foi possível confirmar se a variante encontrada é *de novo*, ou proveniente da herança de progenitores heterozigotos.

Para os demais éxons, nenhuma alteração na sequência de DNA foi verificada.

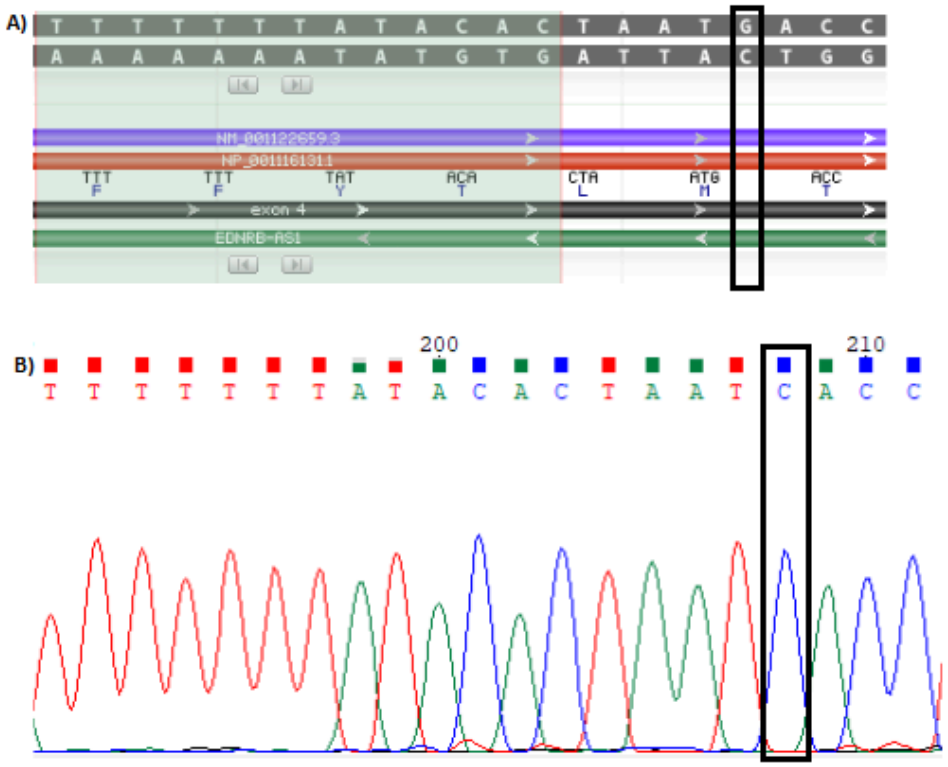

Figura 3. a) Sequência selvagem do éxon 4 do gene *EDNRB*. b) Variante em homozigose encontrada no sequenciamento do indivíduo diagnosticado com síndrome de Shah-Waardenburg. Em ambas imagens, o retângulo preto destaca o nucleotídeo selvagem e alterado, respectivamente.

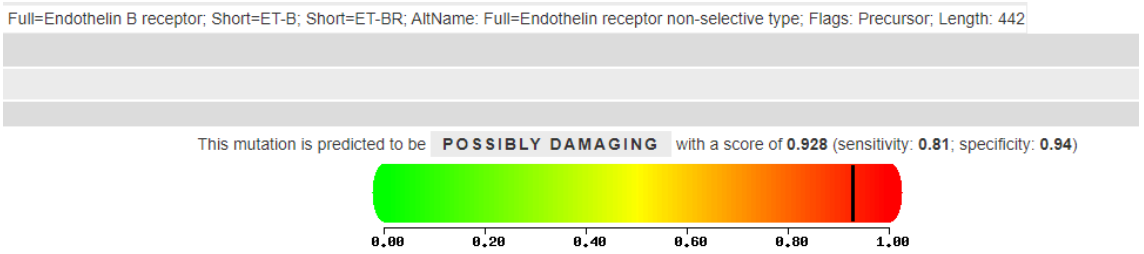

Figura 4. Resultado da predição do algoritmo PolyPhen para a variante M296I.

7.3. Resultado do gene *FGFR2* - Síndrome de Apert

Para o gene *FGFR2* foram desenhados primers para amplificar os éxons selecionados com sítio *hotspot* para variantes associadas a craniossinostoses, uma vez que o gene conta com um total de 26 éxons em sua estrutura. Sendo assim, foram selecionados os éxons 7, 8, 9, 10, 13 e 15 para iniciar a investigação do diagnóstico molecular. Conforme esperado, foi identificada uma variante *de novo* no éxon 7 do gene (NG_012449.2:g.83299C>G, NP_000132.3:p.Pro253Arg, rs77543610) (Figura 5). A alteração genética encontrada já foi descrita na literatura como patogênica e associada à síndrome de Apert, além de outras craniossinostoses.

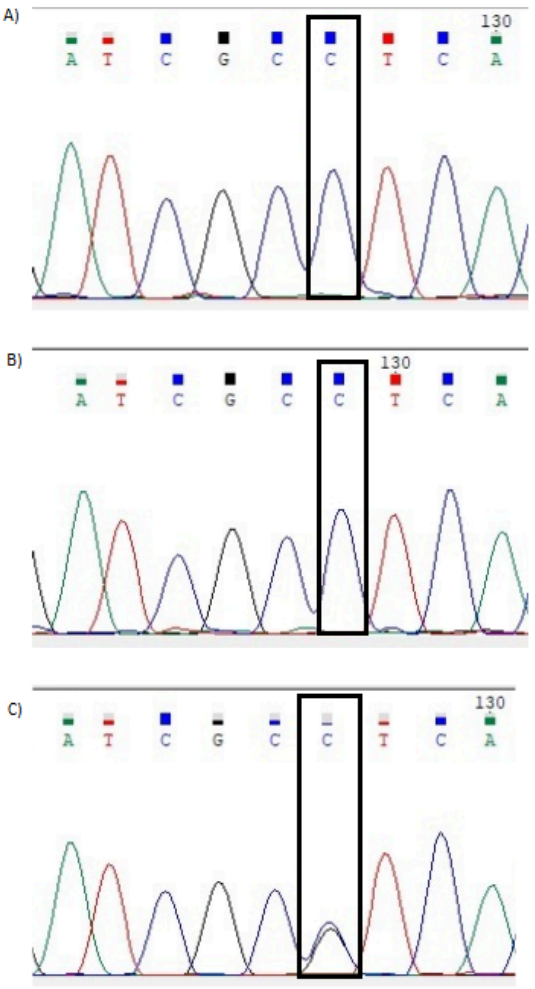

Figura 5. a) Eletroferograma do pai com pico em homozigose do alelo selvagem “C”. b) Eletroferograma da mãe com pico em homozigose do alelo selvagem “C”. c) Eletroferograma do filho com pico heterozigoto: alelo selvagem “C” e variante “G”.

7.4. Resultados do gene *FOXC2*

O gene *FOXC2* é pequeno e composto por um único éxon. Dessa forma, para conseguir cobrir apropriadamente toda a região codificante do gene, quatro pares de primers foram desenhados. Entretanto, infelizmente, apenas algumas regiões do gene foram amplificadas com sucesso:

conseguimos uma boa leitura do início do éxon, mas para as regiões subsequentes encontramos dificuldades de adquirir um eletroferograma de qualidade.

De forma a confirmar a ausência completa de variantes no gene *FOXC2* seria necessário identificar regiões mais suscetíveis ao anelamento para desenhar novos *primers*. Outra possibilidade plausível seria a existência de uma variante na sequência do paciente que acarrete em mudança de fase de leitura, prejudicando, dessa forma, a amplificação e/ou leitura da sequência de nucleotídeos.

7.5. Resultados do gene *SOX9*

Para a análise do gene *SOX9*, pares de primers foram desenhados para todos os três éxons que compõem a sua região codificante. Entretanto, apenas os éxons 2 e 3 foram amplificados com sucesso. Ainda, para as regiões em que o sequenciamento foi possível, não identificamos nenhuma variante considerada patogênica ou de significado incerto e que, portanto, justificasse o fenótipo de displasia campomélica da paciente. No éxon 2 do gene, por exemplo, foi identificada uma variante sinônima - apesar da troca de nucleotídeo, o aminoácido traduzido se manteve (Figura 6).

Para confirmar a ausência completa de mutações no sequenciamento do gene seria necessário a confecção de novo par de *primers* para o éxon não amplificado. Entretanto, sabe-se que alterações genéticas que implicam em mudança da fase de leitura são as mais relatadas no gene (Sock *et al*., 2003; Lecointre *et al.*, 2009). Dessa forma, é possível que a dificuldade de anelamento e/ou amplificação adequada de algumas regiões esteja relacionada

a deleções ou inserções maiores, mimetizando eletroferogramas falhos e de difícil leitura.

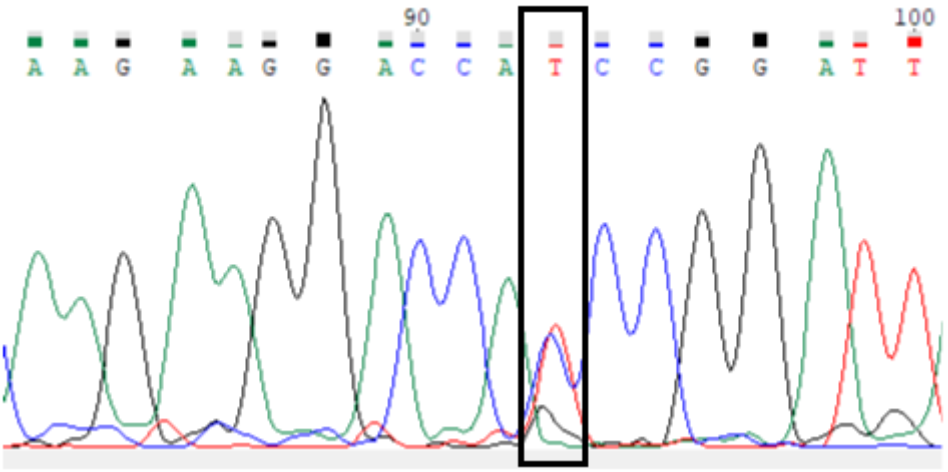

Figura 6. Eletroferograma da paciente ilustrando a variante sinônima encontrada no sequenciamento do gene *SOX9*.

7.5. Revisões Sistemáticas publicadas em Revistas Internacionais

7.5.1. Microarray-Based Comparative Genomic Hybridization, Multiplex Ligation-Dependent Probe Amplification, and High-Resolution Karyotype for Differential Diagnosis Oculoauriculovertebral Spectrum: A Systematic Review

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Microarray-Based Comparative Genomic Hybridization, Multiplex Ligation-Dependent Probe Amplification, and High-Resolution Karyotype for Differential Diagnosis Oculoauriculovertebral Spectrum: A Systematic Review

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Abstract

Oculoauriculovertebral spectrum (OAVS) is a rare class of heterogenous congenital craniofacial malformation conditions of unknown etiology. Although classic OAVS has been described as hemifacial microsomia with facial asymmetry and microtia, there is no consensus regarding clinical criteria for diagnosis or genetic cause. This systematic review aims to assess the applicability of high-resolution (HR) karyotype, fluorescence in situ hybridization, multiplex ligation-dependent probe amplification (MLPA), and microarray-based comparative genomic hybridization (array-CGH) for differential diagnosis of OAVS. A search was performed in PubMed and Web of Science using all entry terms to the following descriptors: Goldenhar's syndrome, cytogenetic analysis, hybridization in situ, fluorescent, comparative genomic hybridization, multiplex polymerase chain reaction, whole genome sequencing, and karyotype analysis methods. After screening, 25 articles met eligibility. Of the included studies, 59 individuals had a genetic alteration identified. Array-CGH, MLPA, and HR karyotype appear to be viable approaches for molecular diagnosis in OAVS. Heterogeneity is a hallmark of OAVS. Establishing an enhanced framework for diagnosis would inform clinical decision making, and better resource utilization could improve health care facility efficiency and economy.

Keywords
- Goldenhar's syndrome
- OAVS
- comparative genomic hybridization

Introduction

Oculoauriculovertebral spectrum (OAVS; OMIM 164210), including Goldenhar's syndrome and hemifacial microsomia, is a rare class of heterogenous craniofacial conditions of unknown etiology characterized by craniofacial malformations derived from the first and second branchial arches during embryonic development.[1] Numerous studies highlighted the clinical and genetic heterogeneity of OAVS,[2,3] and unsurprisingly, the birth prevalence for OAVS is inconsistent and ranges between 1:5,600 and 1:45,000.[4,5] Positive family history has been suggested to be of diagnostic value.[6] Although classic OAVS has been described as hemifacial microsomia with facial

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asymmetry and microtia, there is no consensus regarding clinical criteria for diagnosis[7] or genetic cause.[8] Several genomic and chromosomal alterations have been reported in individuals with clinical diagnosis for OAVS,[6,9–11] and there is no gold standard modality for molecular diagnosis in OAVS.

Heterogeneity is a hallmark of OAVS. Establishing an enhanced framework for diagnosis would inform clinical decision making, and better resource utilization could improve health care facility efficiency and economy. This systematic review aims to assess the applicability of high-resolution (HR) karyotype, fluorescence in situ hybridization (FISH), multiplex ligation-dependent probe amplification (MLPA), and microarray-based comparative genomic hybridization (array-CGH) for differential diagnosis of OAVS.

Methods

The question addressed by the systematic review was: for patients with clinical OAVS, is cytogenetic diagnostic testing with HR karyotype, FISH, MLPA, or array-CGH reliable to confirm diagnosis? The hypothesis was that cytogenetic testing would improve diagnosis. A systematic search of the literature was performed following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines. A search was performed in PubMed and Web of Science using all entry terms to the following descriptors: Goldenhar's syndrome, cytogenetic analysis, hybridization in situ, fluorescent, comparative genomic hybridization, multiplex polymerase chain reaction, whole genome sequencing, and karyotype analysis methods. All articles up until December 31, 2019, were selected for screening. Three reviewers independently performed article selection, and the results were stored in the authors' spreadsheet. The inclusion criteria were as follows: (1) OAVS or a synonym as the main object of the study and (2) OAVS patients with cytogenetic tests. The screening process consisted of three phases. In phase 1, articles were screened by title for inclusion of OAVS or a synonym as the main object of the study, and articles with OAVS or a synonym in the title were screened in the second phase for inclusion of cytogenetic technique in the abstract. The final phase consisted of full-text assessment for results of the cytogenetic testing and the relationship between molecular findings and OAVS. Any discrepancies were resolved by discussion until consensus was reached.

A total of 86 articles were assessed. After duplicate removal and according to the inclusion and exclusion criteria, full-text articles were assessed for eligibility, and data from 25 articles were included (►**Fig. 1**). The following variables were collected from each article: clinical findings, clinical criteria, genetic techniques performed, and molecular findings.

Results

Articles Screened and Reviewed

After screening, 25 articles published between 1988 and 2019 met inclusion criteria. Article types included: original article ($n = 11$), case reports ($n = 9$), short report ($n = 3$), and letter ($n = 2$) (►**Table 1**).

OAVS Clinical Criteria and Range of Clinical Findings

Among the screened articles, 41% did not cite a reference for OAVS clinical diagnostic criteria; 28% cite Tasse et al's criteria[7]; and 31% cite other clinical criteria. As expected, a wide range of craniofacial and other clinical features were described (►**Table 2**). Microsomia (54.5%), mandibular hypoplasia (36.3%), cleft palate (36.3%), macrostomia (27.2%), micrognathia (27.2%), retrognathia (9%), microcephaly (4.5%), and facial hypoplasia (9%) were the most frequently reported craniofacial features. Among the ocular abnormalities, epibulbar dermoid (46.1%) was most often reported, but patients with microphthalmia, anophthalmia, and ptosis ($n = 3$) were also reported. Ear alterations were described in all cases. Preauricular tags were reported in 68% of the articles and unilateral or bilateral microtia were observed in 40%. Abnormal spinal features, developmental delay, and cases of congenital heart disease were rarely reported. Family history for OAVS were described in nine studies,[1,2,6,7,9,10,12–14] and four studies included twin analysis,[1,2,15,16] two of which had no report of family history.[17,18]

Methods Applied in OAVS Investigation

Of the 25 included articles, 22 performed karyotype analysis (G-banding, $n = 14$; HR banding, $n = 6$; and R-banding, $n = 2$), 10 used FISH, 2 used MLPA, and 15 used array-CGH. Overall, of 303 individuals suspected of OAVS, only 59 individuals had associated genetic alterations (►**Table 2**). The remaining 244 individuals had no alterations or were rediagnosed based on the results with a disorder sharing clinical features with OAVS. Half of the 22 articles reporting use of karyotype analysis reported chromosomal alterations, including numerical and structural abnormalities, such as duplications, inversions, and translocations (►**Table 2**). Half of the 10 articles using FISH detected some kind of genetic alteration, including: translocations ($n = 3$), additional chromosome 22 ($n = 1$), and inversion p11.2q22.3 on chromosome 14 ($n = 1$). Both studies that performed MLPA technique found deletions in different regions 22q11.2 (►**Table 2**). Of the 15 articles reporting use of array-CGH technique to investigate OAVS, 13 identified genetic alteration, especially deletions and duplications of different chromosomal regions (►**Table 2**). Whole exome sequencing (WES) was also performed in one article but showed no significant result. All used quantitative polymerase chain reaction (qPCR) to confirm variations found by array-CGH.

Discussion

Clinical Findings

Considerable heterogeneity of clinical findings in individuals with suspected OAVS was described (►**Table 2**), including: craniofacial, limb, and organ system abnormalities. Some clinical findings were consistent in all cases, reinforcing the feasibility for a standardized clinical criteria. While many articles did not cite a diagnostic clinical criterion for OAVS, the most cited was the Tasse et al's criteria,[7] which

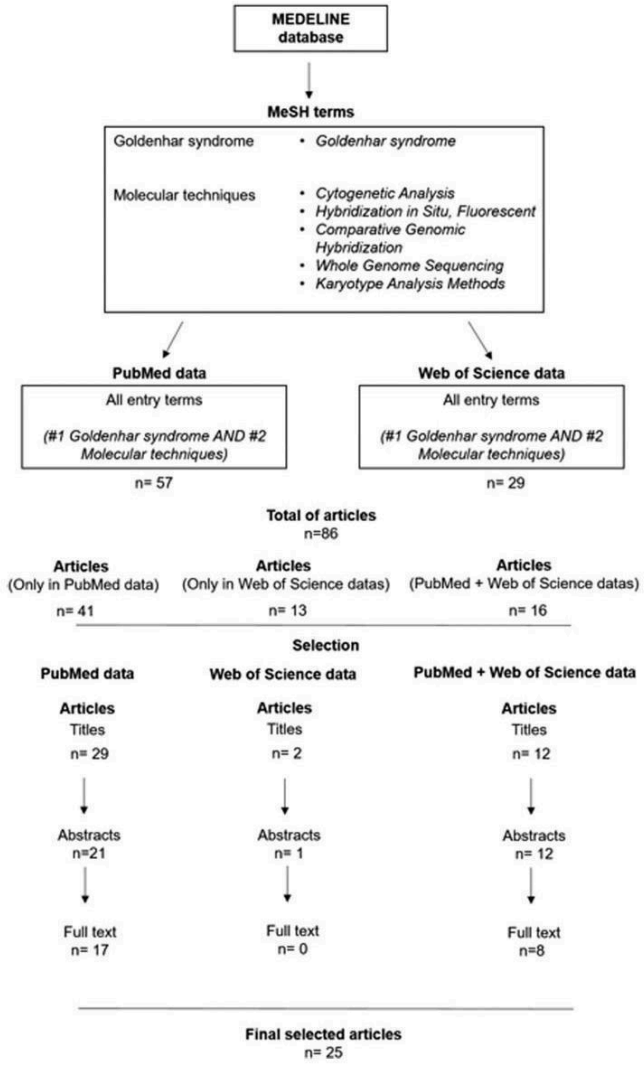

Fig. 1 Flowchart of the study selection process.

required the presence of microtia or hemifacial microsomia with or without other minor ear malformations. After evaluating 51 OAVS patients and their parents, Beleza-Meireles et al (2015) confirmed the validity of the Tasse et al's criteria and suggested adding a positive family history to the original criteria.[6] Huang et al (2010) cautioned that data suggesting an emphasis on positive family history could be skewed.[9]

Problems of Differential Diagnosis

The OAVS phenotype may overlap with other disorders that are important for differential diagnosis. These syndromes include: Treacher Collins' syndrome (OMIM 154500), auriculocondylar syndrome (OMIM 602483),[12] Townes–Brocks' syndrome (OMIM 107480),[9,19] cri du chat syndrome (OMIM 123450),[20] oculoauricular syndrome (OMIM 612109), branchio-otorenal syndrome 1 (OMIM 113650), cat eye syndrome

Table 1 Selected articles information

Author	Year	Category
Ala-Mello et al	2008	Case Report
Ballesta-Martínez et al	2013	Case Report
Beleza-Meireles et al	2015	Original Article
Bragagnolo et al	2018	Original Article
Brun et al	2012	Case Report
Colovati et al	2015	Short Report
Dos Santos et al	2014	Research Letter
Descartes	2006	Original Article
Herman et al	1988	Case Report
Huang et al	2010	Original Article
Huang et al	2010	Short Report
Josifova et al	2004	Original Article
Kobrynski et al	1993	Case Report
Northup et al	2010	Original Article
Puvabanditsin et al	2016	Case Report
Rooryck et al	2010	Original Article
Rooryck et al	2010	Short Report
Rosa et al	2010	Original Article
Silva et al	2015	Original Article
Spineli-Silva et al	2018	Case Report
Tasse et al	2005	Original Article
Verona et al	2006	Case Report
Xu et al	2008	Research Letter
Zielinski et al	2014	Original Article

(OMIM 115470), and otofaciocervical contiguous gene deletion syndrome (OMIM 166780).[1] Deletions and duplications on chromosome 22 in regions 22q11.2 causing DiGeorge's syndrome (DGS; OMIM 188400),[1,6,8,21–23] 22q11.1,[6] 22q13.32-33,[6] and 22q13.3[24] should also be considered.

Genetic Techniques

The authors in the reviewed articles used many molecular methods, and the variety of finding was great. While most molecular findings in individuals with OAVS are located within chromosome 22, it is important to highlight other alterations were also found. Molecular changes not involving chromosome 22 are of uncertain significance. Despite the high-quality information provided by array-CGH, this method does not detect balanced translocations and chromosomal inversions. Thus, it is suggested to begin the investigation through karyotyping techniques, followed by MLPA and array-CGH (►**Table 3**).

Karyotype Analysis

Karyotype was used most, with alterations found in different chromosomes and chromosome regions. The most observed alterations included chromosome 9 (inv 9),[3] t(9;18),[2,25] and chromosome 22 (del 22[23,24,26]). Chromosome banding is an essential technique used for karyotyping to identify normal and abnormal chromosomes. In an individual with suspected OAVS, Xu et al (2008)[23] found no alterations with the lower resolution GTG, but HR karyotype revealed a deletion at 22q11. GTG karyotype have a resolution of 450 to 550 bands, while HR can detect 850 band level.[27] The difference in banding resolution could explain why the GTG karyotype can miss some alterations found with HR karyotype. Therefore, for investigation of microdeletions associated with OAVS, HR and not GTG karyotyping should be used.

Table 2 Clinical and cytogenetic finding in individuals with clinical diagnosis of OAVS

Authors	Main clinical findings	Molecular techniques	Cytogenetic findings	Number of individuals
Ala-Mello et al (2008)[30]	Macrostomy Submucous cleft palate Epibulbar dermoids Preauricular tags Hemifacial microsomy Hypertelorism	G-banding karyotype FISH Array-CGH	45,XX, inv(2) (q32q37)mat, dic(5;21) (p15.3;q22.3)dn 21q and 5p were absent Deletion 5p15.33 Deletion 21q22.3 Duplication 21q22.11-q22.12	1/1 1/1 1/1
Ballesta-Martínez et al (2013)[12]	Micrognathia Macrostomia Preauricular pits and tags Auricular agenesis	High-resolution banding karyotype Array-CGH	Normal Duplication in 14q23.1	1/1 1/1
Beleza-Meireles et al (2015)[6]	Hemifacial microsomia Microtia External ear abnormalities Preauricular skin tags Cleft lip and/or cleft Palate Micrognathia Epibulbar dermoids Vertebral anomalies	Array-CGH	Duplication Xp11.21 Duplication 22q11.21 Duplication 22q13.32-33 Deletion 14q12 Duplication 10p15.33 Deletion 19q13.3 Duplication 22q11.1 Duplication 22q11.1q11.21	10/22

Table 2 (*Continued*)

Authors	Main clinical findings	Molecular techniques	Cytogenetic findings	Number of individuals
Bragagnolo et al (2018)[1]	Mandibular hypoplasia Macrostomia Micrognathia Preauricular tags and pits Epibulbar dermoid	G-banding karyotype Array-CGH	Normal Duplication 4p16.1 Deletion 4p16.3p15.33 Duplication Xp22.33p22.31 Deletion 8q13.3 Duplication 8q24.3 Duplication 10p14 Duplication 10p13. Deletion 10q26.2q26.3 Deletion 16p13.3 Duplication 16p13.11p12.3 Duplication 17q11.2 Deletion 22q11.21 Deletion Xp22.33	72/72 13/72
Brun et al (2012)[17]	Microtia Hemifacial microsomia Mandibular hypoplasia Bilateral conductive hearing loss Cleft palate	G-banding karyotype Array-CGH FISH	Normal Microdeletion in 15q24.1q24.2 Microdeletion in 15q24.1q24.2	1/1 1/1 1/1
Colovati et al (2015)[21]	Hemifacial microsomia Retrognathia Agenesis of the external auditory canal Soft cleft palate	G-banding karyotype MLPA Array-CGH	Normal Deletion in the 22q11.21 Deletion in the 22q11.21	1/1 1/1 1/1
Dos Santos et al (2014)[22]	Hemifacial microsomia Left ptosis Dysmorphic right ear Retrognathia Hearing loss	Array-CGH	Deletion in the 22q11.21	1/1
Descartes (2006)[20]	Macrostomia Midface hypoplasia Ear tags and pits Clinodactyly Frontal hemangioma	G-banding karyotype	46,XX,del(5)(p15.33)	1/1
Herman et al (1988)[24]	Hemifacial microsomia Epibulbar dermoids Macrocephaly Mandibular hypoplasia Preauricular tags Vertebral anomalies	High-resolution banding karyotype	46,XY,del (22)(q13.31)	1/1
Huang et al (2010)[9]	Preauricular tags Epibulbar dermoid Micrognathia Hypertelorism Facial dysplasia Hemifacial microsomia	G-banding karyotype Array-CGH	Normal 153 copy number variations, but without significant result	4/4 13/13
Huang et al (2010)[19]	Hemifacial microsomia Preauricular tags Atresia of the external auricular canal Vertebral anomalies	High-resolution banding karyotype Array-CGH qPCR	Normal Deletion on chromosome 5q13.2 Deletion on chromosome 5q13.2	1/1 1/1 1/1
Josifova et al (2004)[13]	Preauricular tags Right hemiparesis Developmental delay Hearing loss	G-banding karyotype FISH	Normal 46,XY,der(5)t(5;8)(p15.31;p23.1)	2/2 2/2

(*Continued*)

Table 2 (*Continued*)

Authors	Main clinical findings	Molecular techniques	Cytogenetic findings	Number of individuals
Kobrynski et al (1993)[26]	Hemifacial microsomia Epibulbar dermoid Absence of the external auditory meatus Preauricular pits Clinodactyly	G-banding karyotype FISH	47, XX, + 22 47, XX, + 22	1/1 1/1
Northup et al (2010)[14]	Ear abnormality Micrognathia Microtia Asymmetric face	G-banding karyotype FISH	46,XY,inv(14) (p11.2q22.3) 46,XY,inv(14) (p11.2q22.3)	1/1 1/1
Puvabanditsin et al (2016)[11]	Preauricular skin tags Hemifacial microsomia Microtia Absence of the auditory canal Microsomia Micrognathia	Array-CGH	Deletion in 7q21.11	1/1
Rooryck, et al (2010)[2]	Anotia or microtia Preauricular tags or pits Hearing loss Hemifacial microsomia Eye anomalies Vertebral anomalies	R-banding karyotype Array-CGH	47,XXX Isodicentric Y 46,XX,t(9;18)(p23;q12) Deletion 12p13.33 Duplication 18p11.23-p11.31 Duplication 20p12.2 Deletion 14q32.2 Trisomy X Duplication Yp-q11.221 Deletion Yq11.222-q12 Duplication 8q11.23 Deletion 2p11.2 Duplication 9q34.11 Duplication 4q35.1 Duplication 13.q13.1 Deletion 2q11 Amplification Xp22.33 Duplication 11q21	3/95 11/86
Rooryck, et al (2010)[25]	Microtia Preauricular tag Hemifacial microsomia Mandibular hypoplasia	R-banding karyotype FISH Array-CGH	46,XX, t(9;18)(p23;q12) 46,XX, t(9;18)(p23;q12) Duplication 18p11.23p11.31	1/1 1/1 1/1
Rosa et al (2010)[29]	Unilateral mandibular hypoplasia Preauricular skin tags Microtia Rib alterations Growth retardation	High-resolution banding FISH	Normal 22q11 was normal	3/3 3/3
Silva et al (2015)[3]	Orofacial clefts Micro/retrognathia Hemifacial microsomia Microtia/anotia Preauricular skin tags Auricular abnormalities Macrostomia	High-resolution banding karyotype FISH	47,XX, + mar mos47,XX, + mar/46,XX 46,XX,t(6;10)(q13;q24) 46,XX,inv(9)(p11q13) 22q11 and 5p were normal	4/23 23/23
Spineli-Silva et al (2018)[8]	Dysmorphic ears Preauricular tags Malar hypoplasia Bilateral cleft lip	G-banding karyotype MLPA Array-CGH	Normal Deletion in the 22q11.2 distal region Deletion in the 22q11.2 distal region	1/1 1/1 1/1

Table 2 (*Continued*)

Authors	Main clinical findings	Molecular techniques	Cytogenetic findings	Number of individuals
Tasse et al (2005)[7]	Preauricular pits/tags Hemifacial microsomia Microtia Orofacial clefts Anomalies of the eyes Epibulbar dermoids	G-banding karyotype FISH	Gonosomal mosaic 45,X and 47,XXX 22q11 was normal	1/49 20/20
Verona et al (2006)[18]	Facial hypoplasia Mandibular hypoplasia Micrognathia Epibulbar dermoid Preauricular tags	G-banding karyotype	Normal	1/1
Xu et al (2008)[23]	Cleft lip and palate Macrostomia Preauricular tags Microcephalic Facial dysmorphics	G-banding karyotype High-resolution banding karyotype FISH Array-CGH (BAC) Array-CGH (oligo)	Normal Deletion of 22q11.21–q11.23. 22q11 was normal Normal Deletion 22q11.21-q11.22	1/1 1/1 1/1 1/1 1/1
Zielinski et al (2014)[10]	Mandibular hypoplasia Facial asymmetry Preauricular skin tags Microtia Retrognathia	Array-CGH Exome	Duplication in 14q22.3 Normal	4/4 3/3

Abbreviations: Array-CGH, microarray-based comparative genomic hybridization; BAC, bacterial artificial chromosome; FISH, fluorescence in situ hybridization; MLPA, multiplex ligation-dependent probe amplification; OAVS, oculoauriculovertebral spectrum; oligo, oligonucleotide; qPCR, quantitative polymerase chain reaction.

Table 3 Estimated budget of techniques and screening power

Technique	United States (US$)	Brazil (US$)	Detectable chromosomal changes					Resolution
			inv	ins	trans	del	dup	
GTG karyotype	600.00	200.00	x	x	x	x	x	~ 2 Mb
HR karyotype	800.00	240.00	x	x	x	x	x	2–5 Mb
R-banding karyotype	N/A	N/A	x	x	x	x	x	~ 2 Mb
FISH	600.00	800.00				x	x	200 kb–5 Mb
Array-CGH	600.00	1,200.00				x	x	1 kb–1 Mb
MLPA	500.00	600.00				x	x	< 5 Mb

Abbreviations: Array-CGH, microarray-based comparative genomic hybridization; del, deletion; dup, duplication; FISH, fluorescence in situ hybridization; HR, high-resolution; ins, insertion; inv, inversion; kb, kilobases; Mb, megabases; MLPA, multiplex ligation-dependent probe amplification; N/A, not available; trans, translocation.
Note: Resolution according to the International System for Human Cytogenomic Nomenclature (2016).

FISH

FISH uses fluorescent DNA probes that bind to a specific region of interest on the chromosome[28] to detect microdeletions (deletions <5 megabases), which are difficult to detect with conventional cytogenetics methods. All articles that included FISH did so simultaneously with karyotype. None of the four studies using FISH probes for 22q11.2 region found alterations,[3,7,23,29] though one study found a deletion in the 22q11.2 region through HR karyotype.[23] A possible explanation for why HR karyotype was successful when FISH had failed to show the deletion is that FISH probe used may have been for a different region. This outcome highlights the potential pitfalls of using FISH as a primary modality to investigate a poorly understood and genetically heterogenous pathology. In one instance, FISH was used to confirm a normal GTG karyotype and showed instead a translocation between telomeric regions of chromosomes 5p and 8p,[13] however. The other articles describe FISH used to confirm GTG[14,30] and R[25]-banding and array-CGH[17] findings, including: pericentric inversion 14q11-14q24[14]; a translocation between chromosomes 21q and 5p[30]; translocation (9;18)(p23;q12)[25]; and deletion in chromosome 15q.[17]

Due to the variety of chromosomal findings in patients with suspect OAVS, it may be appropriate to begin with banding techniques. Alterations identified through karyotype would facilitate the FISH probe selection to confirm the genetic diagnosis, but in individuals with a normal karyotype, MLPA and array-CGH may be better options.

MLPA

MLPA identifies abnormal copy number variations (CNVs) in different genes and is used to detect microdeletions and microduplications. Spineli-Silva et al (2018)[8] identified a deletion in 22q11.2 involving locus control regions (LCRs) D and E and genes *HIC2*, *PPIL2*, and *TOP3B*. Colovati et al (2015)[21] found a deletion in 22q11.2 involving LCRs B and D and genes *ZNF74*, *KLHL22*, *MED15*, *SNAP29*, and *LZTR1*.

Both studies that performed MLPA used the P250-B2 kit for DGS (MRC-Holland; Amsterdam, The Netherlands). This kit is an option to screen 22q11.2 locus only. While it is possible to develop a customized panel of genes that contain regions of chromosomes that have been already associated with OAVS, no specific MLPA kit for OAVS/Goldenhar's syndrome currently exists. Industry development of a standardized kit would make MLPA a promising approach for genetic screening and as a confirmatory test following another technique.

Array-CGH

Array-CGH uses simultaneously hybridized reference DNA with target DNA arrayed on a glass slide or other solid platform, allowing a HR evaluation of whole genome CNVs and identifying unbalanced chromosomal anomalies.[31] Bacterial artificial chromosome (BAC)-based and oligonucleotide (oligo)-based arrays are the two major types of array-CGH. As oligo-based arrays have better resolution and high coverage of a single chromosome, they are considered the best option for genomic screening.[32] Alterations found in the studies that have chosen the BAC-based array were heterogeneous and located in chromosomes 22[8,21,22] and 14.[10,12] Xu et al (2008)[23] used BAC-based and oligo-based arrays; the oligo-based array yielded a deletion in 21q11.21-q.11.2 region. Neither BAC-based array nor FISH identified any abnormalities,[23] again highlighting the weaknesses of those modalities in the present context. Oligo-based array showed heterogeneous alterations in 12 of 22 patients with suspected OAVS screened in one study,[6] with 10 of 12 located on chromosome 22 and showing changes predicted to be pathologic. Overall, array-CGH shows great strengths for investigating patients with suspected OAVS, with 1 of the 15 articles describing array-CGH did not observe any molecular findings.

qPCR and WES

Real-time or qPCR and WES were used adjunctively to confirm alterations found with other techniques or exclude possible variants associated with the phenotype.[9,10] The study using WES found more than 20,000 exon variants, none of which was considered to be pathologic, but a duplication in 14q22.3 was identified using array-CGH.[10] A study using qPCR confirmed a deletion in 5q13.2 by array-CGH.[19]

Conclusion

This systematic review indicates array-CGH, MLPA, and HR karyotype are reasonable approaches for differential diagnosis of OAVS and should include in the standard work-up of such patients. To reduce the diagnostic complexity caused by phenotypic and genotypic heterogeneity of OAVS, we suggest screening patients with a standardized clinical criteria for OAVS,[6,7] followed by HR karyotype to exclude structural and numerical changes, screening for 22q11 microdeletion by MLPA, and for identification or in the absence of causative molecular changes, array-CGH. Following the step-wise approach suggested by this systematic review for diagnosis can inform clinical decision making, and better resource utilization could improve health care facility efficiency and economy. Especially in developing countries, this suggested workflow conserves scarce resources while not compromising patient care in investigating a spectrum with such heterogeneous clinical and genetic findings.

Authors' Contributions

A.G., B.D., D.D., and A.S. made substantial contributions to the conception or design of the study: the acquisition, analysis, and interpretation of data for the study; drafting the article; and revising it critically for important intellectual content. R.R. and P.Z. revised the article critically for important intellectual content, and all authors (A.G., B. D., D.D., A.S., R.R., and P.Z.) approved the version to be published and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Conflict of Interest

None declared.

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7.5.2. Candidate genes of oculo-auriculo-vertebral spectrum in 22q region: A systematic review

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Candidate genes of oculo-auriculo-vertebral spectrum in 22q region: A systematic review

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ABSTRACT

Oculo-auriculo-vertebral spectrum (hemifacial microsomia/OAVS, OMIM #164210) is a heterogenous and congenital condition caused by a morphogenesis defect of the first and second pharyngeal arches. Etiology includes unknown genetic, environmental factors and chromosomal alterations, which 22q11.2 region is the most frequently reported. Several candidate genes for OAVS have been proposed; however, none has been confirmed as causative of the phenotype. This review aims to sum up all clinical and molecular findings in 22q region of individuals diagnosed with OAVS and to investigate genes that may be involved in the development of the spectrum. A search was performed in PubMed using all entry terms to OAVS and Chromosome 22q11. After screening, 11 papers were eligible for review. Deletions and duplications in the q11.2 region were the most frequent (18/22) alterations reported and a total of 68 genes were described. Our systematic review reinforces the hypothesis that 22q11 region is a candidate locus for OAVS as well as *CLTCL1*, *GSC2*, *HIRA*, *MAPK1*, *TBX1*, and *YPEL1* as potential candidates genes for genotype–phenotype correlation. Complementary studies regarding genes interaction involved in the 22q11 region are still necessary in the search for a genotype–phenotype association, since the diagnosis of OAVS is a constant medical challenge.

KEYWORDS
22q11.2, candidate genes, hemifacial microsomia, oculo-auriculo-vertebral spectrum, systematic review

1 | INTRODUCTION

Oculo-auriculo-vertebral spectrum (OAVS) also known as Goldenhar syndrome or hemifacial microsomia (HMF/OAVS, OMIM #164210) is a congenital condition that leads to a wide variety of malformations. The cause is uncertain, but the most accepted theory is that this spectrum is caused by a morphogenesis defect of the first and second pharyngeal arches during the first 6 weeks of pregnancy (Spineli-Silva, Bispo, Gil-da-Silva-Lopes, & Vieira, 2018). This phenotype is characterized by a broad and heterogeneous spectrum of clinical features, mainly involving ears, mouth, mandible, eyes, and cervical spine (Colovati et al., 2015; Sharma & Passi, 2013). In addition, external environmental factors (vasoactive medications), maternal intrinsic factors (maternal diabetes), genetic factors (gene mutations), and chromosomal alterations may also lead to the development of this disorder (Chen, Zhao, Shen, & Dai, 2018; Hartsfield, 2007; Renkema et al., 2017).

The etiology of OAVS is still unknown, but predicted to be multifactorial, probably comprising variation in multiple genes and environmental factors. Due to the difficulty to establish an exact genetic

cause for this spectrum, the current diagnosis is based in the observation of clinical characteristics of the patient, pregnancy history, with information about twinning, placental disease, medications, drugs or retinoid acid intaken by mother, in vitro fertilization, intrauterine growth restriction, and the exclusion of differential diagnoses, which can be tested for (Chen et al., 2018; Renkema et al., 2017). Recently, descriptions of severe chromosomal rearrangements have been published in individuals with OAVS. However, its molecular basis is still unclear (Beleza-Meireles, Clayton-Smith, Saraiva, & Tassabehji, 2014). Despite the unclear etiology of OAVS, some studies have already detected loci candidates through linkage studies (Beleza-Meireles et al., 2015; Kelberman et al., 2001) and epigenetic inheritance (Fischer et al., 2006). Among chromosomal abnormalities, chromosome region 22q11.2 is the most frequently reported (Beleza-Meireles et al., 2015; Colovati et al., 2015; Derbent et al., 2003; dos Santos et al., 2014; Lafay-Cousin et al., 2009; Spineli-Silva et al., 2018; Tan et al., 2011; Torti, Braddock, Bernreuter, & Batanian, 2013; Xu, Fan, & Siu, 2008). Low copy repeats (LCRs) in 22q11.2 region have been directly implicated in its chromosomal rearrangements. Those small DNA sequences can lead to a genomic instability, mediate nonallelic homologous recombination (NAHR) or stimulate the occurrence of copy number variation that may result in the deletion or duplication of a genomic segment (Colovati et al., 2015; Stankiewicz & Lupski, 2010).

Several candidate genes for OAVS have been proposed; however, none of them has been confirmed as causative of the phenotype (dos Santos et al., 2014). This review aims to sum up all clinical and molecular findings described in 22q region of individuals diagnosed with OAVS, as well as to investigate genes that may be involved in the development of the spectrum's phenotypic characteristics.

2 | METHODS

We performed a systematic review in accordance with the Preferred Reporting Items for Systematic Reviews. The literature search was conducted by searching PubMed, including articles published from 2000 to 2020 with the terms Goldenhar syndrome, OAVS, craniofacial microsomia, HMF, OAVS, Chromosome 22, 22q11. Two independently reviewers screened titles and abstracts. First screening should include OAVS (or any synonymous names) as the main object of the study together with a Chromosome 22 alteration. Letters that were automatically full-text read due to the lack of studies were an exception. Full-text assessment should describe the alteration found on Chromosome 22 by molecular-cytogenetic analysis and also suggest a relationship between molecular findings and OAVS. Abstracts that did not provide enough information to be included or excluded were full-text read. All duplicated articles were removed and discordant selection cases were resolved by consensus. Furthermore, we excluded papers that were not available either in English or Portuguese. After studies selection, the patients' data were extracted directly from the studies and plotted in an Excel spreadsheet. The process for screening and selecting articles for inclusion is provided in Figure 1.

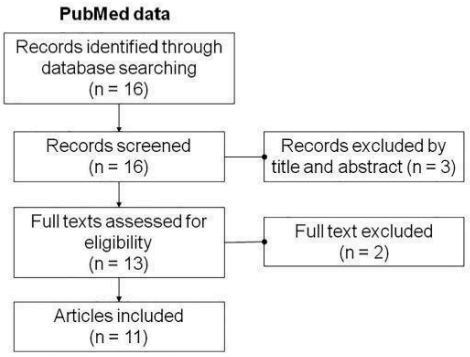

FIGURE 1 Flow diagram of article inclusion

3 | RESULTS

3.1 | Selected information

The database search resulted in 17 papers. After screening, 11 papers were eligible for extensive review. Year of publication ranged from 2003 to 2018 and the studies categories included original article (*n* = 2), case reports (*n* = 5), short report (*n* = 1), and letter (*n* = 3).

3.2 | Clinical characterization of 22 OAVS individuals

Overall, 22 individuals ranging in age from 35 days to 18 years (9 females and 13 males), presenting clinical findings compatible with OAVS and Chromosome 22 abnormalities were described. Four patients showed prenatal risk factors such as gestational diabetes, use of abortive substances and infections. Among the malformations described, ear alterations (preauricular tags, hearing loss and agenesis and atresia of external auditory canal), HMF and congenital heart disease (CHD) were the main clinical features that lead to OAVS investigation (Table 1).

3.3 | Molecular findings

Deletions and duplications in the q11.2 region were the most frequent (18/22) among all molecular alterations involving Chromosome 22 (Table 2). Then, 45 protein-coding genes, 16 pseudogenes, and 7 RNA or noncoding RNA genes were described within this region. Most frequent genes involved in the deletion were *HIC2* (OMIM *607712), *PPIL2* (OMIM *607588), *TOP3B* (OMIM *603582) (Spineli-Silva et al., 2018;Torti et al., 2013; Xu et al., 2008), *YPEL1* (OMIM *608082), *MAPK1* (OMIM *176948) (Tan et al., 2011; Torti et al., 2013; Xu et al., 2008), *UBE2L3* (OMIM *603721), *PRAME*

TABLE 1 Main clinical features of our sample

Main clinical features	Frequency (%)	Reference
HMF	72.7 (16/22)	Beleza-Meireles et al. (2015); Colovati et al. (2015); Digilio et al. (2009); dos Santos et al. (2014); Lafay-Cousin et al. (2009); Tan et al. (2011).
Microtia	36.3% (8/22)	Beleza-Meireles et al. (2015); Colovati et al. (2015); Derbent et al. (2003); Digilio et al. (2009).
Cleft lip, cleft palate, and facial cleft	27.2% (6/22)	Colovati et al. (2015); Digilio et al. (2009); Spineli-Silva et al. (2018); Torti et al. (2013); Xu et al. (2008).
Macrostomia	4.5% (1/22)	Xu et al. (2008).
Malar and mandibular hypoplasia/ asymmetria	45.4% (10/22)	Beleza-Meireles et al. (2015); Derbent et al. (2003); Digilio et al. (2009); Spineli-Silva et al. (2018); Torti et al. (2013).
Preauricular tags	54.5% (12/22)	Beleza-Meireles et al. (2015); Digilio et al. (2009); Lafay-Cousin et al. (2009); Spineli-Silva et al. (2018); Tan et al., 2011; Xu et al. (2008).
Agenesis and atresia of external auditory canal	50% (11/22)	Beleza-Meireles et al. (2015); Colovati et al. (2015); Derbent et al. (2003); Digilio et al. (2009); Torti et al. (2013); Xu et al. (2008).
Epibulbar dermoid	18.8% (4/22)	Beleza-Meireles et al. (2015); Tan et al. (2011); Lafay-Cousin et al. (2009).
Hearing loss	50% (11/22)	Beleza-Meireles et al. (2015); Colovati et al. (2015); Digilio et al. (2009); Tan et al., 2011; Torti et al. (2013).
Vertebral dysmorphia	31.8% (7/22)	Beleza-Meireles et al. (2015); Colovati et al. (2015); Derbent et al. (2003); Digilio et al. (2009); Torti et al. (2013).
Congenital heart disease	60% (13/22)	Beleza-Meireles et al. (2015); Derbent et al. (2003); Digilio et al. (2009); Spineli-Silva et al. (2018); Xu et al. (2008).
Developmental delay	22.7% (5/22)	Balcı and Engiz (2011); Beleza-Meireles et al. (2015); dos Santos et al. (2014); Lafay-Cousin et al. (2009); Spineli-Silva et al. (2018).

Abbreviation: HMF, hemifacial microsomia.

(OMIM *606021) (Torti et al., 2013; Xu et al., 2008), histone cell cycle regulator (*HIRA*; OMIM *600237) (Derbent et al., 2003), and *CLTCL1* (OMIM *601273) (Digilio et al., 2009).

Duplications in the 22q11.2 region were identified in 32% of the patients (07/22), with *GGT2* (OMIM *137181) as the most described gene (Beleza-Meireles et al., 2015). Duplication and deletion of 22q were evidenced concomitantly in an individual, although in distinct regions (q11.1 and q11.2, respectively), involving the following genes: *IL17RA* (OMIM *605461), *CECR1* (OMIM *607575), *CECR2* (OMIM *607576), *SLC25A18* (OMIM *609303), *ATP6V1E1* (OMIM *108746), *IDB*, *MICAL3* (*OMIM *608882*), *PEX26* (OMIM *608666), *TUBA8* (OMIM *605742), *USP18* (OMIM *607057), *HIC2*, *RIMBP3B* (OMIM *612700), *RIMBP3C* (OMIM *612701), *UBE2L3*, *SDF2L1* (OMIM *607551), *PPIL2*, *YPEL1*, *MAPK1*, *TOP3B*, *VPREB1* (OMIM *605141), *PRAME*, *GGTLC2* (OMIM *612339), and *RTDR1* (OMIM *605663) (Torti et al., 2013). A duplication in 22q13.3 region (*FAM19A5*, OMIM *617499) was described in a patient who also had a duplication in 22q11.2 region (*USP18*, *DGCR6*, *GGT2*) (Beleza-Meireles et al., 2015). A third finding was a derivative chromosome (47,XX + der 22t(11;22)(q23;q11)); however, involved genes were not informed (Balcı & Engiz, 2011).

Additionally, there were genes involved in both deletions and duplications in 22q11, as *RIMBP3* (OMIM *612699) (Beleza-Meireles et al., 2015), as well as its paralogous *RIMBP3B* and *RIMBP3C* (Torti et al., 2013), and *TBX1* (OMIM *602054) (Beleza-Meireles et al., 2015; dos Santos et al., 2014).

4 | DISCUSSION

OAVS is probably a group of heterogeneous disorders with the genetic etiology is still unknown.

Chromosomal alterations have been reported in several cases and regions located at 22q were the main findings described in OAVS individuals. In our review, we observed that deletions and duplications in regions 22q11.1 (Beleza-Meireles et al., 2015) and 22q11.2 (Beleza-Meireles et al., 2015; Bragagnolo et al., 2018; Colovati et al., 2015; dos Santos et al., 2014; Spineli-Silva et al., 2018; Xu et al., 2008) were the main findings in individuals with the phenotype. However, these regions comprise a lot of genes with unknown function as many pseudogenes.

The 22q11 region has eight known LCR that contain genes, pseudogenes, and other genomic sequences that are 94–99% identical both individually (within each LCR22) and between them (McDermid & Morrow, 2002). The similarity in their sequences allows the occurrence of NAHRs, a rearrangement mechanism that explains clustered breakpoints and recurrent rearrangements, such as de novo alterations (Ben-Shachar et al., 2008; Shaikh et al., 2000). Thus, the frequent description of deleterious mutations in the 22q11 region of individuals diagnosed with OAVS becomes understandable. However, the relationship between genes within this region and clinical findings remains unclear.

All cases included in this review shared some clinical features. However, phenotypic variability hinders to establish a standard

TABLE 2 Molecular findings and genes involved

Reference	Technique	Molecular findings	Genes involved
P3 Beleza-Meireles et al. (2015)	Array-CGH	**Dup 22q11.2**	*RIMBP3*
P7 Beleza-Meireles et al. (2015)	Array-CGH	**Dup 22q11.2**	***USP18***, ***GGT3P***, and ***DGCR6***
		Dup 22q11.2	*POM121L7*, ***GGT2***, ***BCRP2***, *KB-1592A4.15*, *KB-2A4.13*, and *FAM230B*
		Dup 22q13.3	*MIR4535* and *FAM19A5*
P10 Beleza-Meireles et al. (2015)	Array-CGH	**Dup 22q11.2**	*AK129567*, *AK302545*, and ***GGT3P***
		Dup 22q11.2	*POM121L7*, ***GGT2***, ***BCRP2***, *KB-1592A4.15*, *KB-2A4.13*, and *FAM230B*
P11 Beleza-Meireles et al. (2015)	Array-CGH	**Dup 22q11.2**	***USP18***, *AK129567*, *AK302545*, and ***GGT3P***
		Dup 22q11.2	*POM121L7*, ***GGT2***, ***BCRP2***, *KB-1592A4.15*, *KB-2A4.13*, and *FAM230B*
P14 Beleza-Meireles et al. (2015)	Array-CGH	**Dup 22q11.2**	*POM121L7*, ***GGT2***, ***BCRP2***, *KB-1592A4.15*, *KB-2A4.13*, and *FAM230B*
P15 Beleza-Meireles et al. (2015)	Array-CGH	**Dup 22q11.1**	*CCT8L2*, *FABP5P11*, ***TPTEP1***, *SLC25A15P5*, ***PARP4P3***, *ANKRD62P1-PARP4P3*, *ANKRD62P1*, ***VWFP1***, and ***XKR3***
P16 Beleza-Meireles et al. (2015)	Array-CGH	**Dup 22q11.2**	*POM121L7*, ***GGT2***, ***BCRP2***, *KB-1592A4.15*, *KB-2A4.13*, *FAM230B*, *KB-1592A4.14*, *KB-1183D5.9*, *POM121L8P*, ***BCRP6***
		Dup 22q11.1	*CCT8L2*, *FABP5P11*, ***TPTEP1***, *SLC25A15P5*, ***PARP4P3***, *ANKRD62P1-PARP4P3*, *ANKRD62P1*, ***VWFP1***, ***XKR3***
P17 Beleza-Meireles et al. (2015)	Array-CGH	**Dup 22q11.1**	*CCT8L2*, ***XKR3***, *FABP5P11*, ***TPTEP1***, *SLC25A15P5*, ***PARP4P3***, *ANKRD62P1-PARP4P3*, *ANKRD62P1*, ***VWFP1***
P18 Beleza-Meireles et al. (2015)	Array-CGH	**Dup 22q11.21**	*POM121L7*, ***GGT2***, ***BCRP2***, *KB-1592A4.15*, *KB-2A4.13*, *FAM230B*, *KB-1592A4.14*, *KB-1183D5.9*, *POM121L8P*, ***BCRP6***
P51 Beleza-Meireles et al. (2015)	Array-CGH	**Dup 22q11.1**	***TBX1***+
P1 Colovati et al. (2015)	MLPA and array-CGH	**Del 22q11.2**	*ZNF74*, *KLHL22*, *MED15*, *SNAP29*, and ***LZTR1***
P7 Derbent et al. (2003)	FISH	**Del 22q11.2**	***HIRA***
P1 Digilio et al. (2009)	FISH	**Del 22q11.2**	*CLTCL1*
P2 Digilio et al. (2009)	FISH	**Del 22q11.2**	*CLTCL1*
P3 Digilio et al. (2009)	FISH	**Del 22q11.2**	*CLTCL1*
P1 dos Santos et al. (2014)	Array-CGH	**Del 22q11.2**	***GSC2***, ***TBX1***, ***SETP5***
P1 Lafay-Cousin et al. (2009)	FISH, MLPA, and array-CGH	**Del 22q11.2**	***LZTR1***, *SNRPD3*, *HIC2*, *TOP3B*, *MAPK1*, *YPEL1*, *PPIL2*, *GGT2*, *UBE2L3*, *PRAME*
P1 Spineli-Silva et al. (2018)	MLPA and array-CGH	**Del 22q11.2**	***HCI2***, ***PPIL2***, and ***TOP3B***
P2 Tan et al. (2011)	Array-CGH	**Distal Del 22q11.2**	*HIC2*, *TOP3B*, *MAPK1*, *YPEL1*, *PPIL2*, *GGT2*, *UBE2L3*, *PRAME*
P1 Torti et al. (2013)	Array-CGH	**Dup 22q11.1**	*IL17RA*, *CECR1*, *CECR2*, *SLC25A18*, *ATP6V1E1*, *BID*, *MICAL3*, *PEX26*, *TUBA8*, and ***USP18***
		Del 22q11.2	***HIC2***, *RIMBP3B*, *RIMBP3C*, ***UBE2L3***, *SDF2L1*, *MIR130B*, ***PPIL2***, ***YPEL1***, ***MAPK1***, ***TOP3B***, *VPREB1*, ***PRAME***, *GGTLC2*, and *RTDR1*
P1 Xu et al. (2008)	Array-CGH	**Del 22q11.2**	***HCI2***, *LOC220686*, ***UBE2L3***, *LOC150223*, *CCDC116 SDF2L1*, ***PPIL2***, ***YPEL1***, ***MAPK1***, *PPM1F*, ***TOP3B***, *VPREB1 LOC96610*, *SUHW2*, *SUHW1*, ***PRAME***

Note: The values marked in bold are qualitative data.
Abbreviation: FISH, fluorescence in situ hybridization.

diagnosis. Based on both this review and literature, we suggest that the minimum diagnostic criteria for OAVS in individuals with 22q11 abnormalities should include HMF and auricular alterations. Auricular alterations (preauricular tags, hearing loss, and/or agenesis/atresia of external auditory canal) were described in all individuals included in this review.

CHDs are also a frequent feature described in OAVS. In literature, the percentage of individuals with 22q11.2 deletion that have CHD is 75–80% (Azuma et al., 2015). In this review, CHD was found in 60% of the individuals diagnosed with OAVS that had a deletion and/or duplication in the 22q11 region. Therefore, the presence of any congenital heart malformation could also aid in OAVS investigation.

A total of 68 genes, pseudogenes and RNA genes were listed within all studies. A few genes were altered in individuals who share more than one malformation: HMF, auricular alterations and/or CHD. In duplicate regions, the most evidenced protein-coding genes were *GGT2* (dup22q11.2), *XKR3* (dup22q11.1), and *CCT8L2* (dup22q11.1). However, there are no descriptions in the literature about the association of these genes with HMF or OAVS phenotype.

Deleted regions (del22q11.2) in individuals diagnosed with OAVS had *MAPK1*, *YPEL1*, *HIC2*, *TOP3B*, *PRAME*, *UBE2L3*, *PPIL2*, *HIRA*, and *CLTCL1* as the most reported protein-coding genes. Tan et al. (2011) and Lafay-Cousin et al. (2009) identified two patients with deletions in the same 22q11.2 region involving LCR22-4 to LCR22-7, but they did not describe the genes involved. However, in order to have the information about which genes were comprised in the LCRs, we accessed UCSC Genome Browser assembly ID: hg38 (http://genome.ucsc.edu/). Then, we were able to list the main deleted genes common to both patients: *GGT2*, *MAPK1*, *YPEL1*, *HIC2*, *TOP3B*, *PRAME*, *UBE2L3*, and *PPIL2*.

MAPK1 and *YPEL1* play regulatory functions in cellular processes such as cell division, proliferation, differentiation, and development. Studies with animal model showed that both genes have been found to be possible candidates for the genotype–phenotype relationship with OAVS. *MAPK1* was considered to cause craniofacial and cardiac defects when inactivated at the developing neural crest, while *YPEL1* inactivation resulted in craniofacial cartilage defects and mandibular underdevelopment (Aerts et al., 2006; Newbern et al., 2008). Individuals with a 22q11.2 microdeletion of approximately 1 Mb that also presents a *MAPK1* haploinsufficiency may feature conotruncal and craniofacial anomalies due to neural crest misdevelopment. The clinical characteristics observed in this type of genetic alteration are often similar to the ones described in DiGeorge syndrome spectrum, which is often related to OAVS (Derbent et al., 2003; Digilio et al., 2009). In addition, maternal allele variants of *YPEL1*, when with incomplete penetration trait associated with genetic and/or environmental factors, could lead to OAVS phenotype. Clinical features have not been associated with neither *MAPK1* nor *YPEL1* (Zamariolli et al., 2019). Within the cases included in our review, both genes were always simultaneously deleted when cited (dos Santos et al., 2014; Spineli-Silva et al., 2018; Xu et al., 2008). However, no correlation and/or association between the two genes were found. Presence of craniofacial, auricular, and cardiac malformations in individuals with deletion in *MAPK1* and *YPEL1* may suggest an important role and strong involvement of these genes in the OAVS phenotype.

HIC2 is a transcription factor related to *HIC1* tumor suppressors, which are required for the normal cardiac development (Deltour, Pinte, Guérardel, & Leprince, 2001). The consequences of *HIC2* deletion in individuals with OAVS are still nuclear but this gene could be responsible for specific cardiac malformations when simultaneously deleted with other genes.

TOP3B has already been associated with cognitive impairment and facial dysmorphism in a patient with a minor 22q11.2 deletion (Kaufman, Genovese, & Butler, 2016). The role of *TOP3B* is often described in patients with neurological developmental delay (O'Roak et al., 2012; Stoll et al., 2013). In our review, 40% of the patients with deletion in *TOP3B* presented some developmental delay (Lafay-Cousin et al., 2009; Spineli-Silva et al., 2018). Therefore, *TOP3B* may be a possible gene candidate for OAVS phenotype, especially in cases of cognitive impairment.

PRAME, *UBE2L3*, and *PPIL2* were also found deleted in a 22q11.2 region near well-known functional genes (Lafay-Cousin et al., 2009; Tan et al., 2011; Torti et al., 2013; Xu et al., 2008). Although these genes may be acting through genetic interactions, their function in embryogenesis is still unknown. Therefore, there is no evidence that alterations in *PRAME*, *UBE2L3*, and *PPIL2* are associated with the etiology of OAVS.

Derbent et al. (2003) and Digilio et al. (2009) used TUPLE 1 and N25 probes to identify, respectively, *HIRA* and *CLTCL1* deletions through fluorescence in situ hybridization. *HIRA*, also known as *DGCR1* or *TUPLE1*, is considered the main gene for normal embryonic development and the gold standard marker for DiGeorge Syndrome diagnosis. *HIRA* probably mediates irreversible alterations in the senescent cell cellular cycle (Halford et al., 1993). In animals (mice and chickens), *HIRA* is detected at the neural crest, pharyngeal arches and heart (Gunjan, Paik, & Verreault, 2005).

CLTCL1 is a member of the family of heavy chains of clathrins and plays an essential role in the neural crest development, which is an important component for the morphogenesis of pharyngeal arches (Nahorski et al., 2015). Chromosomal alterations involving *CLTCL1* are also associated with DiGeorge syndrome, velo-cardio-facial syndrome (Long, Trofatter, Ramesh, McCormick, & Buckler, 1996), Down syndrome and cardiac malformations, mainly in typical 3 Mb deletion of LCR22A-LCR22D, a region extensively studied in DiGeorge syndrome (Hou et al., 2020).

Digilio et al. (2009) reported a patient with a 22q11.2 microdeletion in the region of DiGeorge syndrome. This deletion may lead to a phenotype similar to OAVS, which indicates a possible regulatory mechanism in the etiology of the spectrum. In addition, genes mapped in the region 22q11.2 involved in the development of neural crest cells and branchial arches would also be affected. dos Santos et al. (2014) hypothesized an altered genetic nuclear mechanism in this microdeletion carrier. Since nonoverlapping 22q11.2 deletions cause similar phenotypes, a possible regulatory mechanism acting on genes located in the 22q11.2 region and on neural crest cell development was proposed. All individuals that were tested for deletions in

CLTCL1 and *HIRA* had craniofacial malformations, auricular alterations, and CHD. Deletions of *CLTCL1* and *HIRA*, given their important roles in neurological and cardiac development, may be associated with the main clinical characteristics of individuals diagnosed with OAVS. Thus, both genes are strong candidates for genotype–phenotype association in OAVS.

TBX1 was duplicated in one individual and deleted in another (Beleza-Meireles et al., 2015; dos Santos et al., 2014). *TBX1* deficiency in mice caused distinct vascular and cardiac malformations and severe inner ear defects (Vitelli et al., 2003; Vitelli, Morishima, Taddei, Lindsay, & Baldini, 2002). The deletion found in the patient described by dos Santos et al. (2014) also involved *GSC2*. This gene is associated with velocardiofacial syndrome and is homologous to *GSC* (14q32), a gene that plays an important role in branchial arches development. A linkage study proposed that *GSC* could be a candidate gene to explain OAVS phenotype (Kelberman et al., 2001), however a deletion involving *GSC2* was described only in one patient in this review. *TBX1* and *GSC2* are considered candidate genes for the OAVS phenotype due to associations described in the literature.

Asymmetry is a clinical finding commonly described in individuals diagnosed with OAVS. Its severity is variable and affects mainly eyes, ears, and face. The etiology of this phenotype may be associated with genetic and/or environmental factors. Genetics mechanisms that may influence the asymmetric nature of OAVS comprise regulatory and nonregulatory variants as well as topologically associating domain disruption. Candidate genes suggested in this systematic review (*CLTCL1*, *GSC2*, *HIRA*, *MAPK1*, and *TBX1*) are involved in different pathways that could have an important role in the asymmetric nature of OAVS etiology (Figure 2). *YPEL1* still does not have described pathways with evident involvement in OAVS asymmetry. Although some

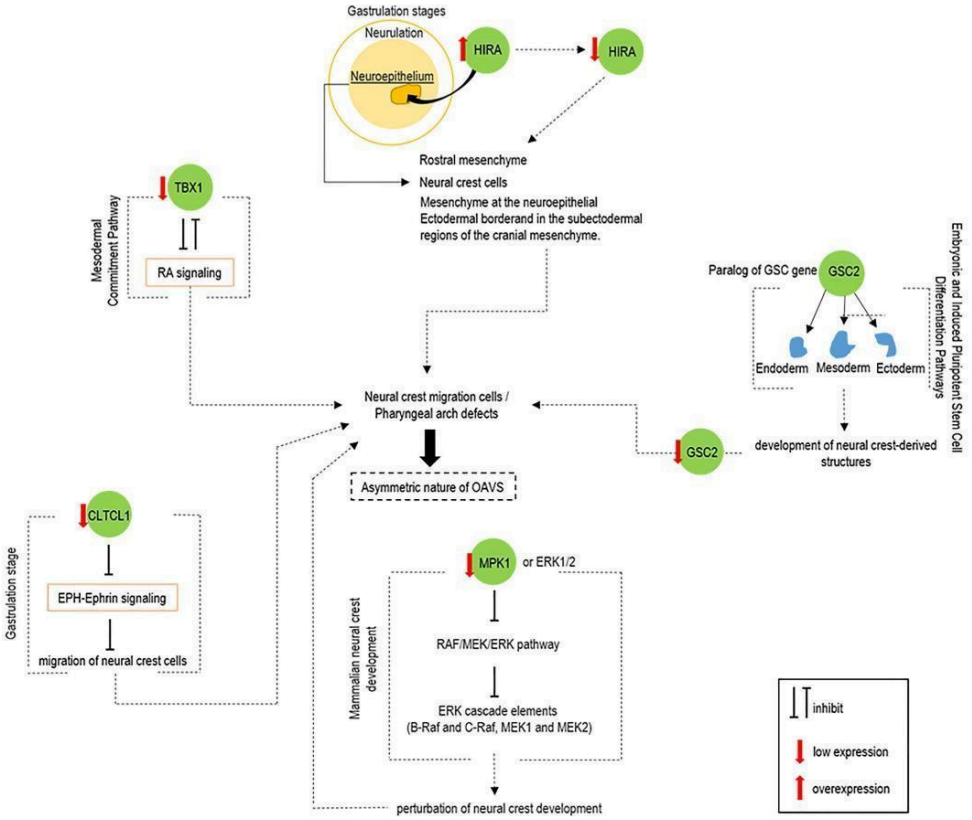

FIGURE 2 Possible pathways involved and mechanisms for oculo-auriculo-vertebral spectrum (OAVS) asymmetry [Color figure can be viewed at wileyonlinelibrary.com]

cause-consequence relationships have been hypothesized, more studies on this matter are necessary to elucidate the proper mechanism and its consequences.

Other genes as *ZNF74*, *KLHL22*, *MED15*, *SNAP29*, *LZTR1*, *RIMBP3*, *RIMBP3B*, *RIMBP3C*, *USP18*, *FAM19A5*, *IL17RA*, *CECR1*, *CECR2*, *SLC25A18*, *ATP6V1E1*, *BID*, *MICAL3*, *SDEX*, *TEXP*, *RTDR1*, *SNRPD3*, *LOC220686*, *LOC150223*, *CCDC116*, *PPM1F*, *SUHW1*, *SUHW2*, *DGCR6*, and *DGCR8* were also described in deleted/duplicated individuals diagnosed with OAVS, but in a lowest frequency. Future research is necessary to elucidate their functionality as well as their genetic interactions. Since a lot of aspects about the genetic background of OAVS is still unknown, these genes should not be ignored while performing a complete investigation of OAVS. It is possible that other genes may still be potential candidates to explain the genotype–phenotype relationship of OAVS as this study focuses only on the ones described in the studies included. For future studies, a genome-wide approach or whole genome sequencing may be important to identify other genes involved in OAVS etiology.

In conclusion, our systematic review reinforces the hypothesis that the 22q11 genomic region is a candidate locus for OAVS as well as *CLTCL1*, *GSC2*, *HIRA*, *MAPK1*, *TBX1*, and *YPEL1* as potential candidates genes for genotype–phenotype correlation. In addition, the authors suggest the possibility of investigating the 22q11 region in patients with the OAVS phenotype. Additional and complementary studies regarding genes interaction involved in the 22q11 region are still necessary in the search for a genotype–phenotype association, since the diagnosis of OAVS is a constant medical challenge.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Andressa Barreto Glaeser, Andressa Schneiders Santos, Bruna Lixinski Diniz, and Desireé Deconte made substantial contributions to the conception or design of the study; the acquisition, analysis, and interpretation of data for the study; drafting the article; and revising it critically for important intellectual content. Rafael Fabiano Machado Rosa and Paulo Ricardo Gazzola Zen revised the article critically for important intellectual content, and all authors approved the version to be published and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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7.6. Revisão de Literatura publicada em Revista Internacional

7.6.1. Fluorescence in situ hybridization (FISH) as an irreplaceable diagnostic tool for Williams-Beuren syndrome in developing countries: a literature review

REVIEW ARTICLE

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Fluorescence in situ hybridization (FISH) as an irreplaceable diagnostic tool for Williams-Beuren syndrome in developing countries: a literature review

Hibridização *in situ* fluorescente (FISH) como ferramenta diagnóstica insubstituível para a síndrome de Williams-Beuren em países em desenvolvimento: uma revisão de literatura

Bianca Soares Carlotto[a], Desirée Deconte[a], Bruna Lixinski Diniz[a], Priscila Ramires da Silva[a], Paulo Ricardo Gazzola Zen[a,b], André Anjos da Silva[c,*]

ABSTRACT

Objective: The aim of this study was to sum up and characterize all Williams-Beuren syndrome cases diagnosed by fluorescence in situ hybridization (FISH) since its implementation, as well as to discuss FISH as a cost-effective methodology in developing countries.

Data source: From January 1986 to January 2022, articles were selected using the databases in PubMed (Medline) and SciELO. The following terms were used: Williams syndrome and In Situ Hybridization, Fluorescence. Inclusion criteria included Williams-Beuren syndrome cases diagnosed by FISH with a stratified phenotype of each patient. Only studies written in English, Spanish, and Portuguese were included. Studies with overlapping syndromes or genetic conditions were excluded.

Data synthesis: After screening, 64 articles were included. A total of 205 individuals with Williams-Beuren syndrome diagnosed by FISH were included and further analyzed. Cardiovascular malformations were the most frequent finding (85.4%). Supravalvular aortic stenosis (62.4%) and pulmonary stenosis (30.7%) were the main cardiac alterations described.

Conclusions: Our literature review reinforces that cardiac features may be the key to early diagnosis in Williams-Beuren syndrome patients. In addition, FISH may be the best diagnostic tool for developing nations that have limited access to new technologic resources.

Keywords: Williams syndrome; Williams-Beuren syndrome; Fluorescence in situ hybridization; Literature review.

RESUMO

Objetivo: Caracterizar todos os casos de síndrome de Williams-Beuren (SWB) diagnosticados por hibridização *in situ* fluorescente (FISH) desde sua implementação, assim como discutir a relação custo-benefício da metodologia de FISH em países em desenvolvimento.

Fontes de dados: Entre janeiro de 1986 e janeiro de 2022 foi realizada uma busca nas bases de dados PubMed (Medical Literature Analysis and Retrieval System Online — Medline) e Scientific Electronic Library Online (SciELO) usando os seguintes termos: síndrome de Williams e hibridização *in situ* fluorescente. O critério de inclusão utilizado foi conter a descrição detalhada de caso(s) de SWB por FISH. Apenas estudos escritos em inglês, espanhol e português foram incluídos. Trabalhos que apresentavam sobreposição de síndromes/condições genéticas foram excluídos.

Síntese dos dados: Após os processos de inclusão, 64 artigos e 205 indivíduos com SWB diagnosticados por meio do método de FISH foram incluídos. O achado mais frequente entre os indivíduos foi a presença de algum tipo de malformação cardíaca (85,4%). A estenose aórtica supravalvar (62,4%) e a estenose pulmonar (30,7%) foram as alterações cardíacas mais descritas. A maioria dos estudos era proveniente dos continentes Europa, Ásia e América do Norte.

Conclusões: A presente revisão de literatura reitera que as malformações cardíacas podem ser a chave para o diagnóstico precoce em pacientes com SWB. Ainda, a técnica de FISH parece ser a melhor ferramenta de diagnóstico para os países em desenvolvimento, cujo acesso às novas tecnologias ainda é escasso.

Palavras-chave: Síndrome de Williams; Síndrome de Williams-Beuren; Hibridização in situ fluorescente; Revisão de literatura.

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INTRODUCTION

Williams-Beuren syndrome (WBS) (OMIM #194050) is a rare developmental disorder with an autosomal dominant trait and numerous clinical findings. WBS is considered a contiguous gene syndrome caused by a microdeletion on chromosome 7q11.23 that ranges in size from 1.5 to 1.8 Mb and encompasses approximately 28 genes.[1,2] The prevalence of the syndrome is estimated to be 1 per 7,500 live births.[3] Distinctive facial features (long philtrum, epicanthal folds, broad forehead, bitemporal narrowness, periorbital fullness, stellate and/or lacy iris pattern, short nose with a bulbous nasal tip, wide mouth, full lips, and mild micrognathia), developmental and intellectual delay, an overly sociable personality, cardiovascular diseases, and idiopathic hypercalcemia comprise the overall WBS phenotype.[4,5]

Fluorescence in situ hybridization (FISH) analysis is considered the gold standard test for precise molecular diagnosis of microdeletion syndromes, such as WBS. Although new technologies such as array-CGH and multiplex ligation-dependent probe amplification (MLPA) outdated FISH, mostly in diagnosing atypical deletion cases, FISH is still a valuable and cost-effective tool to confirm WBS clinical suspicion.[2,6,7]

The area of rare diseases faces a lot of major obstacles with regard to gaining a deep understanding of each syndrome in order to improve patient care. In developing countries, some of these hurdles include difficulty in obtaining a timely and precise diagnosis, shortage of specialized healthcare workers, lack of research, and resource constraint.[8] In Brazil, for example, the Universal Health Service, a large and well-established public healthcare system, does not offer molecular and genetic tests on a daily basis, which directly affects the diagnosis and management of patients with genetic rare diseases.[9] Therefore, an ultimate molecular diagnosis of patients with rare genetic diseases is extremely important. A proper and precise diagnosis aids in the access of proper resources, avoids additional molecular investigation, decreases prognostic uncertainty, allows genetic counseling, and provides psychosocial benefits to both the patient and the family.[10]

The aim of this literature review was to sum up and characterize all WBS cases diagnosed by FISH since its implementation as well as to discuss FISH as a cost-effective methodology in developing countries.

METHOD

This literature review was designed in accordance with the Preferred Reporting Items for Systematic Review and Meta-Analyses guidelines.[11] The literature search was conducted using PubMed (Medline) and SciELO. Mesh and DECS descriptors were used to index articles with the following terms: Williams syndrome and In Situ Hybridization, Fluorescence. The exact search terms for PubMed/MESH terms were "Syndrome, Williams OR Contiguous Gene Syndrome, Williams OR Supravalvar Aortic Stenosis Syndrome OR Williams-Beuren Syndrome OR Syndrome, Williams-Beuren OR Williams Beuren Syndrome OR Beuren Syndrome OR Syndrome, Beuren OR Hypercalcemia-Supravalvar Aortic Stenosis OR Aortic Stenoses, Hypercalcemia-Supravalvar OR Aortic Stenosis, Hypercalcemia-Supravalvar OR Hypercalcemia Supravalvar Aortic Stenosis OR Hypercalcemia-Supravalvar Aortic Stenoses OR Stenoses, Hypercalcemia-Supravalvar Aortic OR Stenosis, Hypercalcemia-Supravalvar Aortic OR Chromosome 7q11.23 Deletion Syndrome OR Williams Contiguous Gene Syndrome) AND (FISH OR Hybridization in Situ, Fluorescent OR FISH Technique OR FISH Techniques OR Technique, FISH OR Techniques, FISH OR Fluorescent in Situ Hybridization OR FISH Technic OR FISH Technics OR Technic, FISH OR Technics, FISH OR Hybridization in Situ, Fluorescence OR In Situ Hybridization, Fluorescent," and for SciELO/DECS, terms were "Williams Syndrome" AND "In Situ Hybridization, Fluorescence."

Included articles were selected in a two-step analysis: title and abstract screening, followed by a full-text read. Authors were categorized into two pairs for independent screening and further discussion of potential disagreements (A.S./B.C. and D.D/P.S.). If the disagreement remained, a "senior reviewer" (B.D.) decided if the study would be included or excluded. Inclusion criteria for the first step were as follows: have a case or cases of WBS as well as any indication of FISH performance. In the second step, the inclusion criteria included WBS cases diagnosed by FISH with a stratified phenotype for each patient. Only studies from January 1986 to January 2022 written in English, Spanish, and Portuguese were included. Studies with overlapping syndromes or genetic conditions were excluded.

Publication metadata were extracted using a data extraction template that was created and modified according to all the studies reviewed. The publication details were captured and summarized in a tabular format developed by the authors of this review. The data extracted from all articles were as follows: article ID, total of cases, case stratification, auditory, behavioral, calcium, cardiovascular, cognitive, connective tissue, dental, endocrine, facial features, gastrointestinal, genitourinary, growth, hematology, integument, musculoskeletal, neurologic, ocular and visual, respiratory, tumor, "typical face," sample type, gender, age at diagnosis with FISH, diagnosis with other molecular techniques beyond FISH, FISH probes, and authors' countries.

RESULTS

After screening, 64 articles were included. A flow diagram of the literature search is depicted in Figure 1. A total of 205 individuals diagnosed with WBS by FISH were included and further analyzed. Demographic analysis showed that 48.5% (66/136) were female and 51.5% (70/136) male. Age at diagnosis ranged from 3 weeks to 37.75 years, with an average of 9.4 years and a median of 6.4 years. Clinical features were evaluated using the Guidance for Clinician in Rendering Pediatric Care.[5] Cardiovascular malformations were the most frequent finding (85.4%), followed by neurological alterations (59.1%), cognitive delay (49.8%), facial dysmorphisms (48.3%), and behavioral changes (46.3%). Clinical findings are described in Table 1. A significant percentage of the patients (40.5%) did not have a clear description of their facial dysmorphisms, terms such as "typical face" or "elfin face" were used instead.

Fluorescence in situ hybridization

Among the included studies, 53.1% did not report the probe(s) used for WBS diagnosis. The described probes are shown in Table 2.[12,13] FISH analysis with more than one probe was performed in seven articles.[14-20] In these studies, a variation in the deletion length was observed among patients since

Table 1. Patients' clinical findings by systems.

Main clinical features	Frequency (%)
Cardiovascular	85.4
Neurologic	59.1
Cognitive	49.8
Facial features	48.3
Behavioral	46.3
Growth	35.6
Connective tissue	29.8
Gastrointestinal	26.3
Integument	24.9
Dental	23.9
Auditory	20.0
Ocular and visual	18.5
Calcium	18.1
Musculoskeletal	13.7
Genitourinary	13.2
Respiratory	7.8
Endocrine	2.9
Hematology	2.9
Tumor	2.9

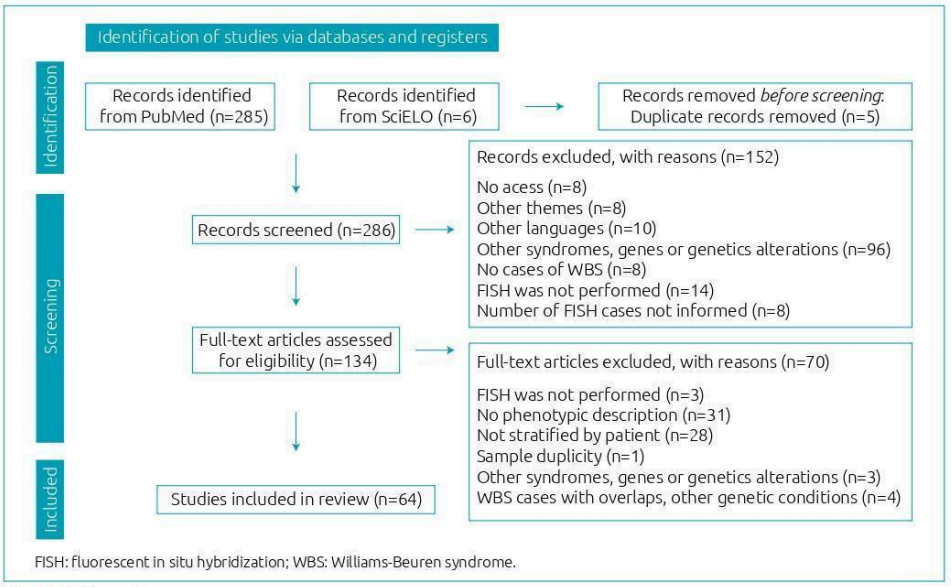


Figure 1. Flow diagram.

different probe sets were used. Other molecular and cytogenetics technologies were used in order to aid in the diagnosis of WBS. Microarray (9.3%) and microsatellite (6.3%) analysis were the most frequent techniques performed alongside FISH. MLPA, PCR, QMPSF, qPCR, and Southern analysis were also performed, and each of them comprised 4.4% of the included studies.

Where, in theworld, are fluorescence in situ hybridization and Williams-Beuren syndrome studies from?

An overview of the authors' locations is described by continents in Table 3. Europe, Asia, and North America comprised the highest percentage of studies included in this review. On the contrary, Latin America and Africa had the lowest numbers, with 4.7 and 3.1% of the studies, respectively. Among the countries, the United States (14.1%), Italy (12.5%), Japan (9.4%), and the United Kingdom (7.8%) were highlighted.

DISCUSSION

Initially, WBS diagnoses were made purely based on the clinical features observed in the patients. The observed phenotype should meet the descriptions provided by Williams et al.[21] and Beuren et al.[22] WBS was primarily described as a syndrome

Table 3. Overview of the authors' locations by continents.

Continents	Frequency (%)
Africa/Europe	3.1
Asia	26.6
Asia/North America	1.6
Eurasia	3.1
Europe	37.5
Europe/Eurasia	1.6
Europe/North America	6.3
Latin America	4.7
North America	15.6

Table 2. FISH probes, BACs, cosmids, PACs, and YACs described and used by all included articles.

FISH probes	Genes	Diagnosed cases (n)
Commercial FISH probes		
WSCR probe (ONCOR, Gaithersburg, MD)	*ELN*	92
Q Biogene, currently MP Biomedicals, probe number CP5155-DC		1
Vysis LSI ELN Kit (Vysis; Abbott Laboratories, Abbott Park, IL)	*ELN+LIMK1*	29
MD Williams-Beuren Kreatech probe	*ELN+LIMK1+CYLN2*	1
Cytocell Williams-Beuren region probe	*LIMK1+EIF4H+RFC2+CYLN2+GTF2IRD1+TBL2+BAZ1B*	3
BACs/Cosmids/PACs/YACs		
cELN-272 and cELN-11D	*ELN*	9
Elastin cosmid		1
Cosmid P5155		1
ELN cosmid 82C and the *ELN*/*LIMK1* cosmid 34B FISH probes	*ELN+LIMK1*	1
Cosmid probes	*ELN+LIMK1+STX1A*	4
CTB-8H17	*FKBP6+FZD9+WSTF**	1
BACs 1008H17, 592D8, P195H06, 1148G03, 054H15; cosmids 182B11, 183E1	*FKBP6+FZD9+ELN+STX1A+GTF2IRD1+CYLN2+GTF2I*	1
Probes B315H11 and CITB51J22	*FZD9+BAZ1B+TBL2+LIMK1+RFC2*†	1
BACs 1008H17, 315H11, 592D8, 155B1, 363B4; cosmids 12915, 82c2, 34b3, 152a8, 128d2, 102f12, 135f3, 82b11, 209c11, 47d1, 160g4, 183e1; PACs 632N4, 391G2, 195H6	*ELN+LIMK1+FZD9+FKBP6+BAZ1B+BCL7B+TBL2+ WBSCR14+STX1A+CLDN3+EIF4H+HSPCO46+RFC2+CYLN2*	3

*Genes referenced according to Korenberg et al.[12]; †Genes referenced according to van Hagen et al.[13].
FISH: fluorescent in situ hybridization; WBS: Williams-Beuren syndrome; BAC: bacterial artificial chromosomes; PAC: P1-derived artificial chromosomes; YAC: yeast artificial chromosomes.

characterized by supravalvular aortic stenosis (SVAS), intellectual disability, facial dysmorphism, dental anomalies, and peripheral pulmonary artery stenosis. Further studies described additional dysmorphic features but without a proper etiology explanation.[23-25] A WBS score (diagnostic index) was developed by Preus[26] in order to assess syndrome features and provide patients' diagnoses.

The term "elfin facies" was described in the 1970s to characterize all recurrent facial dysmorphisms found in WBS individuals.[24,27] WBS facial phenotype is distinguished by a broad forehead, medial eyebrow flare, periorbital fullness, strabismus, stellate iris pattern, flat nasal bridge, malar flattening, full cheeks and lips, a long and smooth philtrum, a rather pointed chin, and a wide mouth.[28] In our review, 40.5% of the included patients were described as having an "elfin face" or a "typical face." The choice of a general description instead of detailed information regarding facial dysmorphisms in WBS patients may hinder a genuine clinical diagnosis of the syndrome. Since 1986, the use of generic terms to report WBS facial features has been discouraged.[28] In our review, studies that provided a detailed description of patients' dysmorphisms showed that the most prevalent features were full lips and a long philtrum (28.3%), followed by periorbital fullness (27.3%), wide mouth (26.3%), full cheeks (25.4%), and broad nasal tip (22.9%). A WBS patient's clinical evaluation is extremely relevant in order to provide a clear and precise diagnosis. Heterogeneity between patients' facial dysmorphism is also broadly described in the literature.[2] The term "elfin face," whose definition is based on a mythological and abstract figure, does not reflect the variety of facial features already described throughout WBS individuals. Therefore, the use of generic terms as part of the syndrome spectrum should be discouraged. Hence, we strongly recommend the use of standardized nomenclature to describe the facial phenotype of WBS patients.

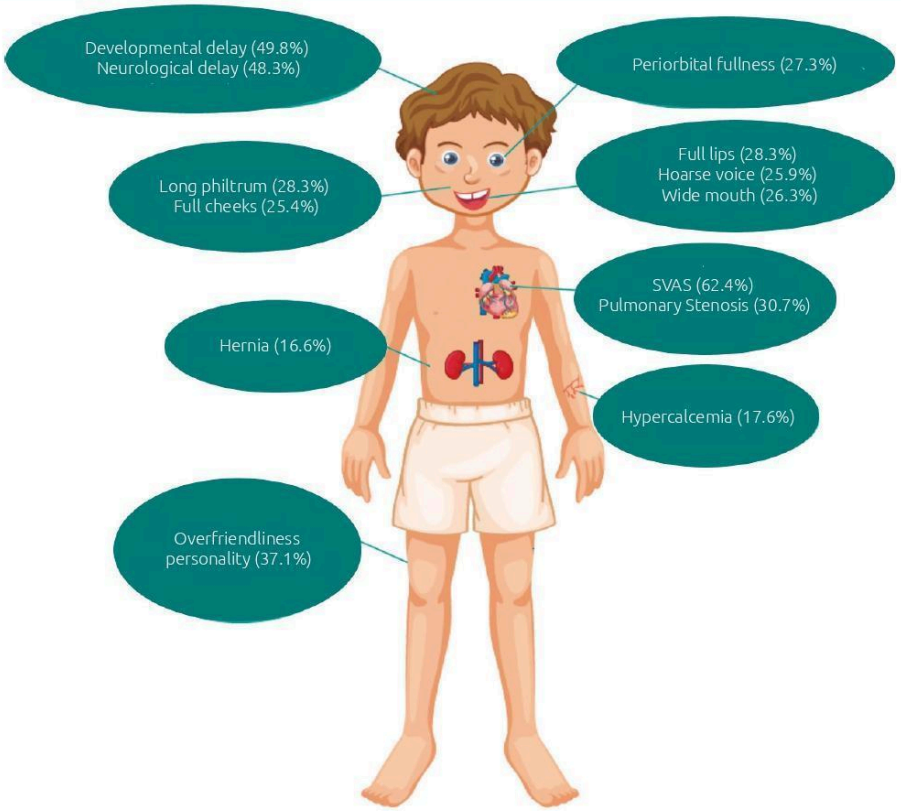
Cardiovascular alterations (80%) are the most frequent features observed in WBS children and are also the major causes of infant morbidity and mortality within the syndrome.[5] SVAS (75%) is considered the main cardiac finding observed in WBS patients, followed by pulmonary artery stenosis (50%).[5,29] In our review, SVAS (62.4%) and pulmonary stenosis (30.7%) were the main cardiac alterations described. The high percentage of cardiovascular malformation among WBS patients points out the value of a detailed cardiovascular screening in an early clinical diagnosis of the syndrome. Although heart features are already known and often described within the syndrome spectrum, WBS patients' diagnoses are still delayed (>1 year, on average).[29] WBS neonatal diagnosis is challenging since some classical features include a friendly personality and facial dysmorphisms that are usually observed only days or months after birth. WBS clinical phenotype is also heterogeneous, and features tend to develop over time, which hinders a proper early clinical diagnosis.[30-32] However, the main congenital heart diseases (CHDs) described in WBS can be screened and diagnosed through routine ultrasonography during the first trimester of pregnancy when performed by expert ultrasonographists.[33] Therefore, we suggest that patients suspicious of WBS should go through a careful examination when looking for cardiovascular findings. As opposed to facial dysmorphisms that are observed over time, congenital heart diseases can be diagnosed early with the aid of prenatal ultrasound. Hence, in order to provide an early diagnosis for WBS patients, CHDs may be the golden key.

Developmental delay (90%) is often observed in WBS individuals. Therefore, referral to early intervention programs such as special education and vocational training is crucial to improve physical, speech, and nutrition features as well as social integration among patients.[4,5] Intellectual delay ranging from light to moderate (75%) and a unique cognitive and behavioral profile are other features frequently described in WBS individuals.[5,34,35] WBS children present an over-friendliness personality characterized by an intense drive for social interaction, a desire to form affectionate bonds, and an increased feeling of empathy. Therefore, an early intervention that aims to enhance social interactions and improve social skills is needed to ease the social inclusion of teenagers and adults with WBS.[35] In our review, developmental delay was found in 49.8% of the patients. Intellectual delay and an over-friendliness personality were described in 48.3 and 37.1% of the individuals, respectively. An overall view of all phenotypic features is shown in Table 1 and Figure 2.

FISH was first performed by Pinkel et al.[36] and Pinkel et al.[37] This cytogenetic technique provides a rapid, precise, and reliable molecular analysis to confirm the suspicion of a clinical diagnosis. FISH is considered the gold standard method for chromosome microdeletion syndromes diagnosis.[4,6,38,39] The WBS diagnosis rate of FISH is over 90% of the cases.[39] WBS molecular etiology was described in the 1990s, the same decade that FISH was implemented as a diagnostic tool for WBS individuals.[40-42] In 1993, Ewart et al.[1] found that the molecular cause of WBS was a microdeletion at chromosome 7q11.23 after observing a elastin gene (*ELN*) hemizygosity by FISH. In our review, peripheral blood was the most commonly collected sample. Surprisingly, we found a case of postmortem diagnosis performed by FISH using both formalin-fixed tissues and paraffin-embedded sections from the kidney. Literature shows that buccal swabs can also be used as samples for FISH microdeletion analysis.[43] Therefore, FISH proved to be an extremely versatile technique when it comes to sample types that can be used as a DNA source.

ELN, *LIMK1*, and *CYLN2* were the main genes designed within FISH probes used in the included studies. *ELN* encodes a structural protein that composes a diversity of body tissues and is the major gene associated with WBS. Therefore, *ELN* deletion can cause some connective tissue abnormalities such as cardiovascular diseases, in particular SVAS in WBS individuals.[1,4,44] *LIMK1* deletion is associated with constructive visuospatial cognition abnormalities as well as neurological features in WBS patients.[45-47] On the contrary, *CYLN2* is a gene associated with cerebellar malformation and neurological impairment that can lead to hippocampal dysfunction and a delay in the development of motor skills.[46,48] Some studies performed other molecular methodologies alongside FISH that allowed a large number of genes to be identified as deleted as well as involved in WBS phenotype. However, the majority of these studies were conducted in developed countries. Therefore, FISH may be the better choice for developing nations that are still lacking in new technologies.

Comparative genomic hybridization (CGH) was first performed by Kallioniemi et al.[49] in order to analyze solid tumor cytogenetics. Nowadays, this technology is widely used to detect chromosome copy number variation.[50] The major advantage of genome-wide array platforms over FISH is the ability to screen for microdeletions and/or duplications throughout the genome that could detect not only WBS but also other syndromes at an earlier age.[50] Gilbert-Dussardier et al.[51] identified a novel microsatellite DNA marker (D7S1870) as a new diagnostic tool for hemizygosity detection in individuals suspected of



WBS: Williams-Beuren syndrome; SVAS: supravalvular aortic stenosis.

Figure 2. Representation of a male WBS patient with classical phenotypic features highlighted.

WBS. Both CGH and microsatellites became valuable methodologies in the investigation of deletion length and characterization of microdeletion syndromes. In addition, MLPA was later added to the pool of molecular technologies that aid researchers in the diagnosis of WBS patientes.[52] In this review, included studies dated from 1993 to 2018 (Figure 3)[1,36,37,49,51,52]. Although CGH, microsatellites, and MLPA were implemented in 1992, 1995, and 2002, respectively, FISH was still performed throughout the years.

Fluorescence in situ hybridization X comparative genomic hybridization cost-effective analysis in developing countries

In the field of genetic rare diseases, molecular diagnosis is essential in order to elucidate the etiology of these conditions as well as provide a genotype-phenotype correlation for uncommon clinical outcomes.[10] Nowadays, different and newer technologies are available to aid molecular investigation and further diagnosis. However, former standard methodologies, such as FISH, are still considered the gold standard for the detection of rare conditions, mainly in developing countries where financial support is limited and affordable technologies are preferred.[2,6,7,53,54,55]

FISH probes covering the *ELN* gene detect the majority of the deletion in children clinically diagnosed with WBS.[39,53,54] Nickerson et al.[39] showed that more than 90% of the patients were hemizygous for the elastin gene, while Souza et al.[53] verified that 83% of the children clinically diagnosed with WBS had the same deletion. Moreover, Ramírez-Velazco et al.[54] analyzed patients clinically diagnosed with WBS and identified that 66% of them had the 7q11.23 deletion detected by FISH. The study also performed CGH analysis in 23 cases where all FISH results were confirmed (18 deletions and 5 negatives). The additional information provided by CGH was the deletion sizes, which enables the patient's classification into typical and atypical deletions.[54] Both studies were conducted in developing countries (Brazil and Mexico), and the conclusion that FISH is the most feasible, effective, and economical approach in those nations was unanimous.[53,54]

In addition, a Brazilian study estimated the techniques' budget in the United States and Brazil and revealed that, on average, US$600 is needed to perform both FISH and CGH analysis in the United States, while in Brazil, FISH analysis would cost US$800 and CGH analysis US$1200.[56] Additionally, CONITEC, a committee that advises the Brazilian Ministry of Health, estimated the financial impact of molecular technologies

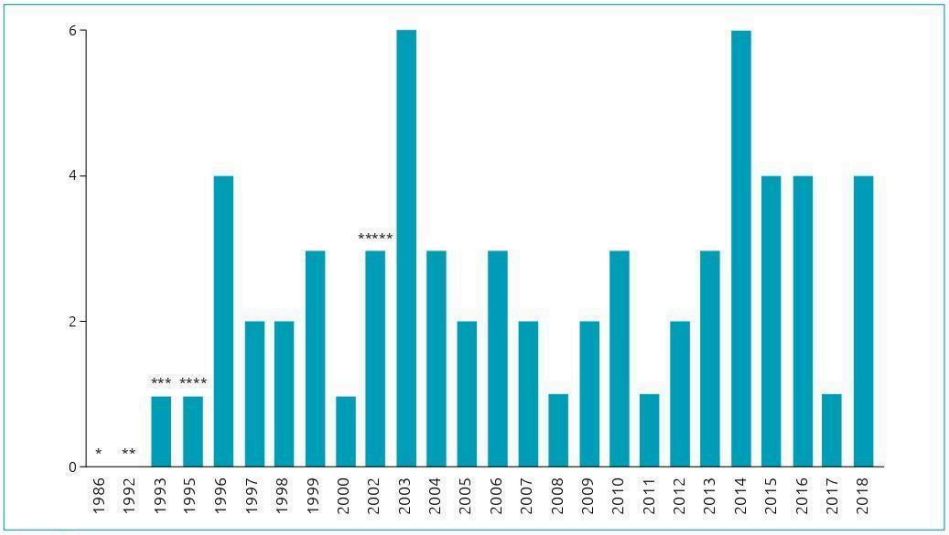


Figure 3. Included studies by year of publication. * FISH's performed for the first time by Pinkel et al.[36,37]; ** CGH's performed for the first time by Kallioniemi et al.[49]; *** Ewart et al.[1] showed that WBS is caused by a microdeletion at chromosome 7q11.23 through FISH; **** a novel microsatellite DNA marker for WBS patients[51]; ***** MLPA is performed for the first time by Schouten et al.[52]. FISH: fluorescent in situ hybridization; WBS: Williams-Beuren syndrome; MLPA: multiplex ligation-dependent probe amplification; CGH: comparative genomic hybridization.

in the country and evaluated the cost of FISH and CGH analysis at R$400 and R$2.000, respectively.[57] Therefore, since all newer technologies are expensive, mainly in countries without proper research support, their use is recommended when FISH is negative in order to investigate atypical deletions.[55] The lack of medical genetic services, health facility limitations, and healthcare access restrictions are also hardships faced by developing nations.[58] Consequently, a proper evaluation by the government is needed in order to seek better healthcare services as well as improved research outcomes, mainly for patients with rare diseases such as WBS.[8]

Although our results are significant, some limitations of this review would be the restriction to access some potential studies that could contribute to our data. To include more articles published around the world, the inclusion of additional databases, such as Embase and Biblioteca Virtual em Saúde, would be interesting.

It is known that the clinical diagnosis alone is insufficient in order to give a proper treatment and follow-up for WBS patients. WBS has a heterogeneity of features described, and a lot of these characteristics are only observed after months or years of life. Therefore, the combination of a successful diagnostic rate for WBS individuals by FISH and a proper cardiac screening (mainly SVAS) may be the key for an early and precise diagnosis in neonatal patients with WBS. An early diagnosis is crucial since these individuals need a multiprofessional healthcare team in order to lessen and soften further complications as well as prevent secondary complications.[4,5] In addition, FISH is a atemporal technology that seems to be irreplaceable for WBS diagnosis, mainly in countries that lack newer methodologies.

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Conflict of interests

The authors declare there is no conflict of interest.

Authors' contribution

Study design: Carlotto BS, Silva AA. *Data collection:* Carlotto BS, Deconte D, Silva PR, Silva AA, Diniz BL. *Data analysis:* Carlotto BS, Deconte D, Silva PR, Silva AA, Diniz BL. *Manuscript writing:* Carlotto BS, Deconte D, Diniz BL. *Manuscript revision:* Carlotto BS, Deconte D, Diniz BL, Silva PR, Zen PRG, Silva AA. *Study supervision:* Zen PRG, Silva AA.

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8. ANEXOS

8.1. Parecer do Comitê de Ética da UFCSPA

UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Estudo de aspectos etiológicos e clínicos de pacientes com doenças genéticas ou síndromes malformativas atendidos no Serviço de Genética Clínica.

Pesquisador: Paulo Ricardo Gazzola Zen

Área Temática: Genética Humana:
(Trata-se de pesquisa envolvendo Genética Humana que não necessita de análise ética por parte da CONEP;);

Versão: 2

CAAE: 69178217.7.0000.5345

Instituição Proponente: Universidade Federal de Ciências da Saúde de Porto Alegre

Patrocinador Principal: Universidade Federal de Ciências da Saúde de Porto Alegre

DADOS DO PARECER

Número do Parecer: 2.230.086

Apresentação do Projeto:
Trata-se de uma proposta de desenvolvimento de múltiplos estudos (série de casos ou mesmo relatos de caso) utilizando dados de prontuário de pacientes atendidos no Serviço de Genética Clínica da UFCSPA/HCSCPA desde a sua criação e que apresentarem diagnóstico de uma doença genética ou uma síndrome malformativa específica cuja descrição ou relato apresente relevância científica. Conforme o livro de registro, até o momento foram avaliados aproximadamente 10.000 pacientes no Serviço.

Objetivo da Pesquisa:
Descrever as características clínicas, laboratoriais e prognósticas de pacientes portadores de doenças genéticas ou síndromes malformativas atendidos no Serviço de Genética Clínica da Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA)/Complexo Hospitalar Santa Casa de Porto Alegre (CHSCPA) na forma de relatos de caso ou séries de casos, a partir de dados clínicos obtidos em seus prontuários médicos.

Endereço: Rua Sarmento Leite ,245
Bairro: Sarmento **CEP:** 90.050-170
UF: RS **Município:** PORTO ALEGRE
Telefone: (51)3303-8804 **E-mail:** cep@ufcspa.edu.br

UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE

Avaliação dos Riscos e Benefícios:

Riscos:

Como o estudo será totalmente realizado em base de dados, não há riscos para os indivíduos. Além disso,os dados coletados e os resultados serão preservados confidenciais e de uso exclusivo dos pesquisadores.

Será assegurado o anonimato dos pacientes envolvidos na pesquisa.

Benefícios:

O benefício se dará pela produção de conhecimento e pela formação acadêmica.

Comentários e Considerações sobre a Pesquisa:

Proposta relevante e metodologicamente adequada para ser desenvolvida.

Considerações sobre os Termos de apresentação obrigatória:

Todos os termos de apresentação obrigatória foram apresentados e estão adequados.

Recomendações:

Sugere-se reapresentar o Termo de entrega de relatório parcial e final com a readequação de cronograma.

Conclusões ou Pendências e Lista de Inadequações:

O projeto está adequado para ser desenvolvido até dezembro de 2022.

Considerações Finais a critério do CEP:

De acordo com o parecer do Relator.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_925489.pdf	04/07/2017 16:24:13		Aceito
Outros	Authorization_image.pdf	04/07/2017 16:21:35	Paulo Ricardo Gazzola Zen	Aceito
Outros	Autorizacao_imagem.pdf	04/07/2017 16:21:02	Paulo Ricardo Gazzola Zen	Aceito
Outros	Autorizacao_uso_de_dados.pdf	04/07/2017 16:20:31	Paulo Ricardo Gazzola Zen	Aceito
Brochura Pesquisa	Projeto_Geral_Doencas_Geneticas_1.pdf	04/07/2017 16:19:02	Paulo Ricardo Gazzola Zen	Aceito
Parecer Anterior	Parc_929_09_498_09_A.pdf	25/05/2017 08:46:21	Paulo Ricardo Gazzola Zen	Aceito

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Continuação do Parecer: 2.230.086

Projeto Detalhado / Brochura Investigador	Projeto_Geral_Doencas_Geneticas.pdf	25/05/2017 08:43:20	Paulo Ricardo Gazzola Zen	Aceito
Outros	Termo_de_anuencia_responsavel_pelo_setor.pdf	25/05/2017 08:41:56	Paulo Ricardo Gazzola Zen	Aceito
Outros	anexo5_termo_compromisso_entrega_relatorio_final.pdf	23/05/2017 11:05:37	Paulo Ricardo Gazzola Zen	Aceito
Outros	Alteracao_coordenador.pdf	23/05/2017 10:45:13	Paulo Ricardo Gazzola Zen	Aceito
Folha de Rosto	Folha_rosto.pdf	23/05/2017 10:43:18	Paulo Ricardo Gazzola Zen	Aceito
Outros	RELATRIO_DE_PESQUISA.pdf	22/05/2017 10:07:51	Paulo Ricardo Gazzola Zen	Aceito
Cronograma	Cronograma_2014.pdf	22/05/2017 10:07:07	Paulo Ricardo Gazzola Zen	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

PORTO ALEGRE, 21 de Agosto de 2017

Assinado por:
Julia Fernanda Semmelmann Pereira Lima
(Coordenador)

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