New *SHH* and Known *SIX3* Variants in a Series of Latin American Patients with Holoprosencephaly

Viviane Freitas de Castro^{a, b} Daniel Mattos^{a, b} Flavia Martinez de Carvalho^{b, c}
Denise Pontes Cavalcanti^d Milagros M. Duenas-Roque^e Juan Llerena Jr^{b, f}
Viviana Raquel Cosentino^g Rachel Sayuri Honjo^h Julio Cesar Loguercio Leiteⁱ
Maria Teresa Sanseverino^j Márcia Pereira Alves de Souza^j Pricila Bernardi^k
Ana Maria Bolognese^l Luiz Carlos Santana da Silva^{b, m} Pablo Barberoⁿ
Patricia Santana Correia^f Larissa Souza Mario Bueno^o
Clarice Pagani Savastano^a Iêda Maria Orioli^{a, b}

^aECLAMC at Departamento de Genética, UFRJ, Rio de Janeiro, Brazil; ^bInstituto Nacional de Genética Médica Populacional INAGEMP, Porto Alegre, Brazil; ^cECLAMC at Laboratorio Epidemiol. Malformações Congênitas, IOC/FIOCRUZ, Rio de Janeiro, Brazil; ^dDepartamento de Medicina Translacional, área de Genética Médica, FCM/UNICAMP, Campinas, Brazil; ^eECLAMC at Servicio de Genética, Hospital Nacional Edgardo Rebagliati Martins/EsSalud, Lima, Peru; ^fECLAMC at Centro de Genética Médica, IFF/FIOCRUZ, Rio de Janeiro, Brazil; ^gECLAMC at CEMIC/CONICET, Buenos Aires, Argentina; ^hUnidade de Genética, HC/FM/USP, São Paulo, Brazil; ⁱECLAMC at HC/UFRGS, Porto Alegre, Brazil; ^jUnidade Neonatal Nicola Albano, HUPE/UERJ, Rio de Janeiro, Brazil; ^kNúcleo de Genética Clínica, Departamento de Clínica Médica/UFSC, Florianópolis, Brazil; ^lDepartamento de Ortodontia, Faculdade de Odontologia/UFRJ, Rio de Janeiro, Brazil; ^mLaboratório de Erros Inatos de Metabolismo, Instituto de Ciências Biológicas/UFP, Belém, Brazil; ⁿRENAC, Centro Nacional de Genética Médica Dr. Eduardo E. Castilla/MS, Buenos Aires, Argentina; ^oComplexo Hospitalar Universitário Professor Edgard Santos, UFBA, Salvador, Brazil

Keywords

Holoprosencephaly · Latin American populations · *SHH* · *ZIC2* · *SIX3* · *TGIF1* · Synonymous variants

Abstract

Holoprosencephaly (HPE) is the failure of the embryonic forebrain to develop into 2 hemispheres promoting midline cerebral and facial defects. The wide phenotypic variability and causal heterogeneity make genetic counseling difficult.

Heterozygous variants with incomplete penetrance and variable expressivity in the *SHH*, *SIX3*, *ZIC2*, and *TGIF1* genes explain ~25% of the known causes of nonchromosomal HPE. We studied these 4 genes and clinically described 27 Latin American families presenting with nonchromosomal HPE. Three new *SHH* variants and a third known *SIX3* likely pathogenic variant found by Sanger sequencing explained 15% of our cases. Genotype-phenotype correlation in these 4 families and published families with identical or similar driver gene, mutated domain, conservation of residue in other spe-

cies, and the type of variant explain the pathogenicity but not the phenotypic variability. Nine patients, including 2 with *SHH* pathogenic variants, presented benign variants of the *SHH*, *SIX3*, *ZIC2*, and *TGIF1* genes with potential alteration of splicing, a causal proposition in need of further studies. Finding more families with the same *SIX3* variant may allow further identification of genetic or environmental modifiers explaining its variable phenotypic expression.

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Introduction

Holoprosencephaly (HPE) is the most common central nervous system (CNS) defect in humans. The prevalence rate is 1/250 conceptions [Matsunaga and Shiota, 1977]. In Latin America, the birth prevalence rate (live and stillbirths) was estimated at 1 per 10,000 [Orioli and Castilla, 2010]. This congenital brain malformation develops during the first month (between the 18th and the 28th day) of gestation and consists of incomplete cleavage of the forebrain into the cerebral hemispheres and subcortical structures along the CNS midline. The brain defects often result in midline facial anomalies and are classified into 3 types, from the most to the least severe: HPE alobar, semilobar and lobar, besides a milder subtype, the middle interhemispheric variant (MIHV). Generally, the severity of the facial defects is associated with the severity of the brain defect [DeMyer et al., 1964]; however, milder forms of facial anomalies may be present without brain malformation and then are called microforms such as the single median maxillary central incisor (SMMCI) or ocular hypotelorism [Cohen, 1989a; Richieri-Costa and Ribeiro, 2006].

Gross numeric and structural chromosomal anomalies cause 25–45% of the HPE cases. Variants in the genes sonic hedgehog (*SHH*) [Roessler et al., 1996], zinc finger protein of the cerebellum 2 (*ZIC2*) [Brown et al., 1998], sine oculis homeobox homolog 3 (*SIX3*) [Wallis et al., 1999], and TG-interacting factor (*TGIF1*) [Gripp et al., 2000] correspond to 20–25% of nonchromosomal/nonsyndromic HPE cases [Mercier et al., 2011]. Small copy number variations (CNV) and these single gene variants explain around 30–35% of the HPE cases [Dubourg et al., 2018; Kim et al., 2019], leaving around 70% of nonchromosomal/nonsyndromic HPE cases without a molecular diagnosis. Identifiable syndromes, variants in minor HPE genes, or more complex etiologies involving genetic and environmental factors also contribute to HPE etiologic heterogeneity [Cohen, 1989b; Roessler et al., 2012]. Few known environmental factors contribute to HPE com-

plex etiology in humans as prenatal maternal exposure to diabetes, alcohol drinking, retinoic acid, and cholesterol deficiency [Cohen and Shiota, 2002; revised by Johnson and Rasmussen, 2010].

SHH was the first gene to be associated with HPE [Roessler et al., 1996] and even now remains the leading cause of nonchromosomal HPE [Dubourg et al., 2016]. *SHH* encodes a signaling protein with a key role in early embryonic development, such as the development of brain and midline facial structures [Belloni et al., 1996]. The Human Gene Mutation Database (HGMD®; <http://www.hgmd.cf.ac.uk/>; access date February 18, 2020) holds 163 disease-causing variants in the *SHH* gene associated with HPE, while ClinVar exhibits 36 pathogenic/likely pathogenic variants (access date October 8, 2020) [Landrum et al., 2018]. The truncation variants are more clearly connected to the impaired signaling function of the *SHH* protein, while missense variants in both *SHH*-N, where they are more frequent, and *SHH*-C, indicate a role in *SHH* auto-catalytic processing throughout mechanisms not fully understood [revised by Roelink, 2018].

ZIC2 has been described as the second identified gene and the second most frequently mutated gene in HPE cases [Brown et al., 1998; Dubourg et al., 2007]. *ZIC2* has several roles in neurological development, including the generation of neural crest, and it acts as a transcriptional activator or repressor. Through its zinc finger domain, *ZIC2* is able to bind to DNA sequences that can also be bound by Gli proteins, which suggests the interaction of *ZIC2* in the *SHH* signaling pathway [Aruga, 2004]. *ZIC2* variants are linked to both classical and MIHV forms of HPE [Fernandes et al., 2007; Maurus and Harris, 2009]. In the MIHV, the error is in the dorsal neural patterning, unlike the classic forms of HPE where the error occurs in the ventral neural patterning. *ZIC2* hypomorphic alleles interacting latter with the *SHH* pathway could cause the MIHV, although the precise *ZIC2* molecular mechanisms are not well understood [reviewed by Barratt and Arkell, 2018].

The *SIX3* gene plays a role in the development of the eyes and anterior forebrain during early vertebrate development. The *SIX3* protein carries a DNA-binding homeobox domain and a *SIX* domain that can enlist factors to activate or repress other signaling pathways. The *SIX3* transcription factor forms complexes with the members of general corepressor Groucho to repress BMP, Wnt, and Nodal targets during the ventral forebrain growth and patterning. *SIX3* additional functions include to directly regulate *SHH* expression, but also be regulated by *SHH* in a feedback loop, acting on critical factors to the

ventral forebrain specification [revised by Domené et al., 2008 and Geng et al., 2016].

Human *TGIF1* encodes a homeobox protein with several alternative splice variants. All variants contain the 3-prime end of exon 10 and all of exon 11 encoding the homeodomain and the carboxyl-termini of the protein [Hamid et al., 2008]. *TGIF1* has several transcriptional repressor and corepressor roles in the retinoid and TGF β /Nodal and can independently regulate the SHH signaling pathway during the ventral midline forebrain patterning. Wotton and Taniguchi [2018] revised the several proposed mechanisms of how *TGIF1* variants can contribute to the HPE phenotype.

Several screenings for variants in patients with HPE gave an estimated rate of 8.2% in *SHH*, 7.4% in *ZIC2*, 3.9% in *SIX3*, and 1.1% in *TGIF1* [Mercier et al., 2011]. The low frequency initially described for *TGIF1* variants in HPE patients, 1.5% (95% CI 0.4–3.8) [Gripp et al., 2000], did not change in more recent cohorts [Mercier et al., 2011; Roessler et al., 2012, 2018a; Dubourg et al., 2016]. *TGIF1* has a minor HPE gene status nowadays [Dubourg et al., 2016; Roessler et al., 2018b] after other genes as *GLI2* (#165230), *FGFR1* (#136350), and *FGF8* (#600483) being recognized as often associated with HPE. Variants in these 3 genes are phenotypically polymorphic, being associated with several syndromes with or without HPE, for example, hypogonadotropic hypogonadism 2 with or without anosmia (#147950), Culler-Jones (#615849), Hartsfield (#615465), and several skeletal dysplasias. So, their frequency in HPE cohorts will depend on how many patients with HPE or microforms have a complete or incomplete form of any of those syndromes. At least 10 other genes, considered minor HPE genes, were also described in HPE patients [Dubourg et al., 2016; revised by Roessler et al., 2018b]. The number of the genes potentially involved are growing due to next-generation sequencing, like the *PPP1R12A* gene recently associated with HPE and urogenital malformations [Hughes et al., 2020].

Most of the HPE-associated gene variants are related to autosomal dominant inheritance [Odent et al., 1998]. The same variant frequently occurs in fully affected, mildly affected, or a normal person of the same family, indicating a complex etiology. There are examples of recessive inheritance in HPE, including compound heterozygous variants or homozygosity [El-Jaick et al., 2007; McCabe et al., 2011], digenic, and oligogenic inheritance with mutations in 2 or more HPE genes [Mouden et al., 2016; Dubourg et al., 2016, 2018]. However, they have been, until now, rare mechanisms in HPE pathogenesis,

and Roessler et al. [2012] showed that the “autosomal dominant with modifier” model better explained the HPE incomplete penetrance and variable expressivity [Roessler et al., 2018b]. Several models proposed in mouse, such as double haploinsufficiency, the interaction of a loss-of-function driver variant with a hypomorphic allele or silent allele, or with a background of modifiers, apply to human [Hong and Krauss, 2018]. Likewise, Roessler et al. [2018a] proposed it is important to consider co-occurring variants, both coding and non-coding, as it is sometimes possible to find evidence of potential gene-gene interactions that are consistent with a modifying effect. Such modifiers are context-dependent and can be silent without the presence of a driver variant [Roessler et al., 2018a].

New advances in studying HPE and other complex diseases to identify the genes acting to cause HPE in each family are those integrating genomic, epigenomic, transcriptomic, proteomic, phenomic, and metabolomic analysis [Kim et al., 2019; revised by Kerr et al., 2020]. Coherently with innovative approaches, Roessler et al. [2018a] said that “the best avenue to further our understanding of HPE lies in increasing numbers of cases rather than the number of genes considered.”

In the present study, we aim to find variants of *SHH*, *ZIC2*, *SIX3*, and *TGIF1* genes that could explain the HPE in a Latin American cohort to enlarge the phenotypic and genetic HPE data and search for families with the same driver variant earlier published. We evaluated 27 families with probands presenting with HPE or SMMCI. The 4 classical HPE genes and clinical data were investigated in these families and the literature reviewed for similar genetic variants.

Material and Methods

The 27 probands with HPE or SMMCI came from Brazil (20), Peru (4), and Argentina (3). Fresh blood or DNA and the clinical information of the probands and relatives were obtained by collaborators of the Latin American Collaborative Study of Congenital Malformations (ECLAMC). ECLAMC is a hospital-based network aiming for collaborative research on congenital anomalies [Castilla and Orioli, 2004]. Familial clinical data received from the patients' physicians included physical examination, genealogical information, prenatal maternal teratogenic exposures, brain imaging, laboratory results, and informed consent. The proband exclusion criterion was chromosomal abnormalities confirmed by a conventional karyotype. There is no karyotype information for 5 of the 6 patients with SMMCI. No patients, but one, were studied for CNV. We included probands with HPE found by prenatal ultrasound, CT scan, MRI, autopsy reports, and those with facial characteristics of HPE or SMMCI.

DNA Extraction and Sequencing

DNA was extracted by using salting out method [Miller et al., 1988]. Double strands of the coding regions and exon-intron borders of *SHH*, *ZIC2*, *SIX3*, and *TGIF1* genes were submitted to Sanger sequencing. Amplification protocols of these regions and primers were described in Savastano et al. [2014]. Amplicons were visualized in 1% agarose gel and further purified by GFX PCR DNA and Gel Band Purification kit (Ge Healthcare, UK), according to the manufacturer's instructions. Fragments were then sequenced using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Products were sequenced at ABI Prism 3130xL (Applied Biosystems). Found variants were confirmed by independent experiments. Sequence data were analyzed using ChromasPro software 1.41 (Technelysium®). Genomic, exonic, and protein sequences were downloaded from the National Center for Biotechnology Information (NCBI; <https://www.ncbi.nlm.nih.gov/>) using the hg19 genome version (online suppl. Table 1; for all online suppl. material, see www.karger.com/doi/10.1159/000515044).

Pathogenicity Prediction

To determine the pathogenicity status of all variants found, they were submitted to VarSome (<https://varsome.com/>), where they were classified according to ACMG criteria, and allele frequencies were annotated from the Genome Aggregation Database (gnomAD Exomes v.2.1.1). Variants were surveyed if already published in HGMD, and only those not found were considered new variants. Variants were evaluated at Human Splicing Finder (HSF; <http://www.umd.be/HSF/index.html>, access date February 18, 2020) for signals of impact on splicing *cis* elements that could alter the transcript [Ohno et al., 2018].

Clinical Presentations and Results

General Clinical Information

In this Latin American series, there were 3 fetuses, 7 newborns, and 17 patients with ages below 15 years. Figure 1 shows the 20 facial images available. Table 1 shows the country and the clinical features of the 27 probands. Twenty-one patients were ascertained due to a cerebral defect and 6 due to SMMCI. Only 1 patient presented with cebocephaly with more severe facial alterations, as cyclopia or ethmocephaly, and did not appear in this series. With or without brain defects, all patients showed developmental delay and/or intellectual disabilities, except the 4-month-old patient, who was not tested.

Patients with *SHH*, *ZIC2*, *SIX3*, and *TGIF1* Likely Pathogenic Variants

Patient 1

A 6-year-old male was born as the first child of a healthy 27-year-old mother and her 42-year-old nonconsanguineous healthy husband. The proband had mild microcephaly, right cleft lip and palate, and SMMCI. Cerebral images were not available, and he presented severe

learning difficulties with preserved social skills (Table 1; Fig. 1). The proband presented a de novo likely pathogenic *SHH* missense new variant (c.437T>G; p.Val146Gly) (Table 2; Fig. 2). He also presented a synonymous single nucleotide variant (sSNV) in *SIX3* (rs78018362; p.Ala30=; c.90G>T), and 3 benign *TGIF1* variants: in the 5' UTR (rs23813; c.-33C>A), a synonymous variant (rs2229337; p.Pro140=; c.420A>G), and a missense variant (rs4468717; p.Pro163Ser; c.487C>T). The proband's mother also presented these 4 variants, and his half-sister only showed the 3 *TGIF1* variants (Table 2; Fig. 2). HSF suggested the *SIX3* synonymous variant rs78018362, with 0.88–9.32% population frequency, potentially produces an alteration of splicing with the creation of an exonic splicing silencer site and change of an exonic splicing enhancer (ESE) site. The missense *TGIF1* variant was predicted to have no impact on splicing.

Patient 2

A 1-year-old male was born at term as the first child of a healthy 23-year-old mother and her healthy 26-year-old nonconsanguineous husband. The proband had microcephaly, ocular hypotelorism, a flat nose, and corrected premaxillary agenesis (Table 1). Cerebral CT showed a MIHV-HPE and corpus callosum hypoplasia. He presented with a new likely pathogenic small *SHH* deletion (c.329_337delCCATCTCGG; p.Ala110_Ser112del), and these 9-bp in-frame deletion induces the loss of 3 amino acids (alanine, isoleucine, and serine) in the polypeptide chain (Table 2; Fig. 2). The proband also presented a benign *ZIC2* in-frame duplication (c.716_718dupACC; p.His239dup) (Table 2; Fig. 2). This small duplication expands the tract from 9 to 10 histidines as previously reported in control individuals [Brown et al., 2001]. HSF estimated this small in-frame duplication, with a population frequency of 0.07–12.70%, potentially produces an alteration of splicing with the creation of an ESE site. Parents' DNA was not available.

Patient 3

A 40-week-old male neonate was born as the first child of a nonconsanguineous 23-year-old father and mother. The proband had microcephaly, premaxillary agenesis, ocular hypotelorism, and bilateral exophthalmia. The cerebral MRI showed semilobar HPE (Table 1). A new likely pathogenic *SHH* missense variant (c.1088G>T; p.Cys363Phe) (Table 2; Fig. 2) was detected in the proband and his sister and mother, both affected with milder craniofacial anomalies. Both children, but not their mother, also carried a *ZIC2* synony-

Fig. 1. The craniofacial aspect of 20 holoprosencephaly (HPE) probands in this series. Patients 2, 3, 9, 23, and 27 are not shown. Patients 8, 13, 24, 25, and 27 with single median maxillary central incisor had no brain defect, and the information was not available for patient 1. Among the HPE group, all the frequent HPE types were diagnosed: 7 alobar HPE in patients 5, 6, 10, 11, 14, 16, and 18; four semilobar in patients 3, 9, 20, and 22; five lobar in patients 4, 12, 17, 21, and 26; and 2 middle interhemispheric variants in pa-

tients 2 and 19; in the remaining 2 patients 7 and 23, the HPE type was not further specified. Patient 15 presented only with corpus callosum agenesis. Twelve patients presented with premaxillary agenesis (2, 3, 5, 6, 10, 11, and 14) or cleft lip and palate (1, 4, 7, 16, and 26). Fourteen patients showed the absence of facial cleft (8, 9, 12, 13, 15, 17–22, 24, 25, and 27). Microphthalmia was described in patients 5, 6, 7, 23, and 27.

Table 1. Overview of the demographic and clinical findings of patients

Patient ID	Country	Sex	Age	NDD	HPE type	Facial type	Other features	Observations
1	Brazil	M	4 ys	+	NFS	SMMCI, uCLP	Microcephaly (<–2 SD), standard height and weight, flat nose, anodontia of teeth 51, mesialized 61 tooth crown, teeth 81 and 82 are fused with one root and two crowns	Mat. half-sister 14 ys, small for age, average academic performance, suspected of ADHD
2	Peru	M	1 year	+	MIHV	Premaxillary agenesis	Small for age, microcephaly (<–2 SD), corpus callosum hypoplasia, hypotelorism, and small flattened nose	
3	Brazil	M	NB 40 w	+	Semilobar	Premaxillary agenesis	Microcephaly (<–3 SD), hypotelorism, and bilateral exophthalmia	Young sister had normal cerebral MRI, normal neurodevelopment, choanal stenosis, reduced maxilla, SMMCI, and ocular hypotelorism; mother with hypotelorism
4	Brazil	F	1.7 ys	+	Lobar	bCLP	Microcephaly (<–3 SD), corpus callosum agenesis, sparse anterior and posterior scalp hair, hypotelorism, flat nose, retrognathia, external genitalia with hypoplasia of labia minora and few sparse pubic hairs, and hypoplastic palmar creases	Mat. exposure to glue vapors
5	Brazil	M	NB 36 w	NA	Alobar	Premaxillary agenesis	Hydrocephalus (<–2 SD), right microphthalmia, hypotelorism, left eyelid coloboma with ipsilateral proptosis, arhinia, right microtia, and left dysmorphic ear, atrial and ventricular septal defects, double outlet right ventricle, and pulmonary trunk hypoplasia	
6	Brazil	M	0.9 y	+	Alobar	Premaxillary agenesis	Microcephaly, hydrocephalus (<+3 SD), hypotelorism, nasal hypoplasia, bilateral microphthalmia, iris coloboma, scrotum hypoplasia, double left ureters, appendicular hypertonía and axial hypotonia, and seizures	Fourth-degree mat. cousin had CLP, mat. Great-aunt had NDD.
7	Brazil	M	SB 26 w	NA	NFS	mCLP	Microcephaly (<–2 SD), oxycephaly, arhinia, hypotelorism, bilateral microphthalmia and microtia, and median cleft lip and soft palate	
8	Brazil	M	0.3 y	NA	No HPE	SMMCI, no facial cleft	Anterior narrowing of the nasal fossa, bilateral stenosis of the piriform opening, lacrimal ducts stenosis, subclinical hypothyroidism	Mat. diabetes and pulmonary tuberculosis
9	Brazil	M	NB 38 w	NA	Semilobar	No facial cleft	Hydrocephalus (>+3 SD), and cranial asymmetry with open cranial sutures	
10	Brazil	M	0.3 y	NA	Alobar	Premaxillary agenesis	Absent nasal bone, hypotelorism, patent foramen ovale, and micropenis	
11	Brazil	F	NB 38 w	NA	Alobar	Premaxillary agenesis	Microcephaly, cerebral parenchyma limited to the frontal region, arhinia, hypotelorism, and a sacral dimple	Mat. exposure to arterial hypertension medication
12	Brazil	F	2.10 ys	+	Lobar	No facial cleft	Microcephaly (<–3 SD), diffuse reduction of brain parenchyma, subependymal heterotopy, and corpus callosum dysgenesis	
13	Brazil	F	11 ys	+	No HPE	SMMCI, no facial cleft	Short nasolabial philtrum, upper lip without cupid bow, micrognathia	
14	Peru	M	NB 37 w	NA	Alobar	Premaxillary agenesis	Hydrocephalus, hypotelorism, nasal hypoplasia, atrial septal defect, bilateral equinovarus feet	
15	Brazil	M	1.9 ys	+	No HPE	No facial cleft	Microcephaly (<–3 SD), global volumetric reduction of the brain parenchyma, mainly affecting supratentorial white substance, and corpus callosum agenesis	

Table 1 (continued)

Patient ID	Country	Sex	Age	NDD	HPE type	Facial type	Other features	Observations
16	Argentina	F	SB 23 w	NA	Alobar	bCLP	Cavum of septum pellucidum absence and fused anterior ventricular horns	Intellectual handicap in three mat. First-cousins
17	Brazil	F	15 ys	+	Lobar	No facial cleft	Microcephaly (<-3 SD), hydrocephalus, craniosynostosis, narrow frontal, hypotelorism, narrow eyelids fissures, high nasal bridge, anteverted nostrils, long nasal filter, micrognathia, malformed ears, ventricular septal defect, right thumb distal phalanx duplication, and right inguinal hernia	Parents are double first-cousins
18	Brazil	M	5 ys	+	Alobar	No facial cleft	Hydrocephalus, hypotonia, prominent frontal, high frontal hair, sparse eyebrows and long lashes, bilateral convergent strabismus, microstomia, deep palmar creases, bilateral second toe hypoplasia, and bilateral third-fourth toes syndactyly	
19	Argentina	M	4.2 ys	+	MIHV	No facial cleft	Hydrocephalus (<+3 SD), Chiari malformation I, corpus callosum dysgenesis, polymicrogyria occipitoparietal, craniofacial asymmetry, hypotelorism, and straight eyebrows	
20	Brazil	F	3.11 ys	+	Semilobar	No facial cleft	Microcephaly (<-2 SD), agenesis of the corpus callosum and septum pellucidum, fusion of midline structure, substance of the radiated crown and frontal lobes, prominent frontal bossing, high frontal hair line, ocular hypertelorism, left preauricular appendage, and internal auditory meatus stenosis	Mat. diabetes and remote parental consanguinity
21	Brazil	F	SB 24 w	NA	Lobar	No facial cleft	Hydrocephalus (<+2 SD), macrocephaly, prominent glabella, prominent occipital, small nose with anteverted nares, and ocular hypertelorism	Mother 47,XXX/46,XX presented bicornuate and septate uterus
22	Brazil	M	0.4 y	NA	Semilobar	No facial cleft	Corpus callosum agenesis, nasal hypoplasia, high frontal hair line, right epicanthus, left preauricular appendage, small nose, retrognathia, and deep palm creases	Mat. diabetes
23	Brazil	F	SB 32 w	NA	NFS	Cebocephaly	Hydrocephalus (<-1 SD), arhinia, bilateral microphthalmia, ascites, complex heart defect, single right kidney, and proximally shortened four limbs	Mat. hypothyroidism and depression
24	Argentina	F	4.6 ys	+	No HPE	SMMCI, no facial cleft	Microcephaly (<-2 SD), flat face, superior labial frenulum absence, hypotelorism, right kidney not visualized, narrow lumbar vertebrae, coccyx absence, bilateral short fifth fingers, and fourth-fifth toes	
25	Brazil	M	5.10 ys	+	No HPE	SMMCI, no facial cleft	Standing height and weight below P3, axial hypotonia, panhypopituitarism, normal cephalic circumference, median face hypoplasia, ocular hypertelorism, eyelid paralysis, blue sclera, high palate, midnasal stenosis, small ears, bilateral auditive loss, microretrognathia, scoliosis, and sacral fold	
26	Peru	M	NB 37 w	NA	Lobar	mCLP	Microcephaly (<-3 SD), hypotelorism, nasal hypoplasia	
27	Brazil	M	11 ys	+	No HPE	SMMCI, no facial cleft	Brachycephaly (<-2 SD), lower frontal and occipital hair line, abundant and dry hair, synophrys, ocular hypertelorism, microphthalmia, iris coloboma, omphalocele, diaphragmatic hernia, anal atresia, hydronephrosis, neurogenic bladder, bilateral cryptorchidism, joint hypermobility, thoracic scoliosis, right lumbar vertebrae foveae, and pes planus	

NB, newborn; SB, stillbirth; ys, years; w, weeks; HC SDS, head circumference standard deviation score (Intergrowth-twenty-first) for age and sex; NDD, neurodevelopmental delay; +, present; NA, not applicable; NFS, not further specified; SMMCI, single median maxillary central incisor; uCLP, bCLP, mCLP, unilateral, bilateral, median cleft lip and cleft palate; MIHV, middle interhemispheric variant; Mat, maternal; hypotelorism = ocular hypotelorism.

Table 2. Variants of *SHH*, *SIX3*, *ZIC2*, and *TGIF1* genes identified in this study

ACMG verdict	Patient ID	Gene	Mutation	Amino acid change	Mutation type	Inheritance	Population frequency range	RefSNPs	Potential splicing alteration (HSF)	References
Likely pathogenic										
	1	<i>SHH</i>	c.437T>G	p.Val146Gly	Missense	de novo	-	-	ESE alt, +ESS	This report
	2	<i>SHH</i>	c.329_337delCCATCTCTCGG	p.Ala110_Ser112del	Deletion	ND	-	-	ECD act, +ESS	This report
	3	<i>SHH</i>	c.1088G>T	p.Cys363Phe	Missense	Mother	-	-	No	This report
	6	<i>SIX3</i>	c.404_407dupGCGC	p.Val137Argfs*18	Frameshift	de novo	-	-	No	Domené et al., 2008 Paulussen et al., 2010
Benign										
	1	<i>SIX3</i>	c.90G>T	p.Ala30=	Synonymous	Mother	0.009–0.093	rs78018362	ESE alt, +ESS	Orioli et al., 2001
	1	<i>TGIF1</i>	c.-33C>A	-	5 prime UTR	Mother	0.096–0.581	rs238132	-	El-Jaick et al., 2007
	1	<i>TGIF1</i>	c.420A>G	p.Pro140=	Synonymous	Mother	0.048–0.292	rs2229337	No	Orioli et al., 2001
	1	<i>TGIF1</i>	c.487C>T	p.Pro163Ser	Missense	Mother	<0.001–0.154	rs4468717	No	Gripp et al., 2000
	2	<i>ZIC2</i>	c.716_718dupACC	p.His239dup	Duplication	ND	<0.001–0.127	rs398124241	ESE alt	Brown et al., 2001
	3	<i>ZIC2</i>	c.1059C>T	p.His353=	Synonymous	ND	<0.001–0.166	rs1831992	ESE alt	Roessler et al., 2009b
	4	<i>SHH</i>	c.897G>C	p.Leu299=	Synonymous	Father	0–0.009	rs9333635	ESE alt	-
	5	<i>SHH</i>	c.630C>T	p.Gly210=	Synonymous	ND	0–0.038	rs9333634	ECD act, +ESS	Nanni et al., 1999
	7	<i>SIX3</i>	c.219C>T	p.Pro73=	Synonymous	ND	<0.001–0.001	rs186163123	ESE alt	Wallis and Muenke, 2000
	8	<i>SIX3</i>	c.576C>T	p.Arg192=	Synonymous	ND	<0.001–0.049	rs182881	ESE alt, ECD act	Wallis and Muenke, 2000
	9	<i>ZIC2</i>	c.716_718dupACC	p.His239dup	Duplication	ND	<0.001–0.127	rs398124241	ESE alt	Brown et al., 2001
	10	<i>ZIC2</i>	c.716_718dupACC	p.His239dup	Duplication	ND	<0.001–0.127	rs398124241	ESE alt	Brown et al., 2001
	11	<i>ZIC2</i>	c.1239+18G>A	-	Intronic	ND	0–0.066	rs139312964	No	Ribeiro et al., 2012

ACMG, American College of Medical Genetics; ND, not determined; Population allele frequency by gnomAD, Exomes v2.1.1; HSF, Human Splicing Findings; ESE alt, exonic splicing enhancer site alteration; + ESS, exonic splicing silencer site creation; ECD act, exonic cryptic donor site activation. Sequence coding access provided in online supplementary Table 1.

Patients with Only Benign Variants

There were 4 exonic synonymous variants in 7 patients, 1 exonic codon duplication, and 1 intronic variant (Table 2). These variants were predicted as benign, but they may have a potential role in HPE due to the possibility of impacting splicing as estimated by HSF. The clinical data and variants of patients 4, 5, 7, 8, 9, 10, and 11 were shown in Figure 1, Tables 1 and 2, and online supplementary Table 2.

Discussion

Cohort Characteristics

We observed some accepted correlations among variants of *SHH* and *SIX3* and the HPE phenotype in our series. The 3 patients with *SHH* variants presented less severe HPE and relatively less-affected facial features than the patient with *SIX3* variants, who also had severe eye defects. However, due to the small number of patients (27), the presence or absence of these characteristics has no statistical significance. The phenotype-genotype correlations in HPE were confirmed in large cohorts by Solomon et al. [2010], Mercier et al. [2011], and Dubourg et al. [2016]. Consistent correlations were the higher prevalence of facial anomalies in patients with *SHH* variants [Solomon et al., 2012], contrasting with no significant facial changes in those with *ZIC2* variants [Solomon et al., 2010]. More severe HPE was predominant in patients with variants in *ZIC2* and *SIX3*, and *SHH* variants were associated with the milder cases or microforms. Keaton et al. [2010] described a wide range of clinical severity in patients with *TGIF1* or *SHH* variants, showing less severe phenotypes than those observed for either the *ZIC2* or *SIX3* genes. Mercier et al. [2011] noted that alobar HPE with severe facial features seems to be more frequent in patients with *TGIF1* variants and suggested to reanalyze in larger series.

Considering the known phenotype-genotype correlations, we found 2 inconsistencies in one patient (2) with MIHV. He presented premaxillary agenesis and a pathogenic variant in the *SHH* gene. It is more frequent to find a *ZIC2* pathogenic variant and a mildly affected face in patients with MIHV [Mercier et al., 2011]. Besides the *SHH* variant, this patient had a *ZIC2* histidine tract expansion predicting changes in the ESE site. A *ZIC2* variant effect would be more in line with the presence of MIHV, although other genes not studied here have been described in patients with MIHV, such as *FGF8* [Hong et al., 2018]. The other patient (19) with MIHV in our series

was without facial dysmorphias, type 4 [Mercier et al., 2011], as expected, and showed polymicrogyria, a defect involving neuronal migration also correlated with *ZIC2* variants [Solomon et al., 2010; Mercier et al., 2011]. However, we did not find any variant or environmental teratogen in patient 19.

Another common characteristic of the HPE cohorts is the female-biased sex ratio, mainly involving the *ZIC2* and *SIX3* variants [Mercier et al., 2011]. Recently, Kruszka et al. [2019] showed 33 HPE cases associated with loss-of-function X-linked cohesin complex variants, of which 32 were females due to potential male lethality from these variants. Those 32 patients had less severe facial types (3 or 4) [Mercier et al., 2011], pointing to a particular subgroup of HPE patients. We found that only ethmocephaly, cebocephaly, and premaxillary agenesis were the facial types associated with the female-biased sex ratio in an earlier Latin American HPE cohort of 195 patients [Savastano et al., 2014]. There is no female excess in the current series with 17 males and 10 female patients, and patients had less severe or normal faces with only 1 patient with cebocephaly and 7 with premaxillary-agenesis, as observed before [Savastano et al., 2014].

Pathogenic Variants and Phenotypes

The variants found in patients 1 and 2, p.(Val146Gly) and p.(Ala110_Ser112del), were located toward the *SHH* N-terminal signaling domain, while the variant in patient 3 was in the *SHH*-C. This fact endorses the current idea that variants in structurally relevant modules in both the N- and C-terminal domain of *SHH* can prevent the processing of SHH pro-protein causing HPE [Traiffort et al., 2004; Roessler et al., 2009b; revised by Roelink, 2018].

Patient 1 exhibited a de novo variant in *SHH* (c.437T>G; p.Val146Gly) involving the Val-146 residue, close to the Asp-147 residue, one of the Zn²⁺ binding site coordinating residues in the SHH N-domain. The other coordinating residues are His-140, Glu-176, and His-182. Traiffort et al., [2004], in their functional study to characterize *SHH* variants, proposed the proximity of their mutated Thr-150 residue, p.(Thr150Arg), to the Asp-147 to explain the impaired production of the active SHH N-fragment. The mutated Val-146 of our patient 1 was in closer proximity to the Asp-147 residues, probably resulting in loose protein-protein interactions in some SHH N-complexes with its partners, but not in others, as proposed by Bosanac et al. [2009] for variants in the Zn²⁺ binding site coordinating resi-

dues. So, this uncertain negative effect of those variants around the Zn^{2+} coordinating residues [Bosanac et al., 2009] could be related to the mild defects in patient 1, as microcephaly (-2 SD) cleft lip, SMMCI, and intellectual disabilities. The patient with the variant in Asp-150Thr [Traiffort et al., 2004] had classical HPE, described by Dubourg et al. [2003] with semilobar HPE, microcephaly, hypotelorism, cerebellar hypoplasia, and diabetes insipidus. It would be interesting to know the patient's phenotype with the p.(Asp147Arg) variant, but a further phenotypic description of the patient is not available [Roessler et al., 2009a].

Patient 1, his mother, and the half-sister bore altogether 4 different nonpathogenic variants in *SIX3* and *TGIF1*, 4 in the proband, besides the pathogenic *SHH* missense de novo variant. Clinically, only the patient presented a mild neuropsychological development delay with mild defects of the HPE spectrum. His half-sister was small for her age, without information about hypophyseal studies, and suspected of ADHD. The mother's intellectual abilities were not tested, and the cerebral images of the proband and half-sister were not available, which hindered a more specific genotype-phenotype correlation in this family. The 3 *TGIF1* variants present in the proband, mother and maternal half-sister, a missense, a 5'UTR, and a synonymous, were described as common by El-Jaick et al. [2007]. Also, El-Jaick et al. [2007] did functional studies on *TGIF1* variants, including a missense variant, p.(Ser162Phe), located near the variant p.(Pro163Ser) found in the patient 1 family. The El-Jaick et al. [2007] patient had encephalocele, corpus callosum agenesis, median cleft lip, and inherited a paternal p.Ser-162Phe variant, whose protein performed normal in the functional studies.

Among the proband's 4 nonpathogenic variants, the synonymous *SIX3* p.Ala30= could potentially create an exonic splicing silencer site and change an ESE site. This variant mean frequency in the general population is 3.5%, which is found in less than 1.5% of Europeans and Africans, 2 important components of the three-hybrid Latin American population. Only the proband had the *SIX3* p.Ala30= synonymous variant combined with de novo *SHH* c.437T>G; p.Val146Gly variant, which could reinforce the pathogenicity of this last variant.

Regarding the *SHH* small in-frame deletion, c.329_337delCCATCTCGG; p.Ala110_Ser112del, found in patient 2, a point variant generating a stop codon in the same region (c.335C>A; p.Ser112*) was described by Roessler et al. [2012]. This fact would indicate that not only the truncation of the protein at this site is patho-

genic, but also the deletion of these 3 amino acid residues (Ala-110, Iso-111, and Ser-112). Patient 2 variant is not present in HGMD; however, other 6 different published missense variants involve these 3 residues [Nanni et al., 2001; Dubourg et al., 2004; El-Jaick et al., 2005; Roessler et al., 2009b, 2012]. Indeed, the *SHH*-N large α helix opposite to the Zn^{2+} coordination domain, from residue 100 to 117, showed 11 different variants found in holoprosencephalic patients [Traiffort et al., 2004; revised by Roelink, 2018]. Three different variants were tested in this region, 2 of which at residue Trp-117 [Maity et al., 2005] impaired the autocatalytic processing reaction, and 1 at residue Ile-111 [Singh et al., 2009] which decreased stability or secretion of *SHH*-N protein. Only the stop codon variant p.(Ser112*) [Roessler et al., 2012] seems incompatible with the complete protein and justifies the severe phenotype alteration, as the patient with this variant exhibited cyclopia. The 3 codon deletions and other missense variants at these residues showed variable phenotypic alterations from just SMMCI in p.(Ile111Phe) [Nanni et al., 2001] or corpus callosum agenesis in p.(Ala110Asp) [Dubourg et al., 2004] to MIHV with premaxillary agenesis or alobar HPE with premaxillary agenesis seen in our patient 2, p.(Ala110_Ser112del), and the patient described by El-Jaick et al. [2005], respectively.

The variant in *SHH*, c.1088G>T, found in patient 3 changed a cysteine to a phenylalanine residue (p.Cys363Phe) in the C-terminal domain. Richieri-Costa and Ribeiro [2006] and Kruszka et al. [2015] reported a similar variant but changing Cys-363 to a tyrosine residue (c.1088 G>A; p.Cys363Tyr). Both substituted the small cysteine with larger amino acids, potentially disrupting the protein conformation at this site. Ser-362, Cys-363, and Tyr-364 (Fig. 2) were among the highly conserved residues in the intein-like elements [Roessler et al., 2009a], and 3 additional variants, c.1085C>A (p.Ser362*); c.1085C>A (p.Ser362Leu), and c.1091A>G (p.Tyr-364Cys) were described by Roessler et al. [2009b] and Solomon et al. [2012], the first one in a patient with alobar HPE, and the others in patients with microcephaly and other minor HPE signs.

Our patient 3 (*SHH* p.Cys363Phe) had semilobar HPE with premaxillary agenesis similar to the patient published by Kruszka et al. [2015], p.(Cys363Tyr), but different from the patient published by Richieri-Costa and Ribeiro [2006], also with the p.Cys363Tyr variant, who presented an HPE-like face, a cyst of the choroidal fissure, and a normal neuropsychological evaluation. All of these 5 patients had affected relatives with minor HPE forms,

like our patient 3, showing that the wide intra- and inter-familial phenotypic variability observed is not constrained by the high phylogenetic conservation of the Ser-362, Cys-363, and Tyr-364 residues. Also, we can note that the most severe case occurred in a truncation variant as expected [Solomon et al., 2012].

The last pathogenic variant identified in this HPE series was a *de novo* small duplication (c.404_407dupGCGC; p.Val137Argfs*18) that occurred inside 6 GC repeats of the SIX-domain of the SIX3 gene. Ribeiro et al. [2006] (c.406_407dupGC; p.Val137Profs*115) and Domené et al. [2008] (c.404_407dupGCGC; p.Val137Argfs*18 and c.405_409dupCGCCG; p.Val137Alafs*116) had already identified 3 truncation variants in this region, with duplication of 1 GC, 2 GC, and a CGCCG duplication. Our patient 6 belongs to the third family described with the SIX3 c.404_407dupGCGC; p.Val137Argfs*18 variant; the second family described by Paulussen et al. [2010]. These 3 frameshift variants changed the Val-137 residue producing a stop codon after the next 18 residues, as in our patient 6, or after the 115 or 116 residues as in the other 2 duplications. Domené et al. [2008] did functional studies using zebrafish assays on these 3 frameshift variants showing that truncated SIX3 proteins lacking DNA-binding site could yet retain some repressive activity. They also showed that all 3 variants affected the SIX3 ability to repress BMD but not to equally repress Wnt [Domené et al., 2008]. Despite the observed functional differences, where the c.406_407dupGC, Val137Profs*115 was more severe than the GCGC or CGCCG duplications, because its mutants lost the capacity to repress both Wnt and BMD signaling, the correlation with the patients' phenotype is not so clear. The patient described by Ribeiro et al. [2006] with dup GC showed the same phenotype as in our patient 6 with dup GCGC, both presenting alobar HPE and premaxillary agenesis, while the patients described by Paulussen et al. [2010] and by Lachawan et al. [2009] (the same case studied by Domené et al. [2008]) with c.405_409dupCGCCG variants presented semilobar HPE. Furthermore, the variant causing the most severe loss of function (c.406_407dupGC, Val137Profs*115), reported by Ribeiro et al. [2006], was present in the patient's father and paternal grandmother, both nonaffected individuals, disclosing further clinical variability. These 3 persons also presented a SIX3 variant (c.206G>A; p.Gly69Asp), tested near-normal functional activity [Domené et al., 2008]. Analyzing the diverse ways SIX3 participates in the ventral telencephalon specification, Geng et al. [2016] showed in a mouse model that different reduction in SIX3 dosage causes different HPE types, as alobar, lobar, semilobar or

normal brain. According to the disclosed ways of SIX3 action, a variant in SHH, PATCHED, FOXG1, or in the SIX3 regulatory region, added to the SIX3 variant, must result in different human HPE forms [Geng et al., 2016]. Additional variants in any of these modifiers could explain the SIX3 functional studies differences between the observed phenotype and the expected. Then, further study of those 5 families with SIX3 variants inside the GC repeats of the SIX-domain using integrated -omics analysis [Kim et al., 2019; Kerr et al., 2020], followed by proper allele-specific functional analysis [Domené et al., 2008; Hong et al., 2016; Soukarieh et al., 2016; Kim et al., 2020] could help to increase our understanding of HPE genes pathogenesis.

Nonpathogenic Variants

Pathogenicity status of variants follows strict rules to not include false-positive variants in curated databases, which could lead to false molecular diagnostics and wrong genetic counseling. We followed ACMG guidelines to classify variants. However, knowledge about variants' impact is still growing, and pathogenicity assigning algorithms may elude potentially pathogenic variants [Hong et al., 2017; Kim et al., 2019]. The exclusion of variants with lesser probabilities of pathogenicity creates a bias toward continuously excluding them from publications. For these reasons, we decided to report 13 variants classified as benign that occurred in 11/27 patients. Seven of these nonpathogenic variants are synonymous, and the others a missense, small duplication, 5'UTR, or intron variant.

Chen et al. [2010] did a broad survey of single nucleotide variants (SNVs) reviewing genome-wide association studies. They found that nonsynonymous SNV (nsSNV) and synonymous SNV (sSNV) exhibited similar likelihood and effect size for disease association. Although acknowledging that these sSNVs might not be causal, they argue that these sSNVs are not likely to be in linkage disequilibrium with another nsSNV and that they are highly likely to be implicated in the disease process [Chen et al., 2010]. sSNV may affect many molecular aspects that could lead to pathogenic dysfunctional proteins. sSNV can disturb transcription as ~15% of human codons simultaneously code for amino acids and transcription factor recognition sites [Stergachis et al., 2013]. They can also alter the expected splicing as exemplified by Pagani et al. [2005] that a quarter of all sSNVs tested resulted in exon 12 skipping in the CFTR protein. sSNVs may also interfere with mRNA secondary structure and stability. Presnyak et al. [2015], studying yeast, showed that codon optimality (the advantage of a codon over another that codes for the same amino acid) contributes to mRNA sta-

bility and thus to fine-tuning the levels of gene expression due to variations in the half-life of transcripts with different sSNV. Codon optimality may also interfere in the rate of elongation during translation in yeast and thus on the early protein stability [Pechmann and Frydman, 2013].

In a recent study, Kim et al. [2020] found sSNV enriched in patients with HPE compared to healthy individual cohorts. Further investigating these sSNV, the authors found no evidence of their impact on splicing by both in silico and in vitro assays. However, 5 sSNV predicted by in silico analyses to impact the translation of *SHH* were also confirmed by in vitro assays to result in a decrease of the *SHH* protein quantity. Furthermore, they presented evidence that this reduction in protein is due to selective degradation by the proteasome. In our series, families 4 and 5 carried 2 of the 5 sSNV studied by Kim et al. [2020]. *SHH* c.897G>C, seen in family 4, reduced the *SHH* protein by 13%, and *SHH* c.630C>T, observed in family 5, reduced the protein by 5%. Both our patients were severely affected, and their cephalic and extracephalic defects have been described in HPE cohorts [Martinez et al., 2018]. To detect sSNV with potential splicing effects, we have used HSF, a bioinformatic tool that was qualified by experimental studies as the minigene splicing assay [Soukarieh et al., 2016]. Our inspection of the mRNA structure of *SHH* c.897G>C variant compared with wild mRNA using VARNA (<http://varna.lri.fr/>) [Darty et al., 2009] revealed structural modifications. However, Kim et al. [2020] inspecting the optimal mRNA structure and base-pairing probabilities did not predict any changes. Then, our preliminary information about the variants not tested by Kim et al. [2020] requires further research to show the effective involvement of these sSNVs in HPE causality, especially functional studies like the splicing minigene reporter assay [Soukarieh et al., 2016; Kim et al., 2020]. In the future, it will need confirmation that the results from the minigene context are the same in the patient cellular and genomic context [Fu and Ares, 2014]. In the series presented here, we could not exclude that patients only carrying benign variants also had a loss-of-function variant in HPE genes that were not studied here.

Conclusion

We studied the *SHH*, *ZIC2*, *SIX3*, and *TGIF1* genes in a Latin American series of 27 patients. We found 4 families with 3 novel variants in *SHH* and a third family with the same published *SIX3* frameshift variant, explaining

15% of our cases. Recent literature shows that the classical 4 genes stay an important source of rare variants. There are novel genes implicated in HPE causality, mainly as modifiers that must be studied in negative or inconclusive cases. The third family we found with a *SIX3* rare variant (p.Val137Argfs*18) deserves a collaborative study including all similar families previously published. We suggest observing the phenotype-genotype correlations based on NGS results and also from other -omics tools in families with the same driver variant as a productive approach to our understanding of HPE pathogenesis. Nine patients, including 2 with *SHH* pathogenic variants, presented benign variants with potential alteration of splicing, a causal proposition in need of further studies. The role of splicing errors from synonymous or other benign variants causing human diseases is well known in the medical literature. However, HPE literature frequently overlooked those variants.

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Statement of Ethics

The study was approved by the Research Ethical Committee affiliated with the Brazilian Ministry of Health (CAAE-59-488716.1.1001.5269). All procedures performed in this study were in accordance with the ethical standards specified in the World Medical Association Declaration of Helsinki. Informed consent was obtained for all patients and relatives for this study and the publication of images.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

I.M.O., V.F.d.C., C.P.S.: study design. V.F.d.C.: manuscript preparation. I.M.O., V.F.d.C., D.M.: revision of the manuscript. V.F.d.C., D.M., I.M.O.: data analysis. F.M.d.C., D.P.C., M.M.D.-R., J.L.Jr, V.C., R.S.H., J.C.L.L., M.T.S., M.P.A.d.S., P.B., A.M.B., L.C.S.d.S., P.B., P.S.C., L.S.M.B., I.M.O.: patient recruitment, clinical examination, and data collection. V.F.d.C., C.P.S.: laboratory work. I.M.O.: grant application, receipt of the funding, and supervision of the research.

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