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Heterozygous rare variants in *NR2F2* cause a recognizable multiple congenital anomaly syndrome with developmental delays

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Nuclear receptor subfamily 2 group F member 2 (*NR2F2* or *COUP-TF2*) encodes a transcription factor which is expressed at high levels during mammalian development. Rare heterozygous Mendelian variants in *NR2F2* were initially identified in individuals with congenital heart disease (CHD), then subsequently in cohorts of congenital diaphragmatic hernia (CDH) and 46,XX ovotesticular disorders/differences of sexual development (DSD); however, the phenotypic spectrum associated with pathogenic variants in *NR2F2* remains poorly characterized. Currently, less than 40 individuals with heterozygous pathogenic variants in *NR2F2* have been reported. Here, we review the clinical and molecular details of 17 previously unreported individuals with rare heterozygous *NR2F2* variants, the majority of which were de novo. Clinical features were variable, including intrauterine growth restriction (IUGR), CHD, CDH, genital anomalies, DSD, developmental delays, hypotonia, feeding difficulties, failure to thrive, congenital and acquired microcephaly, dysmorphic facial features, renal failure, hearing loss, strabismus, asplenia, and vascular malformations, thus expanding the phenotypic spectrum associated with *NR2F2* variants. The variants seen were predicted loss of function, including a nonsense variant inherited from a mildly affected mosaic mother, missense and a large deletion including the *NR2F2* gene. Our study presents evidence for rare, heterozygous *NR2F2* variants causing a highly variable syndrome of congenital anomalies, commonly associated with heart defects, developmental delays/intellectual disability, dysmorphic features, feeding difficulties, hypotonia, and genital anomalies. Based on the new and previous cases, we provide clinical recommendations for evaluating individuals diagnosed with an *NR2F2*-associated disorder.

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INTRODUCTION

Nuclear receptor subfamily 2 group F member 2 (*NR2F2*, also known as *COUP-TF2*) gene encodes a member of the nuclear receptor superfamily of ligand-activated transcriptional factors involved in

several developmental and cellular processes. *NR2F2* is an orphan receptor whose ligand is yet to be identified. Similar to the other nuclear receptors, *NR2F2* protein has three main domains: an N-terminal activation binding motif (1–78aa), a DNA-binding domain

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(79-151aa), and a C-terminal ligand-binding domain (177-411aa) separated by the hinge region [1, 2].

The spatiotemporal expression pattern of *NR2F2* during mammalian development has been studied in mouse models [1, 3, 4]. *Nr2f2* expression is observed between E8.5 and E13.5 in the sinus venosus, umbilical veins, heart, atrium, branchial arches, developing hindbrain, neuroectoderm of anterior midbrain, somites, otocyst, the periocular mesenchyme, optic stalk, olfactory placode, developing testes, the genital tubercle, mesenchyme of the kidney, and the adrenal cortex [1, 4]. High expression of *NR2F2* is observed in the mesenchymal component of several organs during development and organogenesis [1, 3].

Homozygous deletion of *Nr2f2* is embryonic lethal in mice. The *Nr2f2*^{-/-} embryos show growth restriction, edematous cysts, severe hemorrhage in the brain and heart, and die around 10 days of gestation [4]. Heterozygous *Nr2f2* knockout mice are smaller than the wild-type mice and show poor postnatal viability. Furthermore, *Nr2f2* knockout mice show defects in sinus venosus development, dysplastic anterior and posterior cardinal veins, atrial malformations, and angiogenesis defects [4]. A recent study showed that female mouse embryos lacking *Nr2f2* in the Wolffian duct mesenchyme develop as intersex, that is, having both female and male reproductive tracts [5]. Other *Nr2f2* mouse models showed developmental anomalies of the female reproductive system [6–8], anteroposterior patterning of the stomach [9], diaphragm [10], lymphangiogenesis, adipogenesis, limb, eye, and cerebellum [3].

In humans, *NR2F2* is extremely intolerant to loss-of-function (LoF) variants (pLI = 0.99 in Genome Aggregation Database (gnomAD) v2.1.1) [11, 12]. *NR2F2* heterozygous LoF variants have been associated with congenital malformations, including CHD, CDH, and DSD (Supplementary Table 1) [1, 13–24]. However, most of these studies were done using disease-specific cohorts and thus have limited clinical description, especially about the other affected organ systems.

Al Turki et al. (2014) described eight individuals with CHD who had rare LoF, missense, and splice variants, and a balanced translocation in *NR2F2* (Supplementary Table 1) [1]. Subsequently, *NR2F2* rare variants were reported in other CHD cohorts [14–17, 22]. High et al. (2016) identified two individuals with CDH and heterozygous LoF variants in *NR2F2* [24], other CDH-cohort studies have also identified rare *NR2F2* LoF variants and a splice variant [15, 19, 21, 24, 25]. In a cohort of 46,XX SRY-negative ovotesticular DSD cases, Bashamboo et al. (2018) found *NR2F2* LoF variants in three individuals with syndromic clinical features including CHD, CDH and dysmorphic facial features of blepharophimosis, ptosis, and epicanthus inversus syndrome (BPES) [20]. Recently, a rare *NR2F2* de novo missense variant was reported in an individual with 46,XY DSD, micropenis and hypospadias [26].

Two case reports have described small chromosome 15q26.2 deletions (<5Mb) encompassing *NR2F2*: a de novo mosaic 1.7Mb deletion in a fetus with CDH and coarctation of the aorta, and a de novo 3Mb heterozygous deletion in a 46,XX individual reared as male with ovotesticular DSD, dysmorphic features, sinus bradycardia, low weight, blepharophimosis, and ptosis (Supplementary Table 1) [27, 28]. Larger deletions (>5Mb) involving *NR2F2* along with other genes [29–34] and a 15q26.2 deletion including *NR2F2* but without precise molecular breakpoints have also been reported [35].

Here, we describe 17 previously unreported individuals with varied phenotypes, including CHD, CDH, and other affected organ systems including vascular malformations, who have rare heterozygous, mostly de novo variants in *NR2F2*. We review and compare the clinical and molecular data of these new cases with previously reported individuals, to delineate and expand the phenotypic and genotypic spectrum of *NR2F2*-associated disorders.

MATERIALS AND METHODS

Trio whole exome sequencing (WES) was performed for the cases, except for case 8 as the father's testing was pending and case 12, where chromosomal microarray analysis was performed. Detailed methods are provided in the Supplementary information.

RESULTS

Molecular findings

Fifteen of the seventeen newly reported individuals in our cohort had de novo, rare, heterozygous variants in *NR2F2* (NM_021005.4; NP_066285.1), and one (individual 5-1) had a variant inherited from a mosaic, mildly affected mother (individual 5-2, 34% variant allelic fraction, Supplementary Table 2 and Fig. 1). The inheritance for the remaining two individuals was unknown. Sixteen of the seventeen individuals had a heterozygous *NR2F2* single nucleotide variant (SNV) whereas one case (individual 12) had a large deletion. Ten of the fifteen unique *NR2F2* SNVs were rare missense variants; the remaining five were predicted LoF variants. All *NR2F2* variants identified in this study were absent in gnomAD (v2.1.1 and v3) and TOPMed population databases (see Table 1 for details on all variants). The distribution of *NR2F2* variants from our study and previously reported cases on the encoded protein and the gene are shown in Fig. 1 and Supplementary Fig. 1.

Notably, two predicted LoF variants (p.Lys340SerfsTer39, individual 4; p.Glu379Ter, individual 13) map to the last coding exon of *NR2F2* and may escape nonsense-mediated decay; no other downstream truncating variants are reported in the affected individuals. These truncations delete a part of the ligand binding domain; however, whether they lead to the loss of *NR2F2* function is currently unknown.

We also reviewed whole exome sequencing data from the Pediatric Cardiac Genomics Consortium (PCGC) cohort [36, 37], which consists of individuals with cardiac defects and identified one additional case with a rare de novo *NR2F2* variant (p.Gly98Ser). This case is not included in the current cohort, but has been shown in Fig. 1.

In one individual (individual 12) chromosomal microarray identified a novel, large de novo deletion (~1.87Mb, arr[GRCh37] 15q26.2q26.3(96755103_98628389)x1 at chromosome 15q26.2 encompassing *NR2F2-AS1*, *NR2F2*, *SPATA8-AS1*, *LINC02254*, *LINC00923*, *ARRDC4*, and *LINC01582* genes (Supplementary Fig. 1). This deletion results in the loss of the entire *NR2F2* gene and this loss is not seen in the population databases (Database of Genomic Variants (DGV), gnomAD SVs v2.1). *ARRDC4*, the only other protein-coding gene in this deleted region, is not constrained for LoF variants (pLI = 0, gnomAD v2) and currently has no known disease associations. Therefore, the clinical features observed in this individual were ascribed to the single copy loss of *NR2F2*.

Clinical findings

We describe 16 unrelated affected individuals and a mildly affected mosaic mother of one of the individuals with heterozygous variants in *NR2F2*. Clinical case reports for the 17 individuals are described in the Supplementary Information. These individuals had variable clinical features, including developmental delays/intellectual disability, CHDs, dysmorphic facial features, feeding difficulties, hearing impairment, hypotonia, genital anomalies, renal abnormalities, CDH, vascular malformations and prenatal findings, including CHD, IUGR, increased nuchal translucency, and single umbilical artery. The clinical features of these individuals are described in Supplementary Table 2 and the frequencies of the phenotypes observed in our cohort are listed in Table 2. The commonly observed clinical phenotypes in multiple individuals with *NR2F2* variants in our cohort are described below.

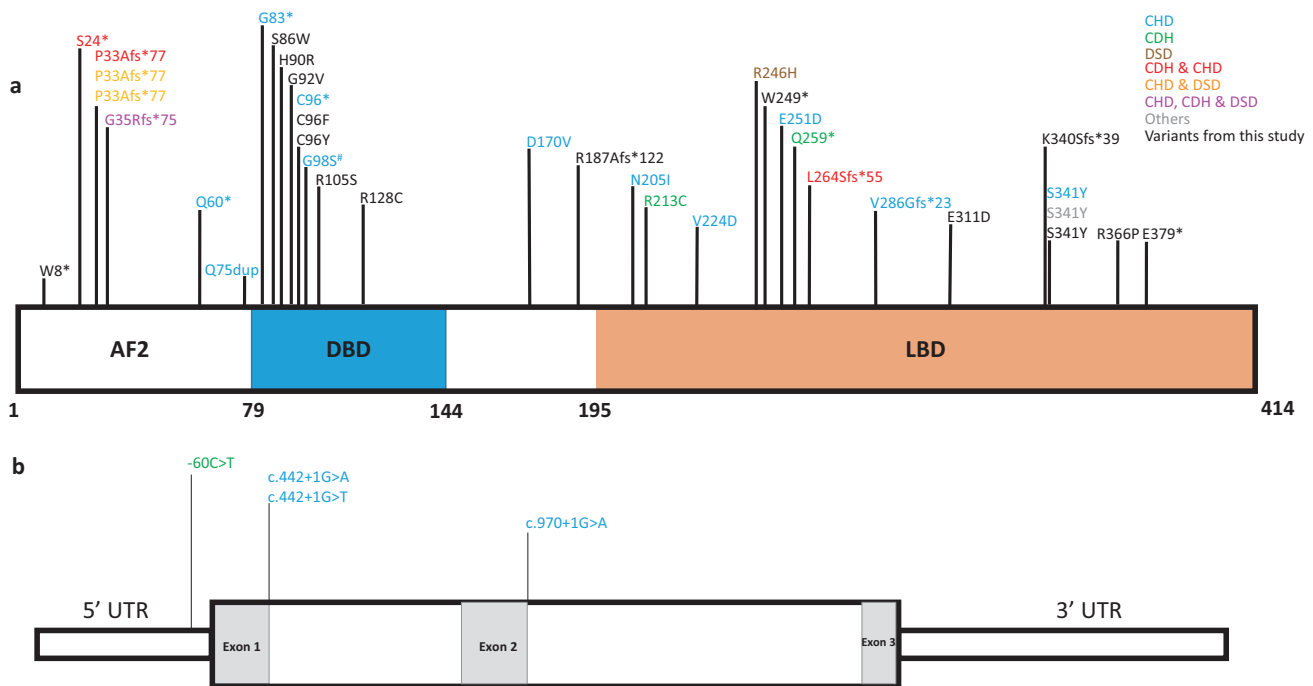


Fig. 1 Variants in NR2F2. **a** Distribution of non-synonymous variants on the NR2F2 protein (NM_021005.4, NP_066285.1). The variants identified in this study are shown in black font, and the previously published variants (except one which is marked with #) are in colored font as per the reported phenotype/clinical features. The location of the functional domains in NR2F2 protein were adapted from Wang et al. 2019 [18]. **b** Schematic representation of the coding exons, the splice variants, and the 5' UTR variant reported in the NR2F2 gene (NM_021005.4). #We also reviewed whole exome sequencing data from the Pediatric Cardiac Genomics Consortium (PCGC) cohort [37, 38], which consists of individuals with cardiac defects and identified one additional case with a rare de novo NR2F2 variant (p.(Gly98Ser), Supplementary Table 4). The cardiac phenotypes, in this case, were secundum atrial septal defect, left aortic arch with normal branching pattern. This case is not included in the current cohort but has been shown in Fig. 1. CHD congenital heart defect; CDH congenital diaphragmatic hernia; DSD disorders of sexual development including 46,XX DSD and 46,XY DSD; "Others" phenotype described in Arsov et al. 2021 [14] includes asplenia. Note that though many of the previously reported affected individuals were ascertained using a disease specific disease cohort, there might be other clinical manifestations which may or may not have been described [1, 14–27] and [Supplementary Table 1].

Prenatal findings. CHDs were detected prenatally in four of seventeen (23.5%) individuals whereas eight of the remaining individuals (47%) had prenatal findings other than CHD, including IUGR, increased nuchal translucency, single umbilical artery, oligohydramnios, hepatic vascular malformation and CDH (Supplementary Table 2).

Postnatal findings

Feeding difficulties. Fourteen of seventeen (82%) individuals had feeding difficulties and/ most with a history of nasogastric tube dependence (13/14).

Developmental delays/Intellectual disability. All 14 individuals for whom data were available had motor delays and thirteen of them also had speech delays. Delays were noted for the age at sitting, walking, and utterance of first words.

Hypotonia was observed in 59% (10/17) of the cohort. Two of the thirteen individuals (15%) for whom brain imaging data were available, showed periventricular white matter abnormalities.

CHD. All individuals (17/17) in our cohort had CHD. The commonly seen anomalies included atrial septal defect (8/17), coarctation of the aorta (8/17), ventricular septal defect (5/17), aortic arch anomalies (3/17), atrioventricular septal defect (2/17), dilated right ventricle (2/17) and pulmonary hypertension (2/17).

CDH. CDH was observed in two of seventeen (12%) individuals, and both died within a few days after birth.

Dysmorphic features. Characteristic facial features were observed in all individuals except for the mosaic mother of individual 5 (16/17; 94%). The commonly observed anomalies (seen in ≥ 3 affected individuals) included upslanted or short palpebral fissures, hypertelorism, low-set and/or dysplastic ears, full cheeks, and retrognathia or micrognathia (Supplementary Tables 2, 4 and Supplementary Fig. 2). Other facial features which were seen in any 2 affected individuals were microcephaly, bilateral epicanthus, deeply set eyes, long eyelashes, long philtrum, facial asymmetry, dysplastic ears, and short neck. Some of the less common features included synophrys, highly arched eyebrows, ptosis, down turned corners of mouth, and tapered finger among others (Supplementary Tables 2, 4).

Hearing loss. Five of fifteen (33%) individuals had hearing loss, which was unilateral in four cases. At least one individual needed hearing aids and another individual had conductive hearing loss.

Strabismus. Divergent strabismus was seen in 6 of 15 (40%) individuals.

Genital anomalies & DSD. Six of seven 46,XY males (86%) in the cohort had cryptorchidism. Out of the nine 46,XX females, one had labial hypertrophy.

One of the 46,XX individuals was diagnosed with DSD (individual 15, SRY negative) and had a de novo NR2F2 LoF variant (c.23G>A, p.(Trp8Ter)). Prenatally, aberrant right subclavian artery, IUGR, and oligohydramnios were detected in this individual and amniocentesis revealed a 46,XX karyotype. At birth, male

Table 1. Molecular details of the NR2F2 variants seen in this study along with in silico predictions for the missense variants (NM_021005.4).

Case	Chr-position-ref-alt (hg19)	HGVS cDNA	HGVS protein	Score		rs IDs			
				SIFT	Provean	REVEL	MetaSVM	CADD	GERP
1	chr15-96877795-G-C	c.933G>C	p.Glu311Asp	0	-2.6	P (0.822)	1.0915	26.9	5.2699
2	chr15-96875621-G-T	c.287G>T	p.Cys98Phe	0.001	-8.48	P (0.9599)	0.8745	32	4.61
3	chr15-96875591-C-G	c.257C>G	p.Ser86Tyr	0	-5.51	P (0.8569)	1.0866	29.9	4.7199
4	chr15-96880621-GA-G	c.1019del	p.Lys340SerfsTer39	-	-	-	-	-	5.12
5 (1 & 2)	chr15-96877608-G-A	c.746G>A	p.Trp249Ter	-	-	-	-	39	5.09
6	chr15-96877420-G-GG	c.588dup	p.Arg187AlafsTer122	-	-	-	-	-	5.05
7	chr15-96875603-A-G	c.269A>G	p.His90Arg	0.001	-6.18	P (0.985)	1.0847	27.3	4.7199
8	chr15-96875621-G-A	c.287G>A	p.Cys96Tyr	0.001	-8.5	P (0.97)	0.8907	31	4.61
9	chr15-96880628-C-A	c.1022C>A	p.Ser341Tyr	0.006	-3.17	P (0.9169)	1.0166	30	5.15
10	chr15-96875647-C-A	c.313C>A	p.Arg105Ser	0	-4.65	P (0.9319)	0.967	28.7	4.61
11	chr15-96875716-C-T	c.382C>T	p.Arg128Cys	0	-6.79	P (0.976)	0.9975	33	4.92
12	arr[GRCh37]15q26.2q26.3(96755103_98628389)x1	-	-	-	-	-	-	-	-
13	chr15-96880740-A-ATA	c.1133_1134dup	p.Glu379Ter	-	-	-	-	-	5.44
14	chr15-96875609-G-T	c.275G>T	p.Gly92Val	0.001	-6.9	P (0.9729)	1.0429	31	4.61
15	chr15-96875357-G-A	c.23G>A	p.Trp8Ter	-	-	-	-	37	4.5599
16	chr15-96880703-G-C	c.1097G>C	p.Arg366Pro	0	-	P(0.951)	-	32	5.44

For the variants when in silico prediction scores were available, they were Damaging (SIFT, Provean, MetaSVM) or Pathogenic (REVEL). The listed single nucleotide variants were not present in gnomAD v2, v3 and TOPMED population databases. Some of the listed variants were submitted to ClinVar by the laboratories which performed the exome sequencing. CADD scores were accessed from <https://cadd.gs.washington.edu/snv>. Bolded variants are predicted to be protein terminating variants.

Table 2. Frequency of clinical features in our cohort.

Clinical Features	Frequency (%)
Prenatal findings	
IUGR/low birth weight	4/17 (23.5%)
Increased nuchal translucency	3/17 (17.6%)
Microcephaly	1/17 (5.9%)
CHD	5/17 (29.4%)
Oligohydramnios	2/17 (11.8%)
Single umbilical artery	3/17 (17.6%)
Postnatal findings	
Microcephaly (at last examination)	6/14 (42.9%)
Dysmorphic features	16/17 (94.1%)
CHD	17/17(100%)
CDH	2/17 (11.8%)
DD/ID	14/14 (100%)
Hypotonia	10/17 (58.8%)
Feeding difficulties/NGT dependence	14/17 (82.4%)
Seizures/staring spells/EEG abnormalities	3/17 (17.6%)
Hearing impairment	5/14 (35.7%)
Strabismus	6/15 (40%)
Asplenia	2/17 (11.8%)
Clinodactyly	5/17 (29.4%)
Genital anomalies	8/17 (47.1%)
Renal anomalies/issues	6/17 (35.3%)
Vascular anomalies	2/17 (11.8%)

external genitalia with perineal hypospadias, pigmented bifid scrotum with penoscrotal transposition and inguinal gonads were observed. There was no history of maternal virilization during the pregnancy. This 46,XX karyotype individual was diagnosed with DSD, severe aortic coarctation, and aberrant right subclavian artery. The abdominal ultrasound showed the presence of both testes, and a testis biopsy revealed testicular parenchyma comprising seminiferous ducts made up of Sertoli cells without the presence of spermatogonia, and the presence of Leydig cells in the testicular interstitium. Subsequently, bilateral orchidopexy, urethroplasty, penoscrotal transposition correction, and scrotal closure were performed.

Renal anomalies. Six of seventeen (35%) individuals had renal findings including renal failure (2/17), renal hypoplasia (1/17), and multicystic dysplastic kidneys (1/17). One of the individuals who had renal hypoplasia and congenital renal failure underwent a renal transplant. Nephrocalcinosis was also noted in one person.

Asplenia. Asplenia was noted in two of seventeen (12%) individuals.

Vascular malformations. Vascular malformations were seen in two of seventeen (12%) individuals, including one with a hepatic vascular malformation and another with vascular lesions on skin.

Skeletal abnormalities. Five out of seventeen (29%) individuals had 5th finger clinodactyly; talipes and clawing of toes were each noted in two individuals, and other skeletal findings were seen in seven out of seventeen individuals (41%; Supplementary Table 2).

DISCUSSION

In this study, we describe 17 previously unreported individuals with rare, potentially disease causing variants in NR2F2 and

provide a comprehensive delineation of the clinical and molecular features of the associated syndrome (Tables 1 and 2, Fig. 1, Supplementary Figs. 3 and 4 and Supplementary Tables 2 and 3). The clinical and molecular details of all the previously reported cases with rare heterozygous *NR2F2* variants are summarized in Supplementary Table 1.

Molecular spectrum

Fourteen of the fifteen *NR2F2* SNVs detected in our cohort are novel variants and have not been previously reported. Nine individuals described in our cohort carry novel rare missense *NR2F2* variants. The missense variant seen in the Pediatric Cardiac Genomics Consortium case (p.(Gly98Ser)) is also not reported before [36, 37]. An additional six missense variants have been previously reported in affected individuals, one of which is recurrent in our cohort (p.(Ser341Tyr); Fig. 1 and Supplementary Table 1). These variants localize to the two conserved functional domains of *NR2F2* protein, the DNA-binding domain and the ligand-binding domain (Fig. 1 and Supplementary Figs. 3 and 4).

Al Turki et al. [1] used an in vitro luciferase assay in HEK293 cells to show the functional effects of *NR2F2* missense variants found in the CHD cohorts (p.(Asp170Val), p.(Asn205Ile), p.(Glu251Asp), p.(Ser341Tyr), and p.(Ala412Ser)). Luciferase assays using transfected expression plasmids of the *NR2F2* variants showed a significant change in the transcriptional activity when compared with wild type *NR2F2* plasmid; the direction of this effect (increased or decreased transcriptional activity) was dependent on the promoter context, that is, *NGFI-A* or *APOB* promoters which are known direct targets for *NR2F2* binding [1]. These results along with the presence of *NR2F2* LoF variants in the affected individuals suggest that the global mis-regulation of transcriptional activities of *NR2F2* targets due to haploinsufficiency or LoF or possibly gain-of-function variants in *NR2F2* during development, may lead to congenital anomalies in multiple organ systems.

Most of the reported *NR2F2* missense variants, however, have not been functionally characterized. Notably, *NR2F2* is constrained for missense changes (missense Z score = 3.6, gnomADv2.1.1). The pathogenicity of the *NR2F2* missense variants detected in the affected individuals is supported by their absence in the population databases, the de novo status, and the pathogenic in silico predictions of their functional impact. In the future, assessing the effect of the *NR2F2* missense variants using in vitro luciferase assays for the transcriptional activity or in vivo chromatin immunoprecipitation (ChIP) experiments using patient-derived cells to assess the changes in levels of *NR2F2* protein binding to target genomic regions, such as gene promoters or regulatory regions, will further elucidate the mechanism of these missense alterations.

Clinical features

The most common clinical features observed in our cohort included developmental delays/intellectual disability (100%), CHD (100%), dysmorphic features (94.1%), feeding difficulties or feeding tube dependence (82.4%), hypotonia (58.8%) and cryptorchidism in 46,XY males (86%, Table 2 and Supplementary Table 3).

All individuals had developmental delays, specifically, language was delayed in all individuals, with a wide range for first words (11 months to 'not yet achieved' at 8 years). Motor milestones were also delayed, with walking achieved at 2–3 years in some cases, but 'not yet achieved' at 8 years in others.

All the affected individuals reported here had CHD and extensive additional medical and developmental issues. The types of heart defects observed were similar to those reported previously, with the majority of individuals having either an atrial or ventricular septal defect or coarctation of the aorta. Three individuals had aortic arch anomalies and two individuals had atrioventricular septal defects. Other less common cardiac defects were valvular and supravalvular pulmonic stenosis, aberrant left subclavian artery, tricuspid regurgitation and bicuspid aortic valve.

Two individuals had pulmonary hypertension and four had cardiac arrhythmias. Interestingly, a de novo *NR2F2* variant p.(Ser341Tyr) was recently reported in a male with developmental delays, asplenia, frequent respiratory infections, dysmorphic facial features, bilateral cryptorchidism, and glandular hypospadias but with no associated CHD, CDH or DSD (Supplementary Table 1) [13]. Echocardiograms and abdominal ultrasound ruled out the presence of CHD and CDH in this individual.

Nearly all individuals in our cohort had dysmorphic facial features, most common being upslanted or short palpebral fissures, micrognathia or retrognathia, low-set or dysplastic ears, hypertelorism, and full cheeks. Other features seen were microcephaly, bilateral epicanthus, long eyelashes, long philtrum, facial asymmetry and short neck. Dysmorphic facial features seen in our cohort and in previously reported individuals with *NR2F2* pathogenic variants [16, 18, 20]; [Supplementary Table 1] overlap with the findings seen in the BPES which is caused by heterozygous loss of function variants in *FOXL2* gene. The shared BPES phenotype in affected individuals with *FOXL2/NR2F2* pathogenic variants suggests, these genes might function together in the developmental pathways leading to eyelid formation. Similarly, both these transcription factors are known to function in mammalian ovarian development and are potential "pro-ovary/anti-testis" genes [20, 38].

Other common findings include feeding difficulties, which required requiring gastric tube due to growth issues. Clinical features that have not been commonly associated with *NR2F2* variants in previously published cases, were seen in our cohort. Five individuals had hypoplastic or dysplastic kidneys, including two with subsequent chronic renal failure, five had hearing impairment, six had strabismus, and three individuals had sleep apnea or breath holding. Laryngomalacia and accessory spleen were also seen in two individuals each (Supplementary Table 2).

Two related individuals (5-1 and 5-2) had asplenia or polysplenia, a phenotypic feature that was recently reported in another affected individual with a de novo *NR2F2* variant [13]. Interestingly, foregut mesenchyme specific conditional *Nr2f2*^{-/-} mice were previously shown to have increased perinatal mortality, CDH, and spleen defects including asplenia [10]. Also, vascular malformations, a phenotype seen in the *NR2F2* mouse model [4], was seen in two individuals of our cohort (individuals 4 and 13). *Nr2f2*^{-/-} mice showed defects in angiogenesis and vascular remodeling, including enlarged blood vessels, abnormal development of the atria and sinus venosus, malformed cardinal veins, and decrease in the complexity of microvasculature in the head and spine regions [4].

Differential diagnoses that were considered for these individuals included Noonan syndrome, Prader-Willi syndrome, Pitt-Hopkins syndrome, Fryns syndrome, Smith-Lemli-Opitz syndrome, peroxisomal disorders, Russell-Silver syndrome, geleophysic dysplasia, Moore Federman syndrome, Myhre syndrome, DSD, and laterality defects.

Genotype-phenotype correlation

Review of all known *NR2F2* SNVs, did not demonstrate any genotype-phenotype correlation or hotspot region(s) where missense variants were significantly enriched (Fig. 1). However, many missense variants are localized to a narrow region in the DNA binding domain (aa 86–105; Fig. 1). Interestingly, the 46,XX DSD associated *NR2F2* LoF variants were all early truncations in coding exon 1 (individual 15, [20]; Fig. 1) or entire gene deletion [27], whereas, on the contrary, all the affected 46,XX individuals with *NR2F2* early truncations [16, 23] or gene deletion (individual 12) did not present with a DSD phenotype (Fig. 1 and Supplementary Table 1).

Our cohort had five predicted LoF *NR2F2* SNVs, and they were present in individuals with a syndromic CHD presentation with neurodevelopmental issues (individuals 5-1, 6, 10 and 13), or 46,XX ovotesticular DSD (individual 15). Similar to our cohort, *NR2F2* LoF variants have been previously reported in cases with

CHD, CDH, and syndromic 46,XX ovotesticular DSD (Supplementary Table 1) [1, 13–25].

In our cohort, there were ten 46,XX individuals, nine of whom were phenotypic females whereas one individual, who carried a heterozygous *NR2F2* LoF variant, was assigned male gender at birth and had a diagnosis of ovotesticular DSD (individual 15; Table 1). *NR2F2* LoF variants and a *NR2F2* gene deletion have been previously reported in individuals with 46,XX ovotesticular DSD [20, 27]. However, individual 12, a 46,XX phenotypic female, who carried a single copy genomic deletion encompassing the entire *NR2F2* gene, did not show evidence of genital anomalies or DSD, but presented with CHD, developmental delays, feeding difficulties, and dysmorphic features. A similar case with a *NR2F2* gene deletion has been reported [28].

The three known deletion CNVs encompassing *NR2F2* also had varied phenotypes, including syndromic CHD (individual 12), syndromic DSD without CHD [27], and syndromic CHD with CDH [28] (Supplementary Fig. 1). These clinical phenotypes highlight the variable expressivity in *NR2F2*-associated disorders. In previous studies the CDH phenotype has only been associated with de novo *NR2F2* LoF SNVs [15, 21, 23, 24] or gene deletion [27], whereas in our cohort, de novo *NR2F2* missense variants in the DNA binding domain were seen in the two individuals affected with CDH (individuals 10 and 11).

A comparison of phenotypes between our cases with missense variants in the DNA binding domain (individuals 2,3,7,8,10,11,14) and in the ligand binding domain (individuals 1,9,16) did not yield any genotype phenotype correlation. Larger cohorts may be needed to recognize any association.

Two of the *NR2F2* variants in our cohort affect amino acid Cys96 (p.(Cys96Phe), p.(Cys96Tyr)), and a nonsense variant at the same amino acid residue has been reported in a CHD cohort [17]. p.(Cys96Ter) variant was identified in a family with six affected individuals with bicuspid aortic valve and fusion of the right and left coronary aortic valve cusps. Two of the affected individuals had aortic stenosis along with aortic regurgitation and another two affected individuals had VSD in addition to bicuspid aortic valve. The two affected individuals in our cohort (individuals 2 and 8) with de novo variants altering Cys98, had CHD, developmental delays, feeding difficulties, and dysmorphic facial features. Individual 2 also had multicystic dysplastic kidneys with chronic renal failure.

Interestingly, two *NR2F2* variants have each been observed recurrently in three unrelated, affected individuals (p.(Pro33AlafsTer77) and p.(Ser341Tyr)). The p.(Pro33AlafsTer77) LoF variant has been reported in two individuals with CHD and 46,XX ovotesticular DSD and in one individual with CHD and CDH [20, 23]. For one of the individuals with 46,XX ovotesticular DSD, the inheritance of p.(Pro33AlafsTer77) was unknown, and for the remaining two cases it was de novo. Similarly, the de novo p.(Ser341Tyr) variant was seen in one individual with an atrioventricular septal defect [1] and in a second individual with asplenia, developmental delays, dysmorphic features, frequent infections, bilateral cryptorchidism, and glandular hypospadias without CHD [13]. In our cohort, this variant was seen in an individual with an atrioventricular defect, feeding difficulties, developmental delays and dysmorphic facial features (individual 9); however, the inheritance of this variant was unknown (not maternal). These variable phenotypic manifestations in individuals carrying identical variants underscore the variable expressivity associated with *NR2F2* sequence alterations. Additional genetic and environmental modifiers may have played a role in the phenotypic expression of the *NR2F2*-related disorder.

Based on the clinical features described in our study, we recommend that any individual with a pathogenic/likely pathogenic *NR2F2* variant should have the following evaluations (1) echocardiogram and electrocardiogram (2) renal-bladder ultrasound (3) assessment of external genitalia and consideration of

imaging if ambiguous (pelvic ultrasound for females/genital tract ultrasound for males) (4) audiology evaluation (5) ophthalmology examination (6) consideration of early placement of gastric tube for problems with weight gain and oral feeding (7) early intervention for speech, occupational, and physical therapy (8) neuropsychological evaluation and developmental assessment as soon as diagnosis is made and (9) confirmation of sex chromosome complement. Testing for *NR2F2* should be considered in any syndromic CHD patient, especially with BPES facial features.

In conclusion, we report findings of 17 unreported individuals and summarize previously reported individuals with heterozygous variants in *NR2F2*, to provide a comprehensive overview of the spectrum of associated features. Based on these data, we provide clinical recommendations for assessment of affected individuals upon diagnosis. These data and recommendations provide guidance for the clinicians and laboratory personnel who encounter this rare disorder.

DATA AVAILABILITY

The data in this study is available in the manuscript, Supplementary information, tables, and figures. The *NR2F2* variants reported in this study are submitted in ClinVar. The following variants have been previously submitted by the clinical testing labs/research groups in ClinVar NM_021005.4: c.1019del:p.(Lys340SerfsTer39), c.746G>A:p.(Trp249Ter), c.558dup:p.(Arg187AlafsTer122), c.269A>G:p.(His90Arg), c.1022C>A:p.(Ser341Tyr), c.1097G>C:p.(Arg366Pro) (Variation IDs: 2429770, 598763, 1805610, 1064859, 128232, 521133). The details of the two variants - c.287G>T:p.(Cys96Phe), c.257C>G:p.(Ser86Trp) which were ascertained as part of the DDD study are available in the DECIPHER website (<https://www.deciphergenomics.org/patient/259383/genotype/191257/browser>; <https://www.deciphergenomics.org/patient/282004/genotype/197133/browser>). The Clinvar IDs for the remaining variants are listed below (Variation IDs: 2570648, 2570646, 2570643, 2570644, 2570645, 2570642, 2570647).

REFERENCES

- Al Turki S, Manickaraj AK, Mercer CL, Gerety SS, Hitz MP, Lindsay S, et al. Rare variants in *NR2F2* cause congenital heart defects in humans. *Am J Hum Genet.* 2014;94:574–85.
- Polvani S, Pepe S, Milani S, Galli A. COUP-TFII in health and disease. *Cells.* 2019;9:101.
- Lin FJ, Qin J, Tang K, Tsai SY, Tsai MJ. Coup d'Etat: an orphan takes control. *Endocr Rev.* 2011;32:404–21.
- Pereira FA, Qiu Y, Zhou G, Tsai MJ, Tsai SY. The orphan nuclear receptor COUP-TFII is required for angiogenesis and heart development. *Genes Dev.* 1999;13:1037–49.
- Zhao F, Franco HL, Rodriguez KF, Brown PR, Tsai MJ, Tsai SY, et al. Elimination of the male reproductive tract in the female embryo is promoted by COUP-TFII in mice. *Science.* 2017;357:717–20.
- Kurihara I, Lee DK, Petit FG, Jeong J, Lee K, Lydon JP, et al. COUP-TFII mediates progesterone regulation of uterine implantation by controlling ER activity. *PLoS Genet.* 2007;3:e102.
- Petit FG, Jamin SP, Kurihara I, Behringer RR, DeMayo FJ, Tsai MJ, et al. Deletion of the orphan nuclear receptor COUP-TFII in uterus leads to placental deficiency. *Proc Natl Acad Sci USA.* 2007;104:6293–8.
- Takamoto N, Kurihara I, Lee K, Demayo FJ, Tsai MJ, Tsai SY. Haploinsufficiency of chicken ovalbumin upstream promoter transcription factor II in female reproduction. *Mol Endocrinol.* 2005;19:2299–308.
- Takamoto N, You LR, Moses K, Chiang C, Zimmer WE, Schwartz RJ, et al. COUP-TFII is essential for radial and anteroposterior patterning of the stomach. *Development.* 2005;132:2179–89.
- You LR, Takamoto N, Yu CT, Tanaka T, Kodama T, Demayo FJ, et al. Mouse lacking COUP-TFII as an animal model of Bochdalek-type congenital diaphragmatic hernia. *Proc Natl Acad Sci USA.* 2005;102:16351–6.
- Karczewski KJ, Francioli LC, Tiao G, Cummings BB, Alfoldi J, Wang Q, et al. The mutational constraint spectrum quantified from variation in 141,456 humans. *Nature.* 2020;581:434–43.
- Lek M, Karczewski KJ, Minikel EV, Samocha KE, Banks E, Fennell T, et al. Analysis of protein-coding genetic variation in 60,706 humans. *Nature.* 2016;536:285–91.
- Arsov T, Kelecic J, Frkovic SH, Sestan M, Kifer N, Andrews D, et al. Expanding the clinical spectrum of pathogenic variation in *NR2F2*: asplenia. *Eur J Med Genet.* 2021;64:104347.
- Qiao XH, Wang Q, Wang J, Liu XY, Xu YJ, Huang RT, et al. A novel *NR2F2* loss-of-function mutation predisposes to congenital heart defect. *Eur J Med Genet.* 2018;61:197–203.

15. Reuter MS, Chaturvedi RR, Liston E, Manshaei R, Aul RB, Bowdin S, et al. The Cardiac Genome Clinic: implementing genome sequencing in pediatric heart disease. *Genet Med*. 2020;22:1015–24.
16. Richter F, Morton SU, Kim SW, Kitaygorodsky A, Wasson LK, Chen KM, et al. Genomic analyses implicate noncoding de novo variants in congenital heart disease. *Nat Genet*. 2020;52:769–77.
17. Wang J, Abhinav P, Xu YJ, Li RG, Zhang M, Qiu XB, et al. NR2F2 loss-of-function mutation is responsible for congenital bicuspid aortic valve. *Int J Mol Med*. 2019;43:1839–46.
18. Upadia J, Gonzales PR, Robin NH. Novel de novo pathogenic variant in the NR2F2 gene in a boy with congenital heart defect and dysmorphic features. *Am J Med Genet A*. 2018;176:1423–26.
19. Kammoun M, Souche E, Brady P, Ding J, Cosemans N, Gratacos E, et al. Genetic profile of isolated congenital diaphragmatic hernia revealed by targeted next-generation sequencing. *Prenat Diagn*. 2018;38:654–63.
20. Bashamboo A, Eozenou C, Jorgensen A, Bignon-Topalovic J, Siffroi JP, Hyon C, et al. Loss of function of the nuclear receptor NR2F2, encoding COUP-TF2, causes testis development and cardiac defects in 46,xx children. *Am J Hum Genet*. 2018;102:487–93.
21. Qiao L, Wynn J, Yu L, Hernan R, Zhou X, Duron V, et al. Likely damaging de novo variants in congenital diaphragmatic hernia patients are associated with worse clinical outcomes. *Genet Med*. 2020;22:2020–28.
22. Li AH, Hanchard NA, Furthner D, Fernbach S, Azamian M, Nicosia A, et al. Whole exome sequencing in 342 congenital cardiac left sided lesion cases reveals extensive genetic heterogeneity and complex inheritance patterns. *Genome Med*. 2017;9:95.
23. High FA, Bhayani P, Wilson JM, Bult CJ, Donahoe PK, Longoni M. De novo frameshift mutation in COUP-TFII (NR2F2) in human congenital diaphragmatic hernia. *Am J Med Genet A*. 2016;170:2457–61.
24. Matsunami N, Shanmugam H, Baird L, Stevens J, Byrne JL, Barnhart DC, et al. Germline but not somatic de novo mutations are common in human congenital diaphragmatic hernia. *Birth Defects Res*. 2018;110:610–17.
25. Schwab ME, Dong S, Lianoglou BR, Aguilar Lucero AF, Schwartz GB, Norton ME, et al. Exome sequencing of fetuses with congenital diaphragmatic hernia supports a causal role for NR2F2, PTPN11, and WT1 variants. *Am J Surg*. 2022;223:182–86.
26. Zidoune H, Ladjouze A, Chellat-Rezgoune D, Boukri A, Dib SA, Nouri N, et al. Novel genomic variants, atypical phenotypes and evidence of a digenic/oligogenic contribution to disorders/differences of sex development in a large North African cohort. *Front Genet*. 2022;13:900574.
27. Carvalho G, Malinverni AM, Moyses-Oliveira M, Ueta R, Cardili L, Monteagudo P, et al. The natural history of a man with ovotesticular 46,XX DSD caused by a novel 3-Mb 15q26.2 deletion containing NR2F2 gene. *J Endocr Soc*. 2019;3:2107–13.
28. Brady PD, DeKoninck P, Fryns JP, Devriendt K, Deprest JA, Vermeesch JR. Identification of dosage-sensitive genes in fetuses referred with severe isolated congenital diaphragmatic hernia. *Prenat Diagn*. 2013;33:1283–92.
29. Poot M, Verrijn Stuart AA, van Daalen E, van Iperen A, van Binsbergen E, Hochstenbach R. Variable behavioural phenotypes of patients with monosomies of 15q26 and a review of 16 cases. *Eur J Med Genet*. 2013;56:346–50.
30. Mosca AL, Pinson L, Andrieux J, Copin H, Bigi N, Puechberty J, et al. Refining the critical region for congenital diaphragmatic hernia on chromosome 15q26 from the study of four fetuses. *Prenat Diagn*. 2011;31:912–4.
31. Dateki S, Fukami M, Tanaka Y, Sasaki G, Moriuchi H, Ogata T. Identification of chromosome 15q26 terminal deletion with telomere sequences and its bearing on genotype-phenotype analysis. *Endocr J*. 2011;58:155–9.
32. Rump P, Dijkhuizen T, Sikkema-Raddatz B, Lemmink HH, Vos YJ, Verheij JB, et al. Drayer's syndrome of mental retardation, microcephaly, short stature and absent phalanges is caused by a recurrent deletion of chromosome 15(q26.2—>qter). *Clin Genet*. 2008;74:455–62.
33. Davidsson J, Collin A, Bjorkhem G, Soller M. Array based characterization of a terminal deletion involving chromosome subband 15q26.2: an emerging syndrome associated with growth retardation, cardiac defects and developmental delay. *BMC Med Genet*. 2008;9:2.
34. Poot M, Eleveld MJ, van 't Slot R, van Genderen MM, Verrijn Stuart AA, Hochstenbach R, et al. Proportional growth failure and oculocutaneous albinism in a girl with a 6.87 Mb deletion of region 15q26.2—>qter. *Eur J Med Genet*. 2007;50:432–40.
35. Rujirabanjerd S, Suwannarat W, Sriro T, Dissaneevate P, Permsirivanich W, Limprasert P. De novo subtelomeric deletion of 15q associated with satellite translocation in a child with developmental delay and severe growth retardation. *Am J Med Genet A*. 2007;143A:271–6.
36. Hoang TT, Goldmuntz E, Roberts AE, Chung WK, Kline JK, Deanfield JE, et al. The congenital heart disease genetic network study: cohort description. *PLoS One*. 2018;13:e0191319.
37. Pediatric Cardiac Genomics C, Gelb B, Brueckner M, Chung W, Goldmuntz E, Kaltman J, et al. The congenital heart disease genetic network study: rationale, design, and early results. *Circ Res*. 2013;112:698–706.
38. Rastetter RH, Bernard P, Palmer JS, Chassot AA, Chen H, Western PS, et al. Marker genes identify three somatic cell types in the fetal mouse ovary. *Dev Biol*. 2014;394:242–52.

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AUTHOR CONTRIBUTIONS

MG collected, analyzed and interpreted the data, drafted the introduction, results, discussion, figures, and tables. MG, LSM, MM, DL, EB, SBS, SMW, KL, PA, AS, LB, ATW, JMS, KS, FD, BC, BI, MFB, AP, JA, EZ, JPS, ADI, JM, VS, SL, SB, IT, JDC, CTG, WKC, SB, EB, NCB, AS provided the clinical data, wrote the clinical case descriptions, methods, critically reviewed and edited the manuscript. EB conceived the study, interpreted the data, and critically reviewed the manuscript.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICAL APPROVAL

The Institutional Review Board of the Children's Hospital of Philadelphia approved this study. Informed consent was obtained from all individual participants included in the study. Families of individuals 2 & 12 consented for publication of images.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41431-023-01434-5>.

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UNDIAGNOSED DISEASES NETWORK

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