



Biomarkers in Duchenne Muscular Dystrophy

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Abstract

Purpose of Review This review highlights the key studies investigating various types of biomarkers in Duchenne muscular dystrophy (DMD).

Recent Findings Several proteomic and metabolomic studies have been undertaken in both human DMD patients and animal models of DMD that have identified potential biomarkers in DMD.

Summary Although there have been a number of proteomic and metabolomic studies that have identified various potential biomarkers in DMD, more definitive studies still need to be undertaken in DMD patients to firmly correlate these biomarkers with diagnosis, disease progression, and monitoring the effects of novel treatment strategies being developed.

Keywords Biomarkers · Duchenne muscular dystrophy · DMD-associated cardiomyopathy · Metabolomics · Proteomics

Introduction

Duchenne muscular dystrophy (DMD) is an X-linked, progressive neuromuscular disorder that affects approximately 1 in 3500–5000 male live births worldwide [1–3]. Caused by mutations on the Xp21 chromosome in the dystrophin *DMD* gene, DMD is characterized by skeletal muscle wasting, diaphragmic weakness leading to chronic restrictive lung disease, and progressive cardiomyopathy [4, 5]. Patients as early as 3 years of age can exhibit signs of DMD, including ambulation problems, calf hypertrophy

(or pseudohypertrophy), difficulty climbing stairs, Gower's sign, fatigue, and dyspnea secondary to diaphragmic weakness as well as progressive cardiomyopathy [6, 7]. Additionally, cardiomyopathy, which has become the leading cause of mortality for DMD patients, is present in the majority of DMD patients by age 10, and almost all DMD patients are expected to have clinical cardiac involvement by age 18 [8–10]. Although in 2021 more DMD patients are living into their 30s and the vast majority of these patients will succumb to complications related to cardiomyopathy, there remains much heterogeneity regarding the age of death among DMD patients.

DMD represents the most severe form of dystrophinopathy and today the majority of suspected cases of DMD are diagnosed by genetic testing with a minority confirmed with a muscle biopsy. Although genetic testing has advanced the diagnostic toolkit to make the diagnosis of DMD, early in the disease process a diagnosis can still be challenging to make with difficulties in distinguishing DMD from Beck muscular dystrophy (BMD), another form of dystrophinopathy. Once a diagnosis of DMD has been made, there remains a paucity of reliable non-invasive measures of disease progression. Many clinical trials use the 6-minute walk test, a measure of the walking distance over a 6-minute time period, as a physical indicator of DMD progression [11, 12]. While physical assessments such as the 6-minute walk test can be useful as a prognostic tool, there are many limitations and confounding factors that make these tests unreliable,

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including a lack of cooperation in pediatric patients [12]. Magnetic resonance imaging (MRI) has been proven to be an accurate, non-invasive measure for disease progression in DMD patients, particularly in monitoring progressive skeletal muscle weakness and cardiac function [13, 14]. Unfortunately, there are limitations related to the accessibility and length of the procedure which reduces the utility of MRIs. Therefore, the use of molecular biomarkers as prognostic indicators has the potential to be more accessible and predictive of disease progression.

Currently, serological levels of creatine kinase (CK) are used as the primary laboratory measure for DMD. The CK level can be used for monitoring DMD myonecrosis and related inflammation [15], while the CK and troponin levels are both very useful as measures of DMD-associated cardiomyopathy, which has become the leading cause of death among DMD patients over the past several decades [10, 16–18]. However, CK and troponin levels do not always correlate reliably with cardiac MRI data, and the CK level especially may not be an ideal biomarker due to its elevated levels in other muscular dystrophies, as well as its variability by age, physical activity, drug treatment, and other factors [19]. Furthermore, CK and troponin levels are not reliable measures for detecting female carrier status or monitoring carrier disease progression, with a specificity of approximately 50% and a sensitivity of 33% [19].

As noted above, very few reliable diagnostic and prognostic measures for DMD exist currently, and many of these measures are costly and inefficient. Therefore, more reliable biomarkers are needed to accurately identify and measure disease progression in both DMD patients and carriers. This review will provide further insight into the current state of biomarker research for DMD. Thus, continued research on discovering biomarkers and metabolomics with high levels of sensitivity and specificity can lead to more accurate tracking of underlying pathophysiological processes as well as facilitate more efficient and effective clinical trials and drug development by more accurately predicting treatment response and efficacy [20].

Current State of Biomarker Research and Potential Biomarkers

By applying various approaches to uncovering potential biomarkers and profiling serum proteins, such as stable isotope labeling, mass spectrometry (MS)-based proteome screening, and multiplexed antibody or aptamer-based assays [20, 21], numerous studies have found differences in protein profiles between DMD patients and healthy controls (Table 1). Many of these studies focused largely on circulating serum muscle-derived biomarkers, which were associated with myonecrosis and inflammation that occur in DMD muscle.

However, biomarkers associated with muscle function, cardiac status, inflammation, and metabolomics can potentially describe different disease states of DMD patients.

Biomarkers Associated with Muscle Function

The heart is responsible for circulating blood throughout the body. A study by Hathout et al. (2014) utilized stable isotope labeling and high-precision mass spectrometry to identify serum biomarkers in both *mdx* mice, a murine model of DMD, and in human DMD patients [12]. Label-free proteome profiling on DMD patients and healthy controls found that of 23 elevated proteins in *mdx* mice, 20 were also significantly elevated in DMD patients, including various myofibrillar proteins, glycolytic enzymes, and transport proteins. Of these potential biomarkers, adiponectin and matrix metalloproteinase-9 (MMP-9) were the only two proteins which increased with age, while all other biomarkers decreased with age, similar to CK levels [12]. MMP-9 is a particular protein of interest and is among the potential biomarkers for DMD that have been the most extensively studied [20]. As a protease involved in extracellular matrix (ECM) remodeling and the fibrotic and inflammatory processes associated with dystrophinopathies, MMP-9 has been shown to be increased not only in DMD mouse models, but also in DMD patients [22–24]. Other proteins of significance included myosin light chain 1/3, filamin-C, and myomesin-3, all of which were increased in serum obtained from DMD patients and the significance of the increase has been further investigated in subsequent studies [19, 25].

Anaya-Segura et al. (2015) also performed analyses on potential biomarkers in DMD, specifically examining serum levels of MMP-9, matrix metalloproteinase-2 (MMP-2), tissue inhibitor of metalloproteinases-1 (TIMP-1), myostatin (GDF-8), and follistatin (FST) [19]. By comparing these potential markers in steroid-naïve DMD patients with healthy controls, the study found particular promise in GDF-8, and FST to distinguish patients with DMD [19]. GDF-8 is involved in a signaling pathway which inhibits skeletal muscle regeneration, and the study found that GDF-8 was significantly decreased in DMD patients, while FST, the inhibitory counterpart to GDF-8, was significantly increased in the DMD study population [19]. MMP-9 was also significantly altered in DMD patients and correlated with the results of the studies previously mentioned. While the evaluation of these specific molecules suggests great promise as potential biomarkers in DMD patients, the authors noted certain limitations. Alterations in MMP-9 levels, for instance, were not exclusive to DMD and occur in a number of other diseases, and so additional biomarkers may be needed to create a more sensitive measure of disease progression [19].

Table 1 Potential circulating biomarkers in DMD

DMD vs control	Notable biomarkers	Relevant studies
Skeletal muscle function		
↑	Matrix metalloproteinase-9 (MMP-9)	Hathout et al. <i>Hum Mol Gen</i> 2014, Nadarajah et al. <i>Neuromuscul Disord</i> 2011, and Zocevic et al. <i>Neuromuscul Disord</i> 2015
↑	Adiponectin (ADPN)	Hathout et al. <i>Hum Mol Gen</i> 2014
↑	Myomesin-3 (MYOM3)	
↑	Filamin-C (FLNC)	
↑	Myosin light chain 1/3 (MLC 1/3)	Hathout et al. <i>Hum Mol Gen</i> 2014, Ayoglu et al. <i>EMBO Mol Med</i> 2014
↑	Matrix metalloproteinase-2 (MMP-2)	Anaya-Segura et al. <i>Molecules</i> 2015, Miyazaki et al. <i>Hum Mol Gen</i> 2011
↑	Tissue inhibitor of metalloproteinases-1 (TIMP-1)	Anaya-Segura et al. <i>Molecules</i> 2015
↓	Growth differentiation factor-8 (GDF-8)	
↑	Follistatin (FST)	
↑	Heat shock protein 70 (HSP70)	Hathout et al. <i>Proc Natl Acad Sci</i> 2015
↑	Mitogen-activated protein kinase 12 (MAPK-12)	
↑	Calcium-calmodulin-dependent protein kinase II α (CaMK-II α)	
↑	Persephin (PSPN)	
↓	Proto-oncogene tyrosine protein kinase receptor Ret (RET)	
↑	Myoglobin (Mb)	
↑	Malate dehydrogenase 1 (MDH1)	
↑	Heart-type fatty acid-binding protein-3 (FABP3)	
↑	Titin (TTN)	Oonk et al. <i>Proteomics Clin Appl.</i> 2016 and Misaka et al. <i>Clin Chem Acta.</i> 2019
↑	Myosin	
↑	Carbonic anhydrase 1 (CA1)	
↑	Fibronectin (FN)	Martin et al. <i>Proteomics Clin Appl</i> 2014
↑	Carbonic anhydrase 3 (CA3)	Ayoglu et al. <i>EMBO Mol Med</i> 2014
↑	Malate dehydrogenase 2 (MDH2)	
↓	miR-1	Cacchiarelli et al. <i>EMBO Mol Med</i> 2011
↑	miR-133	
↑	miR-206	
Cardiac function and inflammation		
↑	Interleukin 1 receptor-like 1 protein (ST2)	Anderson et al. <i>Pediatr Cardiol</i> 2017
↑	Heart-type fatty acid-binding protein (FABPH)	Hathout et al. <i>Hum Mol Gen</i> 2014
↑	Matrix metalloproteinase-1 (MMP-1)	Soslow et al. <i>J Card Fail</i> 2019
↑	Matrix metalloproteinase-7 (MMP-7)	
↑	Matrix metalloproteinase-9 (MMP-9)	
↑	Matrix metalloproteinase-10 (MMP-10)	
↑	Tissue inhibitor of metalloproteinases-1 (TIMP-1)	
↑	Tissue inhibitor of metalloproteinases-2 (TIMP-2)	
↑	Tissue inhibitor of metalloproteinases-3 (TIMP-3)	
↑	Tissue inhibitor of metalloproteinases-4 (TIMP-4)	
↑	Interleukin-6 (IL-6)	Cruz-Guzman et al. <i>Biomed Res Int</i> 2015
↑	Tumor necrosis factor-alpha (TNF- α)	

Table 1 (continued)

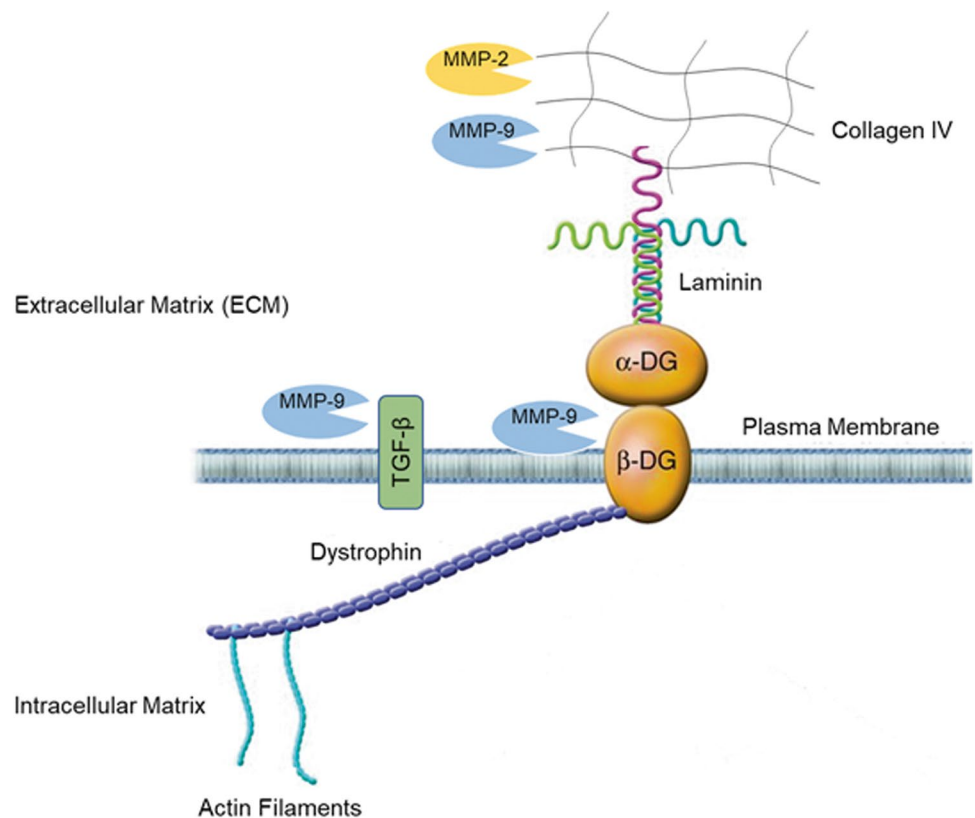
DMD vs control	Notable biomarkers	Relevant studies
↑	Phospholipase A2	Hathout et al. <i>Proc Natl Acad Sci</i> 2015
↑	C-X-C motif chemokine 10	
↓	Growth differentiation factor-8 (GDF-8) or myostatin	
↑	Growth differentiation factor-11 (GDF-11)	
↑	Thrombospondin-4 (TSP-4)	
↑	Troponin I (TnI)	Matsumura et al. <i>Brain Dev</i> 2007
↑	miR-222	Becker et al. <i>J Cardiovasc Magn Reson</i> 2016

It is well-known that MMP-2 and MMP-9 are likely involved in the pathology of dystrophin-deficient muscle (Fig. 1). Dystrophin stabilizes muscle cells by interacting with F-actin, the transmembrane glycoprotein, and the ECM. Lack of dystrophin causes sarcolemma instability and upregulation of MMPs in muscle. MMP-2 degrades collagen type IV and may be associated with ECM remodeling during muscle regeneration and fiber growth. MMP-9 also degrades collagen type I, III, IV, and V in interstitial space and cleaves β -dystroglycan. MMP-9 also converts membrane-bound latent transforming growth factor beta (TGF- β) into an active form by proteolytic cleavage. In contrast, MMP-9 may be involved predominantly in the inflammatory

process during muscle degeneration [26, 27]. Therefore, MMP2/9 could serve as potential DMD biomarkers for disease progression. Future studies are still required to confirm whether serum MMP levels can be used to monitor therapeutic responses to a variety of novel therapeutic modalities proposed to treat DMD.

Another similar study in 2015 by Hathout et al. investigated biomarkers on a larger scale, quantifying over a thousand serum proteins in two separate cohorts of DMD patients [28]. This study utilized the SOMAscan assay, a modified assay based on properties of the SOMAmer (Slow Off-rate Modified Aptamer), to profile serum proteins [28]. Ultimately, the study found 24 proteins elevated and 20

Fig. 1 The role of matrix metalloproteinases in myocytes



proteins decreased in DMD patients, including some novel proteins. The trends in many of these proteins, like in the previous studies, were similar to CK levels in that they were most notably increased at the beginning of disease progression and declined with age. Additionally, most proteins were of muscle origin, and novel proteins of interest included heart shock protein 70 (HSP70), mitogen-activated protein kinase 12 (MAPK-12), and calcium-calmodulin-dependent protein kinase II α (CaMK II α). Several proteins involved in inflammation were also noted as significant, including phospholipase A2 (PLA2) and C-X-C motif chemokine 10 (CXCL10), which could play important roles in monitoring treatment response in DMD patients. Persephin, a protein included in the glial cell line–derived neurotrophic factor (GDNF) family of necrotic factors, was another protein of interest, as it is involved in reinnervation processes in motor neurons. The authors hypothesized that the increase of persephin and decrease in proto-oncogene tyrosine protein kinase receptor Ret (RET) may indicate the processes of reinnervation and denervation that occurs in DMD patients, and that these proteins have great promise as potential biomarkers for therapies targeted towards stabilizing muscle fibers [28].

Many other studies have suggested additional molecular markers that may serve as potential biomarkers for DMD. Oonk et al., in their comparative MS- and immunoassay-based proteome screening of DMD patient sera, verified that titin, myosin, and carbonic anhydrase I (CA1) could serve as potential biomarkers [29]. Martin et al., utilizing a MS-based bottom-up pipeline, found that fibronectin was significantly increased in DMD patients compared to healthy age-matched controls, and may be useful as a biomarker due to the apparent correlation with disease progression [30]. This study also commented on the interaction between fibronectin and MMP-9, a potential biomarker mentioned by several of the previously cited studies and stated that while no correlation was found between the two protein concentrations in this study, this could be due to certain feedback mechanisms resulting from fibronectin's role as a substrate for MMP-9 [30]. Further studies of fibronectin as a biomarker for DMD may provide further insight into its potential as a molecular marker.

A study by Ayoglu et al. utilized multiplexed antibody arrays to profile DMD patient sera, revealing 11 potential biomarker candidates for DMD [31]. Among these, carbonic anhydrase 3 (CA3), myosin light chain 3 (MLC3), and malate dehydrogenase 2 (MDH2) were significantly elevated in DMD patients, all at higher levels as compared to CK levels. While the exact mechanisms resulting in increases in these proteins are unknown, impaired secretion due to tissue leakage and membrane disruption is thought to play a role [31]. CA3 is of particular interest and has been shown to be elevated in healthy individuals with skeletal muscle injury,

potentially a more sensitivity biomarker of skeletal muscle injury as compared to CK levels [31, 32]. In this study, CA3 levels distinguished not only DMD patients from healthy controls, but also DMD patients from BMD patients, suggesting that CA3 may serve as a more sensitive biomarker for DMD [31].

In addition to proteins, many studies have identified the utility of serum and plasma microRNAs (miRNA), or dystromirs, as potential biomarkers for DMD, which are released into the blood as a result of muscle damage and typically correlate with the severity of muscle deterioration [33]. A study by Cacchiarelli et al. proposed that miR-1, miR-133, and miR-206 were enriched in the serum of DMD patients, and had potential as promising biomarkers for both the diagnosis of DMD and probably also for prognostic monitoring and assessment of therapies [33]. The muscle-specific miR-1, miR-133, and miR-206 have been demonstrated to be necessary for proper skeletal and cardiac muscle development and function [34]. Importantly, the study found that these miRNA markers were more sensitive than CK levels, and that high levels of miRNA were found to correlate with lower levels of ambulation in DMD patients [33]. The serum stability of miRNAs was stated to be an additional advantage of miRNAs in comparison to CK levels, and subsequent studies have validated these markers as particularly promising biomarkers for DMD [33, 35].

Biomarkers Associated with Cardiac Function and Inflammation

As cardiomyopathy is present in almost all DMD patients, there has been a push to discover biomarkers for assessing cardiac function, though significant progress still needs to be made to provide concrete cardiac biomarker data. While standard biomarkers of heart failure such as N-terminal pro-b-type natriuretic peptide (NT-proBNP) and left ventricular ejection fraction (LVEF) are often not sensitive enough during the early stages of DMD-associated cardiomyopathy [16, 36], several potentially novel molecular markers associated with cardiomyopathy have been identified, including interleukin-1 receptor-like protein (ST2), thrombospondin-4 (TSP4), and heart-type fatty acid-binding protein (FABPH) [12, 16, 37]. A recent study by Soslow et al. also found that several matrix metalloproteinases (specifically MMP-1, MMP-7, MMP-9, and MMP-10), as well as several tissue inhibitors of metalloproteinases (TIMP-1–4), were elevated in the sera from DMD patients as compared with sera from healthy controls [38]. These proteins function to regulate inflammation and play a role in remodeling the extracellular matrix during fibrosis, both important issues in DMD-associated cardiomyopathy [38]. MMP-7 was of particular interest, as it was found to correlate inversely with LVEF and

previous in vitro studies have corroborated elevated levels of MMP-7 with cardiomyocyte dysfunction [38].

Many potential biomarkers associated with the inflammatory processes have also been identified and studied, including interleukin-1, interleukin-6, tumor necrosis factor- α (TNF- α), C-reactive protein, leptin, and adiponectin. A study by Cruz-Guzman et al. found that serum levels for these cytokines were all relatively high in DMD patients compared to healthy controls at the early stages of disease and decreased with disease progression and loss of ambulation [39]. Growth differentiation factor 11 (GDF-11), a protein believed to play a role in reversing cardiomyopathy and skeletal muscle degradation, has also been identified as a potential cardiac biomarker, and its interaction with myostatin or GDF-8 may shed further insight into the potential of both of these proteins as biomarkers [28].

Just as miRNA or dystromir data has been useful for determining skeletal muscle biomarkers, dystromirs have also shown promise in determining cardiac abnormalities in DMD patients. A study by Becker et al. correlated the upregulation of specific cardiac miRNAs with MRI data and late gadolinium enhancement (LGE) positivity and found that miR-222 may serve as a potential biomarker [40]. Another study by Jeanson-Leh et al. found additional biomarkers associated with cardiac disease dysregulated in the sera of DMD patients, including miR-208a, miR-208b, and miR-499 [41]. miR-208b and miR-499 in particular were significantly elevated in DMD patients [41]. However, the study also utilized a golden retriever muscular dystrophy (GRMD) model of DMD to uncover potential dystromirs and found that there were no correlations between serum levels of these markers and cardiac functional assessment [41]. Limited research has been done on cardiac miRNAs as molecular markers of DMD-associated cardiomyopathy, and so further research is needed to determine the utility of miRNAs as biomarkers in assessing DMD-associated cardiomyopathy.

Metabolomics as Biomarkers in Duchenne Muscular Dystrophy

Metabolomics, a scientific field of study similar to proteomics, can also be utilized in the context of DMD to create a profile of screening agents to more accurately and effectively diagnose, monitor, and treat cardiomyopathy in DMD patients. There have been few comprehensive untargeted clinical metabolomic studies of DMD patients and none of these studies specifically correlated with DMD-associated cardiomyopathy [42–44]. Without cardiac-specific studies, reported metabolomic derangements in DMD-associated cardiomyopathy may not be limited to the myocardium.

One untargeted metabolomic study by Spitali et al. (2018) uncovered 15 potential metabolites, all involved in energy metabolism, mitochondrial alterations, or other metabolic processes associated with DMD, which significantly differentiated DMD patients from healthy controls [42]. These included metabolites involved in testosterone metabolism (such as 5- α -dihydrotestosterone glucuronide and dehydroisoandrosterone 3-sulfate), metabolites involved in amino acid metabolism (such as L-aspartic acid, L-serine, ornithine, p-coumaric acid, 2-hydroxycaproic acid), imidazole acetic acid, guanidinoacetic acid, erythrose, 5-methoxyindoleacetate, citrulline, DL-p-hydroxyphenyllactic acid, creatine, and creatinine [42]. While testosterone metabolites were found to be altered in DMD patients, treatment with glucocorticoids may have been the cause of the decrease and could therefore have been a confounding factor [42]. While low testosterone is associated with chronic glucocorticoid treatment, it is also associated with poor prognosis and increased risk of mortality in male heart failure patients [45, 46]. Furthermore, testosterone replacement therapy after myocardial injury improved myocardial metabolism by PPAR α augmentation [47]. Despite the potential limitation in the study, further studies investigating these specific metabolites could reveal more effective treatment and monitoring measures for DMD.

Another untargeted metabolomic study of DMD patients identified 14 significantly altered serum metabolites, as compared to healthy control patients [43]. Upregulated metabolites included arginine, creatine, and two unknown metabolites with mass/charge (m/z) ratios of 357 and 312. Decreased metabolites included creatinine androgen derivatives and additional unknown metabolites. Increasing creatine/creatinine ratios strongly correlated with age and disease progression in DMD patients, whereas these ratios decreased with age in healthy controls [43]. Likewise, a urine metabolomic study in the *mdx* murine model of DMD identified upregulated creatine, an unknown 357 m/z metabolite, biliverdin and hypusine as well as an upregulated ratio of creatine/creatinine [48]. The study also evaluated effects of corticosteroid treatment upon *mdx* urine metabolites and found the downregulation of creatine, unknown 357 m/z metabolite, hypusine and biliverdin [48]. Biliverdin is a metabolite of the heme oxygenase degradation pathway, a regulator of oxidative stress and has anti-inflammatory properties, which could be related to enhanced myocardial inflammation found in DMD-associated cardiomyopathy [49]. Hypusine is an amino acid exclusive to the eIF5A translation factor family, which modulates cell proliferation and apoptosis and promotes glycolysis during ischaemia [50–52]. Creatine and creatinine are part of the phosphocreatine pathway, in which creatine kinase produces adenosine triphosphate (ATP) from phosphocreatine and adenosine diphosphate (ADP), thereby providing ATP during high

energetic demand. Serum and urinary creatine and creatinine are associated with worsening outcomes in heart failure following myocardial infarction [53, 54]. A caveat of the study was the inability to identify all of the significant metabolites, as well as the low pH of the eluent, which possibly skewed protonation of metabolites in LC-MS [48].

An untargeted metabolic study of isolated skeletal muscle identified enhanced phospholipids in DMD, using ultra-high-resolution mass spectrometry imaging [44]. Specifically, upregulated phospholipids included lysophosphatidylcholine, phosphatidylcholine, phosphatidic acid, sphingomyelin, phosphatidylserine, and triacylglycerol, with diminished ATP levels [44]. A potential limitation of this study was the invasiveness of the *in vitro* technique, requiring isolation of skeletal muscle tissues from patients as well as introduction of potential artifacts from sample processing. Results of the study were consistent with previous studies that have identified deficiencies in ATP and enhancements in phospholipids, reflecting the clinical presentation of dyslipidemia in DMD [55–59].

In addition to the few untargeted metabolomic studies, there have been many targeted metabolite clinical studies of DMD which have aided in the development of potential metabolic biomarkers [55–72]. Overall, these studies have identified alterations in creatine, purines, citrulline, nitric oxide, prostaglandin, L-carnitine, leptin, ferritin, phosphatidylcholine, and other Krebs cycle metabolites [55–72]. Upregulation of urinary prostaglandin metabolites in DMD patients may be pathological rather than compensatory, as administration of inhibitors of prostaglandin D2 (PGD2) in *mdx* mice reduced muscle necrosis [61]. Upregulation of metabolites of the ATP-recycling phosphocreatine pathway, especially creatine, has been extensively studied in DMD and other muscular dystrophies, as discussed earlier. High levels of urinary ferritin in DMD may reflect myoglobin-mediated iron release from necrotic muscle in DMD [62]. Early and late stages of DMD and BMD have demonstrated markers of metabolic syndrome and elevated levels of leptin, indicating altered fat metabolism [39, 60]. Likewise, enhanced circulating and skeletal muscle phospholipids have been identified in DMD [58, 72]. Altered circulating phospholipids and dyslipidemia have cardiovascular complications including atherosclerosis and are considered a major risk factor for adverse cardiovascular events.

Downregulated metabolites identified in DMD include metabolites of citrulline [73], nitric oxide [71], Krebs cycle metabolites [74], L-carnitine, and purine metabolites [66]. As L-carnitine is necessary for transport of fatty acids into the mitochondrial matrix and aids in reduction of oxidative stress [75], its downregulation in early stages of DMD [66, 67, 72] may exacerbate the enhanced levels of circulating phospholipids found in DMD [58, 72]. For nitric oxide metabolism, L-citrulline is converted to L-arginine, which is

then converted to nitric oxide (NO) by neuronal nitric oxide synthase (nNOS) in muscle cells. As dystrophin binds nNOS for the generation of NO from L-arginine, reduced levels of L-citrulline and NO have been found in DMD [76]. Vasodilation is regulated by nitric oxide, lowering blood pressure, and hemodynamic load of the heart. Blood pressure regulation is a therapeutic target for limiting cardiac damage in DMD [77]. In the study, plasma levels of arginine were paradoxically elevated [43], which may be due to leakage from necrotic muscle cells, sample preparation, glucocorticoid administration, or age differences between the studies. DMD patients also have reduced levels of purines (adenosine, guanosine, hypoxanthine, and xanthine), which is evident in diminished ATP generation in DMD muscles [66, 78]. Furthermore, in DMD, the stress-responsive activation of xanthine oxidase converts purine substrates to superoxide, hydrogen peroxide, and uric acid [63, 64]. Extensive studies of xanthine oxidase inhibitors (allopurinol) have shown mixed efficacy in preventing or improving function in DMD patients, possibly due to drug interaction of glucocorticoids as well as the lack of sufficient muscle cell populations for testing effects of enhanced purine levels [68, 69, 79]. Metabolite levels that may be associated with enhanced oxidative stress and reduced ATP generation may be further investigated to determine the role in myocardial dysfunction in DMD.

The murine *mdx* and GRMD models of DMD have been employed for untargeted metabolomic studies [80–85]. Tsunaka et al. identified altered plasma levels of metabolites necessary for energy production (glutamine), amino acid interconversion, nucleotides, and nitric oxide metabolites [80]. The serum lipid profile of *mdx* mice was elevated and the taurine-creatinine ratio was increased, consistent with previous reports [81]. In *mdx* muscle satellite cells and serum, untargeted metabolomics identified elevated levels of purine/pyrimidine metabolites (hypoxanthine, oxypurinol, and adenosine) and fatty acid metabolites [82]. Depleted metabolites included amino acids (D-alanine, thymidine, and D-glutamate) and diminished Krebs cycle associated metabolites (succinic acid, lactic acid, oxoglutaric acid) [82]. Additionally, *mdx*-derived adipose progenitor cells had elevated acylcarnitines, which inhibit adipose progenitor cell proliferation [82]. In a series of metabolomic studies performed in *mdx* mice by Griffin et al., the investigators identified enhanced lipid, lactate, glutamate, lysine, and taurine, whereas diminished metabolites included glucose, choline, malonate, beta-hydroxybutyrate, and cholesterol [83, 86–88]. Gulston et al. found a correlation between metabolomic and proteomic measurements in *mdx* cardiac tissue, ultimately identifying perturbations in phospholipid, amino acid, carbohydrate, and Krebs cycle metabolites [84]. The GRMD untargeted metabolomic study involving DMD skeletal muscle found altered levels of arginine and proline

metabolites, with elevated glutamate and oleic acid [85]. Overall, these metabolite profiles may reflect altered metabolism in DMD, possibly myocardial perturbations, which should be evaluated.

Mitochondrial dysfunction in particular is a major consequence of DMD, primarily due to the disruption of calcium gradients and membrane potentials, and the buildup of intracellular calcium [89]. The mitochondrial dysfunction results in major metabolic alterations, which were observed by Lindsay et al. by examining urinary metabolite levels involved in the Krebs Cycle of *mdx* mice [65]. Profiling metabolites of *mdx* and wild-type mice at 1 and 3 months via MS-based metabolic screening, the study found that nearly all metabolites involved in the Krebs cycle were depleted in *mdx* mice at 3 months [65]. Additionally, of 225 measured metabolites, 8% were reduced and 2% were elevated in the *mdx* cohort after 1 month. After 3 months, the percentages rose to 28% reduced and 11% elevated. Principal component analysis (PCA) was performed to compare wild-type and *mdx* mice metabolite levels and found that citrate, cis-aconitate, AKG, succinate, and malate, all metabolites of the Krebs Cycle, were significantly reduced in *mdx* mice after 3 months, while the remaining Krebs metabolites, isocitrate and fumarate, did not significantly differ. Succinate was especially decreased in *mdx* mice, with a 6.4-fold decrease as compared to wild-type mice. The study also addressed the possibility of reduced exercise capacity in the *mdx* cohort as a confounding factor, stating that there was no difference in physical exercise between the *mdx* and wild-type cohorts. Further studies validating these potential biomarkers, particularly succinate, could play an essential role in improving DMD diagnosis, disease progression monitoring, and treatment strategies [65].

Overall, the untargeted metabolomic and targeted metabolite studies of *mdx* and GRMD models of DMD and DMD patients, have highlighted alterations in lipid metabolism, energy production, and nitric oxide metabolism. Compensatory and pathological mechanisms associated with these alterations continue to be investigated, especially in DMD-associated cardiomyopathy. DMD patients develop dyslipidemia, which may be associated with reduced L-carnitine and elevated phospholipid metabolites, yet further investigations are needed to determine the cardiac involvement of these metabolites. Overactive xanthine oxidase-mediated conversion of adenine and guanine purines to uric acid and reactive oxygen species remains a potential avenue for therapeutic intervention as well as a potential biomarker. Nitric oxide metabolites are also the focus of therapeutic interventions. Challenges for the usage of these metabolites as biomarkers of disease severity or as diagnostic biomarkers include effects of medications upon these metabolites, especially glucocorticoids as well as variability of muscle necrosis in age-matched patients.

Summary

Although there have been a number of proteomic and metabolomic studies that have identified various potential biomarkers in DMD, more definitive studies, particularly studies related to biomarkers associated with cardiac dysfunction, still need to be undertaken in DMD patients to firmly correlate these biomarkers with diagnosis, disease progression, and monitoring the effects of novel treatment strategies being developed.

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Declarations

Conflict of Interest Dr. Pradeep Mammen declares the following conflicts of interests: American Heart Association (AHA) (member of the AHA Career Development Research Grant Committee), California Institute of Regenerative Medicine (member of the Grants Working Group), CareDx Inc. (Site PI for the SHORE Registry), Catabasis Inc. (research grant), NIH (research grants and ad hoc grant reviewer for the NIH MOSS, SMEP, and TDPS Study Sections), Dyne Therapeutics (scientific consultant and member of the Scientific DMD Advisory Board), Novartis Gene Therapies Inc. (member of the Data Monitoring and Safety Committee), PhaseBio Inc. (research grant and member of the Scientific Advisory Board), and Revidia Therapeutics Inc. (member of the Scientific Advisory Board). The other authors have declared that no conflicts of interest exist as it pertains to the subject of the current study.

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