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Deep intronic *TIMMDC1* variant delays diagnosis of rapidly progressive complex I deficiency

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ABSTRACT

Complex I deficiency is the most common pediatric mitochondrial disease. It can cause a wide range of clinical disorders, including Leigh syndrome. *TIMMDC1* encodes an assembly protein of complex I and has been recently associated with early onset mitochondrial disease in three unrelated families. In all three families the same homozygous deep intronic variant was identified leading to inclusion of a new exon resulting in a frameshift and premature stop codon (c.596 + 2146A > G, p.Gly199_Thr200ins5*). Herein, we describe two brothers of Dutch descent, presenting in infancy with hypotonia and respiratory insufficiency and a rapidly progressive and fatal disease course. Laboratory findings and metabolic investigations revealed no specific abnormalities, notably no raised plasma lactate. MRI showed transient lesions in the basal ganglia of brother 1. A muscle biopsy demonstrated complex I deficiency in brother 2. Exome sequencing yielded a novel heterozygous *TIMMDC1* variant: c.385C > T, p.(Arg129*). Targeted sequencing revealed the previously published deep intronic variant c.596 + 2146A > G, p.(Gly199_Thr200ins5*) on the second allele which is not detected by exome sequencing. In summary, we present the fourth family with *TIMMDC1*-related disease, with a novel nonsense variant. This report illustrates the importance of considering mitochondrial disease even when laboratory findings are normal, and the added value of targeted sequencing of introns.

1. Introduction

Mitochondrial diseases are a complex group of clinically diverse disorders that arise as a result of mitochondrial respiratory chain dysfunction (Chinnery et al., 1993). The most common pediatric mitochondrial disease is complex I deficiency, accounting for approximately 30 percent of all mitochondrial diseases among children (Skladal et al., 2003). Complex I, also referred to as NADH dehydrogenase, is a large protein complex that is part of the electron transport chain (Lenaz et al., 2006). A deficiency of complex I can cause a wide range of diseases, such as cardiomyopathy, neurologic diseases (e.g. Leigh syndrome) and myopathy, accompanied by clinical symptoms including hypotonia, respiratory insufficiency and seizures (Fassone and Rahman, 2012; Loeffen et al., 2000). Though the phenotype can be heterogeneous, the prognosis of complex I deficiency is usually poor with rapid progression (Alston et al., 2017).

Complex I consists of 44 subunits that are encoded by either mitochondrial DNA or nuclear DNA and mutations of either can lead to complex I deficiency. The structure of complex I is comprised of 14 core subunits necessary for catalysis, while the remaining 30 subunits are involved in regulation, stability or assembly of the complex. In addition, there are an unknown number of extrinsic assembly factors (Andrews et al., 2013), one of which is the Translocase of Inner Mitochondrial Membrane Domain-Containing protein 1 (TIMMDC1) [OMIM 615534]. Guarani et al. (2014) (Guarani et al., 2014) reported that TIMMDC1 contains four transmembrane domains, is localized in the mitochondrial inner membrane and interacts with several components of complex I throughout the process of assembly. Furthermore, they observed

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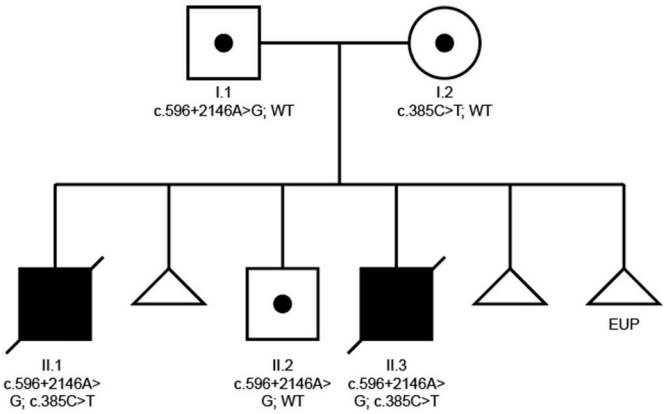

Fig. 1. Pedigree.
Pedigree. Squares represent males, circles represent females. Blackened family members possess both variants of *TIMMDC1* and show symptoms of disease, while a dot indicates the family member to be a carrier of one variant of *TIMMDC1*. Underneath them is specified which variants they are identified with, WT means wild type. Deceased family members are marked by a diagonal line. EUP = extra-uterine pregnancy.

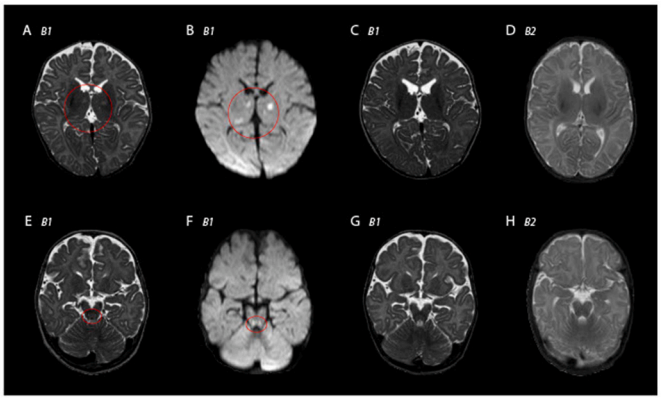

Fig. 2. MRI images.
MRI images of brother 1 (B1) and brother (B2) in axial planes. The upper row shows hyperintense laesions in the right and left thalamus on T2-weighted imaging (A) and DWI (b1000) (B) of brother 1, taken at age 3 months. At age 5.5 months these lesions are normalized (C). The T2-weighted images of brother 2 taken at age 1.5 months show no abnormalities in this region (D). At age 5.5 months hyperintense lesions in the dorsal mesencephalon were apparent, as shown on the bottom row on T2-weighted imaging (E) and DWI (b1000) (F). These were not visible at age 3 months (G). The T2-weighted images of brother 2 show no abnormalities in this region as well (H).

reduced complex I activity, following depletion of TIMMDC1 in human colorectal cancer cells (Guarani et al., 2014).

Pathogenic *TIMMDC1* variants were first described by Kremer et al. (2017) (Kremer et al., 2017). In a cohort of exome-negative patients with mitochondrial disease, they used RNA sequencing in patient-derived fibroblasts to identify the underlying defect (Kremer et al., 2017). In two unrelated individuals with complex I deficiency they identified a homozygous intronic *TIMMDC1* variant leading to inclusion of a new exon resulting in a frameshift and premature stop codon (c.596 + 2146A > G, p.Gly199_Thr200ins5*). Both *TIMMDC1* messenger RNA and protein expression were down-regulated in patient fibroblasts. Re-analysis of an in-house whole genome sequencing (WGS) database revealed a third individual with the same homozygous variant. These three unrelated patients all presented in infancy with muscular hypotonia, developmental delay and neurological deterioration (Kremer et al., 2017).

Deep intronic variants cannot be identified by exome sequencing and/or exome-based gene panels as these only cover the exonic sequences and exon/intron boundaries. Thus far, at least 185 intronic pathogenic variants located at least 100 bp away from the nearest canonical splice site, across 77 different disease genes have been reported to date and this is likely an underrepresentation of its true prevalence (Vaz-Drago et al., 2017). This case report shows the added value of a targeted gene panel over exome analysis in two brothers who presented with a rapidly progressive disorder with hypotonia and respiratory insufficiency. Their symptoms, ultimately indicative of mitochondrial disease, were at first nonspecific and a list of neuromuscular disorders was considered in the differential diagnosis. Ultimately bi-allelic pathogenic variants in *TIMMDC1* were identified: a nonsense variant by an exome-based mitochondrial gene panel and the previously described intronic splice site variant by targeted sequencing of this *TIMMDC1* intron.

2. Patient inclusion and consent

After genetic counselling, parents gave written informed consent to diagnostic exome sequencing of brother 1 and 2 and both parents. They gave informed consent to publication of this report.

3. Clinical reports

The family is of Dutch descent. Brothers 1 and 2 have a healthy male sibling, born after brother 1 (Fig. 1). The mother has had three miscarriages, including one extra-uterine pregnancy. Retrospectively, mother noted reduced fetal movements during the pregnancies of brother 1 and 2 compared to their healthy sibling. No family history of neuromuscular disease was noted.

Brother 1 (II.1) was the first child of non-consanguineous Caucasian parents. He was born at term following an uncomplicated pregnancy and in good condition with Apgar scores of 7 and 9. Birthweight was 3400 g (0 SD). After birth, he exhibited hypotonia, for which he was observed for a day. A day after being discharged, he was hospitalized again due to feeding difficulties. He received nasogastric tube feeding which could be weaned off after two weeks. He was discharged but physical therapy was continued.

At two months old he was hospitalized again because of feeding difficulties and weight loss. Three days after admission he developed frequent apneas and respiratory insufficiency requiring mechanical ventilation. No signs of infection were present. Neurological examination demonstrated generalized hypotonia, weakness more evident distally than proximally, a complete head lag and areflexia. He displayed no dysmorphic features. Cardiac examination revealed no explanation for his condition. Laboratory results showed an increased ASAT/ALAT, yGT and LDH, an abdominal ultrasound was normal, and the laboratory results normalized spontaneously. Metabolic investigations excluded Pompe's disease, N-glycosylation disorders, aminoacidopathy, organic acidemia, fatty acid oxidation disorders and peroxisomal disorders. An MRI showed lesions in the basal ganglia and thalamus (Fig. 2), which was interpreted as recent ischemia due to the apneas. The Concentric Needle (CN) Single Fiber EMG showed increased jitter in the left m. orbicularis oculi, which could be suggestive of a neuromuscular transmission disorder. Pyridostigmin (4 mg/kg a day) was administered; however, no clinical response was observed. Meanwhile, he developed a fever. Blood cultures revealed a *Staphylococcus aureus*, for which flucloxacillin was administered during two weeks. Thereafter, he repeatedly showed raised temperatures, excessive perspiration and tachycardias for which no cause was identified.

He was barely making any spontaneous movements and was unable to abduct his eyes when he was four months old, illustrative of his deteriorating condition. A tracheotomy was performed to facilitate chronic ventilation. At five months he displayed ptosis and he showed no movements in his lower extremities. Considering his condition

pyridostigmin was increased to 8 mg/kg a day and amifampridine was administered (1 mg/kg a day) as well. This did not induce a convincing improvement, thus salbutamol (0.1 mg/kg 3dd) was added. A repeat MRI demonstrated normalization of the lesions in the basal ganglia and thalamus, although now diffusion restriction in the dorsal mesencephalon became visible (Fig. 2). At 6.5 months it was decided to cease treatment due to progressive deterioration without response to therapy, and he passed away as a result of respiratory insufficiency.

Brother 2 (II.3) was born at term, with a birth weight of 3555 g (0 SD) after an uncomplicated pregnancy, nearly four years later. He was hospitalized postpartum because of hypotonia and moaning, which was defined as transitional and was discharged three days later without intervention.

At three weeks he was hospitalized again due to feeding difficulties and failure to thrive. He was tube fed and was transferred for diagnostics to an academic center. Neurologic examination showed an alert, though somewhat hypotonic, neonate demonstrating spontaneous, symmetrical motor skills, as well as anti-gravity movements. The hypotonia was more noticeable axially and in the lower extremities as opposed to the upper extremities. A mild hypospadias was noted. Although when agitated his saturation decreased somewhat, he was able to recover on his own and regarded to be in a condition well enough to be discharged. Three weeks later he presented with tachydyspnea, bradycardias and apneas, for which Optiflow was started. During hospitalization he showed periods of excessive transpiration. Signs of upper respiratory tract infection were present. After a week, he was successfully weaned off Optiflow.

At age seven weeks, his saturation decreased abruptly and he was intubated and reanimated. A cardiac ultrasound showed a temporarily reduced systolic and diastolic function of the heart. Extubation was not possible, because upon trying he became dyspneic and showed a lot of discomfort. Ventilation had to be gradually increased. He showed tachycardia and transpiration, however no signs of infection were present, indicating this to be due to autonomic dysregulation or agitation. Laboratory examination of the muscle biopsy of brother 2 revealed a complex 1 deficiency. While at first he was alert and demonstrated some movement in the upper extremities, later on he only made eye contact and when he was 13 weeks old he barely responded to physical examination. It was decided to cease treatment when he was 14 weeks old, he passed away due to respiratory insufficiency after extubation.

Metabolomics analysis, making use of the 5 bloodspots obtained of these patients during their lives, revealed one component that was consistently elevated (average z-score 3.2) in both brothers in all 5 blood spots with a molecular weight consistent with a molecular formula C3H7NO (either 3-aminopropionaldehyde or aminoacetone). Both are metabolites of unknown significance, that have not been associated with a disease.

4. Genetic investigations

4.1. Exome sequencing

Exome Sequencing quad analysis was performed at the University Medical Center (UMC) Utrecht laboratory on the DNA of both brothers and their parents. DNA from patients and parents was isolated from whole blood. Exomes were enriched using SureSelect Clinical Research Exome V2 (Agilent Technologies) and sequenced on a HiSeq 2500 sequencing system (Illumina). Variant assessment was performed according to diagnostic standards of the University Medical Center Utrecht using Alissa Interpret software (Agilent Technologies). Filtering for coding and splice site variants shared by both brothers and in line with a recessive or *de novo* dominant mode of inheritance yielded only one shared homozygous variant in *ADH3B1*. In silico prediction models indicated it to be damaging and highly conserved (Supplements). Since this variant is listed in Gnomad (https://gnomad.broadinstitute.org/) homozygous in one control individual, and germline *ALDH3B1* variants have not been linked to human disease, we continued searching for another plausible cause of disease.

4.2. Exome-based mitochondrial gene panel

DNA of the family was sent to the Maastricht UMC laboratory for targeted exome analysis of a panel of approximately 400 mitochondrial disease genes. Exome sequencing was performed for brother 1 and his parents. Exome enrichment was performed by the Agilent SureSelectXT Human All Exon 50 Mb Kit version 5 (Agilent Technologies, Santa Clara, CA, USA). Sequencing was performed by an Illumina HiSeq4000 platform (Illumina, San Diego, CA, USA). Read mapping and variant calling were done using BWA (mapping) and GATK (calling). Annotations were added using an in-house developed annotation pipeline, which uses as resources amongst others Gencode V19, dbSNP144, ExAC v0.2, and CADD v1.3. Results were also compared to an in-house database of exomes. Targeted exome analysis of a panel of approximately 400 'mitochondrial disease' genes was performed, containing known mitochondrial disease genes and functionally or clinically related genes. Data were filtered for homozygous and heterozygous non-synonymous variants, insertions and deletions (both in-frame and frameshift), nonsense variants and splice-variants, with allele frequencies <0.01 in the ExAC database and in our in-house database. The impact of missense variants on protein function was estimated using the algorithms integrated in the Alamut software version 2.7. Copy number variant (CNV) analyses from the exome data were performed as described (Pfundt et al., 2017). This resulted in identification of a heterozygous pathogenic *TIMMDC1* variant NM_016589.3: c.385C > T (p.(Arg129*)), in brother 1 and mother. This is an ultra-rare variant with a reported allele frequency of 0.00005463 in the European population (www.gnomad.broadinstute.org). Targeted sequencing revealed the same variant in brother 2. Subsequent targeted Sanger sequencing was performed to search for the presence of the previously reported deep intronic *TIMMDC1* variant NM_016589.3:c.597–1340A > G (= c.596 + 2146A > G; p.(Gly199_Thr200ins5*) (Kremer et al., 2017), which was not covered by exome sequencing. This intronic variant was identified heterozygous in both affected brothers, the unaffected brother and the father. Targeted Sanger sequencing excluded the presence of the c.385C > T (p.(Arg129*)) in the unaffected brother. The c.385C > T variant has been submitted to ClinVar (accession SCV001439290).

In summary, both affected brothers are compound heterozygous for pathogenic variants in *TIMMDC1*, whilst their unaffected family members are heterozygous for one of the two variants (Fig. 1) therefore segregating with disease.

5. Discussion

Here, we describe the case of two brothers, with *TIMMDC1*-related complex I deficiency, both presenting with failure to thrive and progressive hypotonia leading to respiratory insufficiency, passing away at the age of 6.5 and 3.5 months. The atypical clinical presentation in combination with the absence of tell-tale biochemical or radiological clues made diagnosis of a mitochondrial disorder challenging. The prominent muscular hypotonia suggested muscular or neuromuscular transmission disorders, such as spinal muscular atrophy, congenital myopathies, congenital myasthenic syndrome and myotonic dystrophy were considered. However, extensive clinical testing and genetic investigations in this direction did not yield a diagnosis. Subsequently, the results of the MRI of brother 1 first led us to reconsider a mitochondrial disorder.

Laboratory findings indicating mitochondrial disease include raised lactate and alanine in serum and cerebrospinal fluid. These, however, may be only elevated under certain circumstances, such as a metabolic crisis or stress. Elevations of proline, glycine and sarcosine have also been associated with mitochondrial disorders (Mitochondrial Medicine Society's Committee on DHaas et al., 2008). However, all these markers were repeatedly normal, serum glycine was even decreased in brother 2.

Table 1
Summary of the five *TIMMDC1* patients.

	Brother 1	Brother 2	Patient 1 #35791	Patient 2 #66744	Patient 3 #96687
Reference	This paper	This paper	Kremer et al. (2017)	Kremer et al. (2017)	Kremer et al. (2017)
TIMMDC1 variant NM_016589.3	c.385C > T, p.(Arg129*); 596 + 2146A > G, p.(Gly199_Thr200ins5*)	c.385C > T, p.(Arg129*); 596 + 2146A > G, p.(Gly199_Thr200ins5*)	Homozygote c.596 + 2146A > G, p.(Gly199_Thr200ins5*)	Homozygote c.596 + 2146A > G, p.(Gly199_Thr200ins5*)	Homozygote c.596 + 2146A > G, p.(Gly199_Thr200ins5*)
Gender	Male	Male	Male	Male	Male
Ancestry	Dutch	Dutch	Greek	North African	German
Gestational period	40 + 4	40 + 2	At term	At term	At term
Birth					
- Weight (SD)	3400 g (0)	3555 g (0)	2450 g (−2)	NA	4180 g (1.5)
- Apgar	7/9	7/9/10	NA	NA	NA
Age at presentation	10 w	4 w	First year of life	6 m	3 m
Growth	3 m	1 m 2 m			
- Weight g (SD)	4080 (−2.5)	3685 (−1.0) 4160 (−1.75)			
- Length cm (SD)	57.5 (−1)	36 (−1) 56 (−0.5)			
- OFC cm (SD)	40 (0)				
Age of death	6.5 m	3.5 m	30 m	20 m	Alive at 4y
Neurological examination	Alert Hypotonia Complete headlag Areflexia No contractures Opthalmoparesis Ptosis Respiratory insufficiency	Alert Hypotonia Complete headlag Areflexia No contractures No fixation of eyes Dropping hand Respiratory insufficiency	Hypotonia	Hypotonia Nystagmus Peripheral neuropathy Cerebellar syndrome	Hypotonia Severe cognitive language impairment
Epilepsy	Absent	Absent	Absent	One episode with abnormal eye movements and myoclonus	Onset 4y, severe therapy-resistant epilepsy
Cardiac examination	Slight aortic valve insufficiency	Echo 1: reduced systolic and diastolic function Echo 2: normal	NA	NA	NA
Eye examination	No structural abnormal findings	No structural abnormal findings	NA	NA	NA
Hearing		BERA abnormal peak pattern and neural conduction to brainstem	Sensorineural deafness		
Other findings		Hypospadias			
Laboratory results	Lactate twice slightly increased CK normal to slightly increased	Lactate not increased	No abnormalities	One episode of mildly elevated lactate, later repeatedly normal	No abnormalities
Metabolic investigations	No abnormalities	Slightly increased excretion of lactate in urine	No abnormalities	NA	No abnormalities
Muscle biopsy	Fairly small muscle fibers with myopathological features	Fairly small muscle fibers and complex I deficiency	Severe isolated complex I deficiency	Complex I defect	NA
Brain imaging	MRI: Small T2 hyperintense lesions in basal nuclei and thalamus, and later diffusion restriction in the dorsal mesencephalon MRS: No abnormalities	MRI: Normal development and signal intensity	MRI: Enlarged ventricles and megacisterna magna MRS: No abnormalities	CT: Hypersignal in basal ganglia MRI: No abnormalities	MRI: No abnormalities
Family				Parents consanguineous	Two older siblings died due to unexplained neurodegenerative disorders with severe epilepsy

Legend: g = gram(s), Apgar = Activity pulse grimace appearance respiration, NA = not available, w = week(s), m = month(s), cm = centimetre, OFC = occipital frontal circumference, SD = standard deviation, y = year(s), BERA = brainstem evoked response audiometry, CK = creatine kinase, MRI = magnetic resonance imaging, MRS = magnetic resonance spectroscopy.

Yet, their clinical symptoms bore resemblance to the clinical phenotype of Leigh syndrome, an indication to examine mitochondrial disease further. Leigh syndrome usually presents with central nervous system manifestations, such as developmental delay, ophthalmoparesis, ataxia, general weakness, hypotonia and respiratory impairment (Finsterer, 2008). T2-weighted MRI often shows bilateral, symmetric focal hyperintensities in the basal nuclei (Baertling et al., 2014) in Leigh syndrome that are characterized as necrotizing lesions upon pathological inspection (Finsterer, 2008). Brother 1 did show hyperintense signals in the basal nuclei, however these were transient and were attributed to ischemic sequela of his incident with acute respiratory insufficiency. Laboratory examination of the muscle biopsy of brother 2 revealed a complex I deficiency.

The mitochondrial gene panel finally showed a heterozygous

pathogenic variant in *TIMMDC1*, which raised suspicion of a *TIMMDC1*-related complex I deficiency. Due to lack of a second exonic variant, it was filtered out in the first exome sequencing analysis. Overlap in clinical symptoms caused us to investigate this gene further and identify the intronic splice variant. The intronic variant was the same as found by Kremer et al. (2017) (Kremer et al., 2017). They reported similar symptoms, such as muscular hypotonia, poor feeding behavior and developmental delay. Table 1 summarizes the clinical findings of all patients. Notably, marked elevated lactate levels are absent in all. Of note, the patients of Kremer et al. (2017) presented at a slightly later age and were not as rapidly respiratory compromised as the two brothers (Kremer et al., 2017). This might be due to some slipping of the splicing machinery leading to some residual wild type transcript expression of the c.596 + 2146A > G allele.

Lake et al. (2019) (Lake et al., 2019) described a patient with Leigh-like syndrome in whom exome sequencing identified two homozygous truncating variants; a known pathogenic c.1336C > T variant in *PDHX* and the c.673C > T variant in *TIMMDC1*. The authors show the latter variant lies in the penultimate exon and escapes nonsense mediated decay, leading to the expression of a truncated protein. Although the amount of assembled complex I and the complex I activity in fibroblasts were mildly reduced relative to control reference, the mutant protein showed comparable assembly potential of complex I assembly in *TIMMDC1* knock-out cells compared to wild-type protein. Moreover, the clinical features of the patients were similar to those of patients possessing the c.1336C > T variant in *PDHX* only, indicating it to be unlikely that the *TIMMDC1* c.673C > T variant was contributing significantly to the phenotype. Therefore, the authors concluded there was no evidence supporting a second diagnosis of *TIMMDC1*-related disorder in this family (Lake et al., 2019).

Intronic variants can generate disease by creating new splice sites leading to the inclusion of a pseudo-exon. This might disrupt the reading frame, resulting in a premature stop codon (Vaz-Drago et al., 2017). Deep intronic variants are not covered by exome sequencing and are difficult to prioritize when found with genome sequencing. Pathogenic variants are generally characterized by reduced levels of mutant transcripts which can be detected by RNA sequencing (Gonorazky et al., 2016). This method of combining RNA analysis and genome sequencing was used by Kremer et al. (2017) resulting in a diagnosis for 10% (5/48) of previously undiagnosed cases (Kremer et al., 2017). However, this approach is not yet widely available in the diagnostic setting.

In conclusion, this report describes the difficulty in establishing a genetic diagnosis in two brothers presenting with muscular hypotonia and respiratory insufficiency. It illustrates the importance of keeping a mitochondrial diagnosis in mind, even when lactate in serum is normal. Moreover, it shows the challenge in identifying deep intronic pathogenic variants, in an era where exome sequencing is part of routine diagnostic investigations. In clinical practice, performing targeted sequencing of known intronic variants in candidate genes can be of considerable additive value in undiagnosed patients and diagnostic laboratories could offer this as an additional service to increase the diagnostic yield. Laboratories could even include known intronic pathogenic variants in the exome capture sets and subsequent analysis pipelines of gene panels, as to further improve diagnostic abilities.

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CRediT authorship contribution statement

Myrthe Naber: Investigation, Writing - original draft, Visualization, Investigation. **Debby Hellebrekers:** Investigation, Writing - review & editing, Data curation. **Rutger A.J. Nievelstein:** Writing - review & editing, Visualization, Investigation. **Peter M. van Hasselt:** Writing - review & editing. **Richard H. van Jaarsveld:** Writing - review & editing, Investigation. **Inge Cuppen:** Writing - review & editing. **Renske Oegema:** Conceptualization, Writing - original draft, Supervision, Project administration.

Declaration of competing interest

The Authors declare that there is no conflict of interest.

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