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Fatty acid profile of skeletal muscle phospholipid is altered in mdx mice and is predictive of disease markers

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ABSTRACT

The mdx mouse is a model for Duchenne muscular dystrophy. The fatty acid (FA) composition in dystrophic muscle could potentially impact the disease severity. We tested FA profiles in skeletal muscle phospholipid (PL) and triglyceride in mdx and control (con) mice to assess associations with disease state as well as correlations with grip strength (which is lower in mdx) and serum creatine kinase (CK, which is elevated in mdx). Compared with con, mdx PL contained less docosahexaenoic acid ($P < .001$) and more linoleic acid ($P = .001$). Docosahexaenoic acid contents did not correlate with strength or serum CK. Linoleic acid content in PL was positively correlated with CK in mdx ($P < .05$) but not con. α-Linolenic acid content in PL was positively correlated with strength in mdx ($P < .05$) but not con. The FA profile in triglyceride showed less difference between groups and far less predictive ability for disease markers. We conclude that profiling the FA composition of tissue lipids (particularly PL) can be a useful strategy for generating novel biomarkers and potential therapeutic targets in muscle diseases and likely other pathological conditions as well. Specifically, the present results have indicated potential benefits of raising content of particular n-3 FAs (especially α-linolenic acid) and reducing content of particular n-6 FAs (linoleic acid) in PL of dystrophic muscle.

1. Introduction

Duchenne muscular dystrophy (DMD) is an X-linked muscle disease that leads to fragility of the sarcolemma and ultimately to a shorter life span in male humans. Although lack of functional dystrophin is the primary defect, the events downstream of membrane destabilization are potential sites of intervention for novel therapeutic strategies. Skeletal muscle in patients with DMD experiences substantial inflammation and oxidative stress as evidenced by tumor necrosis factor–α (TNF-α) content [1], nuclear factor κB (NFκB) activity [2], and protein carbonylation [3]. Patients with DMD also display elevated intracellular calcium levels in skeletal muscle [4]. The mdx mouse is the primary animal model for DMD and, like humans with DMD, lacks the dystrophin protein and experiences higher NFκB activity [5-10], elevated calcium levels [4,11-14], and higher protein carbonylation (oxidative damage) [15] in muscle. Intervention studies in the mdx mouse suggest that oxidative stress [7,8] and inflammation [5,6] are centrally involved in disease progression, and it is also known that these processes can be mediated by fatty acids (FAs) [16,17]. As well, the membrane characteristics within a tissue can be impacted by the FA composition [18,19]. Thus, there are reasons to suspect that dysregulation of the FA composition in tissue lipids may contribute to the magnitude of skeletal muscle degeneration and dysfunction in DMD.

Author contributions: MAT contributed to study design, performed laboratory analyses, analyzed results, interpreted findings, and wrote the manuscript. GCH conceived of and designed the experiment, performed laboratory analyses, analyzed results, interpreted findings, and wrote the manuscript.

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Corticosteroids are currently the standard treatment of DMD but are associated with undesirable adverse effects, and the degree of efficacy is not satisfactory [20]. A better understanding of the changes in molecular profile within tissues could potentially lead to new hypotheses that may ultimately lead to better treatments. It seems plausible that skeletal muscle FA composition may be altered in DMD; and perhaps, normalization of the profile would be a potential avenue for treatment. Some limited attempts have been made to assess the profile of individual FAs in muscle phospholipid (PL) of boys with DMD [21-23], but studies are inconclusive because of lack of dietary control [21-23], and issues related to age matching and sample handling [22]. In addition, in patients with DMD [24] and mdx mice [25,26], the ratio of 34:2 to 34:1 phosphatidyl choline (PC) was compared between highly diseased vs less-impacted portions of dystrophic skeletal muscle. Results indicated a reduced 34:2 to 34:1 PC ratio in cases of significant muscle pathology, but it could not be known which specific FAs were responsible for the observation. It could be inferred that the 18:2 to 18:1 FA ratio was likely reduced in PL of the diseased muscle fibers; but this could be due to changes in linoleic acid (LA) (18:2), oleic acid (18:1), vaccenic acid (18:1), or a combination of all 3. Furthermore, we expected that other FAs aside from those investigated in these studies would be of interest. To gain specific information, we sought to saponify the FAs from isolated PL and then to perform an extensive chromatographic separation of FAs including resolution of positional and geometrical isomers. The previous studies [21-26] measured a limited profile of FAs in muscle lipids, and we recently developed a procedure to assess a wide range of FAs in muscle PL and triglyceride (TG) by high-performance liquid chromatography (HPLC) [27]; thus, we sought to apply this method to dystrophic and control muscle samples to find new potential mediators of the disease. Control of long-term dietary intake in such studies would be helpful and is difficult in humans, but control of lifelong dietary intake in animal models is easily achieved for initial hypothesis testing. We hypothesized that the FA composition in muscle PL of mdx mice would be different from control mice and that specific FA abundances in PL would predict interindividual variations in severity of muscular dystrophy through correlation with disease markers. As well, we hypothesized that the FA composition of TG would not be different between groups but rather would reflect the dietary FA composition.

2. Methods

2.1. Animals

This protocol was approved by the Rutgers University Animal Care and Facilities Committee. Male C57BL/10ScSn-Dmd^{mdx}/J mice (mdx) (n = 8) and male C57BL/10ScSnJ wild-type control mice (con) (n = 8) were purchased from Jackson Laboratories (Bar Harbor, ME) at 7 weeks of age and housed in the animal facility at Rutgers University. The mice were maintained on a 12-hour light/dark photoperiod with all mice allowed ad libitum access to food and water. For their lifetime, all mice consumed the LabDiet 5K52 diet (Purina Mills, Richmond, IN, USA). Upon arrival at our animal facility, the strains were given the same lot of 5K52 diet as each other. We analyzed the FA composition of the diets that were consumed by the mice before arrival at our facility (provided by Jackson Laboratories), and we analyzed a sample of our lot given to mice while housed at the animal facility at Rutgers University. The FA composition was similar between these diet samples, and we present in Table 1 the FA composition of the 5K52 diet fed at our facility.

2.2. Body composition and grip strength

At 11 weeks of age, mice were weighed and body composition was analyzed by an EchoMRI system (Echo Medical Systems, Houston, TX). Grip strength was measured with a Grip Strength Meter (Columbus Instruments, Columbus, OH) using a modified protocol of Payne et al [28]. Briefly, to measure front-limb grip strength, mice were held by the tail and lowered onto a flat-mesh grid attached to a force transducer until only their front limbs grasped. They were

Table 1 – Dietary FA composition (%)

FA	%
Myristic (14:0)	2.23
Myristoleic (14:1n-5)	0.40
Myristelaidic (*trans*-14:1n-5)	0.28
Palmitic (16:0)	15.27
Palmitoleic (16:1n-7)	1.61
Palmitelaidic (*trans*-16:1n-7)	ND
Stearic (18:0)	3.81
Oleic (18:1n-9)	19.54
Elaidic (*trans*-18:1n-9)	ND
cis-Vaccenic (18:1n-7)	1.31
trans-Vaccenic (*trans*-18:1n-7)	ND
Linoleic (18:2n-6)	46.34
Linoelaidic (*trans*,*trans*-18:2n-6)	ND
α-Linolenic (18:3n-3)	5.89
γ-Linolenic (18:3n-6)	ND
Arachidic (20:0)	0.23
Homo-γ-linolenic (20:3n-6)	ND
Arachidonic (20:4n-6)	ND
Eicosapentaenoic (20:5n-3)	1.60
Docosatetraenoic (22:4n-6)	ND
Docosapentaenoic (22:5n-3)	ND
Docosahexaenoic (22:6n-3)	1.50
Total SFA	21.54
Total USFA	78.18
Total TFA	0.28
Linoleic to α-linolenic ratio	7.87
Total n-3	8.99
Total n-6	46.34
Total n-6 to total n-3 ratio	5.16

Values are molar percentage FA composition in total lipid from 5K52 diet. Means ± SE. Total SFA indicates total saturated FAs (myristic, palmitic, stearic, arachidic); total USFA, total *cis*-unsaturated FAs (myristoleic, palmitoleic, oleic, *cis*-vaccenic, linoleic, α-linolenic, γ-linolenic, homo-γ-linolenic, arachidonic, eicosapentaenoic, docosatetraenoic, docosapentaenoic, docosahexaenoic); total TFA, total *trans*-FAs (myristelaidic, palmitelaidic, elaidic, *trans*-vaccenic, linoelaidic); total n-3, α-linolenic, eicosapentaenoic, docosapentaenoic, docosahexaenoic; total n-6, linoleic, arachidonic, docosatetraenoic; ND, not detected.

then pulled away from the force transducer parallel to the grid until their grip released, and peak tension (grams force) was recorded. To measure total-limb grip strength, an angled-mesh grid attached to the same force transducer was used. Mice were held by the tail and lowered onto the grid and allowed to grasp with all 4 limbs, and then pulled toward the force transducer until release, and peak compression (grams force) was recorded. For both front-limb and total-limb tests, each mouse underwent 5 attempts in immediate succession, and the average of the highest 3 attempts was normalized for fat-free mass (FFM) (grams force per gram FFM). Fatigue index was expressed as percentage decline in force from the sum of the first 2 attempts to the last 2 attempts and was calculated as follows: [1 – (attempt 4 + attempt 5) / (attempt 1 + attempt 2)] * 100.

2.3. *Tissue collection*

At 12 weeks of age, mice were anesthetized with a nonrecovery dose of pentobarbital (100 mg/kg), and blood was collected by cardiac puncture. Blood samples were allowed to clot, centrifuged at 13300 rpm for 8 minutes on an Accuspin microcentrifuge (Fisher Scientific, Pittsburgh, PA) to obtain serum, and then stored at –80°C until analysis. Immediately after cardiac puncture, quadriceps muscles were excised and immediately frozen in liquid nitrogen and then stored at –80°C until analysis.

2.4. *Muscle tissue analyses*

Quadriceps PL and TG were extracted using the method of Folch et al [29] and isolated by thin layer chromatography (TLC) as we described previously [27]. Briefly, samples of approximately 30 mg of quadriceps muscle were homogenized using a Potter S Homogenizer (Sartorius-Stedim, Aubagne, France) in 5 mL of chloroform/methanol (2:1 vol/vol) containing 0.05% butylated hydroxytoluene in 5-mL vessels housed in an ice-water bath. The extracts were dried under nitrogen gas, reconstituted with 40 μL chloroform, and spotted onto TLC plates (20 × 20 cm, silica gel G, Analtech, Newark, DE, USA) along with standards in separate lanes (trioleate for TG and dioleoyl L-α-phosphatidylcholine for PL). The PL and TG were isolated and recovered from TLC plates by the method that we previously reported [27], followed by addition of 40 μg of heptadecanoic acid as internal standard, saponification to free FAs, and then derivatization to their 2-nitrophenylhydrazide derivatives [27]. The derivatized compounds were then reconstituted with 150 μL methanol for analysis of FAs as their 2-nitrophenylhydrazide derivatives by HPLC using the method of Miwa et al [30] as optimized by Henderson and Tuazon [27]. The relative abundance of 22 FAs in PL and TG were calculated as molar percentages based upon the following FA external standards purchased from Sigma-Aldrich (St Louis, MO) or Nu-Check Prep (Elysian, MN): myristic acid (14:0), myristoleic acid (14:1n-5), myristelaidic acid (*trans*-14:1n-5), palmitic acid (16:0), palmitoleic acid (16:1n-7), palmitelaidic acid (*trans*-16:1n-7), stearic acid (18:0), oleic acid (18:1n-9), elaidic acid (*trans*-18:1n-9), *cis*-vaccenic acid (18:1n-7), *trans*-vaccenic acid (*trans*-18:1n-7), LA (18:2n-6), linoelaidic acid (*trans,trans*-18:2n-6), α-linolenic acid (ALA, 18:3n-3), γ-linolenic acid (18:3n-6), arachidic acid (20:0), homo-γ-linolenic acid (20:3n-6), arachidonic acid (20:4n-6), eicosapentaenoic acid (20:5n-3), docosatetraenoic acid (22:4n-6), docosapentaenoic acid (22:5n-3), and docosahexaenoic acid (DHA, 22:6n-3). One of the mdx mice had insufficient intramuscular TG content for successful analysis; and so results are n = 8 for PL in both strains, n = 8 for con TG, but n = 7 for mdx TG.

Protein carbonylation was measured as a marker of oxidative damage. In short, approximately 50 mg of quadriceps tissue was homogenized in 750 μL of 50 mmol/L phosphate buffer containing 1 mmol/L EDTA (pH 6.7) in 2-mL vessels housed in an ice-water bath using a Potter S Homogenizer (Sartorius-Stedim). The homogenate was then centrifuged using an Accuspin microcentrifuge (Fisher Scientific) at 13300 rpm for 15 minutes followed by analysis of supernatant using a commercially available kit (Cayman Protein Carbonyl Assay Kit [10005020], Ann Arbor, MI) with a spectrophotometer. Results were normalized to total protein as determined by a commercially available kit (Bio-Rad Protein Assay [500-0006], Hercules, CA).

To measure total phospholipase A2 (PLA2) activity, approximately 70 mg of quadriceps tissue in 150 μL of 50 mmol/L phosphate buffer containing 1 mmol/L EDTA (pH 7.4) was homogenized and then centrifuged in the same manner described above, followed by analysis of supernatant using a commercially available kit (Cayman cPLA2 Assay Kit [765021]) with a spectrophotometer. Results were expressed per tissue wet weight.

2.5. *Serum assays*

Using commercially available kits, serum creatine kinase (CK) activity (Stanbio CK, Liqui-UV [NAC] Procedure No. 2910, Boerne, TX) was measured as an indicator of sarcolemmal membrane damage [31] and serum TNF-α concentration

Table 2 – Group characteristics

	Control	Mdx
Body weight (g)	29.01 ± 0.38	30.00 ± 1.05
FFM (g)	26.77 ± 0.40	28.16 ± 0.91
% Body fat	7.75 ± 1.59	6.09 ± 1.08 ‡
Front-limb strength (g/g FFM)	3.76 ± 0.21	3.17 ± 0.20 ‡
Total-limb strength (g/g FFM)	6.02 ± 0.52	5.11 ± 0.32 §
Front-limb fatigue index (%)	15.20 ± 9.98	24.22 ± 5.91
Total-limb fatigue index (%)	6.55 ± 10.69	25.96 ± 7.22 §
Serum CK activity (U/L)	458 ± 110	5026 ± 1531 ‡
Serum TNF-α (pg/mL)	4.38 ± 0.39	6.56 ± 0.63 †
Muscle protein carbonylation (nmol/mg)	3.05 ± 0.30	5.47 ± 0.84 ‡
Muscle PLA2 activity (nmol/[min·mg])	0.018 ± 0.001	0.013 ± 0.001 *

Values are means ± SE. Carbonylation is expressed per unit protein; and PLA2, per tissue wet weight. Control mice, n = 8; mdx mice, n = 8. Statistical analyses by independent-samples *t* test.
Superscript denotes statistical significance level vs. control, as follows:
* $P < .01$.
† $P = .01$.
‡ $P < .05$.
§ $P < .10$.

Table 3 – PL and TG FA composition (percentage)

FA	PL		TG	
	Control	Mdx	Control	Mdx
Myristic (14:0)	0.52 ± 0.04	0.36 ± 0.08	2.27 ± 0.29	2.43 ± 0.45
Myristoleic (14:1n-5)	0.58 ± 0.08	0.60 ± 0.03	0.63 ± 0.14	0.82 ± 0.15
Myristelaidic (*trans*-14:1n-5)	0.69 ± 0.05	0.78 ± 0.04	0.64 ± 0.16	0.88 ± 0.17
Palmitic (16:0)	23.09 ± 0.47	21.41 ± 0.73	20.59 ± 0.97	19.96 ± 0.67
Palmitoleic (16:1n-7)	1.22 ± 0.06	1.11 ± 0.07	5.54 ± 0.79	4.48 ± 0.53
Palmitelaidic (*trans*-16:1n-7)	ND	ND	ND	ND
Stearic (18:0)	13.42 ± 0.49	13.38 ± 0.33	4.18 ± 0.36	4.04 ± 0.30
Oleic (18:1n-9)	3.45 ± 0.15	6.18 ± 0.42‡	21.48 ± 0.16	19.95 ± 0.93
Elaidic (*trans*-18:1n-9)	ND	ND	ND	ND
cis-Vaccenic (18:1n-7)	7.96 ± 0.89	7.85 ± 0.64	13.53 ± 3.77	16.35 ± 2.05
trans-Vaccenic (*trans*-18:1n-7)	ND	ND	0.13 ± 0.13	0.15 ± 0.15
Linoleic (18:2n-6)	12.01 ± 0.34	13.79 ± 0.19§	25.51 ± 2.07	23.84 ± 1.48
Linoelaidic (*trans*,*trans*-18:2n-6)	0.73 ± 0.06	0.87 ± 0.87	2.05 ± 0.55	2.94 ± 0.60
α-Linolenic (18:3n-3)	1.44 ± 0.08	1.44 ± 0.13	2.05 ± 0.09	2.38 ± 0.08*
γ-Linolenic (18:3n-6)	ND	ND	0.01 ± 0.01	ND
Arachidic (20:0)	ND	ND	0.08 ± 0.03	ND*
Homo-γ-linolenic (20:3n-6)	0.96 ± 0.09	0.85 ± 0.06	ND	ND
Arachidonic (20:4n-6)	7.88 ± 0.30	7.65 ± 0.50	ND	ND
Eicosapentaenoic (20:5n-3)	0.77 ± 0.04	0.70 ± 0.09	0.02 ± 0.02	ND
Docosatetraenoic (22:4n-6)	ND	0.27 ± 0.12	0.18 ± 0.07	0.10 ± 0.07
Docosapentaenoic (22:5n-3)	4.14 ± 0.30	4.90 ± 0.28	ND	ND
Docosahexaenoic (22:6n-3)	21.14 ± 0.59	17.85 ± 0.38‡	1.12 ± 0.30	1.69 ± 0.35
Total SFA	37.03 ± 0.32	35.15 ± 0.74*	27.12 ± 1.38	26.43 ± 0.86
Total USFA	61.55 ± 0.34	63.20 ± 0.73	70.06 ± 0.69	69.60 ± 0.88
Total TFA	1.42 ± 0.10	1.65 ± 0.11	2.82 ± 0.82	3.97 ± 0.75
Linoleic to α-linolenic ratio	8.53 ± 0.52	10.15 ± 0.96	12.52 ± 1.04	10.16 ± 0.87
Total n-3	27.48 ± 0.80	24.90 ± 0.54*	3.19 ± 0.34	4.06 ± 0.41
Total n-6	19.89 ± 0.31	21.71 ± 0.35†	25.68 ± 2.10	23.94 ± 1.51
Total n-6 to total n-3 ratio	0.73 ± 0.03	0.87 ± 0.02§	9.24 ± 1.73	6.65 ± 1.27

Composition of FAs in lipids from quadriceps muscle. Values are means ± SE. Phospholipid: control, n = 8; mdx, n = 8. Triglyceride: control, n = 8; mdx, n = 7. Statistical analyses by independent samples *t*-test.
Superscript denotes statistically significant difference from control, as follows:
* $P < .05$.
† $P < .01$.
‡ $P < .001$.
§ $P = .001$.

(Quantikine Mouse TNF-α Immunoassay [MTA00B], Minneapolis, MN) as a marker of systemic inflammation using a spectrophotometer.

2.6. Diet analysis

Approximately 17 mg of 5K52 diet was pulverized with a mortar and pestle and then sonicated in 2 mL water followed by heating at 85°C for 5 minutes with intermittent shaking. The lipids were then extracted with 5 mL chloroform/methanol (2:1, vol/vol) by 3 minutes of shaking. Tubes were then centrifuged at 750×*g* for 5 minutes, and then the upper phase was transferred and dried under nitrogen gas. The FA composition of total lipid was then analyzed by HPLC as we performed for the tissue samples using our previously reported method [27] and is reported in Table 1.

2.7. Statistical analysis

All data analysis was conducted using a computerized program (SPSS 16.0, Chicago, IL). Independent samples *t*-tests were used to compare mdx to con. Grip strength and fatigue indices were analyzed by 1-tailed tests, and all other comparisons were by 2-tailed tests. When n FA was detected in one group but undetected in the other group, we tested for statistical difference based upon whether the 95% confidence interval included zero. Initially, Pearson correlation coefficients (*r*) were calculated in an exploratory analysis to determine correlations of FA molar percentages with strength and serum CK activity. This analysis identified ALA and LA from tissue PL as being of interest. Next, a more targeted approach was taken to test if abundances of each of these 2 FAs were correlated with markers of inflammation and oxidative stress. Statistical significance was set at $\alpha = .05$.

3. Results

3.1. Body composition, grip strength, and CK activity

Table 2 summarizes group characteristics of con and mdx mice. Mdx had lower body fat percentage compared with con (6.09% ± 1.08% vs 7.75% ± 1.59%, $P < .05$). Gonadal fat pad weights (sum of left and right) were 115 ± 29 mg for mdx and

Table 4 – Correlation of PL FA percentages vs strength

FA	Control				Mdx			
	Front-limb		Total-limb		Front-limb		Total-limb	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*
Myristic (14:0)	−0.086	.840	0.554	.155	0.126	.767	0.149	.726
Myristoleic (14:1n-5)	−0.277	.506	0.069	.871	−0.146	.730	−0.160	.705
Myristelaidic (*trans*-14:1n-5)	−0.179	.672	0.076	.858	0.433	.283	0.395	.333
Palmitic (16:0)	−0.013	.975	0.425	.294	−0.115	.786	−0.129	.761
Palmitoleic (16:1n-7)	−0.459	.253	0.031	.942	−0.284	.495	−0.163	.699
Palmitelaidic (*trans*-16:1n-7)	–	–	–	–	–	–	–	–
Stearic (18:0)	0.052	.903	−0.078	.855	0.021	.960	0.310	.454
Oleic (18:1n-9)	0.526	.180	0.012	.978	0.140	.742	−0.111	.793
Elaidic (*trans*-18:1n-9)	–	–	–	–	–	–	–	–
cis-Vaccenic (18:1n-7)	0.068	.873	−0.038	.929	0.066	.677	0.033	.939
trans-Vaccenic (*trans*-18:1n-7)	–	–	–	–	–	–	–	–
Linoleic (18:2n-6)	0.189	.654	0.307	.459	−0.113	.780	−0.395	.333
Linoelaidic (*trans*,*trans*-18:2n-6)	−0.519	.187	−0.221	.598	−0.215	.610	−0.404	.321
α-Linolenic (18:3n-3)	0.266	.524	−0.654	.079	0.763	.028 †	0.870	.005 ‡
γ-Linolenic (18:3n-6)	–	–	–	–	–	–	–	–
Arachidic (20:0)	–	–	–	–	–	–	–	–
Homo-γ-linolenic (20:3n-6)	0.030	.943	−0.286	.492	−0.011	.979	−0.140	.741
Arachidonic (20:4n-6)	−0.257	.538	0.418	.303	0.341	.408	0.664	.073
Eicosapentaenoic (20:5n-3)	−0.220	.601	−0.183	.665	0.148	.726	−0.032	.941
Docosatetraenoic (22:4n-6)	–	–	–	–	−0.508	.199	−0.574	.137
Docosapentaenoic (22:5n-3)	−0.227	.589	−0.531	.176	−0.450	.263	−0.562	.147
Docosahexaenoic (22:6n-3)	−0.034	.936	−0.180	.669	−0.196	.642	−0.218	.604
Total SFA	0.050	.906	0.663	.146	−0.090	.832	0.029	.946
Total USFA	0.068	.872	−0.505	.202	0.095	.823	−0.002	.997
Total TFA	−0.387	.343	−0.096	.821	−0.024	.955	−0.182	.666
Linoleic to α-linolenic ratio	−0.147	.728	0.655	.078	−0.706	.050 *	−0.871	.005 ‡
Total n-3	−0.096	.822	−0.407	.317	−0.171	.686	−0.249	.552
Total n-6	0.021	.961	0.642	.086	0.243	.563	0.520	.186
Total n-6 to total n-3 ratio	0.098	.818	0.603	.114	0.337	.415	0.548	.159

Composition of FAs in PL from quadriceps muscle. Strength is average of 3 highest values from 5 consecutive attempts, normalized to FFM. *r*, Pearson correlation coefficient for FA composition vs strength. Control, n = 8; mdx, n = 8. Statistical analyses by linear regression.
Superscript denotes statistically significant correlations, as follows:
* $P = .050$.
† $P < .05$.
‡ $P < .01$.

137 ± 18 mg for con not significantly different. Front-limb grip strength normalized for FFM was lower in mdx vs con (3.17 ± 0.20 vs 3.76 ± 0.21 g/g FFM, $P < .05$). In addition, there were tendencies for nonnormalized front-limb strength (88.33 ± 4.43 vs 100.46 ± 5.40 g, $P = .052$) and total-limb strength normalized for FFM (5.11 ± 0.32 vs 6.02 ± 0.52 g/g FFM, $P = .080$) to be lower and for total-limb fatigue index to be higher (25.96% ± 7.22% vs 6.55% ± 10.69%, $P = .078$) in mdx vs con. Serum CK activity was greater in mdx compared with con (5026 ± 1531 vs 458 ± 110 U/L, $P < .05$).

3.2. PL and TG FA composition

Table 3 shows molar percentages of 22 FAs of skeletal muscle PL and TG in con and mdx. Phospholipid in mdx contained lower abundance of DHA (17.85% ± 0.38% vs 21.14% ± 0.59%, $P < .001$), an n-3 (also known as *omega*-3) FA, as well as lower abundance of total n-3 FAs (24.90% ± 0.54% vs 27.48% ± 0.80%, $P < .05$). Phospholipid in mdx contained higher abundance of LA (13.79% ± 0.19% vs 12.01% ± 0.34%, $P < .01$), an n-6 (also known as *omega*-6) FA, as well as total n-6 FAs (21.71% ± 0.35% vs 19.89% ± 0.31%, $P < .01$). Compared with con, mdx PL had a higher ratio of total n-6 to total n-3 FAs (0.87 ± 0.02 vs 0.73 ± 0.03, $P = .001$). In addition to alterations in n-3 and n-6 FA composition, mdx PL had greater abundance of oleic acid (6.18% ± 0.42% vs 3.45% ± 0.15%, $P < .001$) and lower abundance of total saturated FAs (35.15% ± 0.74% vs 37.03% ± 0.32%, $P < .05$). Triglyceride of mdx had higher abundance of ALA (2.38% ± 0.08% vs 2.05% ± 0.09%, $P < .05$), an n-3 FA, and lower abundance of arachidic acid (no detection vs 0.08% ± 0.03%, $P < .05$) compared with con.

3.3. Correlations for FA percentages with strength and CK activity

Table 4 shows Pearson correlation coefficients for PL FA molar percentages with strength (strength expressed as force per FFM). Phospholipid ALA was positively correlated with front-limb ($r = 0.763$, $P = .028$) and total-limb strength ($r = 0.870$, $P = .005$) in mdx but not in con (front-limb: $r = 0.266$, $P = .524$; total-

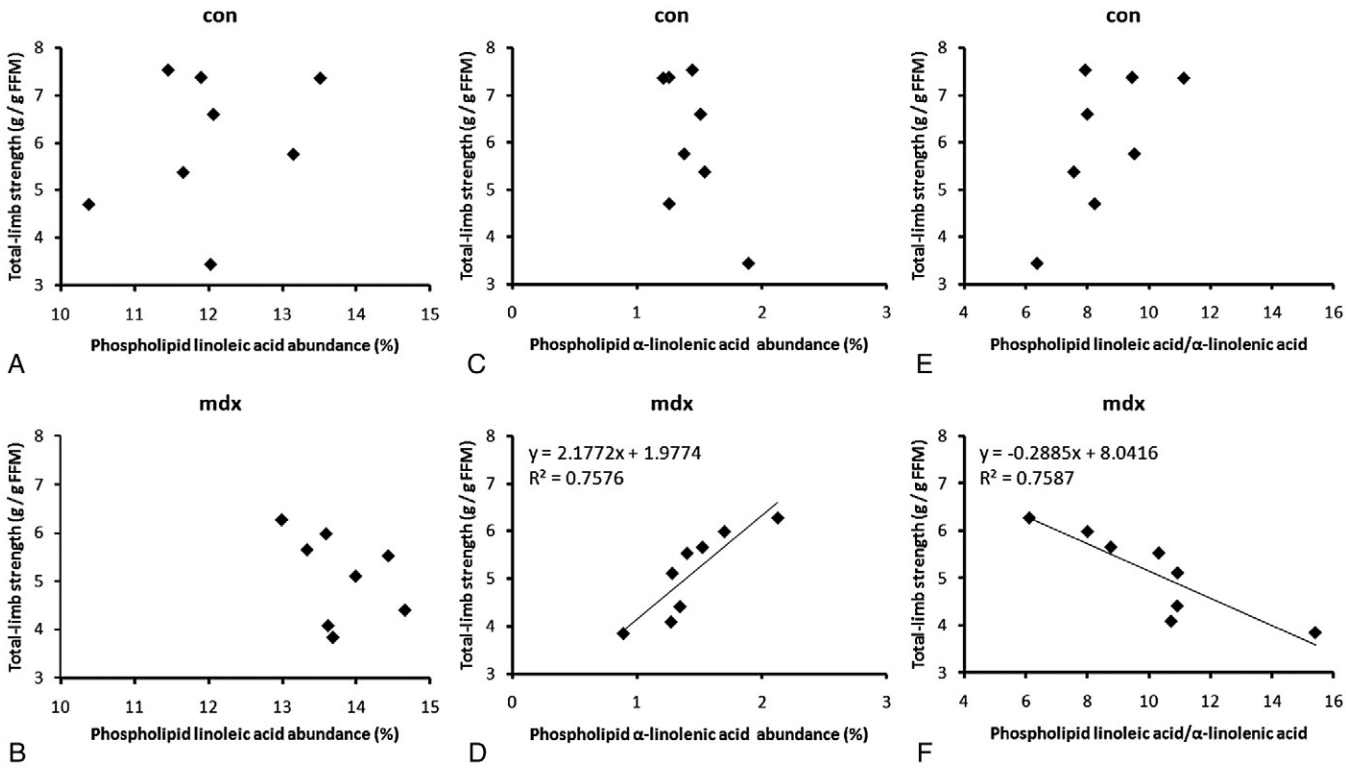

Fig. 1 – Correlations of dietary essential n-3 and n-6 FAs in PL with total-limb grip strength normalized to FFM. A, LA in con mice. B, LA in mdx mice. C, ALA in con mice. D, ALA in mdx mice. E, LA/ALA ratio in con mice. F, LA/ALA ratio in mdx mice. Statistical analyses by linear regression. Regression line and equation only shown in instances of a statistically significant correlation.

limb: r = –0.654, P = .079) (see also Fig. 1 for total-limb strength equations and individual data points). In addition, LA/ALA ratio in PL was negatively correlated with front-limb (r = –0.706, P = .050) and total-limb strength (r = –0.871, P = .005) in mdx but not in con (front-limb: r = –0.147, P = .728; total-limb r = 0.655, P = .078) (Fig. 1). In con, although TG *trans*-vaccenic acid was negatively correlated with total-limb strength (r = –0.708, P = .049), it was not significantly correlated with front-limb strength (Table 5), indicating that the association with strength was not highly robust. To reiterate, strength values used in the correlations discussed above were normalized to FFM. However, when strength was expressed in absolute terms or normalized to body weight, qualitatively similar correlation results were obtained (data not shown). Table 6 shows Pearson correlation coefficients for FA molar percentages in muscle PL with serum CK activity. Linoleic acid content in PL was positively correlated with serum CK activity in mdx (r = 0.776, P = .023) but not in con (r = –0.331, P = .423) (see also Fig. 2 for equations and individual data points). In con, unlike in mdx, PL ALA (r = 0.786, P = .021) and PL LA/ALA ratio (r = –0.799, P = .017) were positively and negatively correlated with CK activity, respectively. There were no significant correlations between serum CK activity and TG FA abundances in mdx or con (Table 7). In summary, results from correlation analyses with strength and serum CK identified the abundance of ALA and LA in PL of mdx muscle as being of special interest.

3.4. *Oxidative damage and inflammation*

To identify potential mediators of the effects of ALA and LA abundances in muscle PL on strength and serum CK activity, respectively, muscle protein carbonylation, muscle PLA2 activity, and serum TNF-α were measured. We specifically tested for correlations of these 3 markers with 2 PL acyl chains (ALA and LA) in a targeted fashion. Compared with con, mdx mice had higher levels of muscle protein carbonylation (5.47 ± 0.84 vs 3.05 ± 0.30 nmol/mg protein, $P < .05$) and serum TNF-α (6.56 ± 0.63 vs 4.38 ± 0.39 pg/mL, P = .01). Total PLA2 activity in muscle was actually lower in mdx than con (0.013 ± 0.001 vs 0.018 ± 0.001 nmol/[min·mg tissue], $P < .01$). Linoleic acid content in PL was positively correlated with protein carbonylation in mdx (r = 0.837, P = .010) but not in con (r = 0.14, P = .741). There were no other statistically significant correlations between the inflammatory and oxidative stress markers with ALA or LA of mdx muscle PL.

4. Discussion

We provide data describing the lipid profile of skeletal muscle of mdx mice in comparison to healthy control mice. As the current strategy to treat DMD, corticosteroid administration, is insufficient, our goal was to generate new hypotheses for subsequent testing of novel therapies. We did pairwise comparison between muscle lipids of mdx and con to test

Table 5 – Correlation of TG FA percentages vs strength

FA	Control				Mdx			
	Front-limb		Total-limb		Front-limb		Total-limb	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*
Myristic (14:0)	−0.303	.466	0.321	.437	0.562	.189	0.690	.086
Myristoleic (14:1n-5)	0.272	.515	−0.427	.291	−0.230	.621	−0.480	.276
Myristelaidic (*trans*-14:1n-5)	0.229	.586	−0.444	.270	−0.230	.620	−0.487	.268
Palmitic (16:0)	−0.142	.738	0.683	.062	−0.113	.810	0.200	.667
Palmitoleic (16:1n-7)	−0.053	.901	−0.049	.909	−0.200	.667	−0.034	.942
Palmitelaidic (*trans*-16:1n-7)	–	–	–	–	–	–	–	–
Stearic (18:0)	−0.050	.906	0.377	.357	−0.121	.797	−0.258	.577
Oleic (18:1n-9)	−0.261	.532	0.424	.295	0.023	.961	0.334	.465
Elaidic (*trans*-18:1n-9)	–	–	–	–	–	–	–	–
cis-Vaccenic (18:1n-7)	0.209	.620	−0.524	.183	−0.288	.531	−0.560	.191
trans-Vaccenic (*trans*-18:1n-7)	−0.053	.901	−0.708	.049 *	0.011	.981	0.026	.955
Linoleic (18:2n-6)	−0.184	.662	0.434	.283	0.365	.421	0.522	.230
Linoelaidic (*trans*,*trans*-18:2n-6)	0.295	.478	−0.478	.231	−0.090	.848	−0.329	.471
α-Linolenic (18:3n-3)	0.293	.481	0.579	.133	0.204	.661	−0.039	.935
γ-Linolenic (18:3n-6)	0.160	.705	−0.176	.676	–	–	–	–
Arachidic (20:0)	−0.212	.614	0.293	.482	–	–	–	–
Homo-γ-linolenic (20:3n-6)	–	–	–	–	–	–	–	–
Arachidonic (20:4n-6)	–	–	–	–	–	–	–	–
Eicosapentaenoic (20:5n-3)	0.160	.705	−0.176	.676	–	–	–	–
Docosatetraenoic (22:4n-6)	−0.399	.327	0.420	.300	−0.449	.312	−0.147	.753
Docosapentaenoic (22:5n-3)	–	–	–	–	–	–	–	–
Docosahexaenoic (22:6n-3)	0.246	.556	−0.200	.636	0.381	.399	0.229	.621
Total SFA	−0.182	.666	0.655	.078	−0.413	.309	−0.091	.830
Total USFA	0.083	.844	−0.686	.061	−0.458	.253	−0.195	.644
Total TFA	0.236	.574	−0.527	.180	−0.362	.378	−0.399	.327
Linoleic to α-linolenic ratio	−0.381	.352	0.182	.667	0.126	.788	0.348	.445
Total n -3	0.308	.458	−0.025	.954	0.364	.422	0.189	.685
Total n -6	−0.195	.644	0.441	.274	0.336	.461	0.503	.250
Total n-6 to total n -3 ratio	−0.396	.332	0.253	.546	−0.303	.509	0.348	.445

Composition of FAs in TG from quadriceps muscle. Strength is average of 3 highest values from 5 consecutive attempts, normalized to FFM. *r*, Pearson correlation coefficient for FA composition vs strength. Control, n = 8; mdx, n = 7. Statistical analyses by linear regression.
* Statistically significant correlations: $P < .05$.

for FAs that may be associated with the presence of the disease state, and we tested for correlations with strength and serum CK activity to see if lipid composition can account for interindividual variations in magnitude of disease markers. Even when control and mdx mice consume identical diets from weaning, the composition of skeletal muscle lipids is different between groups and correlates with physiological parameters. The major findings were with regard to n-3 and n-6 FAs. Mdx mice have relatively lower abundance of DHA in muscle PL (Table 3), and their abundance of ALA in muscle PL is positively correlated with strength (Table 4 and Fig. 1). In addition, LA content in muscle PL was higher in mdx vs con and in mdx mice was positively correlated with a sarcolemmal damage marker (serum CK) (Table 6 and Fig. 2). These findings lead to new hypotheses regarding mechanisms of muscular dysfunction in DMD in the context of positive effects of n-3 FAs and negative effects of n-6 FAs, and provide rationale for future therapeutic interventions. Below, we discuss these findings and the emerging hypotheses that can be tested in future studies.

The current results indicate that DHA content in mdx muscle PL is lower than that in con muscle. However, the significance of the modest relative reduction (~16%) is questionable, as DHA levels were still relatively high in both con and mdx muscle PL, likely because the diet contained DHA (Table 1). The reduced DHA content was associated with the mdx phenotype but cannot explain interindividual differences in magnitude of disease markers. That is to say, we observed a mean difference between groups, but DHA content did not correlate with strength or serum CK activity. A previously investigated mouse model of muscular dystrophy, the dy/dy mouse, is not specifically appropriate for DMD but yet still is an example of skeletal muscle dysfunction. In this mouse model, the content of DHA was lower than that in controls in muscle PL [32,33], similar to the results we have attained for mdx mice. Thus, there may be something about the stress of chronic skeletal muscle pathology that ultimately reduces the content of DHA in PL. Muscles of humans with DMD and mdx mice experience increased oxidative stress [3,15,34]. Phospholipase A2 has a preference for peroxidized membranes [35], and it is possible that oxidative damage to DHA in mdx mice leads to its reduced abundance in PL. It should be noted though that, unlike results reported for human patients with DMD [36], in mdx mice, we did not find elevated PLA2 activity in skeletal muscle (Table 2). As inflammation exacerbates muscle damage and degeneration in mdx mice [5,6] and n-3 FAs exhibit anti-inflammatory properties [16], reduction in DHA content could be unfavorable during intake of a low-DHA

Table 6 – Correlation of PL FA percentages vs serum CK activity

FA	Control		Mdx	
	r	*P*	*r*	*P*
Myristic (14:0)	–0.095	.823	0.089	.835
Myristoleic (14:1n-5)	0.186	.659	–0.171	.686
Myristelaidic (*trans*-14:1n-5)	0.081	.849	–0.207	.622
Palmitic (16:0)	–0.062	.885	0.480	.228
Palmitoleic (16:1n-7)	–0.285	.494	0.325	.432
Palmitelaidic (*trans*-16:1n-7)	–	–	–	–
Stearic (18:0)	0.254	.544	–0.292	.484
Oleic (18:1n-9)	–0.260	.535	0.267	.522
Elaidic (*trans*-18:1n-9)	–	–	–	–
cis-Vaccenic (18:1n-7)	–0.396	.331	–0.567	.143
trans-Vaccenic (*trans*-18:1n-7)	–	–	–	–
Linoleic (18:2n-6)	–0.331	.423	0.776	.023*
Linoelaidic (*trans*,*trans*-18:2n-6)	–	–	0.167	.693
α-Linolenic (18:3n-3)	0.786	.021*	–0.132	.755
γ-Linolenic (18:3n-6)	–	–	–	–
Arachidic (20:0)	–	–	–	–
Homo-γ-linolenic (20:3n-6)	–0.008	.985	0.365	.374
Arachidonic (20:4n-6)	–0.114	.789	–0.413	.309
Eicosapentaenoic (20:5n-3)	0.290	.485	–0.200	.634
Docosatetraenoic (22:4n-6)	–	–	0.261	.532
Docosapentaenoic (22:5n-3)	0.043	.919	0.004	.992
Docosahexaenoic (22:6n-3)	0.573	.138	–0.004	.992
Total SFA	0.287	.490	0.353	.392
Total USFA	–0.318	.442	–0.366	.372
Total TFA	0.154	.716	0.061	.886
Linoleic to α-linolenic ratio	–0.799	.017*	0.124	.770
Total n-3	0.534	.173	–0.064	.879
Total n-6	–0.447	.267	–0.066	.877
Total n-6 to total n-3 ratio	–0.632	.093	0.003	.994

Composition of FAs in PL from quadriceps muscle. Serum CK activity measured as a marker of sarcolemmal damage. r, Pearson correlation coefficient for FA composition vs serum CK. Control, n = 8; mdx, n = 8. Statistical analyses by linear regression.
* Statistically significant correlations: $P < .05$.

diet. A reduction in DHA content could also impair calcium homeostasis, as treatment of endothelial cells with DHA was reported to reduce oxidative stress–induced calcium influx through effects on ion channels [37] that are involved in detrimental calcium entry into mdx muscle fibers [38]. Such calcium influx causes degradation of membrane-bound proteins [31], activation of cytosolic PLA2, as well as amplification of reactive oxygen species (ROS) production [39]. It could be that the related n-3 FA, ALA, is involved in such pathways as well. Although we briefly discuss physiological roles of DHA above, based upon the observation that ALA was correlated with grip strength but DHA was not, it does seem that perhaps ALA is more bioactive in mdx mice, and a discussion of potential relationships between ALA and muscle health in mdx mice follows below.

α-Linolenic acid (the dietary essential n-3 FA) explained much of the interindividual variation in strength in mdx mice in this study. That is, ALA content in muscle PL of mdx (but not con) was highly correlated with grip strength (Table 4 and Fig. 1). This finding is reminiscent of a case report of ALA deficiency in a young girl leading to weakness that was reversed by increased ALA intake [40]. Furthermore, ALA administration to cultured macrophages in vitro inhibits TNF-α expression and NFκB activity [41]. Dietary ALA supplementation in healthy people reduces serum TNF-α [42], which is elevated in DMD [43] and in mdx mice (Table 2). Although we did not observe a significant correlation between serum TNF-α and muscle ALA content, we did confirm the expectation of elevated serum TNF-α in this study in mdx vs control. It may be that greater variation in ALA level in muscle PL is needed to detect this potential correlation. Treatment of mdx mice with EPA, a related n-3 FA, led to decreased skeletal muscle content of TNF-α in a previous study [44], and so there does appear to be some relationship between TNF-α protein expression and n-3 FAs in muscular dystrophy. In rats, reducing dietary ALA intake leads to lower Na^+/K^+ pump activity in the plasma membrane of neurons [45]; and so perhaps higher content of ALA is beneficial for Na^+/K^+ pump activity which is elevated in mdx muscle [46,47], presumably as an adaptation to cope with the increased intracellular sodium concentration [11,12]. What is more, ALA supplementation was recently used successfully in dystrophic hamsters. Though not a model for DMD (instead a model of limb girdle muscular dystrophy), results showed that high intake of ALA in dystrophic hamsters lacking the δ-sarcoglycan protein led to benefits including reduced fibrosis and less centralized nuclei in skeletal muscle fibers [48]. Therefore, there are multiple reasons to believe that higher ALA content in mdx muscle PL could mitigate disease symptoms.

For LA, the dietary essential n-6 FA, content in muscle PL was higher in mdx vs con. Upon first glance, this finding may appear to be at odds with the previous findings that disease in dystrophic muscle is associated with an apparent reduction in the ratio of 18:2 to 18:1 FA content of PC [24-26]. However, it must be noted that interpretation of this ratio is difficult, as we found that dystrophin deficiency impacts both the numerator and denominator, with both LA and oleic acid content higher in muscle PL of mdx vs con (Table 3). Therefore, in these previous studies, changes in oleic acid content might have masked changes in LA content in PL when PL profile was assessed by the ratio of 34:2 to 34:1 PC. In the present study, in addition to the elevated LA levels in PL, mdx muscle also displayed higher levels of protein carbonylation (oxidative damage) as compared with control muscle. Linoleic acid content of muscle PL in mdx mice was highly correlated with serum CK activity (Table 6 and Fig. 2), and the LA content in mdx muscle PL was also significantly correlated with protein carbonylation. Thus, we conclude that mdx mice with higher LA content in muscle PL displayed apparently higher levels of sarcolemmal damage that could be related to oxidative stress. There is some evidence that the n-6 FA content in tissues can impact cell integrity. For example, expression of the *Caenorhabditis elegans* fat-1 gene, which converts LA to ALA and thus reduces the amount of LA, leads to improved survival of rat neurons [49]. As well, it was shown in immune cells that LA, as compared with other FAs, strongly stimulates ROS production [17,50]. Therefore, if LA content in mdx muscle PL is detrimental, it is plausible that the detrimental effects would be through stimulation of ROS production which seems to be elevated in muscle of mdx mice [15] (Table 2) and humans with DMD [3], leading to activation of NFκB [5-10] and other effects. It was reported that patients

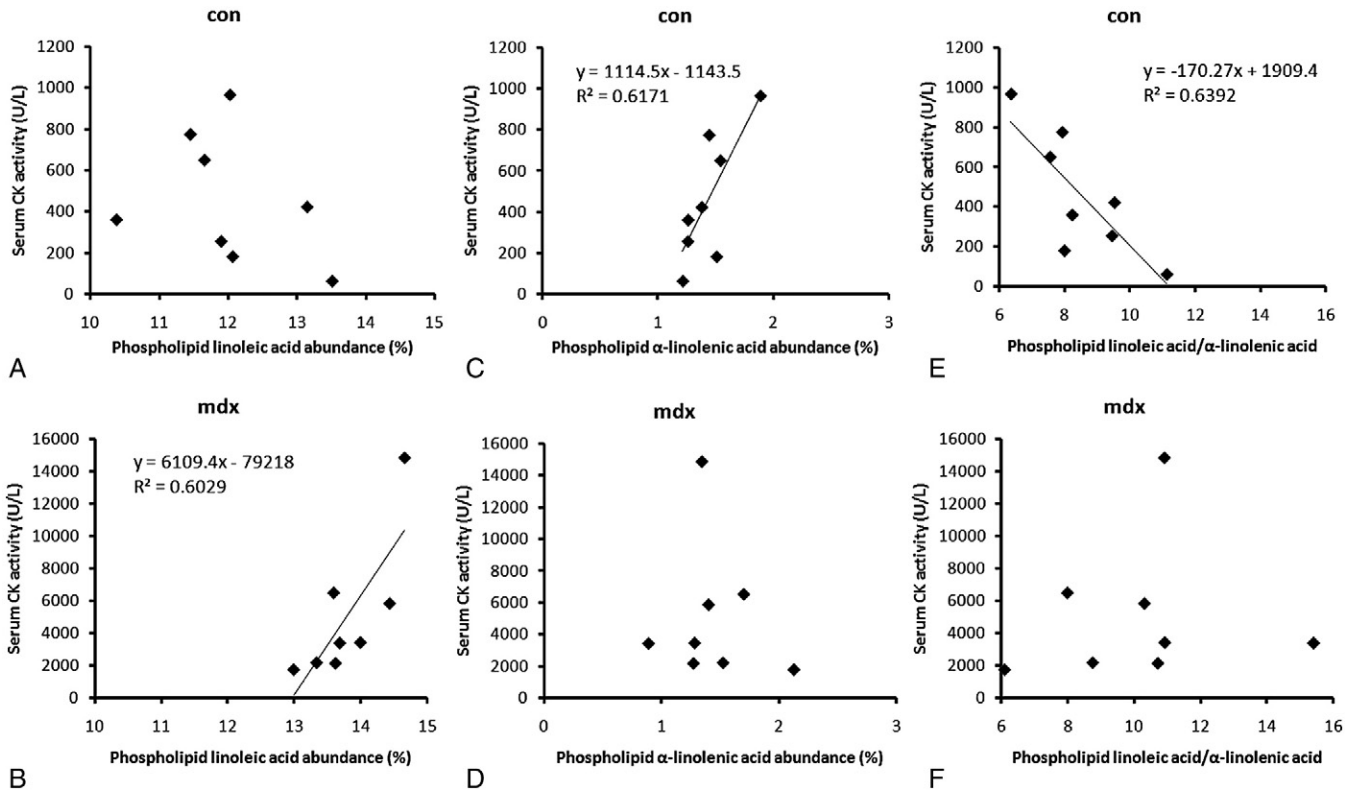

Fig. 2 – Correlations of dietary essential n-3 and n-6 FAs in PL with serum CK activity. A, LA in con mice. B, LA in mdx mice. C, ALA in con mice. D, ALA in mdx mice. E, LA/ALA ratio in con mice. F, LA/ALA ratio in mdx mice. Statistical analyses by linear regression. Regression line and equation only shown in instances of a statistically significant correlation.

with DMD display higher PLA2 activity in muscle [36], and NFκB is a transcription factor for PLA2 [51-53]. Theoretically, PLA2 could exacerbate sarcolemmal damage by production of lysophospholipids which lead to greater influx of calcium into damaged or diseased skeletal muscle fibers [54,55]. However, we were unable to confirm elevated PLA2 activity in dystrophic muscle (as PLA2 enzymatic activity was actually lower in mdx than control mice in the present study). Thus, based upon these results, we are in favor of oxidative stress rather than PLA2 activity as being a potentially important factor in the pathology of mdx mice.

In this experiment, we performed FA profiling to discover new biomarkers of disease severity in mdx mice; the approach was useful, and new potential targets have emerged. We analyzed both the PL and TG of skeletal muscle, and the findings of greatest impact were with regard to the FA profile in PL. It appears that the FA profile in muscle TG is primarily reflective of the dietary FA composition, whereas FA content in muscle PL is more highly regulated and more predictive of disease state. Interventions to reduce the levels of n-6 FAs and increase n-3 FAs in muscle PL may improve disease prognosis, and based upon our results, the dietary essential FAs ALA and LA may be of special importance. Although TG is more directly reflective of diet (Tables 1 and 3), it is known from previous work that changes in dietary intake can still affect PL FA profiles in rodents [56,57] and humans [58]. Thus, to test hypotheses that have emerged from this experiment, dietary intervention will likely be a useful approach. As well, genetic approaches are also being developed such as viral infection with the gene that generates n-3 FAs from n-6 FAs [49,59]. Use of such dietary and genetic approaches in subsequent studies should clarify the potential importance of n-3 and n-6 FAs in the progression and treatment of DMD.

In summary, mdx mice with lower ALA content and higher LA content in muscle PL displayed worsened disease marker levels (strength and serum CK activity). Linoleic acid was additionally associated with oxidative damage in mdx muscle. These positive correlations between ALA and strength, LA and serum CK activity, and LA and oxidative damage were not observed in the control mice. In fact, the relationships between PL FA composition with these outcomes were qualitatively different between strains. Thus, when skeletal muscle is stressed by a disease state or by sarcolemmal lesions per se, it seems that the content of n-3 and n-6 FAs in skeletal muscle PL might become important for the ability to cope with the stressor. In future work testing interventions that manipulate the FA composition of muscle PL in mdx mice, it can be determined if the FA profile has an impact upon the severity of muscular dystrophy or if the FA profile is only a surrogate measure of disease severity. Based upon the present results and considering the well-known health benefits of consuming a diet with a high n-3/n-6 FA ratio, we speculate that raising content of ALA and/or reducing that of LA in muscle PL would decrease the severity of muscle disease in conditions such as DMD. This hypothesis emerged from our

Table 7 – Correlation of TG FA percentages vs serum CK activity

FA	Control		Mdx	
	r	*P*	*r*	*P*
Myristic (14:0)	0.041	.924	−0.454	.306
Myristoleic (14:1n-5)	0.445	.269	0.441	.322
Myristelaidic (*trans*-14:1n-5)	0.492	.216	0.388	.390
Palmitic (16:0)	−0.645	.084	−0.318	.487
Palmitoleic (16:1n-7)	−0.079	.853	0.429	.337
Palmitelaidic (*trans*-16:1n-7)	–	–	–	–
Stearic (18:0)	−0.412	.311	−0.289	.529
Oleic (18:1n-9)	−0.576	.135	−0.198	.670
Elaidic (*trans*-18:1n-9)	–	–	–	–
cis-Vaccenic (18:1n-7)	0.558	.151	0.311	.498
trans-Vaccenic (*trans*-18:1n-7)	0.657	.077	−0.138	.767
Linoleic (18:2n-6)	−0.452	.261	−0.404	.369
Linoelaidic (*trans*,*trans*-18:2n-6)	0.420	.300	0.390	.387
α-Linolenic (18:3n-3)	−0.184	.663	−0.038	.936
γ-Linolenic (18:3n-6)	0.248	.554	–	–
Arachidic (20:0)	−0.386	.345	–	–
Homo-γ-linolenic (20:3n-6)	–	–	–	–
Arachidonic (20:4n-6)	–	–	–	–
Eicosapentaenoic (20:5n-3)	0.248	.554	–	–
Docosatetraenoic (22:4n-6)	0.305	.463	−0.145	.756
Docosapentaenoic (22:5n-3)	–	–	–	–
Docosahexaenoic (22:6n-3)	0.422	.297	0.250	.589
Total SFA	−0.561	.148	−0.586	.167
Total USFA	0.543	.164	0.254	.583
Total TFA	0.489	.219	0.370	.414
Linoleic to α-linolenic ratio	−0.372	.364	−0.294	.522
Total n-3	0.334	.419	0.207	.657
Total n-6	−0.436	.280	−0.400	.373
Total n-6 to total n-3 ratio	−0.349	.397	−0.318	.486

Composition of FAs in TG from quadriceps muscle. Serum CK activity measured as a marker of sarcolemmal damage. *r*, Pearson correlation coefficient for FA composition vs serum CK. Control, n = 8; mdx, n = 7. No statistically significant correlations. Statistical analyses by linear regression.

assessment of the FA profile in lipid classes of dystrophic skeletal muscle, and this exploratory analytical approach may be useful for initial hypothesis generation in numerous other diseases as well.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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