Introduction

Duchenne muscular dystrophy (DMD, OMIM 310200) is one of the more common and well-known genetic disorders, affecting one in 3,500 live male births. It is the commonest and most significant of the muscular dystrophies. The disease is named after Duchenne de Boulogne who described the disease in a series of papers in the 1860s, though the disease was described before that by Meryon in England and others elsewhere, including perhaps the ancient Egyptians. DMD is an X-linked recessive disease, meaning that it mostly affects boys. The disease, like all muscular dystrophies, results from degeneration and loss of muscle fibres. The natural history of DMD includes the affected boys typically becoming wheelchair bound by age 12, though the advent of steroid therapy and advanced ventilatory support has changed this, significantly improving quality of life and life expectancy of the affected boys.

The DMD gene (DMD) was the first major disease gene found using the then ‘new genetics’.

Current Molecular Diagnosis of DMD/BMD

The most common molecular defect in the DMD gene,
accounting for approximately 65% of cases of DMD (and 85% of cases of BMD), is deletion of one or more exons. Duplication of one or more exons accounts for another 6-10% of cases of both DMD and BMD, while the majority of the remaining cases are due to point mutations, small insertions/deletions or splice site changes. Complex rearrangements and deep intronic changes account for approximately 2% of DMD cases.

The minimum level of diagnostic testing that should be undertaken is a screen that detects the majority of exonic deletions. The most basic method still in regular use involves multiplex PCR of the exons known to be most commonly deleted. This method was first published by Chamberlain et al. in 1988 and subsequently further developed by a number of groups to the point where two multiplex sets covering the two mutation hotspots could detect ~98% of all deletions. The advantage of this method is its relative simplicity; however it does not detect duplications, does not characterise all deletion breakpoints, and cannot be used for carrier testing of females.

Newer methods involving quantitative analysis of all exons of the gene have brought about an improvement in mutation detection rate, as they will detect all exon scale deletions as well as duplications. They also fully delineate the exon boundaries of detected mutations, and are able to detect mutations in carrier females. Of the quantitative methods available, multiplex ligation-dependent probe amplification (MLPA – a commercial kit developed by MRC-Holland) is now the most widely used. An important point to note is that with both methods described so far, any apparent mutation indicated by an abnormal reading from a single probe must be confirmed by an alternative method, to account for the possibility of a single nucleotide polymorphism (SNP) under a probe or primer binding site.

A more recent development in quantitative analysis is the use of oligonucleotide-based array comparative genomic hybridisation (array-CGH). This method analyses copy number variation across the entire gene, including intronic and 3' and 5' flanking regions, which has the added advantages of detecting complex rearrangements and large scale intronic alterations and delineating mutation break-points much more closely. The high density of probes (probe intervals are in the order of 144 bp across the entire gene) also means that most mutations will be detected by multiple probes, thereby controlling for the possibility of false positives due to SNPs.

If no deletion or duplication is detected, then, in the case of DMD patients, full sequence analysis should be undertaken. Sequencing can be carried out on either genomic DNA or muscle-derived cDNA. Analysis of genomic DNA has the advantage that it does not require the patient to undergo a muscle biopsy; however because of the large number of separate amplicons required to cover all 79 exons it requires a high level of laboratory automation to be viable, though such methods have been described for some time. Analysis of genomic DNA will not detect mutations in the 2% of cases with complex rearrangements or deep intronic changes. Analysis of muscle RNA therefore has a slightly higher sensitivity, and is more amenable to laboratories with less automation, due to the much more manageable number of fragments (~24); however the requirement for a muscle biopsy is a drawback.

It should be noted that sequence analysis in BMD patients is of limited value, at least from genomic DNA. In one study of 23 non deletion/duplication patients, only three had variants, and these were of uncertain significance.

To summarise, the optimum molecular testing strategy for DMD, current best practice, that best balances technical and patient considerations is an initial screen, preferably quantitative, to detect deletions/duplications, followed by full sequence analysis from genomic DNA. If this is still negative, a muscle biopsy should be performed to enable protein studies and cDNA analysis if warranted. For BMD, if the deletion/duplication screen is negative the next step is muscle biopsy.

Across Australia, the Australasian Neuromuscular Network (ANN) (http://www.ann.org.au) has been established to provide co-ordinated molecular and pathological diagnosis for DMD along with all other neuromuscular disorders.

Experimental Treatments being Investigated for DMD

The size and distribution of expression of the dystrophin gene have posed challenges for the development of therapies for DMD. Cell replacement studies appeared so promising in mouse models, but resulted in disappointing failure when applied to boys with Duchenne. The most recent gene replacement study, using an rAAV delivered dystrophin transgene, resulted in low level, transient dystrophin expression and cell-mediated immune responses.

The lack of success in dystrophin gene replacement in DMD prompted the investigation of ready-to-use and indirect interventions that: a) promote muscle regeneration; b) protect muscle from oxidative damage; c) repair or protect membranes; d) alter protein turn-over; e) suppress inflammatory pathways; and f) suppress atrophy pathways. However, apart from corticosteroids, no other validated pharmacological therapies for DMD have been available, and none of these experimental strategies address...
the primary aetiology of DMD: the absence of functional dystrophin. Despite this limitation, treatments that confer any benefit in DMD, given the relentlessly progressive nature of the disease, are better than doing nothing, and may have additional utility in combination with ‘molecular’ therapeutics that do restore the dystrophin associated complex and a degree of sarcolemmal integrity. 

Although dystrophin restoration in DMD muscle has long been the primary aim, the application of small molecules to up-regulate the dystrophin homologue utrophin aroused interest, not least because unlike other molecular therapeutics under development, this approach is not restricted by the nature of the disease-causing dystrophin gene lesion. However, phase 1 studies on BMN-195 mediated up-regulation of utrophin were suspended due to ‘pharmaceutical and pharmacokinetic challenges’. In addition, utrophin over-expression failed to protect dystrophic (mdx) mouse muscle from exercise-associated injury, and data suggest that full-length utrophin cannot anchor nNOS to the sarcolemma.36

The best outcomes for DMD patients are likely to result from the early application of treatments that restore expression of the various tissue-specific dystrophin isoforms, under appropriate endogenous control. To date, both nonsense mutation read-through and induced exon skipping have shown potential to deliver this outcome. Nonsense mutations cause about 10–15% of DMD cases. Therapies that induce ribosomal read-through of premature termination codons allow production of a full-length functional protein, and both aminoglycosides and Ataluren (PTC124) have undergone clinical evaluation.52 The Ataluren trial failed to meet primary and secondary endpoints but the data is undergoing re-evaluation. Aminoglycoside treatment resulted in dystrophin expression and some functional improvements.38

One of the more promising approaches to therapy for DMD is antisense oligonucleotide-induced exon skipping, where the cellular machinery is fooled into by-passing an exon containing a disease-causing point mutation or altering a deletion or duplication, such that a null mutation is no longer generated and DMD is converted to a milder BMD phenotype.39,40 Theoretically, 83% of Duchenne patients could be treated with anti-sense oligonucleotides.41 Unequivocal, localised dystrophin expression in DMD patient muscle was induced by the removal of exon 51, mediated by direct intramuscular injection of antisense oligomers: PRO0051 (2’-O-methyl modified bases on a phosphorothioate backbone)32 and AVI-4658 (phosphorodiamidate morpholino oligomer).43 The first report of systemic administration of PRO051 has shown wide-spread, low level dystrophin expression, with limited side effects.44 While results from these trials are certainly encouraging, we should remain mindful of the many obstacles ahead and, that for many families, timely implementation of these therapies is an imperative. Antisense oligomer induced exon skipping demands tailored therapies for different mutations. This is predicated upon accurate genetic diagnosis of young patients at an early stage in the disease, as the benefit will be greatest before substantial muscle is lost.45 It is necessary that we consider preclinical development of many different therapeutic compounds for DMD, and deliver exon skipping as a personalised genetic medicine. Clinical development of a compound that will only benefit a small sub-population (in some cases a single individual) will require fundamental shifts in drug manufacture, evaluation, validation, approval and supply.

**Population Screening for DMD**

As stated above, best practice therapy for DMD will require early identification of affected boys, to allow implementation of treatment before muscle tissue is irreversibly lost. This may also include steroid therapy where there is some evidence that early intervention produces highly beneficial results.36,47 One problem in detecting boys with DMD is that due to the mathematics of X-linked genetically lethal diseases48,49 many have no previous family history and therefore appear as sporadic cases with no warning (33% of affected boys and 33% of mothers have de novo mutations).49 Many suggestions have been made as to how to develop ways of identifying affected boys early, including screening boys with developmental delay.50 However, all such attempts have failed to move the time of diagnosis earlier, with the average age of diagnosis, in one of the few studies published, being 4 years and 10 months with a range from 1 year and 4 months to 8 years and 3 months.31 The experience elsewhere is similar, including current experience in Western Australia (unpublished data). The conclusion from published work is that the only way to successfully identify DMD patients early is through population screening.51,52

Population screening is currently only performed in a few places in the world. One of the best published programs is that in Wales, which has been running successfully for 20 years and is performed by newborn screening for Duchenne, using high serum creatine kinase levels as the screening tool.53,54 Newborn screening detects the first affected boy born in a family shortly after birth. It could be argued as to whether this is too early and population screening should be offered at a slightly later age, but newborn screening does seem to work,54 and is being investigated, along with programs aimed at older stages, by more and more jurisdictions; for example, parts of the USA.55,56 Implementation of population screening programs for DMD would change the landscape of best practice diagnosis, but still rely on the same diagnostic tools and allow early intervention with therapy.
Another major effect of early population screening for Duchenne is that it can allow early genetic counselling and the opportunity to avoid secondary cases in the one sibship or the extended family and offers the possibility of prevention of a significant percentage of DMD cases, whereas this is not possible when early screening is not in place, with the result being multiple affected boys in families with no previous family history.

It has also been suggested over many years, that diagnosis of DMD should focus on diagnosing women who are carriers of DMD, before they are identified through having affected boys. This would be a radical departure from current practice of population screening for DMD, but be more in line with current practice of screening for carriers of other common recessive diseases such as cystic fibrosis.

Conclusion
Molecular diagnosis for DMD is complicated by the large size of the gene and the multiple different mutation types. But an optimum molecular testing strategy and best practice guidelines have been established. The current major interest in DMD is that finally, after more than 20 years of trying since the DMD gene was identified, experimental treatments are looking more promising, with multiple experimental treatments recently or currently in clinical trials. One of the most promising appears to be anti-sense oligonucleotide induced exon-skipping. If any of the treatments in clinical trials prove effective, current thinking is that optimum benefit for the patient will be obtained by starting treatments early, before significant muscle pathology develops. Early intervention will require early diagnosis and the most effective early diagnosis appears to be some form of population screening, either newborn screening, or screening at a slightly later age. For those who have worked with DMD over an extended period, this is yet another exciting time to be involved with the disease.

References
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Laing NG et al.


