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Research Article

Molecular analysis of holoprosencephaly in South America

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Abstract

Holoprosencephaly (HPE) is a spectrum of brain and facial malformations primarily reflecting genetic factors, such as chromosomal abnormalities and gene mutations. Here, we present a clinical and molecular analysis of 195 probands with HPE or microforms; approximately 72% of the patients were derived from the Latin American Collaborative Study of Congenital Malformations (ECLAMC), and 82% of the patients were newborns. Alobar HPE was the predominant brain defect in almost all facial defect categories, except for patients without oral cleft and median or lateral oral clefts. Ethmocephaly, cebocephaly, and premaxillary agenesis were primarily observed among female patients. Premaxillary agenesis occurred in six of the nine diabetic mothers. Recurrence of HPE or microform was approximately 19%. The frequency of microdeletions, detected using Multiplex Ligation-dependant Probe Amplification (MLPA) was 17% in patients with a normal karyotype. Cytogenetics or QF-PCR analyses revealed chromosomal anomalies in 27% of the probands. Mutational analyses in genes *SHH*, *ZIC2*, *SIX3* and *TGIF* were performed in 119 patients, revealing eight mutations in *SHH,* two mutations in *SIX3* and two mutations in *ZIC2*. Thus, a detailed clinical description of new HPE cases with identified genetic anomalies might establish genotypic and phenotypic correlations and contribute to the development of additional strategies for the analysis of new cases.

Keywords: holoprosencephaly, ECLAMC, *SHH*, *ZIC2*, *SIX3*.

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Introduction

Holoprosencephaly (HPE, MIM 236100) is a complex brain malformation affecting both the forebrain and the face. This condition can be described according to cerebral malformation severity: lobar, semi-lobar, alobar and middle interhemispheric variant (MIH-HPE) (Demyer and Zeman, 1963; Simon *et al.*, 2002). Facial anomalies are variable, ranging from cyclopia, ethmocephaly or cebocephaly to mild forms, such as ocular hypotelorism or single median maxillary central incisor (SMMCI) (Cohen Jr, 1989; Richieri-Costa and Ribeiro, 2006; El-Jaick *et al.*, 2007a). In HPE families, mild facial anomalies can occur without cerebral malformation, and these anomalies are considered risk factors for HPE in subsequent offspring (Berry *et al.*, 1984; El-Jaick *et al.*, 2005). Cerebral malformations without facial evidence occur in 10-20% of HPE patients (Cohen Jr, 1989).

The etiology of HPE is complex, including both genetic and environmental factors (Barr Jr *et al.*, 1983; Belloni *et al.*, 1996; Cohen Jr and Shiota, 2002; Dubourg *et al.*, 2007). Chromosomal anomalies represent approximately 45% of HPE cases. Multiple malformation syndromes with normal karyotypes, such as Smith-Lemli-Optiz, Pallister Hall and velo-cardio-facial Syndrome, correspond to 25% of HPE cases (reviewed by Dubourg *et al.*, 2007). Numerous mutations in the genes involved in the development of the forebrain and midline face during embryogenesis have been described. The four genes typically associated with HPE cases are *SHH* [HPE3, MIM 142945] (Roessler *et al.*, 1996), *ZIC2* [HPE5, MIM 609637] (Brown *et al.*, 1998), *SIX3* [HPE2, MIM 157170] (Wallis *et al.*, 1999), and *TGIF* [HPE4, MIM 142946] (Gripp *et al.*, 2000). Mutations in one of these genes correspond to 10-20% of non-syndromic cases (Orioli *et al.*, 2001; Dubourg *et al.*, 2004). Other genes associated with HPE, although to a lesser degree, include *PTCH* [HPE7, MIM 610828] (Ming *et al.*, 2002), *TDGF1* [MIM 187395] (De la Cruz *et al.*, 2002), *GLI2* [HPE9, MIM 610829] (Roessler *et al.*, 2003), *DHCR7* [SLOS, MIM 270400] (Shim *et al.*, 2004), *FAST1*/*FOXH1* [MIM 603621] (Roessler *et al.*, 2008), *DISP1* [MIM 607502] (Roessler *et al.*, 2009a), *FGF8* [HH6, MIM 612702] (Arauz *et al.*, 2010), and *CDON* [HPE11, MIM 614226] (Bae *et al.*, 2011).

The phenotypic variability and incomplete penetrance make it difficult to conduct genetic counseling in HPE. Families typically present autosomal dominant, but also autosomal recessive and possibly X-linked inheritance (Ming and Muenke, 1998; Wallis and Muenke, 1999; Muenke and Beachy, 2001). Autosomal dominant inheritance has an estimated penetrance of 80% (Odent *et al.*, 1998), and the same mutation segregating within a family can present asymptomatic, mild and severe forms of the disease (Roessler *et al.*, 1996; El-Jaick *et al.*, 2005). We recently analyzed HPE cases from the ECLAMC (Estudio Latinoamericano de Malformaciones Congénitas-Latin-American Collaborative Study of Congenital Malformations) since 2000 (Orioli *et al.*, 2001; El-Jaick *et al.*, 2005, El-Jaick *et al.*, 2007a), and in the present study, we evaluate the contribution of chromosomal anomalies and mutations in the four main HPE genes (*SHH*, *SIX3*, *TGIF*, and *ZIC2*).

Materials and Methods

ECLAMC

Most of the families studied (140/195; 72%) were derived from the ECLAMC (Estudio Latinoamericano de Malformaciones Congénitas-Latin American Collaborative Study of Congenital Malformations). ECLAMC is a hospital-based registry, which examines births in South America (Castilla and Orioli, 2004). In 1998, the ECLAMC initiated a molecular epidemiological program, called MOLECLAMC, and biological samples from newborns presenting congenital malformations were collected for molecular analysis.

Samples from MOLECLAMC primarily comprise blood spots on filter paper (Schleicher and Schuell no. 903 or IsoCode), and a pediatrician interviewed the mothers of the malformed infants using a form with 50 questions concerning risk factors, including environmental exposure, history of other familial congenital defects, and parental consanguinity (Castilla and Orioli, 2004). The DNA repository of MOLECLAMC also receives samples from pediatricians and geneticists associated with the ECLAMC. These cases occasionally involve older patients, representing 28% (55/195) of the families presented herein.

Patients

The patients were referred based on typical HPE facial dysmorphisms, with or without confirmation through brain imaging, on SMMCI or a family history of HPE. Aneuploidy analyses were performed on some HPE patients with no informed karyotype. Chromosomal microrearrangements were evaluated in some HPE and SMMCI patients without mutations in the four main HPE genes and no known chromosomal anomalies.

The patients referred to the Birth Defects Laboratory at the Federal University of Rio de Janeiro were analyzed in the last 14 years. Initially, we only analyzed mutations in the four main HPE genes. In 2008, the laboratory implemented the MLPA (Multiplex Ligation-Dependent Probe Amplification) technique, and in 2010, we initiated studies to screen for trisomies using QF-PCR. Not all patients could be studied using all techniques, reflecting the lack of adequate DNA quantity and quality, particularly for older samples.

The Ethics Committee of the ECLAMC (CEMIC: Centro de Educación Médica e Investigación Clínica, Buenos Aires, Argentina, IORG-0001315) approved the project, and written informed consent was obtained from adult patients and the parents of infant patients.

DNA extraction

The DNA from blood spotted onto Schleicher and Schuell no. 903 filter paper (Schleicher & Schuell Inc., Keene, NH, USA) was extracted using QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) or DNeasy 96 Tissue Kit (Qiagen, Valencia, CA, USA), and DNA from blood spotted onto IsoCode filter papers (Schleicher & Schuell Inc., Keene, NH, USA) was extracted through boiling in water. The DNA from peripheral blood samples on EDTA was extracted through salting out (Miller *et al.*, 1988).

PCR and direct sequencing

The coding region and exon-intron boundaries of *SHH*, *SIX3, TGIF*, and *ZIC2* were amplified as previously described (El-Jaick 2007a,b; Savastano *et al.*, 2014). Automated sequencing was performed using MegaBACE 1000 (Amersham Pharmacia Biotech In, London, UK) or ABI Prism 377 (Applied Biosystems, Foster City, CA, USA) DNA sequencing systems. The electropherograms were analyzed using Sequencher software (Gene Codes Corporation, Ann Arbor, MI, USA). SIFT and PolyPhen2 online software programs were used to predict the effects of non-synonimous mutations. The gene mutation nomenclature used in this study was consistent with the recommendations of den Dunnen and Antonarakis (2000), and updates are available on the website for the Human Genome Variation Society (HGVS).

Micro-rearrangements analysis

Submicroscopic deletions or gains were screened through Multiplex Ligation-Dependent Probe Amplification (MLPA) (Schouten *et al.*, 2002), using the MLPA Kit P187 or the MLPA Kit P187 B1 (MRC-Holland, Amsterdam, Netherlands). These kits contain probes against the HPE genes *SHH, SIX3, TGIF, ZIC2, PTCH, GLI2,* and candidate genes *TRAPPC10* and *FBXW11.* Normalization and analysis were performed using Coffalyser (Coffa *et al.*, 2008) or GeneMarker (Softgenetics, State College, PA, USA) software programs.

QF-PCR analysis of trisomies

HPE patients without confirmed karyotypes were further tested for aneuploidies on chromosomes 13, 18, 21 and X through QF-PCR, using the Aneufast Multiplex QF-PCR Kit (Genomed AG, Wollerau, Switzerland). Patients with alterations in more than one marker for the same chromosome were subjected to further tests using chromosome specific back-up markers. The analyses were performed using GeneMarker (Softgenetics, State College, PA, USA).

Results

The series

From 1998 to 2012 a total of 195 probands were referred for genetic analysis of the HPE genes. Most of the samples (160/195; 82.1%) were obtained from newborns, livebirths (145/195; 74.4%) or stillbirths (15/195; 7.7%), but the sample also included children of different ages (26/195; 13.3%), adults (3/195; 1.5%), four fetuses (4/195; 2.1%), and two (1.0%) of unknown ages. Females were predominant, with 112 females (58.6%) *vs.* 79 males (41.4%), and this predominance was significant when considering the sex ratio expected at birth ($\chi^2 = 7.10$; $P = 0.008$; $GL = 1$). One patient had no information, and three patients were intersexes. There were 110 patients with isolated HPE or microform ("isolated cases"), and 85 patients (43.6%) with other malformations unrelated to the HPE spectrum ("associated cases"). The occurrence of HPE was verified through pre or postnatal brain imaging or autopsy in 155 probands (79.5%). Forty-one cases with specified HPE types presented with alobar HPE type (41/79; 51.9%), 29 cases presented with semilobar HPE type (29/79; 36.7%), and nine cases presented with lobar HPE type (9/79; 11.4%). In 97 probands HPE was not specified, although there was a suspicion of interhemispheric variant of HPE in three cases. Nineteen patients (19/195; 9.7%) had no HPE in cerebral imaging: one patient presented premaxillary agenesis and anophtalmia, two patients were obligated carrier mothers, and 16 patients had SMMCI. Five of the 16 SMMCI patients exhibited a mild phenotype without mental deficiency and additional defects, such as microcephaly, choanal atresia, lacrimal conduct atresia, or ocular hypotelorism.

The facial descriptions of the 195 patients, including the two obligated carrier mothers, were classified as cyclopia (19 cases), ethmocephaly (6 cases), cebocephaly (17 cases), and premaxillary agenesis (41 cases). The facial types in 172 patients were distributed among seven additional categories: median cleft lip (8 cases), bilateral or unilateral cleft lip with or without cleft palate (8 cases), cleft palate (5 cases), no oral cleft (60 cases), no oral cleft with SMMCI (16 cases), atypical facial cleft (4 cases), and no specified facial type (11 cases). The numbers of patients in these eleven facial type categories are presented in Table 1.

Alobar HPE was frequently observed in almost all facial categories, except for the median, lateral and no oral cleft categories where semilobar HPE was apparently more common (Table 1). Interestingly, the semilobar HPE type was observed in four of five cases of trisomy 13 with HPE type specified. A significant excess of females was observed in the ethmocephaly, cebocephaly, and premaxillary agenesis categories, and the male proportion was normal in the other categories. The proportion of other defects unrelated to the HPE spectrum did not vary among the facial categories. The recurrence of HPE or microform in the 195 families was approximately 19% (37/195; 95% CI: 13.9-25.3) without variation among the facial groups. Parental consanguinity is a rare event, with only three cases among the 158 cases specified for the data (1.9%; 95% CI: 0.5-5.9). The mean maternal age among 149 mothers was 26.9

Table 1 - Frequency of chromosomal anomalies, gene mutations, and type of holoprosencephaly (HPE), classified by facial features.

Facial type			HPE type		Chromosomal anomalies[b]		Gene mutations *SHH/SIX3/TGIF/ZIC2*	
Category	N	% (95% CI)	A/SL/L[a] (N)	Alobar (%)	N	%	N	%
Cyclopia	19	9.7% (6.2%-12.8%)	7/1/0	87.5%	5/11 (2)	63.6%	0/10	0.0%
Ethmocephaly	6	3.1 (1%-4.6%)	0/1/0	0.0%	2/2	100%	0/2	0.0%
Cebocephaly	17	8.7% (5.1%-11.8%)	6/0/1	85.7%	0/3	0.0%	3/10	30.0%
Premaxillary agenesis	41	21% (15.4%-25.7%)	11/4/1	68.7%	8/22	36.4%	3/21	14.3%
Median cleft	8	4.1% (1.5%-6.2%)	0/2/0	0.0%	3/5	60.0%	0/4	0.0%
Lateral cleft lip and palate	8	4.1% (1.5%-6.2%)	0/1/1	0.0%	0/4	0.0%	0/5	0.0%
Cleft palate	5	2.6% (0.5%-4.6%)	2/0/0	100.0%	1/4	25.0%	0/3	0.0%
No oral cleft	60	30.8% (24.1%-36.4%)	12/19/5	33.4%	4/30 (1)	17.9%	3/40	7.5%
No oral cleft+SMMCI	16	8.2% (4.6%-11.3%)	0/0/0	0.0%	1/4	11.2%	2/16	12.5%
Atypical facial cleft	4	2% (0.5%-3.1%)	1/0/0	100.0%	0/2	0.0%	1/3	33.3%
NFS	11	5.6 (2.6%-8.2%)	2/1/1	50.0%	1/4	25.0%	0/5	0.0%
Total	195	100%	41/29/9	51.9%	25/91	29.2%	12/119	10.1%

[a]A = alobar; SL = semilobar; L = lobar.
[b]Number of positive MLPA results in normal karyotypes showed in parentheses. MLPA denominators not showed. MLPA = Multiplex Ligation-Dependent Probe Amplification. NFS = not further specified. SMMCI = Single Median Maxillary Central Incisor.

years (95% CI: 25.8-27.8), and did not vary among the facial groups. There were nine cases of maternal diabetes among the 139 probands (6.5%, 95% CI: 3.2-12.3), and six of these cases exhibited premaxillary agenesis. The proportion of chromosomal anomalies or gene mutations did not differ among the facial categories (Table 1).

Micro-rearrangement analysis and chromosomal anomalies

MLPA screening was performed in 58 patients: ten patients with common trisomies (chromosomes 13 and 18), five patients with structural chromosomal anomalies, 23 patients with normal karyotypes, and 20 patients without karyotype information, showing failure in 15 cases and inconclusive results in eight cases. Among the 35 cases with MLPA results, 28 patients had HPE and seven patients had microforms. We also observed normal MLPA results in 27 cases, in which 14 patients had normal karyotypes, one patient exhibited 46,XX, inv(5) (p14.3; q23.1), and 12 cases showed no karyotype information. Altered MLPA results were observed in three of the 17 cases with normal karyotypes (17.6%; 95% CI: 4.7-44.2) or in three of the 29 cases with or without informed karyotypes (10.3%; 95% CI: 2.7-28.5). We detected two *ZIC2* deletions and one deletion encompassing the *SIX3* and *SIX2* genes. The other altered MLPA results were observed in two cases of trisomy 13, and one case of 18p-, t(6;7) and t(7;14). An affected sibling of the patient with t(6;7) was also examined to verify the occurrence of the *SHH* deletion. Table 2 shows the phenotype and MLPA alterations observed in individuals with gene deletions.

Informed karyotypes were observed in 65 of the probands, and 20 patients had chromosome anomalies. We successfully performed QF-PCR analysis on 26 probands without informed karyotypes and detected chromosomal aneuploidies in five patients: one case of trisomy 21, one case of trisomy 18, and three cases of trisomy 13. Considering informed karyotype and the QF-PCR analysis, we obtained karyotype information for 91 patients (46.7%). Chromosomal anomalies, including aneuploidies or structural anomalies, were observed in 25/91 patients (27.5%; 95% CI: 18.9-38.0). The increased frequency of chromosomal abnormalities was observed in patients with associated HPE (22/51; 43.1%). Only three patients with isolated HPE or microform (3/40; 7.5%) showed chromosomal aberrations: 18p-, inv(5) and an unspecified chromosomopathy. The inv(5) observed in a SMMCI patient, was also observed in her father and brother, both exhibiting a normal phenotype.

Mutation screening

Mutation analyses were successfully performed in 120 patients for *SHH* and *TGIF*, 125 patients for *SIX3*, and 151 patients for *ZIC2*. All four genes were successfully sequenced in 119 patients: 45 patients had HPE, or micro-

Table 2 - Chromosome deletions in patients with holoprosencephaly (HPE).

Patient n.	Sex	HPE type	Facial type	Other features	Karyotype results	MLPA results
439[a]	M	Alobar	Ethmocephaly	microcephaly (CC: 23 cm), cranial fontanels and sutures not palpable, high frontal hair; frontal proboscis; eyes separated by 2 mm skin; fused iris; absent nose; small mouth; short and broad neck; palmar simian creases, 5th fingers clinodactyly, absent flexion creases on left 3rd and 4th fingers, and 3rd right finger; small penis; hypospadias; hypoplasic scrotal folds; right criptorquidia; anal atresia; left renal cyst.	46, XY, t(6;7)(p21; q36)	del *SHH*
568[a]	M	Alobar	Cyclopia	Microcephaly (CC: 22 cm); proboscide over the eye; microstomia; thin lips with down deviated comisssures; low-set ears; short neck with excess skin; bilateral single palmar crease; clinodactyly of 5th finger of both hands; prominent heels; imperforate anus (apparently without fistula); absence of sacrum; X-rays: hemivertebra at L5.	46, XY, t(6;7)(p21; q36)	del *SHH*
3877	F	Unknown	Unknown	Fetal US: triventricular hydrocephalus, and single umbilical artery.	46, XX, -7, +der(7) , t(7;14) (q36;q31) pat	del *SHH*
817	M	Unknown	No oral cleft	Ocular hypotelorism; narrow palate; low insertion of hair on the neck; hirsutism.	46, XY	del *ZIC2*
1233	M	Unknown	Cyclopia	Small mouth; separated ocular globes, proboscide; microstomia; dysmorphic ears with helices folded over the antihelices.	46, XY	del *ZIC2*
6230	M	Alobar	Cyclopia	Microcephaly; fused thalami; arrinia.	46, XY	del *SIX2* and *SIX3*
4772	F	Alobar	Cyclopia	Microcephaly (CC: 23 cm); single ocular globe with two iris; proboscide over the eye; malar hypoplasia; adrenal hypoplasia.	46, XX, del 18p(11.2)	del *TGIF1*

[a]Patient n. 568 is brother of patient n. 439. Chomosomes were reanalyzed after the birth of the second affected son in this family.
MLPA : Multiplex Ligation-Dependent Probe Amplification.

form, and also other malformations not associated with the HPE spectrum, and 74 patients had isolated HPE or microform. Most of the studied patients (86/119; 72.3%) were newborns (81 livebirths, and five stillbirths), but the sample also included 25 children of different ages (25/119; 21.0%) ranging from 4 months to 17 years, three adults (3/119; 2.5%), and four fetuses (4/119; 3.4%). We could not retrieve information for the age of one patient. Among the 119 studied patients, 36 patients had normal karyotypes (18 males and 18 females), 19 patients had normal QF-PCR results, and seven patients had chromosomal anomalies. The remaining 57 patients had unknown karyotypes (18 associated, and 39 isolated HPE or microform), representing 47.9% of the studied cohort.

In total, 47 variants were detected, 35 variants were classified as benign or of unknown significance based on several features, including the presence of the variant in population SNP databases, synonymous benign variants predicted using PolyPhen and SIFT software, or UTR and intronic variants with unknown significance. We detected 12 damaging mutations in 119 probands (10.1%; 95% CI: 5.6-17.3) (Table 3). The pathogenetic mutations included eight *SHH* gene mutations (66%), two *SIX3* gene mutations (17%), and two *ZIC2* gene mutations (17%). All 12 patients presented isolated HPE or microform.

The mutation frequency is similar (10.9%) among the probands with normal karyotype or QF-PCR results (six mutations out of 55 cases). The pedigrees of the six families with mutation recurrences are presented in Figure 1. The parents from six probands were available for study to determine the mutation inheritance. The clinical characterization and mutations identified in the probands and parents are summarized in Table 3. Figure 2 shows clinical photos and eletropherograms from the three most recently studied mutated patients. The other patients have been previously described (Orioli *et al.*, 2001; El-Jaick *et al.*, 2005, 2007a).

SHH mutations

We detected eight mutations in the *SHH* gene. Three patients have been previously described. El-Jaick *et al.* (2005) described a newborn girl presenting the mutation c. 332T > A, p.(I111N), with alobar HPE and premaxillary agenesis, inherited from the mother affected with SMMCI and hypotelorism. Orioli *et al.* (2001) described the other two patients: 1) a 8-year-old male presenting the mutation c. 419A > C, p.(H140P) without specified HPE type, ocular

Table 3 - Clinical characterization and mutations in HPE patients.

Patient n.	ID	Sex	Gene	Mutation	Inheritance	HPE type	Facial type	Other features	Reference
818	Proband	F	SHH	c.214C > T, p.(R72*)	De novo	Lobar	No oral cleft	Microcephaly; frontal lobar hypoplasia; severe hypoplasia of septum pellucidus; depressed nasal tip; thin lips; long philtum.	El-Jaick (2005)
564	Proband	F	SHH	c.332T > A, p.(I111N)	Maternal	Alobar	Premaxillary agenesis	Microcephaly (27.5 cm); ocular hypotelorism; median cleft lip; midface hypoplasia; absence of nasal bones; corpus callosum, fals cerebrum and interhemispheric fissure agenesis; thalami partial fusion; a cystic formation connecting the ventricular system with subarachnoid space; decreased sulci and gyri number and pachygiria.	El-Jaick *et al.* (2005)
566	Mother	F	SHH	c.332T > A, p.(I111N)	Unknown	None	No oral cleft with SMMCI	Ocular hypotelorism; obesity.	El-Jaick *et al.* (2005)
5327	Proband	M	SHH	c.381delC, p.(W128Gfs*58)	Probably Paternal	Alobar	Cebocephaly	Microcephaly; fused thalami; microphtalmia; ocular hypotelorism; median cleft lip; micropenis, and rudimentary scrotum with nonpalpable testes.	This study
2	Proband	M	SHH	c.419A > C, p.(H140P)	Maternal	Unknown	No oral cleft with SMMCI	Ocular hypotelorism; single median maxillar central incisive; bilateral convergent strabism.	Orioli *et al.* (2001)
6	Mother	F	SHH	c.419A > C, p.(H140P)	Unknown	None	No oral cleft	Ocular hypotelorism.	Orioli *et al.* (2001)
576	Proband	F	SHH	c.482T > A, p.(L161Q)	Paternal	Unknown	Premaxillary agenesis	Microcephaly; ocular hypotelorism; absent nose; pectus excavatum.	El-Jaick (2005)
577	Father	M	SHH	c.482T > A, p.(L161Q)	Unknown	None	Normal	Normal	El-Jaick (2005)
2086	Proband	M	SHH	c.548G > A, p.(C183Y)	Unknown	Alobar	Premaxillary agenesis	Microcephaly (CC: 22 cm); cystic area in the region of the cerebellar vermis which extends cranially in the midline; lissencephaly; corpus callosus agenesis, fused thalami; ocular hypotelorism; small palpebral fissures; single nostril with absent columella.	This study
81	Proband	F	SHH	c.548G > T, p.(C183F)	Unknown	Alobar	Cebocephaly	Microcephaly, ocular hypotelorism, bilateral microphthalmia without opening lids; sigle nare, choanal atresia, ogival palate.	Orioli *et al.* (2001)
2815	Proband	F	SHH	c.718A > C, p.(T240P)	Unknown	Unknown	Cebocephaly	Microcephaly; ocular hypotelorism; single nare; severe ogival palate.	This study
1269	Proband	F	SIX3	c.522CA, p.(Y174*)	Unknown	None	No oral cleft	Ocular hypotelorism, almost absent upper frenula; torus palatino; mother of two HPE fetus	El-Jaick (2005)
2885	Proband	M	SIX3	c.686C > T, p.(P229L)	Unknown	None	No oral cleft with SMMCI	Ocular hypotelorism, mild bilateral distichiasis; hyperfolded ears; indistinct and short philtrum; single median maxillar central incisive; mild mandibular prognatism; mammary hypertelorism; normal stature; mild learning delay.	El-Jaick *et al.* (2007a)

Table 3 (cont.)

Patient n.	ID	Sex	Gene	Mutation	Inheritance	HPE type	Facial type	Other features	Reference
469	Proband	F	ZIC2	c.857_858delAC, p.(H286Rfs*80)	De Novo	Semilobar	No oral cleft	Microcephaly; at six months old: trigonocephaly; upslanting palpebral fissures; ligth iris; bilateral epicantic folds; high palate.	Orioli *et al.* (2001)
6721	Proband	M	ZIC2	c.1411_1415delGTGTC, p.(V471Rfs*57)	Paternal	Alobar	Atypical facial cleft	Microcephaly; fused thalami; corpus callosus agenesis; bilateral and symmetrical increase of the echogenicity of the periventricular white matter; ocular hypertelorism; bulky nose with left alar cleft (Tessier n. 1) and a small protuberance at the upper right lateral wall; well delineated philtrum; large mouth.	Savastano *et al.* (2014)
6724	Father	M	ZIC2	c.(=/1411_1415delGTGTC) p.(V471Rfs*57)	Unknown	None	No oral cleft	Normal.	Savastano *et al.* (2014)

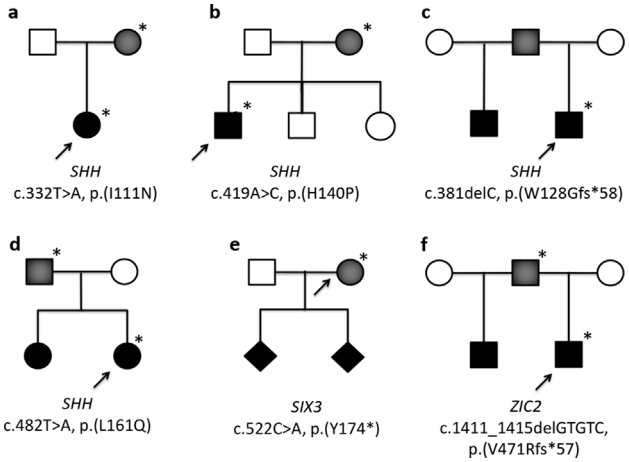

Figure 1 - The pedigrees of families with segregating mutations in HPE genes. Filled symbols indicate frank HPE; partially filled symbols indicate HPE microforms; asterisks indicate mutation carriers confirmed through sequencing; and arrows indicate probands.

hypotelorism, SMMCI, and strabism, inherited from the mother affected with mild ocular hypotelorism; and 2) a newborn male presenting the mutation c. 548G > T, p.(C183F) with alobar HPE, microcephaly, ocular hypotelorism, bilateral microphthalmia without lid opening, a single nostril with choanal atresia, and an ogival palate.

The mutation c. 214C > T, p.(R72*) likely represents a *de novo* mutation in a female infant with lobar HPE, microcephaly, and ocular hypotelorism (El-Jaick, 2005). The mutation c. 381delC, p.(W128Gfs*58) was observed in a male newborn prenatally diagnosed with HPE, presenting a typical face, with microcephaly, ocular hypotelorism, cleft lip, micropenis, and rudimentary scrotum with non-palpable testes (Figure 2 e, f, and g). An analysis of the family history revealed a paternal half-brother with HPE and a father with ocular hypotelorism. Unfortunately, DNA from the father and half-brother was not available. The mutation c. 482T > A, p.(L161Q) was observed in a female proband with HPE, microcephaly, ocular hypotelorism, premaxillary agenesis, flat nose, and pectus excavatum, presenting the karyotype 46,XX, 16qh+. The family history revealed a sister with HPE. The mutation was inherited from the father, who was not affected (El-Jaick, 2005). The mutation c. 548G > A, p.(C183Y) was detected in a male proband with alobar HPE, showing the absence of the corpus callosum and fusion of the thalami, a cystic area in the region of the cerebellar vermis, extending cranially through the midline, lissencephaly, microcephaly, ocular hypotelorism, small palpebral fissures, single nostril, and premaxillary agenesis (Figure 2, a and b). The patient presented CC = 22 cm at birth (< 3rd centile) and 29.8 cm at 13 months of age (< 3rd centile), with a normal karyotype (46,XY). The patient evolved with significant psychomotor retardation, feeding difficulties and difficult to control seizures, dying at age five. The parents were not available for this study. The mutation c. 718A > C, p.(T240P) was present in a fe-

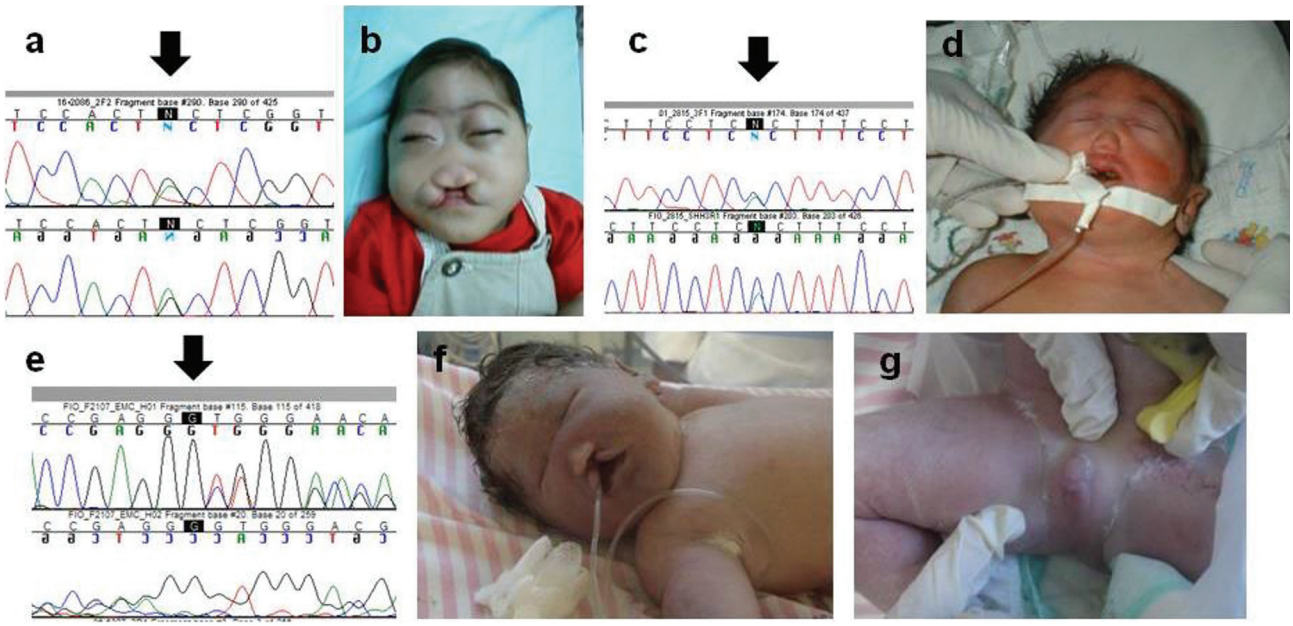

Figure 2 - HPE phenotypes and associated mutations. a) Missense mutation c.548G > A (p.C183Y) in exon 2 of *SHH* in patient ID 2086. b) Male patient (ID 2086) with microcephaly, ocular hypotelorism, small ocular fissures, and premaxillary agenesis. c) Missense mutation c.718A > C (p.T240P in the third exon of *SHH* in patient ID 2815. d) Female newborn (ID 2815) with microcephaly, ocular hypotelorism, single nostril and accentuated ogival palate. e) Deletion (c.381delC) in the *SHH* gene in patient ID 5327, which alters the reading frame and generates a stop codon at 58 residues after the deletion. f) Male newborn (ID 5327) prenatally diagnosed as HPE and presenting single nostril, microphtalmia, ocular hypotelorism, median cleft lip, and microcephaly. g) Picture of patient ID 5327 showing micropenis and rudimentary scrotum with non-palpable testes.

male infant with microcephaly, ocular hypotelorism, single nostril, and pronounced ogival palate (Figure 2, c and d). The family history revealed a nephew of the maternal grandmother with hydrocephalus, and a paternal uncle with a clubfoot. No relatives were available for further analysis in this study.

SIX3 mutations

SIX3 mutations were identified in two probands. The mutation c. 522CA, p.(Y174*) was detected in a woman with ocular hypotelorism and normal intelligence (El-Jaick, 2005). The patient was pregnant with two fetuses affected with HPE. The mutation c. 686C > T, p.(P229L) was observed in an eight-year-old male patient (El-Jaick *et al.*, 2007a) with SMMCI, ocular hypotelorism, mild bilateral distichiasis, mammary hypertelorism, hyperfolded helices, an indistinct and short philtrum, mild mandibular prognatism, normal stature, and a mild learning delay but normal cerebral CT.

ZIC2 mutations

Mutations in the *ZIC2* gene were detected in two probands. Orioli *et al.* (2001) previously described a 6-month-old female, presenting the mutation c. 857_858delAC, p.(H286Rfs*80), with semilobar HPE, microcephaly, trigonocephaly and a relatively non-dysmorphic face.

The mutation c. 1411_1415delGTGTC, p.(V471Rfs*57) was detected in a male infant with a prenatal diagnosis of semilobar HPE, presenting microcephaly, ocular hypertelorism, and a rare cleft nose. The family history revealed a paternal half-sister with HPE. The father presented the same mutation, likely in mosaicism, with mild ocular hypotelorism (Savastano *et al.*, 2014).

Discussion

More than 80% (160/195) of the patients in this study were newborn, and two-thirds of these patients were obtained from the ECLAMC, except for the older patients and those with SMMCI. The controlled epidemiological variables, such as sex, maternal age, maternal diabetes, parental consanguinity, chromosomal or gene defects, and the recurrence of HPE or microform in the family, showed that this sample did not differ from that previously studied at ECLAMC (Orioli and Castilla, 2007).

The phenotypic variables, such as type of HPE, facial types, presence of other cerebral defects or the presence of other defects not associated with the HPE spectrum, differed from those previously observed (Orioli and Castilla, 2007) when newborn patients may not present features, such as SMMCI and/or mental retardation. The expansion of the classical facial classification in HPE (DeMyers *et al.*, 1964) resulted in 11 facial types. We examined some epidemiological and phenotypic variables according to these 11 facial types, to detect useful differences among them, as a facial description is more easily obtained than cerebral imaging or karyotype. The semilobar HPE type was less fre-

quently observed among the four classical facial types, but the sample is too small to access the significance of this finding. Because the semilobar HPE type was more frequently observed in trisomy 13 cases (four among five with HPE type specified), we attempted to characterize the other chromosome anomalies with specified HPE types and observed only alobar HPE in four cases (18 trisomy, 21 trisomy, t(6;7), and del 18p).

The slight female predominance of 1.4:1 (female:male) observed in the present study was consistent with that previously observed in other studies (Croen *et al.*, 1996; Forrester and Merz, 2000; Mercier *et al.*, 2011). This female predominance was restricted to three facial types: ethmocephaly, cebocephaly, and premaxillary agenesis. Patients with cyclopia showed a normal male proportion. This sexual difference might reflect causal differences of HPE associated with facial types. The increased frequency to be confirmed of maternal diabetes in patients with premaxillary agenesis, and *ZIC2* mutations typically associated with normal or mildly affected faces (Brown *et al.*, 2001), might be examples of association between cause and facial type.

Chromosomal anomalies are described in 32% to 41% of HPE cases, and trisomy 13 is the most frequently observed anomaly (reviewed by Solomon *et al.*, 2010a). In the present study, karyotyping or QF-PCR analyses revealed chromosomal anomalies in 27.5% (25/91) of the informative patients. This frequency must be underestimated as 104 patients (53.3%) were not studied or had inconclusive results through QF-PCR. Furthermore, structural anomalies were not detected using this method. As expected, an increased frequency of chromosomal anomalies was observed in patients with HPE and associated malformations.

In addition to the usual cytogenetic study, tracing microdeletions involving genes associated with HPE through techniques, such as MLPA, contributed significantly to the diagnosis. The rate of chromosomal microanomaly detection through MLPA in the present study, ranging from 10.3% (3/29) to 17.6% (3/17 with normal karyotype), is not significantly different from the 5% frequency of microdeletions involving the main four HPE genes in newborns (Bendavid *et al.*, 2006a) or the 9.8% frequency observed in patients without point mutations (Bendavid *et al.*, 2006b). The frequency of microrearrangements and mutations in the four main HPE genes varied between cohorts of fetuses and live-born children. While mutations are more frequent in livebirths, submicroscopic deletions occur more frequently in fetuses (Bendavid *et al.*, 2006a, 2006b, 2009). In the present study, which primarily included livebirths, the frequency of microdeletions, 10.3% (2.7-28.5), and mutations, 12/119 (10.1%; 5.6-17.3), are similar in both groups.

Array-CGH studies have reported frequencies of 17% to 22% losses or gains in the genome of HPE patients, considering known and new HPE loci (Bendavid *et al.*, 2009; Mercier *et al.*, 2011). However, the meaning of these candidate loci in the etiology of HPE was uncertain. In a genotype-first approach study, Rosenfeld *et al.* (2010) identified 136 individuals with deletions in any of the 35 selected HPE loci identified in a cohort of 26,922 patients studied through array CGH. The authors observed HPE in 13 individuals, 11 patients with deletions involving the four primary HPE genes, one patient with a deletion in the HPE8 locus (14q13), and one patient with a deletion in *FGF8*. The other six loci involving HPE genes or candidates were only associated with HPE microforms, but most of the deletions identified in HPE candidate genes showed no HPE, nor microforms of HPE.

The phenotype of patients with microdeletions in one of the four main HPE genes is highly heterogeneous. Similar to intragenic mutations, these patients present phenotypes ranging from alobar HPE to microforms (Bendavid *et al.*, 2007; Rosenfeld *et al.*, 2010). No microdeletions were observed in the seven patients with HPE-microform or the patient with 46,XX, inv(5) (p14.3; q23.1), whose brother and father were carriers with the same inversion, showing normal results using the P-187 HPE Kit for MLPA, where probes against the candidate HPE gene, *FBXW11*, were located in 5q35.1. The patient presented with SMMCI, agenesis of corpus callosum, iris colobome, ocular hypotelorism, and a mild mental deficiency. Additional studies in this family will be important to determine whether the gene in this locus is associated with HPE.

The mutation frequency observed in the present study was 12/119 (10.1%; 5.6-17.3). Dubourg *et al.* (2004) reported a similar rate of 17% in fetuses and living children with normal karyotypes, while Nanni *et al.* (2000) observed mutation in only one case studied, representing 4.3% of the sample of newborn cases in a population from California. Lazaro *et al.* (2004) observed a similar frequency of 16% when considering syndromic and nonsyndromic HPE and patients with midline facial and/or cerebral anomalies without neuroradiological HPE. Although we did not conduct a functional analysis of the identified mutations, these anomalies affected conserved protein regions and therefore can be considered as contributors to the HPE phenotype.

The mutation ratio is higher in liveborn children (20% to 23%) than in fetuses (12.5% to 14%), who usually present more severe phenotypes due to chromosomal anomalies (Dubourg *et al.*, 2004; Bendavid *et al.*, 2009). However, even if most of the sample comprised newborns, the mutational frequency is low compared with the cited literature, likely reflecting the high frequency of cases without karyotype observed in the present study (53.3%).

The frequency of mutations in *SHH*, 8/119 (6.7%; 3.2-13.2), is consistent with the results obtained in previous studies. Nanni *et al.* (1999) observed the same frequency (6.7%) in 344 HPE patients. Bertolacini *et al.* (2009) described a frequency of 8.1%, and Lazaro *et al.* (2004) detected mutations in the *SHH* gene in 8.6% of cases.

Paulussen *et al.* (2010) observed a lower frequency of 3.5% in a Dutch cohort of non-syndromic HPE, including fetuses, neonatal deceased children, children and adults. Roessler *et al.* (2012) included the data from Dubourg *et al.* (2004) and Paulussen *et al.* (2010) in their analyses and observed an aggregated frequency of 5.9% in 475 studied individuals. With the exception of the cohort from Paulussen *et al.* (2010), *SHH* is the most commonly mutated gene among HPE individuals.

Consistent with the study from Solomon *et al.* (2012), we observed the prevalence of missense mutations (6/8, 75%) and a similar prevalence of nonsense (1) and frameshift (1) mutations in the *SHH* gene. A nonsense mutation in codon 72 was detected in the patient 818 (Table 2). A quarter of the mutations described in *SHH* were nonsense or frameshift mutations (Roessler *et al.*, 2009b; Solomon *et al.*, 2012). Except for the mutation p.M457Rfx*18, these anomalies eliminated the autocatalytic processing site from the protein and are considered as null alleles (Roessler *et al.*, 2009b). The mutation observed in the patient 5327 (Table 2) was a deletion of the nucleotide at position c.381 in *SHH*, resulting in a change in the reading frame and generating a stop codon at 58 residues after the alteration. This frameshift mutation resulted in a truncated protein terminating 14 residues before the autocatalytic cleavage site, likely generating a non-functional allele. The variant c. 482T > A, p.(L161Q), detected in the proband 576 (Table 2), was inherited from her father. This mutation is considered damaging (score 1.00). It is located in domain N-SHH, a conserved region that occurs among species, causes the substitution of a hydrophobic amino acid (leucine) to a hydrophilic amino acid (glutamine) and was not observed in the Exome Variant Server. Solomon *et al.* (2012) described the same mutation in a patient with unknown HPE and no other information about the phenotype. Mutation c. 548G > A, p.(C183Y) was identified in patient number 2086 (Table 2) and was also identified in a female HPE patient described by Roessler *et al.* (2009b), with no detailed clinical information, who inherited the mutation from her father (Solomon *et al.*, 2012). This mutation occurred in the same position of the mutation observed in patient number 81 (Table 2) previously described by Orioli *et al.* (2001). Indeed, codon 183 is a hot spot for mutations, as this codon was altered in at least four unrelated families: mutation p.(C183R), detected in a family described by Roessler *et al.* (2009b) and Solomon *et al.* (2012); mutation p.(C183F), detected in a patient previously described by Orioli *et al.* (2001); and the two unrelated probands with mutation p.(C183Y) described in the present study, Roessler *et al.* (2009b), and Solomon *et al.* (2012). The altered region was localized in domain N-SHH and is highly conserved among species. The PolyPhen2 analysis revealed the mutation p.(C183R) as likely damaging (score of 1.00). The variant c. 548G > A was not described in the Exome Variant Server among more than 6,000 sequenced individuals. The alteration c. 718A > C, p.(T240P), detected in patient 2815 (Table 2), affects the C-terminal domain of the SHH protein. Roessler *et al.* (2009b) reported two mutations affecting the adjacent amino acid. This region is conserved among different phyla, and this change leads to the substitution of a threonine, an amino acid with hydrophilic characteristics, with a hydrophobic proline. This mutation was not described in the Exome Variant Server, and the PolyPhen2 analysis considered this mutation as likely damaging (score of 0.773).

In families where the *SHH* gene mutation is segregating, widely variable expressivity among affected members is observed (Solomon *et al.*, 2012). Indeed, in the present study, at least half of the probands with mutations in the *SHH* gene inherited the mutation from one parent who was mildly affected. There was no disproportion of sex in the probands with the *SHH* mutation (proportion was 1:1), consistent with the results of previous studies (Mercier *et al.*, 2011; Solomon *et al.*, 2012). The small sample size prevented comparisons of the distribution of HPE types or facial features.

The *SIX3* gene presented a mutation frequency of 2/119 (1.7%; 0.3-6.5), which is not different from the 4% frequency described by Dubourg *et al.* (2004) in 200 individuals with normal karyotypes, including fetuses and living children and is also not different from the 5.1% frequency reported by Mercier *et al.* (2011). Paulussen *et al.* (2010) observed a higher frequency of 10.5%. Mutations in the *SIX3* gene have been predominantly observed in exon 1 (Lacbawan *et al.*, 2009).

The *ZIC2* gene has been previously described as the second most frequently mutated gene in HPE cases (Dubourg *et al.*, 2007). In the present study, the frequency was only 2/119 (1.7%; 0.3-6.5), a similar frequency of mutations as observed in the *SIX3* gene. Paulussen *et al.* (2010) also observed a 10.5% mutation frequency in *ZIC2* and in *SIX3* genes, although these frequencies were higher than the frequencies observed in the present study. Other studies have reported mutation frequency between 3% and 8.4% (Brown *et al.*, 2001; Dubourg *et al.*, 2004; Solomon *et al.*, 2010b; Mercier *et al.*, 2011; Roessler *et al.*, 2012). Patients presenting mutations in the *ZIC2* gene typically have mild facial defects or no facial anomalies (Brown *et al.*, 2001). It is likely that there was a bias in the present study towards more typical faces, which would lower mutation frequency in gene *ZIC2* and increase chromosomal defect rate. The two mutations in *ZIC2* detected in the present study were observed in patients with semilobar HPE and microcephaly, one patient with a relatively non-dysmorphic face, and another patient presenting a nose cleft (Table 2) (Orioli *et al.*, 2001; Savastano *et al.*, 2014). Interestingly, four patients presented with atypical facial clefts. However, the other patient with a nose cleft showed normal results in the mutational analysis. Recently, there have been attempts to establish genotype-phenotype relations in HPE patients

through studies including hundreds of mutated individuals for *SHH* (Solomon *et al.*, 2012), *ZIC2* (Solomon *et al.*, 2010b) and *SIX3* genes (Lacbawan *et al.*, 2009). Although the number of cases in the present study identified with a chromosomal or gene defect is small (40/195; 20.5%), certain features that were previously identified in the genotype-first approach study of Rosenfeld *et al.* (2010) as the possible common cause between Dandy-Walker anomaly and HPE were observed in the present study. A total of 47 patients presented other cerebral defects besides or instead of HPE, and eight patients showed Dandy-Walker anomalies: two patients with trisomy 13, one patient with trisomy 18, one patient with a normal karyotype, and the remaining four patients did not have informed karyotypes.

The absence of mutations in the *TGIF* gene in our sample likely reflects sample size constraints, as the frequencies of mutations in this gene have been reported as approximately 1% (Dubourg *et al.*, 2004).

The frequency of chromosome anomalies and mutations observed in the present study suggests that patients diagnosed with HPE must first be karyotyped, as previously recommended by other authors (Mercier *et al.*, 2010; Pineda-Alvarez *et al.*, 2010). Molecular methods, such as QF-PCR, are efficient for the diagnosis of associated HPE cases when premature death of newborn precludes cytogenetics studies. Isolated HPE cases might present structural alterations not detected using QF-PCR. In these cases, the MLPA technique is an alternative for the detection of gains or losses in key genes associated with HPE. Current recommendations for the diagnosis of HPE patients also include array-CGH analyses, as a sensitive and efficient technique for the identification of gains and losses in known or new HPE loci, which could clarify the etiology of many additional cases (Mercier *et al.*, 2010). A mutation in one of the four main HPE genes explains only 10% to 20% of cases, and therefore mutation screening is recommended after the exclusion of chromosomal anomalies.

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References

Arauz R, Solomon BD, Pineda-Alvarez DE, Gropman AL, Parsons JA, Roessler E and Muenke M (2010) A hypomorphic allele in the FGF8 gene contributes to holoprosencephaly and is allelic to Gonadotropin-Releasing Hormone Deficiency in humans. Mol Syndromol 1:59-66.

Bae GU, Domené S, Roessler E, Schachter K, Kang JS, Muenke M and Krauss RS (2011) Mutations in CDON, encoding a hedgehog receptor, result in holoprosencephaly and defective interactions with other hedgehog receptors. Am J Hum Genet 89:231-240.

Barr Jr M, Hanson JW, Currey K, Sharp S, Toriello H, Schmickel RD and Wilson GN (1983) Holoprosencephaly in infants of diabetic mothers. J Pediatr 102:565-568.

Belloni E, Muenke M, Roessler E, Traverso G, Siegel-Bartelt J, Frumkin A, Mitchell HF, Donis-Keller H, Helms C, Hing AV, *et al.* (1996) Identification of Sonic hedgehog as a candidate gene responsible for holoprosencephaly. Nat Genet 14:353-356.

Bendavid C, Haddad BR, Griffin A, Huizing M, Dubourg C, Gicquel I, Cavalli LR, Pasquier L, Shanske AL, Long R, *et al.* (2006a) Multicolour FISH and quantitative PCR can detect submicroscopic deletions in holoprosencephaly patients with a normal karyotype. J Med Genet 43:496-500.

Bendavid C, Dubourg C, Gicquel I, Pasquier L, Saugier-Veber P, Durou MR, Jaillard S, Frébourg T, Haddad BR, Henry C, *et al.* (2006b) Molecular evaluation of foetuses with holoprosencephaly shows high incidence of microdeletions in the HPE genes. Hum Genet 119:1-8.

Bendavid C, Dubourg C, Pasquier L, Gicquel I, Le Gallou S, Mottier S, Durou MR, Henry C, Odent S and David V (2007) MLPA screening reveals novel subtelomeric rearrangements in holoprosencephaly. Hum Mutat 28:1189-1197.

Bendavid C, Rochard L, Dubourg C, Seguin J, Gicquel I, Pasquier L, Vigneron J, Laquerrie A, Marcorelles P, Jeanne-Pasquier C, *et al.* (2009) Array-CGH analysis indicates a high prevalence of genomic rearrangements in holoprosencephaly: An updated map of candidate loci. Hum Mutat 30:1175-1182.

Berry SA, Pierpont ME and Gorlin RJ (1984) Single central incisor in familial holoprosencephaly. J Pediatr 104:877-880.

Bertolacini CD, Richieri-Costa A and Ribeiro-Bicudo LA (2009) Sonic hedgehog (SHH) mutation in patients within the spectrum of holoprosencephaly. Brain Dev 32:217-222.

Brown LY, Odent S, David V, Blayau M, Dubourg C, Apacik C, Delgado MA, Hall BD, Reynolds JF, Sommer A, *et al.* (2001) Holoprosencephaly due to mutations in ZIC2: Alanine tract expansion mutations may be caused by parental somatic recombination. Hum Mol Genet 10:791-796.

Brown SA, Warburton D, Brown LY, Yu CY, Roeder ER, Stengel-Rutkowski S, Hennekam RC and Muenke M (1998) Holoprosencephaly due to mutations in ZIC2, a homologue of Drosophila odd-paired. Nat Genet 20:180-183.

Castilla EE and Orioli IM (2004) ECLAMC: The Latin-American Collaborative Study of Congenital Malformations. Comm Genet 7:76-94.

Coffa J, van de Wiel MA, Diosdado B, Carvalho B, Schouten J and Meijer GA (2008) MLPAnalyzer: Data analysis tool for reliable automated normalization of MLPA fragment data. Cell Oncol 30:323-335.

Cohen Jr MM (1989) Perspectives on holoprosencephaly: Part III. Spectra, distinctions, continuities, and discontinuities. Am J Med Genet 34:271-288.

Cohen Jr MM and Shiota K (2002) Teratogenesis of holoprosencephaly. Am J Med Genet 109:1-15.

Croen LA, Shaw GM and Lammer EJ (1996) Holoprosencephaly: Epidemiologic and clinical characteristics of a California population. Am J Med Genet 64:465-472.

De la Cruz JM, Bamford RN, Burdine RD, Roessler E, Barkovich AJ, Donnai D, Schier AF and Muenke M (2002) A loss-of-function mutation in the CFC domain of TDGF1 is associated with human forebrain defects. Hum Genet 110:422-428.

Demyer W and Zeman W (1963) Alobar holoprosencephaly (arhinencephaly) with median cleft lip and palate: Clinical, electroencephalographic and nosologic considerations. Confin Neurol 23:1-36.

den Dunnen JT and Antonarakis SE (2000) Mutation nomenclature extensions and suggestions to describe complex mutations: A discussion. Hum Mutat 15:7-12.

Dubourg C, Lazaro L, Pasquier L, Bendavid C, Blayau M, Le Duff F, Durou MR, Odent S and David V (2004) Molecular screening of SHH, ZIC2, SIX3, and TGIF genes in patients with features of holoprosencephaly spectrum: Mutation review and genotype-phenotype correlations. Hum Mutat 24:43-51.

Dubourg C, Bendavid C, Pasquier L, Henry C, Odent S and David V (2007) Holoprosencephaly. Orphanet J Rare Dis 2:8.

El-Jaick KB (2005) Análise mutacional em pacientes com holoprosencefalia. Doctoral thesis (Doctoral Program in Biological Sciences-Genetics). Instituto de Biologia, Universidade Federal do Rio de Janeiro, 157 pp.

El-Jaick KB, Brunoni D, Castilla EE, Moreira MA and Orioli IM (2005) SHH Ile111Asp in alobar holoprosencephaly in a proposita, whose mother had only a solitary median maxillary incisor. Am J Med Genet A 136A:345.

El-Jaick KB, Fonseca RF, Moreira MA, Ribeiro MG, Bolognese AM, Dias SO, Pereira ET, Castilla EE and Orioli IM (2007a) Single median maxillary central incisor: New data and mutation review. Birth Defects Res A Clin Mol Teratol 79:573-580.

El-Jaick KB, Powers SE, Bartholin L, Myers KR, Hahn J, Orioli IM, Ouspenskaia M, Lacbawan F, Roessler E, Wotton D, *et al.* (2007b) Functional analysis of mutations in TGIF associated with holoprosencephaly. Mol Genet Metab 90:97-111.

Forrester MB and Merz RD (2000) Epidemiology of holoprosencephaly in Hawaii, 1986-97. Paediatr Perinat Epidemiol 14:61-63.

Gripp KW, Wotton D, Edwards MC, Roessler E, Ades L, Meinecke P, Richieri-Costa A, Zackai EH, Massague J, Muenke M, *et al.* (2000) Mutations in TGIF cause holoprosencephaly and link NODAL signalling to human neural axis determination. Nat Genet 25:205-208.

Lacbawan F, Solomon BD, Roessler E, El-Jaick K, Domene S, Velez JI, Zhou N, Hadley D, Balog JZ, Long R, *et al.* (2009) Clinical spectrum of SIX3-associated mutations in holoprosencephaly: Correlation between genotype, phenotype and function. J Med Genet 46:389-398

Lazaro L, Dubourg C, Pasquier L, Le Duff F, Blayau M, Durou MR, de la Pintiere AT, Aguilella C, David V and Odent S (2004) Phenotypic and molecular variability of the holoprosencephalic spectrum. Am J Med Genet A 129A:21-24.

Mercier S, Dubourg C, Belleguic M, Pasquier L, Loget P, Lucas J, Bendavid C and Odent S (2010) Genetic counseling and "molecular" prenatal diagnosis of holoprosencephaly (HPE). Am J Med Genet C Semin Med Genet 154C:191-196.

Mercier S, Dubourg C, Garcelon N, Campillo-Gimenez B, Gicquel I, Belleguic M, Ratié L, Pasquier L, Loget P, Bendavid C, *et al.* (2011) New findings for phenotype-genotype correlations in a large European series of holoprosencephaly cases. J Med Genet 48:752-760.

Miller SA, Dykes DD and Polesky HF (1988) A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16:1215.

Ming JE and Muenke M (1998) Holoprosencephaly: From Homer to Hedgehog. Clin Genet 53:155-163.

Ming J, Kaupas M, Roessler E, Brunner H, Golabi M, Tekin M, Stratton RF, Sujansky E, Bale SJ and Muenke M (2002) Mutations in PATCHED-1, the receptor for SONIC HEDGEHOG, are associated with holoprosencephaly. Hum Genet 110:297-301.

Muenke M and Beachy PA (2001) Holoprosencephaly. In: Scriver CR, Beaudet AL, Sly WS, Valle D, Childs B, Kinzler KW, Vogelstein B (eds) The Metabolic and Molecular Basis of Inherited Disease. McGraw-Hill Companies, Inc., New York, pp 6203-6262.

Nanni L, Ming JE, Bocian M, Steinhaus K, Bianchi DW, Die-Smulders C, Giannotti A, Imaizumi K, Jones KL, Campo MD, *et al.* (1999) The mutational spectrum of the sonic hedgehog gene in holoprosencephaly: SHH mutations cause a significant proportion of autosomal dominant holoprosencephaly. Hum Mol Genet 8:2479-2488.

Nanni L, Croen LA, Lammer EJ and Muenke M (2000) Holoprosencephaly: Molecular study of a California population. Am J Med Genet 90:315-319.

Odent S, Le Marec B, Munnich A, Le Merrer M and Bonaiti-Pellie C (1998) Segregation analysis in nonsyndromic holoprosencephaly. Am J Med Genet 77:139-143.

Orioli IM, Castilla EE, Ming JE, Nazer J, Burle de Aguiar MJ, Llerena JC and Muenke M (2001) Identification of novel mutations in SHH and ZIC2 in a South American (ECLAMC) population with holoprosencephaly. Hum Genet 109:1-6.

Orioli IM and Castilla EE (2007) Clinical epidemiologic study of holoprosencephaly in South America. Am J Med Genet Part A 143A:3088-3099.

Paulussen AD, Schrander-Stumpel CT, Tserpelis DC, Spee MK, Stegmann AP, Mancini GM, Brooks AS, Collée M, Maat-Kievit A, Simon ME, *et al.* (2010) The unfolding clinical spectrum of holoprosencephaly due to mutations in SHH, ZIC2, SIX3 and TGIF genes. Eur J Hum Genet 18:999-1005.

Pineda-Alvarez DE, Dubourg C, David V, Roessler E and Muenke M (2010) Current recommendations for the molecular evaluation of newly diagnosed holoprosencephaly patients. Am J Med Genet C Semin Med Genet 154C:93-101.

Richieri-Costa A and Ribeiro LA (2006) Holoprosencephaly-like phenotype: Clinical and genetic perspectives. Am J Med Genet A 140:2587-2593.

Roessler E, Belloni E, Gaudenz K, Jay P, Berta P, Scherer SW, Tsui LC and Muenke M (1996) Mutations in the human Sonic Hedgehog gene cause holoprosencephaly. Nat Genet 14:357-360.

Roessler E, Du YZ, Mullor JL, Casas E, Allen WP, Gillessen-Kaesbach G, Roeder ER, Ming JE, Ruiz i Altaba A and

Muenke M (2003) Loss-of-function mutations in the human GLI2 gene are associated with pituitary anomalies and holoprosencephaly-like features. Proc Natl Acad Sci USA 100:13424-13429.

Roessler E, Ouspenskaia MV, Karkera JD, Velez JI, Kantipong A, Lacbawan F, Bowers P, Belmont JW, Towbin JA, Goldmuntz E, *et al.* (2008) Reduced NODAL signaling strength via mutation of several pathway members including FOXH1 is linked to human heart defects and holoprosencephaly. Am J Hum Genet 83:18-29.

Roessler E, Ma Y, Ouspenskaia MV, Lacbawan F, Bendavid C, Dubourg C, Beachy PA and Muenke M (2009a) Truncating loss-of-function mutations of DISP1 contribute to holoprosencephaly-like microform features in humans. Hum Genet 125:393-400.

Roessler E, El-Jaick KB, Dubourg C, Velez JI, Solomon BD, Pineda-Alvarez DE, Lacbawan F, Zhou N, Ouspenskaia M, Paulussen A, *et al.* (2009b) The mutational spectrum of holoprosencephaly-associated changes within the SHH gene in humans predicts loss-of-function through either key structural alterations of the ligand or its altered synthesis. Hum Mutat 30:E920-930.

Roessler E, Vélez JI, Zhou N and Muenke M (2012) Utilizing prospective sequence analysis of SHH, ZIC2, SIX3 and TGIF in holoprosencephaly probands to describe the parameters limiting the observed frequency of mutant genegene interactions. Mol Genet Metab 105:658-664.

Rosenfeld JA, Ballif BC, Martin DM, Aylsworth AS, Bejjani BA, Torchia BS and Shaffer LG (2010) Clinical characterization of individuals with deletions of genes in holoprosencephaly pathways by aCGH refines the phenotypic spectrum of HPE. Hum Genet 127:421-440.

Savastano CP, Bernardi P, Seuanez HN, Moreira MAM and Orioli IM (2014) Rare nasal cleft in patient with holoprosencephaly due to mutation in ZIC2 gene. Birth Defects Res A [in press].

Schouten JP, McElgunn CJ, Waaijer R, Zwijnenburg D, Diepvens F and Pals G (2002) Relative quantification of 40 nucleic acid sequences by Multiplex Ligation-Dependent Prove Amplification. Nucleic Acids Res 30:e57.

Shim YH, Bae SH, Kim JH, Kim KR, Kim CJ and Paik YK (2004) A novel mutation of the human 7-dehydrocholesterol reductase gene reduces enzyme activity in patients with holoprosencephaly. Biochem Biophys Res Commun 315:219-223.

Simon EM, Hevner RF, Pinter JD, Clegg NJ, Delgado M, Kinsman SL, Hahn JS and Barkovich AJ (2002) The middle interhemispheric variant of holoprosencephaly. AJNR Am J Neuroradiol 23:151-156.

Solomon BD, Rosenbaum KN, Meck JM and Muenke M (2010a) Holoprosencephaly due to numeric chromosome abnormalities. Am J Med Genet C Semin Med Genet 154C:146-148.

Solomon BD, Lacbawan F, Mercier S, Clegg NJ, Delgado MR, Rosenbaum K, Dubourg C, David V, Olney AH, Wehner LE, *et al.* (2010b) Mutations in ZIC2 in human holoprosencephaly: Description of a novel ZIC2 specific phenotype and comprehensive analysis of 157 individuals. J Med Genet 47:513-524.

Solomon BD, Bear KA, Wyllie A, Keaton AA, Dubourg C, David V, Mercier S, Odent S, Hehr U, Paulussen A, *et al.* (2012) Genotypic and phenotypic analysis of 396 individuals with mutations in Sonic Hedgehog. J Med Genet 49:473-479.

Wallis DE and Muenke M (1999) Molecular mechanisms of holoprosencephaly. Mol Genet Metab 68:126-138.

Wallis DE, Roessler E, Hehr U, Nanni L, Wiltshire T, Richieri-Costa A, Gillessen-Kaesbach G, Zackai EH, Rommens J and Muenke M (1999) Mutations in the homeodomain of the human SIX3 gene cause holoprosencephaly. Nat Genet 22:196-198.

Internet Resources

Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), http://evs.gs.washington.edu/EVS/ (December 10, 2013).

Guidelines for Mutation Nomenclature, Human Genome Variation Society (HGVS), http://www.hgvs.org/mutnomen/ (December 10, 2013).

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/OMIM (November 24, 2013).

Polymorphism Phenotyping v2 (PolyPhen-2), http://genetics.bwh.harvard.edu/pph2/index.shtml (December 10, 2013).

Sorting Intolerant From Tolerant Program (SIFT), http://sift.jcvi.org/ (December 10, 2013).

Universidade Federal do Rio de Janeiro-Electronic theses and dissertations library, http://fenix3.ufrj.br/50/teses/d/CCS_D_KeniaBalbiElJaick.pdf (January 11, 2014).