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RESEARCH ARTICLE

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Infantile hypertrophic pyloric stenosis in patients with esophageal atresia

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Abstract

Background: Patients born with esophageal atresia (EA) have a higher incidence of infantile hypertrophic pyloric stenosis (IHPS), suggestive of a relationship. A shared etiology makes sense from a developmental perspective as both affected structures are foregut derived. A genetic component has been described for both conditions as single entities and EA and IHPS are variable components in several monogenetic syndromes. We hypothesized that defects disturbing foregut morphogenesis are responsible for this combination of malformations.

Methods: We investigated the genetic variation of 15 patients with both EA and IHPS with unaffected parents using exome sequencing and SNP array-based genotyping, and compared the results to mouse transcriptome data of the developing foregut.

Results: We did not identify putatively deleterious de novo mutations or recessive variants. However, we detected rare inherited variants in EA or IHPS disease genes or in genes important in foregut morphogenesis, expressed at the proper developmental time-points. Two pathways were significantly enriched ($p < 1 \times 10^{-5}$): proliferation and differentiation of smooth muscle cells and self-renewal of satellite cells.

Conclusions: None of our findings could fully explain the combination of abnormalities on its own, which makes complex inheritance the most plausible genetic explanation, most likely in combination with mechanical and/or environmental factors. As we did not find one defining monogenetic cause for the EA/IHPS phenotype, the impact of the corrective surgery could should be further investigated.

KEYWORDS

esophageal atresia, exome sequencing, infantile hypertrophic pyloric stenosis, tracheoesophageal fistula, VACTERL

1 | INTRODUCTION

Esophageal atresia (EA), a congenital discontinuity of the esophagus caused by a faulty development of the foregut, can present either as an isolated defect but is often seen in combination with other malformations (Brosens et al., 2014). EA occurs in about 2.5 cases per 10,000 live births within Europe (Oddsberg, Lu, & Lagergren, 2012; Pedersen, Calzolari, Husby, Garne,, & group, 2012) and over three-quarters of patients present with a tracheoesophageal fistula (TEF) (Macchini et al., 2017; Pedersen et al., 2012). Frequently, the malformations seen in combination with EA are part of the VACTERL (Vertebral, Anorectal, Cardiac, Tracheoesophageal, Renal or urinary tract of Limb malformations) association. VACTERL association is a diagnosis of exclusion in which three or more features of the VACTERL spectrum are present and no known genetic syndrome is identified (Solomon et al., 2012). Clustering of one or more associated malformations could also be the result of a shared genetic etiology. Recognizing these clusters might be hampered by variable expressivity and/or reduced penetrance.

Another prevalent, but less well-known, associated malformation is Infantile Hypertrophic Pyloric Stenosis (IHPS) (Rollins, Shields, Quinn, & Wooldridge, 1989). In these patients, the pyloric muscle hypertrophies in the first weeks of life, causing a narrowing of the pyloric channel (Panteli, 2009). Seemingly, healthy-born infants present at week 3–6 of life with projectile postprandial vomiting. This condition requires surgery where the upper layer of the circular smooth muscle of the pylorus will be incised, to release the passage from the stomach to the intestine again. Previously, we have described a 30 times higher prevalence (7.5%) of IHPS in patients with EA compared to the normal population (0.25%) (van Beelen et al., 2014). This increased prevalence has been reported in other retrospective studies (3.3–13%) as well (Deurloo, Ekkelkamp, Schoorl, Heij, & Aronson, 2002; Palacios, Sanz, Tàrranga, San Roman, & Carbó, 2014). The diagnosis of IHPS is more difficult and often delayed in patients with EA. Relatively common complications after EA repair, such as stenosis of the anastomosis, can protect against reflux and lead to just regurgitation. By the time these patients start vomiting, there is a massive gastroesophageal reflux.

The increased prevalence of IHPS in patients EA suggests a relationship. However, no research has been carried out toward the cause of this increased prevalence. It is unclear if IHPS is the consequence of the surgical repair or the result of a shared genetic etiology. As the esophagus and the pyloric sphincter are both foregut derived structures, we hypothesize that genetic alterations affecting genes important for foregut morphogenesis are the main drivers for the combination of defects seen in these patients. Given the low prevalence of the disorder and the high impact on development, we will concentrate on genes intolerant to heterozygous or recessive variation (Lek et al., 2016; Ruderfer et al., 2016) harboring rare putative deleterious single nucleotide changes or large CNVs.

2 | METHODS

2.1 | Patient cohort

This study was approved by the Medical Ethical Review Board of Erasmus Medical Center (MEC 193.948/2000/159). We searched the Erasmus University MC-Sophia Children's Hospital EA cohort and the database of the standardized prospective longitudinal follow up program in our hospital for children with congenital anatomical anomalies (Gischler et al., 2009) for patients born between 1970 and 2017 with a combination of both EA and IHPS in history. Parental informed consent for whole exome sequencing (WES) was obtained for 15 patients.

2.2 | Detection of genetic variation using exome sequencing

Initially, we included all variants with an minor allele frequency (MAF) below 1% in 1000 Genomes phase 3 version 5, Exome Variant Server 6500 v0.0.30, Genome of the Netherlands (Genome of the Netherlands, 2014), ExAC 0.3 and our in-house cohort ($n = 906$), consisting of individuals captured with the SureSelect Human All Exon 50 Mb Targeted exome enrichment kit v4 ($n = 279$), SureSelect Clinical Research Exome v1 ($n = 387$) and Haloplex Exome target enrichment system ($n = 240$), Agilent Technologies, Inc., Santa Clara, California). We aimed at finding variants that could be classified as pathogenic or likely pathogenic by the American College of Medical Genetics and Genomics (ACMG) guidelines (Richards et al., 2015). All nonsense variants,

variants predicted to affect splicing and all heterozygous variants with a Combined Annotation-Dependent Depletion (CADD) score (Kircher et al., 2014) above 20 were selected for individual patient analysis in different downstream tools (see Supporting information S1). Prioritized variants were further classified according to the criteria in Supporting information S2. Next, we focused on variants with a MAF below 5%, and we selected all protein coding and splicing variants in genes sensitive for recessive variation (Prec <0.9) for evaluation in recessive models (see Supporting information S1). Determination of variant segregation and confirmation of de novo of inherited status of variants was done with Sanger sequencing unless otherwise indicated. Variants were considered ultra-rare (Bennett, Petrovski, Oliver, & Berkovic, 2017) when they were absent from the gnomAD (http://gnomad.broadinstitute.org/) (Yandell et al., 2011) dataset. Ultra-rare, X-linked or recessive variants predicted to be deleterious are submitted to the ClinVar database https://www.ncbi.nlm.nih.gov/clinvar/.

2.3 | Pathway enrichment analysis of genes affected by rare variants

To investigate if specific pathways are enriched with ultra-rare variants, Gene IDs with variants in canonical splice sites, nonsense variants, protein altering inframe InDels and missense variants were uploaded to Ingenuity pathway Analysis (Qiagen, Venlo, The Netherlands). Additionally, a more stringent set was uploaded with loss of function variants, predicted to be loss of function intolerant (PLI ≥0.9 or Prec ≥0.9) and protein altering variants with a Z score ≥ 3.

2.4 | Expression of candidate genes

Candidate gene expression was determined at relevant developmental time points in mouse. Gene expression of top-ranking genes derived from the individual patient sample prioritizations were determined using datasets (GSE13040, GSE19873, GSE34278, GSE15872, GSE43381) downloaded from the Gene Expression Omnibus (GEO) (Edgar, Domrachev, & Lash, 2002). From these datasets, we used public data on mice on the endoderm, mesoderm and ectoderm at E8.25, foregut at E8.5 and esophagus, stomach, pyloric sphincter, and intestine at E11.5–E18.5 (https://www.ncbi.nlm.nih.gov/geo/) (Chen et al., 2012; Li et al., 2009; Millien et al., 2008; Sherwood, Chen, & Melton, 2009; Stephens et al., 2013). These datasets were imported into BRB-ArrayTools Version 4.5.0 - Beta_2 (http://linus.nci.nih.gov/BRB-ArrayTools.html), annotated by Bioconductor (www.bioconductor.org), R version 3.2.2 Patched (September 12, 2015 r69372) and normalized. We determined differential expression between tissue types and classified upregulated genes being expressed in the tissue under investigation.

2.5 | Detection of common SNP associated with IHPS

Genome-wide association studies (GWAS) revealed five loci highly associated with IHPS (rs11712066, rs573872, rs29784, rs1933683, and rs6736913), pointing toward *MBNL1*, *NKX2-5*, *BARX1*, and *EML4* as candidate genes (Everett & Chung, 2013;Fadista et al., 2019; Feenstra et al., 2012). Unfortunately, rs673913 proved to be resulting in false positive results (e.g., due to sequencing errors or alignment difficulties) in all patients and controls (see Supporting information S3). As a results, we did not include rs673913 in our calculation of the polygenic risk score (PGRS). With SNP-array we genotyped the data of EA patients without IHPS, patients with EA and IHPS and unaffected controls to determine ancestry as well as proxy SNP prevalence of the four above-mentioned IHPS associated SNPs. The same was done with data of related and unrelated parents of EA patients and parents of EA/IHPS patients. Sanger sequencing was used to confirm the risk allele frequency of these SNPs in our 15 EA/IHPS patients and to validate the chosen proxy SNPs.

Using the odds ratio (OR) of the associated SNPs, we calculated polygenic risk scores (PGRS): PGRS = $\sum$ Ln (OR risk allele) * allele count (Wray, Goddard, & Visscher, 2007). For this, we used the OR found in GWAS studies (Fadista et al., 2019; Feenstra et al., 2012) and the OR we calculated from our SNP array data for EA patients versus EA/IHPS patients (see Supporting information S4b). A paired t test, Kruskal–Wallis test and Mann–Whitney test was used to compare the PGRS within each patient en between the different groups. All statistical analyses were performed in SPSS V.24.0 (IBM, Chicago, Illinois), with a significance level of $p < .05$.

3 | RESULTS

3.1 | Patient cohort

In total, 27 out of 664 patients (4.1%) born with EA between 1970 and 2017 developed IHPS. Twenty-one (77.8%) of them were male. A sacral dimple was present in seven patients (25.9%), anomalies of the vertebrae or ribs in eight patients (29.7%) and genitourinary anomalies in six patients (22.2%) of which two patients (7.4%) had

TABLE 1 Phenotype description

Individual	Gender	EA-type	Phenotype	Remarks
SKZ_0027	Female	C	EA/TEF, IHPS, thin ear helix, seizures	–
SKZ_0096	Male	C	EA/TEF, IHPS, syndactyly second-third finger, radial dysplasia, abnormal fibula	VACTERL association
SKZ_0244	Male	C	EA/TEF, IHPS, anal atresia, intestinal malrotation, sacral dimple, abnormal os coccygis, abnormal vertebrae L1, thenar hypoplasia, both sides hypoplastic "floating" thumbs, both sides dysplastic radii	VACTERL association, mother is a DES daughter
SKZ_0321	Male	C	EA/TEF, IHPS, mild left sided expansion of the pyelocaliceal system, breath holding spells	–
SKZ_0353	Female	C	EA/TEF, IHPS, sacral dimple, thin/slender build, diminished hearing, palpebral fissures slant up, hemolytic anemia, short phalanges	Glucose-6-phosphate dehydrogenase deficiency
SKZ_0399	Male	C	EA/TEF, IHPS, anal atresia, sacral dimple, two umbilical vessels, posteriorly rotated ears, small ears/microtia, flat face, bifid scrotum, small penis/micropenis, small palmar crease, thick fingers, broad thumbs, proximal placement of thumbs, microstomia, thick broad neck, wide nasal bridge, patent ductus arteriosis, fourth toe abnormally placed	VACTERL association
SKZ_0400	Male	C	EA/TEF, IHPS, extra ribs, fusion of vertebrae, macrocephaly, bulbar dermoid cyst, auricular tags, short thick/broad neck	Klippel-Feil syndrome
SKZ_0683	Male	C	EA/TEF, IHPS, sacral dimple	–
SKZ_0760	Male	C	EA/TEF, IHPS, hemivertebrae, bitemporal narrowing of the head, prominent forehead, hyper mobile/extensible fingers, narrow thorax/funnel chest, thin lower and upper lip, spasticity, cerebral palsy	–
SKZ_0788	Male	C	EA/TEF, IHPS, inguinal hernia, jaundice, deafness	–
SKZ_0790	Female	C	EA/TEF, IHPS	–
SKZ_0796	Male	C	EA/TEF, IHPS	Vanishing twin
SKZ_0848	Male	C	EA/TEF, IHPS, sacral dimple, hypospadias, patent ductus arteriosus	–
SKZ_0887	Male	C	EA/TEF, IHPS, abnormal sacrum, fusion of vertebrae, posteriorly rotated ears, small mandible/micrognathia, rocker-bottom feet, sandal gap of toes, open mouth appearance, short neck, jaundice	–

TABLE 1 (Continued)

Individual	Gender	EA-type	Phenotype	Remarks
SKZ_1003	Male	C	EA/TEF, IHPS, abnormal sacrum, cleft jaw, cleft palate, cleft upper lip, depressed/flat nasal bridge, fused ribs	Methyldopa (aldomet) for hypertension during pregnancy
SKZ_1248	Female	C	EA/TEF, IHPS, small large fontanel, deafness, small ears, auricular tags, single palmar crease, small/ hypoplastic deep set ears	–
SKZ_1260	Male	C	EA/TEF, IHPS, syndactyly of second-third toe, bifid/ fused ribs	–
SKZ_1353	Male	C	EA/TEF, IHPS, cleft uvula, epicanthic folds, abnormal dermatoglypic patterns, hyperconvex/clubbed nails, hypoplastic scrotum, hypospadias, bifid scrotum, hydrocele of testis	–
SKZ_1407	Female	A	EA, IHPS	–
SKZ_1472	Male	C	EA/TEF, IHPS, eczema of hands with hyperhidrosis, blisters and erythema, Xerosis cutis	Antibiotics for respiratory infection during pregnancy
SKZ_1961	Male	C	EA/TEF, IHPS, sacral dimple, mild dysmorphic features, small mouth, pointy ears, long fingers	Maternal hypertension
SKZ_2013	Male	A	EA, IHPS, persistent superior vena cava, scoliosis, Horner's syndrome	–
SKZ_2023	Male	C	EA/TEF, IHPS, small chin, sacral dimple	–
SKZ_2050	Male	C	EA/TEF, IHPS, atrial septum defect	
SKZ_2082	Male	C	EA/TEF, IHPS, persistent tracheolaryngeal cleft, anal atresia, atrial septum defect, tracheal-laryngeal anomaly, prostate fistula	VACTERL association
SKZ_2149	Male	C	EA/TEF, IHPS	–
SKZ_2171	Female	C	EA/TEF, IHPS, spina bifida Th10/11, synostoses vertebrae, hydronephrosis, kyphoscoliosis	Unknown medication for headaches and nerves during pregnancy

Note: EA-type classification according to Gross classification (Gross, 1947).
Abbreviations: EA, esophageal atresia; DES, di-ethylstilbestrol; IHPS, infantile pyloric stenosis; TEF, tracheoesophageal fistula.

hypospadias. Four patients (14.8%) had three or more anomalies within the VACTERL spectrum (Solomon, 2011). A full phenotypical description of the 27 EA/IHPS patients is given in Table 1. Twenty patients have been described previously (van Beelen et al., 2014).

3.2 | Detection of genetic variation

Previously, we have described rare Copy Number Variations (CNVs) and their inheritance pattern in patients with EA (Brosens et al., 2016). Seventeen EA/IHPS patients described in that manuscript are included in this study. None of the six large CNVs identified were de novo, all were inherited from one of the unaffected parents. All CN profiles of main EA and IHPS disease genes (Brosens et al., 2014; Peeters, Benninga, & Hennekam, 2012) were normal. All rare CNVs classified as a variant of unknown significance (VUS), likely deleterious or deleterious are described in Supporting information S5.

Exome sequencing resulted in at least 5 Giga-bases of raw sequence data with an average coverage of 70X and 90% of target bases covered over 20X. Quality of the sequence data is listed in Supporting information S6.

3.3 | Mendelian models of inheritance

As none of the parents of the 15 investigated patients were affected, we first considered dominant de novo and recessive modes of inheritance. We could not identify de novo pathogenic variation in known EA and IHPS disease genes (Brosens et al., 2014; Peeters et al., 2012). Subsequently, we searched for rare putative damaging variation exome wide and could detect putative deleterious ultra-rare protein coding or splice site variation ($n = 100$). We did not detect any (likely) pathogenic variants in known disease genes. Twenty-five variants turned out to be sequencing artifacts. Furthermore, we could not confirm the segregation of 15 mutations due to lack of parental DNA. We determined the segregation of all remaining ultra-rare variants predicted to be VUS ($n = 37$), or likely deleterious ($n = 23$). However, all putative deleterious variants tested were inherited from one of the unaffected parents.

We inspected the CN profiles from WES-CN and SNP-array for partial overlap with genes affected by heterozygous variant predicted to be deleterious in recessive loss of function intolerant or missense intolerant genes ($n = 48$). We could not detect unmasking of a recessive mutation by a CNV.

We did not detect putative homozygous recessive, compound heterozygous nor X-linked-variants in known disease genes. Given the small sample size of our cohort, we concentrated our analysis on putative recessive inherited variants with a population frequency below 0.05 in genes intolerant to recessive variation (PLIrec >0.9). Furthermore, for putative compound heterozygous inherited variants, we additionally focused on genes that do not often have rare missense variants (missense Z score > 2). For putative homozygous and X-linked variants, we excluded variants with a similar homozygous variant in GnoMAD and those with a CADD score below 15 (except for variants predicting splicing). Only variants in *COL4A2* (NM_001846:exon22:c.G1438A:p.A480T, NM_001846:exon44:c.G4195A:p.V1399I), *SLC6A2* (NM_001172502:exon1:c.G80A:p.C27Y, NM_001172502:exon2:c.G418A:p.V140I) and *VPS13D* (NM_015378:exon19:c.C4022T:p.S1341L, NM_015378:exon31:c.C7243T:p.H2415Y) were in genes that do not often have rare missense variants. Only the variants in *VPS13D* could both be classified as VUS.

We believe it is difficult to confidently classify the other putative compound heterozygous variants as VUS or higher as neither the gene has a low rate of missense variants, nor it is a missense variation a known disease mechanism (as it is not in a known disease gene).

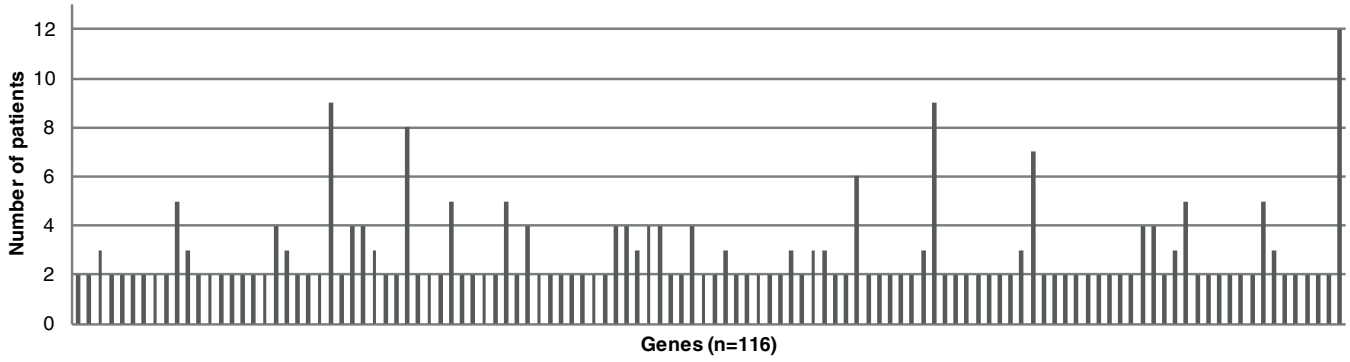

FIGURE 1 Number of patients with variants per gene. Thirty-six genes were found in ≥3 patients of which six genes were present in more than five patients *CNTN2*, *DSPP*, *NOTCH4*, *PRRC2A*, *SEC16B*, *ZNF717*). Four (*AMBRA1*, *ATP2A3*, *DSCAM*, *NOTCH1*) out of 116 genes were predicted to be intolerant for missense variants (Z-score ≥ 3)

TABLE 2 Genetic syndromes and mutated genes with tracheoesophageal and pyloric anomalies as variable features

Syndrome	Esophageal or pyloric anomaly	Inheritance	Loci	Gene(s)	OMIM	References
Esophageal atresia or stenosis						
Epidermolysis bullosa, junctional, with pyloric stenosis or atresia[c]	Esophageal and pyloric atresia or stenosis	AR	2q31.1 17q25.1	*ITGA4* *ITGA6*	226730 226730	Varki, Sadowski, Pfendner, and Uitto (2006); Vivona, Frontali, Di Nunzio, and Vendemiati (1987)) Ruzzi et al. (1997)
Ehlers-Danlos syndrome[c]	EA and IHPS	AD	2q32.2	*COL3A1*	130050	Kroes, Pals, and van Essen (2003); Kuivaniemi et al. (1990)
Trisomy 13	EA/TEF and IHPS	AD	13	Multiple	NA	Brosens et al. (2014); Taylor (1968)
Trisomy 18	EA/TEF and IHPS	AD	18	Multiple	NA	Brosens et al. (2014); Taylor (1968)
Trisomy 21	EA/TEF and IHPS	AD	21	Multiple	190685	Brosens et al. (2014); Freeman et al. (2009)
Fryns syndrome	EA/TEF and IHPS	U	Unknown	Unknown	229850	Ayme et al. (1989)
Fetal alcohol syndrome	EA/TEF and IHPS	NA	NA	NA	NA	Brosens et al. (2014); Lodha, Satodia, and Whyte (2005); Mangyanda et al. (1998)
Motility anomalies of the esophagus						
Epidermolysis bullosa dystrophia[c]	Esophageal strictures and stenosis	AR, AD AR	3p21.31 11q22.2	*COL7A1* *MMP1*	131750 226600	Christiano, McGrath, Tan, and Uitto (1996); Christiano, Suga, Greenspan, Ogawa, and Uitto (1995); Hovnanian et al. (1994)
Cornelia de Lange syndrome[b,c]	Esophageal stenosis and dysmotility and IHPS	AD	5p13.2	*NIPBL*	122470	Cates, Billmire, Bull, and Grosfeld (1989); Gillis et al. (2004)
Apert syndrome	Esophageal stenosis and IHPS	AD	10q26.13	*FGFR2*	101200	Blank (1960); Pelz, Unger, and Radke (1994))
Congenital generalized lipodystrophy	Esophageal dysmotility and IHPS	AR	17q21.2	*PTRF*	613327	Rajab, Heathcote, Joshi, Jeffery, and Patton (2002); Rajab et al. (2010)
Opitz-Kaveggia syndrome	Nutcracker esophagus and IHPS	XL	Xq13	*MED12*	305450	Battaglia, Chines, and Carey (2006); Smith, Edwards, Notaras, and O'Loughlin (2000)
Noonan syndrome[c]	Esophageal dysmotility and IHPS	AD	12q24.13	*PTPN11*	163950	Barberia Leache, Saavedra Ontiveros, and Maroto Edo (2003); Shah, Rodriguez, Louis, Lindley, and Milla (1999)
Visceral neuropathy	Dilated Non-peristaltic esophagus and IHPS	U	Unknown	Unknown	243180	Schuffler, Bird, Sumi, and Cook (1978); Tanner, Smith, and Lloyd (1976)

(Continues)

TABLE 2 (Continued)

Syndrome	Esophageal or pyloric anomaly	Inheritance	Loci	Gene(s)	OMIM	References
Costello syndrome	Loss of elastic fibers in esophagus, IHPS	AD	11p15.5	*HRAS*	218040	Gripp and Lin (1993); Mori et al. (1996)
Other associations						
Chronic idiopathic intestinal pseudo obstruction[b,c]	Gastro-intestinal dysmotility and IHPS	XL	Xq28	*FLNA*	300048	Gargiulo et al. (2007); Tanner et al. (1976)
Fronto-metaphyseal dysplasia[b]	EA/TEF	XL	Xq28	*FLNA*	305620	Franceschini et al. (1997)
X-linked periventricular heterotopia[b]	IHPS	XL	Xq28	*FLNA*	300049	Nezelof, Jaubert, and Lyon (1976)
FG syndrome[b,c]	Esophageal dysmotility and IHPS	XL	Xq28	*FLNA*	300321	Peeters et al. (2012); Unger et al. (2007)
CHARGE syndrome[b,c]	EA/TEF	AD	8q12.1-q12.2	*CHD7*	214800	Brosens et al. (2014)
						Hypogonadotropic hypogonadism with or without anosmia[b,c]
IHPS[a]	AD	8q12.1-q12.2	*CHD7*	612370		Jongmans et al. (2009); Kim et al. (2008)

Note: This table is modified from two reviews on esophageal atresia (Brosens et al., 2014) and infantile hypertrophic pyloric stenosis (Peeters et al., 2012).

Abbreviations: AD, autosomal dominant; AR, autosomal recessive; EA, esophageal atresia; IHPS, infantile hypertrophic pyloric stenosis.; NA, not applicable; TEF, tracheoesophageal fistula; U, unknown; XL, X-linked.

[a]In literature IHPS is associated with other genes responsible for this syndrome.

[b]No overlap in EA and IHPS phenotype for this syndrome, the gene mutated in this syndrome can be responsible for different syndromes in which either EA or IHPS are variable features.

[c]More genes associated to possible several subtypes of this syndrome.

Additionally, we found a homozygous putative splice donor change (*MICAL2*: NM_001346292:exon21:r.spl and a hemizygous change (RPGR: NM_000328:exon14:c.1579_1581del:p.Q527del) we could classify as VUS (see Supporting information A1).

3.4 | Non-Mendelian models

We found variants in the same gene in multiple patients (see Figure 1). Of these 116 genes (VUS = 87, likely deleterious = 30), 36 genes were found in ≥3 patients of which six genes were present in more than five patients. We prioritized all rare variants with three in silico tools (see Supporting information S1). Fifty-four variants in 34 genes were prioritized by VAAST (Hu et al., 2013; Kennedy et al., 2014; Yandell et al., 2011), which prioritizes based on variant deleteriousness as well as by Phevor and PhenIX which prioritize more on phenotype (Singleton et al., 2014; Zemojtel et al., 2014).

We evaluated the number of damaging variants in developmental important pathways and known disease genes using 44 ancestry matched controls sequenced on the same platform as our 15 patients. There were no differences between controls and. However, some genes known to be important for foregut morphogenesis or syndromatically associated with EA or IHPS were affected in patients and unaffected in the healthy controls: *TNXB* (NM_019105.6:c.4444G>A, p.Val1482Met), *WDR11* (NM_018117.11:c.1138G>T, p.Val380Phe), *PEX3* (NM_003630.2:c.1012A>G, p.Ser338Gly), *TBX3* (NM_016569.3:c.506G>A,

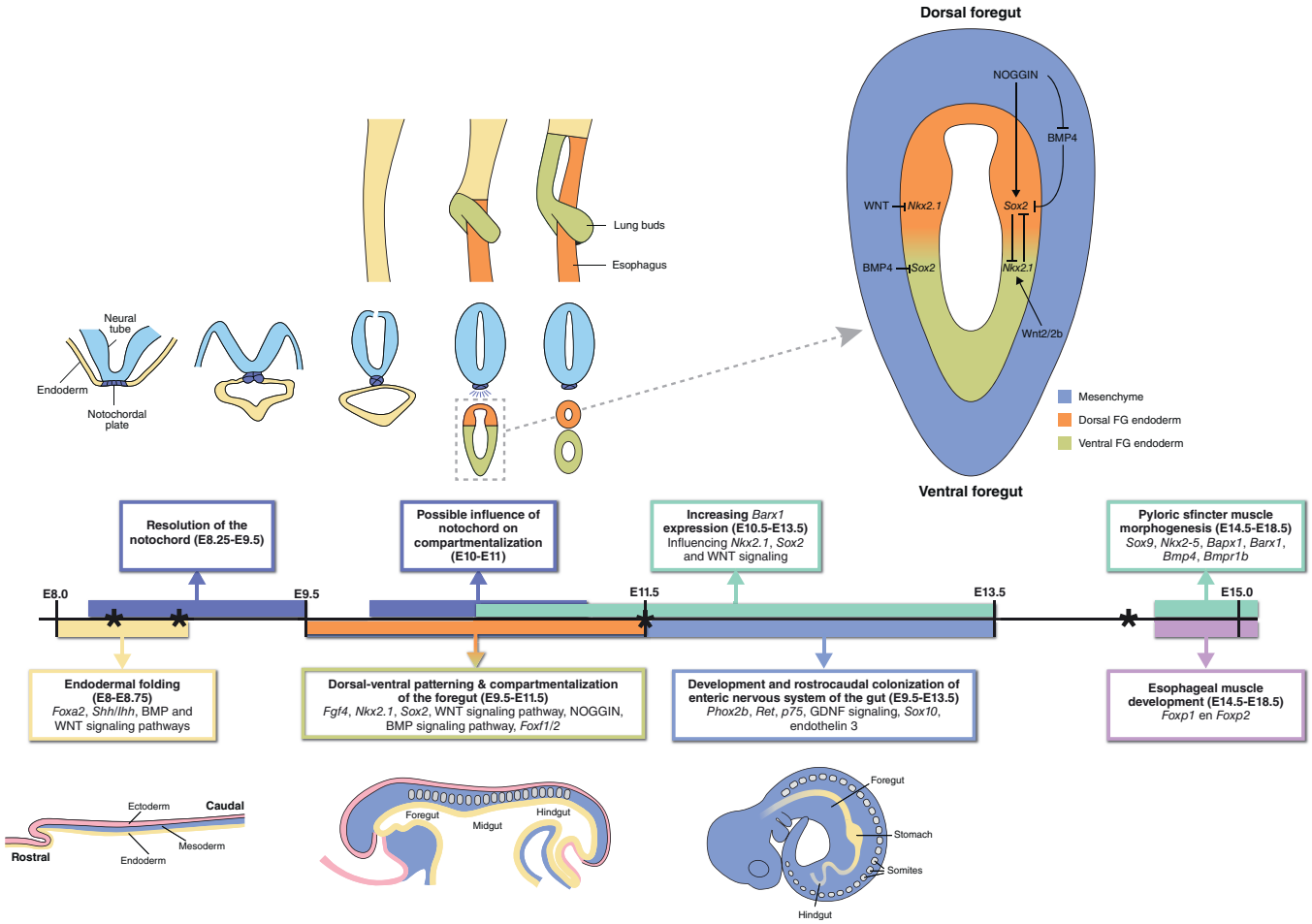

FIGURE 2 Timeline of models and genes known to be important for foregut development in mice (Anderson, Newgreen, & Young, 2006; Fausett & Klingensmith, 2012; Heath, 2010; Perin, McCann, Borrelli, De Coppi, & Thapar, 2017). Visualization of lung bud formation and the genes known to be of importance during tracheoesophageal separation. Timeline of esophageal and pyloric sphincter development. In mice, early foregut formation starts with *Foxa2* stimulation of the anterior endoderm at E8.0 (Heath, 2010). The endodermal sheet folds and forms a tube at E8.75 (Sherwood et al., 2009). Next, signals from the notochord start dorsal-ventral patterning around E9.0, with high *Nkx2.1*/absent *Sox2* in the ventral future trachea and absent *Nkx2.1*/high *Sox2* in the dorsal future esophagus and stomach (Que et al., 2007). These dorsal-ventral patterns lead to compartmentalization of the foregut. Between E9.5 and E11.5, the foregut separates in the primordial esophagus and stomach, and in the primordial trachea. Primordial lung buds become apparent at E9.5 (Sherwood et al., 2009). The separation site is marked by mesenchymal expression of *Barx1* (Woo et al., 2011). The esophagus is completely separated from the trachea at E11.5. Pyloric sphincter formation is mostly studied in chick and mouse models. This formation starts with the thickening of the circular smooth muscle layer between the antrum and the duodenum around E14.5 and the primordial pyloric sphincter is complete around E18.5 (Self et al., 2009; Smith, Grasty, et al., 2000). In addition to its functioning in foregut separation, the *Barx1* homeobox gene is also vital for stomach differentiation and stomach smooth muscle development. It inhibits *Wnt* signaling (Woo et al., 2011) and modulates the expression of *Bapx1*, another important factor required for pyloric sphincter morphogenesis (Jayewickreme & Shivdasani, 2015; Stringer et al., 2008; Verzi et al., 2009). Asterisk represents the time points used in expression analysis

p.Arg169Gln), and *GDF6* (NM_001001557.2:c.281C>G, p.Pro94Arg) (see Supporting information S7). Furthermore, the number of putative deleterious variants between these two groups did not differ (see Supporting information S8) Unfortunately, a burden test comparing the variant profiles of these genes between the patients and their parents was not possible since no WES data of the parents was available.

3.5 | Pathway enrichment analysis of genes affected by rare variants

First, we evaluated genes with variants in canonical splice sites ($n = 16$), nonsense variants ($n = 21$), protein altering inframe InDels ($n = 28$) and missense variants ($n = 557$). Additionally, a more stringent set was used with loss of function variants, predicted to

be loss of function intolerant (PLI ≥0.9, $n = 4$) and protein altering variants with a Z score ≥ 3 ($n = 44$). Only when looking at the selected protein altering variants (Z score ≥ 3, $n = 44$) or loss of function intolerant (PLI ≥0.9, $n = 4$), two pathways were significantly enriched (p value $<1 \times 10^{-5}$): proliferation and differentiation of smooth muscle cells (*INSR*, *ITGB1*, *NOTCH1*, *TCF4*, *PDE4D*, *TERT*, *ANKRD17*, *DICER1*) and self-renewal of satellite cells (*ITGB1*, *NOTCH1*).

3.6 | Expression of main candidate genes during development

With public micro-array transcriptome data we evaluated which genes were upregulated at a specific time-point in the foregut, esophagus or pyloric sphincter and used the output as an indicator of gene expression (see Supporting information S9). Of the genes classified as VUS or likely deleterious in our exome sequencing results, 28 genes were upregulated in both the foregut or esophagus as well as the pyloric sphincter (see Supporting information S9). Seven out of 116 genes with putative deleterious variants in more than one patient were differentially expressed in mice foregut: *Adamtsl4* at E8.5, E14.5 and E16.5; *Ankrd26* at E14.5; *Cntn2* at E8.5, E15.5 and E18.5; *Hspg2* at E8.25, E8.5, E14.5 and E18.5; *Kcnn3* at E8.5 and E15.5; *Ldb3* at E8.5, E14.5 and E15.5; *Sec16b* at E8.5, E14.5 and E16.5.

3.7 | Detection of common SNPs associated with IHPS

We confirmed the selected proxy SNPs found in the SNP array data (see Supporting information S4a) using Sanger sequencing of the four loci highly associated with IHPS (rs11712066, rs573872, rs29784, and rs1933683 near genes *MBNL1*, *NKX2-5*, and *BARX1*, respectively) in the EA/IHPS patient set. ORs for the four risk loci are shown in Supporting information S4b. In total, 28 EA patients (53.6% male), 16 EA/IHPS patients (93.8% male), 80 EA parents (46.3% male, $n = 66$ related), 24 EA/IHPS parents (50.0% male) and 1,297 controls (47.8% male) were compared. We did not find a significantly higher incidence of any risk allele for EA/IHPS patients compared to EA patients. Based on the ORs from the literature, we calculated a median polygenic risk score (PGRS) of 0.56 for EA patients and 0.70 for EA/IHPS patients. When using the OR from the SNP array data, we found a median PGRS of 0.39 for EA patients and 0.58 for EA/IHPS patients. When comparing all groups together, there was no significant difference in PGRS (see Supporting information S4c). When comparing the groups separately, there was a nearly significant difference for the PGRS for EA patients compared to EA/IHPS patients ($p = .08$, see Supporting information S4d). We did not detect rare putatively deleterious variants in *MBNL1*, *NKX2-5*, and *BARX1* in the patient exome sequencing data.

4 | DISCUSSION

We hypothesized that the increased prevalence of IHPS in patients with EA compared to the prevalence of IHPS in the normal population was driven by genetic alterations affecting genes important for foregut morphogenesis. The combination of EA and IHPS makes sense from a developmental perspective as the esophagus and the pyloric sphincter are both foregut derived structures. Organ specification during embryonic development is under tight spatiotemporal control of specific growth factors, transcription factors and signaling cascades (Jacobs & Que, 2013; Li et al., 2009). Disturbances in these pathways could impact proper development. The esophagus, as well as the stomach, starts developing from the fourth week after conception onwards. The stomach turns around its anterior–posterior axis during embryonic development (Cetin, Malas, Albay, & Cankara, 2006). The developing pylorus can be visualized with immunostaining at week six after gestation and differentiates during fetal life (Koyuncu, Malas, Albay, Cankara, & Karahan, 2009).

Environmental (Felix et al., 2008; Feng, Chen, Li, & Mo, 2016; Krogh et al., 2012; Markel, Proctor, Ying, & Winchester, 2015; Sorensen, Norgard, Pedersen, Larsen, & Johnsen, 2002; Zwink et al., 2016) and genetic contributions (Brosens et al., 2014; Peeters et al., 2012; Solomon et al., 2012) have been described for both EA and IHPS as single entities, or in combination with other anatomical malformations. For example, it has been suggested that in utero exposure to diethylstilbestrol (DES) is associated with the development of EA (Felix, Steegers-Theunissen, et al., 2007). Moreover, both malformations are variable features in often phenotypically overlapping genetic syndromes (see Table 2), which indicates a genetic background for EA and IHPS. More evidence for a genetic contribution can be deduced from twin studies and animal models (de Jong, Felix, de Klein, & Tibboel, 2010). The concordance rates in monozygotic twins compared to dizygotic twins is higher for EA (Veenma et al., 2012) and IHPS (Krogh et al., 2010) as single entities. Also, the recurrence risk is elevated for siblings and offspring of affected individuals with EA in combination with other associated anomalies (McMullen, Karnes, Moir, & Michels, 1996; Robert et al., 1993; Van Staey, De Bie, Matton, & De Roose, 1984; Warren, Evans, & Carter, 1979). In contrast, the recurrence risk for isolated EA is low (Schulz et al., 2012) and moderate for IHPS (Elinoff, Liu, Guandalini, & Waggoner, 2005; Krogh et al., 2010). Different than for EA, there has been reported a

male predominance for IHPS (4:1) (MacMahon, 2006). There have been risk loci associated to IHPS (Everett & Chung, 2013; Fadista et al., 2019; Feenstra et al., 2012; Feenstra et al., 2013; Svenningsson et al., 2012). To date, no risk loci have been described for EA.

4.1 | Absence of rare highly penetrant pathogenic changes

As mentioned, EA and IHPS can be part of specific genetic syndromes (see Table 2). None of the 15 patients had a pathogenic alteration in one of those known disease genes. This is in line with previous studies in which limited causal changes could be detected in patients with EA and associated anomalies (Brosens et al., 2016; Hilger et al., 2015; Zhang et al., 2017).

Subsequently, we determining the segregation of heterozygous ultra-rare alterations in genes intolerant to variation and recessive variation in genes intolerant to recessive variation (Lek et al., 2016; Ruderfer et al., 2016). We did not identify ultra-rare de novo dominant, recessive or X-linked deleterious protein coding alterations in these genes. Although we could confirm a compound heterozygous variant in *FAM46A* in one patient and an X-linked variant in *SH3KBP1* in another patient, *FAM46A* and *SH3KBP1* were not differentially expressed at the time points important for foregut morphogenesis. Given the male predominance, it is surprising that no X-linked alterations were identified. Additionally, it is unlikely that a dominant—inherited high penetrant—change is a likely cause of EA and IHPS as the parents of these patients are unaffected. It could be that a rare variant burden exists. However, we have not detected it, likely due to limited sample size. Focusing on known

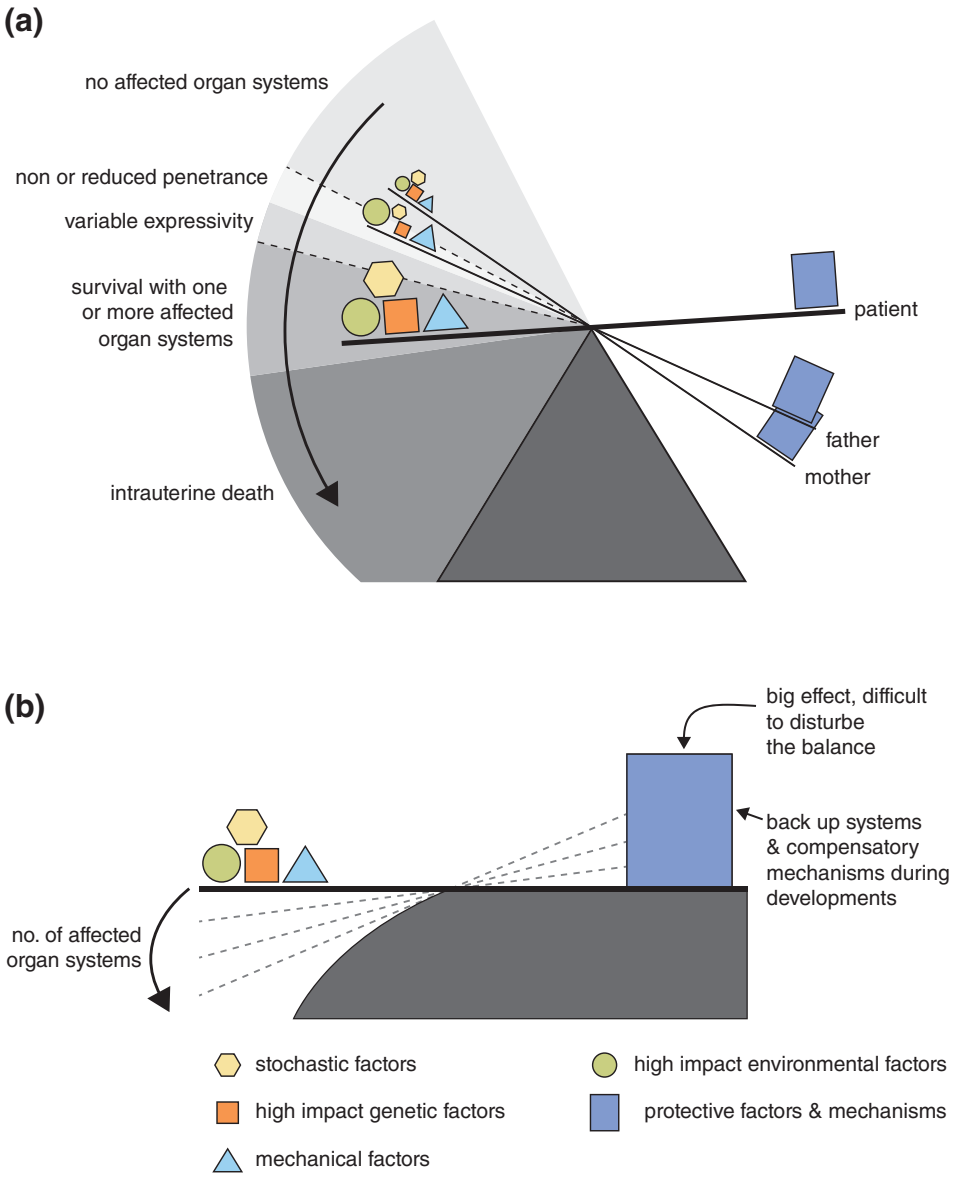

FIGURE 3 Two models for EA/IHPS etiology. (a) Burden model and (b) slippery slope model. The combination of multiple high impact factors (genetic, environmental, mechanical, and/or stochastic) together can modulate the phenotypical spectrum. These risk factors are in balance with protective factors like backup systems and compensatory mechanisms

candidate genes did also not reveal enrichment (Supporting information S7b).

4.2 | Coding sequences of genes crucial in esophageal and pyloric sphincter formation are affected

Subsequently, we focused on genes involved in foregut development by combining the results of literature research (Heath, 2010; Jayewickreme & Shivdasani, 2015; Que et al., 2007; Self, Geng, & Oliver, 2009; Sherwood et al., 2009; Smith, Grasty, Theodosiou, Tabin, & Nascone-Yoder, 2000; Stringer, Pritchard, & Beck, 2008; Verzi et al., 2009; Woo, Miletich, Kim, Sharpe, & Shivdasani, 2011) with data of previous expression studies (Chen et al., 2012; Li et al., 2009; Millien et al., 2008; Sherwood et al., 2009; Stephens et al., 2013) (see Figure 2). Given their described importance in normal development, variations in multiple of these genes might explain the higher incidence of IHPS in patients with EA. Five of these genes (*TNXB*, *WDR11*, *PEX3*, *TBX3*, and *GDF6*) were affected in patients and unaffected in healthy controls. These variants might not be sufficient to result in disease but are predicted to impact the protein and might contribute together with other unknown factors to disease development.

Seven genes (*ADAMTSL4*, *ANKRD26, CNTN2*, *HSPG2*, *KCNN3*, *LDB3*, *SEC16B*) with variants in more than one patient were differentially expressed in the developing foregut, esophagus or pyloric sphincter in mice between E8.25 and E16.5. Most of these variants had a population frequency above the prevalence of EA. If these variants are highly penetrant, they would not be the likely cause. To study reduced penetrance, drastically increased sample sizes are needed for an analysis going beyond known intolerant genes.

4.3 | Haplotypes associated with IHPS development could have an impact in some patients

Additionally, we investigated the IHPS associated risk haplotypes rs11712066, rs573872, rs29784, and rs1933683 (Everett & Chung, 2013; Fadista et al., 2019; Feenstra et al., 2012) in EA/IHPS patients, as well as EA patients, EA parents, EA/IHPS parents and healthy controls. Although we could not identify a significantly higher single risk allele frequency for EA/IHPS patients, we found a slightly higher PGRS for EA/IHPS patients compared to EA patients ($p = .08$). Further research is needed on a larger scale to confirm the impact of this haplotype.

4.4 | Possible contribution of non-genetic factors

Furthermore, previous studies have suggested the contribution of non-genetic factors as an explanation for the combined occurrence of EA and IHPS. The most common thought is that mechanical and/or environmental factors disturb the developmental field. Environmental risk factors like pesticides, smoking, herbicides and periconceptional alcohol or multivitamin use (Felix et al., 2008; Feng et al., 2016; Krogh et al., 2012; Markel et al., 2015; Sorensen et al., 2002; Zwink et al., 2016) have been suggested for both EA and IHPS. Impaired gastric contractility and esophageal relaxation were observed in Adriamycin and doxorubicin induced EA in mice (Tugay et al., 2001; Tugay, Yildiz, Utkan, Sarioglu, & Gacar, 2003). To which extent these factors influence the fetal development, depends on the specific risk factors and their timing.

4.5 | IHPS might be an acquired condition related to surgery or treatment of EA

Last, IHPS could also be the result of the atresia itself, potentially as a result of the surgical procedure or the postoperative treatment. Previous studies have suggested vagal nerve lesions, a gastrostomy and transpyloric feeding tubes as possible causes for an increased incidence of IHPS after correction of EA (Ilhan, Bor, Gunendi, & Dorterler, 2018). IHPS has been suggested to be a neuromuscular disorder with the involvement of smooth muscle cells, interstitial cells of Cajal and the enteric nervous system. The hypertrophy could be the result of discoordinated movements of the pyloric sphincter and the contractions of the stomach (Hayes & Goldenberg, 1957), perhaps as the result of absent nitric oxide synthase activity (Vanderwinden, Mailleux, Schiffmann, Vanderhaeghen, & De Laet, 1992). Mechanistically, this association between EA and IHPS seems plausible. However, it does not explain why IHPS is not fully penetrant in patients with EA. Further research on the cause and other specific clinical risk factors for patients with EA should be considered, for example, the late start of enteral feeding or the long-term tube feeding.

4.6 | Models for EA/IHPS disease etiology

Starting off, we hypothesized that genetic defects, disturbing foregut morphogenesis, would be responsible for

the combination of EA and IHPS. A monogenetic syndromic model is unlikely to explain the increased incidence of IHPS in these patients, since we have not detected a central causative gene. The phenotypical spectrum of our EA/IHPS cohort is very heterogeneous and could be the result of impacts on multiple genes, each gene unique to each individual patient. Therefore, it remains possible that IHPS is a rare and less well-known feature of the syndromic phenotype of EA.

We propose two different multifactorial models in which the combination of CNVs, deleterious protein alterations (Felix, Tibboel, & de Klein, 2007; Brosens et al., 2014), severe changes in the developmental field during the organogenesis (Martinez-Frias, 1994; Martinez-Frias & Frias, 1997) and/or environmental inducing epigenetic changes (Sorensen et al., 2002) together modulates the phenotypical spectrum seen in these patients.

The first is a burden model (see Figure 3a). Genetic, epigenetic, environmental and mechanical factors form a burden of risk factors, which balances with protective mechanisms. In this model, the point of balance is not shifted by a mutation in a central gene. Although each person has certain risk factors, in most individuals this will not lead to affected organ systems. There is an intermediate range between normal and affected in which individuals can have the genetic burden but lack an abnormal phenotype (reduced penetrance) or their symptoms differ in severity (variable expressivity). The latter would fit the results in this study. Mechanical or environmental factors could make the difference in shifting the balance.

The second is a slippery slope model (see Figure 3b) in which the burden of low impact genetic variants and environmental disturbances alone does not impact the balance, until it crosses a certain threshold. The protective mechanisms (e.g., compensatory mechanisms) during development are very strong, making it really difficult to shift the balance. Most fetuses will not develop any malformations despite the combined genetic and environmental burden. Once the threshold is reached, the balance is immediately greatly disrupted and often multiple organ systems are affected. This model also fits with the phenotypical results in this study since four patients had three or more anomalies within the VACTERL spectrum. In this model there is a high tolerance for low impact genetic variation and only high impact variation (aneuploidies, exposure to toxic substances, pathogenic changes in developmental crucial genes) will shift the balance.

5 | CONCLUSIONS

To conclude, the presence of genetic variation in genes involved in foregut development and/or EA or IHPS disease genes might contribute to disease development. We found putative deleterious variation in genes expressed in both the developing esophagus as in the developing pyloric sphincter.

We propose two multifactorial models in which the combination of multiple high impact genetic, mechanical and environmental factors together can shift the balance from normal to abnormal development. A burden model with reduced penetrance or variable expressivity is most likely as genetic factors seem to contribute. Future research should investigate the incidence of IHPS in larger cohorts of patients with EA to further explore this hypothesis. To investigate the role of treatment or surgery, clinical factors related to the surgical correction of EA—for example vagal nerve lesions after surgery, the late start of oral feeding or transpyloric feeding tubes—should be systematically registered.

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CONFLICT OF INTEREST

Authors do not have any potential conflicts (financial, professional, or personal) relevant to the manuscript to disclose.

DATA AVAILABILITY STATEMENT

The identified individual participant data will not be made available. All predicted deleterious variants were submitted to the ClinVar database at https://www.ncbi.nlm.nih.gov/clinvar/.

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