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Significance of incidental copy number variants in the Duchenne muscular dystrophy gene

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ABSTRACT

We report results of laboratory and clinical investigations in 32 cases with incidental findings of large, intragenic deletions and gains in the huge Duchenne muscular dystrophy gene using microarray analysis. The patients and prenatal cases were referred for various reasons unrelated to DMD. Multiplex Ligation-dependent Probe Amplification of the *DMD* gene confirmed and refined deletions (19/32) and duplications (13/32). In 18 of the 32 cases a dystrophinopathy diagnosis could be established; 10 males were found to have dystrophinopathy and eight females were diagnosed as carriers. Sixteen of them had a pathogenic deletion and two had a pathogenic duplication. In three of the 32 cases the variants remained of unknown significance. In one of the 32 cases dystrophinopathy could be excluded. In the remaining 10 cases, the variant was likely benign. Our results show the importance of additional genetic analyses and clinical follow up after potentially incidental findings of copy number variants in the *DMD* gene. Moreover, our study provides insight in the possible effect of intragenic copy number variants in the *DMD* gene. Therefore, the article can provide guidance in the interpretation of copy number variants in the *DMD* gene, for example once DMD is included in newborn screening.

1. Introduction

The introduction of comparative genomic hybridization arrays (aCGH) and single nucleotide polymorphism (SNP)-based array for the detection of copy number variations (CNVs) in genetic diagnostics has led to findings in genes related to phenotypes other than those for which the patient was referred (e.g. incidental findings). Unexpected CNVs consisting of single-exon or multi-exon deletions/duplications have been reported mainly in genes in which they are known to be the most common disease-causing variants, such as the *DMD* gene. In patients referred for conditions such as developmental delay [1,2] and in prenatal cases with abnormalities in first-trimester screening tests [3] intragenic CNVs in the *DMD* gene have been found. The *DMD* gene is located on the short arm of the X chromosome (Xp21.2p21.1). It is the largest known gene in humans, spanning about 2.2 Mb, which explains its high mutation rate. The *DMD* gene (NM_004006.3) is composed of 79 exons and encodes the large 427 kDa dystrophin protein present in skeletal muscle [4]. Pathogenic variants in the *DMD* gene cause the degenerative muscle disorders Duchenne (DMD) and Becker (BMD) muscular dystrophy, so called dystrophinopathies. Large single exon or multiple exon deletions or duplications account for about 65 % of the cases (60 % deletions and 5 % duplications), while 35 % of the cases is caused by single nucleotide variants or small rearrangements [5]. About one-third of the copy number (CNV) variants occur *de novo*; the

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remaining are inherited from a carrier mother or via germline mosaicism [6,7]. DMD is the most common paediatric muscular dystrophy and is more severe than the generally later manifesting BMD. Patients suffering from dystrophinopathies may show great phenotypic variability, ranging from DMD, BMD, an intermediate phenotype to almost asymptomatic or with very mild complaints like cramps [4,8]. Some patients with only cognitive impairment or cardiac involvement but without muscle problems have been shown to have pathogenic variants

Table 1
Deletions at the *DMD* locus (see also supplemental findings).

Case	Sex	Age (yrs)	Motive for referral	Array result	Exons deleted based on array results (IF/OOF)	Exons deleted after MLPA confirmation (IF/OOF)	Inheritance	Segregation analysis in healthy adult males (yrs)	Conclusion
1	M	- (fetus)	Prenatal testing of male fetus due to kidney aplasia and dysplastic kidney (TOP)	arr[GRCh37] Xp21.1 (32,687,712–33,058,441)x0	exon 2–9 (OOF)	exon 2–9 (OOF)	maternal	Asymptomatic grandfather (69) carries deletion	likely benign
2	F	- (fetus)	Prenatal testing of female fetus due to abnormalities in previous pregnancy	arr[GRCh37] Xp21.1 (32,687,512–33,058,582)x1	exon 2–9 (OOF)	exon 2–9 (OOF)	paternal	Asymptomatic father (29) carries deletion	likely benign
3	F	- (fetus)	Prenatal testing of female fetus due to paternal chromosome abnormality	arr[GRCh37] Xp21.1 (32,372,244–32,882,170)x1	exon 2–37 (OOF)	exon 3–37 (IF)	de novo	–	carrier dystrophinopathy
4	M	54	CDD	arr[GRCh37] Xp21.1 (32,437,854–32,627,230)x0	exon 13–29 (IF)	exon 13–29 (IF)	maternal	NI; asymptomatic brother and sister do not carry deletion	dystrophinopathy
5	M	2	Short stature, high CK levels, motor DD, speech/language deficits, hemihypertrophy, clinodactyly	arr[GRCh37] Xp21.1 (32,246,117–32,407,930)x0	exon 33–43 (OOF)	exon 31–44 (IF)	unknown	NA; foster child	dystrophinopathy
6	M	8	Hyperdiploidy, Acute Lymphatic Leukemia	arr[GRCh37] Xp21.1 (32,109,121–32,335,291)x0	exon 42–44 (IF)	exon 42–44 (IF)	unknown	NA	dystrophinopathy
7	M	6	GDD, muscle weakness, CK 20.000 U/L	arr[GRCh37] Xp21.1 (32,149,400–32,235,295)x0	exon 44 (OOF)	exon 44 (OOF)	de novo	–	DMD
8	F	10	Congenital heart defects, ADHD, dyslexia	arr[GRCh37] Xp21.1 (31,974,707–32,085,367)x1	exon 45 (OOF)	exon 45 (OOF)	de novo	–	DMD carrier
9	M	8	GDD	arr[GRCh37] Xp21.1 (31,954,128–32,221,493)x0	exon 45 (OOF)	exon 45 (OOF)	maternal	NA	DMD
10	M	7	Growth retardation and mild MDD	arr[GRCh37] Xp21.1 (31,734,779–31,987,992)x0	exon 45–52 (OOF)	exon 45–52 (OOF)	de novo	–	DMD
11	M	- (fetus)	Prenatal testing of male fetus due to increased risk combined test	arr[GRCh37] Xp21.1 (31,667,683–32,095,241)x0	exon 51–61 (IF)	exon 45–54 (OOF)	de novo	–	DMD
12	F	12	Congenital hypotonia, mild CCD, speech deficits	arr[GRCh37] Xp21.1 (31,836,420–31,976,320)x1	exon 46–50 (OOF)	exon 46–50 (OOF)	de novo	–	DMD carrier; no 2nd hit in *DMD*
13	F	41	CDD; Phelan-McDermid syndrome	arr[GRCh37] Xp21.1 (31,693,192–31,871,341)x1, 22q13.31q13.33 (43,139,556–49,691,432)x1	exon 46–51 (OOF)	exon 46–51 (OOF)	de novo	–	DMD carrier
14	M	6	GDD, partial Gower's sign, microchromosomal abberations, Fragile X	213 kb deletion at Xp21.1 *No specifications received*	NR	exon 48–54 (OOF)	de novo	–	DMD
15	M	4	GDD, obesity	arr[GRCh37] Xp21.1 (31,540,768–31,951,697)x0	exon 46–55 (OOF)	exon 48–55 (IF)	de novo	–	BMD
16	F	- (fetus)	Prenatal testing of female fetus due to UA	arr[GRCh37] Xp21.1 (31,766,645–31,853,992)x1	exon 50–51 (IF)	exon 50–51 (IF)	de novo	–	carrier dystrophinopathy
17	M	6	CDD	arr[GRCh37] Xp21.1 (31,766,645–31,853,992)x0	exon 49/ 50–51 (IF)	exon 50–51 (IF)	maternal	Asymptomatic grandfather (63) carries deletion	BMD
18	F	13	CDD, clinical phenotype of Angelman syndrome	arr[GRCh37] Xp21.1 (31,649,453–31,835,562)x1	exon 51–54 (OOF)	exon 51–54 (OOF)	de novo	–	DMD carrier
19	M	5	Mild CDD and behavioral problems	Deletion at Xp21.1 *No specifications received*	exon 56–60 (IF)	exon 56–60 (IF)	maternal	Asymptomatic grandfather (66) carries deletion	Unknown

Abbreviations: BMD= Becker muscular dystrophy; CDD= Cognitive Developmental Delay; DMD= Duchenne muscular dystrophy; IF= In Frame; GDD= Global Developmental Delay; MDD =Motor Developmental Delay, NA= Not available; NI=Not Informative, NR= Not Reported; OOF= Out-of-Frame; TOP= Termination of Pregnancy; UA= ultrasound abnormalities.

in the *DMD* gene [9]. Most female carriers are asymptomatic but have an increased risk of muscle damage and dilated cardiomyopathy [10] next to the risk of passing on the pathogenic variant to offspring. In 1988 it was postulated that pathogenic CNVs that cause a frameshift in the *DMD* gene (out-of-frame) are usually found in DMD patients, whereas in-frame variants are mainly detected in patients with the milder BMD [11]. The reading frame rule correlates with the clinical phenotype in about 90 % of the cases [5,12]. In-frame deletions in the *DMD* gene can lead to a spectrum of mild symptoms or elevated serum kinase only [13]. About 10 % of the variants do not follow the reading frame rule with certain exceptions occurring frequently [5,14,15], most notably the most common out-of-frame deletion of exons 3–7 [16]. We describe the analysis of CNVs identified as incidental findings in the *DMD* gene in 32 Dutch cases that had been referred for indications unrelated to dystrophinopathy. Our study demonstrates the importance of further genetic testing in case of the identification of new variants in a known disease gene. Diagnosis of DMD patients at an early age is very important in relation to therapeutic intervention. In addition, female relatives may be screened for their risk of carrier status, and prenatal testing becomes accessible. This strategy could also apply for new variants in the *DMD* gene once Duchenne Muscular Dystrophy is added to newborn screening (NBS). Currently, pilot studies for the implementation of Duchenne Muscular Dystrophy in NBS are being set up [17–20] and conducted worldwide.

2. Materials and methods

2.1. Materials

Genomic DNA was extracted from samples of whole blood, skin biopsy, bone marrow or chorionic villi samples using standard procedures.

The Dutch DMD genetic expertise center in Leiden was consulted for 51 patients who presented with clinical phenotypes unrelated to DMD, in whom genome-wide array analysis conducted at various clinical genetics centers in the Netherlands had identified gross rearrangements of the *DMD* gene. Whole blood or genomic DNA from these individuals was sent to the expertise center for MLPA screening of the *DMD* gene to confirm the array results, to specify the exons involved in the deletion or duplication and to perform additional genetic testing, if necessary and possible. Nineteen cases were excluded from the study due to the absence of information on the patients' clinical phenotypes, unavailability of MLPA results or explicit objection to scientific publication. This study includes the remaining 32 patients. Most patients suffered from cognitive and/or motor developmental delay or were prenatal cases with strong evidence of abnormalities in the fetus. A few had multiple congenital abnormalities or were suspected to have a rare genetic disorder, see Tables 1 and 2 and supplemental data.

The study is a retrospective descriptive case series. The Medical Ethics Committee of the Leiden University Medical Centre issued a statement of no objection. All data were pseudonymized to comply with rigorous privacy guidelines. For data collection in this study, informed consent and ethical approval were not required according to Dutch law [21,22].

2.2. Methods

Additional investigations such as MLPA, segregation and FISH analysis were carried out as described below. Subsequent interpretation and classification of the genetic variants were performed and genotype-phenotype correlations were established.

2.2.1. Array analysis

Array analysis using array CGH, 180 K oligo-array (Agilent), CytoScan High-density and 250 K SNP array (Thermo Fisher Scientific) was performed according to the instructions from the manufacturer. Copy number was assessed using several software packages.

2.2.2. MLPA analysis

Multiplex ligation-dependent probe amplification (MLPA) analysis was performed for screening of whole-exon deletions/duplications according to the MRC Holland protocol (MRC Holland; P034- and P035-kits). MLPA data were analyzed using GeneMarker software version v3.0.

2.2.3. FISH analysis

Fluorescence in situ hybridization (FISH) was carried out [23] in most cases with a copy number gain (10/13 cases, Table 2) and in one deletion case (case 13, Table 1).

2.2.4. Segregation analysis

Pathogenicity of novel variants was determined after segregation analysis in the family, if possible. All variants were submitted to the Leiden Open Variation Database (LOVD-DMD)[5].

3. Results

A total of 51 incidental findings were identified over a period of 8 years. We were able to validate, confirm and specify 32 CNVs in the *DMD* gene (19 deletions and 13 duplications/gains). Several results are highlighted in this paragraph. A full overview of the results; phenotypes of the index patients, array and MLPA results, additional DNA analysis and conclusions regarding the deletions and duplications, are shown in Tables 1 and 2 respectively. The details about the patients are provided in the supplementary data.

3.1. DMD deletions (*Table 1* and supplementary data)

DMD-MLPA was performed in all 19 cases: in 12 cases the results of the array analysis were confirmed; in two cases where array analysis had only indicated that the deletions were located in Xp21.1 specification of the exons involved was established; in five cases there was a difference between the array and MLPA results with respect to the total number of exons or which exons were deleted.

In total, sixteen different deletions in the DMD gene (NM_004006.3) were found in the 19 patients and their families. Three of the deletions were detected in more than one family: exon 2–9 (cases 1 and 2), exon 45 (cases 8 and 9) and exon 50–51 (cases 16 and 17). All 16 variants have been published and have been associated with a range of phenotypes: DMD, BMD, Intermediate Muscular Dystrophy (IMD) and Muscular dystrophy (MD). Eleven of our sixteen deletions had arisen *de novo*.

In the 19 patients with deletions in the *DMD* gene, genotype-phenotype correlation was determined, and if possible, segregation analysis was performed. Based on these results five male patients (cases 7, 9, 10, 11 and 14, including one fetus) with out-of-frame (OOF) deletions were diagnosed with DMD, two male patients (cases 15 and 17) with in-frame (IF) deletions were diagnosed with BMD, three male patients (cases 4, 5 and 6) with in-frame deletions were diagnosed with a dystrophinopathy not specified in DMD or BMD. Six females (cases 3, 8, 12, 13, 16 and 18) were diagnosed as carriers of a dystrophinopathy. Patient 12 was referred to a paediatric neurologist. In the remaining three cases (case 1, 2 and 19) the deletion did not result in a dystrophinopathy.

Two (cases 3 and 16) of the six females were fetuses diagnosed as carriers of dystrophinopathy. The remaining four females (cases 8, 12, 13 and 18) were diagnosed as carriers of DMD. Clinical symptoms associated with DMD carrier status were not clearly present in these four females, other clinical symptoms like congenital heart defects (case 8), congenital hypotonia (case 12) were more prominent or patients were diagnosed before with Phelan-McDermid syndrome (case 13) and Angelman syndrome (case 18) by array analysis.

In five of the 19 patients the deletion was classified as variant of uncertain significance (VUS). Additional segregation analysis in these

Table 2
Duplications at the *DMD* locus (see also supplemental findings).

Case	Sex	Age (yrs)	Motive for referral	Array result	Exons duplicated based on array results (IF/OOF)	Exons duplicated after MLPA confirmation (IF/OOF)	Inheritance	Segregation analysis in healthy adult males (yrs)	FISH	Conclusion
20	M	- (fetus)	Prenatal testing of male fetus due to UA (TOP)	arr[GRCh37] Xp21.1 (33,058,382–33,409,990)x2	DP427c + exon 1	DP427c + exon 1	maternal	Healthy brother of maternal grandfather (60) carries duplication	DNA mother: interstitial duplication in *DMD* gene	likely benign
21	F	40	Co-incidental finding in DNA mother of son with IDD	arr[GRCh37] Xp21.1 (32,993,147–33,603,101)x3	Dp427c + exon 1–2	Dp427c + exon 1–2	(maternal)	Healthy brother (39 yrs) carries duplication	Interstitial duplication in *DMD* gene	likely benign
22	F	- (fetus)	Prenatal testing of female fetus due to UA (TOP because of unilateral shortening of femural bone)	arr[GRCh37] Xp21.1 (32,860,747–34,399,257)x3	Dp427c + exon 1–4	Dp427c + exon 1–4	maternal	Asymptomatic brother of mother carries duplication	Interstitial duplication in *DMD* gene	likely benign
23	F	40	Multiple abnormalities	arr[GRCh37] Xp21.1 (33,158,437–33,293,101)x3	exon 1	exon 1	maternal	Asymptomatic brother (37) carries duplication	Interstitial duplication in *DMD* gene	likely benign
24	M	- (fetus)	Prenatal testing of male fetus due to UA, IUGR (TOP)	arr[GRCh37] Xp21.1 (33,150,225–33,292,999)x2	exon 1	exon 1	maternal	Asymptomatic grandfather (61) carries duplication	–	likely benign
25	M	3	CP, soft palate	arr[GRCh37] Xp21.1 (32,508,556–32,581,680)x2	exon 17–20 (IF)	exon 17–20 (IF)	maternal	NI	Did not confirm 73 kb duplication	Unknown
26	F	3	Multiple congenital abnormalities	arr[GRCh37] Xp21.1 (31,990,522–32,285,738)x3	Exon 44 (OOF)	exon 44 (OOF)	unknown (non-maternal)	None	–	Unknown
27	M	6	CDD	arr[GRCh37] Xp21.1 (31,840,701–32,106,875)x2 Xq26.3 (136,510,620–137,027,604) x2	exon 45–49 (IF)	exon 45–50 (OOF)	maternal	–	Extra copy inserted at distant genomic location Xq25–26	no effect in *DMD* gene
28	F	26	Preliminary testing pregnant female after previous pregnancy with IUFD	arr[GRCh37] Xp21.1 (31,756,290–32,190,220)x3	exon 45–51 (IF)	exon 45–51 (IF)	maternal	Asymptomatic uncle carries duplication	–	likely benign
29	F	3	Neuromuscular clinical phenotype (severe hypotonia, myopatic, scoliosis,	arr[GRCh37] Xp21.1 (31,661,789–32,204,021)x3 arr[GRCh37] Xp21.2 (30,860,326–31,278,838)x3	45–54 (OOF) and 64–79	45–54 (OOF) and 63–79	de novo	–	Interstitial duplication in DMD gene	symptomatic DMD carrier?

(*continued on next page*)

Table 2 (*continued*)

Case	Sex	Age (yrs)	Motive for referral	Array result	Exons duplicated based on array results (IF/OOF)	Exons duplicated after MLPA confirmation (IF/OOF)	Inheritance	Segregation analysis in healthy adult males (yrs)	FISH	Conclusion
			positive Trendelenburg, contractures) and ADHD							
30	F	6	Motor problems, decreased strength in calves	arr[GRCh37] Xp21.1.2 (31,273,376–31,784,247)x3	exon 52–63 (OOF)	exon 52–63 (OOF)	de novo	–	Confirmed Xp21.2p21.1 location	symptomatic DMD carrier
31	F	11	Growth retardation, atrial septal defect, vesicoureteral reflux	arr[GRCh37] Xp21.1.2 (30,393,146–31,429,432)x3	exon 61–79	exon 61–79	maternal	Asymptomatic (67) grandfather carries duplication	Confirmed Xp21.2 location	likely benign
32	F	- (fetus)	Prenatal testing due to pregnancy with IUFD	arr[GRCh37] Xp21.2 (31,054,997–31,405,896)x3	exon 61–79	exon 61–79	maternal	Asymptomatic (66) father of mother carries duplication	Confirmed Xp21.2 location	likely benign

Abbreviations: ADHD= attention deficit hyperactivity disorder; CP= Cleft Palate; CDD= Cognitive Developmental Delay; DMD= Duchenne muscular dystrophy; FISH=Fluorescence in situ hybridization; IF= In Frame; IUFD= Intra Uterine Fetal Death; MDD= Motor developmental Disorder; NA= Not Available; NC= Non-Coding; NI= Not Informative, NR= Not Reported; OOF= Out-of-Frame; TOP= Termination of Pregnancy; UA= Ultrasound Abnormalities.

five families was performed to further investigate the pathogenicity of the detected deletion. Segregation analysis in case 4 proved not to be informative, since the in-frame deletion of exons 13–29 was not detected in the only asymptomatic male relative. Based on the information in the LOVD-DMD database, genotype and phenotype information, the patient was diagnosed with dystrophinopathy (see supplemental data for more details).

In four families an asymptomatic older male relative carried the familial deletion. In case 1 and 2 the in-frame deletion of exon 2–9 was identified in asymptomatic male relatives, which is described in more detail below. In case 17 the more common deletion of exon 50–51 was found and in case 19 the relatively unknown in-frame deletion of exon 56–60 was identified. Both cases are described in more detail below (see supplemental data for more details).

Three deletions are worth describing in more detail:

The first is an out-of-frame deletion of exon 2–9 which is predicted to cause a shift in the *DMD* reading frame. It was detected in fetuses in two unrelated Dutch families. In both families the deletion was classified as likely benign because there was an asymptomatic, older male relative with the same deletion. In case 1 it was the maternal grandfather and in case 2 it was the father. However, caution is indicated, particularly in case 2 because the father is only 34 years old, and it cannot be ruled out that he may develop heart or muscle problems later in life. The same deletion has been described by [24] in a male fetus (aborted) and his mother, who turned out to be a mosaic carrier. The variant was not found in DMD variant databases, neither in the normal population [25, 26] nor in dystrophinopathy patients [5].

The second is an in-frame deletion of exons 50–51 which was found in a 6-year-old male (case 17) suffering from cognitive developmental delay and his 63-year-old asymptomatic maternal grandfather. After additional neurological examination of the young index patient some evidence of muscle disease was observed. We therefore classified the variant as BMD causing. In this family, variability in the phenotype associated with this variant is observed. We have previously reported the same variant in a young Dutch boy with normal muscle strength and occasional muscle cramps [27]. This variant has been reported in patients with DMD, BMD and with an intermediate phenotype (LOVD-DMD database) in literature. This data shows that prediction of genotype/phenotype correlations within a family is not always reliable.

The third is an in-frame deletion of exons 56–60 found in a 5-year-old patient with mild cognitive developmental delay and behavioral problems (case 19). The clinical phenotype of the patient does not fit a dystrophinopathy. The 66-year-old asymptomatic maternal grandfather carried the same deletion. However, in contrast to our patient without any muscle complaints, in the LOVD-DMD database three DMD patients are described with the same deletion, however little clinical data was provided [28–30]. The deletion has never been found in Dutch dystrophinopathy families before. We therefore classified the deletion in this case as variant of unknown significance.

To summarize, 10 of the 19 patients were diagnosed with dystrophinopathy (DMD/BMD) and six as carriers for DMD/BMD; in two cases dystrophinopathy could be excluded, in one case the effect of the deletion remained unknown.

3.2. *DMD duplications (Table 2 and supplemental data)*

DMD-MLPA confirmed the results of array analysis in 11 of 13 cases with a gain. For case 27 and 29 there was a difference in outcome between these two genetic tests.

Additional FISH investigation performed in 10 cases demonstrated that the gain was due to a duplication in Xp21 in eight patients and due to an insertion at the distant genomic location, Xq25q26 in one individual, which ruled out dystrophinopathy in this patient (case 27). In one patient (case 25) the gain of 73 kb was probably too small for confirmation by FISH analysis. These results show the importance of investigating the location of the gain by FISH or another technique with similar results (for example short or long-read WGS) for patients with unexpected intragenic gains in the *DMD* gene. In three cases (case 24, 26 and 28) where FISH analysis was not performed, an insertion of the duplicated region outside the *DMD* locus could not be excluded.

In the remaining 12 patients (case 27 excluded after FISH) with duplications in the *DMD* gene, genotype-phenotype correlation was determined and, if possible, segregation analysis was performed. In two symptomatic female DMD carriers (case 29, described in more detail below, and case 30) the duplication in Xp21 had arisen *de novo*. Both out-of-frame duplications were classified as pathogenic (exons 45–54 and exons 52–63).

In two (cases 25 and 26) no conclusion could be drawn (described in more detail below). In the remaining eight families the duplication was considered to be benign because an adult asymptomatic male relative

had the same duplication.

Twelve different duplications were identified. Two novel duplications were found in our study, the duplication of uncertain significance of exons 17–20 (case 25) and the likely benign duplication of Dp427c + exon 1–4 (case 20). Two variants were detected twice: duplication of the non-coding exon 1 (cases 23 and 24) and duplication of exons 61–79 (cases 31 and 32). Both variants were classified as likely benign in our study; duplication of non-coding exon 1 has been reported in the literature as variant of unknown significance (VUS) in Muscular Dystrophy [31], which is also the case for duplication of exons 61–79 [32].

Four duplications are worth describing in more detail:

No conclusion could be drawn about the effect of two duplications: one was the duplication of exons 17–20 (case 25). This was a novel variant found in the index patient (case 25) and his mother. FISH analysis did not confirm the gain in Xp21 and segregation analysis was not informative. This in-frame variant encompasses a small part of the repeat region of dystrophin and has not been found before in our Dutch dystrophinopathy population nor has it been reported in the LOVD-DMD database. The other was a duplication of exon 44 (case 26). It was found in a young female but not in her mother; her father was not available for testing. In the absence of FISH results, it cannot be excluded in both cases that the gain is inserted in another part of the genome.

An in-frame duplication of exons 45–51 (case 28) was classified as likely benign because it was also present in an asymptomatic uncle. It is known that although the variant has been reported in MD/DMD/BMD patients, it is also found in healthy controls (LOVD-DMD database). Moreover, recent MLPA analysis of samples submitted to our laboratory, which are not included in this study, demonstrated the duplication of exons 45–51 in two patients with non-muscle disease related conditions. The variant was an incidental finding in both patients. Later, additional FISH analysis in one of these patients showed that the gain was due to an insertion in chromosome 17, which excluded dystrophinopathy.

Case 29 was a female patient who was found to be a symptomatic carrier of DMD. She had two *de novo* duplications in different regions of the *DMD* gene: a non-contiguous out-of-frame duplication of exons 45–54, which was pathogenic, and duplication of exons 63–79. The latter has been reported in a BMD patient [33], in a patient with dystrophinopathy [30,34] and in a healthy control [32]. It is unknown whether the duplications in our patient are situated in tandem and if they are located on the same or different X-chromosomes (in *cis* or in trans). Additional genetic studies (FSHD and WES analysis) did not identify a second pathogenic variant. She was diagnosed as symptomatic DMD carrier, but the variants cannot explain her severe phenotype. She was referred to a paediatric neurologist.

To summarize, two of the 13 patients were diagnosed as symptomatic carriers for DMD; in nine cases dystrophinopathy could be excluded; in two the effect of the gain remained unknown, but it cannot be excluded that in both cases the variant was inserted in another part of the genome.

4. Discussion

With the introduction of genome wide CNV analysis into genetic diagnostics, an increasing number of incidental findings in various genes has been reported. Through genome wide CNV analysis by array or Next Generation Sequencing approaches, many CNVs in one of the largest genes - the *DMD* gene - have been detected by chance. These have been found in young boys and a girl with nonspecific findings [1], in young girls suffering from developmental delay [2] and in prenatal cases with abnormalities in first-trimester screening tests [35].

In the 32 patients with incidental findings in the *DMD* gene included in this study, we established dystrophinopathy in 18 cases (56 %) of which 16 were caused by deletions. These included nine males, six females, one male fetus and two female fetuses. The majority of the patients were young: eight out of nine males with BMD/DMD were between the ages of 2–8 years and five out of six female carriers of BMD/DMD were between 3–13 years of age. It is clear that further genetic studies are important when incidental findings are detected in the *DMD* gene, particularly in young male patients for early diagnosis and consequently possible treatments (therapeutic intervention). In addition, further family studies for carrier status will become possible and prenatal testing in future pregnancies will become available.

Developmental delay was the most prominent indication for referral of most of the young male patients. DMD or BMD was diagnosed following more extensive neurological examination by a (pediatric) neurologist. It is therefore crucial that young male patients with delayed motor development are referred early to specialists like pediatric neurologists.

Among the eight female carriers two were fetuses, the six others had been referred for various reasons: cognitive developmental delay, congenital heart defects, hypotonia, motor problems to severe neuromuscular complaints. Although a parallel diagnosis of a specific cognitive developmental delay had been identified in two patients (Phelan-McDermid syndrome in case 13 and Angelman syndrome in case 18), these patients were also carriers of DMD. All cases with unexpected variants in the *DMD* gene have been further examined to confirm carrier status and to predict the effect of the variant. However, in one symptomatic female carrier (case 29) the severe (atypical) phenotype could not be explained by her carrier status and additional genetic studies are planned. A novel technique like nanopore genomic long-read sequencing (LRS) is planned. By means of LRS undetected structural variants or cryptic splice sites have been recently reported to cause forms of muscular dystrophies [36].

More patients with a deletion ($n = 19$) than with a duplication ($n = 13$) in the *DMD* gene had been referred. A diagnosis of dystrophinopathy or carrier status was established in more cases with a deletion (16/19), than in cases with a duplication (2/13). In 13 (11 deletions and 2 duplications) of the 32 cases, the variant had arisen *de novo*; in *de novo* cases conclusions about the status of a variant cannot always be drawn as segregation analysis is not possible. Consultation of databases is therefore recommended although one should carefully check the types of tests used for genetic analysis. In addition, one should be aware when looking for predictions or observations of phenotypes that older publications or those on screening large cohorts of patients do not always mention phenotypes in relation to genotypes. For familial cases conclusions about the effect of the variant can be drawn in nearly all cases by using additional segregation analysis. In one of the deletion cases additional segregation analyses (case 19) showed the deletion in an asymptomatic older male relative, however the variant was recently described in a DMD patient [29]. We therefore classified the deletion as variant of unknown significance. If family studies are not informative and FISH analysis is not possible, as was the case with two gains (case 25 and 26), then no conclusion can be reached.

In seven cases CNVs found by MLPA analysis did not match the CNV reported by microarray. One of the explanations may be the location of a breakpoint of a CNV. If a breakpoint of a CNV, detected by array analysis, is situated in close proximity to an exon-intron boundary, confirmation with a second test is advised. To this end the location of the breakpoint of a CNV should always be checked. Especially in cases of our study with various clinical symptoms and where no DMD-like phenotypes were expected. Currently, when using whole genome sequencing (WGS) accurate breakpoints of CNVs can be established. The second explanation may be that with the start of our study older array platforms have been used.

In the recent years various newborn screening (NBS) pilot studies for DMD have been performed in several countries [17–20] or are planned [37]. Data from pilot studies show that it is feasible to include DMD in NBS for newborn males: in a two-tier approach based on CK measurement followed by DMD gene testing once CK level is elevated [18,37]. Although there is no cure for DMD yet, the increasing number of treatment options shows the benefits of early detection of DMD. Our study could be of value during NBS, by showing how to proceed if CNVs in the

DMD gene are found that are not well established.

In our cohort of 28 different CNVs, 16 variants (14 deletions and 2 duplications) were classified as pathogenic. One gain (case 27) was shown by FISH to be due to an insertion in a distant genomic location and had no effect on the *DMD* gene. Seven of the remaining 11 CNVs were classified as likely benign because an adult male relative with the same variant was asymptomatic and four variants were of unknown significance. In two families (cases 1 and 2) an out-of-frame deletion of exons 2–9 was classified as likely benign. However, a supplementary medical examination of the asymptomatic older male relatives was not possible in both families. It is of great importance in such cases to follow-up the asymptomatic male relatives later in life by a cardiologist [38] and a neurologist. Possibly the presence of alternative transcripts in which the original variant has been modified could cause a relatively mild onset in family members, as has been suggested for in-frame deletion of exons 3–9 in this part of the gene [39]. This study also emphasizes that during interpretation it has to be kept in mind that exceptions to the reading-frame rule do exist and that not all CNVs in the DMD gene result in dystrophinopathy. More and more is becoming known about exceptions to the reading-frame rule due to advanced genetic techniques.

5. Conclusion

Our study has shown that when intragenic copy number gains and losses in the *DMD* gene are found by chance in individuals referred for indications other than BMD/DMD, there is a high risk that additional genetic testing and clinical evaluation by a (pediatric) neurologist reveals that the patient is affected by or is a carrier of dystrophinopathy. In some cases, a BMD/DMD phenotype was identified alongside a clinical diagnosis explaining the initial reason for referral. It is therefore important to:

1) Validate, specify, and characterize, if possible, the CNV with another validated technique like DMD MLPA, especially in case of single exon imbalances, in order to better interpret and classify the CNV and predict the phenotype.
2) Perform FISH or (short or long read) WGS analysis in case of gains for mapping the variant.
3) Perform segregation analysis to look for older asymptomatic male relatives, and if possible, carry out medical examination of these male relatives to determine the pathogenicity of the CNV. In some cases, dystrophin analysis of muscle tissue can be helpful.
4) Check (international) *DMD* variant databases for genotypes and correlating phenotypes.
5) Refer the patient to a (paediatric) neurologist for clinical examination.
6) Carry out DNA analysis in female relatives for carrier status if a pathogenic CNV is identified in the index patient.

This study also emphasizes that not all CNVs in the *DMD* gene result in a dystrophinopathy. More knowledge on these CNVs is valuable, for example during the interpretation of DMD NBS results.

CRediT authorship contribution statement

Ieke B. Ginjaar: Writing – review & editing, Writing – original draft, Validation, Methodology, Data curation, Conceptualization. **Marjolein Kriek:** Writing – review & editing, Investigation. **Mariëtte J.V. Hoffer:** Writing – review & editing, Validation, Methodology, Data curation. **Renske Oegema:** Writing – review & editing, Investigation. **Ellen van Binsbergen:** Writing – review & editing, Validation, Data curation. **Karin E.M. Diderich:** Writing – review & editing, Investigation. **Laura J.C.M. van Zutven:** Writing – review & editing, Validation, Methodology, Data curation. **Floor A.M. Duijkers:** Writing – review & editing, Investigation. **Alida C. Knegt:** Writing – review & editing, Validation, Methodology, Data curation. **Corrie E. Erasmus:** Writing – review & editing, Investigation. **Nicole de Leeuw:** Writing – review & editing, Validation, Methodology, Data curation. **Joke B.G.M. Verheij:** Investigation. **Trijnie Dijkhuizen:** Writing – review & editing, Validation, Methodology, Data curation. **Hermine A. van Duyvenvoorde:** Writing – review & editing, Writing – original draft, Validation, Methodology, Data curation, Conceptualization.

Declaration of competing interest

None.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.nmd.2025.106219.

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