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Revisiting the dystrophin-ATP connection: How half a century of research still implicates mitochondrial dysfunction in Duchenne Muscular Dystrophy aetiology

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ABSTRACT

Duchenne Muscular Dystrophy (DMD) is a fatal neuromuscular disease that is characterised by dystrophin-deficiency and chronic Ca^{2+}-induced skeletal muscle wasting, which currently has no cure. DMD was once considered predominantly as a metabolic disease due to the myriad of metabolic insufficiencies evident in the musculature, however this aspect of the disease has been extensively ignored since the discovery of dystrophin. The collective historical and contemporary literature documenting these metabolic nuances has culminated in a series of studies that importantly demonstrate that metabolic dysfunction exists independent of dystrophin expression and a mild disease phenotype can be expressed even in the complete absence of dystrophin expression. Targeting and supporting metabolic pathways with anaplerotic and other energy-enhancing supplements has also shown therapeutic value. We explore the hypothesis that DMD is characterised by a systemic mitochondrial impairment that is central to disease aetiology rather than a secondary pathophysiological consequence of dystrophin-deficiency.

Introduction

Duchenne Muscular Dystrophy (DMD) is the most prevalent muscular dystrophy afflicting ~1 in 3500–5000 live born males [1,2]. Regarded as a debilitating and fatal skeletal muscle disease, it is characterised by muscular weakness, exercise intolerance and progressive deterioration of skeletal muscle. Sufferers are generally confined to a wheelchair by 12 years of age with cardiorespiratory failure ultimately ensuing by the third decade of life [3,4]. 100 years after DMD was first described [5], the cause was identified as a gene mutation on the short arm of the X-chromosome [6]. The product of this mutation is the ablation of dystrophin, a 427 kDa rod-shaped [7] protein usually associated with the sarcolemma of muscle fibres via a complex of glycoproteins. The presence of dystrophin and associated glycoproteins provides integrity and rigidity to the fibre, however dystrophin-deficiency and the secondary reduction of these glycoproteins [8] renders the fibres more susceptible to damage as they become structurally unstable and exceedingly porous to the extracellular environment. As a result, excessive calcium (Ca^{2+}) influx, poor Ca^{2+} handling, activation of proteases/lipases and mitochondrial Ca^{2+} overload precede muscle degeneration. Over time, and as regeneration fails, fatty and connective tissue replacement culminates in non-functional muscle tissue.

In a bid to cure this progressive and fatal muscle wasting, the majority of research since 1987 has focused on genetically manipulating the disease by reintroducing the dystrophin gene (or a miniature version) back into the genome [9–11] and pharmacological intervention [12,13]. While some success has been observed with exon skipping and termination codon read-through trials (as reviewed in [14]), many complications of genetic therapy, including immunological reaction to delivery vectors, affordability and suitability [15] have been reported. As yet, there is no cure. Currently, corticosteroid treatment is used to delay muscular weakness and prolong function but has reported side effects including cardiomyopathy, weight gain, cataracts, hypertension, cushingoid features and osteoporosis [16].

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Prior to the discovery of dystrophin (and by several research groups afterwards), DMD was considered to be a disease of metabolic origin, with a strong body of literature demonstrating deficiency of key metabolic systems and regulators, including the mitochondria. As mitochondria constitute the ubiquitous adenosine triphosphate (ATP)-producing machinery of the cell and consequently play a crucial role in signalling cell death, their dysfunction seemingly induces a myriad of physiological events that underscore, or at least exacerbate, dystrophinopathy. Deficits encompassing the cytosolic enzymes of glycolysis [17–21] and the purine nucleotide cycle (PNC) [22,23], and the mitochondrial enzymes of the Tricarboxylic Acid (TCA) cycle [21,24] and Electron Transport Chain (ETC) [25–27] have been consistently reported in DMD sufferers, female carriers and animal models of the disease. Severely reduced ATP content [28–32] is the downstream consequence of these deficits and has been observed in skeletal muscle from both DMD patients and animal models. Dysregulation of cellular energy homeostasis has a variety of consequences for muscle including (1) impaired contractile apparatus function leading to reduced strength, ambulatory capacity and exercise tolerance; (2) impaired intracellular Ca^{2+} buffering leading to loss of homeostasis and Ca^{2+}-induced degeneration; (3) reduced protein synthesis; and (4) reduced satellite cell activation, replication, migration and differentiation leading to a markedly decreased capacity for regeneration of damaged muscle fibres. The mitochondria are also explicitly involved in maintaining, and are strongly regulated by, cytosolic Ca^{2+} concentration ($[Ca^{2+}]$). Heightened mitochondrial $[Ca^{2+}]$ increases ATP synthesis until overload induces permeability transition, collapses the mitochondrial membrane potential and signals apoptotic cellular death pathways. Thus an important question that could be asked is: What comes first, the chicken (Ca^{2+}-induced pathophysiology) or the egg (mitochondrial dysfunction)? That impaired metabolism has been observed in *mdx* myoblasts independent of dystrophin-deficiency (dystrophin is not phenotypically expressed until myoblastic fusion into myotubes) [33], indeed suggests an intrinsic metabolic deficiency. Metabolic impairment is also evident in a variety of tissues and cells from DMD patients and animal models that express a different dystrophin isoform – these include liver [34,35], heart [36,37] and brain [38–42]. Collectively, the literature strongly suggests that DMD is characterised by a systemic metabolic impairment, which is central to the aetiology of the disease and not secondary to the pathophysiology as currently accepted.

In 1992, and following 30 years of clinical research, Bonsett and Rudman [43] published a timely article in *Medical Hypotheses* that offered compelling evidence to highlight that DMD is predominantly underscored by metabolic impairment at the mitochondrial level, and that this can be anaplerotically "corrected" using high dose adenylosuccinic acid (ASA) treatment. Since this publication, and despite mounting literature indicating the same perturbations in animal models of DMD, metabolic therapy – with the exception of dietary creatine monohydrate supplementation – is still not a mainstay of DMD treatment. We suggest that re-defining DMD as a metabolic myopathy and strategically treating it as such, could improve patient outcomes and quality of life.

Hypothesis

Our hypothesis challenges the currently accepted pathophysiological paradigm describing DMD aetiology, which pinpoints dystrophin-deficiency-induced Ca^{2+} homeostasis de-regulation as the primary defect. We hypothesise that DMD is primarily a mitochondrial myopathy, in which the inability to generate sufficient quantities of ATP to fuel Ca^{2+} buffering from myofibres, induces the pathophysiological cascade of events leading to muscle wasting and fatty and connective tissue infiltration.

Arguments to support the hypothesis

A plethora of metabolic deficiencies in dystrophin-deficient muscle

The earliest literature of Meryon [44] and Duchenne [45] – who are renowned for reporting the first cases of DMD and the collective pathological manifestations of the disease, respectively – described the gross anatomical observations of DMD skeletal muscle fibres. A prominent feature of these fibres was intrafibral lipid accumulation. In whole fibre preparations, lipids are present extensively within the sarcoplasm and attached to the sarcolemma, and leach into the extracellular fluid from damaged fibres (Fig. 1A) [46,47]. This feature has also been reported in histological preparations using fat-specific stains [48,49]. Intracellular lipid droplets are a normal feature of healthy skeletal muscle, albeit in lesser abundance, in which they are located proximal to the sarcoplasmic reticulum and mitochondria to act as energy reservoirs [50]. As skeletal muscle has a high affinity for fatty acid oxidation as ATP demand increases, these reservoirs act as important regulators of cellular energy homeostasis during metabolic stress. The early work of Charles Bonsett's laboratory on cultured human myocytes highlights an equivalent propensity for healthy and DMD cells to produce intracellular lipid droplets when supplemented with nutrient dense 20% foetal bovine serum (FBS) superfluous to cellular nutrient demand i.e. when nutrients are supplied and uptaken in excess of cellular requirements, intrafibral lipid accumulation is a natural consequence [46]. While reducing the FBS concentration induced concurrent reductions in lipid accumulation in the healthy myocytes until lipid accumulation was absent, DMD myocytes continued to produce lipid droplets irrespective of serum concentration [46] highlighting a reduced capacity for metabolism that culminates in enhanced production of lipids at the cellular level (Fig. 1B). [24]. Intramyofibral lipid accumulation is also characteristic of obese, type 2 diabetic patients and aged skeletal muscle [51–55] indicating comparable metabolic dysfunction amongst these disease states. In a subsequent study, the same group provided evidence indicating this phenomenon was due to isocitrate dehydrogenase (IDH) dysfunction/deficiency as the addition of isocitrate to DMD cells induced significant lipid formation [24].

In dystrophin-deficient skeletal muscle from human DMD patients and animal models, however, metabolic dysfunction is not limited to IDH, but is widespread across multiple metabolic pathways [56] and culminates in resting ATP levels that are at least 50% of healthy control levels [28–32]. In intensely exercised, healthy skeletal muscle, physiological fatigue mechanisms ensure that ATP demand does not exceed production capacity – a ~40% drop in resting ATP levels appears to be the critical maintenance threshold such to trigger these mechanisms and reduce demand on the metabolic system (as reviewed in [57]). Thus, compared to healthy skeletal muscle, resting dystrophin-deficient muscle consistently maintains sub-threshold ATP levels which are likely incompatible with long-term cell survival. Taken in context of Bonsett's research, it is not that dystrophin-deficient muscle has a lesser requirement for ATP synthesis, in fact the exact opposite it true. Intrafibral lipid production and accumulation even in the presence of minute nutrient provision seems ostensibly linked to an intrinsic metabolic defect that limits the conversion of nutrients into cellular energy (ATP).

A plethora of isolated deficits in the cellular energy system have been reported in dystrophin-deficient skeletal muscle from human patients and animal models, which would both individually and collectively contribute to this failure of energy homeostasis (summarised in Table 1). Due to the strong and multifaceted allosteric regulation of metabolism by associated up- and down-stream products and reactants, one broken link in the metabolic chain would induce deleterious consequences at multiple levels

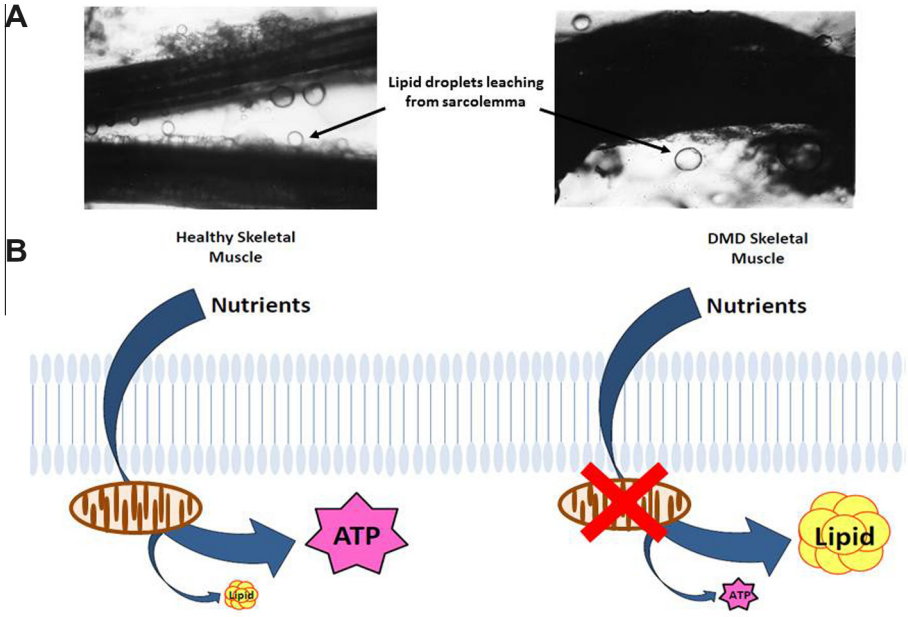

Fig. 1. Accumulation of intramyofibral lipids is a feature of dystrophin-deficient skeletal muscle and a hypothetical consequence of mitochondrial dysfunction. Lipid droplets are evident in the sarcoplasm, the sarcolemma and leaching into the extracellular fluid of isolated dystrophin deficient myofibres (Photographs courtesy of Bonsett [182]; reproduced with the permission of C.C. Thomas Publisher Ltd). In a hypothetical model to explain this phenomenon (B), nutrients are typically oxidised by the mitochondria to synthesise ATP in healthy skeletal muscle (left) with minimal directed to intracellular lipid production. In dystrophic muscle (right), the capacity to utilise nutrients for ATP synthesis is significantly impaired which coincides with an increased propensity to produce intracellular lipid (adapted from Bonsett, unpublished). We hypothesise that this feature is a consequence of mitochondrial dysfunction that is independent of the absence of dystrophin protein.

Table 1
Summary of the metabolic deficits in the metabolic pathways of dystrophic skeletal muscle.

	Defect description	DMD model	References
Macronutrient uptake and availability	Normal glucose uptake but ↓ glucose content	Human DMD and *mdx* mouse	[183–185]
	↓ GLUT4 mRNA and protein expression in aged diaphragm	*mdx* mouse	[75]
	↓ Gluconeogenic precursors (alanine and glutamine)	Human DMD	[183,185,186]
	↑ Fructose content	Human DMD	[187]
Glycolysis	↓ Glucose-6-phosphate	Human DMD	[19]
	↓ Phosphofructokinase activity, ↓ sensitivity to allosteric regulation	Human DMD and *mdx* mouse	[18–20,78,79]
	↓ Aldolase	Human DMD	[17,19,188]
	↓ Pyruvate kinase activity	Human DMD	[20,189]
	↓ Lactate dehydrogenase	Human DMD	[18,19,103]
	↓ Lactate production and acidification	Human DMD and *mdx* mouse	[31,183,190]
Glyocgen storage and utilisation	↑ Glycogen content	Human DMD and *mdx* mouse	[121,124,191]
	↓ Phosphorylase activity	Human DMD and *mdx* mouse	[17–20,99,101,190–195]
	↓ Phosphoglucomutase activity	Human DMD and *mdx* mouse	[20,102]
Fat oxidation	↓ Palmitate oxidation	Human DMD patients and carriers	[95,196,197]
	↓ Palmitoylcarnitine and malate oxidation	Human DMD	[95,183,198–202]
	↓ Total carnitine	Human DMD	[95,183,199–202]
	↓ Fatty acid transport into mitochondria	Human DMD	[202]
Creatine phosphagen system	↓ PCr concentration	Human DMD and *mdx* mouse	[28,84,85,87,183,203–205]
	↓ Cr concentration	Human DMD and *mdx* mouse	[28,183,184,205,206]
	↓ TCr	Human DMD and *mdx* mouse	[28,207,208]
	↓ PCr/Pi	Human DMD	[85,203,209]
	↓ PCr/ATP	Human DMD	[85,117,203]
	↑ Urinary Cr excretion (due to ↓ turnover)	Human DMD	[210]
Purine nucleotide cycle	↓ IMP concentration	Human DMD	[86]
	↓ Adenylate kinase content and activity	*mdx* mouse	[23]
	↑ Uric acid excretion	Human DMD	[86,211,212]

spanning the entire metabolic system – thus pinpointing the precise defect becomes difficult. Indeed, the only established physical link between the dystrophin protein and the metabolic pathways is via neuronal nitric oxide synthase (nNOS). Dystrophin-deficiency results in the secondary loss of nNOS [61]. In skeletal muscle, nNOS generates NO which is a key intracellular signalling molecule with strong metabolic regulatory capacity that has effects on contraction, blood flow, glucose uptake and metabolism [62,63]. In healthy

muscles, nNOS localises to the subsarcolemma bound to the dystrophin protein complex (DPC), and more specifically, the syntrophins. The absence of dystrophin disrupts the formation of the DPC [8] and affects nNOS localisation [58,59]. As nNOS exists unbound in the cytosol of *mdx* mouse (genetically homologous murine model of DMD) skeletal muscle and subsequently becomes a substrate for the calpain proteases, a 25-fold decrease in nNOS activity [59–61] and content is observed [62–65]. In the skeletal muscle of DMD patients, nNOS is absent in the pellet fraction of biopsy samples (confirmed by both enzyme assay and Western blot) [58,59]. Additionally, nNOS mRNA in both human DMD [66] and *mdx* [59,67] skeletal muscle is reduced, and as a consequence, endogenous NO production is significantly decreased [68–70]. nNOS-generated NO appears to play a key role in facilitating glucose uptake by stimulating glucose transporter 4 (GLUT4) translocation at rest [71] and during contraction [72]. Despite the reduction of nNOS in *mdx* skeletal muscle, basal (resting) glucose uptake has been shown to be equivalent to control muscle [21,73,74] with GLUT4 expression also normal in young animals [75,76]. However, GLUT4 expression (and its mRNA) decreases in the diaphragm of older *mdx* mice [75], which is important clinically as the diaphragm is the only *mdx* muscle to undergo progressive degenerative wasting throughout the lifespan as per the human disease [77]. Decreased mRNA expression in the older *mdx* mice suggests that disease progression may affect protein expression of GLUT4 and therefore the ability to bring glucose into muscle fibres sufficient to maintain energy production. nNOS also exerts strong regulatory capacity over the key rate-limiting glycolytic enzyme, phosphofructokinase (PFK) in a non-NO-mediated manner [78]. In both DMD and *mdx* muscle samples, reduced PFK activity is observed [18–20,78,79] and this seems ostensibly linked to the significant down-regulation of both glycogen and glucose metabolism in dystrophin-deficient muscle (summarised in Table 1) [56,80]. In healthy muscle, PFK co-localises at the sarcolemma alongside nNOS and is inhibited by high concentrations of ATP and activated by ADP and other by-products of ATP hydrolysis (as reviewed in [81]). As ATP concentration is diminished in dystrophic muscle, PFK activity should, logically, be increased to promote ATP synthesis and energy balance. However, altered allosteric regulation of PFK has been observed in *mdx* muscle [78] suggesting that PFK fails to respond appropriately to normal stimuli. Despite the soluble and cytoskeleton-bound PFK enzymes being distributed normally, the sensitivity of PFK to its allosteric regulators is reduced [79]. This indicates a functional change in PFK properties and/or its modulation, which significantly reduces its activity and likely contributes to the overall metabolic deficit in dystrophic muscle. Reintroduction of nNOS into *mdx* skeletal muscle has shown some benefit in improving glucose and glycogen metabolism (in addition to reducing membrane degradation and muscle inflammation) [78,82], such to improve exercise tolerance which was attributed to the positive allosteric effect nNOS exhibited on PFK [78].

While the secondary loss of nNOS abundance and function goes toward accounting for the widespread depression of glycolytic and glycogenolytic function observed in dystrophin-deficient muscle, its effect on downstream oxidative ATP production is unclear and yet to be characterised. While glycolytic perturbations would plausibly induce secondary reductions in mitochondrial metabolism as a direct result of reduced pyruvate flux through the system, the subsequent activation of stress-responsive metabotropic transcription factors should theoretically activate fat oxidation and stimulate mitochondrial biogenesis to restore ATP production as a compensatory mechanism. This would alleviate pressure on the creatine phosphagen and purine nucleotide salvage pathways. However, as summarized in Table 1, this is not the case in dystrophin-deficient muscle, and as such, all signs seemingly point toward the mitochondria as the key site of metabolic anomaly.

All signs point to the mitochondria

The fundamental role of the mitochondria is ATP synthesis, thus they are the major cellular regulators of energy homeostasis. More recently mitochondria have emerged as playing an important role in the regulation of initiating apoptotic cell death. Mitochondria are adept at sensing and responding to intracellular changes in energy balance to maintain homeostasis, but once the metabolic insult exceeds regulatory capacity, mitochondrial dysfunction ensues. Prolonged mitochondrial stress can initiate apoptosis when dissipation of the mitochondrial membrane potential, release of cytochrome c and/or caspases and opening of the mitochondrial transition pore occurs (as reviewed in [83]).

Functional abnormalities in dystrophic mitochondria. Mitochondrial dysfunction in dystrophic skeletal muscle is well documented and a key contributor to the reductions (up to 50%) in resting ATP content [27–32,79,84–89], with decreased ATP content in the brain of DMD patients also evident [39]. Impaired handling of substrates including pyruvate [21,25–27,33,90–95], malate [25–27,91–93] and glutamate [26,27,94] (with glutamate content increased in *mdx* diaphragm [96]) have been consistently reported to produce lower oxidation rates compared to healthy controls, even in combination with other substrates. Addition of succinate, on the other hand, has been shown to either restore [21,25,97,98] or at least partially restore oxidation rates to control levels [26,27,91,92]. This is a widely reported feature of dystrophin-deficient muscle metabolism and as published by us recently, indicates that the metabolic deficit may be located at complex I of the ETC [27]. Alternatively, as it appears that some enzymes of the TCA function abnormally – including succinic CoA synthetase, aconitase, malate dehydrogenase and IDH [99–103] – which would result in decreased production of reducing equivalents at the TCA level, the ability of succinate to restore oxidative phosphorylation may lie in its ability to bypass a defective TCA system and stimulate complex II of the ETC directly.

Various enzymes of the TCA cycle (in addition to complex V of the ETC) are regulated by increases in intramitochondrial [Ca^{2+}]. Pyruvate dehydrogenase (PDH) (indirectly activated by Ca^{2+}-activated phosphatase), α-ketoglutarate dehydrogenase and IDH (at higher concentrations) are all allosterically activated as mitochondrial matrix [Ca^{2+}] rises (as reviewed in [104]). This normally results in the anaplerotic expansion of TCA-generated reducing equivalents and a greater chemiosmotic drive for, and faster speed of, ATP production at complex V. These enzymes should theoretically be stimulated in dystrophic muscle (as free intracellular Ca^{2+} is considerably higher at rest and during contraction [105–109]) to increase Ca^{2+} buffering and remove the pathological stimulus. However, normal stimulation of these enzymes by increased [Ca^{2+}] appears to be absent in dystrophic muscle as evidenced by decreased IDH activity [100]. If IDH fails to activate in response to the extremely high [Ca^{2+}] observed in DMD, it may be that other Ca^{2+}-sensitive enzymes are not responding appropriately either. The consequence of this is insufficient ATP production and Ca^{2+} buffering capacity leading to amplification of the pathological stimulus (i.e. [Ca^{2+}]). As it has been recently demonstrated that *mdx* mitochondria hypersensitively respond to a Ca^{2+} load to prematurely open the permeability transition pore (channel that initiates mitochondrial death) [110], we suggest that the inability of dystrophic mitochondria to respond to an overwhelming Ca^{2+} stimulus by ramping up ATP production, favours premature induction of pro-apoptotic pathways such that cell death is the only viable outcome (Fig. 2).

Reduced oxidation rates of the substrates that channel through the TCA cycle appears to culminate at the ETC. In saponin-skinned

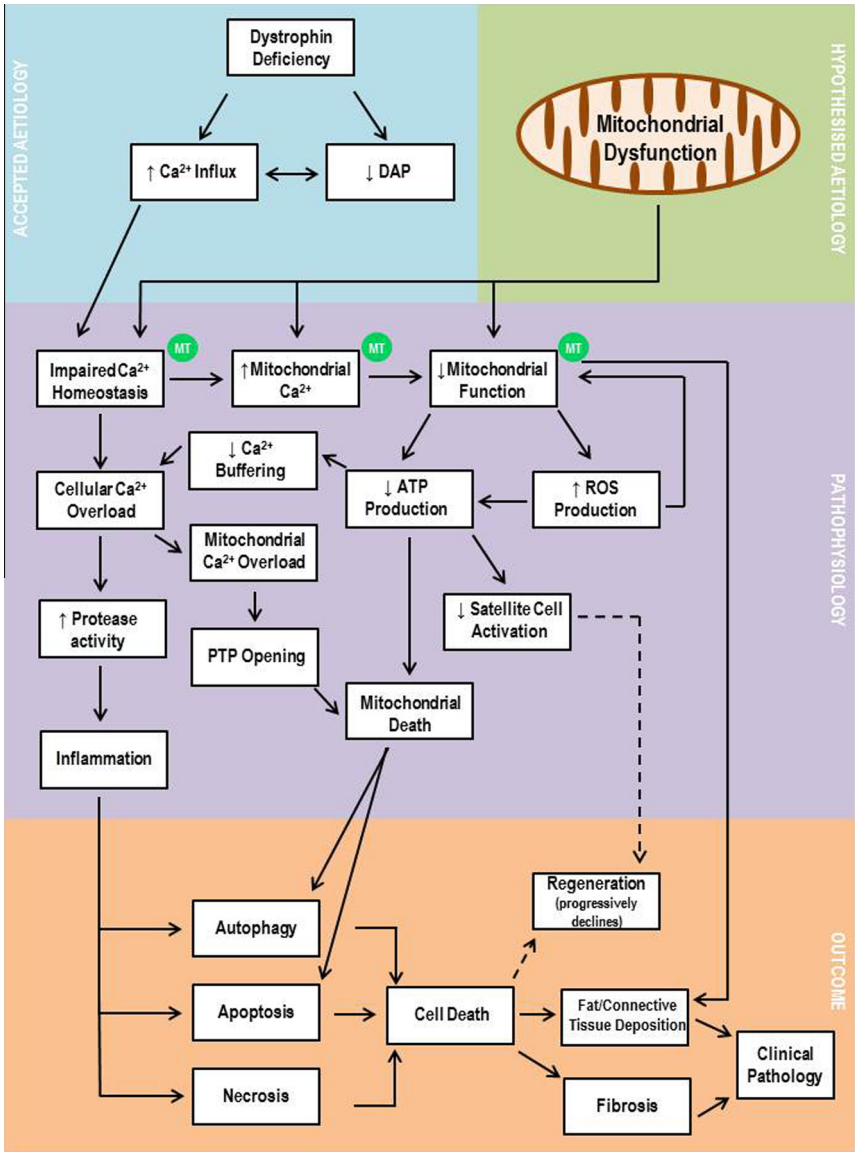

Fig. 2. Challenging the accepted paradigm of DMD aetiology: the potential for metabolic therapy to anaplerotically "correct" dystrophin-deficiency-mediated pathology. In the accepted aetiology of DMD (blue box), the pathophysiology (purple box) and clinical outcomes (orange box) are the result of dystrophin- and DAP-complex-deficiency-mediated Ca^{2+} influx and homeostatis deregulation. Mitochondrial dysfunction is a secondary consequence of cellular Ca^{2+} overload. In our hypothesised aetiology of DMD (green box), inherent mitochondrial dysfunction is the precursor to dystrophic pathophysiology and not a secondary consequence of dystrophin-deficiency as currently accepted. Inherent mitochondrial dysfunction would limit the ATP production required to buffer Ca^{2+} from myofibres and organelles and maintain regenerative capacity, thus driving the clinical phenotype. The application of metabolic therapy (MT) (green circles) would target the impaired Ca^{2+} homeostasis, increased mitochondrial Ca^{2+} load and mitochondrial dysfunction to effectively buffer the Ca^{2+} influx induced by dystrophin/DAP-deficiency and prevent the subsequent pathophysiology. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mdx skeletal muscle fibres, the maximal rate of respiration, as stimulated by the addition of ADP, was nearly 50% lower regardless of the substrate used [26]. Similarly, isolated dystrophic mitochondria function at ~60% of maximal respiration control rates [26,88], while a biopsy from a DMD patient revealed similar respiratory deficits [26]. Additionally, reduced ADP-stimulated [91–93,97,111,112] and basal respiration has been reported [33,111–114], with further reductions observed as the disease progresses [94]. The ability of mitochondria to aptly respond to the increased [Ca^{2+}] and requirements of dystrophic muscle appears to be further impaired as the spare respiratory reserve, which indicates the ability for the ETC to increase ATP production in response to metabolic challenge, is reduced by ~60% [111]. Such a deficit can be accounted for by reduced activities of the ETC enzymes. In *mdx* fibres of the quadriceps, the activities of rotenone-sensitive NADH:cytochrome *c* reductase, succinate:cytochrome *c* reductase and cytochrome *c* oxidase were found to be 50% of that in normal fibres [26], with a 20–35% reduction in the activities of complexes I, II and IV in *mdx* fibres of the tibialis anterior. Moreover, in both the fast-twitch extensor digitorum longus and slow-twitch soleus of the *mdx* mouse, NADH activity is reduced [115], with *mdx* myoblasts expressing a decreased complex III and V content [33]. There is also a significant decrease in the expression of genes encoding the subunits of complexes I, II, III and IV in DMD muscle [99]. Ultimately, the maximal ATP synthesis rate is reduced by up to 75% in mitochondria isolated from dystrophic skeletal muscle [27,88]. Similar respiratory dysfunction is observed in the brain of the *mdx* mouse. Decreased activity of complexes I and IV is observed throughout various sections of the brain [42] indicating that despite not being strongly involved in the pathological progression

of the disease, the brain still manifests similar metabolic deficits as per the skeletal musculature.

In addition to the content and activity of isolated complexes of the mitochondrial respiratory chain, functional measures of mitochondrial performance are challenged in dystrophin-deficient skeletal muscle. Dystrophic mitochondria exhibit reduced respiratory control, ADP/oxygen (O) and P/O ratios [25,88,90,92,94,97,98,113,116], all of which indicate that dystrophic mitochondria are not as tightly coupled as healthy mitochondria, thus reducing the phosphorylation potential [117] as evidenced by the 40% reduction in ATP produced per O_2 molecule consumed [88]. Uncoupling refers to any process that impacts upon the P/O ratio and subsequently depletes the potential energy. This includes loss of protons due to inefficient proton pumping by the ETC complexes, leak of electrons from the respiratory chain and activity of uncoupling proteins – all of which dissipate the mitochondrial membrane potential. While uncoupling is thought to provide protective effects as it can buffer reactive oxygen species (ROS) produced by electron leak from the respiratory chain, prolonged uncoupling can lead to severe mitochondrial impairment and death [118].

The NAD/NADH ratio is an important regulator of metabolism [119]. NAD is a cofactor at multiple sites of the TCA cycle and in glycolysis, where it is reduced to NADH and oxidised at complexes I, III and IV of the ETC. This generates the mitochondrial membrane potential which is the driving force for ATP production. Therefore, maintaining the NAD/NADH ratio is imperative, albeit seemingly difficult in dystrophic muscle due to the decreased total intramitochondrial NAD pool [91]. Moreover, as the NADH produced at the glycolytic level is dependent upon the malate-aspartate and glycerol-3-phosphate shuttles to enter the mitochondria, and these rely on glutamate oxidation (which is notably decreased in dystrophic muscle [26,27,94]), glycolysis-generated NADH may be largely prevented from contributing to respiration. Together, this indicates that the NAD/NADH ratio is unable to suitably modulate metabolic function due to other confounding factors that impair the maintenance of the NAD and NADH pool at the mitochondrial level.

Structural abnormalities in dystrophic mitochondria. Proper mitochondrial structure and locale is also important to function and is compromised in dystrophin-deficient states. Mitochondria exist in two distinct pools – located beneath the sarcolemma (subsarcolemmal) and at the I band and intermyofibrillar space of the contractile apparatus (intermyofibrillar) [120]. Subsarcolemmal mitochondria account for 10–15% of the mitochondrial pool and supply ATP for Ca^{2+} handling, ion transport, membrane function and the peripheral nuclei, while also assisting with glucose homeostasis and lipid utilisation [120]. In contrast, intermyofibrillar mitochondria constitute up to 90% of the mitochondrial pool and provide ATP for contraction. Intermyofibrillar mitochondria differ from subsarcolemmal mitochondria in that they maintain a higher respiratory rate via increased mitochondrial enzyme activity [120]. Despite their differences, both pools of mitochondria share a networking system that allows them to translocate to areas of increased metabolic demand. Thus, mitochondria are extremely responsive to changes in isolated regions of the intracellular environment.

In *mdx* skeletal muscle, a decrease in mitochondrial mass has been reported [114,115]. This is partnered with a decrease in the density of subsarcolemmal mitochondria and the accrual of intermyofibrillar mitochondria around necrotic and regenerating fibres with no change in overall mitochondrial number [88]. This suggests that either the subsarcolemmal mitochondria are translocating to support the intermyofibrillar mitochondrial pool or require the presence of dystrophin for scaffolding to remain at their proper location. Decreased density would be detrimental as the subsarcolemmal mitochondria play a role in Ca^{2+} handling and lipid metabolism, which may partially explain the inability to appropriately handle the stress applied by Ca^{2+} and the deficits observed in β-oxidation. Moreover, in human DMD biopsies, an increased population of dense and dilated mitochondria have been observed [121–123] along with changes in cristae shape and density [49]. Swollen mitochondria are also evident in *mdx* mouse skeletal muscle [110,124,125] along with morphologically abnormal cristae structure [110]. While morphological changes of the mitochondria are generally characteristic of fibres undergoing degeneration and necrosis, it appears this swollen morphology may exist outside of an environment conducive to swelling [27]. Isolated *mdx* mitochondria bathed in a Ca^{2+}-free environment are more swollen than mitochondria isolated from healthy animals [27]. While this could be a residual effect of an extreme pre-isolation *in vivo* Ca^{2+} environment, it may also be an inherent feature of the disease as alterations in mitochondrial architecture, morphology and localisation are apparent in female DMD carriers that express dystrophin and do not manifest dystrophinopathy [126]. If so, this inherent swollen morphology would affect mitochondrial functionality, as changes in cristae shape have recently been shown to alter ETC supercomplex assembly [127] which would deleteriously impact upon their function. Together, this decrease in mitochondrial mass and inherent swollen morphology strongly suggest that metabolic impairments in DMD are an inherent feature of the genotype, and this is exacerbated – but not caused by – the persistent elevation of Ca^{2+}.

Dystrophic muscle does not respond normally to master energy signals

In healthy skeletal muscle, ATP depletion induced by metabolic, nutritional and/or environmental stressors (including intense exercise and hypoglycaemia) stimulates ATP-producing pathways to restore energy homeostasis [128]. One important regulator of this switch from ATP-consuming (anabolic) to ATP-producing (catabolic) pathways is adenosine monophosphate-activated protein kinase (AMPK), a major sensor of cellular energy status. Induced by rises in the AMP/ATP ratio, AMPK stimulates glucose uptake, glycolysis, fatty acid oxidation [128,129] and various TCA cycle and ETC enzymes [130], while also modulating expression of a suite of genes – including PGC-1α – that increase mitochondrial biogenesis. Thus, AMPK activation favours the oxidative fibre phenotype, which is highly beneficial for dystrophic muscle as this fibre type is less affected by the disease [131] and therefore may offer protection from damage. In addition, AMPK appears to play a significant role in muscle remodelling as it stimulates autophagy. Autophagy is a catabolic pathway that breaks down cellular components when they are in excess or damaged, or, to provide fuel sources in times of metabolic challenge [132]. Therefore, AMPK is a positive stimulator of metabolism, controlling the supply of fuel to various metabolic pathways and initiating remodelling to improve muscle structure and function. Considering that AMPK positively modulates metabolism, stimulates targeted remodelling of muscle to improve oxidative capacity and is activated by ATP depletion, AMPK activation should, theoretically, be enhanced in dystrophic skeletal muscle. Indeed, Pauly et al. [110] demonstrate a higher basal AMPK activation in *mdx* diaphragm, highlighting that metabolic stress-induced signalling pathways are appropriately activated in dystrophic muscle. When the AMPK-activator metabolite 5′-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) was given to *mdx* myotubes *in vitro* and to 6 week old *mdx* mice via daily intraperitoneal injection, AMPK activation was further enhanced compared to untreated conditions. In the *mdx* mice in particular, AICAR treatment had several beneficial effects including increased activation of autophagic signalling proteins, maximal force production and time to permeability transition pore opening in response to Ca^{2+} challenge

[110]. Most noteworthy however, was that despite AICAR inducing a ~50% increase in activated AMPK, only minimal increases were observed in acetyl CoA carboxylase phosphorylation (the downstream target of AMPK activity) and this failed to induce any changes in oxidative metabolism including activity of citrate synthase, cytochrome oxidase and mitochondrial O_2 consumption. This data importantly highlights that while dystrophin-deficient muscle can ably detect metabolic stress, the downstream response of the metabolic systems to AMPK phosphorylation fails to improve ATP synthesis to abate this stress.

The plasticity of the skeletal musculature in response to isolated and chronic exposure to metabolic stress is afforded via the induction of a slow-type oxidative phenotype. In a study that compared global gene expression responses between skeletal muscle from metabolically-challenged endurance-trained individuals who had been previously sedentary, and DMD patients, ~90 genes were shown to be modulated identically [56]. This data highlights that strong metabolic challenge is a feature of dystrophinopathy and that it invokes similar responses as per chronic endurance exercise. However, while the expression of genes regulating oxidative phosphorylation was increased following endurance training as expected, they were differentially down regulated in muscle from DMD patients [56]. These included genes of carbohydrate, glycogen and mitochondrial metabolism [56]. Thus, while DMD muscle adapts on a genetic level to metabolic stress as per endurance trained athletes, this stress seems not to induce the regular adaptations at the mitochondrial level to enhance the ATP production capacity of the skeletal musculature. It has been well established in the literature that inducing type I oxidative fibre type transformations pharmacologically and genetically affords therapeutic value to dystrophin-deficient skeletal muscle by reducing the rate of disease progression [133–136], as type II fibres are preferentially affected [131,137], which can be promoted by the activation of AMPK [115,132,138–140] and its downstream targets [114,141–143]. However, it has recently been suggested that the beneficial effects of a slow type I phenotype is functionally related to enhanced utrophin A expression – in dystrophin/utrophin double knock-out mice AICAR administration afforded no benefit – in comparison, therapeutic benefit was observed in *mdx* mice [140]. Thus whether AMPK activation can suitably induce benefits at the mitochondrial level to buffer metabolic demand remains unclear.

Another important role of AMPK is regulation of the targeted removal of dysfunctional organelles/structures via autophagy. It has been observed that *mdx* diaphragm is laden with dysfunctional mitochondria characterised by morphological abnormalities and an increased propensity to open the permeability transition pore [110]. The removal of dysfunctional mitochondria is strongly regulated in healthy skeletal muscle [144], albeit background mitophagic activity is typically low due to the relatively low ratio of unhealthy/healthy mitochondria. However, in dystrophic skeletal muscle, there is reduced propensity for sufficient and/or functional mitophagy leading to the accumulation of defective mitochondria, particularly in the subsarcolemmal pool [145]. Ineffective autophagic signalling induction has been demonstrated in *mdx* [145] and human DMD [110,145] skeletal muscle with AMPK activation seemingly central to the problem. Both AICAR- [110] and low protein diet-induced AMPK activation demonstrably increases the activity of pro-autophagic pathways and ameliorates the dystrophic condition [110], indicating that improving the clearance of dysfunctional mitochondria is beneficial. Thus while Pauly et al. [110] have demonstrated enhanced endogenous AMPK signalling in dystrophin-deficient skeletal muscle, it appears insufficient to match the extent of mitochondrial pathology evident in DMD without therapeutic support.

Revisiting the dystrophin-ATP connection: is a mitochondrial disease at the heart of DMD?

Several hypothetical review and original research papers have both historically and more recently proposed that the lack of dystrophin protein may not be the primary cause of the progressive and fatal degeneration observed in DMD, but rather a co-morbidity [33,43,146,147]. We have described a plethora of mitochondrial defects (in addition to many others of substrate feeder pathways that are allosterically regulated by the functional capacity of the mitochondria) that are also commonly observed in mitochondrial diseases and in senescence. Indeed, DMD shares common metabolic and mitopathological features with various mitochondrial diseases and with aged skeletal muscle, including often comparable symptomology. In addition a more recent study has shown that mitochondrial dysfunction exists in "pre" dystrophin-deficient myoblasts prior to the "typical" cascade of events that are commonly believed to cause the progressive muscle degeneration and wasting evident in DMD [33]. Collectively, this literature importantly suggests a mitochondrial aetiology of DMD.

Because skeletal muscle accounts for ~40–50% of body weight and ~30% of oxygen consumption at rest, it is an important regulator of overall metabolism. As such, mitochondrial deficits manifest vastly in the skeletal musculature and myopathy is thus characteristic of many mitochondrial diseases. Mitochondrial disease can arise from mutations in the maternally inherited mitochondrial DNA (mtDNA), and less commonly in the nuclear DNA. mtDNA resides in the matrix and encodes for the hydrogen pumping regions of the respiratory chain complexes, highlighting its integral role in the regulation of metabolism [148]. However due to its proximity to the respiratory chain, mtDNA is extremely vulnerable to mutation, most commonly by ROS produced by the respiratory complexes [149,150]. Initially, this has minimal effect on mitochondrial function, until the number of mutant mtDNA outnumbers wild-type mtDNA. As mutant mtDNA accumulates, the bioenergetical capacity of the cell diminishes. Various diseases result from mtDNA mutations and manifest themselves as multisystemic diseases. These mitochondrial diseases share common features with DMD including mental impairment, skeletal muscle weakness, cardiomyopathy and multisystem metabolic dysfunction [148,151]. Reduced activities of complex I, III, IV and V of the ETC, increased ROS production and decreased ATP synthesis are common nuances of mitochondrial diseases and DMD [151]. The fact that dystrophin is encoded and expressed normally in these diseases, but that they share clinical features with dystrophinopathy indicates the potential for a common disease origin that is not linked to dystrophin-deficiency.

As the majority of ETC complexes (excluding complex II) are partially encoded by mtDNA and reports exist that describe mitochondrial dysfunction in DMD carriers that express dystrophin normally, maternal mtDNA inheritance would be a likely theoretical origin of such a mitochondrial mutation. Female carriers of the dystrophin gene mutation on one of their X chromosomes commonly express normal levels of dystrophin (albeit sporadic dystrophin-deficient fibres have been reported [152]). As such, they do not manifest DMD. However, despite lacking phenotypic pathology, deficits in mitochondrial responses to exercise have been reported. Carriers are unable to perform muscle work at the same level as controls and their P_i/PCr ratio is higher for corresponding work levels [153,154]. This supports an inability of the mitochondria to sufficiently replenish the Cr/PCr system during activity. Post-exercise recovery of the PCr/inorganic phosphate (P_i) ratio is also much slower in carriers [153], demonstrating that mitochondrial insufficiency is also apparent at rest. Additionally,

sharp increases in serum CK activity are observed following exercise in carriers but are absent in healthy exercised individuals [155,156]. Notably, the co-occurrence of a mtDNA mutation in a family with extensive history of DMD has also been observed [157], which adds further credence to the ideation of mtDNA mutation underscoring DMD pathology.

If not inherited, another likely origin of mtDNA mutation is via the rapidly progressive accumulation of ROS-induced mutations that are not too dissimilar to those that underscore senescence as described in the popular *mtDNA accumulation theory of aging* (reviewed in [158]). Aging muscle shares many symptomatic characteristics of dystrophic muscle including fatigability, muscular weakness and atrophy, and mitochondrial dysfunction. In aging muscle, it appears that accumulation of mutant mtDNA leads to mitochondria characterised by decreased oxidative capacity, increased oxidative stress and decreased ATP synthesis [148,159] which impairs muscular function and viability. Of note, a characteristic feature of senescent mitochondria is a reduction in spare respiratory capacity [160] which renders mitochondria unable to adapt to increased energy demand, thus promoting fatigue, exercise intolerance and progressive muscle wasting (sarcopenia) which are all clinical features of DMD. Indeed, both aged and dystrophic muscles display deregulation of the same genes involved in metabolism [161] which highlights once more the possibility of mtDNA mutation involvement in DMD.

Perhaps one of the more compelling pieces of evidence that mitochondrial dysfunction is an inherent feature of DMD is a recent finding by Onopiuk and colleagues [33]. Using myoblasts from control and *mdx* mice, it was observed that *mdx* myoblasts exhibit changes to several mitochondrial functional parameters including decreased basal oxygen consumption, increased mitochondrial membrane potential and ROS production (~70% higher) and decreased complex III and V content [33]. Remarkably, these metabolic changes are observed at a time when dystrophin is yet to be expressed in myoblasts [162]. In both control and *mdx* myoblasts, dystrophin expression was negligible, despite an mRNA transcript evident in control myoblasts [33]. Myoblasts express a different metabolic phenotype to myotubes including a greater dependence on glycolysis [62]. Lactate production via glycolysis pacifies ~60% of energy demand (due to its conversion to pyruvate via LDH) [62] and *mdx* myoblasts demonstrably produce more lactate [33] indicating heavy reliance on glycolytic flux. This appears to be pertinent to *mdx* myoblasts as the basal rate of respiration following the addition of glucose and pyruvate was depressed compared to controls [33], suggesting that further oxidation of intermediates in the mitochondria is impaired. The authors concluded that the metabolic dysfunction in *mdx* myoblasts is independent of dystrophin-deficiency as deficits were observed in *mdx* myoblasts prior to the time of dystrophin expression.

There are now also several case studies in the literature documenting either dual mtDNA and nuclear dystrophin gene mutations in family pedigrees [157] or dystrophin gene abnormalities with pseudometabolic presentation but dystrophin protein expression [163,164]. A case study by Wong and colleagues [157] describes the presentation of an adolescent male with a strong family history of DMD but who does not express the genotype himself, with complicated seizure disorder, congenital heart disease and developmental delay. Suspected mitochondrial respiratory chain disorder was confirmed in which low levels of heteroplasmic A3243G mutation was detected in the mtDNA. The diagnosis of MELAS disorder was made following respiratory enzyme analysis that revealed significantly elevated complex IV activity without gross mitochondrial cytopathy (albeit some mitochondria displayed altered cristae structure and were morphometrically abnormal). The patient carried low mutant loads in all tissues analysed – 6%, 8%, 12%, 17% and 9% for blood, hair follicle, buccal mucosa, skeletal muscle and skin fibroblast cultures, respectively. The mutation appeared to have occurred *de novo* as it was not detected in the maternal mtDNA from blood, hair follicle or buccal mucosa cells, albeit this could not be confirmed given the relatively low mutant load found in the patient. Similar cases of mtDNA mutation in the background of other, more severe nuclear gene mutations (such as cystic fibrosis and spinal muscular atrophy) have been reported by the same group [165,166] highlighting the propensity for dual mitochondrial and nuclear gene mutations – perhaps as a result of modifier gene induction – that are difficult to diagnose due to broad and often competing symptomologies.

The pseudometabolic presentation of DMD due to missense mutations in the dystrophin gene has also been documented. Romero and colleagues [163] report three male adolescents presenting with exercise-induced myalgia, muscle stiffness, and myoglobinuria following strenuous exercise – all symptoms of metabolic diseases including glycogen storage disorder, fatty acid oxidation disorder and mitochondrial cytopathy. All patients were found to have a hemizygous T-to-C mutation in exon 15 of the DMD gene resulting in an amino acid substitution of leucine to proline at codon 575. Immunohistochemcial staining of dystrophin and other proteins of the DPC was normal as was western blot analysis for dystrophin quantity and size. A further two reports of the same missense mutation inducing recurrent rhabdomyolysis has been reported [167]. These case studies highlight symptoms characteristic of metabolic disease that are seemingly induced by dystrophin gene point mutations but which are not phenotypically associated with dystrophin protein expression abnormalities.

Most recently, several studies by the same group have documented the clinical history of canine models of muscular dystrophy [168–170] and dystrophin-deficient human DMD patients [171] that express a mild disease phenotype and in some instances, a normal lifespan, despite the absence of dystrophin. Zucconi et al. [168] and Zatz et al. [169] describe the clinical history of a golden retriever muscular dystrophy dog and its offspring, who display absent dystrophin production, unremarkable utrophin regulation, hallmark histopathological features of skeletal musculature and extreme elevations in serum CK levels as per phenotypically normal severely-affected dogs, but are seemingly able to buffer this to maintain muscle mass, ambulation and a normal life span. A similar canine colony has been reported in the Labrador retriever muscular dystrophy model [170] which also displays the absence of dystrophin – albeit the precise mutation on the dystrophin gene was not elucidated in this study – and are asymptomatic. This protection seems related to the maintenance of strong regenerative potential throughout the lifespan, albeit the precise mechanism through which muscle function is maintained despite the absence of dystrophin requires further investigation and characterisation. Finally, in human DMD patients, Zatz et al. [171] have reported half-brothers with comparable, minimal (near absent) levels of dystrophin expression, elevated serum CK levels and pathological histological parameters, but who express widely variable phenotypic progression of DMD. While one brother has progressed through a normal disease course with onset of symptoms at 3 years, diagnosis at 7 years and loss of ambulation at 9 years, the older brother shows mild signs of muscle weakness and physical dysfunction with mild calf hypertrophy, but maintains normal ambulatory capacity at 16 years of age. The same paper describes a third case of an unrelated male 16 year old adolescent who displayed normal phenotypic DMD at age 7 years when diagnosis was made, but whom now displays only mild weakness and calf hypertrophy and is fully ambulatory. Other isolated case studies exist documenting the complete absence of dystrophin expression but a mild DMD phenotype [172,173]. While modifier gene regulation likely accounts for such phenotypic differences, it is possible that in these rare cases of DMD, mitochondrial dysfunction is

spared or anomalies are corrected such that ATP demand is met to overcome the pathophysiological insult induced by dystrophin-deficiency – indeed, metabolism and cellular redox status are highly regulated by transcriptional modification and the induction of nuclear factors. At the very least, these cases highlight that the loss of dystrophin expression is not the sole contributor to the pathological deterioration of skeletal muscle in DMD, and while indeed promoting sarcolemmal leakiness and significant damage, dystrophin-deficiency can be effectively buffered by adaptive mechanisms in some instances.

Treating DMD as a metabolic disease

Bonsett & Rudman's seminal study illustrating the critical role of ATP depletion in dystrophinopathy and the vast potential for anaplerotic correction [43] was to be one of several papers documenting the beneficial effects of targeted metabolic therapy on the pathophysiological and clinical course of DMD. ASA is a product/reactant of the purine nucleotide cycle that has the dual function of producing fumarate to stimulate the TCA cycle and ADP resynthesis via purine nucleotide salvage pathway reactions, to ultimately increase mitochondrial ATP production and reduce the loss of purines from the muscle (into the blood stream) as xanthine and hypoxanthine. Comprising a 10 year clinical trial, ASA treatment induced vast improvements in Cr retention within, and histological features and the regenerative capacity of, dystrophic muscle, which was accompanied by improved energy levels, stamina and strength [43]. In cultured DMD cells, the addition of ASA was effective at removing the overwhelming presence of intracellular lipid droplets [24], thus apparently rectifying the metabolic dysfunction either at the allosteric or metabotropic transcriptional level. Notably, cessation of ASA therapy diminished the positive benefits observed during treatment of DMD patients [43], highlighting that ongoing support of the mitochondria is pivotal to mitigating disease progression. Similar clinically beneficial effects have been observed for allopurinol [30,86,174–177] – which inhibits xanthine oxidase activity via its active metabolite oxypurinol, and therefore seems to reduce the flux of xanthine from skeletal muscle during metabolic stress – and Cr [178–181] – which increases the total PCr pool and therefore the phosphorylation potential of skeletal muscle. Notably for allopurinol, the age at which supplementation begins is crucial for induction of beneficial effects, and as with ASA, clinical improvements diminish upon caessation of therapy [176]. These studies importantly highlight the potential for the clinical use of metabolic therapies and the necessity for further investigation into the ways in which such therapies can be enhanced to improve the phenotypic progression of DMD and the quality of life of patients.

Conclusions

Although the collective literature over the past 50 years has carefully documented the plethora of metabolic abnormalities consistently observed in DMD patients, genetic carriers and genotypically identical animal models of the disease, the significance of this data has been largely ignored. As a cure for DMD remains currently elusive, every effort must be made to consider all possibilities for improved characterisation and treatment of the disease. We hypothesise an aetiological nuance at the mitochondrial level that manifests in multiple deficiencies of various metabolic pathways to culminate in severe ATP insufficiency and clinical manifestation of the disease. Of course, it cannot be denied that changes induced by dystrophin-deficiency, including disruption of the DPC and failed Ca^{2+} homeostasis, play a role in the severe and progressive muscle wasting characteristic of DMD. However, if mitochondrial defects do underlie DMD aetiology, then re-defining DMD as a metabolic myopathy and strategically targeting research funding, and treating it clinically as such, could improve patient outcomes and enhance quality of life.

Conflict of interest

No conflict of interest, financial or otherwise is declared by the authors.

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