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Extracerebral manifestations of nonchromosomal, nonsyndromic holoprosencephaly

Ariel F. Martinez, Paul S. Kruszka, and Maximilian Muenke

Medical Genetics Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD

Abstract

Nonchromosomal, nonsyndromic HPE (NCNS-HPE) has traditionally been considered as a condition of brain and craniofacial maldevelopment. In this review, we present the results of a comprehensive literature search supporting a wide spectrum of extracerebral manifestations identified in patients with NCNS-HPE. These manifestations have been described in case reports and in large cohorts of patients with “single-gene” mutations, suggesting that the NCNS-HPE phenotype can be more complex than traditionally thought. Likely, a complex network of interacting genetic variants and environmental factors is responsible for these systemic abnormalities that deviate from the usual brain and craniofacial findings in NCNS-HPE. In addition to the systemic consequences of pituitary dysfunction (as a direct result of brain midline defects), here we describe a number of extracerebral findings of NCNS-HPE affecting various organ systems. It is our goal to provide a guide of extracerebral features for clinicians given the important clinical implications of these manifestations for the management and care of patients with HPE and their mutation-positive relatives. The health risks associated with some manifestations (e.g., fatty liver disease) may have historically been neglected in affected families.

Keywords

Holoprosencephaly; HPE; SHH pathway; extracerebral manifestations; neural tube defect; congenital heart defect; skeletal anomaly; genitourinary anomaly; gastrointestinal anomaly; fatty liver disease; NAFLD

1 INTRODUCTION

Holoprosencephaly (HPE, MIM 236100) is a common malformation of the human brain caused by incomplete midline separation of the developing prosencephalon (Roessler et al., 1996). HPE affects approximately 1 in 250 conceptuses, of which less than 1 in 10,000 reach full-term pregnancy and only 1 in 100,000 live past the first year (Orioli & Castilla, 2010). The etiology of HPE is complex, with both genetic and environmental factors implicated (M. Cohen, Jr. & Shiota, 2002; Heyne et al., 2016; Lipinski, Godin, O’Leary-Moore, Parnell,

Corresponding author: Maximilian Muenke, M.D., National Human Genome Research Institute, National Institutes of Health, 35 Convent Drive, Room 1B203, Bethesda, MD 20892-3717, Phone: 301-402-8167, Fax: 301-496-7184, mamuenke@mail.nih.gov.

CONFLICT OF INTERESTS

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& Sulik, 2010; Muenke & Beachy, 2000). Approximately 25% of patients with HPE comprise monogenic syndromes and 40–50% harbor a chromosomal aberration (Dubourg et al., 2007; Solomon, Gropman, & Muenke, 2013). Analysis of recurrent chromosomal anomalies led to the identification of the first genes implicated in HPE, which participate in key developmental signaling networks such as Sonic hedgehog (SHH), bone morphogenetic protein (BMP), fibroblast growth factor (FGF), wntless/integrated (WNT), Nodal, and retinoid (Petryk, Graf, & Marcucio, 2015; Roessler & Muenke, 1998). Mutations in the *SHH* gene are the most common cause of nonchromosomal, nonsyndromic HPE (NCNS-HPE) in humans (Nanni et al., 1999; Roessler et al., 1996; Solomon et al., 2012) and many of the HPE genes encode proteins that appear to either directly or indirectly regulate SHH expression or signaling (Petryk et al., 2015; Taniguchi, Anderson, Sutherland, & Wotton, 2012). To date, a total of 15 genes have been identified associated with HPE (Arauz et al., 2010; Bae et al., 2011; S. A. Brown et al., 1998; de la Cruz et al., 2002; Dubourg et al., 2016; Dupe et al., 2011; Gripp et al., 2000; Ming et al., 2002; Ribeiro, Quiezi, Nascimento, Bertolacini, & Richieri-Costa, 2010; Roessler et al., 1996; Roessler et al., 2003; Roessler, Ma, et al., 2009; Roessler et al., 2008; Roessler, Pei, et al., 2009; Wallis et al., 1999), of which *SHH*, *SIX3*, *ZIC2*, and *TGIF1* are core components of testing panels (usually referred to as “four-gene screening”), representing roughly 24% of all cases with known etiology (Paulussen et al., 2010; Solomon et al., 2013).

Depending on the severity of brain and craniofacial malformations, HPE can be divided into three main subtypes according to DeMyer’s classification (in order of decreasing severity): alobar, semilobar and lobar (DeMyer, 1987). Alobar HPE is characterized by complete or almost complete interhemispheric fusion, a single ventricle, typically absent olfactory bulbs and corpus callosum, and severe facial features such as synophthalmia (cyclopia) and proboscis. In semilobar HPE, there is partial cortical separation posteriorly, a single ventricle, and absent or hypoplastic corpus callosum. In lobar HPE, the ventricles are separated, but there is incomplete separation of the frontal lobes, especially ventrally. In the rare lobar subtype, septopreoptic HPE, the midline fusion is limited to the septal and/or preoptic regions of the telencephalon without a significant frontorostral fusion (Hahn, Barnes, Clegg, & Stashinko, 2010). A milder form called middle interhemispheric variant (MIHV) has also been characterized, in which the posterior frontal and parietal lobes fail to separate and the corpus callosum may be hypoplastic (Hahn & Barnes, 2010). At the mildest end of the spectrum, microform HPE describes individuals with subtle craniofacial anomalies, such as a single maxillary central incisor, hypotelorism, eye coloboma, narrow nasal bridge, etc., in the absence of typical brain malformations, at least at the level that can be detected by conventional neuroimaging (Mercier et al., 2011; Solomon et al., 2009).

While chromosomal or syndromic HPE can cause malformations of multiple body systems, NCNS-HPE has traditionally been considered as an “above-the-neck” condition. However, a number of extracephalic manifestations have been described over the years in NCNS-HPE that can be present in a significant proportion of patients, accounting for the complexity of genetic and environmental interactions responsible for the HPE phenotype.

2 THE HEDGEHOG PATHWAY IN DEVELOPMENT

The hedgehog (Hh) pathway is evolutionarily conserved from *Drosophila* to superior vertebrates. Hh ligands act as key mediators in fundamental cellular processes of embryonic development, acting as morphogens that promote dose-dependent induction differentiation and determining cell fates. They also have mitogenic effects, controlling cell proliferation, survival, and organogenesis in different anatomical regions of the body. In particular, SHH serves as an induction signal of the ventral neural tube to promote development of the anterior-posterior axis of extremities and somatic ventral structures (Ingham & McMahon, 2001; Ruat, Roudaut, Ferent, & Traiffort, 2012).

2.1 Endocrine glands

Hh signaling participates actively in the development of endocrine glands: pituitary, ovary, testis, adrenal cortex, pancreas and prostate (for reviews see M. M. Cohen, Jr., 2010; P. J. King, Guasti, & Laufer, 2008). In addition to SHH, there are two other vertebrate Hh ligands: Indian hedgehog (IHH) and Desert hedgehog (DHH) (M. M. Cohen, Jr., 2003), each with a specific developmental expression pattern and function. SHH is expressed in the neural tube, IHH in the cartilaginous growth zone, and DHH is mostly restricted to the gonads, including Sertoli cells in the testes and granulosa cells in the ovaries (M. M. Cohen, Jr., 2010; Varjosalo & Taipale, 2008). In HPE, reports of endocrine dysfunction have been limited to the pituitary, adrenal and thyroid glands (Table I). Although the adrenal and thyroid dysfunction are thought to be the result of abnormal pituitary secretions, here we also discuss the role of Hh signaling in the development of these two glands.

2.1.1 Pituitary gland—Pituitary formation begins with an invagination of the anterior pituitary placode within the oral ectoderm to form Rathke's pouch, the primordium of the gland. The dorsal region of this structure makes direct contact with a region of the ventral diencephalon, the infundibulum, and this interaction, along with regions within Rathke's pouch and the surrounding mesenchyme, is required for the specification of pituitary endocrine cell types from progenitor cells within the pouch (P. J. King et al., 2008; Scully & Rosenfeld, 2002). These cell types are defined by the particular hormones they produce, which in the anterior pituitary are the corticotrophs (adrenocorticotrophin secreting), somatotrophs (growth hormone), lactotrophs (prolactin), thyrotrophs (thyrotrophin) and gonadotrophs (luteinizing hormone and follicle-stimulating hormone), and in the intermediate lobes, the melanotrophs (melanocyte-stimulating hormone).

Molecular studies have demonstrated that Hh signaling is essential for the development of the hypothalamic-pituitary axis (M. M. Cohen, Jr., 2010; Treier et al., 2001). In mice, SHH is expressed in the ventral diencephalon and the oral ectoderm and its downregulation triggers induction of the pituitary placode. A dual induction from the diencephalon is required for the formation of Rathke's pouch (Ericson et al., 1998; Takuma et al., 1998; Treier et al., 1998), with both BMP4 and FGF signals from the ventral diencephalon being required for the development of a definitive pouch. Subsequently, a dorsoventral BMP2 gradient, appearing at a SHH boundary, together with an opposing dorsoventral FGF8 gradient, serve to determine ventral/intermediate cell phenotypes (gonadotropes,

thyrotropes, somatotropes, lactotropes) and dorsal cell phenotypes (melanotropes and corticotropes), respectively, with attenuation of the BMP2 signal ultimately required for terminal differentiation. *Shh* null mice (Chiang et al., 1996) and mutations in human SHH or other SHH pathway interacting genes that cause HPE display pituitary agenesis (Odent et al., 1999; Roessler et al., 2003). While this might suggest an indirect requirement for SHH (via disruption of ventral diencephalon development), other experiments indicate SHH signaling is directly involved in pituitary development. Attenuation of SHH signaling by forced expression of antagonist hedgehog interacting protein (Hip) under the control of the *Pitx1* enhancer—which is active in the oral ectoderm and Rathke's pouch—results in pituitary agenesis with the formation of only a rudimentary pouch, with normal development of the ventral diencephalon (Treier et al., 2001). Overexpression of SHH in the pituitary gland under the control of the α -glycoprotein promoter, which is expressed throughout the Rathke's pouch, leads to pituitary hyperplasia and premature expression of the luteinizing hormone β -subunit gene, indicating that SHH promotes proliferation and differentiation of the ventral pituitary cell types (gonadotrophs and thyrotrophs) (Treier et al., 2001).

2.1.2 Adrenal gland—The adrenal gland contains a neuroendocrine medulla inside a steroidogenic cortex. The medulla produces catecholamines in response to sympathetic inputs, while the cortex produces essential steroids in response to hormonal inputs. Glucocorticoid release, which mediates the stress response (Sapolsky, Romero, & Munck, 2000), is modulated by the hypothalamic-pituitary axis. During embryogenesis, the cortex develops from the intermediate mesoderm, but knowledge on cortical specification signals and development is scarce. The adrenal cortex originates from the adrenogenital primordium, a common gonadal and adrenal structure derived from the mesonephric mesenchyme and overlying coelomic epithelium of the urogenital ridge (Hatano, Takakusu, Nomura, & Morohashi, 1996). Some evidence suggests a role for Hh signaling: *in situ* hybridization experiments show that *Shh* mRNA is expressed in the outer adrenal cortex in mice, and human and murine models with Hh pathway mutations manifest adrenal aplasia or dysgenesis (Bitgood & McMahon, 1995; Ching & Vilain, 2009; King et al., 2008). While these findings suggest a requirement for SHH in early adrenal development, adrenal dysgenesis can be the result of SHH-mediated pituitary dysfunction as described above, which can cause loss of pro-opiomelanocortin (POMC)-derived pituitary hormones that are required for the trophic support of adrenal growth in postnatal life (Vila et al., 2005). However, adrenal glands develop normally in *POMC* null mice (Karpac et al., 2006), indicating that pituitary function is likely to affect adrenal development prenatally. In fact, the experiments of King, Paul, & Laufer (2009) demonstrate that SHH signaling has a direct effect on adrenocortical development at multiple levels, including modulation of capsule thickness and proliferation of steroidogenic cortical cells.

2.1.3 Thyroid gland—During development, the foregut forms the esophagus, stomach, proximal duodenum, thymus, thyroid, airways, pancreas, and liver. Thyroid morphogenesis is initiated when the thyroid primordium buds from the ventral wall of the pharyngeal foregut endoderm that lies in close contact with the aortic sac endothelium (Alt et al., 2006; Fagman, Andersson, & Nilsson, 2006). Interestingly, proper development of the thyroid requires exclusion of SHH expression (Fagman, Grande, Gritli-Linde, & Nilsson, 2004).

Although there is no SHH expression at any point in thyroid development, the thyroid does not separate into two distinct lobes in *Shh*^{-/-} mice, and instead becomes a single unilateral mass (Fagman et al., 2004). This single-lobe thyroid gland of *Shh* null mice forms normal follicles that contain apparently normally differentiating thyrocytes. In the study by Alt et al. (2006), it was shown that this thyroid phenotype is indirectly caused by a failure of the aortic arch to cross the midline and both carotid arteries develop on one side of the esophagus.

As mentioned above, overexpression of SHH in the pituitary gland leads to pituitary hyperplasia by promoting proliferation and differentiation of the ventral pituitary cell types (gonadotrophs and thyrotrophs) (Treier et al., 2001). Additional investigation is needed to determine whether the thyroid dysfunction seen in some patients with HPE is a result of abnormal thyroid development or an indirect consequence of an aberrant hypothalamic-pituitary-thyroid axis.

2.2 Organ systems

From the study of *Gli* mutant mice, it was apparent that the spectrum of defects observed remarkably resembled human VACTERL association (Quan & Smith, 1973), which was originally given the VATER acronym to describe a number of associated malformations comprising vertebral defects (V), anal atresia (A), tracheoesophageal fistula with esophageal atresia (TE), radial and renal dysplasia (R). The further observation of cardiac anomalies by Temtamy and Miller (1974) within a VATER cohort broadened the definition and the expanded acronym VACTERL was coined to also include limb defects (Kaufman, 1973; Nora & Nora, 1975). Below, we describe the role of SHH signaling in the development of organ systems based on the phenotypic evaluation of *Shh* and *Gli* mutant mice.

2.2.1 Skeletal system—SHH is secreted by the notochord and floor plate cells, which are involved in the patterning of the somites into the sclerotome and the dermamyotome. The sclerotome, gives rise to the vertebral column, and the dermamyotome develops into muscle and skin (Christ, Huang, & Wilting, 2000). In *Shh*^{-/-} and *Gli2*^{-/-}/*Gli3*^{-/-} mice, lack of SHH signaling leads to severe disruption of the patterning of somites and results in a complete absence of the vertebral column (Chiang et al., 1996). More subtle defects in somite patterning were observed in the *Gli2* and *Gli3* mutant mice: the ventral and dorsal aspects of the vertebral column were defective in the *Gli2*^{-/-} and *Gli3*^{-/-} mice, respectively (Mo et al., 1997). In the developing limb buds, SHH is expressed in a posterior region called the zone of polarizing activity, which is involved in limb patterning along the anteroposterior axis (Dudley & Tabin, 2000). In *Shh*^{-/-} mice, limb buds form initially, although most distal structures do not develop (Kraus, Fraidenraich, & Loomis, 2001; Mo et al., 1997). In contrast, *Gli3*^{-/-} and *Gli2*^{-/-}/*Gli3*^{-/-} limb buds develop extra digits, resulting in severe polydactyly (Mo et al., 1997). In a more recent study, inhibition of the SHH pathway in rats using vismodegib (a drug for basal-cell carcinoma) showed a spectrum of malformations, including absent/fused digits in the hindlimb and unossified or incompletely ossified skeletal elements (Morinello, Pignatello, Villabruna, Goelzer, & Burgin, 2014).

2.2.2 Gastrointestinal and respiratory systems—SHH signaling plays essential roles in the development of the foregut and the hindgut (Grindley, Bellusci, Perkins, & Hogan,

1997; Kimmel, Mo, Hui, & Kim, 2000; Litingtung, Lei, Westphal, & Chiang, 1998; Mo et al., 2001; Motoyama et al., 1998; Pepicelli, Lewis, & McMahon, 1998; Ramalho-Santos, Melton, & McMahon, 2000). *Shh*^{-/-} mice exhibit severe lung hypoplasia and various defects in the trachea and esophagus, indicating that SHH is an endodermal signal essential for the development of these foregut derivatives (Litingtung et al., 1998; Pepicelli et al., 1998). In less severe cases, *Shh*^{-/-} mice develop esophageal and tracheal stenosis, which are strikingly similar to the foregut defects of the *Gli2*^{-/-} mice (Motoyama et al., 1998). More severe *Shh*^{-/-} phenotypes include esophageal atresia and tracheoesophageal fistula, which resemble those of the *Gli2*^{-/-}/*Gli3*^{-/-} mice (Chiang et al., 1996). These observations clearly illustrate the importance of gene dosage of *Gli2* and *Gli3* in SHH signaling during foregut development and suggest that defective SHH signaling can lead to a spectrum of foregut malformations commonly observed in human disease, including NCNS-HPE. Others have shown that *Shh* and *Gli* mutant mice also display distal hindgut defects (Kimmel et al., 2000; Mo et al., 2001) that represent the full spectrum of anorectal malformations observed in humans, including anal stenosis, imperforate anus and cloaca. In *Shh*^{-/-} mice, the distal hindgut fails to develop into separate alimentary and urinary tracts, resulting in a persistent cloaca (Mo et al., 2001). These observations suggest that SHH signaling plays similar roles in epithelial-mesenchymal interactions underlying the development of the foregut and hindgut. Therefore, defects in this signaling pathway could be a molecular basis for the rarely associated gastrointestinal and respiratory malformations observed in NCNS-HPE.

2.2.3 Cardiovascular system—SHH signaling participates in heart development. SHH is required for both cardiac progenitor specification and subsequent differentiation in *Drosophila* and in vertebrates, and is known to play a role in the specification of left-right asymmetry in early vertebrate embryogenesis (Ahmad, 2017). Accordingly, *Shh*^{-/-} mice display a variety of laterality defects including alteration of heart looping (Meyers & Martin, 1999; Tsukui et al., 1999). The *Gli2*^{-/-}/*Gli3*^{-/-} mice exhibit persistent truncus arteriosus (Chiang et al., 1996), one of the components of VACTERL association that may also manifest, although rarely, in NCNS-HPE.

2.2.4 Urogenital system—*Shh*^{-/-} and *Gli2*^{-/-}/*Gli3*^{-/-} mice also show anomalies of renal development. *Shh*^{-/-} mice develop a solitary kidney and *Gli2*^{-/-}/*Gli3*^{-/-} mice develop a horseshoe kidney. It is possible that these renal defects are secondary to the laterality defects seen in the *Shh*^{-/-} mice or the midline defects seen in the *Gli2*^{-/-}/*Gli3*^{-/-} mice. The study by Morinello et al. (2014) of SHH signaling inhibition in rats using vismodegib showed evidence of a dilated renal pelvis or ureter. As mentioned above, *Shh*^{-/-} mice shows failure of the distal hindgut to separate into alimentary and urinary tracts, which results in the formation of a cloaca (Mo et al., 2001).

In addition to the systemic consequences of pituitary dysfunction as a result of brain maldevelopment, here we describe a spectrum of “below-the-neck” manifestations reported for NCNS-HPE, with a focus on studies involving large cohorts of patients. The findings of a comprehensive review of the literature are summarized in Table I. Reports of patients with a complex phenotype suggestive of chromosomal or syndromic HPE and without a molecular diagnosis were excluded.

3 ENDOCRINE DYSGENESIS IN NCNS-HPE

The first report of endocrine dysfunction in HPE dates back several decades. In 1961, two cases of alobar HPE were described that lacked a pituitary gland, and had hypoplasia of the adrenal cortex and the thyroid gland (Haworth et al., 1961). Over the years, additional clinical reports supported a relationship between NCNS-HPE and endocrinopathies, in particular posterior pituitary dysfunction, suggesting that the same midline defects implicated in the disease somehow affect the formation and function of the pituitary gland (Antonini, Grecco Filho, Elias, Moreira, & Castro, 2002; Hahn et al., 2005; Hintz et al., 1968; Traggiai & Stanhope, 2002). Because the endocrine dysfunction associated with other glands (adrenal cortex, thyroid, gonads) are likely the result of pituitary insufficiency, our discussion below will focus on the latter.

As mentioned above, SHH signaling is essential for the development of the hypothalamic-pituitary axis (M. M. Cohen, Jr., 2010; Treier et al., 2001) and, thus, pathogenic mutations in HPE genes are expected to cause pituitary anomalies. Endocrine disorders were frequently reported in patients with solitary median maxillary central incisor (SMMCI) syndrome due to SHH mutations (MIM 147250), even before SMMCI was considered part of the HPE spectrum (Fulstow, 1968; Rappaport, Ulstrom, & Gorlin, 1976; Rappaport et al., 1977). The molecular etiology for HPE-related endocrinopathies began to expand later when our group reported pituitary dysfunction associated with *GLI2* mutations in patients with a distinctive HPE-like phenotype characterized by defective anterior pituitary formation and hypopituitarism, with or without apparent midline forebrain abnormalities (Roessler et al., 2003). Several years later, Franca et al. described patients with *GLI2* mutations that presented with a similar phenotype characterized by hypopituitarism and ectopic neurohypophysis (Franca et al., 2010). Another study done in a cohort of 30 patients with pituitary stalk interruption syndrome (PSIS) or isolated pituitary hypoplasia found mutations in *SHH* or *TGIF1* in three individuals with a single central incisor, a common feature of NCNS-HPE (Tatsi et al., 2013). Subsequently, additional reports of mutations in *SHH*, *GLI2*, *ZIC2*, *TGIF1* and *CDON* were described in patients with PSIS or pituitary hormone deficiencies with or without overt HPE (Bashamboo et al., 2016; L. E. Cohen, 2012; Flemming et al., 2013; Keaton et al., 2010; Lazaro et al., 2004; Solomon et al., 2012; Tasdemir et al., 2014; Voutetakis, Sertedaki, & Dacou-Voutetakis, 2016). Based on this abundance of evidence, pituitary anomalies have been established as part of the spectrum of midline defects and more specifically, those related to NCNS-HPE definitely constitute mild forms of an expanded NCNS-HPE spectrum (Table I).

3.1 Posterior pituitary dysfunction

Posterior pituitary dysfunction seems to be the most frequently associated pituitary anomaly in HPE and manifests predominantly as diabetes insipidus (DI) (Hasegawa et al., 1990; Traggiai & Stanhope, 2002; Van Gool et al., 1990). Although a formal metaanalysis has not been performed and many times reports do not specify what pituitary hormones are deficient in HPE, the incidence of DI in HPE can be roughly estimated at 3–5% (Dubourg et al., 2004; Lachawan et al., 2009; Paulussen et al., 2010; Solomon et al., 2012). Central DI is usually due to a midline structural abnormality that affects the hypothalamus or posterior pituitary

gland, and results in vasopressin deficiency. In neuroimaging studies of classic HPE, the hypothalamic nuclei showed the highest incidence of non-separation among the deep gray nuclei (Simon et al., 2000), and the severity of DI was found to be correlated with the degree of hypothalamic non-separation (Hahn et al., 2005). However, there was no correlation between the severity of DI and an abnormality of the pituitary gland, suggesting that the deficiency in vasopressin secretion is somehow caused by a hypothalamic defect (Hahn et al., 2005). One proposed mechanism of DI in HPE is an abnormal development of the supraoptic and paraventricular nuclei of the hypothalamus, or in the release of vasopressin via the infundibulum and the posterior pituitary gland (M. M. Cohen, Jr., 1989). The defect in induction or patterning of the hypothalamic nuclei could cause a reduction in the number of vasopressin secretory neurons.

3.2 Anterior pituitary dysfunction

Anterior pituitary dysfunction is less common in NCNS-HPE. Hahn et al. (2005) found in a cohort of 117 children with NCNS-HPE that 11% had hypothyroidism, 7% hypocorticism, 5% growth hormone deficiency, and 6% multiple pituitary hormone deficiency. These incidence values are lower than those reported by Traggiai and Stanhope (2002), who found growth hormone deficiency in 42% and multiple pituitary hormone deficiency in 21% of their patients with HPE, possibly due to a selection bias as their patients were referred by an endocrinologist. In many clinical settings around the world patients with HPE do not undergo a comprehensive endocrine evaluation; therefore, the true incidence of endocrinopathies in NCNS-HPE is likely underestimated.

4 SYSTEMIC ANOMALIES IN NCNS-HPE

4.1 Extracranial skeletal anomalies

Extracranial skeletal defects are frequent in NCNS-HPE. The overall spectrum of skeletal anomalies described across studies include scoliosis, costal and/or vertebral abnormalities, radial dysplasia, talipes equinovarus, absent thumb, polydactyly, brachydactyly, clinodactyly, camptodactyly, and brachymetacarpalism. Our study of 157 NCNS-HPE patients with mutations in *ZIC2* identified extracranial skeletal anomalies in 14% of patients (Solomon et al., 2010), and the study of 645 NCNS-HPE probands and 699 relatives by Mercier et al. (2011) found defects in 16%, 9% and 22% of patients with mutations in *SHH*, *ZIC2* and *SIX3*, respectively. Mutational analysis of about 400 patients with NCNS-HPE spectrum identified 112 individuals with mutations in *GLI2*, of which those with truncating mutations were more likely to have pituitary anomalies and postaxial polydactyly (Bear et al., 2014). A similar study of 110 patients with NCNS-HPE found three individuals with likely pathogenic *GLI2* variants with associated postaxial polydactyly (Bertolacini, Ribeiro-Bicudo, Petrin, Richieri-Costa, & Murray, 2012). Because many mutations in *GLI2* do not result in frank HPE but instead are associated with specific findings including pituitary insufficiency and/or polydactyly, this phenotype was recognized as Culler-Jones syndrome (MIM 615849). Recently, our group published a study of adolescents and adults with NCNS-HPE that proposed an expansion of the NCNS-HPE phenotype to include clinical manifestations not typically considered as part of the spectrum. In that study, one patient

with NCNS-HPE and negative four-gene screening results presented with scoliosis, hemivertebrae, and missing ribs (Weiss et al., 2018).

4.2 Spinal neural tube defects

The study by Mercier et al. (2011) of 645 NCNS-HPE probands and 699 relatives found neural tube defects (NTDs) in 4% and 8% of individuals more frequently associated with *ZIC2* (rachischisis) and *TGIF1* mutations, respectively. However, no overall statistical correlation was found between NTDs and the molecular data. Similarly, we reported an incidence of NTDs of 4% in a cohort of 157 NCNS-HPE patients with mutations in *ZIC2* (Solomon et al., 2010). Interestingly, an association between a histidine track polymorphism in *ZIC2* and NTDs had been previously proposed (L. Y. Brown et al., 2002), although a causal effect has not been demonstrated. Functional studies in a murine model of *Zic2* downregulation showed delayed neurulation resulting in HPE, spina bifida and skeletal abnormalities (Nagai et al., 2000). While the mechanisms by which *ZIC2* mutations cause NTDs are not well understood, they are thought to be associated with the role of this gene in axial midline establishment during early development, even before it appears to influence the development of the dorsal telencephalon (Cheng et al., 2006; Warr et al., 2008).

Other reports have shown an association between spinal neural tube defects and mutations in *SHH* and *SIX3* (Lazaro et al., 2004; Paulussen et al., 2010).

4.3 Congenital heart anomalies

Congenital heart defects have been reported in NCNS-HPE patients with *SHH* (8%), *ZIC2* (14%) and *TGIF1* (25%) mutations (Mercier et al., 2011). To the best of our knowledge, there has been only one single report of cardiac anomaly, in particular a septal defect, associated with *SIX3* mutations (Poelmans et al., 2015). Also, we found in our study of 157 probands with *ZIC2*-associated HPE that approximately 9% had a cardiac anomaly (Solomon et al., 2010). A mutant *Zic2* mouse model has shown association with random cardiac situs and demonstrated that this gene is required for left-right axis formation, likely because of its role controlling the expression of genes driving the formation and function of node cilia (Barratt, Glanville-Jones, & Arkell, 2014). We have previously suggested a link between NODAL signaling, heart anomalies and NCNS-HPE, but to date there have been no reports of patients with mutations affecting this pathway that have both HPE and a congenital heart defect (Karkera et al., 2007; Roessler et al., 2008; Roessler, Pei, et al., 2009). However interesting, a recent work has demonstrated that *ZIC2* mutations cause HPE by disrupting NODAL signaling (Houtmeyers et al., 2016).

4.4 Other congenital visceral anomalies

Visceral defects involving the gastrointestinal, pulmonary and genitourinary systems may occur in NCNS-HPE. Some of the studies mentioned above have described malformations in these systems in patients with NCNS-HPE with identifiable mutations (Bear et al., 2014; Dubourg et al., 2004; Mercier et al., 2011; Paulussen et al., 2010; Solomon et al., 2012; Solomon et al., 2010; Weiss et al., 2018). For example, a significant correlation was found between renal/urinary malformations and mutations in *SHH* and *ZIC2* (Mercier et al., 2011). In our study of 396 individuals with mutations in *SHH*, 5% had genitourinary/renal

abnormalities including hypoplastic penis, cryptorchidism, renal hypoplasia, hypospadias, and ambiguous genitalia (Solomon et al., 2012).

4.5 Fatty liver disease

Nonalcoholic fatty liver disease (NAFLD) was only recently proposed by our group to be part of the HPE spectrum (Guillen-Sacoto et al., 2017). The role of SHH signaling in liver homeostasis has long been established, with vast evidence supporting activation of the pathway during acute and chronic liver injury (Machado & Diehl, 2018). Elicited tissue responses to SHH ligand include expansion of liver progenitor populations, myofibroblast accumulation and fibrogenesis, repair-associated inflammation, and vascular remodeling (Machado & Diehl, 2018). In addition to its role in liver repair, several animal studies have suggested an involvement of the SHH signaling in the regulation of lipid metabolism in adipocytes (Moisan et al., 2015; Suh et al., 2006) and more recently, in hepatocytes (Matz-Soja et al., 2016). However, the effect of human SHH pathway germline mutations on the development and progression of NAFLD had not been investigated. Analysis of a large pediatric and adult NCNS-HPE cohort revealed a significant association between HPE and liver steatosis that was independent of the severity of brain malformation, demographic characteristics, obesity or hepatotoxic influences such as special diets, alcohol or medications (Guillen-Sacoto et al., 2017). This observation was recapitulated in a *Gli2* heterozygous null mouse model of diet-induced fatty liver disease that showed increased liver steatosis with reduced or absent fibrosis and inflammation when, compared to wildtype animals (Guillen-Sacoto et al., 2017). While this finding may appear counterintuitive based on the established paradigm of NAFLD progression otherwise involving upregulation of fibrosis and inflammation (Cai et al., 2016), the long-term effects of liver steatosis in the context of Hh signaling attenuation are unknown and, therefore, patients with HPE and their mutation-positive relatives must be followed up for NAFLD-associated health risks.

5 SUMMARY

While NCNS-HPE has traditionally been considered as an “above-the-neck” condition primarily involving forebrain and craniofacial structures, substantial clinical evidence supports an association between mutations in NCNS-HPE genes and congenital anomalies affecting various organ systems. HPE must be considered as a complex Mendelian disorder in terms of both clinical presentation and molecular etiology that is determined by an intricate network of interacting genetic and environmental factors. It has been suggested that NCNS-HPE involves a multiple-hit process (Ming & Muenke, 2002; Rosenfeld et al., 2010) and animal models have provided evidence supporting at least possible digenic mechanisms that implicate either the same or two different signaling pathways. This hypothesis is supported by observations of low penetrance and/or variable expressivity in NCNS-HPE multiplex families and reports of patients with two different mutations (Ming & Muenke, 2002; Nanni et al., 1999; Rahimov, Ribeiro, de Miranda, Richieri-Costa, & Murray, 2006; Wannasilp et al., 2011). In order to better understand the pathophysiology of the broader NCNS-HPE spectrum, it is critical to perform a thorough evaluation of probands and their relatives to include extracephalic manifestations, especially in mildly affected individuals. Because of the complex interaction between Hh signaling and other developmental

pathways, and the incomplete penetrance and variable expressivity associated with many pathogenic variants, it is possible that a proportion of patients with NCNS-HPE belong to different diagnostic labels of known syndromes or yet unidentified ones. A better understanding of the molecular effects of HPE-associated mutations and the identification of modifier genes will help us to dissect the complex pathogenesis of NCNS-HPE and related disorders. It is our ultimate goal to directly translate research findings into better care for affected patients and their families.

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Biographies

Ariel F. Martinez, PhD, is a research biologist at the Medical Genetics Branch, National Human Genome Research Institute, National Institutes of Health (Bethesda, MD) with eight years of experience working on human developmental diseases, including holoprosencephaly (HPE). He is an Active Candidate of the American Board of Medical Genetics and Genomics in the specialty of Clinical Molecular Genetics and Genomics. Current work in Dr. Muenke's laboratory involves genetics testing of patients with HPE, where he serves as CLIA Laboratory Supervisor. His research is focused on understanding the molecular mechanisms underlying HPE pathogenicity.

Paul S. Kruszka, MD, MPH, is a board-certified clinical geneticist at the Medical Genetics Branch, National Human Genome Research Institute, National Institutes of Health (Bethesda, MD) who has been involved in HPE research with Dr. Martinez and Dr. Muenke since 2013. His research includes searching for new HPE candidate genes, helping establish genotype-phenotype correlations, and identifying environmental factors influencing HPE manifestation.

Maximilian Muenke, MD, is a board-certified pediatric geneticist and Chief of the Medical Genetics Branch, National Human Genome Research Institute, National Institutes of Health (Bethesda, MD). He has worked on the genetics of HPE for the past 25 years. His group has discovered numerous genes contributing to HPE, including the first and best-known gene, Sonic hedgehog. His work has contributed to understanding how the brain develops in humans.

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Table I

Extracephalic manifestations of nonchromosomal, nonsyndromic HPE.

System	Anomaly	Associated Gene(s)*	References
Endocrine	Posterior pituitary anomaly/diabetes insipidus	<i>SHH, SIX3, ZIC2, TGIF1, GLI2, ZIC2/GLI2, ZIC2/GAS1, GAS1, FGF8, FGFR1</i>	4, 5, 7, 13, 15, 16, 20, 26–28, 33, 34, 37–41, 45, 50, 52, 53, 54
	Anterior pituitary anomaly/hypopituitarism	<i>SHH, ZIC2, TGIF1, GLI2, PTCH1, CDON</i>	2–4, 6, 9, 10, 12, 14, 16–22, 29–31, 34, 38, 47, 51, 52, 54
	Short stature	<i>SHH, ZIC2, GLI2,</i>	4, 9, 10, 17, 18, 20, 33, 37, 42
	Growth hormone deficiency	<i>GLI2, CDON</i>	2, 4, 8, 9, 12, 18, 20, 28, 29, 38, 42, 51
	Thyroid dysgenesis	<i>CDON</i>	1
	Hypothyroidism	<i>ZIC2, TGIF1, GLI2</i>	9, 12, 18, 21, 22, 28, 37, 41
	Adrenal hypoplasia/insuff.	<i>SIX3, CDON</i>	14, 16, 18, 20, 21, 24, 51
	Hypogonadotropic hypog.	<i>FGFR1</i>	52
	Precocious puberty	-	41
	Hypoglycemia	<i>GLI2, CDON</i>	3, 4, 10, 12, 18, 21, 28
	Temperature dysregulation	<i>SHH, TGIF1, GAS1</i>	5, 21–23, 41, 48, 49
CNS	Neural tube defect (rachischisis/spina bifida)	<i>SHH, SIX3, ZIC2, TGIF1</i>	19, 24, 27, 35
Cardiovascular	Septal defect	<i>SHH, SIX3, GLI2</i>	4, 32, 34
	Patent ductus arteriosus	<i>SHH</i>	7, 34
	Tricuspid atresia	<i>SHH</i>	34
	Interrupted inferior vena cava	<i>SHH</i>	34
	Situs ambiguous	<i>SHH</i>	34
	Complex vascular anomaly		18
	Other (not specified)	<i>GLI2</i>	24, 35
Extracranial skeletal	Costal/vertebral abnormalities	<i>SHH, SIX3</i>	7, 24, 27, 32
	Scoliosis	<i>ZIC2</i>	41
	Clubhand or foot	<i>SIX3</i>	19, 24
	Syndactyly	<i>SIX3, TGIF1</i>	32, 46
	Absent thumb or 1st metacarp	<i>GLI2</i>	24
	Polydactyly	<i>SIX3, ZIC2, GLI2</i>	9, 10, 12, 24, 29, 30, 46, 52
	Brachydactyly	<i>GLI2</i>	6, 24
	Clinodactyly	<i>SHH</i>	7, 24
	Camptodactyly	<i>SHH, ZIC2</i>	7, 46
	Ungual hypoplasia	<i>GLI2</i>	24
	Brachymetacarpalism	<i>SHH</i>	7, 19
	Other (not specified)	<i>ZIC2</i>	35
Gastrointestinal	Anal atresia	<i>SHH, TGIF1</i>	46
	Esophageal atresia	-	24
	Intestinal stenosis	-	24

System	Anomaly	Associated Gene(s)*	References
	Cholestasis	<i>CDON</i>	3, 51
	Hyperbilirubinemia	-	18, 21
	Fatty liver	<i>SHH, SIX3, FGF8</i>	11, 41
	Other (not specified)	<i>ZIC2</i>	35
Genitourinary	Gallbladder agenesis	<i>GLI2</i>	24
	Cholelithiasis	-	41
	Ureteral dilatation	<i>SHH</i>	24
	Double ureter	<i>GLI2</i>	4
	Renal anomalies	<i>SHH, ZIC2</i>	7, 24, 34, 41
	Vesicoureteral reflux	<i>GLI2</i>	4, 10
	Hypospadias	<i>SHH, GLI2, TGIF1</i>	4, 24, 34, 46
	Cryptorchidism	<i>SHH, GLI2, CDON</i>	4, 10, 24, 34, 51
	Micropenis	<i>SHH, ZIC2, TGIF1, GLI2, GAS1</i>	4, 12, 24, 34, 46, 54, 56
	Ambiguous genitalia	<i>SHH, SIX3</i>	34, 46
	Other (not specified)	<i>SHH, ZIC2</i>	7, 34, 35
Respiratory	Abnormal lung lobulation	<i>GLI2</i>	24
	Left isomerism of lung	<i>SHH</i>	27
	Lung hypoplasia	-	24
	Laryngomalacia	-	20
	Other (not specified)	<i>ZIC2</i>	35
Other visceral malformations	Thymus hypoplasia	-	24
	Common mesentery	-	24
	Single umbilical artery	<i>GLI2</i>	4, 24
	Diaphragmatic hernia	-	24
	Exomphalos	-	24
	Inguinal hernia	-	24
	Umbilical hernia	-	21
	Diastasis recti	-	16
	Polysplenia	<i>CDON</i>	51

¹ Arlis and Ward, 1992;

² Artman and Boyden, 1990;

³ Bashamboo et al., 2016;

⁴ Bear et al., 2014;

⁵ Berry et al., 1984;

⁶ Cohen, 2012;

⁷ Dubourg et al., 2004;

⁸ Fleming et al., 1990;

- ⁹Flemming et al., 2013;
- ¹⁰Franca et al., 2010;
- ¹¹Guillen-Sacoto et al., 2017;
- ¹²Hamilton et al., 1998;
- ¹³Hasegawa et al., 1990;
- ¹⁴Haworth et al., 1961;
- ¹⁵Hayward, 1979;
- ¹⁶Hintz et al., 1968;
- ¹⁷Huang et al., 1998;
- ¹⁸Kjellin et al., 1999;
- ¹⁹Lazaro et al., 2004;
- ²⁰Lo et al., 1998;
- ²¹Matthai and Smith, 1996;
- ²²Keaton et al., 2010;
- ²³Kruszka et al., 2015;
- ²⁴Mercier et al., 2011;
- ²⁵Nanni et al., 2001;
- ²⁶Parker and Vann, 1985;
- ²⁷Paulussen et al., 2010;
- ²⁸Rappaport et al., 1977;
- ²⁹Roessler et al., 2003;
- ³⁰Roessler et al., 2005;
- ³¹Simon et al., 2000;
- ³²Poelmans et al., 2015;
- ³³Hong et al., 2017;
- ³⁴Solomon et al., 2012;
- ³⁵Solomon et al., 2010;
- ³⁷Tasdemir et al., 2014;
- ³⁸Traggiai and Stanhope, 2002;
- ³⁹Van Gool et al., 1990;
- ⁴⁰Wannasilp et al., 2011;
- ⁴¹Weiss et al., 2018;
- ⁴²Yassin and El-Tal, 1998;

⁴⁵Gripp et al., 2000;

⁴⁶Orioli et al., 2001;

⁴⁷Ming et al., 2002;

⁴⁸Richieri-Costa and Ribeiro, 2008;

⁴⁹Ribeiro et al., 2010;

⁵⁰Arauz et al., 2010;

⁵¹Bae et al., 2011;

⁵²Dubourg et al., 2016,

⁵³Lacbawan et al., 2009;

⁵⁴Pineda-Alvarez et al., 2012.

Reports of patients with a complex phenotype suggestive of chromosomal or syndromic HPE and without a molecular diagnosis were excluded. Gene names separated by a forward slash represent individuals with digenic mutations.

* Genes currently reported as associated. New associations may be reported in the future.