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Melanie L. Leitner
Accelerating NeuroVentures, LLC

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Electrical impedance myography for reducing sample size in Duchenne muscular dystrophy trials

Melanie L. Leitner¹, Kush Kapur², Basil T. Darras², Michele Yang³, Brenda Wong⁴, Laura Dalle Pazze⁵, Julaine Florence⁶, Martin Buck⁷, Laura Freedman⁷, Jose Bohorquez⁷, Seward Rutkove⁷ & Craig Zaidman⁶

¹Accelerating NeuroVentures, LLC, Needham, Massachusetts
²Department of Neurology, Boston Children’s Hospital, Boston, Massachusetts
³Department of Neurology, Children’s Hospital Colorado, Denver, Colorado
⁴Department of Pediatrics, University of Massachusetts Medical School, Worcester, Massachusetts
⁵Charley’s Fund, New York, New York
⁶Department of Neurology, Washington University in St. Louis, St. Louis, Missouri
⁷Myolex Inc, Brookline, Massachusetts

Abstract

Objective: To evaluate the sensitivity of electrical impedance myography (EIM) to disease progression in both ambulatory and non-ambulatory boys with DMD. Methods and Participants: A non-blinded, longitudinal cohort study of 29 ambulatory and 15 non-ambulatory boys with DMD and age-similar healthy boys. Subjects were followed for up to 1 year and assessed using the Myolex® mView™ EIM system as part of a multicenter study. Results: In the ambulatory group, EIM 100 kHz resistance values showed significant change compared to the healthy boys. For example, in lower extremity muscles, the average change in EIM 100 kHz resistance values over 12 months led to an estimated effect size of 1.58. Based on these results, 26 DMD patients/arm would be needed for a 12-month clinical trial assuming a 50% treatment effect. In non-ambulatory boys, EIM changes were greater in upper limb muscles. For example, biceps at 100kHz resistance gave an estimated effect size of 1.92 at 12 months. Based on these results, 18 non-ambulatory DMD patients/arm would be needed for a 12-month clinical trial assuming a 50% treatment effect. Longitudinal changes in the 100 kHz resistance values for the ambulatory boys correlated with the longitudinal changes in the timed supine-to-stand test. EIM was well-tolerated throughout the study. Interpretation: This study supports that EIM 100 kHz resistance is sensitive to DMD progression in both ambulatory and non-ambulatory boys. Given the technology’s ease of use and broad age range of utility it should be employed as an exploratory endpoint in future clinical therapeutic trials in DMD. Trial Registration: Clinicaltrials.gov registration #NCT02340923

Introduction

Duchenne muscular dystrophy (DMD) remains a devastating disease with limited therapeutic options. One key challenge to identifying therapy efficacy is the limited availability of sensitive and objective measures for evaluating the drug response throughout the disease course. For example, the 6-minute walk test (6MWT) has been one of the standard outcome measures.¹ It generally shows improving or stable values until approximately 7 years in boys with DMD² and cannot be used in non-ambulatory children, restricting its use to a narrow age range. Other functional outcome measures are hindered by requiring subjective evaluations by the evaluators and/or dependence on task completion.³ Moreover, measuring progression in non-ambulatory children is also difficult given the small set of similarly limited outcome measures available.⁴,⁵

A basic approach for circumventing these issues is to use biomarkers that neither rely on subjective assessments nor are impacted by motivation.⁶ While these include...
muscle biopsy\(^7\) and blood-based analytes,\(^8\) imaging approaches can also be used.\(^5,10\) For example, muscle magnetic resonance imaging (MRI) is sensitive to disease progression;\(^11\) quantitative ultrasound also shows promise.\(^10\) Yet MRI is expensive, challenging to scale, and inconvenient; ultrasound likewise requires image system standardization and specific training to perform.\(^12\)

Another technology offering promise as a potential therapy-response biomarker is electrical impedance myography (EIM). In EIM, a weak, high-frequency electrical current is applied across a muscle of interest and the resulting voltages measured.\(^13\) Changes in the voltage characteristics provide insights into the health and integrity of the muscle, the impact of disease, and the effect of therapy.\(^14\) Several studies have shown that EIM has high inter- and intrarater reliability.\(^15-17\) It has practical advantages as well. EIM only takes seconds to perform, is painless, less expensive relative to standard imaging options, and is minimally impacted by respiratory status or body contractures. In DMD, a single-site longitudinal study provided evidence that EIM parameters are sensitive to disease progression as well as to the beneficial effects of corticosteroid therapy.\(^18\) However, that study, as well as a subsequent reanalysis of the data, using more advanced analytical approaches\(^19\), were limited since that study utilized an off-the-shelf impedance-measuring system not intended for muscle assessment and did not assess non-ambulatory boys.

Here we report results from a multisite, longitudinal cohort DMD study exploring a dedicated EIM device to assess DMD progression, with a focus on identifying a single EIM parameter sensitive to disease progression across a wide age range regardless of ambulatory status.

**Methods**

**General**

We performed a five-site multicenter study including Boston Children’s Hospital, Washington University in St. Louis, Colorado Children’s Hospital, Cincinnati Children’s Hospital, and Skulpt/Myolex, Inc. Four clinical sites enrolled both healthy and DMD boys, while the Skulpt/Myolex site only enrolled healthy boys. Institutional review board approval was obtained at each of the five sites. Parental written informed consent and participant verbal or written assent was also acquired for all participants. The dates of recruitment and data collection were 31 March 2014–27 June 2016.

**Participants**

**DMD boys**

All boys were required to have genetic confirmation of disease or to have a brother with genetically confirmed DMD and a characteristic clinical picture. DMD boys were excluded if they were enrolled in a therapeutic clinical trial or had a concomitant condition that substantially impacted health. Boys were enrolled regardless of corticosteroid use or ambulatory status.

**Healthy boys**

Healthy boys had no history of neuromuscular disease or any other disorder that would be anticipated to affect muscle health and were recruited via IRB-approved advertisement and word-of-mouth.

**Study design**

Study visits included baseline (0), 3, 6, and, 12 months. At each visit, medications were reviewed, interim medical history obtained, and weight and height measured. In addition to the EIM measurements, a standard set of age- and ability-appropriate motor function tests were also performed in the DMD boys only. We sought to enroll approximately 60 boys with DMD and 60 healthy controls, all between the ages of 5 and 17, based on initial sample size estimates.

**EIM measurements**

EIM was performed using the Myolex mView® system (Myolex (formally Skulpt), Inc, Boston, MA); see Figure 1, consisting of a handheld EIM device with disposable electrode pads. Each electrode array contains three electrode-paired configurations, and thus three sets of data are obtained virtually simultaneously across 41 applied electrical current frequencies between 1 kHz and 10 MHz. The handheld device is connected via a cable to a power converter box, which itself is directly connected to a laptop. After wetting the skin with saline, the electrode array is applied and an EIM measurement taken. This entire process is briefly repeated two times on each muscle to ensure stability/consistency of the data. Seven muscles were studied unilaterally: lateral deltoid, biceps brachii, forearm flexors, forearm extensors, quadriceps, tibialis anterior, and medial gastrocnemius. The right side was chosen for measurement unless clear left side dominance was present, in which case the left side was measured. All evaluators were trained in proper use of the system.

**Functional measurements**

In addition to EIM data, several functional measures were assessed longitudinally in the DMD boys where possible. These included: the 6MWT,\(^1\) North Star Ambulatory Assessment,\(^9\) Handheld Dynamometry (HHD)\(^20\)
performed unilaterally using a standard HHD device on the corresponding muscles to those assessed with EIM, the timed supine-to-stand test,\textsuperscript{21} and the Brooke upper extremity scale.\textsuperscript{22}

**Data analysis**

**Preliminary data processing**

Prior to a formal data analysis, raw multifrequency EIM data were assessed for artifacts, or other technical factors negatively impacting data quality, using an automated algorithm. This algorithm was sensitive to noise across the frequency spectrum, and to extreme or negative values at low frequencies (under 30 kHz) typical of poor electrode contact.

As our goal was to assess the potential for EIM parameters to effectively assess the disease progression in both ambulatory and non-ambulatory children over a period out to 1 year, prior to the data analysis we removed data from children who switched steroid or ambulatory status.

Extensive reliability testing (inter and intra-rater) has been performed previously for EIM across a variety of disease indications including specifically in DMD,\textsuperscript{15,18} and thus we chose not to repeat that analysis here.

Longitudinal analysis was performed using a linear mixed effects model with random intercept and slope terms to account for within-subject correlations and between-subject variability under the missing-at-random assumption. The main result of interest was the slope difference between healthy and DMD boys, since this would enable sample size estimation (effect sizes). Univariate correlations were also performed comparing EIM parameter changes with functional changes in the DMD boys out to 1 year. Thus, our main focus was to identify those EIM parameters that showed the largest effect sizes, thereby providing the greatest sensitivity to disease-related change and enabling the use of smaller patient sample sizes. We then sought to determine whether these EIM parameters correlated with known functional measures.

A total of 40 different EIM parameters were selected for assessment (across frequencies, electrode configurations, and impedance features) for 10 different muscles/muscle combinations (i.e., seven individual muscles plus upper, lower, and whole-body averages), resulting in a total of 400 possible outcomes assessed per patient/subject visit.

Missing data were not imputed, and all measurements were included. Sample size estimates for a potential clinical trial were obtained using the effect sizes observed in our current study. Specifically, for EIM analyses, effect size was computed as \( \frac{\text{mean slope difference}}{\text{slope difference standard deviation}} \), where the difference is between the healthy and DMD boys. For our sample size calculations, we assumed that the EIM measurements would be obtained every 3 months and modeled 80% power to identify significant differences between the two groups at 12 months with \( P < 0.05 \).

**Results**

Table 1 shows the demographic data for the DMD and age-matched healthy control subjects used in the
following analyses. Figure 2, a CONSORT flowchart, summarizes the overall enrollment for the study. A total of 53 DMD boys and 57 healthy controls were initially screened and enrolled. However, six ambulatory boys who changed steroid status over the course of the study and three boys who changed ambulatory status over the course of the study were excluded from the analysis. We divided the remaining group of DMD boys into two cohorts for analysis: an ambulatory cohort of 29 DMD boys stably on or off steroids and a non-ambulatory cohort of 15 DMD boys (stably on or off steroids).

Fifty-seven healthy age-similar controls were also recruited across the five sites and enrolled over the same time period. Forty four of them were matched to the ambulatory boys and 13 to the non-ambulatory boys, based on age.

Importantly, due to unexpected funding limitations, the study was terminated prematurely, allowing only a subset of the children to complete all 12 months, as indicated in the CONSORT flow chart.

System tolerability/adverse events
There were no serious or relatable adverse events in the entire study and all children tolerated the EIM procedure well.

Exclusion of data due to artifact/noise
About 3.0% of EIM data were excluded due to artifact detected by our automated algorithm prior to analysis.

Overall results
We first sought to determine the significant differences in EIM parameter slopes between DMD and healthy boys to establish the sensitivity of different EIM parameters to disease-related change. Table 2 shows the number of parameters with different longitudinal slope values at different levels of significance. The ambulatory children showed many significantly different EIM parameters when comparing slopes with healthy boys (approximately 38% of the EIM parameters assessed), whereas the number of significantly different EIM parameters was smaller in the non-ambulatory group.

The longitudinal changes (slopes) in the 50 and 100 kHz resistance values assessed across muscles/muscle groups were found to be significantly different from the changes in the muscles of age-similar healthy controls ($P < 0.05$) across all four groups (ambulatory and non-ambulatory at 6 and 12 months), with a slightly higher number of muscles showing significant changes in the 100 kHz values. Accordingly, for purposes of the analysis that follows we focused on this EIM parameter since it appeared to be the most robust and sensitive to DMD-related muscle changes across disease state out of the various EIM parameters we explored.

EIM outcomes
Twenty-nine ambulatory DMD boys with mean age (range) 8.65 (5.89–12.37) years and 44 healthy controls with 8.65 (5.03–12.81) years had data included out to 6 months and, given the funding issues noted above, we were also able to obtain 12-month data for seven ambulatory DMD boys and 10 healthy controls out of these original cohorts. For the non-ambulatory data set, 15 DMD boys (14.40 (9.57–17.01) years) and 13 healthy boys (14.9 (13.52–16.93) years) were included in the 6-month analysis; and out of these five DMD boys and four healthy controls contributed data at 12 months.

Tables 3 and 4 shows the differences in the rate of change in the resistance slope comparing the DMD to healthy boys for the 100 kHz resistance parameter over 6 and 12 months. Based on these differences, the effect sizes and needed sample sizes for a potential clinical trial using the 100 kHz resistance measure can be calculated, and these are also shown in Tables 3 and 4 for both the ambulatory and non-ambulatory cohorts. Significant differences are present in the upper extremity muscles of the non-ambulatory boys, whereas the ambulatory boys show the greatest differences in lower extremity muscles. In the non-ambulatory boys, while many muscles differed between boys with DMD and controls, only the 100 kHz resistance in biceps data shows significance in both the 6- and 12-month analyses.

Table 1. Demographic data for the ambulatory and non-ambulatory DMD and age similar healthy control cohorts.

|                      | Ambulatory Cohort | Age Similar Healthy Controls | Non-ambulatory Cohort | Age Similar Healthy Controls |
|----------------------|-------------------|-------------------------------|-----------------------|-------------------------------|
| N                    | n = 29            | n = 44                        | n = 15                | n = 13                        |
| Mean Age (range)     | 8.65 (5.89–12.37) | 8.65 (5.03–12.81)             | 14.40 (9.57–17.01)    | 14.9 (13.52–16.93)            |
| Height + S.D. (in)   | 122.39 ± 8.75     | 134.33 ± 14.36                | N/A                   | 171.75 ± 7.65                 |
| Weight + S.D. (kg)   | 31.84 ± 14.79     | 31.25 ± 11.79                 | 57.51 ± 19.96         | 64.42 ± 21.82                 |
| % Steroid            | 100%              | 0                             | 100%                  | 0                             |

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We note that resistance values increase over time in the DMD boys and decrease in the healthy subjects (Figs. 3 and 4), so the observed slope differences correspond to a combination of both effects. For example, for the 12-month ambulatory analysis, the seven muscle average slope in DMD boys is 0.0617 (S.E. +/- 0.0166) whereas that for healthy boys is -0.0232 (S.E. +/- 0.0141). Similarly, for the non-ambulatory boys, biceps slope in DMD boys is 0.0529 (S.E. +/- 0.0302) and that for healthy boys is -0.0858 (S.E. +/- 0.0316).

Functional changes and correlations

In the ambulatory DMD cohort, neither the North Star Ambulatory Assessment nor the 6-MWT showed significant change over 6 months or 1 year compared to baseline (North Star, baseline 24.8 ± 6.4 points, 23.3 ± 8.9 points at 1 year, 6-MWT, 397 ± 71 m baseline, 398 ± 68 m at 1 year). However, the supine-to-stand test showed significant worsening at both 6 months and 1 year (baseline, 4.7 ± 2.0s, 6 months 5.7 ± 2.9s, 1 year 5.0 ± 1.6s), $P = 0.0004$ and $P = 0.026$, respectively.

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**Table 2.** Rates of significance out of the 40 EIM parameters x 10 muscle groups evaluated when comparing longitudinal EIM slopes for DMD vs healthy boys.

|                | Ambulatory | Non-Ambulatory | 6 months | 12 months | 6 months | 12 months |
|----------------|------------|----------------|----------|-----------|----------|-----------|
| $P < 0.05$     | 132        | 4              |          |           |          |           |
| $P < 0.01$     | 85         | 0              |          |           |          |           |
| $P < 0.001$    | 45         | 0              |          |           |          |           |
| $P < 0.0001$   | 17         | 0              |          |           |          |           |
We performed a correlation analysis for the entire ambulatory cohort between longitudinal changes (out to 12 months) in supine-to-stand and longitudinal changes (out to 12 months) in 100 kHz resistance across muscles and muscle groups. We identified highly significant correlations between longitudinal changes in supine-to-stand and resistance for both individual muscles and muscle groups, with the highest correlation ($R = 0.644$, $P = 0.00068$) between supine-to-stand and the 100 kHz resistance seven muscle average.

The only functional measure assessed longitudinally in the older boys was the Brooke upper limb assessment. This showed little-to-no change over the 6- or 12-month periods in the majority of boys. Thus, we did not pursue a correlation analysis between EIM measures and Brooke scores.

**Baseline differences based on longitudinal mixed effect model**

In the ambulatory DMD boys, at baseline the 100 kHz resistance values were uniformly higher than those of controls in all muscles studied (as above in Figure 4), although those values reached significance in only a small subset of muscles (e.g., quadriceps of $5.45 \pm 0.36$ vs. $4.50 \pm 0.29$ ohms, $P = 0.042$ for DMD vs. healthy boys). In contrast, in the non-ambulatory boys, there were significant differences across virtually all muscles studied at baseline, with the DMD boys again having higher values (e.g., quadriceps $13.40 \pm 0.43$ ohms vs. $8.70 \pm 0.44$ ohms, $P < 0.0001$ for DMD vs. healthy boys).

**Discussion**

This study extends our original observations that EIM is sensitive to changes over time in DMD across multiple ages and stages of disease (ambulatory to non-ambulatory) and when performed by different investigators across institutions. In addition, we identified specific EIM parameters that (1) differentiated healthy boys from DMD boys at baseline, (2) were sensitive to disease-related change over time with large effect sizes (and thus excellent potential power to detect drug effects in a clinical trial), and (3) correlated to meaningful functional change over time. The sample size estimations included here parallel both those identified in the earlier single-site study of EIM$^{18}$ and those identified for MRI.$^{11}$

A key finding of these studies is that 100 kHz resistance — in lower limb muscles (especially quadriceps and tibialis anterior) for ambulatory boys and upper limb muscles (especially biceps brachii) for non-ambulatory boys — is the most sensitive and reliable EIM-based measure of DMD disease progression across disease stage. Further assessment of this EIM parameter as a sensitive measure of disease progression and potentially treatment effect will be very valuable in future studies. Resistance values not

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**Table 3. 100 kHz resistance slope differences (+standard errors), effect sizes, and sample size estimates for a 6-month clinical trial at 50% and 25% treatment effects**

| Measure               | Slope difference (+S.E.) | Slope difference $P$-value | Effect size | Sample size at 50% | Sample size at 25% |
|-----------------------|--------------------------|----------------------------|-------------|--------------------|--------------------|
| **6-month analyses Ambulatory** |                          |                            |             |                    |                    |
| Seven muscle average  | 0.0876 (0.0260)          | 0.0011                     | 0.7904      | 101                | 403                |
| Lower muscle average  | 0.1201 (0.0314)          | 0.0002                     | 0.8975      | 78                 | 312                |
| Upper muscle average  | 0.0609 (0.0287)          | 0.037                      | 0.4936      | 258                | 1031               |
| Quads                 | 0.1482 (0.0337)          | 0.1024                     | 8.1         | 547                | 2188               |
| Tibialis              | 0.1115 (0.0304)          | 0.0006                     | 0.8843      | 81                 | 322                |
| Gastroc               | 0.0561 (0.0389)          | 0.1539                     | 0.3389      | 1590               | 6360               |
| Wrists extensors      | 0.1021 (0.0286)          | 0.0006                     | 0.8361      | 90                 | 360                |
| Wrists flexors        | 0.0864 (0.0340)          | 0.0133                     | 0.589       | 181                | 724                |
| Biceps               | 0.0334 (0.0392)          | 0.3977                     | 0.1987      | 1590               | 6360               |
| Deltoid               | 0.0238 (0.0492)          | 0.6296                     | 0.1119      | 5017               | 20067              |
| **6-month analyses Non-ambulatory** |                          |                            |             |                    |                    |
| Seven muscle average  | 0.0426 (0.0445)          | 0.3436                     | 0.3559      | 496                | 1983               |
| Lower muscle average  | 0.0440 (0.0638)          | 0.4939                     | 0.2561      | 958                | 3830               |
| Upper muscle average  | 0.0350 (0.0532)          | 0.5131                     | 0.2446      | 1050               | 4200               |
| Quads                 | 0.0018 (0.0693)          | 0.9791                     | 0.0103      | 502310             | 2369238            |
| Tibialis              | −0.00045 (0.0740)        | 0.9521                     | 0.024       | 109395             | 437577             |
| Gastroc               | 0.1629 (0.1130)          | 0.1619                     | 0.5601      | 201                | 801                |
| Wrists extensors      | 0.0492 (0.0760)          | 0.5242                     | 0.2498      | 1007               | 4025               |
| Wrists flexors        | −0.0687 (0.0652)         | 0.2974                     | 0.3973      | 398                | 1592               |
| Biceps               | 0.1321 (0.0630)          | 0.0412                     | 0.784       | 103                | 409                |
| Deltoid               | 0.0712 (0.0813)          | 0.3854                     | 0.3251      | 594                | 2376               |

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only reflected differences in disease progression between the two groups; in the ambulatory boys the changes over time (as measured by slope) also correlated significantly across several muscles with the supine-to-stand test.

Importantly, other EIM parameters also show disease change. As noted in Table 2, for the ambulatory cohort well over 100 EIM parameter-muscle pairs demonstrated significant differences in longitudinal trajectories in healthy versus DMD boys. These parameters included single and multifrequency reactance and phase values, as well as other resistance-related parameters. However, many of these parameters did not reach significance in the admittedly small non-ambulatory cohort comparisons. Given the relatively small patient numbers in both non-ambulatory DMD and age-similar healthy cohorts out to 12-months, and the known disease heterogeneity, this is perhaps not surprising. It remains conceivable that these parameters could still fare well in a larger study and could be valuable for detecting drug-related effects on DMD muscle.

Table 4. 100 kHz resistance slope differences (+/- standard errors), effect sizes, and sample size estimates for a 12-month clinical trial at 50% and 25% treatment effects.

| Measure                  | Slope Difference (+S.E.) | Slope Difference P-value | Effect Size | Sample Size at 50% | Sample Size at 25% |
|--------------------------|--------------------------|--------------------------|-------------|-------------------|-------------------|
| 12-month analyses Ambulatory |                          |                          |             |                   |                   |
| Seven muscle average     | 0.0849 (0.0217)          | 0.0002                   | 1.2137      | 43                | 171               |
| Lower muscle average     | 0.1025 (0.0234)          | 0                        | 1.5838      | 26                | 101               |
| Upper muscle average     | 0.0637 (0.0253)          | 0.0145                   | 0.7801      | 104               | 413               |
| Tibialis                 | 0.0912 (0.0206)          | 0                        | 1.7724      | 20                | 80                |
| Quads                    | 0.1383 (0.0284)          | 0                        | 1.7233      | 22                | 85                |
| Gastroc                  | 0.0529 (0.0314)          | 0.0998                   | 0.6315      | 158               | 630               |
| Wrist extensors          | 0.1028 (0.0243)          | 0                        | 1.3288      | 36                | 143               |
| Wrist flexors            | 0.0764 (0.0273)          | 0.0077                   | 1.0145      | 24                | 95                |
| Biceps                   | 0.0365 (0.0306)          | 0.2415                   | 0.4192      | 358               | 1430              |
| Deltoid                  | 0.0355 (0.0446)          | 0.4298                   | 0.2551      | 965               | 3859              |
| 12-month analyses Non-ambulatory |              |                          |             |                   |                   |
| Seven muscle average     | 0.0567 (0.0349)          | 0.1194                   | 0.9631      | 68                | 271               |
| Lower muscle average     | 0.0643 (0.0526)          | 0.2375                   | 0.7004      | 129               | 513               |
| Upper muscle average     | 0.0510 (0.0375)          | 0.179                    | 0.827       | 92                | 368               |
| Quads                    | 0.0110 (0.0570)          | 0.8486                   | 0.1088      | 5301              | 21201             |
| Tibialis                 | 0.0413 (0.0612)          | 0.5078                   | 0.3783      | 439               | 1756              |
| Gastroc                  | 0.1117 (0.0829)          | 0.2057                   | 0.7703      | 106               | 424               |
| Wrist extensors          | 0.0885 (0.0531)          | 0.1136                   | 0.9557      | 69                | 276               |
| Wrist flexors            | -0.0245 (0.0461)         | 0.5981                   | 0.3283      | 583               | 2331              |
| Biceps                   | 0.1387 (0.0438)          | 0.0027                   | 1.919       | 18                | 69                |
| Deltoid                  | 0.0784 (0.0562)          | 0.1689                   | 0.8353      | 90                | 360               |

Figure 3. Example of longitudinal differences in EIM Resistance values at 100 kHz averaged across seven muscles for DMD ambulatory vs age-similar healthy control cohorts over 6 months, $R = 0.088 (0.026), P = 0.0012$ (left) and 12 months, $R = 0.085 (0.022), P = 0.00025$ (right).
Figure 4. Example of multifrequency resistance data of a DMD boy (right) and similarly aged healthy boy (left) measured over time in both upper (deltoid) and lower (vastus lateralis) limb muscles, in order to provide a qualitative sense as to how the multifrequency data changes over time. Note markedly different baseline values and increasing resistance in the DMD example with stable or slightly decreasing resistance in the healthy example (baseline curves are in blue vs 6- and 12-month curves). As supported by the cohort data presented in Tables 3 and 4, the relatively larger change between 0 and 6 months as compared to the relatively smaller change between 6 and 12 months in this particular boy with DMD is not representative of the entire group.
Compared to off-the-shelf impedance devices, the array used for this study was designed to capture muscle-related effects on impedance signals and to be less impacted by subcutaneous fat.\textsuperscript{23} Intramuscular fat deposition will increase resistance values and it is likely this, in combination with a loss of myofibers, that is contributing to the observed change in EIM parameters. We have also observed changes in resistance in recent EIM studies in the D2-mdx mouse which, unlike the standard mdx mouse, develops substantial fat infiltration of muscle.\textsuperscript{24} Changes in EIM-based resistance values are likely not specific to DMD and are likely to occur in other neuromuscular diseases characterized by increased intramuscular fat and myofiber loss.

Resistance tends to be the most stable of the impedance measurements, since at 100 kHz it is about $10 \times$ the value of the measured reactance. While it remains to be determined whether resistance at 100 kHz will ultimately be the most robust EIM parameter for determining candidate therapeutic efficacy in future DMD clinical studies, analyzing resistance across lower limb muscles in ambulatory boys and across upper limb muscles in non-ambulatory boys represents a useful starting point.

In the earlier study\textsuperscript{18} that was neither optimized for clinical use nor for measuring muscle impedance rather than fat, 100kHz resistance was not as robust as some of the other EIM parameters assessed, but it did perform in a similar manner to the current study. For example, in that study, the 100 kHz resistance measured in six muscles also showed higher mean baseline values in boys with DMD than healthy controls ($159 \pm 5.7$ ohms vs. $124 \pm 5.3$ ohms, $P = 0.0001$) and also increased over 12 months in boys with DMD compared to decreases in healthy subjects ($+0.71 \pm 0.34$ vs. $-0.2 \pm 0.34$ ohms/month, $P = 0.043$) giving an effect size of 0.96 for a 12-month study (vs. 1.2 for the seven muscle average at 12 months in this multisite study).

There were several limitations to this study, with the most obvious of these being the limited amount of 12-month data obtained. This was entirely due to the unexpected funding limitations that required us to close the study several months earlier than anticipated and not due to any technical or procedural concerns. For the most part, as our results show (Tables 3 and 4), although more limited the results of the 12-month analyses mirror the 6-month analyses.

The question arises whether the missing data for the 12-month time point should be considered missing completely at random, or whether early termination of the study also implies that the participants who contributed 12-month data were those who enrolled in the study first, and the earliest enrollers might be a somewhat different population than those who enroll late. Although it is possible that differences in the populations who completed the 6- vs. 12-month study visit could have affected our results, we found no differences in these two cohorts to suggest this.

Based on our data and sample size estimates, we would recommend that 12 months be the time course of a clinical study utilizing EIM as a study outcome. While it would be preferable to have an earlier time point, and this would be possible using EIM, as shown in Table 3 the number of subjects necessary to reliably detect a treatment effect at 6 months would be significantly larger than the number of subjects required at 12 months. We selected 12 months as the recommended time point for a relatively small clinical study with relatively liberal inclusion criteria including boys across multiple ages and genetic backgrounds, as this was the population we tested. However, our analyses also support the option of using EIM and running a shorter clinical trial using a larger population of boys.

A second major limitation was the absence of other dedicated upper extremity functional measures outside of the Brooke. Specifically, we did not include the PUL,\textsuperscript{5} which only became available after this study was designed. Third, we were not able to assess the impact of steroid initiation given the very few children who switched status during the study. Fourth, many of the functional measures we assessed changed minimally in both groups over the relatively short study time duration of 6–12 months, so seeking correlations between EIM changes and functional changes over time was a challenge. But this limitation also speaks to the limitations in the sensitivity and high variability of several of the current set of widely used functional measures and suggests the need to go beyond functional measures alone in assessing DMD progression. Finally, we have assumed linearity and the absence of ceiling or floor effects; clearly, the appropriateness of these assumptions can only be determined with additional future study.

While it can be reasonably argued that these data do not prove conclusively that EIM is sensitive to disease progression per se, the fact that EIM parameters change over time in a manner that is consistent with the known ongoing pathology strongly supports this contention. Importantly, the EIM changes also correlate with the longitudinal changes in accepted functional measures like the timed supine-to-stand test. Furthermore, there is substantial animal data to support the strong relationship between EIM alterations and tissue status. Finally, the fact that EIM appears to capture muscle changes across a wide variety of other neuromuscular diseases and morbidities (ALS, SMA, FSHD, disuse atrophy, etc), particularly in muscle groups affected by disease, gives us additional confidence that the changes that EIM is detecting are due intrinsically to DMD progression.
EIM remains a relatively new technique and its application in DMD even more recent. Only through the incorporation of this technology into future clinical trials can we fully understand and refine EIM’s role in DMD in finding effective therapies for this disease. Based on our promising results here, we encourage academic researchers and the pharmaceutical industry alike to incorporate the use of EIM into their future DMD clinical trials.

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**Conflict of Interests**

Drs. Rutkove and Bohorquez hold equity in Myolex, Inc, have or currently serve on the board of directors, have received salary or consulting income from the company, and are named as inventors on patents owned or licensed to Myolex, Inc. Laura Freedman holds equity in Myolex and receives a salary. Martin Buck similarly receives a salary. None of the other authors have any specific conflict to report. Dr. Melanie Leitner and Ms. Laura Dalle Pazze receive income from Charley’s Fund (a nonprofit Duchenne muscular dystrophy foundation).

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