Whole genome sequencing in transposition of the great arteries and associations with clinically relevant heart, brain and laterality genes

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Background The most common cyanotic congenital heart disease (CHD) requiring management as a neonate is transposition of great arteries (TGA). Clinically, up to 50% of TGA patients develop some form of neurodevelopmental disability (NDD), thought to have a significant genetic component. A "ciliopathy" and links with laterality disorders have been proposed. This first report of whole genome sequencing in TGA, sought to identify clinically relevant variants contributing to heart, brain and laterality defects.

Methods Initial whole genome sequencing analyses on 100 TGA patients focussed on established disease genes related to CHD (n = 107), NDD (n = 659) and heterotaxy (n = 74). Single variant as well as copy number variant analyses were conducted. Variant pathogenicity was assessed using the American College of Medical Genetics and Genomics-Association for Molecular Pathology guidelines.

Results Fifty-five putatively damaging variants were identified in established disease genes associated with CHD, NDD and heterotaxy; however, no clinically relevant variants could be attributed to disease. Notably, case-control analyses identified significantly more predicted-damaging, silent and total variants in TGA cases than healthy controls in established CHD genes ($P < .001$), NDD genes ($P < .001$) as well as across the three gene panels ($P < .001$).

Conclusion We present compelling evidence that the majority of TGA is not caused by monogenic rare variants and is most likely oligogenic and/or polygenic in nature, highlighting the complex genetic architecture and multifactorial influences on this CHD sub-type and its long-term sequelae. Assessment of variant burden in key heart, brain and/or laterality genes may be required to unravel the genetic contributions to TGA and related disabilities. (Am Heart J 2022;244:1–13.)

Background

Congenital heart disease (CHD) affects approximately 0.6% to 0.8% live born children globally.[1,2] The most common cyanotic congenital heart disease (CHD) in the neonate, constituting 5% to 7% of all heart defects, is D-loop/dextro-transposition of the great arteries (d-TGA), which is characterised by atrioventricular concordance and ventriculoarterial discordance. Survival is dependent on the arterial switch operation which is conducted within the first days to weeks of life, with 90% of patients surviving to adulthood.[3]

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Submitted May 20, 2021; accepted October 8, 2021

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0002-8703

https://doi.org/10.1016/j.ahj.2021.10.185

The pathogenesis of d-TGA is poorly understood with early research implicating environmental risk factors and teratogenic substances, in disease causation.[4-6] TGA is conventionally thought of as a conotruncal defect, with failed rotation of the great vessels leaving persisting parallel great arteries and resulting ventriculoarterial discordance.[3] However, in the last decade, it has been suggested that a subset of TGA may arise from an error in specification of "laterality" or left-right patterning, with associated variants identified in genes, such as *NODAL, ZIC3* and *CFC1*, that determine the direction and architecture of cardiac looping during development and the left-right morphology of the atria and abdominal organs.[7-11] Indeed, d-TGA is seen among patients with heterotaxy syndrome. The low familial recurrence[12,13] and absence of extra-cardiac anomalies compared to other conotruncal lesions, provide further evidence for a shared genetic basis with laterality disorders.[14,15]

While copy number variants (CNV) have been associated with disease and broadened the list of candidate genes,[16] the genetic contributions in TGA remain largely unknown for the majority of presenting cases, which tend to be isolated and sporadic in nature.

Recently, the contribution of cilia-related genes has become increasingly relevant in CHD pathogenesis[17] and more specifically in TGA. In the first exome-based study of TGA, conducted on 65 patients with both d-TGA and l-TGA, despite the very different structural and clinical presentations, associations between airway ciliary dysfunction, respiratory symptoms and genetic variants in established primary ciliary dyskinesia (PCD) genes were sought.[18] While the mutational load was significantly higher than in controls; surprisingly, no clinically relevant variants were identified in known PCD-associated genes. This finding was echoed in a more recent exome analysis in patients with TGA, comprising d-TGA, l-TGA and double outlet right ventricle-type TGA.[19] While 82 candidate genes enriched for cilia-related pathways were identified, providing further evidence for the role of ciliary genes in CHD pathogenesis,[20] and a novel candidate gene for TGA, *DYNC2LI1*, proposed; no clinically relevant, disease-causing variants in known PCD genes were identified.[19]

Characteristic of patients undergoing the arterial switch operation is the later onset of neurodevelopmental disorders (NDD). NDD in TGA has been extensively studied with studies demonstrating that patients with d-TGA are particularly vulnerable to NDD with impairments affecting key neurocognitive areas including psycho-motor, visual-spatial and executive functioning which continue across the lifespan.[21-23] Associations with impaired psychosocial health and quality of life is also becoming increasingly apparent, particularly during adolescence, with notable attention deficits in childhood highly predictive of worse psychosocial health in adolescents.[21,24,25] Known risk factors, including perioperative events, currently explain less than 30% of the variability in NDD outcomes, implicating genetic and other patient-specific factors in disease development.[26] Evidence supporting the previously suggesting genetic link between heart and brain development in patients with CHD and NDD has recently been presented.[27] Furthermore, the critical role of primary cilia in neurodevelopment is becoming increasingly apparent[28,29] and provides an attractive explanation for the NDD often accompanying patients with TGA.

In this study, we sought to define the translational relevance of these recent findings. We performed whole genome sequencing in 100 patients with isolated and only d-TGA. We developed purpose-designed virtual gene panels, comprising genes reproducibly associated with human disease, to identify clinically relevant genetic variants associated with heart, brain and laterality defects. Specifically, we sought to address three questions for this homogeneous patient group: (1) can a subset of d-TGA be attributed to clinically relevant genetic variants in key heart expressing genes; (2) can clinically relevant genetic variants in laterality genes further support d-TGA being considered a defect in laterality; and, finally, (3) can actionable genetic variants in key brain expressing genes be identified, that may be associated with adverse neurodevelopmental outcomes in d-TGA patients.

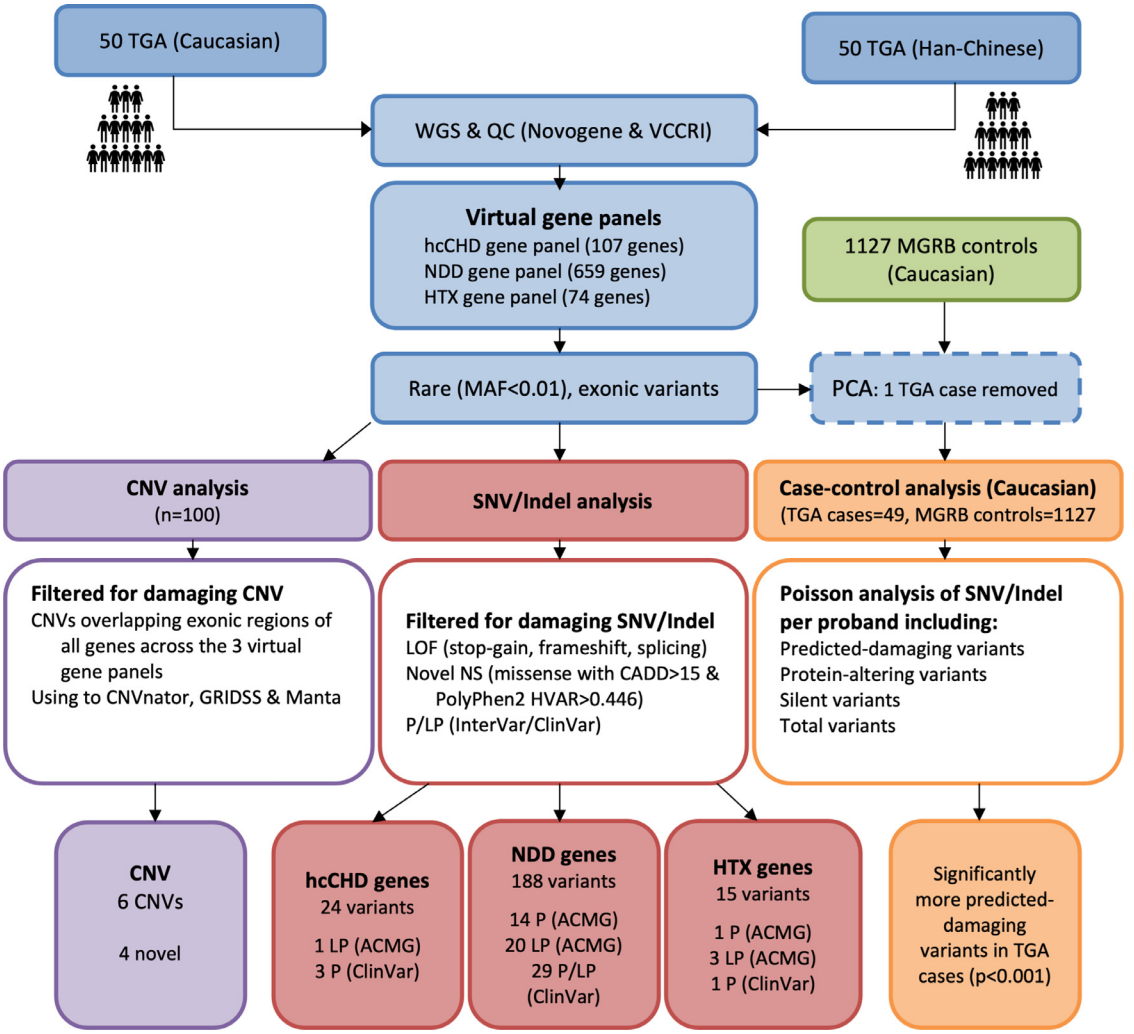
Methods

An outline of the study methods is presented in Figure 1, the full description of the methods is available as part of the Supplementary Materials (Supplemental Methods, Supplemental Table I, Table II, Supplemental Figure 1–4). The data that support the findings of this study are available from the corresponding author upon reasonable request. Ethical approval was obtained from the Sydney Children's Hospitals Network Human Research Ethics Committee (2019/ETH03951) and all study participants consented to their participation in whole genome sequencing research.

Sources of funding

This work is supported by a HeartKids Grant-in-Aid (2018). The MGRB was funded by the NSW State Government. GMB is supported by a National Heart Foundation Postdoctoral Fellowship (101894). EG is supported by a NSW Health Early-Mid Career Fellowship, a National Heart Foundation Future Leader Fellowship (101204). SLD is the recipient of a National Health and Medical Research Council (NHMRC) Project Grant ID1102373, Principal Research Fellowship ID1135886, and NHMRC Synergy Grant ID1181325, and a NSW Health Cardiovascular Research Capacity Program Senior Scientist Grant.

Figure 1



Study outline and summary of findings. Flowchart depicting the study design, including the WGS process, virtual gene panel design and variant filtering, as well as the main findings of the study. *WGS*, whole genome sequencing.

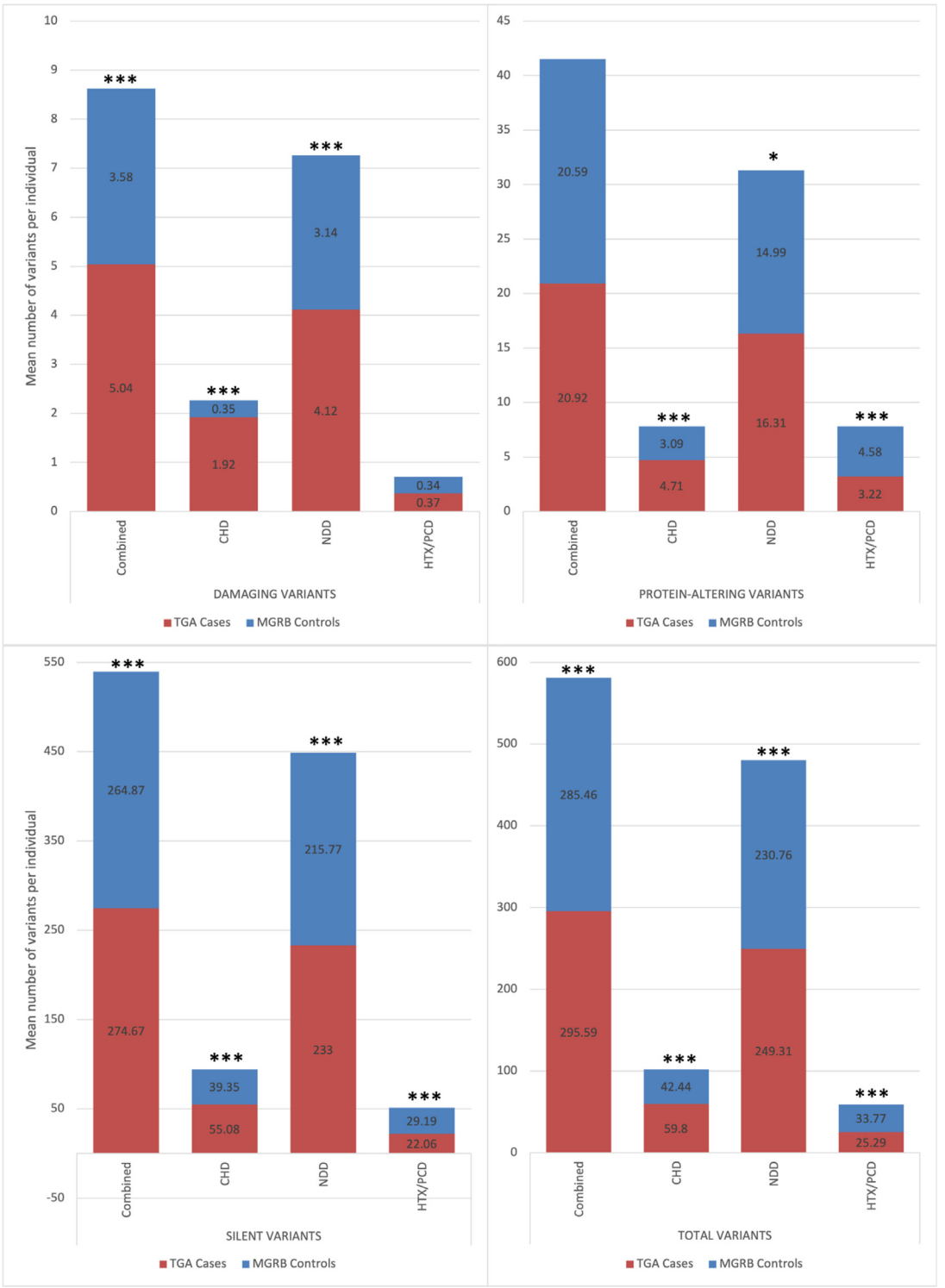
RPH has a National Health and Medical Research Council Australia Fellowship (573705), a Senior Principal Research Fellowship (APP1118576), and is supported by funds from the National Heart Foundation of Australia and HeartKids Australia (G11S5629), the Australian Research Council (ARC) Special Research Initiative into Stem Cell Science (CT0094B; SR110001002), the New South Wales (NSW) Government Ministry of Health (Cardiovascular Disease Senior Scientist Grant; 20:20 campaign) and the NSW Cardiovascular Research Network (LM373). JG is supported by NHMRC Senior Principal Research Fellowship (APP1155224).

Results

Study participants

Study particpants comprised 50 Han Chinese and 50 Caucasian patients with d-TGA. Clinical and demographic information is presented in Supplemental Table III. Low cardiac output syndrome developed post-surgery in 20 Caucasian paitents (40%) and in 15 Han Chinese patients (33% - excluding the 4 patients who did not undergo surgery). Of the 50 Caucasian patients with detailed clinicla data, six patients (12%) had neurological events including infarcts, seizures, haemorrhages and encephalopathies, with three presenting pre-operatively and three post-operatively. Twenty-three of the 50 Caucasian participants had available neurodevelopmental as-

Figure 2



Case-control analyses showing the mean number of variants between Caucasian TGA cases and healthy Caucasian controls across the CHD (n = 107), NDD (n = 659) and HTX/PCD (n = 74) gene panels and all genes combined (n = 779). (**A**) Predicted-damaging variant analysis; (**B**) Protein-altering variant analysis; (**C**) Silent variant analysis; and (**D**) Total variant analyses. *$P < .05$; ***$P < .001$. *CHD*, congenital heart disease; *HTX*, heterotaxy; *NDD*, neurodevelopmental disabilities; *PCD*, primary ciliary dyskinesia; *TGA*, transposition of great arteries.

sessments at age 3 years using the Bayley Scale of Infant and Toddler Development-III (BSID-III).[30] Patients with scores ≤7 in two or more of the five BSID-III domains, were classed as having NDD. Of those 23 participants, only 1 participant was identified as having mild NDD with the remaining 22 having normal scores. This was surprising following reports that up to 50% of TGA patients have NDD,[21,31] although our sample size is small and developmental tests were uniformly offered but not undertaken. Patients from remote locations or lowere socioeconomic strata may face obstacels to testing and are alos acknowledged to be at higher risk of NDD.[24] Speech delays were reported in the medical records of 2 patients despite a normal BSID-III in 1 patient. The majority of the Caucasian cohort comprised sporadic CHD (ie, no family history of CHD), except 1 participant who had a sibling with a spontaneously closed ventricular septal defect. All surgeries were performed using conventional techniques with continuous full flow cardiopulmonary bypass.

Cardiac analysis – CHD gene panel

Prior to variant filtering, a total of 1518 rare variants (MAF ≤ 0.01) were identified in the 100 TGA patients using the high confidence CHD (hcCHD) gene panel. After QC, filtering and prioritization, 24 candidate variants remained comprising 3 stop-gain, 5 splicing and 16 novel missense (Figure 1, Supplementary Table IV). Two likely pathogenic variants in *MYBPC3* and *MYH7* (both in Han Chinese participants) were identified by InterVar with both variants previously associated with hypertrophic cardiomyopathy (HCM) not congenital heart disease (Table 1). The stop-gain variant in NM_000256.3(*MYBPC3*):c.2526C>G (p.Tyr842Ter) is frequently observed in Han Chinese patients with HCM[32]; and while the patient is reportedly doing well with no signs of HCM in routine surveillance echocardiography, this information will be used to guide future clinical management and monitoring for possible late-onset HCM. Despite being listed as likely pathogenic according to InterVar, the non-synonymous variant NM_257.2(*MYH7*):c.5459G>A(p.Arg1820Gln) is classed as a variant of uncertain significance (VUS) in ClinVar and upon manual variant curation using ACMG-AMP guidelines, due to conflicting *in silico* predictors and observations in population databases. Two additional missense variants in *GJA1* and *ZFPM2,* previously associated with CHD, were identified[33,34]; however, despite classed as "pathogenic" by some submitters to ClinVar, both InterVar and manual curation class these variants as VUS due to conflicting evidence (Table 1).

Neurodevelopmental analysis – NDD gene panel

Prior to variant filtering, a total of 8243 rare variants (MAF ≤ 0.01) were identified in the 100 TGA patients using the purpose-designed NDD gene panel. After QC, filtering and prioritisation, 188 candidate variants remained comprising 15 stop-gain, 1 stoploss, 17 frameshift, 51 splicing and 104 novel missense variants (Figure 1, Supplementary Table IV). Of the 188 candidate variants, 14 were classified as pathogenic and 20 as likely pathogenic according to ACMG-AMP guidelines. Twenty variants were classified as pathogenic and/or likely pathogenic in ClinVar (Table 1).

In 1 patient, 2 heterozygous variants were identified in the *POLG* gene, with both variants previously observed in patients with POLG-related disorders[35,36] (https://tools.niehs.nih.gov/polg/). The NM_002693.2(*POLG*):c.2554C>T (p.Arg852Cys) variant has been reported in multiple patients with POLG-related disorders who were compound heterozygous for this and a second pathogenic variant. While the NM_002693.2(*POLG*):c.32G>A (p.Gly11Asp) variant has also been observed in patients with POLG-related disorders, it typically occurs in *cis* with NM_002693.2(*POLG*):c.2554C>T (p.Arg852Cys) and another pathogenic variant on the other allele.[35,37] Stewart et al concluded that NM_002693.2(*POLG*):c.32G>A (p.Gly11Asp) is probably a benign polymorphism or disease modifier and that NM_002693.2(*POLG*):c.2554C>T (p.Arg852Cys) in conjunction with another pathogenic variant resulted in disease. According to the available clinical information, the patient does not have any symptoms of POLG-related disorder.

A heterozygous stop-gain variant was identified in the *RARB* gene, which is associated with syndromic microphthalmia type 12. The NM_000965(*RARB*):c.553C>T (p.Arg185Ter) variant has not been previously reported and is not present in gnomAD; however, there was no mention of microphthalmia, pulmonary hypoplasia or diaphragmatic hernia in the patient's medical record. Whereas microphthalmia is usually a recessive disease, dominant forms have been described in patients with *RARB* variants; however, they were all missense gain of function variants[38] so it is more likely that this individual is a carrier.

Finally, a variant previously thought to cause X-linked mental retardation 58, NM_004615.3*(TSPAN7)*:c.515C>A (p.Pro172His),[39] was identified in a male patient. While this variant was initially classified as pathogenic, the high frequency of this variant in gnomAD, including in 66 males, is highly suggestive of this being a benign variant. Furthermore, the patient with this variant had normal BSID-III assessments at both 1 and 3 years of age (Supplemental Table I).

Various other known pathogenic and likely pathogenic variants associated with recognised neurodevelopmental disorders were identified; however, they are all heterozygous variants in genes associated with recessive conditions. No important variants were identified in genes overlapping with the hcCHD gene panel and/or the heterotaxy (HTX) panel.

Table. Pathogenic and likely pathogenic variants according to InterVar and as reported in ClinVar (with additional manual curation using ACMG-AMP guidelines) using the three virtual gene panels

Panel	Variant type	Gene	Variant detail*	Patient origin (ID)	gnomAD	ACMG	InterVar	ClinVar	Disease (Inheritance)
hcCHD									
	Stop-gain	*MYBPC3*	NM_000256.3:c.2526C>G (p.Tyr842Ter)	Han Chinese (A0814K1N63292735T4D1)	-	LP	LP	P	HCM (AD)
	Missense	*MYH7*	NM_000257.4:c.5459G>A (p.Arg1820Gln)	Han Chinese (A0814K1N63243638T4D2)	2.03E-05	VUS	LP	VUS	HCM, distal myopathy (AD)
	Missense	*GJA1*	NM_000165.5:c.1127G>A (p.Arg376Gln)	Caucasian (SCHN_4181)	0.0002	VUS	VUS	P/VUS/LB/B	HLH & AVSD (AD)
(HTX/PCD)	Missense	*ZFPM2*	NM_012082.4:c.2107A>C (p.Met703Leu)	Han Chinese (A0815K1N63357551T4D1, A0815K1N63447656T4D2)	0.0004	VUS	VUS	P/VUS/B	DORV (AD)
NDD									
	Stop-gain	*ACADM*	NM_001127328:c.739C>T (p.Arg247Ter)	Han Chinese (A0815K1N63357551T4D1)	3.23E-05	LP	P	-	MCAD deficiency (AR)
	Stop-gain	*IFT172*	NM_015662:c.3130C>T (p.Arg1044Ter)	Han Chinese (A0815K1N63343431T4D1)	1.22E-05	LP	P	-	Retinitis pigmentosa 71, short-rib thoracic dysplasia (AR)
	Stop-gain	*IFT172*	NM_015662:c.1028C>G (p.Ser343Ter)	Han Chinese (A0814K1N63235188T1D1)	-	LP	P	-	Retinitis pigmentosa 71, short-rib thoracic dysplasia (AR)
	Stop-gain	*NDUFS1*	NM_005006.7:c.1669C>T (p.Arg577Ter)	Caucasian (SCHN_2011)	1.22E-05	P	P	P	MC1DN5 (AR)
	Stop-gain	*RARB*	NM_000965:c.553C>T (p.Arg185Ter)	Caucasian (SCHN_4250)	-	LP	P	-	Microphthalmia, syndromic 12 (AD, AR)
	Stop-gain	*MMAA*	NM_172250.3:C.586C>T (p.Arg196Ter)	Han Chinese (A0815K1N63450195T4D1)	8.13E-06	P	P	P	Methylmalonic aciduria (AR)
	Stop-gain	*MCPH1*	NM_024596.5:c.2145G>A (p.Trp715Ter)	Caucasian (SCHN_4294)	0.0001	P	P	P	Microcephaly 1 (AR)
	Stop-gain	*PIGO*	NM_032634:c.884C>A (p.Ser295Ter)	Caucasian (SCHN_4139)	-	LP	P	-	Hyperphosphatasia with mental retardation syndrome 2 (AR)
	Stop-gain	*SLC6A5*	NM_004211:c.376C>T (p.Arg126Ter)	Han Chinese (A0815K1N63495691T4D2)	-	LP	P	-	Anxiety-related personality, Obsessive-compulsive Disorder (AD, polygenic)
	Stop-gain	*BBS2*	NM_031885.4:c.823C>T (p.Arg275Ter)	Caucasian (SCHN_441)	0.0002	P	P	P	Bardet-Biedl syndrome (AR)
	Stop-gain	*CRB2*	NM_173689:c.714C>A (p.Cys238Ter)	Han Chinese (A0200495172 11)	-	LP	LP	-	Ventriculomegaly cystic kidney disease, Focal segmental glomerulosclerosis (AR)
	Stop-gain	*GABRB3*	NM_001191321:c.5G>A (p.Trp2Ter)	Han Chinese (A0814K1N63141879T4D1)	6.22E-05	LP	LP	-	Epilepsy, early infantile epileptic encephalopathy (AD)
	Frameshift insertion	*CRB2*	NM_173689.7:c3089_3104dup(p.Gly1036fs)	Caucasian (SCHN_441)	0.0009	P	-	P	Focal segmental glomerulosclerosis (AR)
	Frameshift insertion	*BBS10*	NM_024685.4:c.271dup (p.Cys91fs)	Caucasian (SCHN_3351)	0.0006	P	-	P	Bardet-Biedl syndrome (AR)

(continued on next page)

Table. (*continued*)

Panel	Variant type	Gene	Variant detail*	Patient origin (ID)	gnomAD	ACMG	InterVar	ClinVar	Disease (Inheritance)
	Frameshift deletion	*ELAC2*	NM_018127.7:C.2009del (p.Cys670fs)	Caucasian (SCHN_4340)	3.61E-05	P	-	P	Combined oxidative phosphorylation deficiency 17 (AR)
	Frameshift insertion	*BCKDHA*	NM_000709:c.111dupC (p.His37fs)	Han Chinese (A0815K1N63372104T4D2)	2.03E-05	P	-	P	Maple syrup syndrome (AR)
	Frameshift deletion	*LRP2*	NM_004525:c.2514_2517del (p.Gly838fs)	Caucasian (SCHN_3271)	-	LP	-	-	Donnai-Barrow syndrome (AR)
	Frameshift insertion	*SETBP1*	NM_001130110:c.681_682insTT (p.Leu227fs)	Han Chinese (A0814K1N63232006T1D1)	1.34E-05	LP	-	-	Mental retardation 29, Schinzel-Giedion midface retraction syndrome (AD)
	Splicing	*MCC1*	NM_020166.5:c.c.639+2T>A	Han Chinese (A0815K1N63419356T4D2)	1.08E-05	P	-	P	3-Methylcrotonyl-CoA carboxylase 1 deficiency (AR)
	Splicing	*ACADM*	NM_000016.5:c.611+1G>A	Caucasian (SCHN_2572)	4.06E-06	LP	-	LP	MCAD deficiency (AR)
	Missense	*POLG*	NM_002693.2:c.2554C>T (p.Arg852Cys)	Caucasian (SCHN_2053)	4.47E-05	P	P	P/VUS	POLG-related disorders (AR)†
	Missense	*SCO2*	NM_005138.2:c418G>A (p.Glu140Lys)	Han Chinese (A0200573172l1)	9.03E-05	P	P	P	SCO2-related disorders (AR)
	Missense	*HEXB*	NM_000521.4:c.1250C>T (p.Pro417Leu)	Caucasian (SCHN_3616)	0.0006	LP	P	P/LP	Sandhoff disease (AR)
	Missense	*PHGDH*	NM_006623:c.586C>T (p.Ala76Ser)	Han Chinese (A0815K1N63475143T4D1)	9.02E-05	VUS	LP	-	Neu-Laxova syndrome, Phosphoglycerate dehydrogenase deficiency (AR)
	Missense	*TREX1*	NM_033629.6:c.340C>T (p.Arg114Cys)	Han Chinese (A0814K1N63170114T4D2)	8.14E-06	LP	LP	LP	Aicardi-Goutieres syndrome (AR)
	Missense	*HADH*	NM_005327.5:c.643C>A (p.Pro215Thr)	Caucasian (SCHN_3802)	0.0018	VUS	LP	LB/VUS	3-hydroxyacyl-CoA dehydrogenase deficiency hypoglycaemia (AR)
	Missense	*MUT*	NM_000255.4:c.1663G>A (p.Ala555Thr)	Han Chinese (A0814K1N63088490T4D1)	2.85E-05	LP	LP	LP	Methylmalonic aciduria (AR)
	Missense	*ERCC5*	NM_000123:c.2383G>A (p.Ala795Thr)	Caucasian (SCHN_3271)	2.89E-05	VUS	LP	-	Cerebrooculofacioskeletal syndrome 3, Xeroderma pigmentosa (AR)
	Missense	*LIG4*	NM_001098268:c.833G>T (p.Arg278Leu)	Han Chinese (A0815K1N63357551T4D1)	3.23E-05	VUS	LP	-	LIG4 syndrome (AR)
	Missense	*GCH1*	NM_000161.3:c.610G>A (p.Val204Ile)	Han Chinese (A0200654171l1)	0.0002	VUS	LP	LP/VUS	Dopa-responsive dystonia (AD, AR)
	Missense	*PMM2*	NM_000303.3:C.442G>A (p.Asp148NAsn)	Caucasian (SCHN_3088, SCHN_3351)	0.0001	LP	LP	P/LP	Congenital disorder of glycosylation type 1a (AR)
	Missense	*NPC1*	NM_000271.5:c.3560C>T (p.Ala1187Val)	Han Chinese (A0814K1N63235188T1D1)	0.0001	VUS	LP	VUS	Niemann-Pick disease (AR)
	Missense	*SUMF1*	NM_01164674:c.341C>T (p.Ala114Val)	Caucasian (SCHN_440)	4.06E-06	VUS	LP	-	Multiple sulfatase deficiency (AR)
	Missense	*PCCB*	NM_000532.5:c.872G>A (p.Cys291Tyr)	Caucasian (SCHN_1470, SCHN_3668)	0.0013	VUS	LP	LP/VUS/LB	Propionicacidemia (AR)

(*continued on next page*)

Table. (*continued*)

Panel	Variant type	Gene	Variant detail*	Patient origin (ID)	gnomAD	ACMG	InterVar	ClinVar	Disease (Inheritance)
	Missense	*SMO*	NM_005631:c.431G>A (p.Arg144His)	Han Chinese (A0200573172 11)	0.0001	VUS	LP	-	Basal cell carcinoma, Curry-Jones syndrome (somatic)
	Missense	*NAGA*	NM_000262.3:c.973G>A (p.Glu325Lys)	Caucasian (SCHN_4299)	0.0025	LP	LP	P/LP/VUS	Alpha-NAGA deficiency (AR)
	Missense	*EZH2*	NM_004456:c.688A>G (p.Met230Val)	Han Chinese (A0815K1N63343431T4D1)	4.06E-06	LP	LP	-	Weaver syndrome (AD)
	Missense	*NAGA*	NM_000262.3:c.479C>G (p.Ser160Cys)	Caucasian (SCHN_2053)	0.0008	LP	LP	P/LP/VUS	Alpha-NAGA deficiency (AR)
	Missense	*BBS2*	NM_031885.4:c.98C>A (p.Ala33Asp)	Caucasian (SCHN_4253)	8.21E-06	VUS	-	P/LP/VUS	Non-syndromic retinitis pigmentosa (AR)
	Missense	*TBCD*	NM_005993.5:c.3365C>T (p.Pro1122Leu)	Han Chinese (A0814K1N63235188T1D1)	6.91E-05	LP	-	P/VUS	Encephalopathy with brain atrophy and thin corpus callosum (AR)
	Missense	*GALC*	NM_000153.4:c.956A>G (p.Tyr319Cys)	Caucasian (SCHN_4181)	0.0014	B	-	P/VUS/B	Krabbe disease (AR)
	Missense	*ACADM*	NM_000016.6:c.985A>G (p.Lys329Glu)	Caucasian (SCHN_4146)	0.0033	P	-	P	MCAD deficiency (AR)
	Missense	*UNC80*	NM_032504.1:c.1806G>C (p.Gln602His)	Caucasian (SCHN_4172)	0.0015	LB	-	LP/VUS/LB	Hypotonia, infantile, with psychomotor retardation and characteristic facies (AR)
	Missense	*SLC22A5*	NM_003060.4:c.77G>A (p.Ser26Asn)	Caucasian (SCHN_3271)	1.23E-05	LP	-	P	Carnitine deficiency, systemic primary (AR)
	Missense	*PMM2*	NM_000303.3:c.422G>A (p.Arg141His)	Caucasian (SCHN_2698)	0.0040	P	VUS	P	Congenital disorder of glycosylation type 1a (AR)
	Missense	*TSPAN7*	NM_004615.3:c.515C>A (p.Pro172His)	Caucasian (SCHN_4139)	0.0010	LB	-	P/VUS/LB	X-linked mental retardation 58 (XLR)
	Missense	*RARS2*	NM_020320.5:c.1A>G (p.Met1Val)	Han Chinese (A0815K1N63343431T4D1)	0.0001	P	-	P	Pontocerebellar hypoplasia type 6 (AR)
HTX/PCD									
	Splicing	*ARMC4*	NM_018076.2:c.576-2A>T	Caucasian (SCHN_2771)	3.990e-05	LP	-	-	PCD (AR)
	Splicing	*RSPH1*	NM_080860.4:c.275-2A>C	Caucasian (SCHN_4088)	3.608e-04	P	-	P	PCD (AR)
	Stop-gain	*ARMC4*	NM_018076.2:c.866C>G (p.Ser289Ter)	Han Chinese (A0814K1N63170114T4D2)	9.990e+02	LP	LP	-	PCD (AR)
	Frameshift	*CENPF*	NM_016343:c.1386_1389del: (p.Arg462fs)	Caucasian (SCHN_4203)	9.990e+02	LP	-	-	Stromme syndrome (AR)

ACMG-AMP, American College of Medical Genetics – Association for Molecular Pathology; *AD*, autosomal dominant; *AR*, autosomal recessive; *AVSD*, atrioventricular septal defect; *B*, benign; *DORV*, double outlet right ventricle; *hcCHD*, high confidence CHD; *HCM*, hypertrophic cardiomyopathy; *Het*, heterozygous; *HLH*; hypoplastic left heart; *Hom*, homozygous; *HTX*, heterotaxy; *LB*, likely benign; *LP*, likely pathogenic; *MCAD*, Medium-chain acyl-CoA dehydrogenase; *MC1DN5*, mitochondrial complex I deficiency nuclear type 5; *NDD*, neurodevelopmental disabilities; *P*, pathogenic; *PCD*, primary ciliary dyskinesia; *VUS*, variant of unknown significance, *XLR*, X-linked recessive.
*All variants are heterozygous.
†Variant observed in combination with another known POLG variant, NM_002693.2:c.32G>A (p.Gly11Asp), resulting in a patient with a complex allele.

A specific focus on those participants for which neurodevelopmental data, specifically BSID-III 3-year assessments, were available, revealed that 15 of the pathogenic variants occurred in 9 patients with normal development and in 1 patient with mild NDD (Supplemental Table V). In the patient with mild NDD, SCHN_3351, two pathogenic/likely pathogenic variants were identified, NM_24685.4(*BBS10*):c.271dup (p.Cys91fs) and NM_000303.3(*PMM2*):c.442G>A (p.Asp148Asn). Both variants are associated with different recessive NDD disorders and are therefore unlikely contributors of the mild NDD evident in the patient. Furthermore, the variant in *PMM2* was also identified in a patient with normal development, SCHN_3088. As only a quarter of patients had available neurodevelopmental assessments, and only 1 patient reported mild NDD, we were unable to conduct any association studies between variant burden and neurodevelopmental outcomes.

Laterality analysis – HTX/PCD gene panel

As the heterotaxy/PCD panel had 19 genes in common with the hcCHD gene panel, the findings discussed will focus on the remaining 55 genes. Prior to filtering, 325 rare variants (MAF < 0.01) were identified. Following QC, filtering and prioritisation, 15 variants remained, comprising 2 stop-gain, 2 splicing, 1 frameshift and 10 novel missense variants (Figure 1, Supplemental Table IV). One pathogenic variant in the *RSPH1* gene was identified as well as two likely pathogenic variants in *ARMC4* gene and one in the *CENPF* gene (Table 1). The splicing variant NM_080860.4(*RSPH1*):c.275-2A>C, is a known pathogenic variant associated with PCD; however, it is recessive and requires biallelic mutations to cause disease.[40,41] Similarly, while variants in the *ARMC4* gene are associated with PCD,[42] and both the *ARMC4* variants are predicted to be likely pathogenic, they are heterozygous with no additional *ARMC4* variants identified in these 2 patients. Finally, a likely pathogenic heterozygous frameshift variant NM_016343(*CENPF*):c1386_1389del (p.Arg462fs) was identified, which is associated with Stromme syndrome, a recessive condition.[43]

Case-control analysis – hcCHD, NDD and HTX/PCD genes

Following filtering for putatively damaging variants in the TGA cases and controls across the three gene panels (779 genes), the TGA cases had significantly more predicted-damaging variants per proband (Mean = 5.04, SD = 0.321) than controls (Mean = 3.58, SD = 0.056; $P < .001$, Exp(B) = 1.404) (Figure 2A). Limiting the analyses to each gene panel, revealed that the TGA cases had significantly more predicted-damaging variants in CHD genes (Mean = 1.92, SD = 0.198; $P < .001$, Exp(B) = 5.460) and NDD genes (Mean = 4.12, SD = 0.290; $P < .001$, Exp(B) = 1.314) than controls (CHD genes: Mean = 0.35, SD = 0.018; NDD genes: Mean = 3.14, SD = 0.053). While the TGA cases had more predicted-damaging variants in HTX/PCD genes (Mean = 0.37, SD = 0.087) than controls (Mean = 0.34, SD = 0.017) the difference was not significant (Figure 2A).

Additional case-control analyses categorising the variants as "protein-altering" (variants that alter the protein product or amino acid sequence) or "silent" (variants that do not alter the protein product or amino acid sequence) variants were conducted (Figure 2B and C). This found that TGA cases had significantly more "protein-altering" variants in CHD and NDD genes compared with controls, but they did not differ in the total number of "protein-altering" variants (Figure 2B). The TGA cases also had significantly more "silent" and "total number" of variants among CHD, NDD and in the combined gene list than controls. Interestingly, the TGA cases had significantly less "protein-altering" and "silent" variants in HTX/PCD genes than controls (Figure 2C and D).

CNV analysis – hcCHD, NDD and HTX/PCD genes

Prior to filtering, 317 CNVs were called, of which 56 overlapped exonic regions. Following inspection of the bam files on IGV, six CNVs were called as true positives (Supplemental Table VI). Two of these CNVs, duplications involving the genes *SIK1* and *CBS*, have been reported multiple times in gnomAD and other databases and are likely to be benign. The four remaining CNVs involve genes associated with recessive disorders and none of the patients had any other variants in these genes to constitute compound heterozygosity. However, one of these genes, *DEAF1*, has also been associated with the autosomal dominant condition Vulto-van Silfout-de Vries syndrome, in which *de novo* heterozygous variants have been reported to cause disease.[44,45] Reported variants are missense, with likely gain of function; loss of function variants in this gene are associated with an autosomal recessive condition. Vulto-van Silfout-de Vries syndrome is characterised by moderate to severe intellectual disability, impaired expressive speech, behavioral issues and mild dysmorphism[45]; however, examination of this patient's medical record, revealed no learning difficulties or developmental delay at his most recent cardiac review at 9 years of age. As such, it is likely that this finding represents carrier status for the recessive condition associated with *DEAF1* and is unlikely to be relevant to this patient's phenotype. No large structural variants (>1 Mb) were detected in any of the samples.

Discussion

In this first report of whole genome sequencing (WGS) in TGA we sought to identify clinically relevant variants contributing toward cardiac malformations, disturbances in laterality and neurocognitive dysfunction, representing three key areas of research in this patient group. As

an initial approach, we focussed on established genes associated with CHD, NDD, HTX and PCD known to be associated with human disease in an attempt to identify clinically relevant and meaningful information for these patients.

The overall finding of this work is that the majority of d-TGA does not result from monogenic variants in known disease genes. It is possible that other as-yet-unidentified factors may be responsible; however, the findings from the case-control analysis are suggestive that the genetic contribution to d-TGA causation may be oligogenic or polygenic in nature. To an extent this is expected as d-TGA is generally regarded as sporadic with low familial recurrence – which was also observed in our cohort; and as such, identifying a single causal variant is less likely than in familial forms of disease.[12] The expected diagnostic yield for isolated, sporadic CHD is currently ~10% to 20%[46,47]; however, here we demonstrate this to be much lower for patients with d-TGA, with no monogenic causal variants identified in this cohort.

The case-control analysis provides additional evidence that a oligo-/polygenic inheritance likely contributes to TGA development with TGA cases harbouring significantly more variants, and more importantly, predicted-damaging variants, in established disease-causing genes than healthy controls. The significant increase in predicted-damaging and protein-altering variants specific to CHD and NDD genes, further implicates variants in these genes as possible contributors to disease. While significant differences between TGA cases and controls were also noted in the silent variant analysis across the various gene panels, interpretation and understanding of silent variation and its contribution toward disease is unknown, limiting the potential relevance of these findings at present.

Our findings are supported by recent publications on genomic analysis in TGA, which included the targeted sequencing of established disease genes as well as more comprehensive, exome-wide analyses.[18,19] While one study generated 82 candidate genes requiring further validation, no disease-causing variants were identified in either study with a combined total of 324 TGA patients. Similarly, in a study on 97 families with various forms of CHD, no causal variants were identified in the single patient with isolated TGA.[48] Furthermore, 33% of TGA patients harboured potentially damaging variants in more than one candidate gene[19] and a significant increase in overall mutational load, including in rare coding PCD genes, was observed in TGA patients compared to controls.[18] Taken together, and in line with our findings on an additional 100 TGA patients undergoing WGS, these data are strongly suggestive of an oligo- and/or polygenic inheritance underlying TGA development.

Previous studies on TGA patients have identified causal variants in a handful of established CHD and/or candidate TGA genes[7-11]; however, no disease-causing variants were observed in any of these candidate genes in the present study. It is worth noting that most of the previously reported disease-causing variants were identified in familial forms of TGA in which a monogenic cause was more likely.[8] Echoing our findings, and unlike other studies focussing on familial disease, no disease-causing variants were identified in 7 candidate TGA genes in 102 sporadic cases of TGA.[49] So, while disease-causing variants in established CHD genes have been reported in TGA, this tends to be limited to familial forms of disease; with a molecular diagnosis in established CHD genes unlikely for the majority of presenting TGA cases, which are sporadic.

Prior genomic studies have identified increased variant burden in genes highly expressed in the developing brain and heart, providing evidence for the previously suggested genetic link between NDD and CHD.[27,50,51] Notably, the implicated genes tend to have an established association with relevant phenotypes, which prompted our focus on known disease genes. While multiple pathogenic variants, including known variants associated with recognised neurodevelopmental disorders, were identified, most of these require biallelic variants to cause disease and are therefore unlikely to be contributing toward an abnormal neurodevelopmental phenotype. A recent study identified 12 NDD genes in which damaging *de novo* variants were overrepresented in CHD patients compared with controls.[50] Whereas 10 of these 12 genes were included in our analyses, no pathogenic variants were identified in any of these genes in our cohort. Together with previous studies, including targeted gene analyses and exome-based studies focussing on NDD genes,[27,50,51] this study further supports the hypothesis that unlike severe developmental delay and intellectual disability, CHD-associated NDD, which tends to be milder in presentation, is most likely explained by variant burden in key brain and/or cardiac genes thereby limiting diagnostic potential with currently available tools and analysis. Alternatively, structural and functional changes arising from intrauterine hypoperfusion or hypoxia, and/or peri-procedural events, may be more relevant than genetic variants.[24,26]

Additionally, no pathogenic variants were identified in the 38 genes associated with both a cardiac and neurodevelopmental phenotype, providing further support that CHD-associated NDD is unlikely to be due to highly penetrant, rare variants in the utilized gene sets. Large, phenotypically well-defined cohorts with documented NDD outcomes will be required to achieve clinically relevant results.

A central role for cilia in the pathogenesis of CHD has been demonstrated in a large-scale mouse mutagenesis screen,[20,52] the findings of which suggest that CHD may constitute a new class of ciliopathies.[20] Unlike traditional ciliopathies which are rare and exhibit an autosomal recessive inheritance pattern, Klena et al hypothesize that

CHD may result from variants in the "ciliome" conferring an additive or polygenic effect.[20]

More recently, the contribution of cilia-related genes, and more specifically genes associated with PCD, were investigated in patients with TGA. While individual variants in known PCD genes were not considered disease-causing, overrepresentation of variants in PCD-associated genes and pathways in TGA patients was observed.[18,19] Surprisingly, while there were more predicted-damaging HTX/PCD variants in TGA patients in our cohort compared with controls, the difference was not statistically significant, suggesting that many more cilia-related genes beyond established disease-causing genes likely contribute toward disease development.

TGA patients exhibit airway ciliary dysfunction as well as increased respiratory symptoms similar to patients with heterotaxy and/or PCD[18]; however, no biallelic causal variants were identified in known PCD genes in our cohort or in previous studies. This together with findings from previous studies, suggests that while the respiratory symptoms and ciliary dysfunction in patients with TGA are similar but milder than in overt PCD, TGA may constitute a different form of ciliary dysfunction, distinct from classic autosomal recessive PCD.

Limitations

Whereas our analyses were limited to known disease genes, based on the findings from recent studies in TGA,[19] extending the analysis to the exome is unlikely to reveal any clinically relevant results in probands alone without additional functional and familial studies.

Potentially causal variants may have been missed due to lacking family data which would have assisted in identification of *de novo* variants, a known mechanism of disease; and provided important additional information to assist with pathogenicity determination, especially for missense variants.

Issues associated with sequencing cases and controls on different platforms need to be acknowledged. However, the same bioinformatics pipeline was applied to both case and control data. While every attempt was made to enable comparative analyses between cases and controls, biases may still exist and need to be considered. Furthermore, the case-control analysis is limited to the Caucasian cohort due to the lack of suitable Han Chinese control data.

Similarly, the underrepresentation of the East Asian ancestry in population databases needs to be acknowledged. While filtering according to MAF was based on the HIGH_AF_POPMAX frequency to address possible disparities in allele frequencies due to the underrepresentation of various ethnic groups, this alone may not be sufficient and needs to be considered during result interpretation.

Finally, to optimise the search for disease-causing variants, we limited our analysis to rare variants (MAF < 0.01) thereby disregarding the contribution of common variants to disease; however, the evaluation of common variants and their contribution toward disease would require thousands of participants in order to achieve statistically meaningful findings and is beyond the scope of this study.

Conclusion

We present compelling evidence that the majority of d-TGA cases are not caused by highly penetrant rare variants in established and/or novel genes and that oligogenic, polygenic and/or multiple other undefined factors underlie disease development and later brain manifestations. Large, phenotypically well-defined cohorts, incorporating a battery of neurodevelopmental assessments, combined with variant burden analyses to enable large-scale association analyses are required to obtain clinically relevant information for this patient group in terms of their cardiac and neurological development.

Further, polygenic risk scores may in time provide insight into the complex genetic aetiology underlying TGA; but importantly, they may also identify patients at risk of developing comorbidities associated with disease, including NDD. Finally, assessing the contribution of oligo- and polygenic factors will be important in understanding disease aetiology in this genetically complex patient group in the future.

Disclosures

None. The authors are solely responsible for the design and conduct of this study, all study analyses, the drafting and editing of the paper and its final contents.

Conflict of interest

None.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ahj.2021.10.185.

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