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# Biallelic *DAW1* variants cause a motile ciliopathy characterized by laterality defects and subtle ciliary beating abnormalities

**Joseph S Leslie**[1,*], **Rim Hjeij**[2,*], **Asaf Vivante**[3,4,*], **Elizabeth A Bearce**[5], **Laura Dyer**[6], **Jiaolong Wang**[7], **Lettie Rawlins**[1,15], **Joanna Kennedy**[1], **Nishanka Ubeyratna**[1], **James Fasham**[1,15], **Zoe H Irons**[5], **Samuel B Craig**[5], **Julia Koenig**[2], **Sebastian George**[2], **Ben Pode-Shakked**[3,8], **Yoav Bolkier**[3,9], **Ortal Barel**[10,11], **Shrikant Mane**[12], **Kathrine K. Frederiksen**[7], **Olivia Wenger**[13], **Ethan Scott**[13], **Harold E. Cross**[14], **Esben Lorentzen**[7], **Dominic P Norris**[6], **Yair Anikster**[3,8,11], **Heymut Omran**[2], **Daniel T Grimes**[5,#], **Andrew H Crosby**[1,#], **Emma L Baple**[1,15,#]

[1.] Institute of Biomedical and Clinical Science, RILD Wellcome Wolfson Centre, University of Exeter Medical School, Royal Devon & Exeter NHS Foundation Trust, Barrack Road, Exeter, EX2 5DW, UK

[2.] Department of General Pediatrics, University Hospital Muenster, 48149 Muenster, Germany

[3.] Sackler Faculty of Medicine, Tel Aviv University, Israel.

[4.] Department of Pediatrics B and Pediatric Nephrology Unit, Edmond and Lily Safra Children's Hospital, Sheba Medical Center, Tel Hashomer, Israel

[5.] Institute of Molecular Biology, University of Oregon, Eugene, OR, USA

[6.] MRC Harwell Institute, Harwell Campus, Oxfordshire, OX11 0RD, UK

[7.] Department of Molecular Biology and Genetics, Aarhus University, Gustav Wieds Vej 10c, DK-8000 Aarhus C, Denmark

[8.] Metabolic Disease Unit, Edmond and Lily Safra Children's Hospital, Sheba Medical Center, Israel

[#]Corresponding authors: Emma Baple E.Baple@exeter.ac.uk, Clinical Senior Lecturer (Genomic Medicine), Consultant in Clinical Genetics, Medical Research (Level 4), RILD Wellcome Wolfson Centre, Royal Devon & Exeter NHS Foundation Trust, Barrack Road, Exeter, EX2 5DW, UK, Andrew Crosby A.H.Crosby@exeter.ac.uk, Professor of Human Genetics, Medical Research (Level 4), RILD Wellcome Wolfson Centre, Royal Devon & Exeter NHS Foundation Trust, Barrack Road, Exeter, EX2 5DW, UK, Daniel T Grimes DTGrimes@uoregon.edu, Assistant Professor, Institute of Molecular Biology, University of Oregon, Eugene, OR, 97403, USA.
Conceptualization: A.H.C., E.L.B., D.T.G., H.O, Y.A., D.P.N, E.L.; Formal Analysis; J.S.L., R.H., A.V., E.A.B., L.D., J.W.; Investigation: J.S.L., R.H., A.V., E.A.B., L.D., J.W., L.R., J.K., S.G., Z.H.I., S.B.C., J.K., B.P.S., Y.B., O.B., S.M., K.K.F, O.W., E.S., H.E.C; Writing – original draft: J.S.L., R.H., E.A.B., B.P.S., E.L., D.P.N., Y.A., H.O., A.H.C., D.T.G., E.L.B., Writing - review and editing: J.S.L., R.H., E.A.B., A.H.C., D.T.G., E.L.B., L.D., E.L., D.P.N., H.O., A.V. and Y.A.

[*]These authors contributed equally

Ethics Declaration

Ethical approval for this project is from Akron Children's Hospital (#986876), University of Arizona (IRB – 1000000050), University of Exeter Medical School, and the Institutional Review Board of Sheba Medical Center (#7786–10). Written informed consent was obtained in all cases from patients or from a responsible parent in the case of children. Ethical approval for all zebrafish studies was obtained from the international Association for Assessment and Accreditation of Laboratory Animal Care (approved by the University of Oregon Institutional Animal Care and Use Committee). Ethical approval for all mouse work was obtained from the UK Home Office and experiments were carried out in accordance with the Medical Research Council (MRC) Harwell Ethics Committee. All mouse colonies were maintained in a pathogen-free environment at the Mary Lyon Centre, MRC Harwell Institute.

Conflict of interest statement

The authors declare no conflict of interests.

[9.] Pediatric Heart Institute, Edmond and Lily Safra Children's Hospital, Sheba Medical Center, Tel-Hashomer, Israel

[10.] The Genomic Unit, Sheba Cancer Research Center, Sheba Medical Center, Tel-Hashomer, Israel

[11.] The Wohl Institute for Translational Medicine, Sheba Medical Center, Tel-Hashomer, Israel

[12.] Department of Genetics, Yale School of Medicine, New Haven, Connecticut, USA

[13.] New Leaf Center Clinic for Special Children, Mt Eaton, Ohio, United States.

[14.] Department of Ophthalmology, University of Arizona College of Medicine, Tucson, AZ, USA.

[15.] Peninsula Clinical Genetics Service, Royal Devon & Exeter Hospital, Gladstone Road, Exeter, EX1 2ED, UK

## Abstract

**Purpose—**The clinical spectrum of motile ciliopathies includes laterality defects, hydrocephalus and infertility, as well as primary ciliary dyskinesia (PCD) when impaired mucociliary clearance results in otosinopulmonary disease. Importantly, ~30% of PCD patients lack a genetic diagnosis.

**Methods—**Clinical, genomic, biochemical, and functional studies were performed alongside *in vivo* modelling of *DAW1* variants.

**Results—**Here we identify biallelic *DAW1* variants associated with laterality defects and respiratory symptoms compatible with motile cilia dysfunction. In early mouse embryos, we show that *Daw1* expression is limited to distal, motile ciliated cells of the node, consistent with a role in left-right patterning. *Daw1* mutant zebrafish exhibit reduced cilia motility and left-right patterning defects including cardiac looping abnormalities. Importantly, these defects were rescued by wild type but not mutant *daw1* gene expression. Additionally pathogenic *DAW1* missense variants display reduced protein stability, while DAW1 loss of function is associated with distal type 2 outer dynein arm assembly defects involving axonemal respiratory cilia proteins, explaining reduced cilia-induced fluid flow in particle tracking velocimetry experiments.

**Conclusion—**Our data define biallelic *DAW1* variants as a cause of human motile ciliopathy, and determine that the disease mechanism involves motile cilia dysfunction explaining the ciliary beating defects observed in affected individuals.

### Keywords

DAW1; primary ciliary dyskinesia; left-right asymmetry; heterotaxy; motile cilia

## Introduction

Cilia are hair-like microtubule-based projections from the plasma membrane extending into the extracellular space to perform wide-ranging developmental and physiological functions. Two main forms of cilia are defined according to their function; motile cilia which induce extracellular fluid movement, and immotile (or sensory) cilia important for chemo-/mechano-sensory signalling[1]. Disorders arising due to ciliary malfunction ('ciliopathies')

comprise a genetically and clinically heterogeneous group of conditions typically involving variants in genes encoding core ciliary molecular components[2]. Due to the near ubiquitous expression of cilia and their functional diversity, ciliopathies may manifest a broad range of clinical features.

Disorders arising from defects in motile cilia are termed 'motile ciliopathies', characterized by varying combinations of clinical features dependant on the specific genes involved and their tissue expression[2]. Motile cilia, positioned throughout the epithelium of the respiratory tract, sinuses and middle ear, uniformly orientate to generate a monodirectional flow for mucociliary clearance[3]. Motile cilia dysfunction is frequently associated with primary ciliary dyskinesia (PCD), the most commonly recognized motile ciliopathy, characterized by severe recurrent otosinopulmonary disease, caused by persistent mucus retention[4]. PCD is strongly associated with male infertility and laterality defects including *situs inversus* (mirror-image reversal) and *situs ambiguous* (heterotaxy)[4]. Male infertility is caused by defects in sperm flagella, whereas laterality defects arise due to disruption of left-right (L-R) internal organ asymmetry, an evolutionarily conserved process in vertebrates initiated by motile cilia at the ventral node during embryonic development. These specialized cilia rotate to induce a leftward flow of embryonic fluid detected by immotile cilia around the node periphery[5], inducing left-sided activation of the NODAL cascade[6,7].

Many pathomolecular processes underlie non-syndromic laterality defects including impaired NODAL signalling (eg *DAND5*[8,9]), and motile (eg DNAH9[10]) and sensory cilia dysfunction (eg PKD2[11]). However, when alongside otosinopulmonary PCD symptoms laterality defects are invariably due to defective assembly, motility, or organisation of motile cilia. Cilia motility is driven by inner and outer dynein arms (IDAs and ODAs); motor complexes that power sliding interactions between microtubules, with ODAs being the primary force generator. Ciliary import of pre-assembled ODAs is required for ciliary beating, a process shown to be mediated in green algae *Chlamydomonas reinhardtii* by intraflagellar transport component IFT46 and cargo adaptor ODA16[12–14]. The zebrafish ODA16 homolog (Wdr69) is required for motile cilia function in zebrafish[15], as are its planarian (smed-DAW1)[16] and mammalian (DAW1) homologs. Human and green algae DAW1/ODA16 crystal structures reveal highly similar β-propeller regions, with a conserved C-terminal ODA binding domain[17,18]. *Chlamydomonas* ODA16 associates with IFT46 via an N-terminal region including the β-propeller, although it remains unclear whether DAW1 exhibits a similar association[17,18]. Here we show biallelic *DAW1* variants cause a novel motile ciliopathy characterized by laterality defects and subtle ciliary beating defects.

## Material and Methods

### Clinical and genetic studies

Affected individuals from family 1 were identified through their clinician as part of a larger study of laterality disorders in Anabaptist communities. In order to further characterize the genetic and clinical spectrum of *DAW1*-ralated disease, we explored GeneMatcher (https://genematcher.org) identifying a further Palestinian child (family 2). All genotyped individuals underwent echocardiography. Additional phenotypic information was obtained through the clinical care provider using a targeted clinical questionnaire, with

written informed consent. DNA was extracted from blood/buccal samples using standard techniques. Following dideoxy sequencing exclusion of established Amish/Mennonite PCD and laterality defect founder variants (family 1, see supplementary methods)[19], exome sequencing (ES, family 1: 1-VII-8, 1-VII-1, 1-VII-5, family 2: 2-IV-1) was performed on Illumina platforms. Full sequencing methodology for each family is described in supplementary methods.

Variants with <5 reads, a frequency >0.5% in gnomAD (Genome Aggregation Database, v2.1.1) and/or in-house databases, were excluded. Exonic or intron/exon boundary (±6 nucleotides of the splice junction), *de novo* (family 2), homozygous or compound heterozygous variants were evaluated and prioritized by call quality, allele frequency (MAF), predicted functional consequence and segregation with disease. All variants were then assessed for correlation with the clinical phenotype.

Unique primers were utilized for amplification and bidirectional dideoxy sequencing of all *DAW1* variants identified, which were deposited in ClinVar (SCV002025271 - SCV002025275).

### Immunofluorescence analysis

Nasal brushings were obtained from all affected individuals. Immunofluorescence analysis was performed as previously described[20] (see supplementary methods for details).

### *In situ* expression analysis

C57BL/6J mouse embryos were dissected following confirmation of mating by vaginal plugs. Fixed embryos (4% paraformaldehyde) were dehydrated with methanol. Whole mount *in* situ hybridization (WISH) with an antisense digoxygenin-labelled *Daw1* riboprobe (594–1326 of sequence ENSMUST00000065436.9, Ensembl release 101) followed standard protocols. Alkaline phosphatase-coupled anti-digoxygenin antibody (Roche) was used to localise hybridized probes using NBT/BCIP (Roche). Zebrafish *dand5*/*myl7 in situ* hybridization[21] used digoxygenin-labelled antisense probes generated from synthesized DNA templates by *in vitro* transcription (Roche) following standard procedures for embryo imaging (THUNDER stereoscope, Leica).

### Zebrafish

Embryos from natural matings (AB strain zebrafish) were incubated (28°C). *daw1*$^{b1403}$ CRISPR-Cas9 generated mutants harbouring an Isoleucine-140/Alanine-141 deletion exhibit loss-of-function phenotypes as previously described (Bearce et al., *in press in Development*). Daw1 mRNA synthesized from Daw1-encoding linearized plasmid (mMessage mMachine SP6 Transcription Kit, ThermoFisher Scientific) was purified using lithium chloride for injection (20 pg of Daw1$^{WT}$ or variant mRNA; site-directed mutagenesis, ThermoFisher) at the 1-cell stage. Heart looping was assessed at 36–48 hours post fertilization. For KV cilia imaging, embryos (somite stage 8–10) were dechorionated and mounted in #1.5 coverslip-bottomed Mattek chambers in low-melt 0.5% agarose with the dorsal-posterior side down. Live imaging (Nikon Ti2 inverted microscope), captured 512 × 512 differential interference contrast images at 250 frames per second (ImageJ). Motility was visualized

using temporal image correlation spectroscopy (TICS), a Fourier transform based strategy for time course data where areas of an image with periodic fluctuations are brighter in intensity and correspond to motile cilia. The density of cilia motility, called “motile area”, was then calculated as the area of a region of interest which showed periodic fluctuations, corresponding to the area habroring motile cilia.

### High speed video microscopy and particle tracking velocimetry

We obtained cell cultures from two affected individuals (1-VII-1, 1-VII-5) homozygous for the *DAW1* NM_178821.2:c.427A>G; p.(Asn143Asp) variant. In 1-VII-1 we performed high speed video microscopy (HSVM) to analyze ciliary beating. In 1-VII-5 we undertook particle tracking experiments to analyze the ability of affected cilia to generate monodirectional flow. Particle tracking velocimetry experiments / high speed video microscopy were performed as previously described[22] (see supplementary methods for details).

## RESULTS

### Genetic and clinical studies

Four individuals from two families, with rare predicted damaging biallelic DAW1 variants were identified. The first family as part of a larger study investigating the spectrum and causes of laterality disorders in the Anabatist community and the second through GeneMatcher. (See figure 1 and supplementary table 1 for family pedigrees and ES variant lists).

**Family 1—**We initially investigated three Mennonite female siblings (63–76 years) with features suggestive of mild PCD. Individual 1-VII-8 displayed *situs inversus* (SI) without otosinopulmonary symptoms. Individual 1-VII-1 displayed SI and had a history of recurrent otitis media in childhood. Individual 1-VII-5 has normal *situs* but is reported to have had multiple episodes of lower respiratory tract infections throughout her life, and a chronic wet cough since childhood which is now productive, consistent with dysfunction of respiratory cilia. She also reports recurrent episodes of otitis media in early life. All three affected women had children, with no apparent reduced fertility or history of ectopic pregnancy. Systemic examination was otherwise unremarkable and there was no evidence of craniofacial dysmorphism.

Dideoxy sequencing excluded all previously established Amish/Mennonite causes of PCD and/or laterality defects. To define the genetic cause of disease ES was undertaken using DNA from all three affected individuals. Filtering of ES data using standard metrics (call quality, allele frequency <0.05 in gnomAD) identified a homozygous missense variant (*DAW1*, NM_178821.2:c.427A>G; p.(Asn143Asp), Chr2(GRCh38):g.227893904A>G) as the only likely candidate cause of disease. The *DAW1* c.427A>G; p.(Asn143Asp) variant is absent from population databases (gnomAD v2.1.1, v3.1.2 accessed on 20/05/2022), results in the substitution of a highly conserved residue (figure 1), and is predicted to be damaging by multiple *in silico* tools. Modelling the variant in the crystal structure of human DAW1 determines that Asn143 is positioned on the β-propeller N-terminal face,

and that the Asn143Asp substitution leads to loss of hydrogen-bonds between β-propeller blades 2 and 3, potentially disrupting local structure and impacting association with binding partners (supplementary figure S1). The variant co-segregates as appropriate in all family members, and as expected for a founder variant it is also present in in the Anabaptist (Amish/Mennonite) variant server (AVS a population specific database comprising of >10,000 exomes) at low allele frequency (0.002) and only in heterozygous state.

**Family 2—**An 8 year old Palestinian child with heterotaxy (2-IV-1) presented at 8 months with hypoplastic left heart, mitral atresia, transposition of the great arteries (TGA), double outlet right ventricle (DORV), total anomalous pulmonary venous return (TAPVR), pulmonic stenosis and bronchial situs inversus. Abdominal ultrasound showed a transverse liver and right-sided spleen (table 1, figure 1, supplementary figure S2). Clinical examination was otherwise unremarkable and chromosomal microarray analysis was normal. Trio ES analysis excluded variants in all known laterality disorder/PCD genes and identified a candidate homozygous nonsense *DAW1* variant (NM_178821.2: c.357G>A; p.(Trp119*), Chr2(GRCh38):g.227893834G>A) as the likely cause of disease (figure 1). The variant is absent from population databases (gnomAD v2.1.1, v3.1.2 accessed on 20/05/2021) and is only present in a single heterozygote in a database of >8000 regional exomes. Furthermore, no loss-of-function *DAW1* variants have ever been observed in the general population in a homozygous state.

The genetic findings in both families described here align with those of a previous study investigating potential genetic causes of unexplained congenital heart disease in 2,871 individuals[23]. In this study biallelic *DAW1* variants identified in two individuals were proposed as the candidate cause of disease. A homozygous *DAW1* missense variant (NM_178821.2:c.1091G>C; p.(Ser364Thr), Chr2(GRCh38):g.227921439G>C) was identified in a single individual with heterotaxy. This variant affects a highly conserved residue (figure 1), is predicted damaging by multiple *in silico* tools and is present at a low frequency in gnomAD v2.1.1 (0.00001591 accessed on 20/05/2022) and v3.1.2 (0.000006661 accessed on 20/05/2022), but not in homozygous state. Modelling the variant in the DAW1 crystal structure revealed that the side-chain hydroxyl group of Ser364 interacts with the side- and main-chain of Asp366 within blade 7 on the C-terminal face of the β-propeller, which is thought to bind ODAs (supplementary figure S1). A second individual with left ventricular obstruction was found to have inherited compound heterozygous *DAW1* (NM_178821.1:c.197T>A; p.(Leu66*), Chr2(GRCh38):g.227889939T>A and NM_178821.1:c.1116G>T; p.(Trp372Cys), Chr2(GRCh38):g227921464G>T) variants. Importantly, left ventricular obstruction may commonly occur in patients with heterotaxy, consistent with this individual being affected by a laterality defect. The Leu66* variant is present at a low frequency in gnomAD v2.1.1 (0.000008168, accessed on 20/05/2022) and v3.1.2 (0.000006571, accessed on 20/05/2022) but not in homozygous state, and is likely to result in nonsense mediated decay. The Trp372Cys variant affects a highly conserved residue (supplementary figure S3), is predicted to be damaging by multiple *in silico* tools and is absent from gnomAD (v2.1.1, v3.1.2, accessed on 20/05/2022). Notably, heterotaxy is frequently associated with defects in nodal cilia function and is observed in ~6–12% of PCD patients[24,25].

Ciliary beating is frequently impaired in motile ciliopathies. Therefore, we performed high speed video microscopy to analyse ciliary beating of a 15-day spheroid culture from individual 1-VII-1 (family 1), (see supplementary materials). Compared to a control spheroid culture (video 1), multiciliated respiratory cells of 1-VII-5 show a subtle reduction of the beating amplitude due to an impaired recovery stroke, which results in a stiff beating pattern (video 2). Moreover, to mimic the process of particle lung clearance *in vitro*, fluorescent particles were added to the apical compartment of the ALI-Transwell inserts to perform particle-tracking experiments with the cultivated respiratory epithelial cells from 1-VII-5, and a healthy control subject. Prior to every particle tracking experiment, a ciliary beat frequency (CBF) measurement of each insert under physiological conditions (37°C) was performed to provide an overall impression of the inserts and to analyse the CBF. The CBF of 1-VII-5 with a mean of 9.21 ± 0.09 Hz was slightly increased compared to the healthy control (8.52 ± 0.09 Hz), p=0.035. Particle tracking velocimetry experiments including a group of 5 healthy controls resulted in a weighted mean particle velocity of 61.5 ± 3.7 μm/s. 1-VII-5 demonstrated a particle velocity of 43.3 ± 2.2 μm/s, which is reduced by almost 30% compared to the healthy control group. The difference in velocity between the healthy control group and 1-VII-5 is 18.2 ± 4.3 μm/s. This translates into a significance of p=0.000027. Overlay and polar graphs revealed that the particle flow was directed (supplementary figure S4) for both the healthy control group as well as for 1-VII-5. In summary, we undertook two independent assessments investigating integrity of respiratory cilia function, which both show subtle respiratory beating defects consistent with abnormal cilia clearance of the airways.

### *DAW1* variants impact protein stability *in vitro*

To assess the impact of the DAW1 variants on protein expression and stability, wild type (WT), p.Asn143Asp and p.Ser364Thr missense alterations were expressed in insect cells and purified to assess unfolding temperature using nano differential scanning fluorimetry (see supplementary methods). Both alterations exhibited lower protein expression than WT in insect cells, indicative of impaired folding or stability. The third variant, p.Trp372Cys, was completely insoluble when over-expressed in insect cells, consistent with Trp372 being part of the DAW1 WD-repeat structure. Wild-type DAW1 displayed an unfolding temperature of 75.4 °C (S.D. of 0.30 °C), while the p.Asn143Asp mutant DAW1 displayed a small reduction in unfolding temperature of 74.0 °C (4 S.D below WT; supplementary figure S1B), indicating that protein stability is only modestly impacted by the p.Asn143Asp substitution. Conversely, the p.Ser364Thr mutant DAW1 was found to be significantly destabilized, unfolding at a greatly reduced temperature of 59°C (supplementary figure S1B).

### *Daw1* is expressed in the motile ciliated cells of the embryonic node in mice

PCD associated genes that cause laterality defects are required for motile cilia function in the embryonic node at ~3 weeks of gestation. To investigate *DAW1* expression, RNA *in situ* hybridisation analysis was performed in mouse embryos at an equivalent developmental stage (early headfold to 6 somite stage). Mouse *Daw1* expression was evident in the node by the late headfold stage, reducing over the next few hours, consistent with a role in L-R determination (supplementary figure S5). Analysis of histological sections revealed *Daw1*

expression to be limited to the ventral node cells (supplementary figure S5). These cells exhibit motile cilia, consistent with DAW1 being directly involved in nodal cilia motility rather than other elements of the L-R determination pathway.

## *daw1* mutant zebrafish embryos exhibit reduced cilia motility and left-right patterning defects

To determine the impact of *DAW1* loss-of-function in an animal model, L-R patterning in zebrafish *daw1*$^{b1403}$ mutants was investigated. *daw1*$^{b1403}$ mutants, generated by CRISPR/Cas9 mutagenesis (Bearce et al., *in press*) harbour a 2-amino acid deletion in a conserved region (figure 2A). Mutant embryos phenocopied previously published *daw1* morphants (embryos injected with morpholino oligonucleotides targeting *daw1*;[15]). At 2 days post fertilization (d.p.f.), *daw1*$^{b1403}$ mutants exhibited abnormal laterality of cardiac looping, indicative of defective L-R patterning (figure 2B). While control embryos showed dextral heart looping, *daw1*$^{b1403}$ mutant clutches contained embryos exhibiting either dextral or sinistral looping or, in some cases, failure of directional looping (figure 2B). In zebrafish, organ asymmetry is determined by an L-R symmetry-breaking event that occurs in Kupffer's vesicle (KV), equivalent to the ventral node of mammals. Motile cilia within KV generate an asymmetric fluid flow that is faster on the anterior-left side of the vesicle[26,27]. This results in repression of *dand5* on the left side of KV, causing an R>L bias in *dand5* expression. This asymmetric expression was observed in control embryos (figure 2C; n=28/30). By contrast, *daw1*$^{b1403}$ mutants exhibited bilaterally equal *dand5* expression (figure 2C; n=21/30), demonstrating that the L-R patterning defect in *daw1*$^{b1403}$ mutants lies upstream of asymmetric gene expression. To directly assess the cilia motility responsible for establishing L-R asymmetry, KV cilia were imaged with high-speed differential interference contrast microscopy at the 10-somite stage. In control embryos, KV cilia beat at 35.5 ± 2.4 Hz. In *daw1*$^{b1403}$ mutants, motility was almost entirely abolished (figure 2D and video 3). Together, this demonstrates that KV cilia motility is significantly disrupted in *daw1*$^{b1403}$ mutants, resulting in abnormal *dand5* asymmetric expression and cardiac laterality defects. These findings cohere with a previous zebrafish Daw1 knockdown study reporting motile cilia-associated developmental defects in Daw1 morphants[15].

## *In vivo DAW1* variant modelling

To assess the impact of *DAW1* variants on *in vivo* function, variant mRNAs were expressed in zebrafish embryos by 1-cell injection and, following 36–48 hrs incubation, cardiac looping laterality was determined. As above, uninjected *daw1*$^{b1403}$ mutants exhibited high levels of looping defects (58.9 ± 8.3% [mean ± standard deviation]; n=198; figure 2E). These defects were robustly rescued by mRNA injection encoding wild-type zebrafish Daw1, such that only 12.0 ± 8.2% of injected mutants exhibited looping laterality defects (n=78). By contrast, zebrafish mRNA containing the p.Asn143Asp human variant failed to rescue mutants (59.0 ± 10.7%; n=101; figure 2E). Similarly, expression of p.Ser364Thr also failed to rescue mutants (66.4 ± 4.0%; n=21; figure 2E), while expression of p.Trp372Cys led to a partial but minor rescue (47.0 ± 3.7%; n=70; figure 2E). To directly assess the impact of human variant *daw1* expression on cilia motility, we imaged cilia in KV. As above, *daw1*$^{b1403}$ mutants lacked KV cilia motility, but this was rescued by injection of *daw1*$^{WT}$ mRNA (videos 4–5). In agreement with our analysis of cardiac looping, expression

of *daw1*$^{Asn143Asp}$ and *daw1*$^{Ser364Thr}$ failed to restore motility while *daw1*$^{Trp372Cys}$ partially restored motility, resulting in cilia that beat slowly in KV (videos 6–8). Our rescue data is summarized in figure 2F. Overall, these data demonstrate that all three missense mutations are loss-of-function, with p.Asn143Asp and p.Ser364Thr behaving as nulls in this context while p.Trp372Cys exhibited reduced but not completely abolished function.

### *DAW1* variants result in a variable partial defect in outer dynein arm assembly to ciliary axonemes.

Defects in ODAs are the most frequently reported defects in PCD[28]. We have previously reported two types of ODAs in human: type 1 proximal containing DNAH5 and DNAH11 and type 2 distal containing DNAH5 and DNAH9[29]. As DAW1 is reported to be implicated in the transport of ODAs to the axonemes in lower organisms, we analyzed respiratory cilia from the four affected individuals with biallelic *DAW1* variants for defects in ODA assembly using immunofluorescence analysis (IF). Analysis with antibodies targeting ODAs DNAH5[30], DNAH9[10] and the axonemal ODA intermediate chains DNAI1[31] and DNAI2[32] confirmed normal axonemal localisation of the four ODA proteins in respiratory cilia of controls, and in affected individual 1-VII-8 (*DAW1* p.(Asn143Asp) homozygote). Conversely, in respiratory cells from individual 2-IV-1 (*DAW1* p.(Trp119*) homozygote), DNAH5, DNAI1 and DNAI2 assembled only to the proximal part of the ciliary axonemes in 62% (n[proximal]=23; n[total]= 37), 56% (n[proximal]=19; n[total]= 34) and 61% (n[proximal]=16; n[total]= 26) of cells respectively, and DNAH9 was absent from 71% (n[mislocalized)]=15; n[total]= 21) of the cells (figure 3), indicating a variable defect in type 2 ODA assembly in respiratory cells of this individual. We also analyzed cells from all four individuals with antibodies targeting CCDC114, an ODA docking component that anchors ODAs to microtubules[33]. We observed normal localisation in all individuals (supplementary figure S6). Normal axonemal localisation of nexin dynein regulatory complex (N-DRC) component GAS8 (figure 3) and the inner dynein arm (IDA) light chain DNALI1 as well as the outer dynein arm heavy chain DNAH11 (supplementary figure S7) confirmed absence of other ciliary ultrastructure defects due to *DAW1* variants. Additionally, as affected individuals presented mild respiratory symptoms with laterality defects, we analyzed CFAP53[34], ENKUR and MNS1[19,35] localisation whose deficiency causes a similar human phenotype. All three proteins localized normally in 1-VII-8 and 2-IV-1 (supplementary figure S8), indicating that DAW1 most likely resides within a different molecular complex.

Together these findings indicate that DAW1 variants may result in a variable defect in ODA assembly to the distal portion of the axonemes, explaining the mild respiratory symptoms in some affected individuals and the modestly reduced speed and displacement of particles in particle tracking analysis.

## Discussion

The most common and widely recognized motile ciliopathy is PCD, characterized by severe recurrent otosinopulmonary disease caused by impaired mucociliary clearance, in combination with other variable features including laterality defects and male infertility[1,4]. A combination of diagnostic tests including nasal nitric oxide measurement, ciliary biopsy

with electron microscopy, and ciliary beat analysis using high-speed video microscopy may be used to diagnose PCD. However, none are comprehensive, and mild presentations will frequently go undiagnosed. Patients who lack otosinopulmonary features of PCD but present with *situs inversus* or heterotaxy further complicate diagnosis[36].

While >40 ciliary genes are associated with PCD and laterality defects, variants in these genes explain only ~70% of PCD cases[37]. Recently, an increasing number of novel genetic disorders have been described in which motile cilia dysfunction is associated with varying combinations of clinical features seen in PCD[38]. Indeed, several disease genes including *MNS1, CFAP53* and *DNAH9* have been associated with motile ciliopathy disorders typified by laterality defects and male infertility, but do not fulfil PCD diagnostic criteria[10,19,34,35].

Here we present genetic, clinical, functional and animal model data delineating a novel motile ciliopathy caused by DAW1 deficiency and characterized by laterality defects and subtle ciliary beating defects. Due to its known role in cilia motility and the phenotype in mouse and zebrafish models, *DAW1* variants have previously been proposed as a potential cause of PCD[15], although no conclusive association with human disease has been made. The variable otosinopulmonary features in affected family members in our study included a chronic wet cough and otitis media. Laterality defects were present in 3/4 individuals with 2/4 presenting with *situs inversus* and 1/4 presenting with heterotaxy. Particle tracking analysis of respiratory cilia revealed slightly reduced particle speed and displacement in one individual with recurrent lower respiratory tract infections (1-VII-5) when compared with healthy controls, indicative of impaired mucociliary clearance. Similar mild otosinopulmonary phenotypes with prominent laterality defects have previously been reported in individuals with CFAP53[34] and DNAH9-related ciliopathy disorders[10]. Jin *et al.* previously described two individuals presenting with congenital heart disease compatible with a laterality defect, in whom biallelic *DAW1* (homozygous p.(Ser364Thr), compound heterozygous p.(Leu66*)/p.(Trp372Cys)) variants were proposed as candidate causes of disease[23]. It is not known if either patient exhibited otosinopulmonary features or reduced fertility. All four patients recruited to the present study were female, and thus male infertility, a frequent feature of motile ciliopathies, could not be assessed. However, *DAW1* is highly expressed in the testis (GTex, accessed on 11/11/2021), and clinical phenotypes of similar ciliary genes often include male infertility. It is therefore possible that the full spectrum of DAW1-related disease may include male infertility, this will be clarified as further affected individuals are identified.

Our data shows that *Daw1* is expressed at the node in mouse embryos, consistent with a role in determining L-R asymmetry[39]. Furthermore, *Daw1* expression is limited to the motile ciliated cells in the node pit and does not extend to the sensory cilia at the node periphery. Congruous with this, *daw1*$^{b1403}$ zebrafish mutants exhibit anatomical and molecular L-R defects owing to significantly reduced KV cilia motility, confirming an essential role for DAW1 in L-R asymmetry through motile cilia function and consistent with the human phenotype. Importantly, while wild-type Daw1 robustly rescued mutant *daw1*$^{b1403}$ zebrafish laterality defects, neither the Asn143Asp, Ser364Thr nor the Trp372Cys substitutions displayed rescue ability confirming that they result in loss-of-DAW1 function.

Previous studies in mice, zebrafish and algae identified DAW1 as a likely contributor to the intraflagellar transport (IFT) of ODAs[13,15,17,40,41]. In algae, DAW1 interacts with IFT46, which together bind and chaperone assembled ODAs into the ciliary axoneme[17]. Given the high sequence conservation and structural similarity of algae and human DAW1, it seems likely that it performs a similar functional role in mammalian motile cilia. In humans, 2 types of ODAs exist: type 1 proximal composed of heavy chains DNAH5 and DNAH11, and type 2 distal composed of heavy chains DNAH5 and DNAH9. Here, we found that DAW1 deficiency led to a variable defect in the assembly of human type 2 ODAs to ciliary axonemes, with some cells displaying normal ODA assembly, indicating that DAW1 function in humans is likely partly compensated by other molecular components. As recently reported, DNAH9 variants leading to a complete loss of type 2 ODAs from ciliary axonemes result in milder forms of PCD[10]. The occasional absence of type 2 ODAs due to biallelic *DAW1* variants is thus consistent with the mildly impaired mucociliary clearance and respiratory symptoms observed.

Previously *Daw1*-deficient mice have been shown to exhibit a mild PCD phenotype, including laterality defects with striking similarities to those observed in the affected individuals described here. Solomon *et al.* observed that the ciliated area in $Daw1^{-/-}$ mice was not reduced compared to wild-type animals, with high-speed video microscopy showing robust ciliary motion[42]. Despite this, $Daw1^{-/-}$ mice displayed impaired mucociliary clearance, postulated to be due to dyskinetic ciliary motion, with an abnormal stroke pattern. Additionally, $Daw1^{-/-}$ mouse cilia displayed a reduced angle of effective stroke. The subtle ciliary beating defects and reduced mucociliary clearance observed in affected individuals in our study are consistent with these mice findings. Together, the data presented here conclusively define pathogenic biallelic *DAW1* variants as a new motile ciliopathy disorder characterized by laterality defects and mild respiratory symptoms caused by subtle ciliary beating defects. Thus, *DAW1* gene analysis should be performed in individuals with heterotaxia and features suggestive of otosinopulmonary disease. Awareness of DAW1-related disease and the wider growing group of motile ciliopathies, where heterotaxia is associated with mild but chronic respiratory symptoms, will improve treatment and reduce preventable lung disease.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Data availability

The variants listed in this paper have been submitted to ClinVar (SCV002025271 - SCV002025275).

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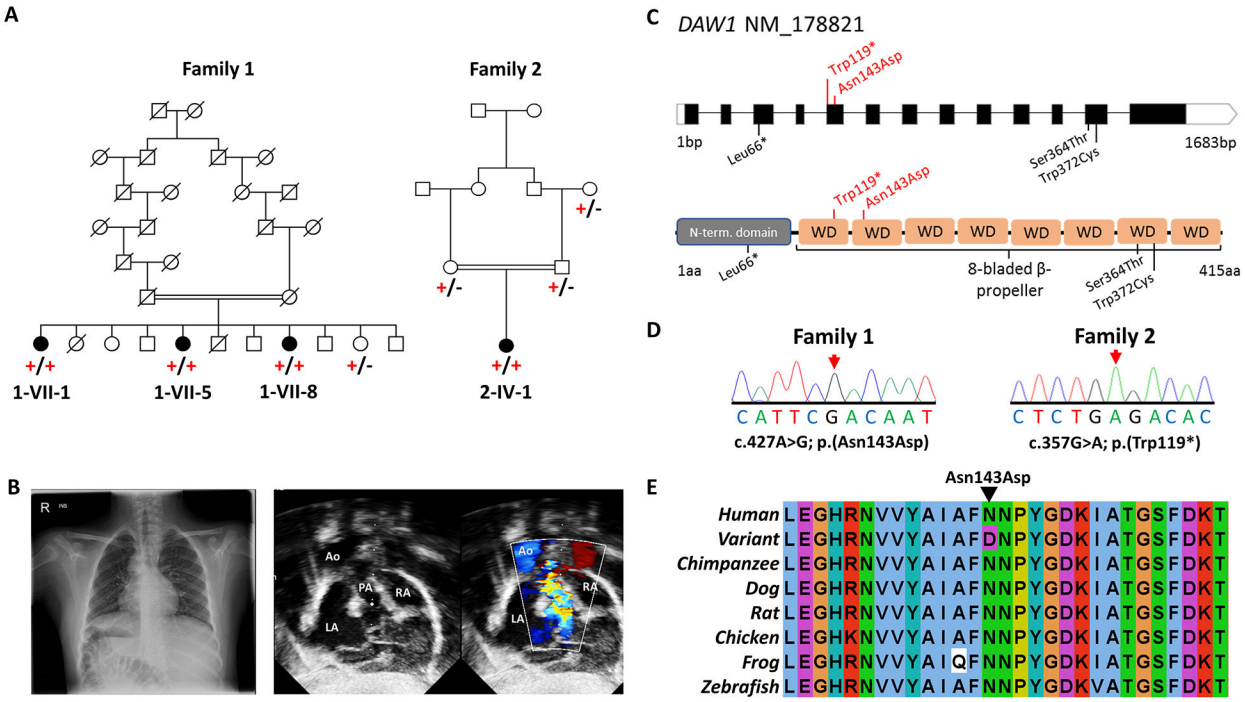
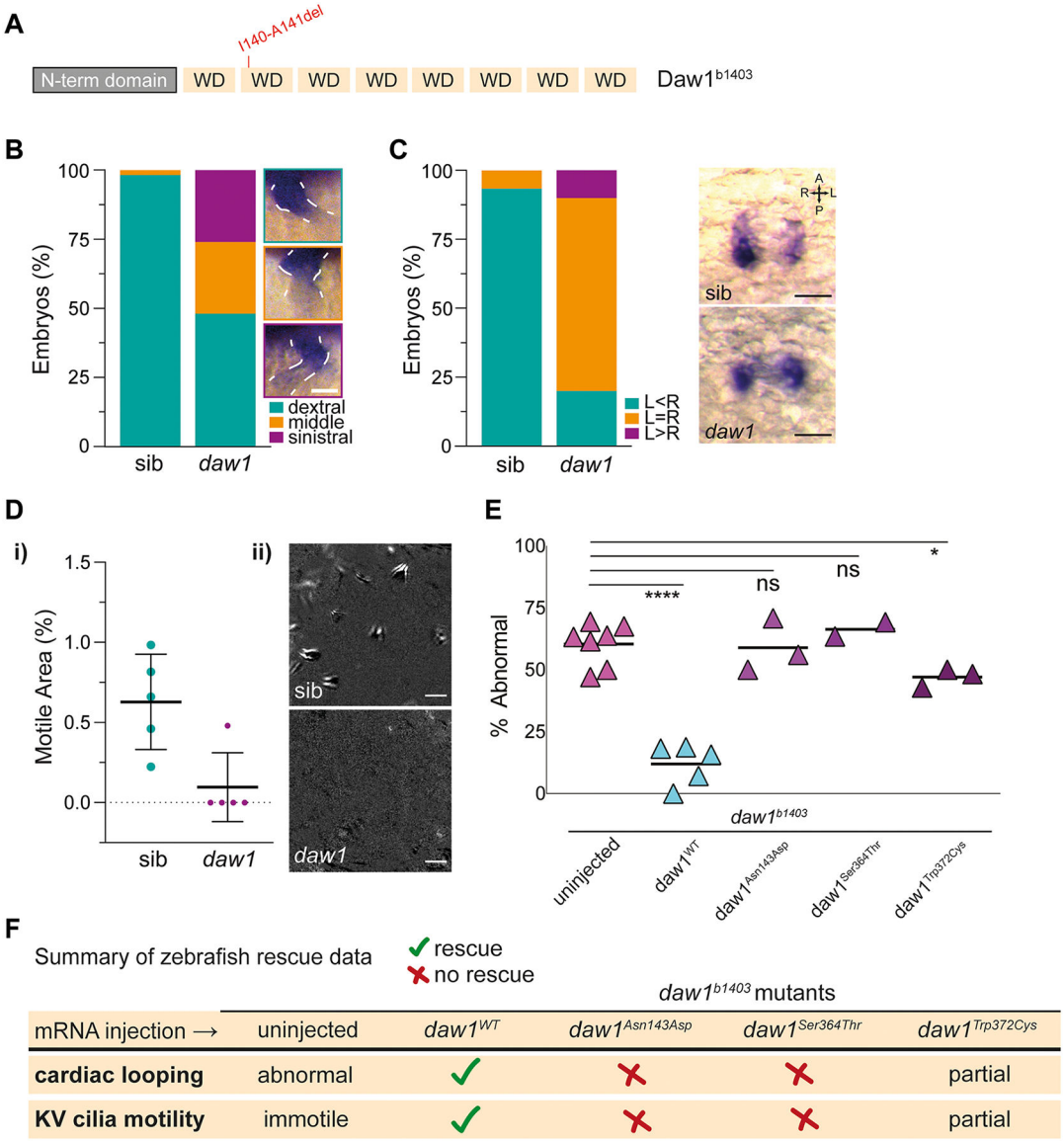



**Figure 1.**

*DAW1* variants, family pedigrees and clinical images

**A** Simplified pedigree of the extended Mennonite family (family 1) and Palestinian family (family 2) in which segregation of the [NM_178821.2:c.427A>G; p.(Asn143Asp)] and [NM_178821.2: c.357G>A; p.(Trp119*)] *DAW1* variants are indicated (+; variant, –; wild type). **B** Chest radiograph from individual family 2-IV-1 illustrating the right sided aortic arch, right sided stomach and *situs inversus*. Echocardiogram imaging from the same individual illustrating atrial *situs inversus*. Ao; aorta, LA; left atrium, PA; pulmonic artery, RA; right atrium. **C** Schematic showing *DAW1* intron-exon genomic organisation (upper panel) and polypeptide domain architecture (lower panel), with position of DAW1 variants indicated. Variants identified in the present study are indicated above the schematic in red, variants identified in previous studies are indicated below the schematic in black. **D** Sequencing electropherograms from individuals homozygous for the Asn143Asp and Trp119* variants. **E** Multiple sequence alignment of the Mennonite *DAW1* NM_178821.2:c.427A>G p.(Asn143Asp) variant, the substituted residue is indicated by the black arrow.




| mRNA injection → | uninjected | *daw1*$^{WT}$ | *daw1*$^{Asn143Asp}$ | *daw1*$^{Ser364Thr}$ | *daw1*$^{Trp372Cys}$ |
|---|---|---|---|---|---|
| **cardiac looping** | abnormal | ✓ | ✗ | ✗ | partial |
| **KV cilia motility** | immotile | ✓ | ✗ | ✗ | partial |

**Figure 2.**

*In vivo* modelling of *DAW1* variants using zebrafish

*In vivo* modelling of Daw1 variants using a zebrafish mutant. A Schematic of zebrafish Daw1 protein showing the location of the 2 amino acid deletion present in *daw1*$^{b1403}$ mutants. **B** Quantitation of cardiac looping phenotypes with representative images in *daw1*$^{b1403}$ mutants (n=54) and sibling controls (n=118). **C** Quantitation of *dand5* expression with representative images in *daw1*$^{b1403}$ mutants (n=30) and sibling controls (n=30). **D** Quantitation of the area occupied by motile cilia in KV at the 10-somite stage. Each datapoint represents a distinct embryo. **Dii** Representative temporal image correlation spectroscopy images of KV cilia motility where white/gray pixels show regions of periodic motion. **E** Dot plot showing the percentage of *daw1*$^{b1403}$ mutant embryos exhibiting abnormal cardiac looping laterality after injections with mRNA encoding either WT or

variant Daw1. * P<0.05; **** P<0.001; ns – not significant; unpaired t test applied. **F** Summary table of zebrafish rescue assay data. Scale bars: **B** and **C** - 50 μm; **Dii** - 5 μm.

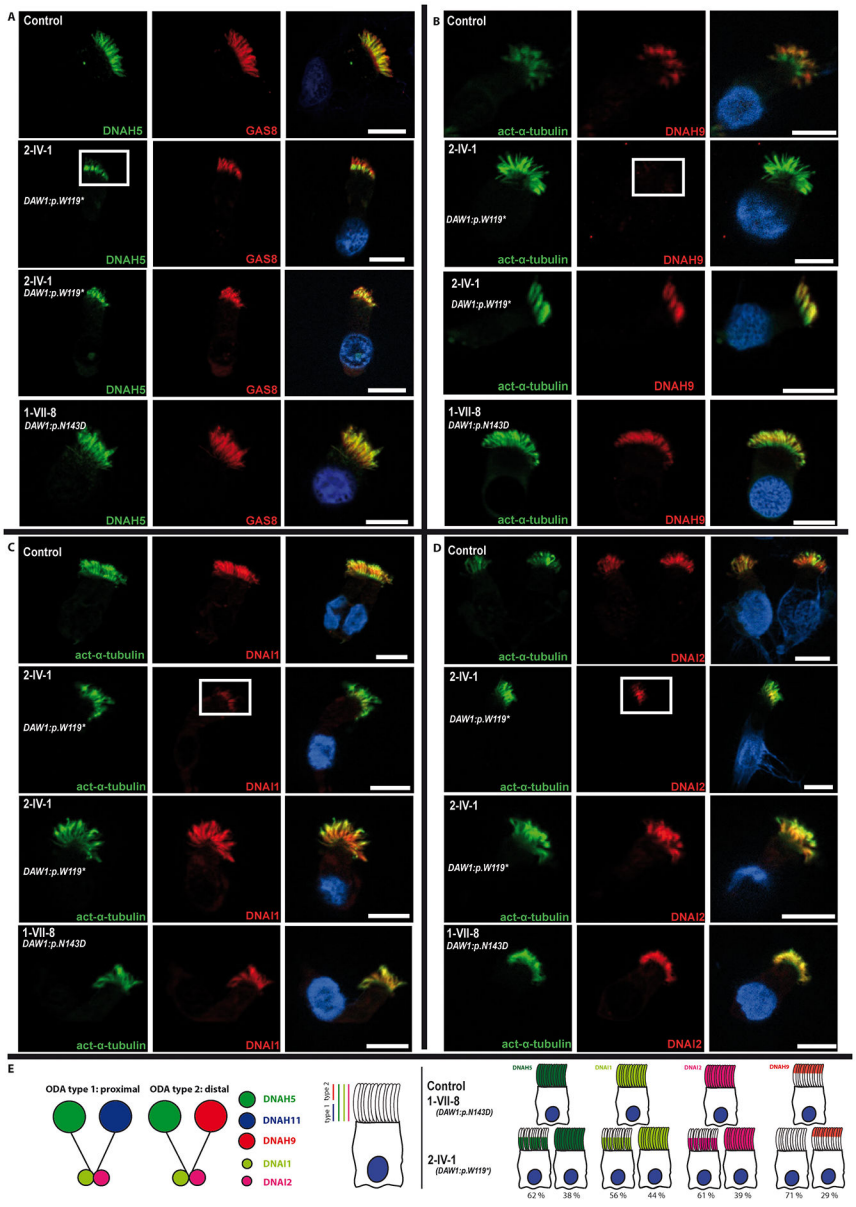



**Figure 3.**

*DAW1* loss-of-function variants cause a variable partial defect in the assembly of the outer dynein arm components DNAH5, DNAH9, DNAI1 and DNAI2 to the ciliary axonemes. **(A-D)** Respiratory epithelial cells derived from the affected Palestinian child (2-IV-1) homozygous for *DAW1* NM_178821.2: c.357G>A; p.(Trp119*), a Mennonite individual (1-VII-8) homozygous for *DAW1* variant NM_178821.2:c.427A>G; p.(Asn143Asp) and an unrelated control. Cells were double-labeled with antibodies directed against DNAH5 (green, **A**), GAS8 (red, **A**), DNAH9 (red, **B**), DNAI1 (red, **C**), DNAI2 (red, **D**) and acetylated alpha-tubulin (green, **B**, **C** and **D**). Nuclei were stained with Hoechst 33342 (blue). In an unaffected control and affected individual 1-VII-8, DNAH5, DNAI1 and DNAI2 localized to the whole axonemal length and DNAH9 localized to the distal compartment of the axonemes. However, in affected individual 2-IV-1, DNAH5, DNAI1

and DNAI2 localized in more than half of the cells only to the proximal part of the ciliary axonemes with 62%, 56% and 61% respectively and DNAH9 was absent in 71% of the cells (white box), indicating that recessive loss-of-function variants in *DAW1* variably affect the distal localization of ODA proteins. Scale bars, 10μm. **E. (Left side)** Schematic illustrating the two types of ODAs in human: ODA type 1 localized to the proximal part of the cilia and containing DNAH5 and DNAH11, ODA type 2 localized to the distal part of the cilia and containing DNAH5 and DNAH9. Both types contain the intermediate chains DNAI1 and DNAI2. **(Right side)** Schematic highlights the distal loss of DNAH5, DNAI1, DNAI2 as well as the loss of DNAH9 from the ciliary axonemes of affected individual 2-IV:1 with the corresponding percentages.

**Table 1.**

Clinical findings of affected individuals homozygous or compound heterozygous for *DAW1* variants

| Identifier | Ethnicity | Sex | Age | Laterality defect | Congenital heart disease | Otosinopulmonary features | Infertility | *DAW1* genotype | Reference |
|---|---|---|---|---|---|---|---|---|---|
| 1-VII-1 | Mennonite | F | 76y | Situs inversus | ✗ | Recurrent otitis media in childhood | ✗ | p.(Asn143Asp)/p.(Asn143Asp) | Present study |
| 1-VII-5 | Mennonite | F | 74y | ✗ | ✗ | Chronic wet cough, recurrent lower respiratory infections Recurrent otitis media in childhood | ✗ | p.(Asn143Asp)/p.(Asn143Asp) | Present study |
| 1-VII-8 | Mennonite | F | 63y | Situs inversus | ✗ | ✗ | ✗ | p.(Asn143Asp)/p.(Asn143Asp) | Present study |
| 2-IV-1 | Palestinian | F | 8y | Situs ambiguous, transverse liver, right-sided spleen | Hypoplastic left heart, mitral atresia, transposition of the great arteries, double outlet right ventricle, total anomalous pulmonary venous return, pulmonary stenosis | ✗ | NK | p.(Trp119*)/p.(Trp119*) | Present study |
| 1–06817 | NK | NK | NK | NK | Hypoplastic aortic annulus, hypoplastic left ventricle, left atrial hypoplasia, left sided patent ductus arteriosus, patent formaen ovale, sinus venosus septal defect, ventricular septal defect | NK | NK | p.(Leu66*)/p.(Trp372Cys) | (23) Jin *et al* 2017 |
| 1–01687 | NK | NK | NK | Situs ambiguous, abdominal situs inversus | Atrial septal defect, biventricular discordant atrioventricular connection, discordant venticuloarterial connection, double outlet right ventriculoarterial connection, double outlet right ventricle, pulmonary stenosis, ventricular septal defect | NK | NK | p.(Ser364Thr)/p.(Ser364Thr) | (23) Jin *et al* 2017 |

**Abbreviations:** F, female; y, years; NK, not known; (✗) indicates absence of a feature in an affected subject.