Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Analysis of thirty-three pairs of twins with Duchenne Muscular dystrophy (DMD): study from a single center in India

Mary Shoba Das Christu Dhas
Molecular Diagnostics, Counseling, Care and Research Centre
Priya Karthikeyan
Molecular Diagnostics, Counseling, Care and Research Centre
Manikandan Suraj
Molecular Diagnostics, Counseling, Care and Research Centre
Ragav Krishna
Molecular Diagnostics, Counseling, Care and Research Centre
Arati Khanna-Gupta
Yale University
Lakshmi BR
drlakshmi@mdcrcindia.org

Molecular Diagnostics, Counseling, Care and Research Centre

Research Article

Keywords: Duchenne muscular dystrophy, Twins, Monozygotic, Dizygotic, Variants

Posted Date: November 18th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11149696/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations:
No competing interests reported.

Tables 1 to 3 are available in the Supplementary Files section.

Analysis of thirty-three pairs of twins with Duchenne Muscular dystrophy (DMD): study from a single center in India

Mary Shoba Das Christu Dhas[1], Priya Karthikeyan [1], Manikandan Suraj [1], Ragav Krishna [1], Arati Khanna-Gupta [1,2], Lakshmi BR [1*]

[1]Molecular Diagnostics, Counselling, Care and Research Centre (MDCRC), Located at Royal Care Super Speciality Hospital, Neelambur, Coimbatore, Tamil Nadu, 641062, India

[2] Department of Immunobiology, Yale University School of Medicine, TAC Building S625, 330 Cedar St., New Haven CT, 06519, USA

*Corresponding author:

Lakshmi BR, Ph.D.

Molecular Diagnostics, Counselling, Care and Research Centre (MDCRC),

Royal Care Super Speciality Hospital

Neelambur

Coimbatore, 641062,

Tamil Nadu,

India

Phone: +91 9444402686

email: drlakshmi@mdcrcindia.org

ABSTRACT

Purpose: Given that twin studies have been invaluable in assessing the role of genetic variation and the environment with disease susceptibility, we analyzed the genetic disease manifestations of DMD in 33 sets of twins.

Methods: Genomic DNA obtained from twin pairs and potential carrier mothers was subjected sequentially to multiplex PCR, MLPA or NGS to identify disease causing variants in the *DMD* gene.

Results: Of these, 18 were male twin sets, 15 were male/female twin sets, of which 2 twin sets were born following assisted reproductive procedures. The majority of twin pairs were dizygotic (DZ), 4 sets were monozygotic (MZ) and the zygosity was unknown in 7 twin pairs. A difference in disease severity between MZ and DZ twins was noted. Of the twenty-two *DMD* gene variants identified in this cohort of DMD twins, no duplication variants was identified and the majority of deletion variants (88%) were found in known *DMD* gene hot-spot regions. Three rare variants were found exclusively in DMD twins. Compared to 1181 DMD singletons, an enrichment of deletions in exons 46-48 and 51-54 was observed in DMD twins. Carrier analysis of mothers of DMD twins showed that the majority of DMD among twins occurs sporadically.

Conclusions: This is the first concerted study of DMD twins done to date. Despite the limited number of twin pairs, our observations provide insights into better understanding DMD through variant distribution and inheritance patterns in twins that contribute to disease severity.

KEY WORDS:

Duchenne muscular dystrophy; Twins; Monozygotic; Dizygotic; Variants

BACKGROUND

Duchenne muscular dystrophy (DMD; OMIM: 310200) is a rare X-Linked recessive degenerative neuromuscular disorder (Muntoni et al. 2003), with an incidence of 1 in 3300-5000 live births (Moat et al. 2013) (Deconinck 2007). DMD affected young males begin to show symptoms early, including frequent falls, calf muscle hypertrophy, walking on toes, waddling gait, difficulty in climbing stairs and Gower's sign. Progressive muscular degeneration with increasing age is a hallmark of the disease. Patients become wheelchair dependent around the age of twelve and succumb to cardiopulmonary failure in their early twenties (McDonald 2018). Currently no curative treatment options are available to patients with DMD (Guiraud 2017), even though several new drugs are currently in the developmental pipeline (Sun et al. 2020). The use of glucocorticoid anti-inflammatory drugs including prednisone or deflazacort are the current standard of care, and disease management has played a central role in disease outcomes (Birnkrant 2018).

Both DMD and Becker Muscular dystrophy (BMD, OMIM: 300376), a less severe form of the disease, are caused by variants in the X-linked *DMD* (*dystrophin)* gene. Loss of the dystrophin protein in muscle cells leads to recurrent myofiber damage, which in turn causes chronic inflammation leading to progressive muscle dysfunction and fibrosis (Rosenberg 2015).

In a previous report from our institution, a study of 961 clinically diagnosed unrelated DMD patients revealed 816 (84%) patients, with a genetically confirmed diagnosis. Among the 816 patients, deletions were found to be the most frequent variant type (~79%), followed by point variants (~12%) and duplications (~9%) in the *DMD* gene (Kumar et al. 2020), similar to percentages of variant types previously reported (Tuffery-Giraud 2009) (Bladen 2015).

In the recent past, despite a better understanding of the genetic defects in the *DMD* gene, a clear correlation between specific genetic variants and their clinical manifestations has not emerged with complete clarity.

Such genotype-phenotype correlates, if identified, could pave the way to a better understanding of the disease and provide a road map to address the unmet need for new and effective treatments for DMD. In the past, studies on twins have provided valuable insights into the contribution of genetics and environmental factors to disease (Boomsma et al. 2002).

Objectives and Design: Comparative analysis of monozygotic (MZ) and dizygotic (DZ) twins has proven to be among the best resources available to evaluate and enable researchers to unravel the roles of genetic factors from shared environmental influences (Johnson et al. 2009). The current work. We hypothesized that through the study of DMD twin sets a clearer picture of genotype-phenotype correlates would emerge. In this study we will address this hypothesis, we will compare DZ with MZ twins diagnosed with DMD. Despite the limited numbers of twin pairs (n=33), Our objectives will include a comparison of DMD disease parameters including the age at onset of disease, age at loss of ambulation and an investigation of the possible enrichment of twin-specific *DMD* gene variants. Additionally, we will address if DMD in twins appear to be related to the phenomenon of twinning within families. The analysis of carrier status of mothers of DMD (an X-lined disease) affected twins will be undertaken to delineate the role of genetics in DMD twins, thereby offering opportunities for disease prevention through counselling in these families. Our study has the potential to advance our understanding of the multifaceted interplay between genetic predisposition and environmental factors that influence the manifestation of DMD.

PATIENTS AND METHODS

Identification of twins with DMD/BMD in the community:

Between the years 2010-2022, 33 pairs of twins were identified at MDCRC, a non-profit organization dedicated to the molecular diagnosis, care and counselling of patients with muscular dystrophies (www.mdcrcindia.org), in the state of Tamil Nadu (TN), India, where one or more of the twins was clinically suspected of having DMD/BMD. The majority of these patients (20 twin pairs) were referred to MDCRC through physicians in clinics and hospitals in Tamil Nadu (TN) state, while the remaining DMD patients (13 twin pairs) were identified through MDCRC's community outreach program designed to identify, diagnose and counsel patients in rural districts of TN state (to be detailed elsewhere). All suspected DMD twins presented with one or more of the following symptoms including frequent falls, waddling gait, calf muscle hypertrophy, difficulty in climbing stairs and Gower's sign. Each twin pair identified was categorized into the following groups: a) both twins affected, b) one twin affected and the second twin unaffected, c) one twin affected the second deceased; d) one twin affected and the second twin female and e) twins born following assisted reproductive procedures (*In-vitro* fertilization, IVF) (Figure 1).

The Institutional Review Board of the Molecular Diagnostics, Counselling, Care & Research Centre (MDCRC) reviewed and approved this work in accordance with the 1964 Helsinki Declaration and its later amendments. Approval for this study was obtained following review by the MDCRC Ethics Review Board and was granted Approval Number MDCRC/03/IEC-DMD016. We confirm that all study participants or their legal representatives gave written or verbal informed consent prior to participation in this research study.

Genetic analysis of twin samples for diagnosis of DMD

Three milliliters of blood were collected in EDTA vacutainers from twin pairs where at least one twin was suspected of having DMD. Consented family members were also

sampled, particularly mothers, for carrier status analysis. Genomic DNA was next extracted using a simple desalting protocol as previously described (Miller et al. 1988). DNA was stored at -80^{o}C until further use. Sequencing of genomic DNA was performed using the algorithm previously suggested by our group (Kumar et al. 2020). Briefly, DNA samples were sequentially subjected to a) multiplex PCR (Kumar et al. 2020), b) Multiplex ligation-dependent probe amplification (MLPA), and lastly c) Next generation sequencing analysis (NGS) (Kumar et al. 2020).

Carrier testing of *DMD* gene variants in mothers and female relatives of twins with DMD: Samples from suspected carrier female relatives, including sisters (of twins with one male and one female) and mothers of DMD positive probands were considered for carrier testing through MLPA or Sanger sequencing.

Determination of zygosity of male twin pairs:

The STR multiplex assay designed to amplify 15 tetranucleotide repeat loci and Amelogenin, a gender determining marker, was used to determine zygosity of the male twin pairs (Yang et al. 2006). All sample preparation, PCR amplifications and electrophoresis were carried out using standard protocols. In the event that all the 15 unlinked loci as well as the gender-determining marker, amelogenin, were identical in the PCR-amplified STR analysis, the twins were considered to be monozygotic (MZ). If on the other hand, the STR markers were different within twin pairs, the twins were considered to be dizygotic (DZ).

Data sharing

All twin related data generated in this study has been submitted to the LOVD repository (Supplementary Table 1A & 1B; (Leiden Open Variation Database; www.lovd.nl: LOVD 00311559-583).

Statistics

Results are expressed as means +/-SD. Standard statistical analysis has been used throughout. P-values</= 0.05 are considered to be statistically significant between two groups.

RESULTS

Thirty-three pairs of twins with DMD identified

Over a period of ten years (2010-2022) thirty-three pairs of twins were identified and counselled for DMD at MDCRC. As depicted in Figure 1, the thirty-three twin sets were classified into three general categories: male twins (17 pairs), male/female twins (14 pairs) and a unique set of twins (2 pairs) born following assisted reproductive procedures (IVF; 1 pair male twins and 1 pair male/female twins). The male twins were further categorized into sets including six twin pairs where both males were afflicted with DMD; five sets of twins where one male had DMD while the other was unaffected and five sets where one twin was affected while the other had died at birth. The male/female twin pairs were also subdivided into two groups: thirteen pairs of male/female twins and one pair where the female twin was deceased (Figure 1).

Zygosity and phenotypic characters

We next determined the zygosity of the twin pairs using the STR multiplex assay designed to amplify 15 tetranucleotide repeat loci and Amelogenin, a gender determining marker. Our analysis revealed 4 sets of male monozygotic twins, 22 sets of dizygotic twins (DZ) and 6 sets of twins where the zygosity remained unknown due to the demise of one twin at birth (Figure 2A and Appendix). In order to determine whether zygosity played a role in disease severity, age at onset of symptoms and age at loss of ambulation were measured for MZ and DZ twins. It was noted that while the age of disease onset for DZ twins appeared to be lower (5.52 +/-2 years) compared to MZ twins (6.75+/-0.8years; $p<0.05$), all MZ twins had

lost ambulation by age 10 years, whereas 35% of DZ DMD affected twins remained ambulant at age 10y (Figure 2B and Appendix), suggesting a possible difference in disease severity between MZ and DZ twins in our cohort.

Twenty-two *DMD* gene variants identified in thirty-three twin pairs

Having identified perhaps the largest set of twin pairs with clinically suspected DMD in the world at a single institution (given that only fourteen sets of DMD twins have been reported in the LOVD database thus far, we sought to genetically confirm the presence of *DMD* gene variants in all the males within this cohort, since none of the female twins demonstrated any clinical symptoms of DMD. Of the twenty-two *DMD* gene variants identified by mPCR, MLPA or NGS analysis (see Methods) amongst the thirty-three twin pairs (Table 1), no duplication variants was found and the majority of twin *DMD* gene variants identified (88%) were deletions in two well defined hot spot-regions in the *DMD* gene (exons 3-16 and exons 45-55) (Figure 3).

Almost all the variants identified in the twin DMD samples were ones that had been previously documented (Table 1) in the LOVD database (www.lovd.nl) and in our MDCRC database. However, compared to over 7000 DMD affected singletons in the two databases (Bladen 2015), three rare and novel variants were discovered in our twin cohort including Exon 45-58 del; and two nonsense variants: Exon: 31 (c.4312C>T) and Exon: 41 (c.5899C>T). Additionally, a rare but previously identified Exon 3-16 del variant was also found in our cohort of twins (Table 1). This rare variants has been reported in 21 patients in the LOVD database.

Some *DMD* gene deletion variants appear to be enriched in twins compared with singleton DMD patients

The most commonly encountered *DMD* gene variant in our cohort of thirty-three twin pairs (n=39) and in 1181 singleton DMD patients in the MDCRC database was Exon 45 del. This variant had an equal representation of 15% in both the twin and singleton populations (Table 2; twins n=5, singletons n=169; Figure 4). Unlike Exon 45 del, which appears to be equally distributed in the singleton and twin populations, a number of variants were found to be enriched in DMD singletons compared to DMD twins, these included Exons 45-52del (5-fold enrichment), Exon 45-50 del (2.5 fold) and Exon 49-50 del (3 fold). In contrast, two *DMD* gene deletions appeared to be significantly enriched in the twin DMD population compared to DMD singletons. These included Exon 46-48 del (2.6-fold increase) and Exon 51-54 del (3.1-fold increase). In addition, the presence of the very rare Exon 3-16 del was noted in a pair of DZ twins. This deletion variant was also found in two related singleton DMD patients in the MDCRC database of over 4000 DMD patients (Figure 4).

The symptomatic severity of DMD has been attributed to the "reading frame rule" which stipulates that variants in the *DMD* gene which interrupt the mRNA translational reading frame lead to out-of-frame-variants and loss of dystrophin protein, likely through nonsense mediated mRNA decay (NMD), resulting in severe disease. In contrast, in-frame *DMD* gene variants lead to the milder Beckers Muscular Dystrophy (BMD), which is likely due to the generation of a partially functional dystrophin protein. This reading frame rule has been found to hold true 90% of the time. While 19 of the 22 twin variants were found to be out-of-frame variants leading to severe disease with respect to age of onset and age at loss of ambulation, two in-frame variants were found in the twin DMD cohort (Table 2). Of these, Exon 3-16 del. appeared to be extremely rare, as noted above, and appeared to defy the reading frame rule, being a predicted in-frame variant but behaving phenotypically like an out of frame variant

(Table 2). The second in-frame variant, Exon 45-53 del, demonstrated BMD features with a late age of onset in both twins (23 years) as well as in singletons patients examined (5-30 years). Twins with the Exon 3-7 deletion, despite having an out of frame variant, demonstrated in-frame BMD-like features (Table 2). Our group has previously reported singleton DMD patients with Exon 3-7 del variants exhibiting milder BMD clinical features (Kumar et al. 2020).

Analysis of both male twins affected with DMD

As indicated in Figure 1, we identified six sets of twins where both males were afflicted with DMD. Of these, 4/6 were MZ, while one twin pair was found to be DZ and the zygosity of one pair remained unconfirmed. The age at diagnosis of this category of twins ranged from 7-14 years, with frequent falls being the most common clinical symptom (in 4/6 twin pairs). While all the twin pairs developed clinical symptoms at the same age (within each twin pair), a delay of one week in symptom manifestation was reported in one MZ twin pair as reported by the parents (Table 3A, twins 3A and 3B). At the time of this writing, all four pairs of MZ twins and zygosity- not- known pair had lost ambulation between 9-11 years of age. In each case, ambulation was lost at the same age for each twin pair. Variants within the *DMD* gene were concentrated in the distal hot spot region (Figure 3) for all MZ and zygosity- not- known twins. The single pair of DZ twins (Table 3A; twins 5A and 5B) on the other hand, remain ambulant (current age 11 years) and harbor the rare Exon 3-16 variant in the proximal hotspot region of the *DMD* gene. This rare variant has been noted to be an in-frame variants, which could potentially bestow milder BMD-like features on the DZ twins. This pair of dizygotic twins was found to have a familial history of DMD (Table 3A).

While twin 1A passed away at the age of 19 years, his twin remains alive, but is currently non-ambulant (Table 3A). This age discordance at the time of death between MZ

twins, who have shared a common living environment, must be explored further to provide valuable data on the "nature versus nurture" question with respect to DMD.

Since DMD is an X-linked disease, the carrier status of mothers of the DMD twin patients was determined to establish the inheritance pattern, if any, of the *DMD* gene variant in each case. Of the four MZ twin mothers, three consented to be tested for carrier status. Of these, two were found to harbor normal *DMD* alleles, while the mother of twins (2A and 2B) was found to be a carrier. Even though the DZ twins' mother did not consent to be tested for carrier status, the fact that a cousin in the family had been diagnosed with DMD suggests that the mother was very likely a carrier (Supplementary Table 2).

Analysis of twins with a single male affected with DMD

This category of twins represents the largest in our cohort (n=20 pairs) with all twins being DZ. The age at diagnosis of this category of twins ranged from 5-21 years with frequent falls being the most common first clinical symptom (Table 3B). While most of the twins were either semi-ambulant or non-ambulant (13/20; 65%), five out of twenty (5/20; 25%) remained ambulant, while the ambulation status was not available for two patients (2/20; 10%). Of note, all ambulant twins were under that age of 8 years (Table 3B). All the DMD deletion gene variants in this group of DZ patients were localized to the distal hot spot region of the *DMD* gene (Table 3B, Figure 3) with the largest number of variants involving Exon 45 del. Two rare nonsense point variants were also found in this group of DZ patients: Exon: 31 (c.4312C>T) and Exon: 41 (c.5899C>T). Both these variants appear to be unique to twins. Three of the twenty twins in this category are deceased; they were aged 11, 18 and 20 years. The carrier status of mothers in this category of twins was not clearly examined as 3/20 mothers could not be examined due to inability to obtain consent or for other reasons. Of the remaining 17 mothers, 11 tested normal for variants in the *DMD* gene, while 6 mothers were found to be

carriers (Supplementary Table 2). Additionally, only seven sets of twins in this group had twin sisters above the age of 18, who consented to be tested for carrier status. All of them were found to be negative for the *DMD* gene variants, and hence were deemed carrier negative.

A third category of twins was identified in our cohort in which one of the twins had died at birth and consequently zygosity of this group could not be determined (Figure 1). The ages of the five twins in this group at disease onset was recorded to be 10, 9, 19, 23 and 7 years (Table 3C) with their current ages being 20, 26, 35, 27 and 11 respectively. The first symptoms observed included frequent fall (2 twins), toe walking (1 twin) and calf muscle hypertrophy (2 twins). Ambulation status varied among the twins, with two being ambulant, one semi-ambulant and two non-ambulant. It is therefore not surprising that two of the twins (10A & 12A) in this category harbored the BMD variant Exon 3-7.

Twins born with DMD following assisted reproductive procedures:

Two sets of DZ twins were identified in our cohort of thirty-three twin pairs that were born following *in vitro* fertilization protocols (Figure 1, IVF twins; Table 3D). IVF is an assisted reproductive procedure used primarily to address issues of infertility. With increasing worldwide trends of decreased fertility in the recent past, IVF has gained considerable popularity. The birth of twins with DMD following IVF procedures is an extremely rare phenomenon.

The first case, a 7-year-old male, one of a pair of twin males, was brought to MDCRC with suspected DMD. He showed symptoms of progressive proximal muscle weakness, calf muscle hypertrophy, Gower's sign, difficulty in climbing stairs, frequent falls and toe walking. No other delay in motor milestones was noted. Confirmation of DMD was achieved following DNA variant analysis which revealed a deletion of exons 51-54 in the *DMD* gene. His twin brother remained free of all symptoms and lacked the *DMD* gene variant. Clinical

history and genetic counselling of the patient and his family revealed that the proband was born following IVF treatment. No family history of DMD was found to be associated with the extended family. *DMD* gene carrier testing of the mother revealed that she was not a carrier, hence the occurrence of DMD in this patient was likely sporadic.

The second IVF case involved a 6-year old boy with Gower's sign, difficulty in climbing stairs, frequent falls and toe walking who was brought to MDCRC with a diagnosis of suspected DMD. Molecular analysis confirmed a deletion variant involving exons 49-52 in the *DMD* gene. No family history of DMD was noted. *DMD* gene carrier testing of the mother revealed that she was not a carrier, hence the occurrence of DMD in this patient was also likely sporadic.

DMD in twins appears to be independent of family history of twinning:

Of the thirty-three pairs of twins in our cohort, we found that five pairs of twins had a family history of twinning. In the first case, the mother of MZ DMD twin boys with a del 46-48 *DMD* gene variant, was herself found to be a twin. This mother was found to not be a carrier of the *DMD* gene variant. Her paternal male cousins were also found to be twins (Supplementary Figure 1). In a second case of MZ twins, the father was found to have a twin brother. The mother of this twin pair, who were afflicted with the del 46-55 in the *DMD* gene, was found to be a non-carrier (Supplementary Figure 2). A third pair of DZ twins, where one twin was affected with the *DMD* (del 46-48) gene variant, had a pair of unaffected male/female twin siblings. No other twins were found in the extended family (Supplementary Figure 3). In the fourth twin pair, one twin was DMD affected (del 3-7) while the other had died at birth. This twin pair also had a pair of unaffected twin brothers (Supplementary Figure 4). A fifth pair of twins (male/female, male: DMD del 45-50) was found to have maternal uncles who were twins (Supplementary Figure 5). Our observations have led us to conclude that the

manifestation of DMD in twins appears to be independent of the phenomenon of twinning within the family.

DISCUSSION

In this manuscript we report the analysis of thirty-three pairs of twins with DMD/BMD from a single institution in India. In general, twin births are known to vary widely worldwide from 1-3% of all births (Smits 2011) (Martin et al. 2019) (Esteves-Pereira et al. 2021) and have been steadily increasing at an annual rate of 2% in the United States (Martin et al. 2012). It has been suggested that rising maternal age following the increased use of contraception and infertility procedures have played a role in increasing twin pregnancy rates (Kulkarni 2013).

Reports of DMD twins, especially MZ twins, in the literature have been scarce (Kulkarni 2004 and references therein), and only fourteen sets of twins have been reported in the LOVD database to date from eight countries. These include DZ: 3; MZ: 2 Zygosity unknown: 9 pairs of twins. Our data set of 33 twin pairs has been deposited in the LOVD database and represents the largest contribution for DMD twins in the world from a single institution, and the first from India. Despite the limited number of twin pairs in our study, our observations provide some novel insights on the variant distribution, inheritance patterns, and disease nature of this lethal disease.

Our analysis of DZ versus MZ twins revealed that while the age of DMD disease onset for DZ twins appeared to be lower compared to MZ twins, all the MZ twins had lost ambulation by the age of 10y, whereas 35% of DZ DMD affected twins remained ambulant at age 10y. While this suggests differences in disease severity between MZ and DZ twins, additional study in larger numbers of twin sets will be needed to verify this observation.

Twenty-two deletion variants in the *DMD* gene were identified in our cohort of 33 twin pairs. Interestingly, no *DMD* gene duplication variants were found in our twin cohort. This might reflect the limited number of twins in our study particularly since duplications in the *DMD* gene occur less frequently than deletion variants, and have been reported to occur at a frequency of around 8% (Kumar et al. 2020) in DMD singletons. Upon examining the LOVD database, a twin with an Exon 12 duplication was reported from Saudi Arabia.

1181 singletons of the over 4000 DMD patients in the MDCRC database were found to share 30 of the 33 *DMD* gene deletions identified in our twin cohort. The Exon 45 del variant appeared to be equally common in both twins and singleton groups. In contrast, deletions in exons 46-48 and 51-54 appeared to be enriched in twins compared to singletons, while other deletion variants appeared to be enriched in singleton DMD patients (Exon 45-42 del, Exon 45-50 del and Exon 48-52 del). This specific enrichment of variants in the two populations of DMD patients could not be correlated with phenotypically distinguishable traits. The importance of this observation needs to be understood from the viewpoint that the number of twins considered in this study (n=33) is statistically small.

Over the years, studies on twins have provided valuable information on the genetic basis and nuances of complex traits and disease correlations (Rev in (Boomsma et al. 2002)). Reports of twins with DMD have been scarce in the literature. A recent study with a focus on treatment of DMD with the drug Eteplirsen described a pair of identical twins with a deletion of exon 45-50 in the *DMD* gene (Alfano 2019). It was reported that this pair had lost ambulation around the age of ten, similar to our observations with MZ twins reported here.

It has previously been documented that discordant MZ twins demonstrate different disease outcomes, as has been illustrated in the case of MZ twins with cleft palate and schizophrenia ((Boomsma et al. 2002) and references within). This phenomenon is likely due

to the finding that even though all MZ twins develop from a single zygote, heterokaryotypic MZ co-twins exhibit discordant karyotypes compared to homokaryotypic co-twins (da Silva Francisco 2019). While all four MZ twins in our cohort harbored deletion variants in the distal hot spot of the *DMD* gene (involving exons 45 to 55) and appeared to be concordant with respect to age at onset of disease and age at loss of ambulation, we noted that the age of demise in one set of MZ twins varied by three years. A second MZ twin set is also poised to demonstrate this trend, with the demise of one twin while the other remains alive. Detailed genetic analysis of such MZ twin pairs could yield valuable and insightful details on genetic factors, such as DNA methylation signatures influenced by environmental factors that may contribute to morbidity in the context of a *DMD* gene variant.

Since DMD is an X-linked disease which currently remains incurable, carrier screening of mothers in their reproductive years followed by genetic counselling remains crucial for prevention of this disorder. We analyzed the carrier status of consented mothers of twenty four of the thirty-three twin sets and found that 29.2% were carriers while 70.8% were not. This finding is in contrast to our previous carrier analysis of singleton DMD patients where 43.7% of mothers were found to be carriers (da Silva Francisco 2019). Our data suggest that mothers of DMD singletons are twice as likely to be carriers of this disease compared with mothers of twins; the caveat in this interpretation being that the number of twins considered in the carrier analysis is small and therefore may not be statistically relevant.

Given that our cohort of thirty-three twin pairs, included two sets of twins born following assisted reproductive procedures. Families with a history of DMD who opt for assisted reproductive procedures must mandatorily undergo first-trimester prenatal diagnosis by transabdominal chorionic villus sampling as part of a preimplantation-genetic diagnosis protocol to prevent the propagation of DMD. Our observations have further led us to conclude

that the manifestation of DMD in twins appears to be independent of the phenomenon of twinning within the family.

CONCLUSIONS

Over a period of ten years, thirty-three pairs of genetically confirmed DMD twins were identified at MDCRC, representing the largest set of DMD twins in the world from a single institution. We used this rare opportunity to compare monozygotic (MZ) and dizygotic (DZ) twins with DMD and our results provide evidence that DZ twins on average appear to develop DMD earlier than MZ twins but lose ambulation relatively later. Discordant MZ twins found in our cohort are likely to provide new insights into the nuances of disease susceptibility. An enrichment of specific *DMD* gene variants appearing in twins and the complete lack of duplication variants is intriguing and requires further investigation. Complete lack of DMD symptoms in female twins was noted and a two- fold reduction of carrier status in twin mothers compared to singleton DMD mothers, suggests that the majority of twins in our cohort arose from sporadic *DMD* gene variants. Further studies on larger cohorts of DMD twins would be needed to confirm the observations made in this study.

FOOTNOTE

Just prior to submission of this manuscript, we became aware of three additional sets of twins (one male-male and two male-female) where one male had DMD while the other twin was no more (Supplementary Table 4). Twin 34A has an Exon 46-51 deletion variant, twin 35A has an Exon 49-50 deletion and 36A has an Exon 60 deletion variant in the *DMD* gene. All these variants are not specific to twins. With the addition of these twin sets the total number of twin pairs analyzed in this study increases to 36.

ACKNOWLEDGEMENTS

We would like to thank all the twin DMD patients and their families who shared their clinical histories and donated blood samples to facilitate this study. We also thank the clinicians associated with MDCRC for sending consented patient samples. In addition, we are grateful to Dr. K. Thangaraj (CCMB, Hyderabad, India) and Mr. Jagamohan for assistance with zygosity analysis.

FUNDING

This research did not receive any specific grant from funding agencies in the commercial or not-for-profit sectors.

REFERENCES

1. Alfano *et al.*, "Long-term treatment with eteplirsen in nonambulatory patients with Duchenne muscular dystrophy," *Medicine (Baltimore).*, 2019.
2. Birnkrant *et al.*, "Diagnosis and management of Duchenne muscular dystrophy, part 2: respiratory, cardiac, bone health, and orthopaedic management," *The Lancet Neurology*. 2018.
3. Bladen *et al.*, "The TREAT-NMD DMD global database: Analysis of more than 7,000 duchenne muscular dystrophy mutations," *Hum. Mutat.*, 2015.
4. Boomsma, D, Busjahn, A. and Peltonen, L, "Classical twin studies and beyond," *Nature Reviews Genetics*. 2002.
5. Da Silva Francisco Junior *et al.*, "Pervasive Inter-Individual Variation in Allele-Specific Expression in Monozygotic Twins," *Frontiers in Genetics*, vol. 10. p. 1178, 2019.
6. Deconinck N and Dan, B, "Pathophysiology of Duchenne Muscular Dystrophy: Current Hypotheses," *Pediatric Neurology*. 2007.
7. Esteves-Pereira *et al.*, "Twin pregnancy and perinatal outcomes: Data from 'Birth in Brazil Study,'" *PLoS One*, vol. 16, no. 1, p. e0245152, Jan. 2021.

8. Guiraud S and Davies, K.E. “Pharmacological advances for treatment in Duchenne muscular dystrophy,” *Current Opinion in Pharmacology*. 2017.
9. Johnson, W, Turkheimer, E, Gottesman, I.I. and Bouchard, T.J, “Beyond heritability: Twin studies in behavioral research,” *Curr. Dir. Psychol. Sci.*, 2009.
10. Kulkarni *et al.*, “Fertility Treatments and Multiple Births in the United States,” *N. Engl. J. Med.*, 2013.
11. Kulkarni ML, “Duchenne muscular dystrophy in monozygotic twins.,” *Indian Pediatr*, vol. 41(3), pp. 290–291, 2004.
12. Kumar *et al.*, “Comprehensive genetic analysis of 961 unrelated Duchenne Muscular Dystrophy patients: Focus on diagnosis, prevention and therapeutic possibilities,” *PLoS One*, vol. 15, no. 6, pp. e0232654–e0232654, Jun. 2020.
13. Martin, J.A, Hamilton, B.E, Osterman, M.J.K and Driscoll, A.K, “Births: Final data for 2018,” *Natl. Vital Stat. Reports*, 2019.
14. Martin, J.A., Hamilton, B.E. and Osterman, M.J.K “Three decades of twin births in the United States, 1980-2009.,” *NCHS Data Brief*, 2012.
15. McDonald *et al.*, “Long-term effects of glucocorticoids on function, quality of life, and survival in patients with Duchenne muscular dystrophy: a prospective cohort study,” *Lancet*, 2018.
16. Miller, S.A, Dykes, D.D, and Polesky, H.F, “A simple salting out procedure for extracting DNA from human nucleated cells,” *Nucleic Acids Res.*, 1988.
17. Moat S.J, Bradley, D.M, Salmon, R, Clarke, A. and Hartley, L, “Newborn bloodspot screening for Duchenne Muscular Dystrophy: 21 years experience in Wales (UK),” *Eur. J. Hum. Genet.*, 2013.
18. Muntoni F., Torelli, S. and Ferlini, A, “Dystrophin and mutations: One gene, several proteins, multiple phenotypes,” *Lancet Neurology*. 2003.

19. Rosenberg *et al.*, "Immune-mediated pathology in Duchenne muscular dystrophy," *Science Translational Medicine*. 2015.
20. Smits, J. and Monden, C. "Twinning across the developing world," *PLoS One*, 2011.
21. Sun, C, Shen, L, Zhang, Z. and Xie, X, "Therapeutic strategies for duchenne muscular dystrophy: An update," *Genes (Basel).*, 2020.
22. Tuffery-Giraud *et al.*, "Genotype-phenotype analysis in 2,405 patients with a dystrophinopathy using the UMD-DMD database: A model of nationwide knowledgebase," *Hum. Mutat.*, 2009.
23. Yang, M.J., Tzeng, C.H, Tseng, J.Y. and Huang, C.Y, "Determination of twin zygosity using a commercially available STR analysis of 15 unlinked loci and the gender-determining marker amelogenin – a preliminary report," *Hum. Reprod.*, vol. 21, no. 8, pp. 2175–2179, Aug. 2006.

FIGURE LEGENDS

Figure 1: Identification and classification of 33 DMD affected twin pairs:

Thirty three twin pairs with confirmed DMD were classified as both males affected; one male and one female and twins following IVF procedures. The numbers indicate the number of twin pairs in the category indicated in the color coded key. Testing for carrier status of female twins is indicated.

Figure 2: Determination and comparison of zygosity in 33 DMD affected twin pairs

- A. Identification of MZ, DZ and zygosity unknown twins partitioned into the twin groups: Both males affected, one male affected, one affected one deceased, one male and one female and IVF twins. The numbers indicate the twin pairs for each colour coded zygosity indicated in the Key.
  B. Comparison of disease onset (age at onset) and progression (Age at ambulation loss) in MZ and DZ twins. Mean age in years is indicated +/- standard deviation. Comparison of ages between MZ and DZ twins was done using a standard Student's t-test. P values equal to or less than 0.05 are considered significant.

Figure 3: Twenty-two variants mapped in the *DMD* gene identified from 33 DMD twins. The figure represents the *DMD* gene with 79 exons and functional domains. Twin identified

deletion variants are indicated by a solid line indicating the loss of exons. Out of frame variants are shown in blue and in-frame variants are in yellow. Parentheses represent numbers of twins with the deletion detected. Point variants are indicated by an arrow in the *DMD* gene. All deletions map to two hotspot regions: proximal (exons 1-22) and distal (exons 43-55) of the *DMD* gene.

Figure 4: Enrichment of some *DMD* gene variants in twins with DMD: The percentage of deletions found in twins and singletons was compared. Each percentage was calculated as the ratio of the number of times a given variant was encountered in the DMD twin cohort or in DMD singletons divided by the total number of variants found in the DMD twin cohort or in the DMD singletons. Fold changes were calculated based on this ratio for a given variant. The arrows point to variants enriched in the twin cohort.

Table 1: Age at onset and ambulation status of 33 DMD twin pairs

OF – Out of frame variant; IF – In frame variant; DZ – Dizygotic; MZ - Monozygotic

Table 2: Frequency of variants in DMD twins compared to DMD singletons (MDCRC)

Table 3A: Category of DMD twins where both male twins are affected with DMD

Table 3B: Category of DZ DMD twins where one twin is affected

Table 3C: Category of DMD twins where the zygosity remains unknown

Table 3D: Category of IVF DMD twins

Supplementary Figures and Tables

Supplementary Table 1A and 1B: Twin data on 33 twin pairs submitted from MDCRC to the LOVD database

Supplementary Table 2: Carrier data on mothers of twins in the cohort

Supplementary Table 3: Determination of zygosity in twin sets

Note: One example of a Monozygotic, Dizygotic and Zygosity unknown twin set has been represented. The STR multiplex assay designed to amplify 15 tetranucleotide repeat loci and Amelogenin, a gender determining marker, was used to determine zygosity of the male twin pairs

Supplementary Table 4: Three additional sets of dizygotic twins

Supplementary Figure 1: Maternal twining observed in a family of monozygotic DMD twins

Supplementary Figure 2: Paternal twining observed in a family of monozygotic DMD twins

Supplementary Figure 3: Sibling twins observed in a family of Dizygotic Twin: Case 1

Supplementary Figure 4: Sibling twins observed in a family of Dizygotic Twin: Case 2

Supplementary Figure 5: Maternal uncle twining observed in a family of Dizygotic Twin

LIST OF ABBREVIATIONS

DMD: Duchenne Muscular Dystrophy
DMD gene: Dystrophin gene
MZ: Monozygotic
DZ: Dizygotic
IVF: *In-vitro* fertilization
NGS: Next generation sequencing

Title: Analysis of thirty three pairs of twins with Duchenne Muscular dystrophy (DMD): study from a single center in India

Figure 1: Identification and classification of 35 DMD affected twin pairs

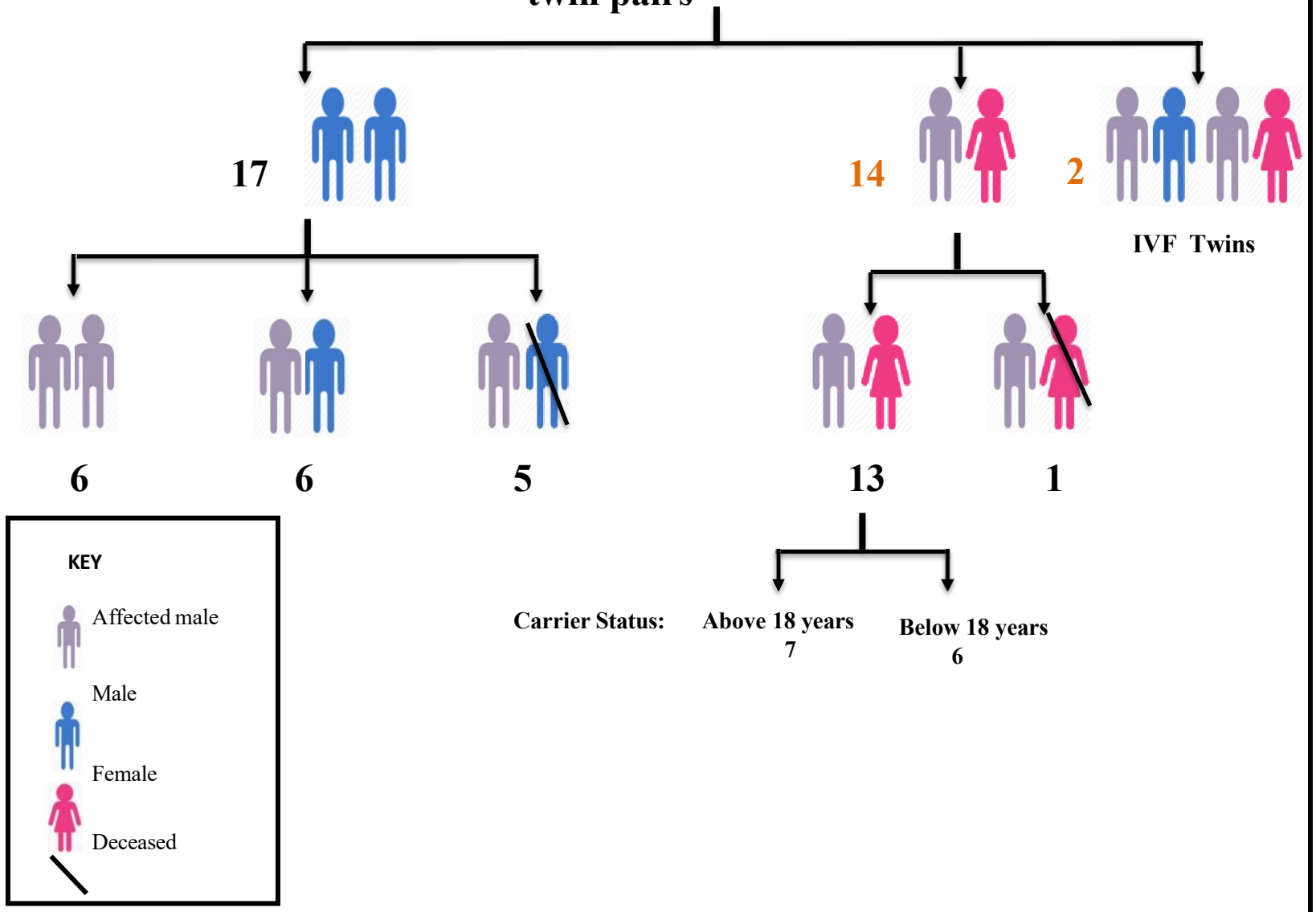


Figure 2A: Determination and comparison of Zygosity in 33 DMD affected twin pairs

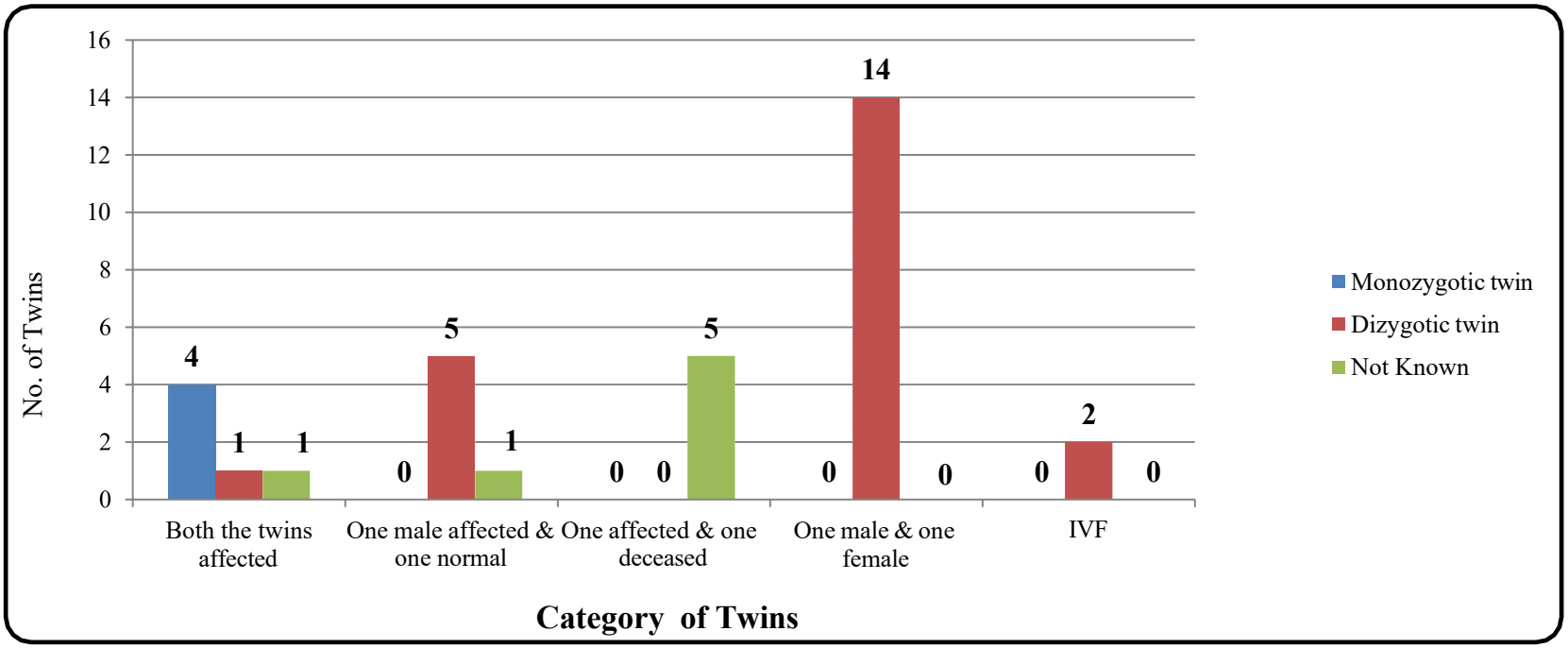


Figure 2B: Comparison of Monozygotic and Dizygotic twins

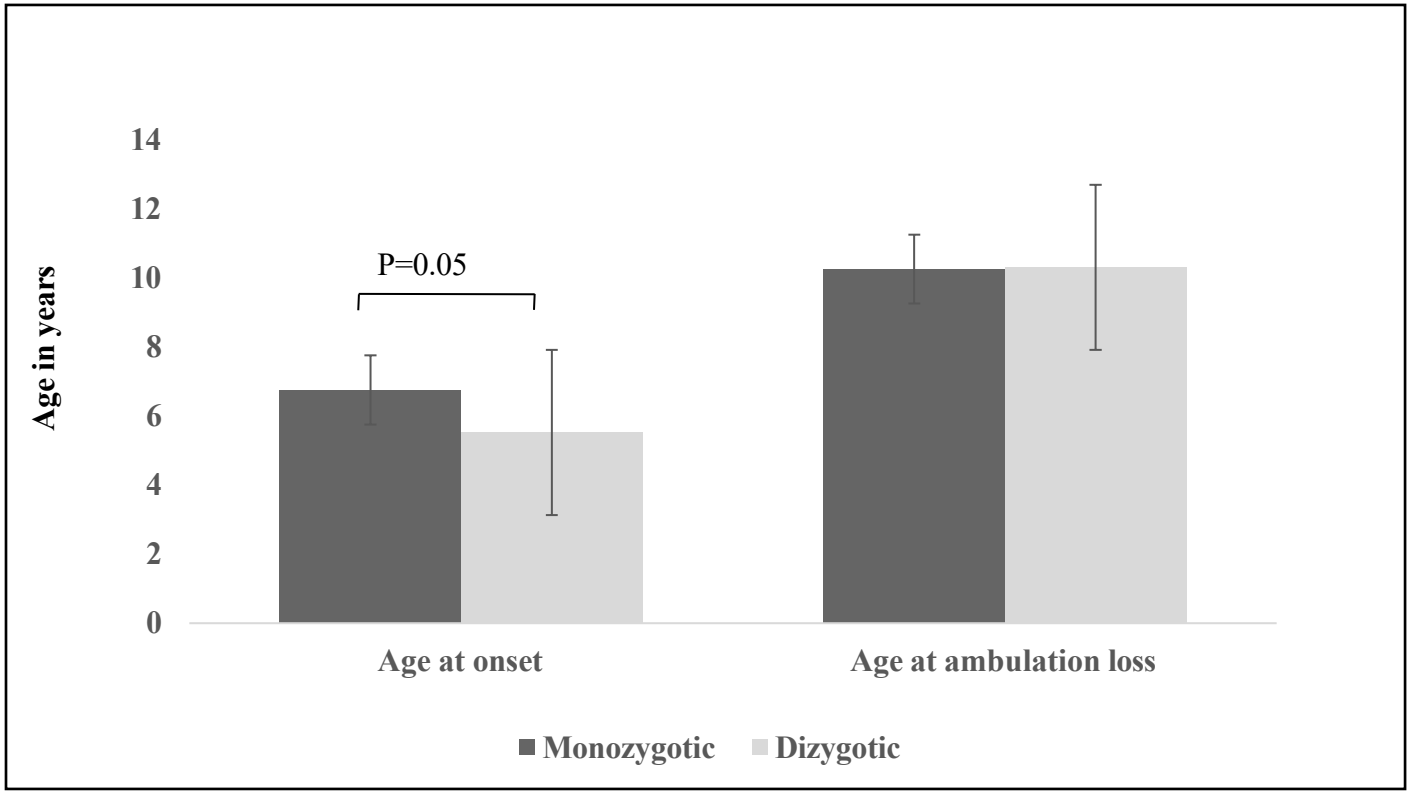


Figure 3: Twenty-Two variants mapped in the *DMD* gene identified from 33 DMD twins

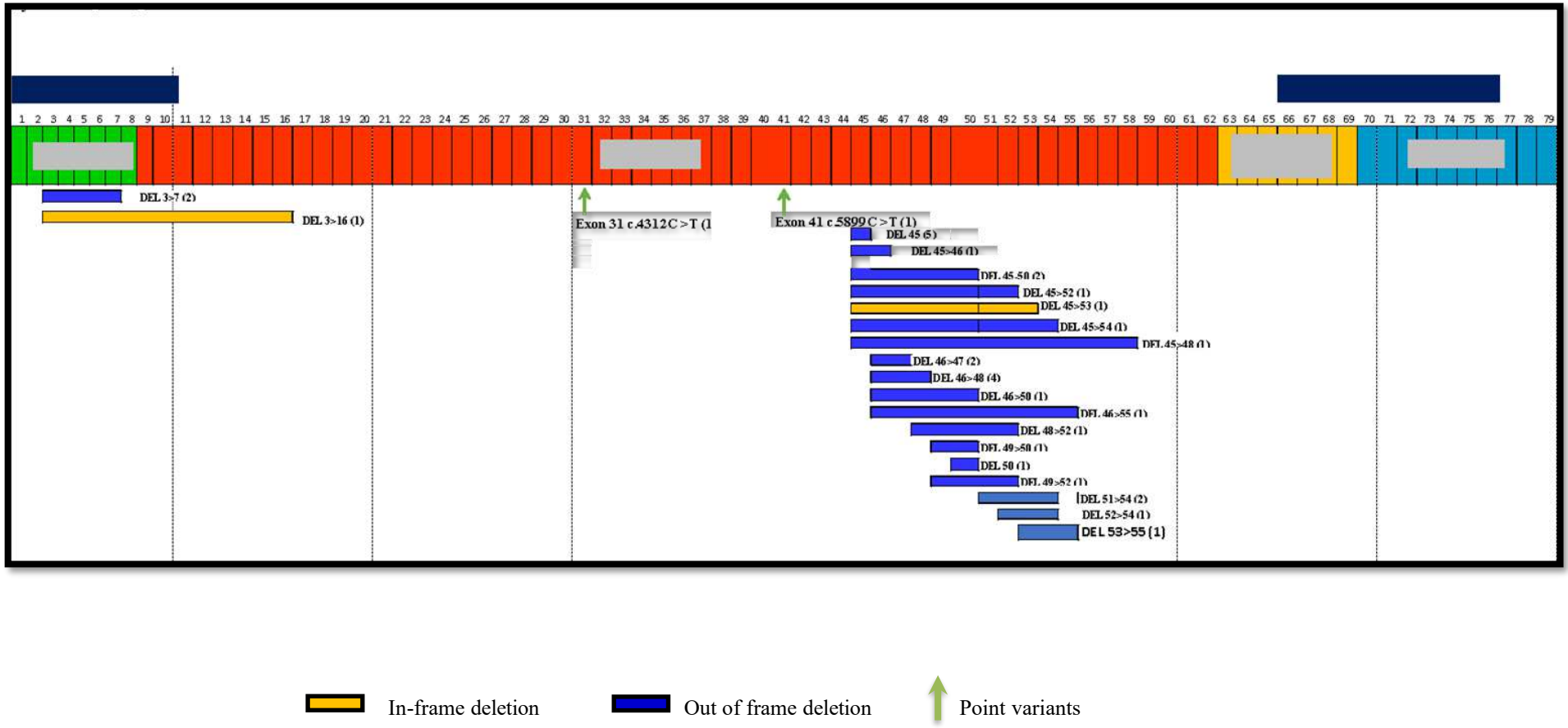
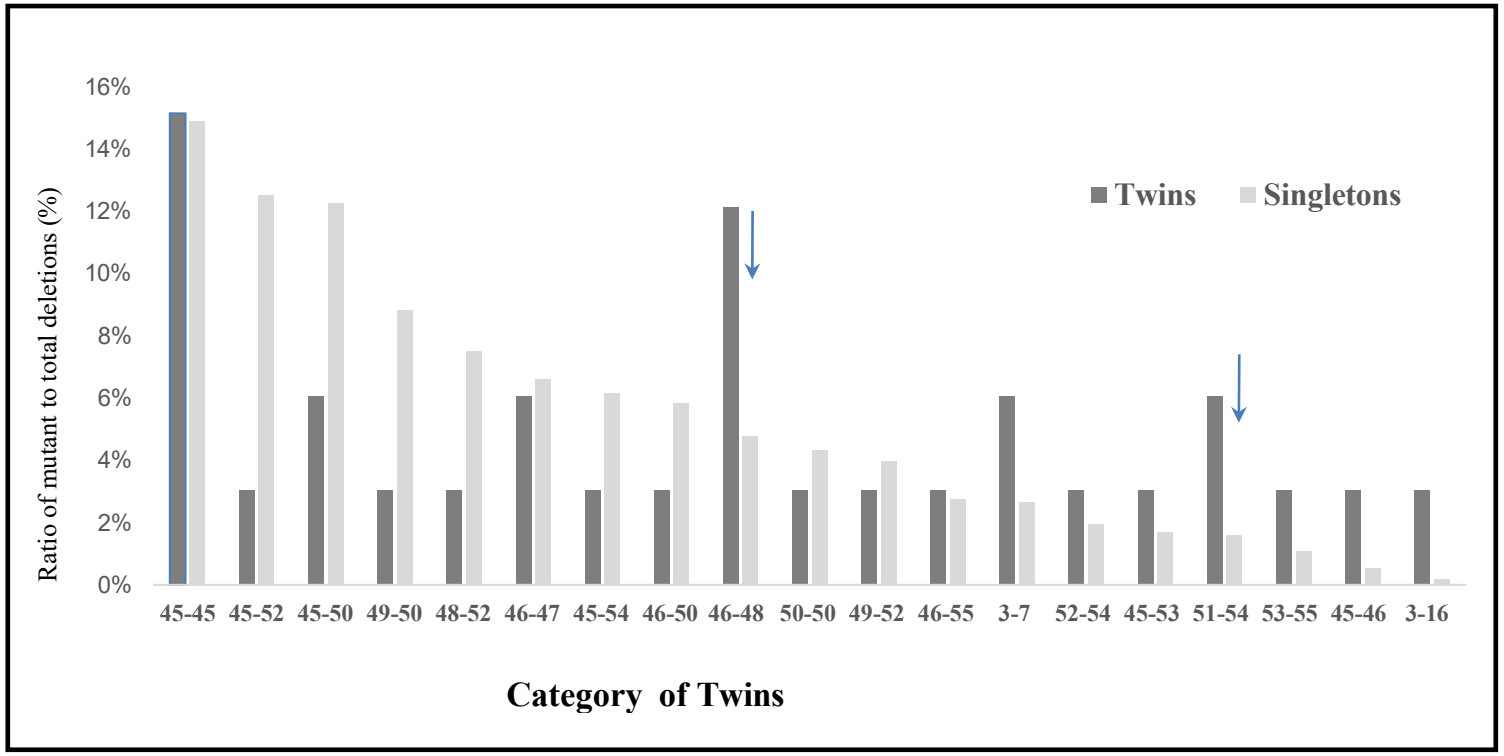


Figure 4: Enrichment of some *DMD* gene variants in twins with DMD



Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TwinManuscriptTables.docx
- SupplementaryFiguresandTables.docx