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Beyond Copy-Number and Coding-Region Analysis: Resolving the Molecularly Unresolved Dystrophinopathy

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Exon-level copy-number analysis followed by sequencing of all 79 *DMD* exons and their flanking intronic sequence achieves a cumulative sensitivity approaching 99%, and no current protocol reaches 100% sensitivity. In clinical series, however, approximately 2–5% of patients with convincing dystrophinopathy remain molecularly unresolved after such testing. This review addresses that residual group. The reasons for failure lie in the architecture of the locus: more than 99% of the 2.22 Mb locus is intronic, dosage-based assays cannot detect balanced rearrangements, and short reads resolve repeat-embedded breakpoints poorly. Published studies identify two main categories — deep intronic splice-altering variants and complex structural variants — but their relative proportions across populations remain uncertain. The evidence required to classify such a variant as causative depends on its class: transcript analysis is effectively indispensable for a novel deep intronic variant, whereas a rearrangement that directly disrupts coding exons may be resolved from breakpoint, phenotype and dystrophin findings. I set out how splicing evidence enters classification under the ClinGen Sequence Variant Interpretation Splicing Subgroup recommendations, a stage at which the same RNA result can inadvertently be counted twice. I also set out how the evidence for a balanced structural variant must be assembled where neither the copy-number nor the splicing scoring framework covers it, and propose a staged approach to the unresolved patient. Resolving the variant is a precondition for assessing genotype-dependent therapeutic eligibility and for variant-specific carrier testing in the family.

Key words: Duchenne muscular dystrophy, Deep intronic variants, RNA splicing, Long-read sequencing, Variant classification

REVIEW ARTICLE

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INTRODUCTION

The European Molecular Genetics Quality Network (EMQN) best-practice guidelines report a cumulative sensitivity approaching 99% for exon-level copy-number analysis followed by sequencing of all 79 *DMD* exons and their flanking intronic sequence [1]. The same guidelines add that no molecular testing protocol currently reaches 100% sensitivity [1]. Most laboratories proceed stepwise, screening for exon-level copy-number change and moving to small-variant analysis only if the copy-number screen is negative [2]; others detect both copy-number change and small variants from a single sequencing run. The scope of the small-variant step varies among centers: Sanger sequencing of all 79 exons with their flanking intronic sequence in some, exome or targeted panel sequencing in others. This standard testing pathway is described in detail elsewhere and is not revisited here [1,2]. In clinical series, approximately 2%–5% of patients still have no molecular diagnosis after such testing [3]. These two percentages are not contradictory: the size of the residual group depends on assay design and on the referral setting. This review concerns the patients who remain unresolved after such testing.

A patient should be regarded as molecularly unresolved when three conditions hold. First, the clinical phenotype is convincing. Where a muscle biopsy has been performed, absent or reduced dystrophin provides strong supporting evidence.

Second, a copy-number assay with validated exon-level resolution across the locus has been negative. Third, sequencing of the coding region with its flanking intronic sequence has been negative. Each condition matters. Without the first, the appropriate next step is to reconsider the diagnosis rather than to pursue rare *DMD* variant classes. Without the second or the third, an apparently unresolved case may reflect incomplete or unvalidated testing rather than a variant class the assays cannot detect. If a copy-number method does not cover every exon, as multiplex PCR does not, or has not been validated at single-exon resolution, it can report a true single-exon deletion as normal [1,2]. If an apparent single-exon deletion was dismissed without independent confirmation, it should be re-examined, since the apparent deletion may conceal a pathogenic small variant, as described below [1]. A negative sequencing result is less informative if the sequencing did not include the intronic sequence flanking each exon. Such cases should be resolved at the assay level before the patient is considered molecularly unresolved.

The first condition is more important than is usually recognized. Other muscular dystrophies can produce a clinically indistinguishable presentation: a man followed for years with a diagnosis of Duchenne muscular dystrophy was found instead to have *FKRP*-related limb-girdle muscular dystrophy when a second muscle biopsy showed normal dystrophin and sequencing identified biallelic *FKRP* variants [4]. Dystrophin immunostaining usually distinguishes dystrophinopathy from these conditions, but not always, since abnormal dystrophin has been described as a secondary phenomenon in sarcoglycanopathy [5]. In three situations — where dystrophin deficiency was sought but not demonstrated, where it is equivocal, or where the clinical and protein findings disagree — the exome or genome data already available should be reanalyzed systematically before rare *DMD* variant classes are pursued. In 101 unsolved neuromuscular families, reanalysis resolved 17; in five of these the causative variant was intronic and lay in a gene other than *DMD* [6].

Leaving the variant unidentified now has greater clinical consequences than in the past, because both mutation-specific therapy and variant-specific cascade testing require a defined variant [1,2]. Zhao and colleagues described a family in which the proband was diagnosed by biopsy but neither multiplex ligation-dependent probe amplification (MLPA) nor exome sequencing identified the variant; carrier testing was therefore unavailable, and a second affected child was subsequently born before combined long-read and RNA sequencing provided the diagnosis [3]. The sections that follow consider first why the assays failed in such a case and then what would be required to identify the variant.

DIAGNOSTIC BLIND SPOTS OF EXON-CENTRIC TESTING

The failure modes follow from the architecture of the locus rather than from poor practice (Table 1). *DMD* spans more than 2.22 Mb, of which over 99% is intronic; the coding sequence of the full-length muscle isoform totals 11,058 bases across 79 exons [7].

Dosage-based assays detect only unbalanced events, regardless of size. A balanced inversion or translocation that disrupts the gene produces no change in copy number, so it is undetectable by MLPA and by array comparative genomic hybridization whether it spans a few kilobases or most of the chromosome. An event that separates *DMD* exons from their promoter without deleting them is equally undetectable by these methods.

Probe-based methods have an artifact of their own. An MLPA kit provides only one probe per exon across the 79 ex-

Table 1. Diagnostic blind spots of exon-centric testing in dystrophinopathy

Blind spot	Mechanism	Variant class missed
Exon-centric capture	More than 99% of the 2.22 Mb locus is intronic, and intronic interrogation is limited to the sequence immediately flanking each exon	Deep intronic variants creating cryptic splice sites, disrupting splicing regulatory elements, or introducing polyadenylation signals
Dosage-based detection	MLPA and array CGH register only unbalanced change	Balanced inversions and translocations; events separating exons from the promoter without deleting them
Probe-binding artifact	A sequence variant beneath a single MLPA probe causes allele dropout by preventing hybridization	Small variants that mimic single-exon deletions, and true single-exon events dismissed as artifact
Short-read alignment	Reads covering a breakpoint inside a repetitive element match many near-identical copies and cannot be uniquely aligned	Structural variant breakpoints; complex rearrangements; intronic repeat expansions

CGH, comparative genomic hybridization; MLPA, multiplex ligation-dependent probe amplification.

ons of the full-length muscle isoform. A sequence variant under a probe-binding site can prevent the probe from hybridizing, and the resulting allele dropout is indistinguishable from a single-exon deletion. The EMQN guidelines therefore require any apparent single-exon deletion, and likewise any apparent single-exon duplication, to be confirmed by an independent method before it is accepted or dismissed [1]. Array comparative genomic hybridization is less susceptible to this artifact, because most exon-level events are covered by several oligonucleotide probes rather than one [1].

Coding-region sequencing reaches only the intronic sequence immediately flanking each exon. Deep intronic variants that create cryptic splice sites, disrupt splicing regulatory elements, or introduce premature polyadenylation signals all lie outside the interval captured by panel and exome assays [3,7].

Short-read data resolve breakpoints within repetitive sequence poorly. Short-read sequencing can infer structural variants from read-pair, split-read, and coverage signals, but that inference requires the reads to be assignable to a unique position in the genome. Breakpoints inside a repetitive element such as a LINE-1 retrotransposon may therefore be missed or poorly resolved, and large intronic repeat expansions may be missed or inaccurately sized. Long-read sequencing largely overcomes this limitation by spanning the repetitive sequence together with its unique flanking regions [3].

A negative standard workup is therefore not strong evidence against dystrophinopathy. The variant classes that the standard workup cannot detect are those that remain possible in an unresolved patient. Where the diagnosis is clinically convincing, such a result is better explained by the limitations of the assays than by a wrong diagnosis.

WHAT IS FOUND IN UNRESOLVED CASES

If the negative result reflects the assays rather than the diagnosis, the question that follows is which variant classes those assays failed to detect. Several studies now answer that question directly.

Waddell and colleagues studied 13 affected males with elevated creatine kinase, from seven families in which MLPA and exome sequencing had failed to yield a diagnosis, and identified a causative *DMD* variant in every family. Two families carried deep intronic single-nucleotide variants causing inclusion of a pseudoexon — an intronic sequence aberrantly spliced into the mature transcript. The remaining five harbored structural rearrangements: an insertion of approximately 118 kb of chromosome 8 sequence into intron 43, and four inversions, the largest spanning 119.8 Mb of the X chromosome [8]. Such an insertion is readily missed by exon-centric assays, which interrogate *DMD* sequence rather than sequence inserted from elsewhere in the genome.

Okubo and colleagues analyzed muscle transcripts from 20 Japanese patients who had dystrophin deficiency on biopsy but no exon variant on standard testing, and detected an abnormal *DMD* transcript in 19 of them. Exonization of intronic sequence accounted for 15 of these; the remainder comprised exon skipping, aberrantly spliced and polyadenylated transcripts, and transcription termination. The genomic causes were intronic single-nucleotide variants, chromosomal rearrangements, and, in one patient, a nucleotide repeat expansion that terminated transcription within intron 62 [9]. Such repeat expansions are not reliably detected by exon-centric testing.

Pan and colleagues independently applied a transcript-led strategy to 13 males with a clinical diagnosis of Duchenne or Becker muscular dystrophy but no definitive molecular result. Muscle RNA sequencing showed aberrant splicing or expression in 12 of them, and targeted reanalysis of DNA then identified the underlying deep intronic variants and complex rearrangements [10]. These two transcript-led studies therefore converged on the same principle: transcript analysis can direct the subsequent genomic search.

The largest series applied targeted long-read sequencing together with muscle RNA sequencing to 50 biopsy-confirmed Chinese patients in whom MLPA and exome sequencing had been negative, and reached a molecular diagnosis in all 50 [3]. Thirty of the 50 patients (60%) carried deep intronic single-nucleotide variants that led to pseudoexon inclusion or exon skipping. In the remaining 20 (40%) the variant was a complex structural rearrangement: 13 inversions, 5 translocations, and 2 insertions or deletions. Twenty-three of the variants (46%) had not previously been recorded in disease databases.

The same series also illustrates mechanisms that conventional testing cannot detect: small intronic inversions that activate pseudoexons, large repeat-rich insertions that introduce a polyadenylation signal and truncate the transcript, and balanced rearrangements that disrupt *DMD* without changing copy number [3].

Across the published unresolved cohorts, two categories recur most often: deep intronic splice-altering variants and complex structural variants. These studies go beyond what the guidelines anticipated. According to the EMQN guidelines, combined copy-number and coding-region analysis misses

only deep and mid-intronic splicing variants, and complex rearrangements are rare [1]. In contrast, the cohorts above suggest that structural variants form a proportion of the residual group comparable to that of deep intronic variants, at least among patients ascertained by a convincing dystrophinopathy phenotype and negative standard testing. Their relative frequencies, however, should not yet be treated as established. The only series large enough to give proportions is single-center, ethnically homogeneous and biopsy-confirmed [3], and ascertainment, referral pattern and population would all be expected to influence which variant classes are found. Both categories are sufficiently common that a workup addressing only one of them will leave a substantial proportion of patients molecularly unresolved.

MATCHING THE TECHNOLOGY TO THE VARIANT CLASS

The available modalities are complementary rather than hierarchical, and the choice follows from which variant class is suspected (Table 2).

Short-read genome sequencing covers intronic sequence and therefore captures deep intronic single-nucleotide variants inaccessible to exome sequencing. It has two limitations. The first is interpretive: intronic variants are numerous, and prediction alone rarely establishes pathogenicity. The second is technical: because of the alignment problem described above, repeat-embedded breakpoints may remain unresolved even when the event is detected.

Long-read sequencing, targeted or genome-wide, directly addresses this alignment limitation. It also detects deep intronic single-nucleotide variants: in the largest series, targeted long-read sequencing identified both the intronic variants and the structural variants [3]. Its distinctive advantage, however, is that it resolves structural variants and repeat expansions at nucleotide resolution and determines phase — whether two variants lie on the same allele — both of which matter for complex rearrangements. In the same series, 13 inversions of 1.9 kb to 95 Mb were mapped to the base pair and confirmed by Sanger sequencing [3]. A targeted assay is generally less costly and less computationally demanding, but it interrogates only the selected region: it cannot identify a causative variant in another gene, a limitation that matters most when the diagnosis itself is in question. Genome-wide long-read sequencing removes that restriction, at correspondingly greater cost. Both forms require genomic DNA of high integrity (a stricter requirement than for MLPA or short-read sequencing) and specialized bioinformatics for alignment and structural variant calling. These requirements, together with cost and limited availability outside specialized centers, remain obstacles to routine clinical adoption [3].

Optical genome mapping detects large structural rearrangements, including balanced inversions and translocations, at a resolution substantially higher than that of conventional karyotyping. A single case illustrates what it adds beyond karyotyping. A boy with clinically diagnosed Duchenne muscular dystrophy and negative standard testing was found on karyotyping to have a paracentric X-chromosomal inversion, but karyotyping could not determine whether the inversion involved *DMD*. Optical genome mapping localized the proximal breakpoint to *DMD* intron 41 and the distal breakpoint to an intergenic region 18.9 Mb away, so that the inversion splits

Table 2. Contribution of each modality after a negative standard workup

Modality	Resolves	Limitations	Sample requirement
Short-read genome sequencing	Deep intronic single-nucleotide variants; many structural variants	Repeat-embedded breakpoints at nucleotide resolution; functional consequence	Genomic DNA (blood)
Long-read sequencing (targeted or genome-wide)	Deep intronic single-nucleotide variants; greatest advantage for repeat-mediated, balanced, or complex rearrangements, repeat expansions, breakpoint resolution, and phasing	Functional consequence; a targeted assay interrogates only the selected region; high-integrity DNA, cost, limited availability, and bioinformatic demands	High-molecular-weight genomic DNA
Optical genome mapping	Large structural rearrangements, including balanced inversions and translocations	Single-nucleotide variants; functional consequence	Ultra-high-molecular-weight DNA
RNA sequencing / RT-PCR	Pseudoexon inclusion, exon skipping, intron retention, transcription termination	The underlying genomic lesion	Muscle RNA; blood not a reliable substitute
Dystrophin immunohistochemistry/ western blot	Pattern and extent of protein loss by domain	The genomic lesion	Muscle biopsy

the transcription unit and separates the exons beyond intron 41 from the promoter [11]. Optical genome mapping does not detect single-nucleotide variants, however, and therefore addresses only the structural category.

The three genomic methods above identify the variant; whether it alters the transcript is a separate question. Transcript analysis — RNA sequencing or targeted reverse transcription PCR (RT-PCR) — answers that question directly, showing pseudoexon inclusion, exon skipping, intron retention, or transcription termination. Dystrophin immunohistochemistry and western blot answer a different question. They show how much protein is lost and from which domains, but not what lesion caused the loss. Their contribution to the evidence is discussed below. For transcript analysis, muscle is the reference tissue, and blood is not a reliable substitute: only low-level illegitimate *DMD* transcription occurs in lymphocytes, and the splicing pattern there may differ from that in muscle [1]. Muscle, however, is not always available. Molecular testing now usually precedes biopsy, so some unresolved patients have never had one, and tissue stored years earlier may no longer yield usable RNA. Where no suitable material exists, two options remain. The first is a repeat biopsy taken specifically for transcript analysis; this may be considered once a candidate variant has been identified, but is considerably more difficult to justify as a speculative procedure in a child. The second is a surrogate tissue; several have been explored, but none has been validated against skeletal muscle [1].

RNA AS FUNCTIONAL EVIDENCE: WHEN IS IT INDISPENSABLE?

Identifying a candidate variant does not determine how much evidence is then needed to classify it as causative. In the published unresolved cohorts, DNA and RNA analyses were routinely performed together [3,8–10]. As a universal requirement, however, this does not fit every variant class: the need for transcript evidence depends on how much the genomic finding establishes by itself (Table 3).

A novel deep intronic single-nucleotide variant provides limited evidence on its own. A single base has changed within an intron that may exceed 100 kb; the same intron also contains many rare variants with no functional effect; and in silico tools predict an effect on splicing rather than demonstrate one. Transcript evidence supplies what the genomic position alone cannot establish: it shows the abnormal mRNA. Without transcript evidence, such a variant will usually remain of uncertain significance. For this variant class, RNA analysis is therefore effectively indispensable. The published series show this in practice: the genomic result alone did not establish the consequence, and transcript analysis was needed to do so [3,8–10].

A structural variant that does not cut the gene in two presents a similar problem. An inversion or an insertion that lies entirely within one intron leaves the promoter and all 79 exons in their normal order; a rearrangement whose breakpoints both lie outside the gene leaves it untouched. Such a variant is suspicious because of where it is, but its position does not show whether it changes the transcript: an intronic rearrangement may do nothing, or it may create a new splice site or a premature polyadenylation signal, and the DNA sequence alone cannot tell which. Transcript analysis is therefore usually decisive.

A rearrangement that directly disrupts coding exons, or separates them from the promoter, is itself direct evidence of gene disruption: the genomic architecture accounts for the absence of dystrophin without further inference. In this situation three findings may together be sufficient: breakpoints characterized at nucleotide level, concordance with the clinical phenotype, and a compatible pattern of dystrophin loss on protein stud-

Table 3. Evidentiary requirement according to variant class

Variant class	What the genomic finding establishes on its own	Role of transcript evidence
Novel deep intronic single-nucleotide variant predicted to create a cryptic splice site or activate a pseudoexon	Limited: the genomic position alone does not establish a functional effect, the interval contains many rare variants with no functional effect, and prediction is not demonstration	Effectively indispensable; without it the variant will usually remain of uncertain significance
Structural variant that does not cut the gene in two: inversion or insertion lying entirely within one intron, or a rearrangement whose breakpoints lie outside the gene	Little on its own: the variant is suspicious because of its position, but position does not show whether the transcript is changed	Usually decisive; without it the variant remains a candidate
Rearrangement directly disrupting coding exons, or separating them from the promoter	Direct: disruption of the coding exons, or their separation from the promoter, is in itself strong evidence of loss of function	Supportive rather than obligatory, where breakpoints, phenotype and dystrophin findings concur

ies. Transcript analysis is then supportive rather than obligatory. Where no suitable muscle RNA remains, insisting on it would only delay the diagnosis.

FROM DETECTION TO CLASSIFICATION

Classification is the step most often left implicit in reports of novel non-coding *DMD* variants. The original ACMG/AMP framework gives limited guidance on how predicted and experimentally observed splicing effects should be combined; the ClinGen Sequence Variant Interpretation (SVI) Splicing Subgroup has since published recommendations for how each should be weighted within that framework [12]. Two points are of particular relevance to a non-coding *DMD* candidate variant.

The first is that RNA evidence modifies the strength of PVS1 rather than entering as generic functional evidence. Where the observed aberrant transcript causes loss of function — by introducing a premature termination codon, or by removing clinically relevant protein sequence — the subgroup proposes that this evidence be captured by PVS1_Strength, a graded form of the loss-of-function criterion. The criterion is further annotated, for example as PVS1_Strength (RNA), to mark a call based on an assay rather than on prediction alone.

The second is that the same splicing evidence should not be counted again elsewhere. PS3 and BS3, the functional-evidence criteria, are reserved for well-established assays that measure an effect which the splicing assay itself does not capture. The in silico prediction that raised the suspicion is replaced by the experimentally derived weight rather than counted in addition to it. The evidence is overstated when a report describes an RNA result and then also applies a general functional criterion, or when it retains the in silico prediction code alongside the RNA-derived weight. Because later decisions are based on the classification rather than on the raw result, double-counting can affect downstream clinical interpretation.

Structural variants are less fully addressed by these recommendations. The ACMG/ClinGen technical standards score copy-number gains and losses [13], and the ClinGen splicing recommendations score splicing evidence. A balanced inversion or translocation changes no dosage, and it need not act through splicing, so neither document offers a framework for scoring it.

Where no framework applies, the evidence has to be assembled case by case, and the elements are the same as those set out in the previous section (Table 3). The genomic finding is considered first. A rearrangement that breaks the coding sequence, or separates the coding exons from the promoter, is strong evidence by itself; a rearrangement confined to one intron, or lying outside the gene, is not sufficient evidence on its own. The transcript result then supplies what the genomic finding lacks, and how much it is needed depends on that finding. Finally, the predicted consequence is compared with the dystrophin and clinical findings. The published cases show the range of practice: in the series of Waddell and colleagues, structural variants were confirmed by transcript analysis of muscle RNA [8], whereas the balanced inversion described above, which separates the exons beyond intron 41 from the promoter, was accepted as the diagnosis on the breakpoint and the phenotype alone, without transcript or protein studies [11].

The last of these elements, concordance with the dystrophin findings, applies to every variant class and is considered in more detail here. Dystrophin immunostaining shows how much protein is lost and from which domains; an aberrant transcript predicts what that pattern should be. If the transcript carries a premature termination codon and is degraded by nonsense-mediated decay, little protein is made, and staining for every domain should be uniformly absent or markedly reduced. If the transcript escapes decay and a truncated protein is made, staining should be lost only for the domains beyond the truncation point. In a patient who has already had a muscle biopsy, the prediction can be checked against the existing immunostaining without any further investigation. Agreement supports the candidate variant; disagreement means that the candidate should be re-examined.

CLINICAL CONSEQUENCES OF RESOLVING THE VARIANT

Molecular diagnosis is now a requirement for eligibility for several disease-modifying approaches: exon-skipping agents are prescribed according to exon amenability, and gene-transfer therapy requires a confirmed *DMD* mutation. An unresolved patient is therefore ineligible for the agents that require a defined mutation, and exon amenability cannot be assessed. Individual indications and age restrictions change over time and are not reviewed here. Beyond the approved agents, deep intronic variants that activate pseudoexons may themselves become targets for individualized antisense oligonucleotides designed to suppress the aberrant exon inclusion [14,15]. Such treatment is developmental rather than established, but defining the variant increasingly has therapeutic as well as di-

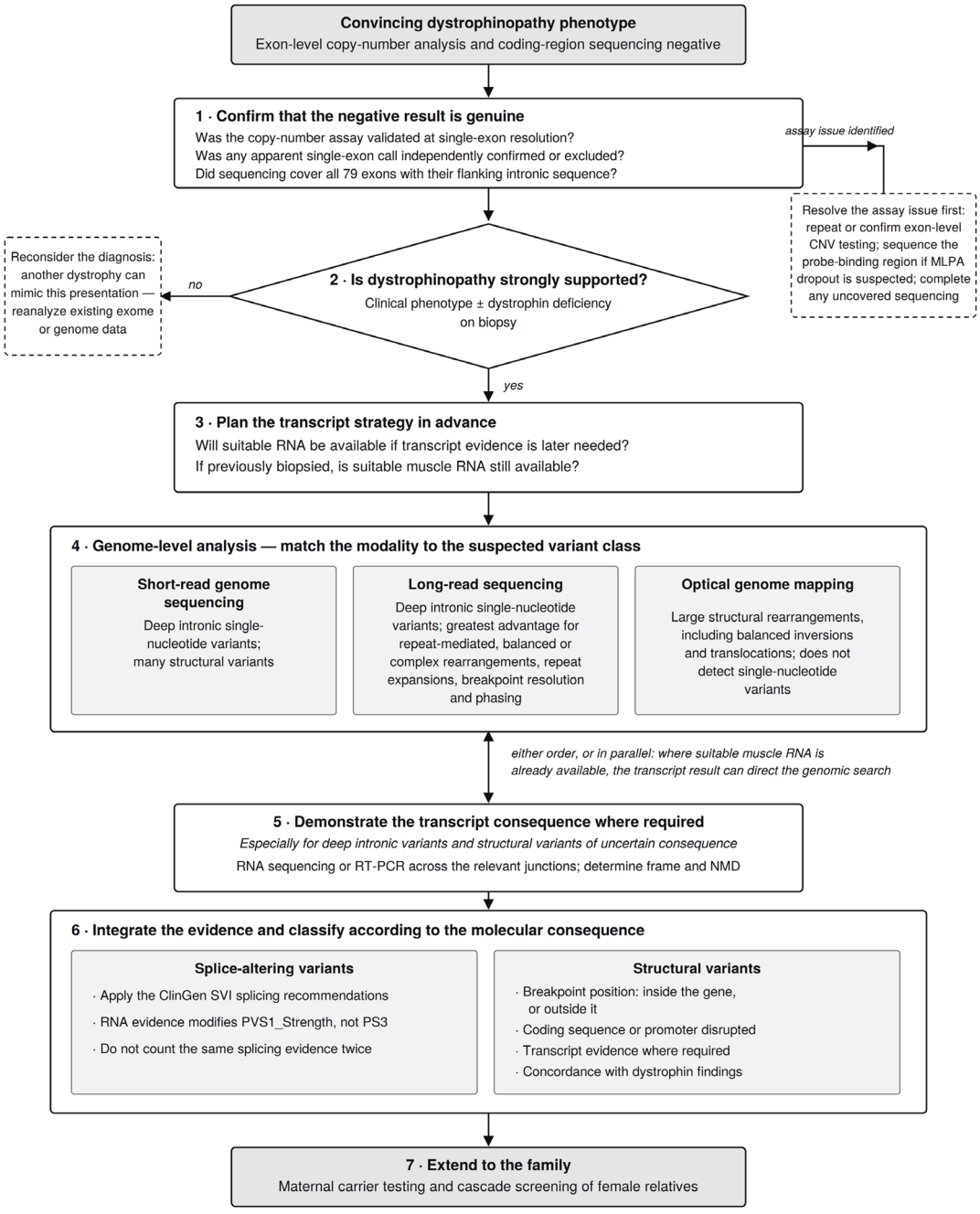


Fig. 1. Staged approach to the patient with a convincing dystrophinopathy phenotype in whom exon-level copy-number analysis and coding-region sequencing are negative. Steps 1 and 2 establish that the negative result reflects a variant the assays cannot detect rather than incomplete testing, and that dystrophinopathy remains the diagnosis under consideration; an assay issue at Step 1, or an unsupported diagnosis at Step 2, diverts the patient from this pathway (dashed boxes). Dystrophin deficiency on biopsy is supporting evidence at Step 2 rather than a prerequisite, since molecular testing is now usually requested before biopsy. Step 2 is needed because another muscular dystrophy can produce the same clinical presentation. Step 3 settles whether suitable RNA will be obtainable before a candidate variant emerges. Steps 4 and 5 are drawn in sequence but are not fixed in that order: where suitable muscle RNA is already available the transcript result can be obtained first and used to direct the genomic search, and the two analyses are often performed in parallel. Step 5 is conditional: it is needed where the genomic finding does not itself establish the consequence — usually for deep intronic variants and for structural variants of uncertain effect. Step 6 assembles the evidence and classifies it; the coding rules for splicing evidence, and the elements of evidence required for a structural variant, are given in the text. Step 7 extends the result to the family. NMD, nonsense-mediated decay; SVI, Sequence Variant Interpretation.

agnostic consequences.

Variant-specific carrier and cascade testing requires a defined familial variant; without one, only indirect approaches such as haplotype analysis remain, and these are feasible in only a few families [1]. Once the variant is defined, maternal carrier testing and cascade screening of female relatives become possible, allowing appropriate cardiac surveillance and informed reproductive counseling [1,16].

A POST-NEGATIVE APPROACH AND RESEARCH GAPS

Fig. 1 summarizes a staged approach. The first priority is to confirm that the negative result is genuine and that dystrophinopathy remains strongly supported; a patient whose testing was incomplete should be retested rather than investigated for rare variant classes. The availability of suitable RNA is determined in advance, so that transcript analysis remains possible when a candidate variant emerges. The genomic modality is then chosen according to the suspected variant class. Classification follows from the molecular consequence, and family testing proceeds once the variant is defined. Similar stepwise approaches incorporating RNA sequencing have recently been proposed [17].

Fig. 1 places genomic analysis before transcript analysis, but the order is not fixed: either analysis may be performed first. Where muscle RNA is already available, starting from the transcript is efficient: the aberrant splicing event defines the genomic region to be examined, and the search becomes targeted rather than exploratory. Both of the transcript-led studies cited above began in this way [9,10]. Where no muscle is available, or where a structural variant is suspected from the outset, the genomic analysis is the better starting point. In practice, the order depends on which material and which platforms are available, and the two analyses are often performed in parallel rather than in sequence.

Three questions remain open. The composition of the residual group in other populations is unknown: the published cohorts are few, and population differences are largely unexplored. Long-read sequencing and optical genome mapping have not been compared directly in this setting. The cost-effectiveness of a combined genomic and transcriptomic workup has not been formally examined. Leaving the case unsolved also has a cost: in the family described in the Introduction, a second affected child was born before the diagnosis was completed.

CONCLUSIONS

In the cohorts published so far — small, mostly biopsy-confirmed, and drawn from single populations — deep intronic splice-altering variants and complex structural variants account for most molecularly unresolved dystrophinopathies. It is not yet established whether the same distribution applies more widely, since cohort selection and population differences would both be expected to affect it. Short-read or long-read genome sequencing, with optical genome mapping for structural variants, can identify these variants, but pathogenicity must be established by evidence appropriate to the variant class. For a novel deep intronic variant, transcript evidence is effectively indispensable. For a structural variant that directly disrupts coding exons or separates them from the promoter, transcript evidence may be unnecessary if the breakpoint, phenotype and dystrophin findings concur. The remaining challenges are to define the composition of the residual group in other populations, to establish how long-read sequencing and optical genome mapping compare in this setting, and to establish the cost-effectiveness of this combined genomic and transcriptomic workup.

NOTES

Conflicts of interest

The author declares no conflicts of interest.

Use of artificial intelligence

During the preparation and revision of this manuscript, the author used Claude (Anthropic) to translate the author's draft into English and edit it, to draft revisions under the author's direction, including the response to reviewers, to render Figure 1 from the author's design, to help identify and verify references, and to format the manuscript according to the journal's instructions. The author decided the scientific content and the final wording, reviewed every passage prepared with the tool, verified all references against their original sources, and takes full responsibility for the content of the publication.

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