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Whole exome sequencing in 18 Brazilian families with vertical transmission of non-syndromic oral clefts

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ABSTRACT

This study describes the results of whole exome sequencing in the etiological investigation and genetic counseling of families presenting with non-syndromic oral clefts with vertical transmission recorded in the Brazilian Database on Craniofacial Anomalies. Whole exome sequencing was performed in 18 families presenting with non-syndromic oral clefts with vertical transmission, and variant filtering was used to identify rare, and also possibly pathogenic variants in genes associated with oral clefts. Overall, our study identified seven families (38.88%) presented with segregating variants possibly related to oral clefts in our cohort: *SETX*, *NOTCH1*, *FRAS1*, *ARHGAP29*, *KMT2D*, *ANKRD11*, *SIX1*, *BMP6*, *LRP2* and *TFAP2A*. In another family, all affected members (5.55%) presented a rare variant in *FAM193A*, which has no recognized function yet, but has prediction of deleterious effect. Our study highlights oral clefts clinical and etiological heterogeneity and shows the complexity of using whole exome sequencing for genetic counseling in non-syndromic oral clefts with vertical transmission.

1. Introduction

Orofacial clefts (OC) are the most common craniofacial birth defects with an estimated global prevalence of 1:700–1:1.000 newborns (Mossey et al., 2011). OC can be classified based on the phenotype, ranging from microforms to a complete facial cleft. The three most common OC types include cleft lip (CL), cleft palate (CP) and cleft lip and palate (CLP). The microform phenotype is a minimal manifestation or subclinical sign detected in noncleft subjects, and may involve either lip and/or palate. The main microforms include: *orbicularis oris* muscle discontinuation (could be detected through ultrasonography), notches in the vermilion border, insufficiency velopharyngeal and bifid uvula. The occurrence of microform indicates a greater propensity to cause OC in the offspring (Klotz et al., 2010; Mossey et al., 2010).

Approximately 70% of OC are non-syndromic (NSOC), in which the etiology is complex in most cases. Therefore, genetic counseling is still based on populational studies (Saleem et al., 2019). Although genome-wide association studies have identified many genetic variants related to the risk of NSOC so far, the effect of each variant alone depends on the ethnicity, as a result of different risk allele frequencies in diverse populations (Saleem et al., 2019). In this group, 80% of these patients represent sporadic cases, and about 20% are from multiplex families (Mossey and Modell, 2012; Saleem et al., 2019). Among the latter, there are some cases with proved vertical transmission (Basha

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et al., 2018).

Complex diseases such as OC seem to emerge from the additive effect of disease-causing variants with relatively high population frequencies (minor allele frequency – MAF less than 5%) (Panagiotou et al., 2010). Approximately 45 genetic loci related to NSOC risk have been identified so far and it accounts for only 25% of the estimated heritability (Bishop et al., 2020). Studies with different approaches have been performed to search for rare coding deleterious variants that could explain this missing heritability.

The use of whole exome sequencing (WES) has allowed the identification of rare genetic variants segregating in affected individuals from multiplex NSOC families (Bureau et al., 2014; Aylward et al., 2016; Basha et al., 2018; Awotoye et al., 2022) including those in genes already associated with syndromic orofacial clefts (SOC) (Basha et al., 2018). In some multiplex families, OC present as part of syndromes with variable expressivity and they can mimic NSOC in individuals with very subtle signs, particularly in families in which the proband has an a priori isolated OC, as it was recently demonstrated (Slavec et al., 2023).

In this article, we describe segregating variants and genetic counseling in 18 Brazilian families diagnosed as NSOC with vertical transmission at the latest clinical evaluation selected from Brazilian Database on Craniofacial Anomalies (BDCA).

2. Material and methods

2.1. Cohort

The Brazil's Craniofacial Project (BCFP) is a multicentric, multiprofessional and voluntary group, with the aim to provide scientific information on many aspects of craniofacial conditions. Since 2013, the Brazilian Database on Craniofacial Anomalies (BDCA) is one of the approaches of the BCFP, which records etiology and clinical follow-up of OC, among other data (Gil-da-Silva-Lopes et al., 2020).

This study used primary data recorded through the BDCA and biological samples from its associated biorepository from 10 BDCA-collaborating hospitals located in the Northeast, Southeast and Southern regions of Brazil (Volpe-Aquino et al., 2018; Gil-da-Silva-Lopes et al., 2020). Inclusion criteria were families with diagnosis of NSOC with vertical transmission recorded at BDCA, in which the probands were at least 2 years old at time of cohort selection and had normal neuropsychomotor development. Exclusion criteria were families with incomplete data records and families with SOC, including history of intellectual disability (ID). The final cohort was composed of 18 multiplex families with a diagnosis of NSOC at the time of cohort selection.

2.2. DNA samples

Genomic DNA samples stored in the BDCA biorepository were obtained from peripheral blood samples using phenol/chloroform extraction, following a standard protocol at the Human Cytogenetics and Cytogenomics Laboratory - School of Medical Sciences – UNICAMP. All samples were purified by Microcon-30kDa Centrifugal Filter Unit with Ultracel-30 membrane (Millipore, Billerica, MA, USA).

2.3. Whole exome sequencing (WES)

WES was performed by Macrogen, Inc.© (Seoul, Republic of Korea) using 1 μg of genomic DNA, which was fragmented into a library of small segments, and capture was performed using the Agilent SureSelectXT Human All Exon Kit V6 (Agilent Technologies®, Santa Clara, CA, USA). Sequencing was performed on Illumina NovaSeq 6000 Sequencing System (Illumina, Inc.©, San Diego, CA, USA) to generate paired-end, 2x150 bp reads, and with on-target 140x coverage. The raw data was extracted to a Fastq format.

2.4. WES data analysis

Individuals were analyzed using a standard procedure conducted at the Human Genetics Laboratory of the de Duve Institute in Belgium. Raw data (.fasta files) were aligned to the reference human genome assembly (GRCh38) using BWA 0.7.15. Aligned sequences (.bam files) were processed using Samtools 1.12 "MarkDup" (for marking duplicates) and GATK 4.2 "BQSR" (for base quality scores recalibration). Variant calling was then performed using GATK 4.2 "Haplotype Caller" (following Broad Institute best practices). The variant calls (.vcf) files generated were annotated, imported and further analyzed on Highlander 17.18 (https://sites.uclouvain.be/highlander/), the in-house bioinformatics framework of the Genomics and Bioinformatics platform of UCLouvain, with a local database currently containing more than 4300 WES categorized by pathology. Highlander provides extensive variant-annotation, filtering and visualization. Filtering was retained for variants that satisfied the following criteria:

(i) pass GATK standard quality-control filters;
(ii) within a list of 544 candidate genes for oral clefts;
(iii) 1% allele frequency in the population with the maximum allele frequency in GnomAD WES samples (https://gnomad.broadinstitute.org/help/popmax);
(iv) detected in <1% samples from individuals with unrelated pathologies (or unaffected controls) in the in-house database of 3274 germline WES;
(v) missense, nonsense, frameshift and changes affecting splicing;
(vi) for missense variants, predicted to affect protein function by at least 6 out of 20 prediction tools (DAMAGING in Mutation Taster, FATHMM, FATHMM-XF, Polyphen2 (HDIV), Provean, SIFT4G, Mutation Assessor, MCAP, LRT, Lists2, Deogen, ClinPred, BayesDel (with MaxMAF), PrimateAl and MetaSVM, or a score >20 in CADD phred, >0.5 in VEST, >0.5 in REVEL, >0.75 in MVP, >0.75 in MutPred).

After selection of the variants by Highlander (https://sites.uclouvain.be/highlander/) a preliminary classification was performed using what is known about protein function (animal studies, scientific literature), alignment, constraint scores of the protein (pLI, z-score for missense variants), frequency in databases such as LOVD (https://www.lovd.nl/), Clinvar (https://www.ncbi.nlm.nih.gov/clinvar/), DECIPHER (https://www.deciphergenomics.org/), and predictions by Varsome (https://varsome.com).

A second analysis of all cases was performed using Varstation (Cervato et al., 2021) as this platform also makes use of the ABraOM database (https://abraom.ib.usp.br/), which contains genomic variants obtained with WES and whole genome sequencing (WGS) from the Brazilian population (Naslavsky et al., 2017). In addition, an automatic variant pre-classification was performed, according to the recommendations and guidelines of the ACMG, Association of Molecular Pathology (AMP) and the College of American Pathologists (CAP) (Richards et al., 2015). Each variant pre-classification was verified by the analyst, using medical records, public databases (ClinVar and DECIPHER) and scientific literature. Variants were reclassified when necessary.

After these procedures, the files of all cases were clinically reviewed, and two participants were reevaluated during a follow-up consultation (families 5 and 16).

2.5. Validation by sanger sequencing

The variants were confirmed by Sanger sequencing. The Ensembl Genome Browser (assembly GRCh37/hg19 and GRCh38/hg38) was used to design the primers. The fragments of interest were amplified by Polymerase Chain Reaction and purified using Exo-Sap enzyme (Applied Biosystems). Sequencing reaction was performed with BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). Sequence reading was

performed through automated capillary electrophoresis (ABI 3500xL Genetic Analyzer, Applied Biosystems) and the results were analyzed using the CodonCode Aligner software (CodonCode Corporation, Dedham, MA, USA).

3. Results

Out of 18 families identified with NSOC with vertical transmission at BDCA, trio analysis was available and performed in 14 families (families 1, 2, 3, 4, 5, 6, 9, 10, 11, 12, 14, 15, 17 and 18). In the other 4 families, the individuals analyzed varied according to DNA sample availability as follows: WES was performed in the proband and the affected mother in families 7 and 8; in the proband and the affected brother in family 13; and in the proband, the affected sister and the affected mother in family 16.

3.1. Results of exome sequencing

WES results showed that 7/18 families had one or more segregating variants of interest possibly related with NSOC (families 5, 6, 8, 9, 10, 11 and 15); 1/18 families had a single variant segregating in all affected members, which has no recognized function yet (family 18); and 10/18 of families had no results of interest from WES (families 1, 2, 3, 4, 7, 12, 13, 14, 16 and 17).

Seven families had one or more segregating variants: the likely pathogenic variant in *SETX* (family 5), the VUSes in *NOTCH1* (family 6), *FRAS1* (family 8), *ARHGAP29*, *ANKRD11* and *KMT2D* (family 9), the likely pathogenic variant in *SIX1* (family 10), the VUS in *BMP6* (family 11), the likely pathogenic in *LRP2* and the VUS in *TFAP2A* (family 15). All variants segregated in affected individuals from each respective family. In one family (family 18) all affected members had a single VUS in *FAM193A*, which has no unknown function and not related to OC to date. This is a rare variant, with a frequency of 0.0013% in Gnomad aggregated and less than 0.01 in all population studies; there are no homozygotes in the population studies. The REVEL is 0.48, but the Franklin aggregated prediction is 0.78 (with deleterious prediction).

After molecular diagnosis and clinical reevaluation, two families were reclassified as SOCs (families 5 and 16). However, in family 16, no abnormalities were found in WES.

Fig. 1 shows the pedigree of the 18 families with NSOC, and the respective variants detected. Detailed clinical data, WES results and ACMG classification/criteria are depicted in Table 1.

4. Discussion

Genetic counseling of NSOC is challenging, since it has multifactorial etiology, with contribution of diverse genetic variants and environmental factors. A limited number of causal genes directly responsible for the phenotype have been described so far (Cheng et al., 2023). Furthermore, multiple gene interactions seem to take part in different craniofacial conditions (Saleem et al., 2019).

In NSOC families, GC has been usually performed through empiric populational risk, usually established before the knowledge of OC's microform (Mossey and Modell, 2012). Commonly, the informed risk of

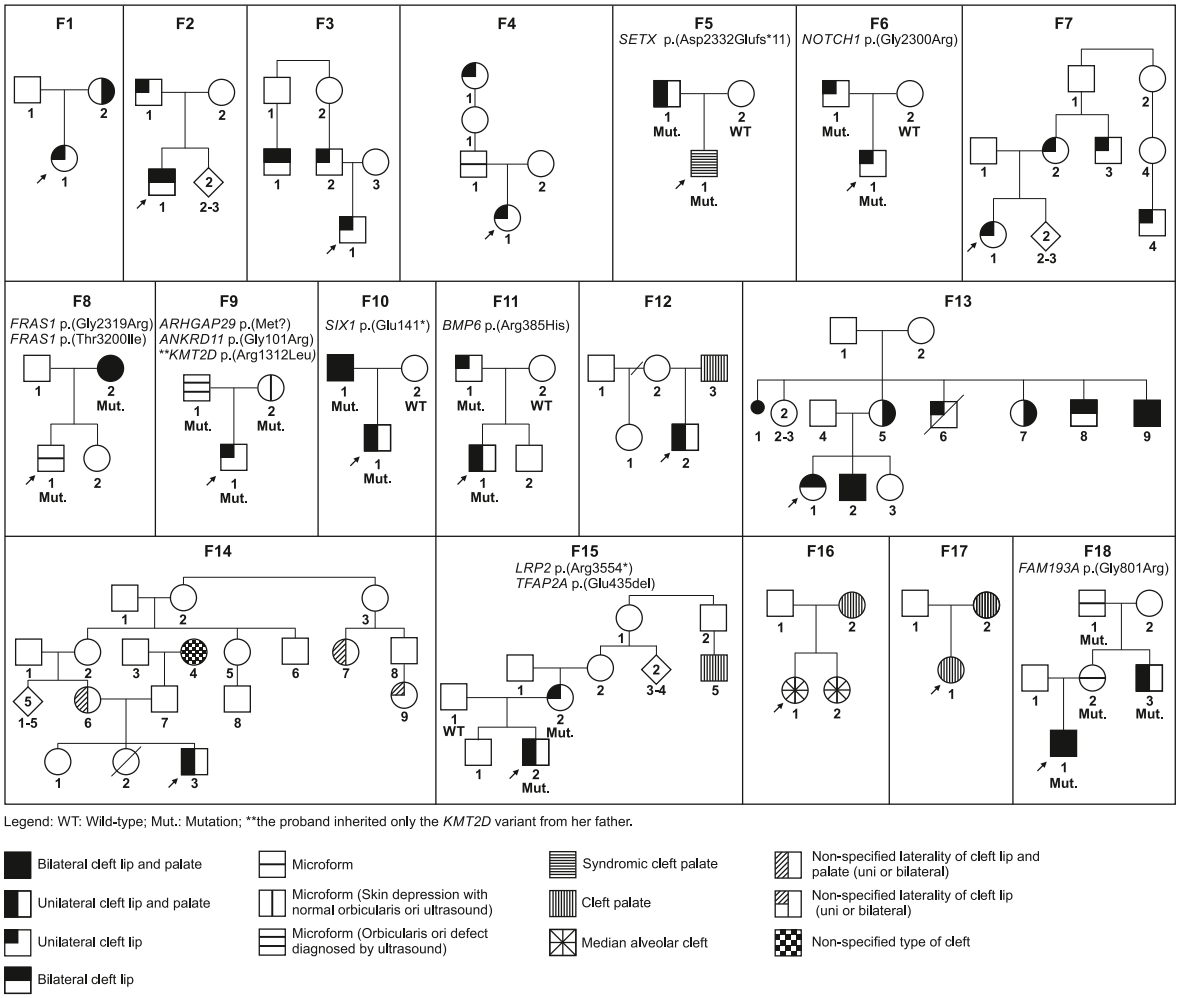

Fig. 1. Pedigrees of 18 multi-case families with NSOC and the respective segregating genetic variants.

Table 1
Variants of interest detected in the present sample.

ID Family	Variants identified by WES	gnomAD (aggregated)	gnomAD pLI score	REVEL score	ACMG Classification/ Criteria	Gene: OMIM-related phenotype (s)	ID Pedigree	OC type
F5	NM_015046.7:*SETX*: c.6996_7002del:p.(Asp2332Glufs*11)	0%	0.95	N/A	Likely Pathogenic	Amyotrophic lateral sclerosis 4, Juvenile, AD; Spinocerebellar ataxia, autosomal recessive, with axonal neuropathy 2, AR	II-1	CP
	Het.				PVS1 (Very strong); PM2 (Moderate)		I-1	CLPu
F6	NM_017617.5:*NOTCH1*: c.6898G > A:p.(Gly2300Arg)	<0.01%	N/A	0.39	VUS	Adams-Oliver syndrome 5, AD; Aortic valve disease 1, AD	II-1	CLu
	Het.			Uncertain	PP2 (Supporting); BP6 (Supporting)		I-1	CLu
F8	NM_025074.7:*FRAS1*: c.6955G > A:p.(Gly2319Arg)	<0.01%	N/A	0.76	VUS	Fraser syndrome 1, AR	II-1	Microform CLPb
	Het.			Deleterious	PM2 (Moderate); PP3 (Supporting)		I-2	
	NM_025074.7:*FRAS1*: c.9599C > T:p.(Thr3200Ile)	<0.01%	N/A	0.79	VUS	Fraser syndrome 1, AR		
	Het.			Deleterious	PP3 (Moderate)			
F9	NM_004815.4:*ARHGAP29*: c.2T > C:p.(Met1?)	<0.01%	1	N/A	VUS	CL ± P, AD**	II-1 I-2	CLu Microform
	Het.				PVS1 (Moderate); PM2 (Moderate)			
	NM_001256183.2: *ANKRD11*:c.301G > A:p.(Gly101Arg)	0%	N/A	0.43	VUS	KBG syndrome, AD		
	Het.			Uncertain	PM2 (Moderate)			
	NM_003482.4:*KMT2D*: c.3935G > T:p.(Arg1312Leu)	0%	N/A	0.61	VUS	Kabuki syndrome 1, AD	II-1	CLu
	Het.			Uncertain	PM2 (Moderate); PP2 (Supporting)		I-1	Microform
F10	NM_005982.4:*SIX1*: c.421G > T:p.(Glu141*)	0%	0.65	N/A	Likely Pathogenic	Branchiootic syndrome 3, AD	II-1	CLPu
	Het.				PVS1 (Very strong); PM2 (Moderate)		I-1	CLPb
F11	NM_001718.6:*BMP6*: c.1154G > A:p.(Arg385His)	<0.01%	N/A	0.43	VUS	–	II-1	CLPu
	Het.			Uncertain	PM2 (Moderate)		I-1	CLu
F15	NM_004525.3:*LRP2*: c.10660C > T:p.(Arg3554*)	0%	1	N/A	Likely Pathogenic	Donnai-Barrow syndrome, AR	IV-2 III-2	CLPu CLu
	Het.				PVS1 (Very strong); PM2 (Moderate)			
	NM_001372066.1: *TFAP2A*:c.1303_1305del:p.(Glu435del)	<0.01%	N/A	N/A	VUS PM2 (Moderate); PM4 (Moderate)	Branchiooculofacial syndrome, AD		
	Het.							
F18	NM_003704.4:*FAM193A*: c.2401G > A:p.(Gly801Arg)	<0.01%	N/A	0.48 Uncertain	VUS	–	III-1 II-2 II-3	CLPb Microform CLPu
	Het.				PM2 (Moderate); PP3 (Supporting)		1–1	Microform

Legend: Het: Heterozygous; N/A: Not applicable; AD: Autosomal dominant; AR: Autosomal recessive; XLD: X-linked dominant; CLu: Cleft lip unilateral; CLP: Cleft lip and palate; CLPu: Cleft lip and palate unilateral; CLPb: Cleft lip and palate bilateral; CP: Cleft palate; **: Definitive classification by GenCC.

recurrence (RR) of CL/P is approximately 3.6–4.7% in future siblings or if one parent is affected (Sivertsen et al., 2008). However, populational studies have shown that the RR of CL/P may be diverse for different populations. Furthermore, advances in genotyping technologies, mainly genome-wide association studies, demonstrated that there are many genetic risk loci possibly responsible for a small portion of the heritability of NSOCs depending on the genetic background of each population (Moreno et al., 2004; Nikopensius et al., 2009; Mossey and Modell, 2012; Leslie et al. 2016, 2017a, 2017b; Ludwig et al., 2017; Yu et al., 2017). Since the Brazilian population has a mixed genetic background (Souza et al., 2019), these empiric RR probably have a different reality.

Although most cases of multiplex families with NSOC seem to have a complex etiology, there is still very little information in literature about the genes involved in the etiology of NSOC with vertical transmission. In these cases, WES was useful to screen out some candidate genes (Bureau et al., 2014; Aylward et al., 2016; Basha et al., 2018; Manojlovic et al., 2023). Even so, the lack of a genomic database for OC, and that there must be unique variants in population still impact the GC based on WES.

Also, variants in genes previously described as causative of SOC were also reported (Basha et al., 2018). However, it is important to consider that, in some families, SOC with variable expressivity, can mimic NSOC (Slavec et al., 2023).

This article provides some molecular information to contribute to new studies and improve GC for families with vertically-transmitted OC. In the present cohort, WES identified genetic variants segregating in affected individuals in seven families (families 5, 6, 8, 9, 10, 11 and 15).

4.1. *WES identified variants possibly related with NSOCs etiology*

Some rare variants in genes related with OC were classified as likely pathogenic and VUSes according to the current criteria of the guidelines of ACMG/AMP (Richards et al., 2015). More recently, there has been a growing tendency to consider the phenotype as important evidence for variant classification (Johnson et al., 2022). This is particularly true when considering craniofacial anomalies, which comprise a group of conditions with etiological and phenotypic heterogeneity. In addition, ACMG criteria were tailored primarily to monogenic conditions analysis. NSOC are predominantly of complex etiology, and there is very little information on monogenic etiology of NSOC in literature and in databases. Therefore, variants herein detected might be related to the phenotypes described and may contribute for the discussion of variants interpretation. According to ACMG guidelines, families 5, 6, 8, 9, 10, 11 and 15 presented with segregation of variants of interest.

In family 5, the heterozygous likely pathogenic variant *SETX*: c.6996_7002del:p.(Asp2332Glufs*11) segregates in the proband, with CP, and in the affected father, with unilateral CLP. This family was reevaluated during follow-up in BDCA posterior to the moment of cohort selection. Both have not neurological findings, but the proband's diagnosis of NSOC was updated to SOC, since he presented with intellectual disability, and interventricular septal defect lately detected. This variant could be an incidental finding, but the *SETX* gene interacts with other proteins with known associations with heart and palatal embryogenesis, like RNApolII, CHD4 and Histone H3/H4. Also, there is a report of a proband with global developmental delay, tetralogy of Fallot, and bilateral CLP, with two heterozygous missense variants in *SETX*, both classified as VUSes (Munabi et al., 2022). The likely pathogenic variant detected in the proband of the present study may be at least partially causative of the phenotype. It would be possible that other patients with concomitant OC and congenital heart disease (CHD) might also have deleterious variants in *SETX* gene. Therefore, populational and functional studies, as well as reporting information in genetic databases would be necessary to corroborate the hypothesis.

In family 6, the heterozygous VUS in *NOTCH1*:c.6898G > A:p.(Gly2300Arg) segregates in the proband and in the affected father, both with unilateral CL. The Notch pathway is involved in facial patterning and intercommunicates with Shh, Bmp and Wnt pathways (Pakvasa et al., 2020). The *Notch1* plays a role in palate formation during embryogenesis and disruption in *Notch1* expression may result in defects in apoptosis and palate fusion leading to CP in mice (Zhang et al., 2016). Data from family 6 present in BDCA shows the exclusive presence of CL. This family was registered in the BDCA, but there was no follow-up, which limits the genotype-phenotype correlation. The presence of VUS in *NOTCH1* highlights the importance of a complete clinical evaluation and limited information about OC in the genomic databases. Similar descriptions, populational records, and functional studies would contribute to a better characterization of the role of this gene in NSOC and, consequently, to the GC for families with vertical transmission of this congenital defect.

In family 8, two heterozygous VUSes in *FRAS1* gene (related to autosomal recessive Fraser syndrome - OMIM #219000) segregate in the proband, with cleft microform and in the affected mother, with bilateral CLP: *FRAS1*:c.6955G > A:p.(Gly2319Arg) and *FRAS1*:c.9599C > T:p.(Thr3200Ile). *FRAS1* is related to autosomal recessive Fraser syndrome (OMIM #219000), which is characterized by severe findings including cryptophthalmos, urogenital and digital malformations, as well as CL and CP (van Haelst et al., 2008). Since both variants are inherited, we expect them to be *in cis* and not exert the compound heterozygote effect. However, we cannot rule out the hypothesis that the two variants segregating together can alter the protein function and can influence the phenotype slightly. This is a clear example that families are unique and functional studies must be done to make GC informative. Recording these in public genomic database would be also useful.

In family 9, the proband presents with unilateral CL; his mother has a cleft microform (mild scar in the upper lip), the heterozygous, and his father was diagnosed with a cleft microform (discontinuity of *orbicularis oris* muscle detected by ultrasound). The heterozygous VUS *ARHGAP29*: c.2T > C:p.(Met1?) which segregates in the proband and in his affected mother could be causative of the phenotype of this family. *ARHGAP29* has been associated with NSOC risk in diverse populations (Leslie et al., 2012). Recently, a paper in which the family herein described is included reinforced the importance of the *ARHGAP29* in NSOC. In this article, up to 2% of investigated individuals with NSOC presented variants in this gene, with a penetrance of 88% (Ranji et al., 2024).

In addition, there are other inherited VUSes that may also contribute to the phenotype of family 9. The variant *ANKRD11*:c.301G > A:p.(Gly101Arg) also segregates in the proband in the affected mother. Another variant, *KMT2D*:c.3935G > T:p.(Arg1312Leu), segregates in the proband and his affected father. Both genes have strong evidence for a certain role in OC, more commonly CP. *ANKRD11* has a role in craniofacial complex ossification during embryogenesis. Heterozygous or homozygous deletions in murine homologous *Ankrd11* result in diverse craniofacial alterations in murine models, including secondary palate malformation and CP (Roth et al., 2021). *KMT2D* was described as a potential cleft candidate gene in a cohort of 130 African case-parent trios with non-syndromic CL/P, evaluated by WGS (Awotoyeet al. 2022). Loss of function of murine homologous *Kmt2d* in neural crest cells drives alteration in the differentiation of mesenchymal cells during secondary palate formation, leading consequently to CP formation (Shpargel et al., 2020). Although CP isolated is not observed in this family, all the VUSes inherited may be contributing to the greater severity of the proband.

In family 10, the likely pathogenic heterozygous variant *SIX1*: c.421G > T:p.(Glu141*) segregates in the affected father, with bilateral CLP, and in the proband, with unilateral CLP. This gene (OMIM*601205) is related to Branchiootic syndrome type 3 (OMIM #608389), and deafness dominant autosomal 23 (OMIM #605192). Despite Family 10 not being affected by these two conditions, *Six1* has a recognized role in craniofacial structures embryogenesis, including the ear, the nasal cavity, the maxilla, and the mandibular skeleton in mice. It works in conjunction with *Six2* and loss of function of both results in abnormal apoptosis in developing medial nasal processes in animal models. (Liu et al., 2019). In addition, *Six1* and *Six* seem to participate in a regulatory network with the *Alx* gene family to control frontonasal development (Liu et al., 2019). The clinical presentation herein described could be part of expanded phenotype but, according to current knowledge, the association of *SIX1* with the phenotype of this family is purely speculative. However, this report can open a front for genomic investigation and functional approaches that could contribute to the elucidation of the role of this gene in OC.

In family 11 the heterozygous VUS *BMP6*:c.1154G > A:p.(Arg385His) segregates in the proband with unilateral CLP and in affected father with unilateral CL. *BMP6* gene is part of Bone Morphogenetic Proteins (BMPs), a group of signaling molecules that belongs to the Transforming Growth Factor-β (TGF-β) superfamily of proteins. TGF-β superfamily encompasses a large group of signaling ligand, including the subfamilies BMP and the TGF-β, which play a key role in lip and palate formation through BMP/TGF-β signaling pathway (Reynolds et al., 2020). *BMP6* is expressed in cartilaginous tissue, stimulating mesenchymal cell differentiation into chondrocytes (de Mara et al., 2013), indicating an important role of *BMP6* in bone formation. *BMP6* was considered a candidate gene for NSOC in a meta-analysis of 13

genome wide association studies (GWAS) with families of diverse populations (Marazita et al., 2004). Additional case reports, functional studies, and information in different populational genetic databases would contribute to clarify impact of this variant in the OC phenotype.

In family 15, two heterozygous variants were detected segregating in the proband, with unilateral CLP, and in the affected mother, with unilateral CL: the likely pathogenic variant *LRP2*:c.10660C > T:p.(Arg3554*) and *TFAP2A*:c.1303_1305del:p.(Glu435del). *LRP2* has been described to cause the autosomal recessive Donnai-Barrow syndrome (OMIM #222448). Additionally, two homozygous variants in *LRP2* were described segregating in two siblings of a consanguineous family with clinical features of the ocular phenotype of Stickler syndrome and mild features of Donnai-Barrow syndrome (Nixon et al., 2022). *TFAP2A* is better known as a gene related with SOC, like in the autosomal dominant Branchiooculofacial syndrome (OMIM #113620). Bishop et al. (2020) performed WES of European, Latin, and Taiwanese trios and demonstrated that *de novo* mutations in this gene have a critical role in an individual's risk for NSOC. Despite the autosomal recessive inheritance related to *LRP2*, also the relationship with SOC of *TFAP2A*, we hypothesize that both variants may have an additive effect in this family, since these genes are related to OC pathogenesis.

4.2. Family 18 and the FAM193A gene

In family 18, the VUS *FAM193A*:c.2401G > A:p.(Gly801Arg), which has not been related with any phenotype so far, segregates in the proband, with bilateral CLP, in the affected maternal uncle, with unilateral CLP, in the mother and maternal grandfather, both with microform. The lack of information regarding this gene does not allow us to attribute any contribution to the genesis of OC. However, recurrence as a single and variant segregating in all affected family members led to its description in this study. This can be a reference for future information based on population genomics and, if relevant, functional studies. For the present family, the information did not add knowledge that would modify the CG.

4.3. Genetic counseling

Genetic counseling for families with NSOC is highly challenging. From a clinical point of view, the number of known affected individuals may be limited by family size and by undiagnosed microforms. From an etiological point of view, there are an extensive number of genes related to the OC phenotype, in addition to gene-environment interactions (Saleem et al., 2019). The investigation through WES has brought new information which contributes to build a new paradigm for genetic counseling in NSOC but is still far from modifying GC in many families.

In the case of families with vertical transmission, the literature is limited and suggests that monogenic transmission is related to a number of different pathogenic variants in a large number of different genes, often unique and specific to each of the families studied. These genes, in most cases, are also involved in syndromic disorders, reinforcing the need for careful and complete clinical assessment before proceeding to determine the risk for each individual. Even in those cases, environmental influence should not be excluded. In our data, most of our findings are still VUSes and did not modify GC. It is still important to report the findings to the family so that in the future, these variants can be reclassified to be more informative with new data. Furthermore, recent information and data suggesting an additive effect of VUSes are still subject to scientific studies and need to be better understood to see their value in genetic counseling (Holzinger et al., 2017; Diaz-Perez et al., 2023; Manojlovic et al., 2023).

Since NSCL/P is often a multifactorial condition, many variants detected by WES are still difficult to interpret. Therefore, despite WES not being very useful in this study to improve GC, other studies have shown that WES can aid GC. WES should therefore still be performed and recorded in public databases for OC.

5. Conclusion

Overall, our study shows that out of 18 families with NSOC investigated through WES, seven (38.88%) families presented with segregating variants. In all families with two or more variants, we hypothesize that these variants could contribute as an additive effect in the genesis of the NSOC. Even though WES identified variants of interest in 7/18 families, none of these variants were able to give a definite diagnosis for these families. In addition, the contribution of environmental factors to the phenotypes could not be excluded.

The real contribution of the variants detected by WES in this cohort to the phenotypes of each family is still not completely understood. Additional information about these variants' frequencies in different ethnicities and functional studies, tailored by WES results in families with more than one VUS would improve knowledge on NSOC cases like the ones presented here. Therefore, WES added complexity in GC in this cohort.

Thus, this study highlights the clinical and etiological heterogeneity of OC and shows the complexity of using WES for GC in vertical transmissions of NSOC, where one would expect to find more convincing variants of interest.

Ethical Approval

The study follows the recommendation of the Declaration of Helsinki (1975) and was approved by the Ethics Committee of Unicamp, São Paulo, Brazil (CAAE #35316314.9.1001.5404 and CAAE #85020018.8.0000.5404). All patient data were fully anonymized.

Author contributions

Conceptualization, V.L.G.S.L and T.P.V.; methodology, M.M.C., E.P and V.L.G.S.L., software, R.H.; validation, M.M.C., E.P., M.A.T, T.P.V., G.R.C.C., and V.L.G.S.L.; formal analysis, M.A.T., M.M.C., E.P., and V.L.G.S.L.; investigation, M.A.T., M.M.C., T.H.S. and V.L.G.S.L.; resources, M.V. and V.L.G.S.L.; data curation, M.M.C., E.P., M.A.T. and V.L.G.S.L.; writing-original draft preparation, M.M.C., M.A.T., E.P., and V.L.G.S.L.; writing-review and editing, M.M.C., M.A.T., E.P., T.P.V., M.V., and V.L.G.S.L.; visualization. M.AT., and V.L.G.S.L.; supervision, M.V. and V.L.G.S.L.; project administration, M.V. and V.L.G.S.L.; funding acquisition, M.V. and V.L.G.S.L.

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