A METABOLOMICS APPROACH TO INVESTIGATE DISEASE PROGRESSION IN DUCHENNE MUSCULAR DYSTROPHY

By

BRITTANY A LEE

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2016
To my family
ACKNOWLEDGMENTS

I would like to first and foremost acknowledge my husband, partner and chef, Nathan McMullen for his patience and support throughout my graduate studies. I would also like to acknowledge my mentors, Glenn Walter, Arthur Edison and Krista Vandenborne for advice, guidance and for pushing me to be a better person and scientist. I would also like to thank my committee for taking the time to give me feedback and guidance. I would like to thank my parents, sister, brother-in-law, friends and lab mates for their constant love, support and confidence in me.

I would like to acknowledge the support for this work by the National Institute of Arthritis and Musculoskeletal and Skin Diseases/National Institute of Neurological Disorders and Stroke R01AR056973/R01AR065943. I appreciate training grants including the T32 Neuromuscular Training Program (HD043730) and the TL1 Clinical and Translational Training Program (TR000066) for supporting me financially and giving me all of the opportunities. I thank our collaborators at Oregon Health & Science University, Southeast Center for Integrated Metabolomics (NIH/NIDDK 1U24DK097209-01A1), National High Magnetic Field Laboratory’s AMRIS Facility and Drs. Stephen Gardell and Christopher Petucci at Sanford-Burnham-Prebys Medical Discovery Institute at Lake Nona for targeted LC-MS metabolomics.

Finally, I would like to acknowledge and thank all of the participants in the ImagingDMD study that contributed urine and serum. I would like you to know that you inspire me every day.
# TABLE OF CONTENTS

## ACKNOWLEDGMENTS

Page 4

## LIST OF TABLES

Page 8

## LIST OF FIGURES

Page 9

## LIST OF ABBREVIATIONS

Page 11

## ABSTRACT

Page 15

## CHAPTER 1  GENERAL BACKGROUND

Page 17

- History of Duchenne Muscular Dystrophy .......................................................... 17
- Clinical Manifestation of DMD ........................................................................... 19
- Pathology of DMD ............................................................................................ 20
- The *mdx* mouse as a model for DMD .............................................................. 21
- Clinical Trials in DMD ..................................................................................... 23
- Outcomes in DMD ........................................................................................... 27

## INTRODUCTION TO METABOLOMICS

Page 29

- Techniques ........................................................................................................ 31
- Nuclear Magnetic Resonance ........................................................................... 32
- One-Dimensional NMR ................................................................................... 33
- Two-Dimensional NMR ................................................................................... 34
- TOCSY and HSQC ............................................................................................ 34
- Mass Spectrometry ........................................................................................... 35
- MRI ................................................................................................................... 36
- Feature Identification ......................................................................................... 38
- Metabolism in DMD ......................................................................................... 39

## FIGURES ........................................................................................................... 41

## CHAPTER 2  QUALITY CONTROL FOR NMR METABOLOMICS DATA

Page 46

- Introduction ........................................................................................................ 46
- Materials and Methods ..................................................................................... 48
  - Study design ..................................................................................................... 48
  - NMR sample preparation and acquisition .................................................... 49
  - NMR data processing ..................................................................................... 50
- Quality Control Workflow ................................................................................ 51
  - Assessment of Instrumentation Variation ..................................................... 51
  - Assessments of Sample Variation .................................................................. 53
  - Assessment of Feature Variation .................................................................... 53
- Results and Discussion .................................................................................... 54
3 \( ^{13}C \) AND \( ^{1}H \) METABOLOMICS COMBINE TO IMPROVE ANALYSIS AND IDENTIFICATION IN COMPLEX MIXTURES: APPLIED TO CROSS SECTIONAL MDX SERUM STUDY

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>64</td>
</tr>
<tr>
<td>Experimental Methods</td>
<td>67</td>
</tr>
<tr>
<td>Synthetic Mixtures</td>
<td>67</td>
</tr>
<tr>
<td>Drosophila melanogaster tissue extracts</td>
<td>67</td>
</tr>
<tr>
<td>Mouse Serum</td>
<td>68</td>
</tr>
<tr>
<td>NMR Data Collection and Processing</td>
<td>68</td>
</tr>
<tr>
<td>Data Analysis</td>
<td>70</td>
</tr>
<tr>
<td>Results and Discussion</td>
<td>72</td>
</tr>
<tr>
<td>Workflow development</td>
<td>72</td>
</tr>
<tr>
<td>(^{13}C ) NMR data yields improved group discrimination</td>
<td>75</td>
</tr>
<tr>
<td>(^{13}C ) NMR offers improved confidence in metabolite identification</td>
<td>77</td>
</tr>
<tr>
<td>Future Improvements</td>
<td>79</td>
</tr>
<tr>
<td>Conclusion</td>
<td>80</td>
</tr>
<tr>
<td>Figures</td>
<td>82</td>
</tr>
</tbody>
</table>

4 A LONGITUDINAL ANALYSIS OF LIPIDS IN MDX MICE

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>86</td>
</tr>
<tr>
<td>Methods</td>
<td>87</td>
</tr>
<tr>
<td>Animals</td>
<td>87</td>
</tr>
<tr>
<td>Study Design</td>
<td>87</td>
</tr>
<tr>
<td>Solution NMR on serum</td>
<td>88</td>
</tr>
<tr>
<td>HR-MAS NMR on ex vivo gastrocnemius muscle</td>
<td>89</td>
</tr>
<tr>
<td>NMR processing</td>
<td>89</td>
</tr>
<tr>
<td>In vivo imaging and spectroscopy</td>
<td>90</td>
</tr>
<tr>
<td>Statistics</td>
<td>91</td>
</tr>
<tr>
<td>Results</td>
<td>91</td>
</tr>
<tr>
<td>Multivariate global analysis of control and dystrophic mice using (^{13}C ) spectra</td>
<td>91</td>
</tr>
<tr>
<td>Quantification of metabolites in serum</td>
<td>92</td>
</tr>
<tr>
<td>Tissue metabolite comparison at different ages</td>
<td>92</td>
</tr>
<tr>
<td>Correlations of metabolites from serum to tissue</td>
<td>92</td>
</tr>
<tr>
<td>Discussion</td>
<td>93</td>
</tr>
<tr>
<td>Figures</td>
<td>97</td>
</tr>
</tbody>
</table>

5 A CROSS SECTIONAL ANALYSIS OF THE URINE METABOLOMЕ IN BOYS WITH DUCHENNE MUSCULAR DYSTROPHY IN PERSUIT OF BIOMARKER DISCOVERY

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>103</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>106</td>
</tr>
<tr>
<td>Study Design</td>
<td>106</td>
</tr>
</tbody>
</table>

Figures: 59

Results and Discussion: 72

Future Improvements: 79

Conclusion: 80

Figures: 82

Introduction: 86

Methods: 87

Animals: 87

Study Design: 87

Solution NMR on serum: 88

HR-MAS NMR on ex vivo gastrocnemius muscle: 89

NMR processing: 89

In vivo imaging and spectroscopy: 90

Statistics: 91

Results: 91

Multivariate global analysis of control and dystrophic mice using \(^{13}C \) spectra: 91

Quantification of metabolites in serum: 92

Tissue metabolite comparison at different ages: 92

Correlations of metabolites from serum to tissue: 92

Discussion: 93

Figures: 97

Introduction: 103

Materials and Methods: 106

Study Design: 106
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMR Study Design</td>
<td>108</td>
</tr>
<tr>
<td>Targeted Mass Spectrometry of Organic Acids</td>
<td>110</td>
</tr>
<tr>
<td>LC/MS/MS Quantitation of Organic Acids in Urine</td>
<td>110</td>
</tr>
<tr>
<td>Targeted Mass Spectrometry of Amino acids</td>
<td>111</td>
</tr>
<tr>
<td>Sample Preparation</td>
<td>111</td>
</tr>
<tr>
<td>Extraction and Derivatization of Amino Acids from Urine</td>
<td>111</td>
</tr>
<tr>
<td>LC/MS/MS Quantitation of Amino Acids in Urine</td>
<td>112</td>
</tr>
<tr>
<td>Serum Protein Analysis</td>
<td>112</td>
</tr>
<tr>
<td>MRI data collection and analysis</td>
<td>113</td>
</tr>
<tr>
<td>Functional tests</td>
<td>113</td>
</tr>
<tr>
<td>Statistics</td>
<td>114</td>
</tr>
<tr>
<td>Results</td>
<td>114</td>
</tr>
<tr>
<td>Muscle-associated metabolites increase in DMD urine</td>
<td>114</td>
</tr>
<tr>
<td>Energy metabolites in DMD urine</td>
<td>116</td>
</tr>
<tr>
<td>Relationship of urine creatine and citrate to muscle and serum biomarkers</td>
<td>117</td>
</tr>
<tr>
<td>Associations between urine metabolites and functional tests</td>
<td>118</td>
</tr>
<tr>
<td>Global associations of urine metabolites and MR parameters</td>
<td>118</td>
</tr>
<tr>
<td>Discussion</td>
<td>119</td>
</tr>
<tr>
<td>Conclusion</td>
<td>126</td>
</tr>
<tr>
<td>Figures</td>
<td>128</td>
</tr>
<tr>
<td>6 CONCLUSIONS AND FUTURE DIRECTIONS</td>
<td>134</td>
</tr>
<tr>
<td>Methods for furthering the metabolomics field</td>
<td>134</td>
</tr>
<tr>
<td>Lipid saturation in DMD</td>
<td>137</td>
</tr>
<tr>
<td>Urine metabolites as biomarkers in DMD</td>
<td>139</td>
</tr>
<tr>
<td>Conclusion</td>
<td>140</td>
</tr>
<tr>
<td>APPENDIX</td>
<td></td>
</tr>
<tr>
<td>A URINE SAMPLE PRERPARATION AND DATA ACQUISITION STANDARD OPERATING PROCEDURES</td>
<td>141</td>
</tr>
<tr>
<td>B SERUM SAMPLE PREPARATION AND DATA ACQUISITION STANDARD OPERATING PROCEDURES</td>
<td>143</td>
</tr>
<tr>
<td>C HR-MAS SAMPLE PREPARATION AND DATA ACQUISITION STANDARD OPERATING PROCEDURES</td>
<td>145</td>
</tr>
<tr>
<td>D QUALITY CONTROL WORKFLOW AND MATLAB SCRIPTS</td>
<td>149</td>
</tr>
<tr>
<td>E SUPPLEMENTARY MATERIAL</td>
<td>156</td>
</tr>
<tr>
<td>LIST OF REFERENCES</td>
<td>157</td>
</tr>
<tr>
<td>BIOGRAPHICAL SKETCH</td>
<td>177</td>
</tr>
<tr>
<td>Table</td>
<td>Description</td>
</tr>
<tr>
<td>-------</td>
<td>------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>4-1</td>
<td>P-values from comparisons of control and \textit{mdx} mice across age</td>
</tr>
<tr>
<td>E-1</td>
<td>Composition and summary results for the synthetic metabolite mixtures used in this study</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>Dystrophin protein with binding domains.</td>
<td>41</td>
</tr>
<tr>
<td>1-2</td>
<td>MR images from boys with DMD.</td>
<td>42</td>
</tr>
<tr>
<td>1-3</td>
<td>Characterization of disease state with MRI T₂.</td>
<td>43</td>
</tr>
<tr>
<td>1-4</td>
<td>Previously studied metabolic pathways in DMD.</td>
<td>44</td>
</tr>
<tr>
<td>1-5</td>
<td>Overview of the study.</td>
<td>45</td>
</tr>
<tr>
<td>2-1</td>
<td>Schematic of Quality Control Workflow</td>
<td>59</td>
</tr>
<tr>
<td>2-2</td>
<td>Instrumental variation for NMR parameters.</td>
<td>60</td>
</tr>
<tr>
<td>2-3</td>
<td>Run order Regression.</td>
<td>61</td>
</tr>
<tr>
<td>2-4</td>
<td>Example BA plots</td>
<td>62</td>
</tr>
<tr>
<td>2-5</td>
<td>Flag summary from Bland-Altman</td>
<td>63</td>
</tr>
<tr>
<td>3-1</td>
<td>Overview of approach to ¹H and ¹³C NMR Statistical Analysis of Synthetic Mixtures</td>
<td>82</td>
</tr>
<tr>
<td>3-2</td>
<td>Identification of <em>D. melanogaster</em> metabolites using ¹D ¹³C.</td>
<td>83</td>
</tr>
<tr>
<td>3-3</td>
<td>PCA scores separated hardy from susceptible flies much better in ¹³C data when compared to ¹H data</td>
<td>84</td>
</tr>
<tr>
<td>4-1</td>
<td>PCA of ¹³C serum spectra is able to differentiate control and <em>mdx</em> at different ages</td>
<td>97</td>
</tr>
<tr>
<td>4-2</td>
<td>Peaks of interest in ¹H spectra.</td>
<td>98</td>
</tr>
<tr>
<td>4-3</td>
<td>Quantification of serum metabolites.</td>
<td>99</td>
</tr>
<tr>
<td>4-4</td>
<td>Correlation matrices to compare serum and tissue measurements.</td>
<td>101</td>
</tr>
<tr>
<td>5-1</td>
<td>Urine creatine and 1- and 3-methylhistidine levels in DMD and in control.</td>
<td>128</td>
</tr>
<tr>
<td>5-2</td>
<td>TCA cycle metabolite levels in DMD and control urine.</td>
<td>129</td>
</tr>
<tr>
<td>5-3</td>
<td>Relationship between urine creatine levels to the vastus lateralis MRI T₂</td>
<td>130</td>
</tr>
<tr>
<td>5-4</td>
<td>ROC curves comparing citrate and serum protein MMP-9 levels in DMD-GC and DMD+GC</td>
<td>131</td>
</tr>
</tbody>
</table>
5-5  Relationship between urine creatine and citrate to functional measures in DMD. .................................................................................................................................................................................. 132

5-6  Correlation matrix of metabolites and MR parameters from control, DMD-GC and DMD+GC subjects ............................................................................................................................................................................................................................... 133
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1D</td>
<td>One dimensional</td>
</tr>
<tr>
<td>2D</td>
<td>Two dimensional</td>
</tr>
<tr>
<td>6MWT</td>
<td>6 minute walk test</td>
</tr>
<tr>
<td>6MWD</td>
<td>6 minute walk distance</td>
</tr>
<tr>
<td>AON</td>
<td>Antisense oligonucleotide</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BFLH</td>
<td>Biceps femoris long head</td>
</tr>
<tr>
<td>BMD</td>
<td>Becker muscular dystrophy</td>
</tr>
<tr>
<td>BMRB</td>
<td>Biological magnetic resonance bank</td>
</tr>
<tr>
<td>COLMAR</td>
<td>Complex mixture analysis by NMR</td>
</tr>
<tr>
<td>COSY</td>
<td>Correlation spectroscopy</td>
</tr>
<tr>
<td>CPMG</td>
<td>Carr-Purcell-Meiboom-Gill</td>
</tr>
<tr>
<td>DAG</td>
<td>Dystrophin associated glycoprotein complex</td>
</tr>
<tr>
<td>DGC</td>
<td>Dystrophin glycoprotein complex</td>
</tr>
<tr>
<td>Dko</td>
<td>Double knock out</td>
</tr>
<tr>
<td>DMD</td>
<td>Duchenne muscular dystrophy</td>
</tr>
<tr>
<td>DNP</td>
<td>Dynamic nuclear polarization</td>
</tr>
<tr>
<td>DSS</td>
<td>4,4-dimethyl-4-silapentane-1sulfonic acid</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FFT</td>
<td>Fast Fourier transform</td>
</tr>
<tr>
<td>FID</td>
<td>Free induction decay</td>
</tr>
<tr>
<td>FOR-DMD</td>
<td>Finding the Optimal Regimen for DMD</td>
</tr>
<tr>
<td>FWHM</td>
<td>Full width of half the max</td>
</tr>
<tr>
<td>GC</td>
<td>Glucocorticosteroids</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>GRMD</td>
<td>Golden retriever muscular dystrophy model</td>
</tr>
<tr>
<td>HMBC</td>
<td>Heteronuclear multiple-bond correlation spectroscopy</td>
</tr>
<tr>
<td>HMDB</td>
<td>Human metabolome database</td>
</tr>
<tr>
<td>HR-MAS</td>
<td>High-resolution magic-angle spinning</td>
</tr>
<tr>
<td>HSQC</td>
<td>Heteronuclear single quantum correlation spectroscopy</td>
</tr>
<tr>
<td>HTS</td>
<td>High temperature superconducting</td>
</tr>
<tr>
<td>IACUC</td>
<td>Institutional animal care and use committee</td>
</tr>
<tr>
<td>KBR</td>
<td>Potassium Bromide</td>
</tr>
<tr>
<td>KLF15</td>
<td>Kruppel like factor 15</td>
</tr>
<tr>
<td>LASER</td>
<td>Localization by adiabatic selective refocusing</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass to charge ratio</td>
</tr>
<tr>
<td>mCK</td>
<td>Muscle isoform of creatine kinase</td>
</tr>
<tr>
<td>MFC&lt;sub&gt;DMD&lt;/sub&gt;</td>
<td>Manifesting female carriers of DMD</td>
</tr>
<tr>
<td>MG</td>
<td>Medial gastrocnemius</td>
</tr>
<tr>
<td>MMCD</td>
<td>Madison metabolomics consortium database</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Matrix metallopeptidase 9</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MRS</td>
<td>Magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>ms</td>
<td>Milliseconds</td>
</tr>
<tr>
<td>MS/MS</td>
<td>Tandem mass spectrometry</td>
</tr>
<tr>
<td>MSD</td>
<td>Meso Scale Discovery</td>
</tr>
<tr>
<td>NDA</td>
<td>New drug application</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institute of Health</td>
</tr>
</tbody>
</table>
NIPALS  Non-linear iterative partial least squares
NMR    Nuclear magnetic resonance
nNOS   Neuronal nitric oxide synthase
NO     Nitric oxide
NOESY  Nuclear Overhauser effect spectroscopy
PAFFT  Peak alignment fast Fourier transform
PCA    Principle component analysis
PDE5   Phosphodiester type 5
PER    Peroneal
pi     Polyunsaturation index
PLS-DA Partial least squares discriminant analysis
PQN    Probabilistic quotient normalization
QC     Quality control
RF     Radio frequency
s      Seconds
SHY    Statistical heterospectroscopy
SOL    Soleus
STOCSY Statistical total correlation spectroscopy
SW     Spectral width
TA     Tibialis anterior
TCA    The citric acid cycle
TE     Echo time
Temp   Temperature
TOCSY  Total correlation spectroscopy
TP     Tibialis posterior
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TR</td>
<td>Repetition time</td>
</tr>
<tr>
<td>Trp</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>ui</td>
<td>Unsaturation index</td>
</tr>
<tr>
<td>VL</td>
<td>Vastus lateralis</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
</tbody>
</table>
Duchenne muscular dystrophy (DMD) is the most common dystrophinopathy; however, with a prevalence of one in every 5,000 male births, it is classified as an orphan disease. The loss of a functional dystrophin protein results in muscle damage and repair. Unfortunately, the repair processes eventually cannot sustain through multiple bouts of damage where infiltration of fat and fibrosis eventually render the muscles unusable. In response to an unstable muscle environment, alterations in cellular processes, metabolism and genetic modifiers exacerbate the disease.

This study combines both new methodologies and the application of these methods to better understand metabolic changes that occur in DMD. I determined the differences in the metabolic profiles longitudinally in the DMD murine model, and cross sectionally in human urine samples. Lipid saturation levels in the *mdx* serum varied at different stages of the disease, suggesting metabolism pathways are affected by the loss of dystrophin but can renormalize with correction. I then investigated the urine from boys with DMD and compared these results to a combination of serum protein markers, function and magnetic resonance measurements. Further we found that urine
metabolites were altered by glucocorticosteroid intervention. The metabolic profile of boys with DMD that were steroid naïve were significantly altered as compared to controls. Moreover, urine metabolites from boys taking steroids were approaching near control levels as compared to boys that were steroid naïve. These findings are potential biomarkers for disease in DMD, but further, these results may be able to give insight into potential mechanisms still unknown to be occurring in the dystrophic tissue.
CHAPTER 1
GENERAL BACKGROUND

History of Duchenne Muscular Dystrophy

Guillaume-Benjamin Duchenne de Boulogne\textsuperscript{1} was a researching clinician whose contributions to science include advancing the field of electrophysiology, initiating the use of muscle biopsies for diagnosis and characterizing many neuromuscular diseases. In 1865, he characterized the most common of these diseases, Duchenne muscular dystrophy (DMD). Duchenne’s initial observations detailed the progressive proximal to distal muscle weakness in the lower limbs, the initial muscle hypertrophy followed by gradual atrophy, and the distinct histological pattern\textsuperscript{2}. Using muscle biopsies and histology throughout the course of the disease, Duchenne discovered that DMD is a disease of muscle rather than of the nervous system\textsuperscript{3}. In 1888, Gower\textsuperscript{4} further characterized DMD by describing a compensatory maneuver (Gowers maneuver) used by many boys to get up from the ground, a consequence of proximal weakness. Gower also made the observation that DMD characteristics were usually in males and seemed to be inherited maternally\textsuperscript{4}.

For 100 years after Duchenne’s description of DMD, little was learned of the pathogenesis of DMD. Functional tests were the primary diagnostic and prognostic determinants for DMD. However, in the 1980s, important breakthroughs for DMD began to occur. In 1983, Davies et al. identified two genetic sequences linked to DMD from genomic libraries of the human X chromosome\textsuperscript{5}. Soon after, ribosomal genes in close proximity to the DMD gene were cloned allowing investigators to identify some of the deletions found in DMD. By 1987, the dystrophin gene was sequenced and was found to be 2.4 megabases and 79 exons, one of the largest genes in humans\textsuperscript{6}. To date, there
is still no cure for DMD, but uncovering the genetic basis of DMD was instrumental in making advances in diagnosis and development of potential therapies.

DMD is the most common muscular dystrophy and dystrophinopathy in children, but it is not the only dystrophinopathy. Becker’s muscular dystrophy (BMD)\textsuperscript{7,8} has a later onset and is a less severe form of DMD as a result of a partially functioning dystrophin protein. Men with BMD usually live until their 60's but can have functional problems throughout life.\textsuperscript{9} A study showed that there may be two patterns of progression for BMD; 1) earlier onset near ages 7-9 with more severe cardiac involvement and difficulty climbing stairs by age 20, or 2) onset in the early teens with milder cardiac involvement.\textsuperscript{10} However, it is difficult to truly define these two subgroups as there is true heterogeneity in the progression of both DMD and BMD. Although, quality of life for BMD is still impacted by loss of muscle function and the need for assistive devices, they are often able to still maintain independence and relatively active lifestyles.\textsuperscript{10}

Manifesting female carriers of Duchenne muscular dystrophy (MFC\textsubscript{DMD}) are also considered to have a dystrophinopathy. Although DMD usually occurs in males, about 12-22% of females that contain the mutation on one of their X chromosomes may still display symptoms.\textsuperscript{9,11} The presentation of disease is much more mild with onset in early adulthood.\textsuperscript{12,13} It is suggested that MFC\textsubscript{DMD} may be due to X-autosomal translocation, which can cause X-inactivation of the chromosome with the non-mutated dystrophin gene, but many genetic mechanisms are still unknown.\textsuperscript{14}
Clinical Manifestation of DMD

The dystrophin protein is absent from birth, but the loss of a functional dystrophin protein does not prevent the muscle from growing and regenerating. Therefore diagnosis often doesn’t occur until the age of 3-5\(^2\) once damage affects physical function. At this stage, delayed gross motor milestones are often the first indication of disease for parents, and this is usually followed by a blood test to check for elevated serum muscle specific isoform of creatine kinase (mCK) levels\(^{15}\). In fact, many advocate for the addition of serum mCK to newborn screens for earlier diagnosis \(^{16-21}\). Although slower than their age-matched counter parts, boys with DMD still tend to show functional improvement until between the ages of 7-9\(^2\). At the onset of the early teens, boys often lose ambulation and use a wheelchair/powerchair exclusively for mobility. Shortly after loss of ambulation, boys begin to lose upper arm function. In the late teen/early twenties, weak heart, thoracic, and diaphragm muscles lead to respiratory and cardiac declines, and boys often need to begin respiratory assistance at night. Ultimately, death from DMD is caused from respiratory and/or cardiac muscle failure\(^3\).

Although there is no cure, there are palliative medications and clinical treatments that can prolong survival. Glucocorticosteroids (GC) have become the current standard of care and have been shown to delay the rate of disease progression by 2 to 5 years\(^{22}\). However, steroids are associated with negative side effects including weight gain, attention deficits, and kidney damage\(^{22}\). Unfortunately, there are no therapies other than GCs that have reached approval by the United States Food and Drug Administration (FDA) (explained in more detail in section Clinical Trials in DMD).
Pathology of DMD

DMD is caused by any number of mutations of the 2.4 Mb dystrophin gene of the X-chromosome\(^5\) that ultimately results in the loss of functional dystrophin protein. Genetic mutations that can result in DMD are quite heterogeneous where about 60-65% of the mutations are caused by large deletions, 5-10% are caused by large duplications, and the remaining mutations are a combination of point mutations, small deletions, or duplications\(^{23}\). The sub-sarcolemmal dystrophin protein (Figure 1-1) acts as an anchor between filamentous actin and the membrane embedded dystrophin glycoprotein complex (DGC)\(^{24-26}\), which connects to the extracellular matrix\(^{27,28}\). Many proteins comprise the DGC, such as sarcoglycans, sarcospan, syntrophins, dystrobrevin, and dystroglycans, each having a vital role in adequate muscle function\(^{29,30}\).

Sarcoglycans\(^{31,32}\) are a transmembrane subgroup of the DGC responsible for maintaining a connection from the cytoskeleton to the extracellular matrix\(^{30,33}\). The loss of any single sarcoglycan results in a clinically described limb-girdle muscular dystrophy\(^{34-37}\). Dystroglycans play the vital role of linking dystrophin to the extracellular matrix. When dystroglycans are mutated or non-functional, the connection to the extracellular matrix is lost, leading to various forms of congenital muscular dystrophy or myopathy\(^{38,39}\).

This project specifically focuses on Duchenne muscular dystrophy, where loss of functional dystrophin connecting the cytoskeleton to the DGC causes sarcolemmal instability. Dystrophin has additional binding sites regulating signaling pathways in the cell (Figure 1-1) such as nNOS. When dystrophin is no longer in the cell, secondary effects such as incorrect cell signaling to regulate nitric oxide cause impaired blood flow and further pathology\(^{28,40}\). Because of the compromised sarcolemma, small molecules
and large proteins leak both in and out of the muscle cells, leading to imbalances in calcium\textsuperscript{15}. Calcium imbalances activate the calpain/caspase pathways\textsuperscript{41,42} resulting in an influx of exogenous immune cells\textsuperscript{43-45} causing further damage. As a result, damaged muscle repair mechanisms recruit satellite cells\textsuperscript{46}. The damage – repair paradigm continues until regenerative mechanisms are exhausted, leading to a predictable time course of inflammation, fatty infiltration, and ultimately fibrosis\textsuperscript{27,47}, rendering the muscles non-functional. The widespread replacement by non-contractile tissues can be readily visualized and quantified by MRI (Figure1-2) which I will discuss further later in this chapter.

**The mdx mouse as a model for DMD**

The *mdx* mouse model is the most common murine model used for DMD preclinical studies. In 1984, the *mdx* mouse was found to have elevated serum mCK and histology that revealed muscle damage\textsuperscript{48}. The *mdx* mouse is similar to the human condition as it also has a mutation in the dystrophin gene that results in a dystrophin knock out. *Mdx* mice have muscle damage early in life (3-4 weeks old) which causes an inflammation response of M1 and later M2 macrophages\textsuperscript{49}. There are also signs of regeneration and repair after this initial wave of damage\textsuperscript{50}. Unfortunately, the *mdx* phenotype does not entirely recapitulate DMD exactly as it presents in humans. For example, the dystrophin protein is located on the X chromosome on the 23\textsuperscript{rd} exon, however, through years of inbreeding both male and female mice are able to be dystrophin deficient. Often the female mice are not used in order to reduce the confounding problem of sex in the results and interpretation of the data. Although female *mdx* mice have reported a more severe phenotype than the males\textsuperscript{51}. As a compensatory mechanism, the *mdx* mouse upregulates utrophin\textsuperscript{52-55}. Utrophin is a
membrane protein with similar structural functions to dystrophin but is smaller and is lacking important binding sites, specifically those to nNOS\textsuperscript{54}. Utrophin is most prominent in fetus muscle but is reduced dramatically when dystrophin begins to be expressed\textsuperscript{56}. In healthy muscle, utrophin is still present predominantly at the neuromuscular junction in the skeletal muscle and intercalated discs in cardiac muscle\textsuperscript{56}. When utrophin is upregulated in the absence of dystrophin, the phenotype is mitigated due to semblance of a stable membrane as the mice become adults\textsuperscript{54,55}. The Tidball lab has shown that the peak time of the disease in the \textit{mdx} is at around 4-6 weeks of age\textsuperscript{44}. The mice then tend to show a recovery phenotype due to the upregulation of utrophin\textsuperscript{50,55}.

However, the \textit{mdx} is not the only mouse model used to study DMD. There is also the double knock-out model (dko) is both dystrophin and utrophin null and the mice that are dystrophin null and heterozygous for utrophin. The dko mice have a more severe form of disease where they are not able to regenerate muscle to the same degree as the \textit{mdx}, and they have a much lower life expectancy at 20 weeks\textsuperscript{57}. Although this model does mimic the human condition a bit more closely, there is still the question of whether the mechanisms of disease are altered by a loss of a second protein. Others have altered the \textit{mdx} model to try to mimic the human condition crossing the \textit{mdx} mouse with models from a different background, such as the DBA/2-\textit{mdx} mouse\textsuperscript{58-60}. The DBA/2-\textit{mdx} is known to be a better model of the human disease because the mice have significantly more fibrosis and less regeneration. Unfortunately, it is very difficult to breed these mice due the severity of the disease. Another method is to delete the \textit{Cmah} gene, which is responsible for the biosynthesis of sialic acid derivative Neu5Gc, which
has been lacking in humans for millions of years but can potentially be protective towards the loss of dystrophin in mice\textsuperscript{61}.

As there is no non-human primate model for DMD, the most common and most appropriate larger animal model for DMD is the golden retriever with muscular dystrophy (GRMD)\textsuperscript{62}. The GRMD model is consistent with the human phenotype in progression, functional loss, and even heterogeneity and variation, which is why it is often used to test potential new drugs and biomarkers. In recent years, they have expanded large animal models to a DMD pig model\textsuperscript{63} and a beagle DMD model\textsuperscript{64}, but further characterization of these models are still necessary before they can be used as confidently as the GRMD model. Overall, there are many other models used to study DMD, each with similarities and inconstancies with the human condition; however, the \textit{mdx} model is currently most genetically relevant model, which is why we use this model in the current study. However, novel studies are beginning to use a humanized mouse model using patient deletions\textsuperscript{60}.

**Clinical Trials in DMD**

There is no drug, therapy, or cure for DMD, but the one current standard of care for managing DMD is GCs, such as Prednisolone and Deflaxacort, which have been shown to prolong ambulation and maintain muscle strength\textsuperscript{22,65,66}. Exact mechanisms for this phenomenon are unknown\textsuperscript{29,67}, although GCs are thought to involve both protective methods that decrease NF-kB signaling but also activate steroid receptors\textsuperscript{68}. One recent study found that steroids were able to upregulate a protein called Krupple-like factor 15 (KLF15), which was shown to alter transcription of proteins involved in metabolic pathways of amino and fatty acids in the \textit{mdx} mouse and human tissue. Much debate still exists in regards to the use of GCs because of the many negative side
effects that exist with prolonged GC use, such as stunted growth, weight gain, cataract formation, kidney problems and behavioral issues\textsuperscript{33,67}. A new trial, Finding the Optimum Regimen for DMD (FOR-DMD), is underway to optimize the regime and type (Prednisone or Deflazacort) of GC for maximal muscle strength but with the fewest side effects (clinicaltrials.gov, NCT01603407).

Past preclinical studies have shown promising results in treating DMD, even implementing a usable dystrophin protein with many forms of gene therapy\textsuperscript{37,43,69,70}. Unfortunately, the success of the preclinical studies has not translated well into the clinical trials. Currently, new research has shifted to potentially ease the disease by treating symptoms or consequences of the disease.

One approach is to ameliorate the inflammatory response to minimize damage to the muscle but without the side effects that come with GC treatment. Specifically, Catabasis is beginning a trial on the drug edasalonexent (previously known as CAT-1004) (clinicaltrials.gov; NCT02439216) which aims to reduce the inflammatory response to prevent damage to the muscle tissue. Other drugs, Santhera (clinicaltrials.gov; NCT01027884) and Eplerenone\textsuperscript{71} seek to treat the cardiac and respiratory effects that are usually the cause of death in this population. These approaches have shown early efficacy and are continuing to be studied. Another approach was to use phosphodiesterase type 5 (PDE5) inhibitors, Tadalafil (Cialis) and Sildenafil (Viagra), to improve blood flow to the muscles of boys with DMD due to the loss of fully functioning nNOS (clinicaltrials.gov, NCT01359670)\textsuperscript{72,73}. Unfortunately, PDE5 inhibitor studies have had limited success. Lastly, there is a new phase 1 / 2 trial testing the safety, tolerability and immunogenicity of anti-myostatin adnectin (BMS-
Anti-myostatin adnectin is a protein with binding affinity to myostatin that inhibits the functionality of myostatin. When myostatin is inhibited, muscle growth is less restricted leading to significantly larger muscles. Because this trial is still in the recruiting phase, the results are still unclear. However, mouse studies with myostatin inhibition were successful in creating larger muscles but have minimal enhances in muscle health, strength and function.

Although treatments to ease symptoms and prolong progression are important to the field, there has been a large effort to address the genetic basis of DMD. Clinical trials have focused on innovative gene replacement therapies or small molecule drugs that “skip over” or “read through” the genetic mutation, resulting in internally truncated, partially functional dystrophin. Therapies, such as viral-mediated gene therapy have all shown success in animals, but demonstrated limited success in human studies.

“Read-through” drugs such as Ataluren, use complimentary nucleotide sequences to bind to RNA acceptor sites allowing transcriptional machinery to maintain the correct reading frame and ignore inappropriate nonsense mutations. Ataluren has had mixed results. The phase 2b clinical trial initially reported inadequate six minute walk test (6MWT) results, leading to a termination of the clinical trial; however, further analysis after subgrouping patients suggested statistical significance. This allowed for a phase 3 trial (clinicaltrials.gov; NCT01826487), ACT DMD in 2014/2015, but the FDA appears unconvinced Ataluren is beneficial for boys with DMD and issued a “Refuse to File” letter in response to the new drug application (NDA) in February of 2016. In Europe, Ataluren (Translarna in Europe) was given accelerated conditional...
approval in 2014. Unfortunately, with the latest results from the phase 3 trial in the United States, there is concern that the European drug regulations may reconsider the approval.

Another gene correction technique is exon skipping antisense oligonucleotides (AON) which offered unprecedented success in animal models\textsuperscript{67,77,78}. AONs bind on pre-processed dystrophin RNA at splice regulatory element binding sites, effectively “hiding” the splice sites, which allows for skipping of the exon with the mutation. As a result, AONs preserve colocalization, membrane stability, and cell signaling cascades within muscle cells\textsuperscript{67,79}. There are currently three AON drugs that have made it to human clinical trials: Drisapersen, Eteplirsen and PRO-044. Unfortunately, phase 2 and 3 studies have had limited success. The NDA for Drisapersen was declined by the FDA who concluded that the research did not provide substantial evidence of the drug’s effectiveness or that the drug has an acceptable risk-benefit to people (clinicaltrials.gov,NCT1890798). There is a current study in the recruiting phase to investigate the long-term use of Drisapersen (clinicaltrials.gov NCT01803412) The Sarepta drug, Eteplirsen, has also been under consideration by the FDA, but has currently been given a three month extension for further review of the submission (clinicaltrials.gov, NCT02255552).

Despite these complications in the field, promising animal studies continue to bring hope of potential therapies for DMD, including peptide-conjugated PMO mediated exon skipping and combination stem cell therapy and exon skipping, which are in preliminary studies for clinical trials\textsuperscript{67,80,81}. Most recently, groups are beginning to move
forward with CRISPR/Cas 9 treatments in DMD\textsuperscript{69}, with encouraging results in rodent models\textsuperscript{82,83}.

**Outcomes in DMD**

Years of preclinical work has helped to identify potential therapies for DMD. Clinical trials are beginning to progress, but only for the subset of boys with DMD that are still ambulatory. The only FDA accepted outcome measure of clinical trials is the 6MWT, which requires adequate ambulation in order to enroll in such trials\textsuperscript{47,84-87}. Functional outcomes show benefit for individuals with DMD and their caregivers in quality of life, but most functional outcomes require ambulation. However, in normal disease progression, post pubescent boys are non-ambulatory, and these functional tests are no longer applicable. Non-ambulatory population outcomes include upper extremity functional testing and general quality of life assessments\textsuperscript{33,37}. Because of current FDA standards, maintenance of upper extremity function is not recognized as a beneficial outcome measure of clinical trials, when in actuality, it should be recognized that maintaining upper extremity function would still improve quality of life for this population. It would be beneficial to have a marker for disease progression and drug efficacy irrespective of the state of disease.

Despite all of the research that has occurred, a robust, sensitive, specific, and non-invasive biomarker eludes researchers. Serum mCK is markedly elevated (100 fold increase) in the DMD population, but mCK unfortunately is a non-specific indicator of muscle pathology\textsuperscript{2,53} and does not change with progression of disease\textsuperscript{88}. One serum metabolomics study revealed differences in creatine as a major difference between DMD and controls\textsuperscript{89}. This is in part due to the fact that mCK is based on total muscle volume, therefore it is significantly decreased during the latter stages of DMD when
muscle mass is lost. Other serum compounds, vitamin D, 1- and 3- methylhistidine and metalloproteinase-9 (MMP-9) have also been investigated as progression biomarkers, but have only been shown to correlate with general muscle states such as muscle degeneration and regeneration. More recently, extensive research in microRNA shows diagnostic and prognostic potential. Microarray analysis has shown several miRNA’s specific to DMD, known as dystromirs, are seen to correlate with different stages of DMD. Many of the miRNA found to be upregulated in DMD, such as miR-1, miR-133a and miR-206, are associated with myogenesis, fibrosis, regeneration and inflammation. Most notably miR-31, the most upregulated in mdx adductor, has been correlated to regeneration and regulation of dystrophin. In serum specifically, miR-1, 21, 31, 29c, and 133a show significant elevation in the mdx. Although promising, it has yet to be shown that these increases in miRNA are sensitive to particular states of disease.

Other studies have investigated urine for markers of disease in DMD. Titin fragments in the urine are shown to associate to disease progression. Others have found that increases in prostaglandin D2 in urine is strong indicator of disease in DMD. Finally, early labeling studies have shown that the turnover of creatine and creatinine in the urine are much higher in boys with DMD than not only controls but other neuromuscular diseases.

We and others have shown that magnetic resonance imaging (MRI) and spectroscopy (MRS), can be used to track disease progression in DMD. MRI has the advantage of visualizing specific muscle involvement and tissue heterogeneity in a non-invasive manner (Figure 1-2), where MRS can be used to quantify the fat that
has infiltrated into the muscle. Further, MRI parameters can detect disease progression in periods as short as 3 months\textsuperscript{103}. Boys with DMD are still improving according to the 6MWT in younger ages before the decline of ambulation begins at around 7-9 years old. However, MRI parameters can still detect muscle pathology declining during these early stages\textsuperscript{104}. Although magnetic resonance is continually progressing as an outcome for DMD in clinical trials, MRI is still expensive and limited by the facilities that are able to perform enhanced MRI sequences. There is still a need for an objective, non-invasive and reliable outcome(s) for DMD progression and therapeutic efficacy to use in clinical trials.

**Introduction to Metabolomics**

In 1999 a paper by Jeremy Nicholson, John Lindon and Elaine Holmes coined the term metabonomics\textsuperscript{105}. In this paper they defined metabonomics as “…the quantitative measurement of the dynamic multi-parametric metabolic response of living systems to pathophysiological stimuli or genetic modification.” A few years later, in 2002, Oliver Fiehn coined the more commonly used term metabolomics with a very similar definition\textsuperscript{106}. These papers paved the way for a new field of –omics that studies small molecules (below 1500 Daltons) that are produced or present in a biological system.

In the past 20 years, there has been a strong push to learn how a group of metabolites in a living system are correlated with phenotype and disease\textsuperscript{107}. The central dogma of biology shows the interconnectedness of genes, RNA and proteins. This interconnectedness is beneficial for attempting to learn if the machinery of a pathway is present. However, biology is more complicated. Increases in transcription of a gene does not mean the entire pathway is upregulated. RNA must be spliced, translated and
the resulting proteins must be functional for a system to work. Genomics, transcriptomics or proteomics cannot answer these complicated questions alone. Although it does not completely resolve these complications, the addition of metabolomics is able to help answer if a system is functioning. Metabolites are not only substrates to enzymes but products of the reactions\(^\text{108}\). Monitoring the quantifications and ratios of metabolites can be used to determine a functioning system by a metabolic profile compared between health and disease. It is for these reasons that metabolomics is so often used for biomarker discovery\(^\text{108}\).

A biological marker, more commonly referred to as biomarker, has been defined by many regulatory institutions. As defined by the National Institute of Health (NIH), biomarkers are “objective indications of medical state observed from outside the patient – which can be measured accurately and reproducibly\(^\text{109}\).” The World Health Organization (WHO) defined biomarker as “any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease” (Retrieved from http://www.inchem.org/documents/ehc/ehc/ehc222.htm). “A biomarker that is measured in an analytical test system with well-established performance characteristics and for which there is an established scientific framework or body of evidence that elucidates the physiologic, toxicologic, pharmacologic, or clinical significance of the test results,” is how the FDA defines a valid biomarker (http://www.fda.gov/Safety/Recalls/IndustryGuidance/default.htm). Although there are small alterations between all of these definitions, the basis is the same and
metabolomics is a valuable technique to determine states of health and disease for biomarker discovery.

Metabolomics has focused heavily on diseases that were already associated with metabolic dysregulation such as diabetes\textsuperscript{110}, cancer\textsuperscript{111} and obesity\textsuperscript{112,113}. More recently, metabolomics has expanded to diseases where the cause of disease is still unknown or has no direct association to metabolism. Interestingly, these studies have shown that even if the disease is without a direct association to metabolism there are often still metabolic alterations to the system such as, Alzheimer’s\textsuperscript{114} and Parkinson’s disease\textsuperscript{115}.

Metabolomics has flourished as a biomarker discovery tool. Metabolomics is becoming more attractive and gaining traction as it has expanded into uses such as precision medicine\textsuperscript{116} and investigation of pathway mechanisms\textsuperscript{117}. Although in its infancy, better integration of -omics platforms will advance the field immensely. Also, metabolomics grows as the technology grows around it. Some of the greatest feats in metabolomics are finding new, efficient and innovative ways to diagnose diseases, determine disease progression or even separate healthy from disease tissue in real time. For example, the iKnife is a state-of-the-art method of detection of pathology. The iKnife send the cauterized tissue during surgery directly to a mass spectrometer for analysis. A user friendly read out then informs the surgeon if the tissue is healthy or cancerous\textsuperscript{118,119}. This has been used in breast cancer\textsuperscript{118}, brain\textsuperscript{118} and most recently, colonoscopies\textsuperscript{119}. However, most studies in metabolomics use relatively established methods in nuclear magnetic resonance (NMR) and mass spectrometry (MS).

**Techniques**

NMR and MS are each essential to metabolomics but are important in unique ways. While NMR cannot compete with MS in sensitivity, it is preferred for structural
information and quantification of metabolites. Using NMR, samples often do not need to be extracted for global profiling. Extracting samples can force a study to target more towards polar or nonpolar metabolites. Using NMR, samples are not destroyed and can potentially be used again for further studies. Global MS is the most common method to study metabolomics and provides an accurate mass with high sensitivity. Although MS has many beneficial characteristics, some disadvantages include difficulties with lower reproducibility than NMR, unambiguous identification, and absolute quantification. Targeted MS is able to overcome many of these disadvantages by using internal standards and tandem MS which allow for better identification. Overall, each is separately a very robust technique but these methods can be very powerful when used together.

**Nuclear Magnetic Resonance**

The basic premise behind magnetic resonance is that nuclei with a spin 1/2, such as $^1$H, $^{13}$C, and $^{31}$P. When in a magnetic field, these nuclei can be found in one of two states; the alpha or the beta state, which are nearly equal in distribution (Boltzmann distribution). When stimulated with an external frequency, energy is added to the nuclei, allowing them to switch states and cancel out equal amounts of nuclei. The detected signal comes from the excess nuclei that are not cancelled from the opposite state. The summation of all frequencies giving signal are measured over time as the free induction decay (FID). In order to decompose the signals into individual frequencies; a fast Fourier transform (FFT) is applied to the FID. The result is an NMR spectrum where peak location on the x-axis is the precessional frequency and peak integrals are relative to the quantity of nuclei precessing at that frequency.
The frequency relates to a specific chemical environment of the nuclei. Electrons also possess spin 1/2, which add or subtract from the magnetic field produced by the nuclei. These small effects are known as shielding or deshielding effects on nearby nuclei. Shielding effects, as well as others caused by solvents, anisotropy, and hydrogen bonding, can alter the frequency at which a specific nucleus resonates. Changes in frequency are measured in Hz and converted to ppm (Hz per MHz), standardized to the frequency of the radio frequency (RF) pulse. The chemical shift in units of ppm is the x-axis on one dimensional (1D) NMR spectra. The peaks at specific chemical shifts is the main way metabolites are identified in both 1D and 2D NMR spectra.

**One-Dimensional NMR**

In this study, NMR experiments are performed on solutions (i.e. serum and urine) and tissue. Solution NMR is very common and has advantages in homogenizing the magnetic field (shim) and often using cold probes allows for greater sensitivity. Unfortunately, to use solution NMR on tissue, one would have to extract the tissue, but potentially lose either polar or nonpolar metabolites. In the case of not wanting to lose either, high-resolution magic-angle spinning (HR-MAS) minimizes the anisotropy by tipping the sample at an angle of 54.7 degrees ($\cos^2\theta=1/3$). The tipping of the sample and spinning at 5,000-6,000 Hz reduces broad peaks caused by anisotropy and gives a detailed spectra of the metabolites within the tissue.

The two most common 1D NMR experiments used in metabolomics are the 1D with nuclear Overhauser effect spectroscopy (NOESY) superscript[127,128] presaturation and Carr-Purcell-Meiboom-Gill (CPMG) superscript[129,130]. Most biofluids contain mostly water whose protons can interfere with the analysis of a solution. The 1D with NOESY presaturation
experiment is often used in metabolomics due to the ability to reduce the water signal\textsuperscript{128}. The CPMG sequence is also a very beneficial sequence for metabolomics. Some biofluids still contain proteins such as serum. One way to remove proteins is to extract them from the solution\textsuperscript{121,131}. In NMR, signals from large molecules decay very quickly creating very broad peaks distorting the spectra. This can be $T_2$ filtered by using a CPMG experiment. To take advantage of the short decay of large molecules signals, the CPMG experiment is a combination of refocusing pulses that maintains signal while large molecules’ signal decay. Once these decay, the resulting signal is from metabolites. In general, if a sample is not expected to have proteins (i.e. urine) then a 1D with NOESY presaturation experiment is used, and if the sample likely contains proteins (i.e. serum) than a CPMG experiment is used. These will all be labeled in the methods of each experiment.

**Two-Dimensional NMR**

Two-dimensional (2D) NMR is defined by two frequencies rather than one, giving much more information\textsuperscript{122,127,132}. There are many 2D experiments, each that elucidates different information. Specific for these studies, we used heteronuclear single-quantum correlation spectroscopy (HSQC)\textsuperscript{133} and total correlation spectroscopy (TOCSY)\{Kumar:1980ui\} to help identify metabolites in complex mixtures.

**TOCSY and HSQC**

An HSQC experiment detects correlations between two different nuclei, most commonly between $^1$H and $^{13}$C\textsuperscript{133} for metabolomics purposes. A peak in a 2D spectrum resides at the coordinates of the chemical shifts of the pair of coupled nuclei. HSQC spectra also can show multiplicity of the protons with a directional differentiation between CH2 groups and CH/CH3 groups, which are phased 180 degrees apart\textsuperscript{133}. 
HSQC spectra are peak picked, giving a list to then compare to a database (explained further in Section Feature Identification) to create an original list of potential metabolites to verify.

TOCSY experiments give information about spin systems throughout a molecule. A spin system is comprised of protons that are coupled through covalent bonds in a molecule. As a TOCSY gives information about protons in association with other protons, a spectrum includes a diagonal of peaks that represents each peak correlated to itself and cross peaks. Cross peaks are the important information as they represent correlations between couplings of nuclei. Therefore, we use TOCSY spectra to verify that proton-carbon correlations found in the HSQC are contained in the same molecule.

**Mass Spectrometry**

Mass spectrometry is the formation of positive and negative ions that are then separated based on their mass to charge ratio (m/z). Most commonly in metabolomics studies, samples are extracted and liquid chromatography (LC) acts to separate the sample prior to m/z detection. MS has many great attributes, most prominently high sensitivity. However, ion suppression (multiple ions eluting and not all being detected), sample degradation, and sample interactions with the instrument can make it difficult to analyze the data. Due to the high sensitivity, global MS results in thousands of peaks, and information limited to elution time and m/z. This can make it difficult to annotate and unambiguously identify features. To overcome these shortcomings, targeted MS uses isotopically labeled internal standards and tandem MS (MS/MS). Concentration curves of isotopically labeled internal standards are included in targeted MS to identify and quantify specific compounds that are of an expected m/z and of a known concentration. MS/MS uses multiple steps of MS creating several...
unique fragments improving identification of a specific compound. For this dissertation, NMR spectroscopy was primarily used. As previously stated, NMR and MS are more powerful when used together. For Chapter 5, some metabolites were difficult to identify and quantify in the NMR spectra, therefore we used targeted LC-MS/MS to improve and expand our data.

**MRI**

MRI is not a widely recognized metabolomics technique and can greatly complement a metabolomics study. It was a common technique used in these projects and necessary to introduce. NMR and MRI work according to the same physical principals, however MRI uses gradients in the x, y, and z directions to detect signal over space in addition to time. $^1$H are the most commonly and easily measured nuclei. 99.98% of hydrogens are $^1$H, providing an ample signal. The abundance of protons ($^1$H$_2$O) in the human body makes imaging very convenient, and different tissue types and compositions can be differentiated using MRI relaxation properties based on their tissue concentrations and biophysical properties.

One of the most advantageous aspects of MRI is the high spatial resolution that it provides, allowing the imaging of tissue heterogeneity. Once a specific area has been chosen to image, this area is separated into small slices where data is acquired. An RF pulse is applied to a particular slice while a gradient is also applied. This is called phase encoding. The gradient causes spin differences dependent on their position and a second gradient in the orthogonal direction during signal acquisition detects these different frequencies over the space, which is called frequency encoding. This is then done for multiple slices to create multiple images over an area of interest. This data is
then recorded in k-space, temporary image space, that can then be Fourier transformed into an image.

Using different delays and pulse sequences, MRI can exploit differences in nuclear relaxation times of protons in different molecules or environment (i.e. fat and water, intracellular vs. extracellular water) to create contrast. We can then use these relaxation parameters to quantify and qualify the type and even health of a particular tissue.

There are two relaxation times: $T_1$ is the magnetization recovery in the longitudinal direction, and $T_2$ is the signal decay in the transverse (xy) plane\textsuperscript{136}. Longitudinal magnetization (in a three coordinate system) would be along the z-axis aligned with the $B_0$ field (static magnet field). When an external RF pulse is applied, the nuclei are perturbed from this initial magnetization. At this point, the Z magnetization is lost as magnetization is tipped into the xy plane. The amount of time necessary to recover the initial z magnetization plane is characterized by the $T_1$ relaxation time, usually measured in seconds (s). When the magnetization is tipped into the xy plane, the initial vector is very large, but as time goes on the nuclei dephase and precess at different rates, causing net transverse magnetization to decay. The time constant at which it decays is the $T_2$ relaxation time, measured in ms. Relaxation times can be useful in separating out specific tissue types and tissue environments\textsuperscript{136}. For example, in DMD the $T_2$ of the muscle is much higher than controls due to the inflammation, damage, and fat infiltrating into the muscle (Figure 1-2). In this project, muscle $T_2$ is used as a MR biomarker to track the fat infiltration and muscle inflammation in DMD.
We chose our time points in the mdx mice to take advantage of the different stages of disease similar to those seen in humans. A more severe phenotype is seen earlier in the mouse (4 to 6 weeks) characterized with more distinct lesions and higher T2 relaxation times throughout the muscle\textsuperscript{50,53,135}(Figure 1-3). At 24 weeks, the damage to the diffuse is more diffuse, marbled through the muscle and T2 regresses closer to control levels

**Feature Identification**

One of the most difficult areas of metabolomics is the identification of metabolites and assignment of peaks. In NMR, chemical shift information for every (or as many as possible) resonance in the molecule must be determined. This section discusses how both 1D and 2D spectral information can be used for annotating NMR spectra.

NMR spectra, especially $^1$H 1D spectra, are complicated by 1) overlapping peaks from multiple metabolites and 2) multiple peaks for a single metabolite. Techniques including statistical or differential analysis of peaks\textsuperscript{137-139}, deconvolution\textsuperscript{140}, multivariate analysis of full-resolution\textsuperscript{141} or binned data\textsuperscript{142}, are all used to analyze spectra and differentiate peaks belonging to specific metabolites.

If a compound is identified, then further steps can be taken for verification. One common method is a spike-in experiment. A known concentration of the compound of interest is added to the original sample (spike-in) to verify that the peaks increase by the appropriate amount. Another approach is to purify or separate a sample by chromatography to limit the amount of metabolites in the sample and learn biophysical properties from when it elutes. Finally, one method that is gaining interest in the metabolomics field is to use 2D NMR spectra to automatically assign features. The Brüschweiler Lab in Ohio State University has created the complex mixture analysis by
NMR (COLMAR)\textsuperscript{143,144}, an online resource to compare peaks from experimental NMR spectra to one of the following online databases: the human metabolome database (HMDB)\textsuperscript{145}, the biological magnetic resonance data bank (BMRB)\textsuperscript{146}, or the Madison metabolomics consortium database (MMCD)\textsuperscript{147}. COLMAR has many tools to database match based on an experiment including a 1D spectra\textsuperscript{143}, TOCSY\textsuperscript{148,149} and even HSQC\textsuperscript{150}. Currently, our lab uses HSQC\textsuperscript{150} peaks to search for metabolites that match the proton-carbon correlations. We then verify these correlations are within the same spin system using proton-proton correlations (TOCSY).

**Metabolism in DMD**

Previous metabolomics studies of dystrophic muscle have mainly focused on a subset of tissues and metabolites. Largely, the DMD mouse model, \textit{mdx}, and healthy control mice were used to show metabolic differences between the two mice. McIntosh et al in 1998, reported that higher taurine levels correlated with regeneration of skeletal muscle in the \textit{mdx} mouse in an elegant experiment displaying how \textit{mdx} mice, MyoD mice, and a crossbreed of \textit{mdx}:MyoD mice’s tissue taurine concentrations correlated with the amount of proliferating muscle cells\textsuperscript{94,123}.

Energy metabolism has also been found to be altered in both \textit{mdx} and human samples. Previous NMR studies from Griffin et al in 2001 and 2009 and others demonstrated that \textit{mdx}-extracted muscles are higher in glutamate, succinate, taurine, and glutamine, which may be due to regenerating muscle\textsuperscript{151-153}. Lower levels of creatine and carnosine are thought to result from prolonged inflammation and degeneration of the muscle. On the other hand, it has been shown that creatine levels are increased in \textit{mdx} serum\textsuperscript{97,154}, which may be attributed to leaky membranes resulting from the
absence of dystrophin. Also, there are alterations of energy metabolites and lipids found in human muscle biopsies\textsuperscript{65,155,156}.

\textit{In vivo} MR spectroscopy animal studies follow similar metabolite trends, but injured dystrophic muscle show that taurine and choline are significantly increased in \textit{mdx} mice over controls\textsuperscript{102,135}. Others have used a lipidomics approach to investigate dystrophic muscle and serum using mass spectrometry\textsuperscript{157} and NMR\textsuperscript{155,158}, where different lipid aggregation was found differentiating degeneration, damaged, and stable tissue\textsuperscript{126,159}. Due to success in the animal studies, recent human studies have looked at \textit{in vivo} spectroscopy and found a significant correlation between trimethylamine to total creatine ratio and muscle function\textsuperscript{102,160}. Targeted mass spectroscopy of urine from boys with and without DMD showed significant changes in prostaglandin D\textsubscript{2}, but was not sensitive to the use of steroids\textsuperscript{52,136}. These studies are very informative and hypothesis forming; however, many studies normalize to creatine, which has been shown to change between DMD and control populations, possibly affecting the analysis. Figure 1-4 shows a compilation of the different metabolic pathways that have previously been investigated.

Many previous studies involve tissue extraction, where the metabolites are released from the original tissue matrix using chemical assays, which limits the viewable metabolites based on solvent. \textit{In vivo} spectroscopy and metabolomics on \textit{ex vivo} tissue and serum that has not been extracted still needs further investigation. Some studies show that limited sample handling may be more physiologically representative and may provide a more accurate global view.
Although there have been many studies on metabolism in DMD, there are two holes in the literature we address in this dissertation. First, \textit{mdx} mice have not been studied using a non-invasive technique such as serum or MRI while also investigating \textit{ex vivo} tissue. There have been no correlations using multiple modes of metabolite investigation both while \textit{in vivo} and \textit{ex vivo} at different ages. Second, there has yet to be a comprehensive analysis of urine metabolites in boys with DMD. We used MRI, MRS and metabolomics techniques such as, NMR and MS to analyze the metabolite content in tissue, serum, and urine to begin making comparisons across different tissues at different states of disease (Figure 1-5).


despite this, there is no comprehensive analysis of urine metabolites in boys with DMD. We used MRI, MRS and metabolomics techniques such as, NMR and MS to analyze the metabolite content in tissue, serum, and urine to begin making comparisons across different tissues at different states of disease (Figure 1-5).

**Figures**

![Dystrophin Protein Domains with Binding Sites](image)

Figure 1-1. Dystrophin Protein with binding domains. Figure is a cartoon of a dystrophin protein labeled with relative locations for bonding domains to actin (green), Par-1b (blue), nNOs (red), as well as other protein domains including the rod domain (dark blue), coiled-coil domain (orange), DBD (purple) and SBS (pink).
Figure 1-2. MR images from boys with DMD. The light areas within the muscle (dark areas) are fat and damage. It can be seen that the disease is heterogeneous in nature. There are differences from muscle to muscle but also differences from subject to subject.
Figure 1-3. Characterization of disease state with MRI T$_2$. MRI images of the hind limb (A-D) of 6 week control, 6 week mdx, 24 week control and 24 week mdx, respectively. MRI T$_2$ was taken from an area of interest in the posterior compartment of the hind limb (E). A voxel was placed in the posterior compartment of the lower hind limb for quantification of in vivo metabolites using MRS (F). Cre represents creatine, Tau represents taurine peaks, and HCD is for histidine containing derivatives.
Figure 1-4. Previously studied metabolic pathways in DMD. Metabolic pathway from the KEGG database. Pathways overlaid in grey with black labels are known to be associated with DMD.
Figure 1-5. Overview of the study. Overall goal of the study is to identify biomarkers and investigate biological differences between DMD populations compared to controls. Samples will be investigated using multiple methods where as spatial information decreases the spectral information increases. Established and novel analysis will be used to make correlations between different levels of systems biology ranging from comparing images to genetic mutations.
CHAPTER 2
QUALITY CONTROL FOR NMR METABOLOMICS DATA

The identification of unknowns or changes in metabolites both by mass spec and NMR requires extremely high instrument stability during large sample runs. In metabolomics it is common to look at all the data at once and identify those candidate samples that are abnormal. Changes in instrument performance throughout a run can cause false positives or obscure differences in metabolomics analysis. Even small changes can affect these results unbeknownst to the examiner unless specifically looking for these variations. While identifying metabolites using NMR, there are routine manual checks that are performed on the acquired spectra to check the quality of the data before the sample is taken out and prior to moving on to the next sample. As the number of samples continue to grow for metabolomics analysis, the standard method of handling them has shifted to using robotic sample changers and automatically acquiring the data. These updated modifications often cause manual inspection of the data difficult to accomplish. In this chapter, I demonstrate a workflow that I have developed to replace manual inspection of the data with an automated and unbiased form detecting variation in the data, whether it be from the sample or caused by the instrument. Implementing this workflow into metabolomics allows for an objective and high-throughput way to maintain quality control for large sample sizes.

Introduction

The United States Food and Drug Administration (FDA) defines quality control (QC) as, “the steps taken during the generation of a product or service to ensure that it meets requirements and that the product or service is reproducible.” In the field of metabolomics, there is not a universal method or establishment that has defined the
requirements for NMR or MS data. Most metabolomics studies have used PCA and other forms of multivariate analysis to determine if instrumentation was contributing to the variance as a confound to the study design\textsuperscript{162-164}. These studies brought to attention the importance of determining instrumental and sample-handling effect from biological variation. However, PCA is a data reducing technique which can obscure the exact cause for high variance. Also, to see these discrepancies in PCA this variance has to be substantial, therefore small deviations that can still effect the spectra are overlooked. PCA is useful as a global search, but is unable to determine 1) specific instrumental variation and 2) sample variation or 3) feature variation.

In 2011, Dunn et al, published a protocol for large scale metabolic profiling using QC samples to assess both run order drift and batch differentiation for LC-MS\textsuperscript{165}. In metabolomics, many investigators use standard samples intermixed throughout a run to compare to experimental samples to detect variation. Standard samples are currently used throughout the run and in each batch as a way to control for instrumentation drift or biological changes over time. To assess changes in the samples of a particular study, small aliquots of each sample are pooled together to act as an average of that study and can correct for that particular run. Common practice is to use commercially available biofluids to use throughout a single study that may involve multiple runs to correct for a batch effect.

Both nuclear magnetic resonance (NMR) and mass spectrometry (MS) have parameters optimized for the identification of specific features. However, parameters often change between samples due to drifts in instrument performance. In large automated sample acquisitions these are not always carefully reviewed individually.
Unintentionally, changes in the instrumentation and drift over time can influence data analysis and interpretation. Also, samples from an individual subject or specimen can have variation from others due to differences in extraction conditions and residual macromolecules that can affect the NMR spectrum baseline or line broadening. It is important to acknowledge these differences prior to any sample analysis. As previously shown\textsuperscript{165}, the use of QC standards repeated throughout a run can be used to assess instrument and the sample integrity. This can be taken further by measuring individual parameters of each experimental spectrum and compared them to the QC samples. By testing these spectral variations among all samples and features, outliers can be detected. The identification of outliers can then be indicated by a flag and referenced post analysis or removed.

The challenge with metabolomics is that there are tens of thousands of different metabolites that could be changing in an uncorrelated manner and we need to identify these changes within a limited number of complex mixtures. With the field of metabolomics growing into ever evolving complicated mixtures, it is critical to have objective, high throughput, efficient tests to perform QC. In this study I demonstrate a workflow to verify data usability and flag potential outliers prior to analysis and interpretation of NMR data.

**Materials and Methods**

**Study design**

This workflow can be used for any NMR samples. For this particular study I have chosen urine samples from DMD and control boys to illustrate the utility of this approach. Within the individual experimental samples, I integrated QC pooled urine standards (n=10) and buffer blanks (n=3) to QC throughout sample preparation and
data acquisition. Samples and QC standards were randomly prepared and assigned a run order with QC blanks (buffer only) as the first, last, and middle samples. QC pooled urine standards were placed after the first blank and before the last blank of each run. The rest of the QC samples are randomized along with the DMD and control boy samples using an excel randomizer. Quality of the data was then verified using a custom-written QC script (APPENDIX D), which uses the blanks and QC samples to verify no significant changes in shim, temperature or the metabolites occurred during the NMR run.

**NMR sample preparation and acquisition**

Urine samples (DMD on steroids n=30; DMD steroid naive n=8; healthy controls n=7) were removed from the UF CTSI Biorepository, allowed to thaw at 4°C, and centrifuged at 12,000g for 5 minutes at 4°C. 400 μl of urine was added to a 1mL eppendorf tube for each sample and mixed with 200 μl of 100 mM phosphate buffer including and 0.1M Sodium 2,2-Dimethyl-2-Silapentane-5-Sulfonate (DSS-D6) (Cambridge Isotope Laboratories, Inc) to serve as a reference standard. Eppendorf tubes were vortexed for 2 minutes and 590 μl of the supernatant were transferred into 5 mm NMR tube (Bruker 5mm tubes for SampleJet Samples. Spectra were acquired using the vendor supplied “noesypr1d” pulse sequence (Chapter 1) with 32K complex data points, 15 ppm spectral width, and 100 millisecond NOESY mixing time with a total acquisition time of 1.817 seconds and a 4 second relaxation delay. 128 scans with 16 dummy scans were acquired. The 90-degree pulse in μs was calculated for each sample using the vendor supplied routine “pulsecal” implemented into the ICON NMR automated scheme. Temperature was regulated to maintain the sample at 300 K. All
NMR parameters were recorded and stored in the “acqus” text file that is stored along with the primary FID data in the Bruker data structure. Data acquisition and a more in depth explanation of the sample preparations SOP in APPENDIX A.

**NMR data processing**

Raw NMR data were initially pre-processed using NMRpipe\textsuperscript{166}. NMRpipe was used for implementing a cosine window function, zero filling, fast Fourier transformation, phasing, and baseline correction\textsuperscript{166}. Fourier transformed data were then transferred into MATLAB for further processing using an in-house Metabolomics Toolbox\textsuperscript{141}. Water was removed by replacing spectral intensity with zeros between 4.7 - 5.08 ppm as to not interfere with further analysis. The ends of the spectra were truncated where there was no information leaving only -0.2 ppm to 10 ppm. A guided alignment script, “guide_align1D”, used a ‘spearman’ cluster method and peak alignment fast Fourier transform (PAFFT) alignment method\textsuperscript{167} in MATLAB. The spectra were normalized using probabilistic quotient normalization (PQN)\textsuperscript{168}. PQN works by comparing a reference spectrum (mean of all spectra) and a subset (test) group of spectra to create a distribution of the amplitudes. Each spectrum is then compared to the reference spectrum and using the distribution of the test set find the most probable dilution factor for that spectrum. Finally, the data are peak picked (max amplitude of each peak) using an in-house script\textsuperscript{141} that detects peaks in 1D NMR spectrum and constructs two matrices; peak intensities and chemical shifts of each peak maximum.

This workflow was designed to track deviations in the 90-degree pulse length, temperature (temp), line broadening and integral of the standard DSS. NMR instrumentation parameters (temperature and 90-degree pulse) were taken directly from the acquisition parameter data file or the “acqus” text file that is stored along with the
primary FID data in the Bruker data structure using an in-house Matlab script called “acqu”. The acqu script creates a structure of select parameters. Each parameter is a vector that contains a value for each sample. To assess line broadening and integration of a standard, the MATLAB workflow script includes taking the area under the curve (AUC) and the full width of the half max (FWHM) of the internal standard, DSS.

Throughout the workflow description below, data used in each portion will be specified as one of the following: aligned (full resolution), normalized (full resolution), peak-picked, NMR parameters or quantifications. All data are accompanied with a corresponding metadata file including information such as the sample name, group information and the order the samples were run. This can then be easily imported into Galaxy for other QC or analysis.

**Quality Control Workflow**

This QC workflow evaluates the degree of NMR instrumentation variation during the acquisition of data for all samples in a study. Post instrumentation variation, the QC workflow can be used for both NMR and MS to measure the samples and feature variation (Figure 2-1.). However, for the purpose for this dissertation, I will only discuss the NMR portion of the workflow, as that is the portion that I worked with Dr. McIntyre to create. Since minor changes can affect the line shape and the quantification of peaks, any variation outside of one standard deviation is flagged.

**Assessment of Instrumentation Variation**

Instrumental performance of the NMR spectrometer was assessed using the following parameters: temperature, the 90-degree pulse calibration, and shimming (magnetic field homogeneity). The NMR parameter data (acqus file) was used in the instrumentation variation assessment.
Temperature is usually regulated and consistent throughout the run by the NMR software (Topspin 3.2, in this case), but temperature changes can still occur, resulting in many changes in the spectrum. Therefore, I monitored for loss of temperature regulation. Reported temperature from each sample acquisition is compared to all other samples as well as tested for significant regression over time. If the temperature altered more than one standard deviation from the rest of the samples or there is a significant correlation with the run order, then the data are flagged.

The 90-degree pulse length, must be calibrated for each sample to be quantitative. The ICON NMR (automation software for NMR) automated sequence commonly used for large studies includes the Bruker command, “pulsescal”, which can automatically calibrate the pulse length and is documented it in the acqus file in the Bruker data file structure. To verify that each 90-degree pulse length is within a realistic range for the NMR acquisition and the set of samples, the 90-degree pulse length is compared by group and flagged if it is outside one standard deviation of the mean of the group.

Using internal standards (DSS), instrument reproducibility and changes throughout the run can be monitored. Shimming, or optimizing the homogeneity of the magnetic field, is usually automated using a gradient shim sequence, but this often does not result in an ideal shim for each spectrum. The quality of the shim can be evaluated by resonance line shape. This workflow uses the full width of half of the max (FWHM) of the internal standard, DSS peak at 0 ppm, to determine shim values. FWHM values that are outside one standard deviation of the mean they are flagged.
Internal standards are also useful to assess variation that could affect analysis, for example DSS integration and line broadening changes if proteins are in the sample due to binding between the DSS and the protein. To test for changes over time or protein (extraction efficacy), integration of DSS at 0ppm peak is plotted against the run order similar to Crockford et al\textsuperscript{169} using run order regression where the internal standard is flagged if there is a significant trend. We also flag the samples that have standardized residuals greater than one standard deviation.

**Assessments of Sample Variation**

To compare sample variation, the initial step is to plot the distribution of the data. The distribution tools plots the scaled data (log transformed) as a histogram and a box and whiskers plot. Ideally the histogram will be shaped as a normal distribution and the box plots will be centered at zero with similar variation throughout all samples. If there is an skewed pattern of the distribution of all of the data points per sample, we can then flag the samples of question. The box and whiskers plot should align at zero and have small and consistent amount of variation amongst all samples. For NMR, full resolution aligned and normalized spectra were used separately, which allows for comparison of the normalization method that was used.

**Assessment of Feature Variation**

To investigate the feature variation within and across samples assessments including run order regression and Bland-Altman\textsuperscript{170} plots are used. Run order regression assesses if each feature has a dependency on the order the data were acquired on the NMR. NMR peak picked data were used to analyze the peak intensity of all peaks across the spectrum against the run order. Similar to the internal standards, the run order regression flags features with a regression that is significantly changing
throughout a run and samples of the feature if the standardized residual was larger than one standard deviation.

Bland-Altman plots were used to compare all combinations of spectra of the QC samples and all combinations of samples within a treatment group. Instrument noise can cause spurious and arbitrary points so the peak-picked data were used for NMR.

Bland-Altman plots compare the average between two reproduced points against the difference between the same two reproduced points\(^{170}\). As there are many biological variations in sample data, it is ideal to first use Bland-Altman plots on the QC samples dispersed throughout the run. As these are different aliquots of the same sample at different times throughout the run, these will give the best information of reproducibility throughout the run. Any peaks that are outside the 95% confidence intervals will be flagged and summed. Flagged data are only labeled for further investigation. Apart from the flag text files, the Bland-Altman MATLAB tool will also output a file (PDF) of each Bland-Altman plot (labeled according to the samples being compared). If using the Bland Altman tool on Galaxy (https://galaxyproject.org/), you will also be provided with two summary graphs, one that shows the proportion of features that were outliers in each sample and the other shows the proportion of samples that a feature was an outlier. These summary figures only show a portion of the total features but the text files have the full data to recreate these plots.

**Results and Discussion**

This workflow is comprised of multiple tests that are regularly performed on NMR spectra to characterize the usability of the data in a semi-automated and quantitative manner. Finding variation introduced by instrumentation, or between samples and features will not only help detect large changes in the data that are within normal ranges
but can also help reduce the chances of making misinterpretations based on any abnormal data.

Using this NMR QC workflow, I was able to rapidly and automatically detect that there was a loss of temperature regulation during a run of my DMD urine samples on the NMR. The effect of temperature changes on metabolite chemical shift for creatine and urea are shown in Figure 2-2A. Using this workflow, 22 of samples were flagged as acquired at the incorrect temperature and removed from the group sample analysis.

In order to perform quantitative NMR measurements, the RF excitation pulse length should be carefully monitored. Ideally the excitation pulse length is optimized and remains constant along with metabolite relaxation throughout a sample run, eliminating the need to account for sample-to-sample corrections due to differences in sample excitation and relaxation. In this experiment the excitation pulse length was found to be regular with a variation of 0.2 μs. However, a quarter (25%) of the samples were flagged for having a pulse length that was greater than one standard deviation. Likely, these 25% of samples had altered because there was no auto tune and match on this NMR. This number would likely reduce with the use of autotune and match for future studies. After further investigation, it did not seem that the change in pulse length of the flagged samples seemed to alter the spectra in any detectable way. However, each peak from each metabolite can have a different $T_1$ rate. If the correct pulse length is not used and the nuclei is not tipped to its optimum 90 degrees then the quantification may be altered. Therefore, it is important to have a correct and consistent pulse length that will tip the nuclei 90 degrees.
Internal standards in NMR are crucial to reference the spectra and evaluate shim. Here, we were able to automate these manual, subjective and time consuming assessments. DSS should always be a singlet, align the spectra at 0 ppm, with a known concentration, which can act as normalization or concentration reference. To test the shimming, or homogenization of the magnetic field, we calculate the full width of the half max (FWHM) (i.e. the lower the FWHM the better the shimming). Traditionally, this is calculated for each spectrum by hand but this can be time consuming and subjective. In this workflow, we automated the calculation of the FWHM and objectively compared all of the FWHM of other samples in the same treatment group. In Figure 2-2B, we show shimming was also very consistent with the exception of one sample that was flagged.

As a final assessment of the instrumentation variation that could be introduced in NMR, we analyzed the integration of the DSS peak in all spectra over the run of the experiment. If there were any significant outliers, then they would be flagged. There was no significant trend seen in the integration of the DSS peak over time however, a few samples were flagged as the standardized residuals of the integrations differed by larger than one standard deviation (data not shown). We also looked at all integrations of the DSS peak and compared to all other DSS peaks to find that five samples were flagged overall (Figure 2-2C). These data suggest that if there is a problem within the NMR acquisition run then this workflow is able to detect any changes.

To investigate sample variation, the distribution of the data is visualized in two different ways; a histogram and a box plot of the log fold change versus the median values of each sample. The normalized samples acquired by NMR have normal
distributions but samples 15 though 20 have a variation larger than a one-fold change.
The histogram of the samples shows alignment across all NMR samples.

NMR feature variation was evaluated using run order regression and the Bland-Altman plots from the normalized peak picked data. In NMR, we used the peak intensities of all peaks across each spectrum. Peak intensities were plotted against the run order and assessed using a linear regression. Of the 550 features, 3.2% were flagged as having a significant trend. We also found that about a fifth (18.9%) of the samples were flagged across all features. Figure 2-3 gives example features of both a feature with a significant trend (Figure 2-3A) and one without (Figure 2-3B). Further there can also output a table of run order regressions of quantifications detailing each sample and if there are any significant trends.

Bland-Altman (BA) plots were used to determine features that changed across samples. Figure 2-4 shows two example of BA plots, both between QC samples. Figure 2-4A shows a BA plot expected between two QC samples taken at the same temperature resulting in minimal variation (samples 84 and 20. Figure 2-4B shows a BA plot between two QC samples but at different temperatures resulting in larger variation between samples 84 and 88. As we know, temperature can affect changes in chemical shifts. Using these Bland-Altman plots we can verify which peaks were altered by temperature change by looking though the flagged features. Figure 2-5 summarizes flagged features and samples. Figure 2-5A shows a plot of the QC pooled samples that had the largest proportions of flags, after verification, we find these samples acquired without temperature regulation. Figure 2-5B plotted peaks that have the largest proportion of flags also verified to be peaks that are changing with temperature, similar
to Figure 2-2 A. Overall, less than 4 % of features were flagged where Sample 20 and 30 had the most and were consistently flagged throughout the workflow. Of the features that were commonly flagged, most were within the 5.7-5.8 ppm region. This region associates to the urea peak which is show to be unaligned due to temperature at differences.

In conclusion, this workflow is a semi-automatic way to quickly and objectively QC NMR data for metabolomics studies. As compared to other QC studies, I did not take a multivariate approach. Here, I described common tests for assessing the quality of the data that are usually performed manually in a more automated way. The quality of the data is determined by measuring the variation in each sample and each feature either biological or introduced by the instrument. This workflow was applied to a run of urine from boys with DMD with results that will be described further in Chapter 6. I found that our QC workflow was able to detect potential problem samples and features that could affect the analysis and interpretation of the results in a high-throughput and objective manner.
Figures

Figure 2-1. Schematic of Quality Control Workflow
Figure 2-2. Instrumental variation for NMR parameters. (A) Urea and creatine peaks are plotted to show the irregularity on chemical shift at different temperatures. DSS FWHM (B) and integrations (C) are compared within each treatment group, where red dots represent flagged samples for variation larger than one standard deviation.
Figure 2-3. Run order Regression. NMR peak picked features were plotted along their run order. Linear regression was performed on each feature where significant regressions of a feature were flagged as well as residuals outside one standard deviation (red dots). (A) An example of a feature with a significant trend over the run order, so it has been flagged. (B) An example of no significant regression between the run order and peak intensity which means it has passed QC.
Figure 2-4. Example BA plots. Comparison of all peak picked features of Sample 18 and Sample 13 show nice correlation and small variation in A. Comparison of Samples 24 and 18 show many features vary and lower correlation of the features.
Figure 2-5. Flag summary from Bland-Altman. In A, the proportion of flags is given for all QC samples. In B, the proportion of flags is represented for the 20 most flagged features.
CHAPTER 3

13C AND 1H METABOLOMICS COMBINE TO IMPROVE ANALYSIS AND IDENTIFICATION IN COMPLEX MIXTURES: APPLIED TO CROSS SECTIONAL MDX SERUM STUDY

This chapter discusses the use of natural abundance 13C NMR in addition to 1H NMR to enhance metabolomics findings. 13C spectra add advantages such as a large spectral dispersion, narrow singlets, and direct measure of the backbone structures of metabolites. These advantages improve group discrimination and confidence in metabolite identification. I was co-first author with Chaevien Clendinen on this paper. Although I did not contribute to all parts of the study, I was significantly involved in 1) designing experiments, 2) writing MATLAB scripts for the analysis workflow, 3) collecting, preparing and analyzing all of the mouse serum studies, and 4) contributing to writing the paper. Although not all portions of this study are directly related to the project on DMD, all portions of this paper are included to complete the full validation of 13C in addition to 1H metabolomics and are used throughout in later chapters in the DMD project. ¹

Introduction

NMR-based metabolomics is most commonly done with 1H detection. Although 13C is often used for compound identification, it is usually recorded indirectly using experiments such as HSQC (heteronuclear single quantum correlation), HMBC (heteronuclear multiple bond correlation), or their variants, all of which employ 1H detection. These indirect methods are powerful, but suffer from the unreliable detection

¹This chapter is published as Chaevien S. Clendinen, Brittany Lee-McMullen, Caroline M. Williams, Gregory S. Stupp, Krista Vandenborne, Daniel A. Hahn, Glenn A. Walter, and Arthur S. Edison, Analytical Chemistry 2014 86 (18), 9242-9250.
of quaternary carbons and correlations that are sometimes difficult to assign, especially with HMBC. Indirect methods also suffer from limited digital resolution along the large $^{13}$C chemical shift dimension. Despite its wide spread use, $^1$H NMR metabolomics studies of mixtures is often complicated by resonance overlap from a relatively narrow 10 ppm diamagnetic chemical shift range. This overlap is exacerbated by $^1$H homonuclear coupling, which creates multiplets that can complicate analysis, although 2D J-resolved experiments can improve that problem. Many $^1$H chemical shifts are sensitive to pH, temperature, or salts, complicating spectral alignment and database matching. In addition, water reduction can obscure nearby resonances and distort baselines, which can make $^1$H-based metabolomics analyses difficult.

$^{13}$C NMR offers many advantages for a metabolomics study, either alone or as a complement to $^1$H NMR: 1) $^{13}$C spectral windows are typically 200 ppm, providing much greater chemical shift dispersion than $^1$H; 2) at natural abundance, $^{13}$C resonances of small metabolites are narrow singlets (with $^1$H decoupling) resulting in less spectral overlap; 3) biogenic metabolites predominantly contain a carbon backbone, and $^{13}$C NMR can detect this directly, including quaternary carbons. These advantages raise the possibility of easier identification of metabolites and better separation between experimental groups using multivariate analyses.

The major obstacle to $^{13}$C NMR metabolomics compared to $^1$H is low sensitivity due to low natural abundance of $^{13}$C (~1.1%) combined with a decreased gyromagnetic ratio $\gamma$(one quarter of that of $^1$H). The energy of an NMR resonance transition is proportional to $\gamma^3$, and the sensitivity is roughly proportional to $\gamma^2$. Though solutions such as isotopic labeling exist$^{148,171-174}$, they can be expensive and are often restricted to
targeted metabolomics. Untargeted studies with $^{13}$C labeling are especially challenging for organisms that cannot be cultured. In addition, isotopic $^{13}$C labeling reintroduces the problem of homonuclear couplings, which complicate $^{13}$C spectra. The other solution is to improve $^{13}$C sensitivity, either through higher magnetic field strengths that improve the Boltzmann polarization\textsuperscript{175}, a variety of hyperpolarization techniques\textsuperscript{176,177}, or improved detection with an optimized probe\textsuperscript{178}. We have designed and built a 1.5-mm high temperature superconducting (HTS) $^{13}$C optimized probe. This probe has a minimal sample volume of 35 μL and over 2x greater $^{13}$C mass-sensitivity than any commercially available $^{13}$C-optimized cryogenic probe, which translates to well over 4x less measurement time for the same sample and same signal-to-noise ratio\textsuperscript{178}. This new technology makes $^{13}$C metabolomics at natural abundance technically feasible.

In the present study, we show that 1D $^{13}$C data enhance metabolite identification and facilitate better separation of groups for comparison using multivariate statistical analysis when compared to 1D $^1$H data alone. We analyzed the $^{13}$C and $^1$H 1D datasets with $^{13}$C-$^{13}$C statistical total correlation spectroscopy (STOCSY)\textsuperscript{179,180} and $^{13}$C-$^1$H statistical heterospectroscopy (SHY)\textsuperscript{181-183} to compile correlated peak lists that were then used in database searches for compound identification. We developed and validated the workflow using synthetic mixtures of 20 common metabolites, defined the limits of detection using current technology, and compared the peak lists generated using both $^{13}$C and $^1$H data to those generated using $^1$H data alone. We then applied our workflow to comparisons of two types of biological mixtures: extracts of cold hardy and cold susceptible \textit{Drosophila melanogaster} flies, to demonstrate the superior performance of 1D $^{13}$C compared to 1D $^1$H in multivariate statistical analysis and mouse
serum taken from control and the Duchenne muscular dystrophy mouse model, \textit{mdx}^{93} to demonstrate the power of $^{13}\text{C}$ metabolomics to not only aid in metabolite identification, but also to prevent misidentification.

\textbf{Experimental Methods}

\textbf{Synthetic Mixtures}

Synthetic mixtures with known metabolite compositions were constructed to test the limits of detection of the probe and our ability to correctly identify known compounds. Two mixtures (A and B) were made using 5 replicate samples per mixture, each using 20 common synthetic metabolites ranging in concentration from 1 to 5 mM (Appendix E). The first ten metabolites were at equal concentrations in both mixtures, and the remaining 10 metabolites differed between mixtures with half higher in mixture A, and half higher in mixture B (Appendix E). To simulate normal sample variation between replicates, each metabolite was pipetted individually into each tube. Each sample was brought to 50 μL using 99% D$_2$O (Cambridge Isotope Labs), of which 40 μL was pipetted into a 1.5-mm NMR tube (Norell).

\textbf{Drosophila melanogaster tissue extracts}

Flies were selected in the laboratory for high (hardy) or low (susceptible) cold hardiness (Williams et al., unpublished results). Flies were lyophilized and weighed prior to metabolite extraction. Polar-phase metabolites were extracted from homogenized fly tissue using an aqueous-optimized metabolite extraction protocol$^{121}$. Twenty replicates of each genetic line (cold hardy and cold tolerant) were collected with 40 flies per replicate. Polar phase extracted metabolites were dried and reconstituted in 40 μL of 99% D$_2$O (Cambridge Isotope Labs) and pipetted into 1.5-mm NMR tubes (Norell).
Mouse Serum

The Duchenne muscular dystrophy (DMD) mouse model, *mdx*, has previously been investigated with metabolomics using tissue extracts, but to our knowledge, serum has not been investigated using both $^1$H and $^{13}$C. One technical difficulty is the small quantity of sample available for NMR in mouse serum studies. Mouse serum measurements were performed on 6 month old C57/B10 control and *mdx* mice. Two hundred μL of blood was collected by submandibular puncture, clotted and centrifuged to extract serum. The mice were then humanely euthanized under 2 % isofluorene anesthesia according to approved IACUC protocols. Once the serum was separated from the red blood cells, it was flash frozen in liquid nitrogen and stored at -80 °C. Approximately 100 μL serum was lyophilized and re-suspended in 40 μL 99% D$_2$O and transferred into 1.5 mm NMR tubes (Norell).

NMR Data Collection and Processing

One-dimensional $^1$H and $^{13}$C spectra were collected on an Agilent VNMRS-600 spectrometer using a custom 1.5 mm $^{13}$C high temperature superconducting (HTS) probe. Synthetic mixture and fly extract $^1$H 1D data were collected in ~2.5 min using a simple pulse sequence with presaturation of residual water, a spectral width of 12 ppm (7183.9 Hz), an observe frequency of 599.68 MHz, a 2.0 s relaxation delay, a 45-degree pulse, and 2.3 s acquisition time. Mouse serum $^1$H data were collected in 18 min using a Carr-Purcell-Meiboom-Gill (CPMG) sequence, to remove the protein signal contribution resulting in a flat baseline. Mouse data were recorded with a spectral width of 16 ppm (9615.4 Hz), a 2.0 s relax delay, a 90-degree initial pulse, a train of 124 180-degree pulses with a 1 ms inter-pulse delay, and a 2.0 s acquisition time. All $^{13}$C spectra were collected under conditions that favor nuclei with short $T_1$ relaxation times to maximize
overall sensitivity and minimize measurement time: a 60° pulse with a 0.1 sec relaxation delay and a 0.8 sec acquisition time. The total time for each $^{13}$C spectrum was ~2 hours, with a 212 ppm (32051.3 Hz) spectral window, a carrier frequency of 98.0 ppm at a frequency of 150.79 MHz. The mouse serum $^{13}$C CPMG sequence used a 90-degree excitation pulse followed by a train of 18 180-degree pulses with a 1 ms inter-pulse delay. All $^{13}$C spectra were recorded using continuous $^1$H decoupling at 599.68 MHZ with a power of 37 dB using WALTZ-16. All NMR spectra were processed in NMRPipe$^{166}$. All $^1$H spectra were processed using a cosine window function, zero-filled 2x, Fourier transformed, and baseline corrected. $^{13}$C spectra were processed using an exponential window function with a 2 Hz line broadening, zero-filled 2x, Fourier transformed, and baseline corrected. The synthetic mixture and fly extract $^1$H spectra were referenced to TSP (0.0 ppm); mouse serum $^1$H spectra were referenced to the lactate peak at 4.1 ppm, because TSP binds to proteins. $^{13}$C spectra were referenced to the anomeric carbon glucose peak at 98.64 ppm.

Fourier transformed and referenced spectra were imported into the MATLAB Toolbox and aligned. The Toolbox has several options for alignment, but we found that the star alignment algorithm where an individual spectrum is chosen as a “star” to which all other spectra are aligned$^{141}$, gave the best results. For the statistical correlations, we binned the $^1$H spectra using an open source optimized bucketing algorithm described by Sousa et al$^{184}$ and peak picked the $^{13}$C spectra. Optimal normalization and scaling methods were specific for each dataset: $^{13}$C and $^1$H spectra of the synthetic mixtures and mice serum were normalized using probabilistic quotient normalization (PQN)$^{168}$ and scaled using pareto scaling$^{185}$. The $^1$H fly spectra were normalized to TSP and
scaled using range scaling\textsuperscript{185}. Principal component analysis (PCA) was conducted on the 1D \textsuperscript{1}H and \textsuperscript{13}C datasets of the synthetic mixture and the flies. Partial least squares-discriminant analysis (PLS-DA) was conducted on the 1D \textsuperscript{1}H and \textsuperscript{13}C datasets of the mouse serum. All NMR raw data, processing scripts, and MATLAB code were deposited in the Metabolomics Workbench database (http://www.metabolomicsworkbench.org/) supported by the NIH Common Fund.

Data Analysis

Our workflow consisted of computing statistical correlations to compile peak lists, followed by database searching to identify metabolites (Figure 3-1). All of the steps described below were implemented in an in-house MATLAB Metabolomics Toolbox\textsuperscript{143} that was used to analyze all the datasets.

1) Statistical correlations: We calculated 2D statistical correlation maps for \textsuperscript{13}C-\textsuperscript{13}C and \textsuperscript{1}H-\textsuperscript{1}H homonuclear (STOCSY;\textsuperscript{179,180} Figure 3-1C) and \textsuperscript{1}H-\textsuperscript{13}C heteronuclear spectra (SHY;\textsuperscript{181-183} Figure 3-1D). In 1D versions of STOCSY and SHY, correlations and covariance are determined for a specific “driver” peak of interest. In the 2D implementation used here, each peak serves as a driver for all other resonances in the spectra, as shown in panels C and D in Figure 3-1.

2) Generation of peak lists: The STOCSY and SHY maps were analyzed by an in-house script written in MATLAB according to the following steps. We first systematically evaluated all \textsuperscript{13}C correlations from the STOCSY and removed any that were not greater than 0. Thus, all peak lists were constructed from the positive \textsuperscript{13}C 2D correlation map, as shown in Figure 3-1C. We systematically examined all \textsuperscript{13}C frequencies along the vertical STOCSY axis—now considered “driver peaks”—for every \textsuperscript{13}C and \textsuperscript{1}H correlation along the horizontal axes of the STOCSY and SHY, respectively.
This generated the starting point for peak lists for database searching. Peaks that did not significantly correlate with the driver peak after FDR correction\(^{186}\) (Pearson’s linear correlation coefficient, Q<0.05) were removed to further eliminate resonances that are unlikely to be part of the same molecule (Figure 3-1E–G). The remaining peaks had both significantly high correlation and a strong covariance with the driver peak. Peak lists from all driver peaks were compared, and those sharing more than 3 peaks were combined and classified as part of the same metabolite.

The \(^1\)H-\(^1\)H STOCSY results were not as useful as the \(^{13}\)C-\(^{13}\)C STOCSYs, but we used these with the synthetic mixture to compare the results with \(^{13}\)C to traditional approaches with just \(^1\)H.

3) Database searching: At this stage in the analysis, the peak lists from step 2 could be analyzed several ways including \textit{de novo} compound identification using chemical knowledge or, for known compounds, matched to a database. We chose to use the COLMAR query\(^{173}\) search engine and the BMRB database\(^ {146}\), because the BMRB has a large number of metabolites with assigned \(^{13}\)C spectra. However, any search protocol and database with both \(^1\)H and \(^{13}\)C NMR data could be used for this step. We note that our database searches in this study were sequential, namely we first searched the \(^{13}\)C data and then searched for \(^1\)H data. It would be advantageous in the future to simultaneously search using all the data. Like all database searches, several possible matches for each query were returned, and the final step in our analysis was manual inspection of BMRB spectra overlaid onto our primary data and covariance maps for final identification.
Results and Discussion

The goal of this paper was to determine the extent to which 1D $^{13}$C NMR can significantly enhance an untargeted metabolomics study. The specific algorithms—STOCSY, SHY, COLMAR query, and PCA or PLS-DA—were all known and used with little or no modification, but were combined into a workflow that incorporates the $^{13}$C data. We used three different test cases to evaluate the workflow and overall approach. To develop and validate the method, we first used a simple synthetic mixture of known compounds. This allowed us to optimize parameters and define the steps of the overall workflow, as well as allowing us to estimate a lower practical limit for the amount of material required with current technology. In order to demonstrate the robustness of this approach, we applied the workflow to two very different biological systems, comparing genetically distinct fruit fly lines with different phenotypic responses to cold and comparing control mice to those with a known mutation in a gene that causes muscular dystrophy. The fly data provided an example of several relatively abundant metabolites that change between groups, and similar animals have recently been studied using traditional $^1$H-based NMR methods, allowing for some comparisons. The mouse serum data validates that this technique can be used when only very limited quantities of starting material are available and shows the value of $^{13}$C measurements to confirm or reject assignments from $^1$H data alone.

Workflow development

We collected and analyzed 1D $^{13}$C (Figure 3-1A) and $^1$H (Figure 3-1B) spectra of two groups of synthetic mixtures to determine the limit of detection for this method and to test our ability to identify metabolites that we knew were in the samples. The concentration ranges of metabolites in these mixes were between about 1 and 5 mM;
thus, the total quantity of compounds in each experiment ranged from 40 to 200 nmoles. The metabolites at the lower concentrations (indicated with an asterisk* in Figure 3-1A) were detectable, but only with relatively low S/N. However, we were unable to obtain $^{13}$C correlations for the resonances with the lowest S/N and were only able to obtain reliable correlations from compounds at or above about 1.5 mM. Thus, we conclude that 60 nmoles is the lower limit of material that works for this workflow with the current $^{13}$C probe, measurement time, and other NMR parameters. Several ideas to improve this overall sensitivity are given below.

The overall workflow is illustrated here with the identification of tryptophan (Trp) in the synthetic mixtures. The $^{13}$C-$^{13}$C STOCSY and $^1$H-$^{13}$C SHY maps were used to generate peak lists, as described above. One of the correlated peak lists had a good match to Trp, following COLMAR query of the BMRB database. The 1D BMRB reference spectra of Trp are shown in black on the axes of plots in Figure 3-1 to illustrate how the statistical correlations were used to match the database. A closer look at the $^{13}$C-$^{13}$C STOCSY region between 109.5 and 141.5 ppm showed the strong correlations between the aromatic tryptophan peaks (red cross-peaks). The trace through the $^{13}$C-$^{13}$C STOCSY at one of the aromatic Trp resonances (114.8 ppm) illustrates the correlations with other Trp peaks (Figure 3-1E).

To evaluate the extent to which $^{13}$C data improved our identification of compounds, we compared results using the entire workflow outlined in Figure 3-1 with results that just used $^1$H-$^1$H STOCSY. Using all the data ($^{13}$C and $^1$H), our workflow returned 36 peak lists but using the $^1$H data alone returned only 26 peak lists. It must be noted that a peak list is defined as the set of correlated peaks that pass a given
information-quality threshold, as described above in Methods. Peak lists can be fragments of molecules, so it is not surprising to have a greater number of peak lists than detected metabolites in the sample. When we searched the appropriate BMRB database using COLMAR query (i.e. $^{13}$C or $^1$H), using all of the data we found 15 of the correct compounds in the list of the top 10 hits from the query, but using just the $^1$H data we only found 7 of the correct matches (APPENDIX G). Because the $^1$H and $^{13}$C data were collected from the same samples and the $^1$H data had better overall signal-to-noise, we believe that the major factor underlying the better performance of the $^{13}$C data for compound identification was that the $^{13}$C workflow generated fewer false correlations—defined as a peak in the generated peak list that is not in the database-matched molecule. In the $^1$H-$^1$H STOCSY 60% of the peaks were falsely correlated, but the $^{13}$C-$^{13}$C STOCSY had only 20% false correlations, as indicated in columns 4 and 5 of Appendix E. This large difference in the false correlations results from the improved resolution in the $^{13}$C 1D data. PCA performed on both 1D $^{13}$C and $^1$H datasets gave predictably good separation considering these were artificially made groups. However, it can also be seen that the loadings from the $^{13}$C PCA are much better resolved than those from the $^1$H PCA. This is demonstrated more clearly below in the fly data. In general, for the synthetic test dataset, we found that the $^{13}$C data were much more useful than $^1$H in generating comprehensive peak lists leading to metabolite identification. In most instances, the peaks generated from $^{13}$C data alone were enough to successfully match metabolites in the database and the $^1$H data were reserved primarily for verification of results.
**13C NMR data yields improved group discrimination**

To test our workflow on samples from a biological system we utilized two isogenic lines of *D. melanogaster*, one hardy and one susceptible to cold exposure. *D. melanogaster* is a model organism and the subject of many NMR-based metabolomics studies\(^{187-189}\), making it desirable to optimize the performance of NMR-based metabolomics in this species. The lines used here originated from the same base population as the Drosophila Genetic Reference Panel\(^ {190}\), but were selected for either rapid (hardy) or slow (susceptible) recovery from a cold-induced coma (a common metric of cold tolerance in insects and other ectotherms). We used these strains because they have a well-annotated metabolome as well as robust and stable differences in the metabolome based on previous \(^1\)H NMR measurements (Williams et al., unpublished results), against which we can evaluate the power of \(^{13}\)C-NMR to identify: A) NMR-visible metabolites, and B) metabolites that discriminate the lines (i.e. a biomarker approach). Detailed physiological analysis of metabolic changes upon cold exposure will be presented elsewhere.

The \(^{13}\)C (Figure 3-2A) and \(^1\)H (Figure 3-2B) spectra are plotted in red (cold hardy) and blue (cold susceptible). As described above, peak lists defining spin systems from the \(^{13}\)C-\(^{13}\)C STOCSY (Figure 3-2E) and \(^1\)H-\(^{13}\)C-SHY (Figure 3-2F) correlations were used to search the BMRB, which yielded the 13 metabolites given in Table S1. As above, the \(^{13}\)C data were more useful than \(^1\)H for database matching. As an example, a slice of the 2D \(^{13}\)C-\(^1\)H SHY at \(^{13}\)C peak 31.69 ppm (Figure 3-2D), which is the β carbon of proline, shows correlations with many \(^1\)H resonances other than the reference proline resonances (black), making unambiguous proline identification using \(^1\)H alone difficult. In contrast, the slice from the same (31.69 ppm) trace in the \(^{13}\)C-\(^{13}\)C STOCSY (Figure 3-
2C) showed very strong correlations with all other proline peaks and matched those from the BMRB perfectly. Metabolites such as glutamine and glutamate could only be distinguished using the improved resolution and chemical shift differences in $^{13}$C and not with the highly overlapped $^1$H spectra. In the absence of $^{13}$C, many of these metabolites would have been ambiguous using 1D $^1$H alone. Metabolites identified in Table S1 were consistent with previously collected and analyzed data using conventional methods (Chenomx), 2D NMR, and spiking experiments (Williams et al., unpublished results).

PCA performed on both the fly $^{13}$C and $^1$H 1D spectra yielded clear separation of cold-hardy flies and cold-susceptible flies on PC1 (Figure 3-3A) and PC2 (Figure 3-3B), respectively. We were able to show, however, that the $^{13}$C data provided much better separation between hardy and susceptible flies and had stronger loadings than those from the $^1$H data. Annotated $^{13}$C loadings are given in Figure 3-3. Histidine, AMP, phosphocholine, taurine, and methionine sulfoxide all loaded with the hardy flies, and glucose (possibly also other sugars), proline, alanine, and arginine loaded with the susceptible flies. All of these compounds were identified and verified as altered by selection in a parallel study using 1D and 2D $^1$H NMR and mass spectrometry, and the direction of alteration was confirmed in 78% of cases (all but AMP and taurine, which were higher in susceptible flies in the parallel study: Williams et al., unpublished results). The differences in the direction of the effect may be due to using only a subset of lines in the present study, and the flies in the present study were also not exposed to cold. These findings support the primary conclusions that hardy flies have alterations to pathways involved in membrane lipid metabolism, oxidative stress, and energy balance.
These alterations are consistent with the hypothesis that cold-hardy flies maintain aerobic metabolism in the cold, preventing the (potentially deleterious) accumulation of alanine and sugars.

**13C NMR offers improved confidence in metabolite identification**

The *mdx* mouse is an animal model for Duchenne muscular dystrophy (DMD)—a muscle degenerating disease found in young males. The inability to produce dystrophin results in a pathology in mice similar to that seen in humans\(^1\). Tracking disease progression in children requires difficult and painful muscle biopsies\(^2\) and effort based functional testing\(^3\). Importantly, current methods may be insensitive to therapeutic intervention. Identifying biomarkers of disease progression for DMD using an easily obtained sample such as serum or urine is essential for improving our ability to treat and diagnose this debilitating disease.

Although the overall sensitivity with the small volume of mouse serum prevents the detection of large numbers of metabolites, we were able to obtain high quality NMR data on the most concentrated compounds, suggesting that 13C NMR metabolomics using isotopically labeled compounds for flux studies on survival bleeds is also possible with this probe. Using the 13C workflow developed here, we have found that the addition of 13C helps in making specific identifications and, perhaps more important, in preventing the misidentification of metabolites suggested by 1H NMR alone. For example, 1H resonances at 0.9, 1.3 and 2.75 ppm are often associated with fatty acids, but the specific fatty acid species is typically not annotated. Here, with the addition of the 13C-13C STOCSY and 13C-1H SHY correlation maps, we were able to identify linoleic acid as the specific fatty acid abundant in our 1H and 13C spectra.
Figure 3-4 shows representative $^1$H and $^{13}$C 1D spectra from the mouse serum. Previous NMR studies in the muscle of mdx vs. control mice have reported a decrease in muscle creatine that varies with age and tissue, thought to result from increased inflammation and degeneration of the muscle$^{94,97}$. It is hypothesized that this leads to an in increase in serum creatine of mdx mice through dystrophin deficient “leaky” muscle membranes$^{97}$. In our data, the $^1$H PLS-DA results indicated a resonance at 1.30 ppm that separates the control from mdx. Analysis of the $^1$H STOCSY using 1.30 ppm as a driver peak indicated a correlating resonance or overlapping resonances at 3.02 ppm, often assigned to creatine. However, other creatine $^1$H resonances were overlapped, making it difficult to analyze. To further investigate whether creatine is associated with disease development in the mdx mouse model, we examined the $^{13}$C-$^1$H SHY data and found correlations with the 3.02 ppm resonance to the $^{13}$C peaks at 56.5 ppm and 41.2 ppm; the correlation at 38.9 ppm we would expect to see for creatine was not present (Figure 3-4). Creatine is often assumed to be the largest contributor to the peak at $^1$H 3.02 ppm$^{134,193}$. To determine if the absence of the creatine peak at 38.9 ppm in the $^{13}$C 1D was due to sensitivity limitations, we compared the relative integrations of the $^1$H resonance at the 3.02 ppm (-CH$_3$) to the succinate at peak 2.39 ppm (CH$_2$-CH$_2$) (Figure 3-4A). Given the relative abundance of the creatine compared to the succinate resonance, we expect to see a $^{13}$C peak at 38.9 ppm comparable to the succinate resonance seen at 36.5 ppm in the $^{13}$C spectra. Therefore, using $^{13}$C data we conclude that creatine is not present at concentrations that we can measure in this experiment. This was verified by a spiking experiment with synthetic creatine, which did not correspond to resonances observed from the mice (data not shown). Despite extensive
precedent in the literature assigning $^1$H resonances at 3.02 ppm in metabolomics mixtures, the $^{13}$C data clearly shows that creatine would have been an incorrect assignment in our case.

Citrulline (Figure 3-4B) has a resonance near 3.02 ppm and is another possible assignment of this peak. $^{13}$C-$^1$H SHY and $^{13}$C-$^{13}$C STOCSY data show correlations between the resonance at 3.02 ppm in the $^1$H spectra and suspected citrulline resonances 56.5 ppm and 41.2 ppm. We do not, however, observe strong correlations to other citrulline $^{13}$C peaks. In addition, the $^1$H chemical shifts do not agree well with citrulline (Figure 3-4A). Thus by using both $^1$H and $^{13}$C spectra of the same sample, we were able to rule out two previously associated compounds as unlikely to be contributing to our NMR spectra. Further analysis is needed to identify the compound(s) associated with these peaks, but this result further demonstrates the utility of $^{13}$C NMR in metabolomics studies.

**Future Improvements**

There are several possible improvements that could be made in the future. First, a larger volume NMR probe with $^{13}$C optimization would provide greater absolute sensitivity. This, however, would result in lower mass sensitivity because the sensitivity of NMR probes is roughly inversely proportional to the diameter of the coils\textsuperscript{194}, but for samples that are not mass-limited this could be a helpful option. Second, a $T_1$ relaxation agent could be added to the samples to further reduce the relaxation time and also better recover slowly relaxing quaternary carbons that we attenuated in this study. Finally, dissolution dynamic nuclear polarization (DNP) is an emerging technique that can transiently enhance the $^{13}$C signal of a sample by >10,000 fold for about 1 or 2 minutes\textsuperscript{175}. If some or all of these techniques could be used to improve sensitivity of the
NMR measurement even more, then the benefits of $^{13}$C metabolomics that we have demonstrated could be fully realized.

**Conclusion**

We have shown that by using a $^{13}$C-optimized NMR probe$^{178}$, we were able to acquire high-quality $^{13}$C NMR data in a reasonable amount of time and with relatively small sample volumes. The overall workflow allowed for more accurate identification of compounds through database matching by utilizing both the standard 1D $^{13}$C and $^1$H NMR data. There is a cost of about 2 hours per sample in terms of extra measurement time, but inspection of the NMR spectra clearly shows the increased spectral dispersion of the $^{13}$C compared to the $^1$H data from the same sample (e.g. Figure 3-1A and B) that in turn leads to many desirable results in database matching and ultimately better separation of groups through multivariate analysis. Using the synthetic mixture, we determined that limit of analysis through correlations was about 60 nmoles, although this could be improved even more with more sensitive NMR measurements.

When statistically combined with $^1$H NMR data on the same samples, the $^{13}$C spectra enable new approaches that produce: 1) peak lists for better spectral annotation, 2) improved group separation using multivariate statistical analysis because of reduced spectral overlap, and 3) improved confidence in identified metabolites and the ability to reject identifications based on $^1$H data alone. We developed and validated our approach using a synthetic mixture and then applied it to two different biological examples: *D. melanogaster* cold tolerance models in which metabolites that significantly changed between hardy and susceptible lines of flies were easily identified, and mass-limited mouse serum where metabolite identification using $^{13}$C spectral analysis could not be done with $^1$H analysis alone. In the case of the mouse serum, the addition of $^{13}$C
data not only enhanced our ability to identify specific compounds but it also prevented mistakes that could have been made with $^1$H data alone.
Figure 3-1. Overview of approach to $^1$H and $^{13}$C NMR Statistical Analysis of Synthetic Mixtures. The 1D $^{13}$C (A) and 1H (B) spectra were processed, aligned, normalized, and scaled. Compounds below 1.5 mM (indicated by * in A) showed no significant correlations. 13C-13C STOCSY (C) and 13C-1H SHY (D) maps were then made from all of the data in A and B. An expansion of the aromatic region of the 13C-13C STOCSY (C) is shown in H. 13C-13C STOCSY traces of resonances (*) (E) were used to generate peak lists as described in the text. Negatively correlated peaks were removed (F) and positively correlated peaks with significant correlations as described in Methods (G) were used as inputs for COLMAR query searches, which for example, found tryptophan (Trp). Tryptophan 1D 1H and 13C reference spectra from the BMRB (black) are overlaid on both correlation matrices (C and D) and the expansion region in H.
Figure 3-2. Identification of *D. melanogaster* metabolites using 1D $^{13}$C (A) and $^1$H NMR (B) data. $^{13}$C-$^{13}$C STOCSY (E) and 2D $^{13}$C-$^1$H SHY (F) correlation maps were generated as described in the text. Slices at 31.69 ppm from the $^{13}$C-$^{13}$C STOCSY (B) and $^{13}$C-$^1$H SHY (D) show the correlations and co-variances with that resonance, which yielded proline in a database search. Proline spectra from the BMRB (black lines in C and D) are overlaid onto the 1D $^{13}$C STOCSY and $^{13}$C-$^1$H SHY to confirm identity.
Figure 3-3. PCA scores separated hardy from susceptible flies much better in $^{13}$C data when compared to $^1$H data PCA of $^{13}$C (A) and $^1$H (B) 1D spectra from cold hardy and cold susceptible flies. $^{13}$C loadings plot (A) showed more changes between cold susceptible (blue) and cold hardy (red) flies. $^1$H loadings plot (B) showed little differences between the groups, though sugars seemed to load better with the cold susceptible flies. Red and positive peaks indicate resonances that were correlated with cold hardy flies and blue and negative resonances indicate resonances that correlated well with the cold susceptible. Annotations are given for 1D $^{13}$C loadings plot (A).
Figure 3-4. Analysis of specific resonances in mouse serum for metabolite identification or error detection. The mdx data are in red and the control in blue. All the spectra are overlaid in A, with the average data for each group shown as a bold line. Resonances at 3.02 ppm (unknown) and 2.39 ppm (succinate) in the $^1$H (A) are expanded and the relative intensities are given in the boxplot. Metabolites indicated in B are overlaid onto the 1D spectra (A and B). Expansion of the carbon 1D from ~20 ppm to 60 ppm show the absence of creatine peak at 38.9 ppm but the presence a peak at 36.5 ppm confirms the possibility of succinate. Using just the $^1$H data, the peak at 3.02 ppm would likely be assigned to creatine, which we can rule out with the $^{13}$C data.
CHAPTER 4
A LONGITUDINAL ANALYSIS OF LIPIDS IN MDX MICE

Introduction

As previously discussed, the mdx mouse model is the most commonly studied murine model in DMD, lacking a functional dystrophin protein, but displays a phenotype that is much less severe than in humans due to an upregulation of utrophin\textsuperscript{93}. Despite this shortcoming, mdx mice still go through well characterized stages of muscle damage and recovery. First, mdx mice go through an initial bout of damage/inflammation at 4-6 weeks followed by a “plateau” stage between 24 to 65 weeks when muscle regeneration can match the damage until eventually the regenerative capacity is exhausted at later stages of life\textsuperscript{195}.

Previous studies have shown the loss of a functional dystrophin protein directly affects the muscle both structurally and metabolically. Dystrophin acts as an anchor to the muscle cell membrane as a means for stabilization through muscle contraction\textsuperscript{24-26}. Without dystrophin to stabilize the sarcolemma, eccentric contractions lead to membrane damage initiating chronic bouts of repair and damage\textsuperscript{24}. This cycle continues until the healthy muscle is replaced with fat and fibrosis and the cell environment is no longer conducive for muscle repair mechanisms\textsuperscript{196}. Many studies have investigated the changes of metabolism brought on by loss of dystrophin. When dystrophin is no longer present, there are significant alterations in nitric oxide synthesis\textsuperscript{197}, energy metabolism\textsuperscript{152,153,198-201}, and lipid synthesis. Interestingly, serum\textsuperscript{155,158} and muscle biopsies\textsuperscript{156,157,202}, have shown alternations in lipid metabolism in both dystrophic humans and mice. However, more work is needed to understand the mechanisms of lipid infiltration as
these are still unknown in the field. To our knowledge, nobody has investigated the influence of serum lipids in tissue of the *mdx* mouse at different stages of disease.

The goal of this study was to investigate longitudinal changes in lipids of serum and tissue from *mdx* mice at 6 weeks and 24 weeks of age. This study provides abnormalities found in lipid and glucose metabolism of the *mdx* mouse which can potentially prove useful for mechanistic insights for translational investigations in DMD.

**Methods**

**Animals**

C57BL/10ScSn-DMD *mdx* mice (*mdx*) and age matched C57BL/10ScSn (controls) were purchased from Jackson Laboratories (Bar Harbor, Maine). The University of Florida’s institutional animal care and use committee (IACUC) approved the experimental protocol for this study. All animals were maintained with a 12 hour light:dark cycle, at 72 degrees Fahrenheit and 42% humidity at the University of Florida Animal Facilities, which are approved by the Accreditation of Laboratory Animal Care.

**Study Design**

Both *mdx* and control mice were obtained at 4 weeks of age and grown to either 6 weeks old (*mdx* (n=6), control (n=5)) or 24 weeks old (*mdx* (n=10), control (n=8)). At each time point (6 or 24 weeks), the mouse hindlimbs were imaged *in vivo*, with T2-MRI of the lower leg followed by blood and tissue collection when the animals were sacrificed. At sacrifice, 200 μL of blood was collected by submandibular puncture, blood was allowed to sit at room temperature for 30 minutes to clot and then was centrifuged to extract serum. Under 2% isoflurane anesthesia, lower leg muscles (gastroc was used specifically in this study) were extracted, flash frozen and stored at -80 °C until
further analysis. The mice were then euthanized under 2 % isoflurane anesthesia according to approved IACUC protocols.

**Solution NMR on serum**

Serum was separated from the red blood cells, by sitting at room temperature for 30 minutes and centrifuged for 15 minutes at 10,000g at 4 °C. Serum was separated, transferred to an Eppendorf tube, flash frozen in liquid nitrogen and stored at -80 °C. At time of analysis, samples were thawed and approximately 100 μL serum was lyophilized and re-suspended in 40 μL 99% D₂O and transferred into 1.5 mm NMR tubes (Norell). One-dimensional ¹H and ¹³C spectra were collected on an Agilent VNMRS-600 spectrometer using a custom 1.5 mm ¹³C high temperature superconducting (HTS) probe.¹⁷⁸

1D ¹H serum data were acquired in approximately 18 minutes using a Carr-Purcell-Meiboom-Gill (CPMG) sequence, which was able to remove the lipoprotein signal contribution due to T₂ decay, resulting in a flat baseline. Data were recorded with the following parameters: SW of 16 ppm (9615.4 Hz), a 2.0 s relax delay, a 90-degree initial pulse, a train of 124 180-degree CPMG pulses with a 1 ms inter-pulse delay, and a 2.0 s acquisition time and 128 scans.

1D ¹³C spectra were collected in approximately 2 hours under conditions that favor nuclei with short T₁ relaxation times in order to maximize overall sensitivity and minimize measurement time, as described previously.²⁰³ We used a 60° pulse with a 0.1 sec relaxation delay and a 0.8 sec acquisition time, a 212 ppm (32051.3 Hz) spectral window, a carrier frequency of 98.0 ppm at a frequency of 150.79 MHz. We found that protein contribution still affected the baseline in ¹³C spectra and therefore used a ¹³C CPMG sequence with a 90-degree excitation pulse followed by a train of 18
individual 180-degree pulses with a 1 ms inter-pulse delay. \(^1\)H was decoupled at 599.68 MHZ with a power of 37 dB using WALTZ-16 continuous recording of \(^{13}\)C spectra.

**HR-MAS NMR on ex vivo gastrocnemius muscle**

The gastrocnemius muscle of the control and \(mdx\) mice at 6 weeks (\(mdx=6,\) control=5) and 24 weeks (\(mdx=10,\) control=8) were analyzed using high-resolution magic-angle spinning (HR-MAS) NMR. Data were acquired using a 4 mm HR-MAS probe on a Bruker 600 MHz spectrometer (AVIII) running Topspin 3.2 software. Sample preparation and data acquisition followed the protocols from Beckonert et al. In addition, the wet weight of the muscle was measured and used for normalization. The magic angle was optimized and set for all samples using potassium bromide (KBr).

Pulse calibration, tune, match and manual shimming were performed for each muscle sample. One dimensional NOESY presaturation (noesypr1d) was used with 128 scans, a spectral width (SW) of 12 ppm, 16k complex data points, a relaxation delay of 2 sec and a NOESY mixing time of 90 ms.

**NMR processing**

HR-MAS and solution NMR spectra were processed using NMRPipe. Spectra were appodized (cosine for \(^1\)H and exponential with 2 Hz line broadening for \(^{13}\)C) zero-filed 2x, Fourier transformed, phased, and baseline corrected. \(^1\)H spectra were referenced to the lactate peak at 4.1 ppm. \(^{13}\)C spectra were referenced to the anomeric carbon glucose peak at 98.64 ppm. Further processing and analysis of NMR spectra were performed using an in-house MATLAB metabolomics toolbox. Spectra were referenced, aligned using the peak alignment fast Fourier transform (PAFFT) algorithm and normalized using Probabilistic Quotient Normalization (PQN). Metabolites were quantified using integration of resolved \(^1\)H resonances with chemical
shift ranges defined in Supp Table 1. The polyunsaturation index (pi) and unsaturation index (ui) were calculated according to Mosconi et al\textsuperscript{204} using the following equations:

\[
pi = \frac{\text{Lipid B}}{\text{Lipid A}} (Eq 1)
\]

\[
u i = \frac{\text{Lipid C}}{\text{Lipid A}} (Eq 2)
\]

where Lipid A is the peak at 0.9 ppm, Lipid B is the peak at 2.75 ppm, and Lipid C is the peak at 5.4 ppm.

**In vivo imaging and spectroscopy**

Magnetic resonance imaging and spectroscopy (MRI/MRS) were performed on an 11.1-T horizontal bore magnet (Magnex) equipped with an Agilent Spectrometer. Mice were initially anesthetized using an oxygen and 3% isoflurane mixture, but isoflurane was reduced to 0.5-1% to maintain anesthesia throughout the scan. Temperature (maintained with a warm water heating pad) and respiration were monitored throughout the scan as well. Lower legs of the \textit{mdx} (6 week n=6, 24 week=10) and control (6 week n=5, 24 week=8) were inserted into a 2.0-cm- internal-diameter, custom-built \textsuperscript{1}H solenoid coil, up to the knee. For T\textsubscript{2}-weighted images, multiple slice, single spin-echo images were acquired with a repetition time (TR) of 2,000 ms, echo times (TE) of 14 ms and 40 ms, a field of view of 10-20 mm\textsuperscript{2}, slice thickness of 0.5-1 mm, and with an acquisition matrix of 128x256 for 2 signal averages. The T\textsubscript{2} decay was fit to a single-exponential decay curve. A 1 X 2 X 2 mm voxel was used in the posterior compartment of the lower leg (in the gastrocnemius) for spectroscopy. Spectroscopy was performed using a localization by adiabatic selective refocusing (LASER) pulse sequence.
Statistics

Multivariate analyses were conducted using Nonlinear Iterative Partial Least Squares (NIPALS) PCA\textsuperscript{205} and PLS-DA\textsuperscript{206} using our in-house MATLAB Metabolomics toolbox\textsuperscript{141}. Targeted comparisons were analyzed using Graphpad Prism version 6. Quantified metabolites were tested for normality using the Shapiro-Wilk test\textsuperscript{207}. Comparisons between controls and \textit{mdx} at both ages analyzed using a one-way ANOVA and a Bonferonni correction. Comparisons between \textit{mdx} and controls at both ages that were not determined as normal using the Shapiro Wilk normality test were compared using a Mann-Whitney test corrected for multiple comparisons. Metabolite and MR parameters were correlated using a linear correlation matrix, in MATLAB (corrcoef), which calculated both the correlation coefficients and p-values.

Results

Multivariate global analysis of control and dystrophic mice using $^{13}$C spectra

$^{13}$C spectra of control and dystrophic serum at both 6 and 24 weeks of age were used to perform PCA in order to identify the largest variation between the cohorts. Figure 4-1A shows the separation of all four groups using PCA. To determine changes in just the \textit{mdx} mice, PCA was performed at 6 weeks and 24 weeks on only the $^{13}$C spectra of the \textit{mdx} mouse serum (Figure 4-1B). The 6 week \textit{mdx} serum spectra separated from the 24 week \textit{mdx} serum spectra using PCA (verified with PLS-DA with a Q$^2$ = 0.82, R$^2$=0.98). The loadings plot for the second component reveals that peaks related to linoleic acid (fatty acids) showed a greater association with 6 week \textit{mdx} and glucose was more associated with the 24 week old mice (Figure 4-1C). As a reference to guide the eye, BMRB\textsuperscript{146} spectra of linoleic acid and glucose are displayed above and below the loadings spectrum, respectively\textsuperscript{146}.
Quantification of metabolites in serum

Figure 4-2 is the overlay of all of the spectra from dystrophic and control mice at both 6 and 24 weeks of age with inset expansions of lipid and glucose peaks. Integration of A, B and C lipid peaks were used to calculate the pi and total ui in the lipids. Total lipids (Figure 4-3A) and unsaturated fatty acids (Figure 4-3B) significantly (p<0.0001, p<0.01, respectively) increased in the 6 week old mdx mice as compared to age matched controls, whereas, both 24 week controls and mdx were observed to have significantly decreased lipids compared to 6 week old mdx and controls (p<0.01). The pi in the 6 week old mice were lower (p<0.01) than that of the 6 week old controls, but still higher than both the 24 week and mdx and controls (p<0.0001 for both) (Figure 4-3C). Glucose levels in the 6 week old mdx mice were significantly (p<0.01) lower than 6 week old controls (Figure 4-3D). Conversely, 24 week old control and mdx mice had significantly higher glucose then controls and mdx at 6weeks (Figure 4-3D).

Tissue metabolite comparison at different ages

The metabolic profile of gastrocnemius tissue from both 6 week and 24 week old control and mdx mice was determined using HR-MAS NMR. Table 1 summarizes the metabolites, the ppm range used to integrate each metabolite and p-values comparing the mdx and controls at each age. Each red p-values represents significant changes between the ages. Alanine, glycine, creatine, ATP, histidine and HC=CH peak at 5.4 ppm, were all found to significantly change with age in the control group. Lactate, the taurine:creatine ratio and ui significantly changed with age in mdx mice only.

Correlations of metabolites from serum to tissue

A correlation matrix was used to find relationships between metabolites within the serum, tissue and in vivo measurements in both controls (left) and mdx (right) (Figure 4-
Each variable is labeled and on the right hand side, the variables are grouped by either tissue, serum or in vivo, representing where the variable was acquired. The control matrix showed strong correlation ($r > 0.50$, $p$-value$<0.05$) between serum and tissue metabolites, except for creatine and taurine. The mdx correlation matrix revealed the strongest correlations ($r > +/-$ 0.60, $p$-values $<0.02$) between lipids in the serum and in tissues with the in vivo MRI $T_2$.

**Discussion**

In this study, an untargeted NMR screen of mdx and control mouse serum revealed an age and a strain differences in serum glucose, total lipid levels and the degree of lipid saturation. We found a positive correlation between serum glucose levels and saturation of lipids with an in vivo indicator of muscle damage (MRI $T_2$) and negative correlation with serum lipids. These results indicate the complex interplay between lipid and carbohydrate metabolites in the tissue and serum during chronic bouts of muscle damage and regeneration that occur in dystrophic muscle.

We used an unbiased and untargeted approach using multivariate analysis, PCA, to determine differences in the mdx, controls at different ages. As we previously demonstrated, $^{13}$C NMR spectra provide better group metabolite discrimination than $^1$H spectra (Figure 4-1A and 1B) alone. PCA analysis revealed that lipids in the serum were more abundant in 6 week old mice, whereas glucose was more abundant in 24 week old animals (Figure 4-1C). These metabolite assignments were verified using a 2D $^1$H-$^{13}$C HSQC which matched with both resonances to glucose and linoleic acid that were all later quantified in the 1D spectra (Figure 4-2).
Previously, studies have shown alterations in lipids in the muscle and blood of patients with DMD. Similarly, we show that total lipids (Figure 4-3A) are increased in the severe form of muscular dystrophy at 6 weeks. Unexpectedly, not all lipids were increased equally. In 6 week old mdx mice, ui (Figure 4-3B) increased but the pi (Figure 4-3C) of the lipids decreased compared to the 6 week old controls. These findings are in agreement with Gillet et al. They used a 2D NMR sequence (COSY) spectra of the hind leg at 3 weeks of age to show that C57BL10 (control to mdx mice) have linoleic acid, in contrast to mdx mice have linolenic acid, a more unsaturated fatty acid. Similarly, a study by Peperi et al. showed alterations in polyunsaturated fatty acids in erythrocytes in DMD.

Despite the integral impact lipid replacement of healthy muscle has on disease phenotype of DMD, the exact mechanism of this pathology has not been fully characterized. It has been shown that lipids in both muscle and serum are unregulated, however, it has never been shown that lipids in the blood affect the lipid profile in the tissue. Altered serum lipids associate with the tissue lipid profile, that also differ from control. Table 1 shows that there is a significant difference (p=0.051) of the pi of mdx compared at 6 weeks and 24 weeks of age. We find that at 6 weeks of age, the severe pathology, there is an increase in muscle pi compared to 24 weeks, where utrophin has upregulated, the muscle has regenerated and the lipid metabolism return to control levels. These data suggest that 1) alterations in serum lipid profiles influence the tissue lipid profile and 2) if the membrane can be re-stabilized with a therapy (i.e. upregulation of utrophin or dystrophin reintroduced with gene therapy) the metabolic alterations will stabilize to control levels. Nevertheless, these finding are
preliminary and further studies are needed to determine if the lipid alterations found systemically in the blood contribute to muscle pathology or mechanisms related to disease burden.

Serum glucose levels are altered in dystrophic mice. We found that glucose is decreased during the period of severe muscle damage in \textit{mdx} as compared to controls and at older ages when the mice have recovered from this initial bout. These results are in agreement with many previous studies in the literature. Rodriguez-Cruz et al. showed that boys with DMD had a high frequency of insulin resistance and obesity, even without the palliative use of steroids\textsuperscript{209}. Further, they showed that in DMD subjects with specific genetic mutations, deletions in exons 45 or 50, there was a 9 fold higher likelihood that they would become insulin resistant\textsuperscript{209}. They also found aggregates of GLUT4 in the cytoplasm of myofibers possibly limiting glucose uptake in muscle and potentially altering glucose metabolism systemically\textsuperscript{209}. Further support of this the observation that the loss of dystrophin and plectin at the sarcolemma destabilizes the membrane and reduces the uptake of glucose\textsuperscript{210}. The decrease in glucose could also be explained by the altered glycogen metabolism not only in \textit{mdx} muscle but liver as well\textsuperscript{211}. We hypothesize that the altered glucose metabolism in the \textit{mdx} mouse is due to the combination of these compensatory mechanisms.

We then used correlation matrices to compare how metabolites changed from 6 to 24 weeks in both the \textit{mdx} and control mice. We found that control mice showed high correlation between tissue and serum lipid metabolites and glycine. The peak at 2.1 ppm associates to linolenic as compared to linoleic acid\textsuperscript{202}. \textit{Mdx} mice do not have as strong or significant correlations with the peak at 2.1 ppm. However, the peak at 2.1
ppm does show negative and significant correlation with MRI T\textsubscript{2}. These data suggest that as DMD is in a more severe form, the systemic lipids change saturation. Potentially the changes in lipid metabolism, increase in phospholipids and alteration in saturation of levels, may contribute to the role of lipid infiltration in DMD.

In conclusion, the systemic saturation of lipids in \textit{mdx} animals differ from controls. This difference is dependent on disease severity. We found correlations between serum lipids to tissue lipids and \textit{in vivo} MRI T\textsubscript{2} suggesting detection of saturation of lipids in serum as a potential means to monitor changes in a disease that is easy to acquire, minimally invasive and translational. These data also show that more work is needed to understand the mechanism of lipid metabolism in DMD and its role in disease progression.
Figure 4-1. PCA of $^{13}$C serum spectra is able to differentiate control and mdx at different ages. A. PCA plot of all groups, B. PCA of mdx at 6 weeks and 24 weeks of age show the separation on the second component. In C the loadings plot showing the 6 week (positive) separate based on lipids and the 24 week (negative) separate based on glucose. D and E are BMRB standard spectra that reveal the similar peaks to the compounds separated out in the loadings plot.
Figure 4-2. Peaks of interest in $^1$H spectra. Serum spectra from control (teal and blue) and mdx (pink and red) mice at 6 and 24 weeks. Large changes found in the $^1$H are magnified to show the differences. Thin lines are from individual mice, and the bold lines are the mean for each group.
Figure 4-3. Quantification of serum metabolites. Quantifications from the $^1$H spectra of lipids and glucose in the serum of control and *mdx* mice at both 6 weeks and 24 weeks of age. Significance was determined by p-value of 0.05. Comparisons to 6 week controls with a p-value < 0.01 are denoted with *, Comparisons to 6 week controls with a p-value < 0.001 are denoted with **, Comparisons to 6 week *mdx* with a p-value < 0.001 are denoted with #, Comparisons to 24 week controls with a p-value < 0.01 are denoted with †.
<table>
<thead>
<tr>
<th>Metabolite</th>
<th>ppm</th>
<th>Control 6 week vs 24 week</th>
<th>mdx 6 week vs 24 week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate</td>
<td>1.3 - 1.34</td>
<td>0.256</td>
<td>0.013</td>
</tr>
<tr>
<td>Alanine</td>
<td>1.44 - 1.51</td>
<td>0.030</td>
<td>0.025</td>
</tr>
<tr>
<td>Taurine</td>
<td>3.36 - 3.46</td>
<td>0.094</td>
<td>0.494</td>
</tr>
<tr>
<td>Myoinositol</td>
<td>4.04 - 4.065</td>
<td>0.205</td>
<td>0.072</td>
</tr>
<tr>
<td>Glycine</td>
<td>3.538 - 3.55</td>
<td>0.003</td>
<td>0.003</td>
</tr>
<tr>
<td>Creatine</td>
<td>3.9 - 3.94</td>
<td>0.042</td>
<td>0.276</td>
</tr>
<tr>
<td>Glucose</td>
<td>5.2 - 5.24</td>
<td>0.152</td>
<td>0.291</td>
</tr>
<tr>
<td>Glucose-1-phosphate</td>
<td>5.38 - 5.45</td>
<td>0.417</td>
<td>0.180</td>
</tr>
<tr>
<td>ATP</td>
<td>6.11 - 6.17</td>
<td>0.025</td>
<td>0.059</td>
</tr>
<tr>
<td>Lipid C-H groups</td>
<td>0.84 - 0.94</td>
<td>0.324</td>
<td>0.404</td>
</tr>
<tr>
<td>Lipid C-H2</td>
<td>2.73 - 2.81</td>
<td>0.381</td>
<td>0.469</td>
</tr>
<tr>
<td>Lipid HC=CH</td>
<td>5.26 - 5.38</td>
<td><strong>0.041</strong></td>
<td>0.174</td>
</tr>
<tr>
<td>Carnosine</td>
<td>3.53 - 3.58</td>
<td>0.466</td>
<td>0.486</td>
</tr>
<tr>
<td>Anserine</td>
<td>3.81 - 3.87</td>
<td>0.453</td>
<td>0.198</td>
</tr>
<tr>
<td>Tau:Cre polyunsaturation</td>
<td>-</td>
<td>0.174</td>
<td><strong>0.036</strong></td>
</tr>
<tr>
<td>index</td>
<td>-</td>
<td>0.374</td>
<td>0.429</td>
</tr>
<tr>
<td>unsaturation index</td>
<td>-</td>
<td>0.219</td>
<td><strong>0.051</strong></td>
</tr>
</tbody>
</table>
Figure 4.4. Correlation matrices to compare serum and tissue measurements. The lower left of each correlation matrix are the correlation coefficients for each comparison which are labeled on the left and top. The upper right portion of each correlation matrix are the p-values for the paired comparison. The scale bar on the bottom of the figure corresponds to the correlations and the one on the right represents the corresponding p-values. Each metabolite is grouped into tissue, serum or in vivo denoted on the left hand side.
CHAPTER 5
A CROSS SECTIONAL ANALYSIS OF THE URINE METABOLOME IN BOYS WITH DUCHENNE MUSCULAR DYSTROPHY IN PERSUIT OF BIOMARKER DISCOVERY

As previously stated in Chapter 1, clinical trials in DMD have had limited success due in part to ineffective outcome measures. This chapter describes a study to determine if urine metabolites could potentially be a viable surrogate outcome for DMD. In accordance with the parent study, ImagingDMD, I was in charge of setting up an affiliated study whose purpose was to collect and store urine and serum for each boy with DMD at their annual appointment that included MRI/MRS, strength, function, updated medical history and demographics data. We collected biofluid samples and all other measurements from control boys at a single time point. I used urine samples from 46 boys to run a cross sectional metabolomics study. Initial analysis of the samples was performed using NMR. Interesting findings then led to a targeted LC-MS/MS study of organic and amino acids to verify discoveries and investigate metabolites difficult to quantify in NMR data. My contribution to this study included 1) helping to write the addendum to the Institutional Review Board to acquire serum and urine samples from human participants, 2) creating an SOP and determining logistics to collect samples with the CTSI Clinical Research Center and store the samples at the UF CTSI Biorepository, 3) collecting and storing samples, 4) optimizing NMR procedures for urine analysis, 5) creating the study design, 6) analyzing all urine samples both on the NMR and the resulting data, 7) writing additional MATLAB scripts to QC (Chapter 2) and analyze the data when necessary, and 8) writing the majority of the manuscript with considerable input from the other authors.
Introduction

Duchenne muscular dystrophy (DMD) is a progressive muscle wasting disease caused by the loss of the dystrophin protein resulting in muscle membrane weakness, inflammation, and failed muscle regeneration. DMD is an X-linked disorder and the most common of the dystrophinopathies, occurring 1 in every 5,000 live male births. Initial observation of clinical symptoms occurs around the age of 5 with proximal weakness (Gowers’ maneuver) progressing from proximal to distal weakness and loss of ambulation in the early teens. People with DMD typically succumb to the complete loss of dystrophin in the third decade of their life from respiratory and cardiac complications.

Dystrophin has several roles within the muscle cell. Structurally, dystrophin binds intracellular filamentous actin on the C-terminus and the dystrophin associated glycol-proteins (DAG) complex at the N-terminus. The DAG complex binds to extracellular matrix components, stabilizing the muscle cell membrane during contractions. Metabolically, dystrophin binds and localizes neuronal nitric oxide synthase (nNOS) to the membrane. Nitric oxide (NO) created from the subsarcolemmal nNOS is important in regulation of glycolysis, inflammation, and blood flow. The loss of dystrophin renders the sarcolemmal membrane mechanically weak and susceptible to muscle damage during eccentric contractions. The loss of membrane integrity results in the ingress of large serum proteins (i.e. albumin) and egress of muscle proteins (i.e. creatine kinase). Likewise, it is believed that the loss of localization of nNOS at the sarcolemma decreases the amount of bioavailable NO in the muscle. Lower amounts of NO attenuate dilatation of vasculature depleting the blood flow and nutrients to the muscles during
exercise\textsuperscript{197}, rendering dystrophic muscle metabolically altered with increased susceptibility to exercise induced ischemia. Ongoing bouts of mechanical injury expose the muscle cell to chronic inflammation, repair, and declining capacity for regeneration. The environment of the damaged muscle is not conducive to retain its regenerative capacity\textsuperscript{196} and the muscle is eventually replaced with fat and fibrous components, ultimately reducing the functional capacity of dystrophic muscle.

Several studies have found significant metabolic alterations in DMD tissue and serum\textsuperscript{156,160,199,216,217}. A study performed in human biopsies of the vastus lateralis\textsuperscript{156}, found that there were significant alterations in small molecules related to energy metabolism, including creatine and metabolites associated with lipid biosynthesis pathways. Because of the elevated permeability of the fragile DMD sarcolemma, calcium influx into the cell initiates an array of signaling cascades, resulting in cycles of damage, inflammation, regeneration and repair. During these cycles, the cell undergoes a number of compensatory mechanisms in an attempt to maintain basic homeostasis. In the mouse model (\textit{mdx}) of DMD, it has been found that mitochondrial respiration is reduced 2-fold and ATP synthesis is inhibited\textsuperscript{153,199,200} despite enzymes involved in the citric acid (TCA) cycle\textsuperscript{218} and creatine synthesis being upregulated\textsuperscript{97}. With the loss of fully functioning nNOS, these metabolic deficiencies are exacerbated by the constriction of blood vessels, preventing nutrients and oxygen from reaching and waste products being removed, from muscle\textsuperscript{197}.

The current standard of care for boys with DMD are high doses of glucocorticosteroids (GCs), which have shown to prolong ambulation by
approximately 2-5 years\textsuperscript{22}. Despite these positive effects, GC lead to an array of several negative side effects, including weight gain, osteoporosis, behavioral issues, and weakened immunity \textsuperscript{22,219,220}. Although the exact mechanism by which steroids ameliorate DMD is unclear, Morrison-Nozik et al. have demonstrated that GCs directly induce Krupple-like factor 15 (KLF15) and reduce disease severity in the \textit{mdx} mouse\textsuperscript{65}. Furthermore, they determined the most robust genes regulated by the GC-KLF15 axis are involved in amino acid and fatty acid metabolism in both the \textit{mdx} mouse model and human samples\textsuperscript{65}.

Currently, there is no cure for DMD despite extensive efforts put forth to develop innovative therapies. Over the last 5 years, many promising clinical trials have attempted to mitigate disease burden, with treatment strategies ranging from gene therapy strategies to improve anti-inflammatory drugs\textsuperscript{34,75,221,222}. Despite the increasing number of clinical trials, success beyond phase 2b has not been realized. One reason for these shortcomings has been attributed to the lack of a robust outcome measure. The primary clinical endpoint of many recent trials have utilized the six-minute walk test (6MWT) which has not proven to be ideal\textsuperscript{223}. Alternatively, muscle biopsies to perform histology are invasive and absolute quantification of dystrophin levels has been challenging\textsuperscript{224}. Therefore, there is a need for surrogate endpoints that are sensitive to disease state and therapeutic intervention, which are noninvasive, easy to acquire, objective in nature, and independent of effort or subject compliance.

In the past, surrogate biomarker studies for DMD have included serum/urine proteins\textsuperscript{76,90,91,95,225,226}, micro-RNA\textsuperscript{77,227}, and imaging biomarkers\textsuperscript{98,101,103,228-232}, but
none are commonly used as primary outcomes in current clinical trials. In this study, we sought to determine if urine metabolites have promise to serve as prognostic and predictive biomarkers for DMD. We hypothesized that metabolites indicative of muscle breakdown would be elevated in the urine from boys with DMD. We investigated urine metabolites involved in oxidative metabolism—largely the TCA cycle intermediates—for possible association with disease progression and response to steroid treatment. Finally, to estimate the robustness of the metabolomics results, we compared urine metabolite levels to markers that have been shown to be associated with DMD disease progression: magnetic resonance imaging (muscle T₂ and fat fraction;¹⁰⁴,²²⁸) and a standard serum (MMP-9) biomarker⁷⁶.

**Materials and Methods**

**Study Design**

Subjects were recruited at the University of Florida (UF) and Oregon Health & Science University (OHSU) to participate in an optional ancillary study of ImagingDMD natural history study (imagingdmd.org, ClinicalTrials.gov Identifier: NCT01484678). The study was approved by the Institutional Review Boards from each participating institution. As this is a pediatric population, parents and/or guardians of all subjects provided informed consent, and subjects provided written assent before any part of the study began.

Inclusion criteria at baseline for boys with DMD include: 1) between the ages 5 to 12.9 years; 2) confirmation of DMD by genetic confirmation, a lack of dystrophin expression on muscle biopsy, or elevated serum creatine kinase levels before the age of 5; and 3) able to walk at least 100 m and climb 4 stairs. Exclusion criteria for
recruitment of boys with DMD include: 1) boys with any known medical problems not relating to DMD, 2) behavioral or cognitive issues prevented valid data collection, and 3) an additional medical condition which could affect muscle function or functional performance. Inclusion criteria for control subjects include: 1) age between 5 and 12.9 years old, 2) normal developmental milestones, and 3) no injury to lower limb requiring immobilization within the last year. Control subjects were excluded if they participated in sport specific training for more than 8 hours per week. More information of the study and design can be found at clincialtrials.gov (Study Identifier: NCT01484678).

Urine and serum samples were acquired during an annual visit to the regularly scheduled ImagingDMD appointments. Urine samples were collected in the morning before the boys went into the MRI for scanning. Samples were put on ice, barcoded, aliquoted, and stored at -80° C until needed. Serum samples were collected later in the day after the MRI scans and functional testing. Blood samples were taken using standard phlebotomy practices and procedures. Up to 20 ml of blood was taken in up to three red top tubes. Tubes were placed upright at room temperature until clotted (usually 30-45 minutes), and samples were centrifuged at 2000g for up to 10 minutes at 4 degrees. Serum was separated from red blood cells, barcoded, and aliquoted. All samples, urine and serum, were stored at -80 °C in the University of Florida CTSI Biorepository, where they remained under constant conditions until analysis. Inflammatory proteins and other clinical markers of muscle damage (serum creatine kinase levels) were measured in the serum using multiplexed and standard ELISA methods. Metabolite levels in the urine were
determined by the both targeted mass spectrometry and $^1$H NMR at SECIM (secim.ufl.edu). All the samples were analyzed without a freeze thaw except for a repeated measurement of the serum samples for additional inflammatory markers.

**NMR Study Design**

Urine samples from DMD and control boys were integrated with QC pooled urine standards and buffer blanks to reassure QC during the samples preparation and data acquisition. Samples and QC standards were randomly prepared and assigned a run order with QC blanks (buffer only) as the first, last, and middle samples. QC pooled urine standards were placed after the first blank and before the last blank of each run. The rest of the QC samples are randomized along with the DMD and control boy samples using an excel randomizer. Quality of the data was then verified using a QC script (unpublished), which uses the blanks and QC samples to verify no significant changes in shim, temperature or the metabolites occurred during the NMR run.

Urine samples (DMD on steroids n=30 (mean age: 10.9 +/- 3 years); DMD steroid naive n=8 (mean age: 7.4 +/- 1.1 years); healthy controls n=7 (mean age: 10.4 +/- 2.8 years)) were removed from the biorepository, allowed to thaw at 4°C, and centrifuged at 12,000g for 5 minutes at 4°C. 400 μl of urine was added to a 1mL eppendorf tube for each sample and mixed with 200 μl of 100 mM phosphate buffer including and 0.1M Sodium 2,2-Dimethyl-2-Silapentane-5-Sulfonate (DSS-D6) (Cambridge Isotope Laboratories, Inc) to serve as a reference standard. Eppendorf tube were vortexed for 2 minutes and 590 μl of the supernatant were transferred into 5 mm NMR tube (Bruker 5mm tubes for SampleJet). Data were acquired on a 600
MHz Bruker using Avance II NMR, with Topspin 3.2 and automated using ICON
NMR 4.7.5 (Build 1, for Topspin, ©Bruker Biospin 2012). Spectra were acquired
using a 1D NOESY sequence with water pre-saturation (noesypr1d) pulse sequence
with 64K data points, 20 ppm spectral width, and 100 millisecond NOESY mixing
time with a total acquisition time of 2.72 seconds and 4 second relaxation delay, for
128 scans with 16 dummy scans. The 90-degree pulse was calculated for each
sample using a series of 1D with variable excitation pulses (pulsecal) implemented
into the ICON NMR automated scheme. Temperature was regulated to maintain the
sample at 300 K. Sample preparation protocols were based on Beckonert et al. 122.

Raw NMR data were initially processed using NMRpipe166. Frequency domain
data were imported into MATLAB for further processing using an in-house
Metabolomics Toolbox 141,203. Water was removed between 4.7 - 5.08 ppm, and the
ends of the spectra were removed, leaving the spectral region from -0.2 ppm to 10
ppm. A baseline correction was fitted to the spectra before spectral alignment. A
guided alignment script, guide_align1D, was used a ‘spearman’ cluster method and
‘CCOW’ alignment method167. The spectra were normalized using probabilistic
quotient normalization (PQN)168, and peak picked using an in house algorithm that
detects peaks in 1D NMR spectrum and constructs two matrices; peak intensities
and chemical shifts of each peak maximum. Only metabolites with no “visually”
apparent overlap from other peaks were used. Quantification of metabolites was
performed from integration under the curve. Metabolites were identified and verified
by overlay spectra from the BMRB database146.
Targeted Mass Spectrometry of Organic Acids

**Sample Preparation.** Frozen urine was thawed and centrifuged briefly to pellet debris. Samples (DMD on steroids n=26 (mean age: 10.3 +/- 2.9 years); DMD steroid naive n=9 (mean age: 7.8 +/- 2 years); healthy controls n=11 (mean age: 9.0 +/- 1.8 years)) were then aliquoted and stored at -80°C.

**Extraction and Derivatization of Organic Acids from Urine.** A 50-μL aliquot of urine was spiked with a 10-μL mixture of heavy isotope-labeled organic acid internal standards (lactate, pyruvate, 3-hydroxybutyrate, succinate, fumarate, malate, α-ketoglutarate, and citrate; Sigma-Aldrich, St Louis, MO; Cambridge Isotopes, Cambridge, MA; CDN Isotopes, Quebec, Canada). This was followed by the addition of 50 μL of 0.4 M O-benzylhydroxylamine and 10 μL of 2 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. Samples were vortexed thoroughly and derivatized at room temperature for 10 min. The derivatized organic acids were then extracted from the urine by liquid-liquid extraction using 100 μL of water and 600 μL of ethyl acetate. Samples were vortexed for 5 seconds and then centrifuged at 18,000 x g for 5 min at 10°C. A 100-μL aliquot of the ethyl acetate layer was dried under nitrogen and reconstituted in 1 mL of 50/50 methanol/water prior to LC/MS/MS analysis.

**LC/MS/MS Quantitation of Organic Acids in Urine.**

Derivatized organic acids were separated on a 2.1 x 100 mm, 1.7 μm Waters Acquity UPLC BEH C18 column (T = 45°C) using a 7.5-min linear gradient with 0.1% formic acid in water and 0.1% formic acid in acetonitrile at a flow rate of 0.3 mL/min. Quantitation of derivatized organic acids was achieved using multiple reaction
monitoring on an Dionex UltiMate 3000 HPLC/Thermo Scientific Quantiva triple quadrupole mass spectrometer (Thermo Scientific, San Jose, CA).

A standard calibration curve (1-5000 μM for lactate; 0.2-1000 μM for 3-hydroxybutyrate; 0.05-250 μM for pyruvate, succinate, fumarate, malate, and citrate; 0.02-100 μM for α-ketoglutarate) for derivatized organic acids was prepared by spiking 10-μL aliquots of organic acids (Sigma-Aldrich, St. Louis, MO) and internal standards (Sigma-Aldrich, St Louis, MO; Cambridge Isotopes, Cambridge, MA; CDN Isotopes, Quebec, Canada) into 50-μL aliquots of water. Calibration samples were derivatized and extracted similarly to organic acids in urine. Data for urine were normalized to creatinine levels as determined by a MicroVue Creatinine Assay (Quidel; San Diego, CA).

**Targeted Mass Spectrometry of Amino acids**

**Sample Preparation**

Frozen urine was thawed and centrifuged briefly to pellet debris. Samples (DMD on steroids n=26; DMD steroid naive n=9; healthy controls n=11) were then aliquoted and stored at -80°C.

**Extraction and Derivatization of Amino Acids from Urine.**

A 100-μL aliquot of urine was spiked with a 10-μL mixture of heavy isotope-labeled amino acid internal standards (Sigma-Aldrich, St Louis, MO; Cambridge Isotopes, Cambridge, MA; CDN Isotopes, Quebec, Canada). This was followed by the addition of 800 μL of ice-cold methanol. Samples were vortexed thoroughly and then centrifuged at 18,000 x g for 5 min at 10°C. A 100-μL aliquot of the methanolic extract was dried under nitrogen and reconstituted in 80 μL of Borate Buffer and 20 μL of MassTrak AAA Reagent (both provided in MassTrak AAA Derivatization Kit;
Waters Corp., Milford, MA). The samples were then derivatized at 55°C for 10 min prior to LC/MS/MS analysis.

**LC/MS/MS Quantitation of Amino Acids in Urine.**

Derivatized amino acids were separated on a 2.1 x 100 mm, 1.7 μm Waters AccQ·Tag column (T = 55°C) using a 9.55-min linear gradient with eluents proprietary to Waters Corp. at a flow rate of 0.7 mL/min. Quantitation of derivatized amino acids was achieved using multiple reaction monitoring on an Agilent 1290/6490 HPLC/triple quadrupole mass spectrometer (Waters Corp., Milford, MA).

A standard calibration curve (1-1000 μM for Gly, Ala, Pro, Val, Arg, Thr, Lys and Gln; 0.5-500 μM for Ser, Leu, Ile, Met, His, Phe, Tyr, Asn, Asp, Gly, Orn and Cit; 0.25-250 μM for Trp) for derivatized amino acids was prepared by spiking 10-μL aliquots of amino acids (Sigma-Aldrich, St. Louis, MO) and internal standards (Sigma-Aldrich, St Louis, MO; Cambridge Isotopes, Cambridge, MA; CDN Isotopes, Quebec, Canada) into 100-μL aliquots of water. Calibration samples were derivatized and extracted similarly to amino acids in urine. Data for urine were normalized to creatinine levels as determined by a MicroVue Creatinine Assay (Quidel; San Diego, CA).

**Serum Protein Analysis**

Serum samples were used to analyze the circulating MMP-9 (DMD on steroids n=40; DMD steroid naive n=6; healthy controls n=6) and mCK (DMD n=30; healthy controls n=4) levels using Meso Scale Discovery (MSD, Rockville, MD). MSD uses electrochemiluminescence technology to detect and quantify proteins. Each sample uses 10 μL of serum and 150 μL of MSD blocker A. Each well is then washed with PBS, diluted and left for 2 hours. After another PBS wash, 25 μL of
detection antibody is added to each well and also incubated for 2 hours. There is one more final wash, the Read buffer is added and the wells are then imaged.

**MRI data collection and analysis**

MRI data were collected on a 3 Tesla Philips Achieva Quasar (Phillips Medical Systems) scanner using a SENSE 8 channel volume knee coil on the lower leg and a 16-coil channel for the right upper leg. Total scan time was approximately 1.5 hours. To improve compliance during MRI scans, boys were provided with video entertainment and were accompanied in the MRI room by both a researcher and a parent for the scan duration. Data were acquired and analyzed according to the methods described by\textsuperscript{100}. Fat fraction was calculated from the MR spectroscopy. Fat fraction was calculated by integrating the MRS fat and water peaks, and dividing the integrated fat value by the sum of the integrated values of fat and water. Water and fat signals were corrected for relaxation time effects.

**Functional tests**

For the 6-minute walk test, subjects are asked to walk as far as they can for six minutes. Subjects can take standing breaks, but were encouraged to continue as soon as possible. As a safety precaution, a researcher trailed subjects. The researcher was only allowed to assist boys if they fell. The outcome measurement of the 6-minute walk test is the total distance walked (6MWD). For the 10-m W/R, subjects were asked to go ten meters as fast as they could by either walking or running. The outcome measurement was the amount of time it took them to go ten meters (in seconds). The fastest time from three trials for the 10-m W/R was used for analyses.
Statistics

All data were normalized to creatinine. NMR data were normalized by the integrals of the assigned resonances. MS data were normalized based on the UV absorbance assay of creatinine for each sample. Therefore, all metabolite scales are unitless. Data were tested for normality using the Shapiro-Wilk Test on Matlab. Targeted comparisons were analyzed using Graphpad Prism version 6. Comparisons between DMD and controls that were determined normal through the Shapiro Wilk normality test were compared using an unpaired t-test with a Bonferroni multiple comparisons correction. Comparisons between DMD and controls that did not pass the Shapiro Wilk normality test were compared using a Kruscal Wallis test. Comparisons between controls, DMD-GC and DMD+GC groups were analyzed using a one-way ANOVA and a Bonferonni correction. Creatine and citrate associated to other outcomes using Pearson correlation on Prism. Metabolite and MR parameters were correlated using a linear correlation matrix, corcoef, on Matlab, which calculated both the correlation coefficients and p-values. MetaboNetworks scripts\textsuperscript{233} were used to create the network maps but correlations were used from the correlation matrix. For differentiation of on vs off GC, ROC curves were created by plotting Sensitivity\% vs. 100-Specificity\%. The area under the curve was calculated with a p-value set at 0.05 using Graphpad Prism 6.

Results

Muscle-associated metabolites increase in DMD urine

\textsuperscript{1}H nuclear magnetic resonance (NMR) and mass spectrometry (MS) were used to evaluate metabolic differences in the urine of boys with and without DMD. Because of the limited number of controls, conventional untargeted multivariate
analyses were not possible. Therefore, we performed a targeted metabolomics approach examining specific resonances from known metabolites for significant changes and quantitative targeted mass spectrometry. The largest differences in the NMR spectra were in the muscle-associated metabolites, creatine and creatinine in the urine (Figure 5-1A). Here, we follow the common practice of normalizing metabolite signal levels to those of creatinine. We found that urine creatine levels in the DMD population was significantly higher than that from controls (p<0.001; Figure 1B).

Increased urine creatine levels have previously been associated with muscle changes in DMD, but it is unclear if high urine creatine levels are due to increased muscle leakage or impaired muscle uptake of creatine. 100-fold increase of the muscle isoform of creatine kinase (mCK) in serum is a diagnostic test for DMD. However the specificity of this test is questionable because the increased mCK levels may merely indicate general muscle damage not specific to DMD and does not correlate well with other measures of DMD disease progression. If the increased urine creatine (Figure 5-1B) were due entirely to muscle damage, one would also expect to find a significant correlation between urine creatine levels and serum mCK (Figure 5-1C). As seen in Figure 5-1D, there is no significant correlation (r=-0.02, p=0.9) between the two, suggesting other mechanisms. However, similar to serum mCK levels in DMD, urine creatine levels were not different between DMD-GC (DMD glucocorticosteroid naïve) and DMD+GC (DMD on glucocorticosteroid treatment) subjects.
Muscle associated metabolites 1- and 3- methylhistidines are associated with actin and myosin turnover\textsuperscript{238}, increase in response to muscle breakdown\textsuperscript{239} and neuromuscular diseases\textsuperscript{92,240}. Consistent with previous studies, 1- and 3- methylhistidines were found to be significantly elevated in DMD urine (p=0.0012 and 0.0009 –GC and +GC respectively), and our results show there is no dependence on GC status (Figure 5-1E and 5-1F), similar to urine creatine levels.

**Energy metabolites in DMD urine**

Mitochondrial damage and metabolic deficits within muscle has been previously identified in DMD\textsuperscript{199,200,241-244}. Further, Morrison-Nozick et al. \textsuperscript{65} showed that proteins involved in amino acid and fatty acid metabolic pathways are regulated by GCs. Therefore, TCA cycle metabolites were measured to investigate metabolism and the effect of GC treatment on metabolism in DMD (Figure 5-2).

Succinate, malate, fumarate, and glutamate were not significantly different between control, DMD-GC, or DMD+GC (Figure 5-2). Compared to controls lactate was significantly elevated in DMD-GC (p=0.043) and DMD+GC (p=0.015), but not significantly different (p=0.99) between DMD-GC and DMD+GC (Figure 5-2). Glutamine was significantly elevated in control vs. DMD-GC (p=0.015) but was not significantly different between the control vs. DMD+GC or DMD-GC vs. DMD+GC (Figure 5-2). In contrast, citrate and alpha-ketoglutarate were both significantly elevated in DMD-GC relative to both control (p=0.0065 and p=0.0015, respectively) and DMD+GC (p=0.0006 and p=0.029, respectively). In summary, lactate was elevated in control vs. both DMD-GC and DMD+GC, glutamine was elevated in DMD-GC vs. control, and citrate and alpha-ketoglutarate were elevated in DMD-GC.
vs. both control and DMD+GC, suggesting that urine metabolites from this part of the TCA cycle are sensitive indicators of response to GC treatment in DMD.

**Relationship of urine creatine and citrate to muscle and serum biomarkers**

MRI $T_2$, is a relaxation time constant associated with MRI signals from water and lipid protons, which can be quantified and mapped with high spatial resolution and three dimensional coverage. Elevated MRI $T_2$ values have been shown to correspond with DMD disease progression and is one of the most promising biomarkers for DMD in recent years$^{98,101,230,232,245}$ and has become the primary outcome measure in at least two clinical studies. Specifically, changes in MRI $T_2$ of the vastus lateralis (VL) has been shown to be associated with disease progression and GC treatment. Although modest, we found that there was a significant correlation ($r=0.42; p=0.008$) between urine creatine and VL MRI $T_2$ (Figure 5-3), indicating that urine creatine levels associate with disease progression in DMD.

Serum levels of matrix metalloproteinase-9 (MMP-9) has been one of the most promising biofluid surrogate biomarkers in DMD, reflecting both ambulation status and GC treatment$^{76}$. Similar to previous studies, we found that serum levels of MMP-9 were significantly ($p=0.028$) increased in DMD compared to control subjects. However, in our study, urine citrate levels rather than serum MMP-9 levels, were more strongly associated with steroid status within the DMD population. Based on a ROC analysis, citrate levels differentiated DMD-GC from DMD+GC with an AUC of 0.80 ($p=0.0014$) (Fig 5-4A), whereas MMP-9 had an AUC of 0.69 ($p=0.15$) for the same group comparison (Fig 5-4B).
Associations between urine metabolites and functional tests

Surrogate biomarkers should correlate with clinically meaningful outcomes. Indices of ambulation, such as the distance a subject walks in six min (6MWD) and the time that it takes a subject to walk or run 10 meters (10-m W/R), are some of the most commonly utilized primary outcome measures in DMD 47. We found that urine creatine levels were negatively correlated with the 6MWD (r=-0.44 and p=0.005) and positively correlated with the 10-m W/R (r=0.47 and p=0.002) (Figure 5-5A and D). In addition, a urine creatine/creatinine ratio of 1 emerged as a threshold for functional loss, as none of boys below this level lost ambulation. Citrate levels did not significantly correlate with function, likely due to dependence on GC treatment. However, there was still a trend for DMD-GC to have higher citrate and worse function than the DMD+GC (Figure 5-5B and C). These results suggest that creatine and perhaps other urine metabolites relate to clinically meaningful outcomes in DMD.

Global associations of urine metabolites and MR parameters

Global associations of targeted urine metabolites and MR measurements were organized into a correlation matrix for each group (Control, DMD+GC, DMD-GC) (Figure 5-6). The bottom-left portion of each matrix displays the linear correlation coefficients, represented by the rainbow color bar below. The top-right portion of each matrix displays the corresponding p-value correlation, colored according to the color bar to the right. The white squares indicate p-values greater than 0.05.

The correlation matrix for the DMD-GC (right) population was the altered from controls as represented by 1) high positive correlations (r>0.6) between amino acids
(besides arginine and aspartate), 2) high positive correlations \((r > 0.6)\) between lactate, pyruvate and 3-hydroxybutyrate to amino acids, 3) high positive correlations \((r > 0.6)\) between the multiple MR measurements, 4) positive correlations \((r > 0.4)\) between aspartate, ornithine, phenylalanine, proline, to most MRI \(T_2\) measurements (excluding the gracilis and tibialis anterior muscles), and 5) negative correlations \((r < -0.4)\) between arginine and MR parameters. The correlation matrix of the DMD+GC (middle) contained more negative correlation findings between TCA intermediates and amino acids compared to DMD-GC group but overall the DMD+GC metabolite correlations were similar to the controls. These data suggest that the urine metabolic profile in DMD is significantly altered, and GCs use in DMD altered these correlations.

**Discussion**

The goal of this study was to determine whether the urine metabolome could differentiate unaffected boys from boys with DMD on and off GCs. As a consequence of losing functional dystrophin protein, membrane instability causes a myriad of cell signaling cascades, resulting in both structural and metabolic pathway perturbations. We hypothesized that these altered pathways would be reflected in the differences in urine metabolite levels. For this reason, we focused our targeted analysis on metabolites involved in creatine metabolism, muscle protein turnover, and the TCA cycle. We found the metabolic profiles were significantly different in the DMD cohorts compared to unaffected boys. Additionally, we were able to identify specific urine metabolites that might be useful indicators of disease and therapeutic intervention.
We hypothesized that metabolites indicative of muscle breakdown would be elevated in the urine of boys with DMD. Others have reported an increase in creatine and methyl histidine in urine of boys affected with DMD (Figure 5-1B), but this has commonly been assumed to result from leaky muscle cells due to sarcolemmal disruption and muscle turnover. This assertion that muscle metabolites should be higher in the urine through excretion/leaky muscles is supported by studies of the DMD mdx mouse model, human muscle biopsies, and in vivo MR spectroscopy all of which have shown decreased creatine levels in muscle tissues. Conversely, other studies using 14-carbon labeled glycine and creatine have shown that newly synthesized creatine was not taken up as rapidly in DMD compared to control muscle. Benedict et al. were able to show that the creatine half-life in the boys with muscular dystrophy was half as long as controls suggesting higher excretion. These results suggest that there is a limitation in the uptake of creatine into dystrophic muscle. Also, studies in the mdx mouse have shown that dystrophic mouse muscle has a compensatory mechanism by which de novo muscle creatine synthesis is upregulated by the overexpression of guanidinoacetate methyltransferase and arginine:glycine amidinotransferase in the muscle. At least two scenarios are consistent with previous data: 1) creatine is leaking out of the muscle before turning into creatinine or 2) creatine is inhibited from entering the muscle and is rapidly excreted through the urine. In this study, we found that creatine in urine does not correlate with serum mCK levels (Figure 5-1D), which is also thought to leak from the muscles. We propose that from the previous studies and our data, it is potentially the combination of these two
scenarios working collectively in attempt to maintain muscle creatine homeostasis. Our data are supported by a Boca et al. that show the creatine:creatinine ratio increasing in the serum of boys with DMD as they age, and there was no correlation between mCK and the creatine:creatinine ratio in DMD participants over the same age range as those in the current study \(^{89}\). In combination, the serum and the urine findings indicate that creatine:creatinine has the potential to become a biomarker for disease progression in DMD. Urine sampling has the additional advantage of a higher subject compliance, for instance in this study, boys with DMD are 2.3 times more likely to volunteer to give urine compared to a blood draw.

Urine creatine and the methlyhistidine isomers, were strong indicators of disease in DMD but appeared insensitive to GC treatment (Figure 5-1). It has been shown that in healthy volunteers treated with GC, 3-methylhistdines are found to increase \(^{246}\) in the urine. Interestingly, a previous study by Kawai et al, show the opposite effect in DMD with a 51-63% decrease in 3-methylhistidine in DMD patients on GCs\(^{247}\), while a study by Tarnopolsky et al, showed no difference in 3-methylhistidines between GC status\(^{201}\). The interpretation of GC use in our student is complicated due to the lack of clear guidelines for GC use in DMD related to dosing regime, preferred GC, and the length of treatment. A study design specific for this purpose is needed to further investigate the influence of these GC parameters and is the topic of current clinical trial (NCT01603407). To the best of our knowledge, no previous studies have related urine creatine levels to GCs treatment in DMD. In agreement with the explanation by Burch et al., who hypothesized that circulating levels of muscle proteins may not be sensitive to the changes occurring
from steroids\textsuperscript{225}, our study indicates that this also holds true for urine creatine levels. Our data, along with previous published results\textsuperscript{201,225}, suggest that creatine and methylhistidine isomers are products of muscle protein catabolism occurring in DMD, but they are likely not involved in the physiologic axis affected by steroid treatment.

Citrate and alpha-ketoglutarate were uniquely sensitive to GC treatment in boys with DMD. In contrast to the creatine and methylhistidine isomers, TCA cycle intermediates were altered in DMD and at control levels in DMD on GC treatment. Citrate and alpha-ketoglutarate increased in the DMD-GC (steroid naïve) group, but were similar to control levels in the DMD+GC treated group (Figure 5-2). Citrate is in a unique position in the TCA cycle minimally involved in anaplerosis, which can contribute to both the TCA cycle and to lipid synthesis\textsuperscript{248-250}. It has been shown, in healthy volunteers using GCs for 15 days, that urine citrate and isocitrate are decreased, but lipid metabolism metabolites are not affected. One plausible explanation is that citrate levels were increased in our DMD-GC cohort, in response to up regulation in lipid membrane repair pathways, as one of its functions is to assist in lipid synthesis.

To further investigate the role of citrate in DMD, we explored metabolite ratios within the TCA cycle. One process within the TCA cycle is the enzyme aconitase, converting citrate into alpha-ketoglutarate. Metabolite ratios revealed that the DMD-GC population has a greater citrate:alpha-ketoglutarate ratio than in controls, indicating that less citrate is converting to alpha-ketoglutarate. Similarly, we found less citrate remained in the TCA cycle as compared to pyruvate in the DMD+GC group. These data suggest that changes in urine metabolites do not simply reflect a
leaky muscle membrane, but instead dystrophic muscle may push the metabolites into alternative metabolic pathways. Metabolic flux studies in the *mdx* mouse coincide with these findings, where it was found that aconitase activity is lower in *mdx* than control\textsuperscript{153}. Also, relative to citrate synthesis, anaplerosis and beta-oxidation were reduced in *mdx* while production of acetyl-CoA, a necessary precursor for lipid synthesis\textsuperscript{250}, was significantly higher in *mdx* as compared to control\textsuperscript{153}. Interestingly, subjects that were on GCs demonstrated reduced citrate and alpha-ketoglutarate levels, suggesting that 1) GC treatment reduces the need for citrate and alpha-ketoglutarate to contribute to lipid synthesis and/or 2) GC treatment reduces metabolic demand and decreases utilization of lipid substrates\textsuperscript{65}. Collectively, these data propose that the lack of dystrophin can cause both metabolic and structural deficits, which can be modified through the use of therapeutic interventions and detected using quantitative analysis of the urine of boys with DMD.

Noninvasive biomarkers have shown great promise to measure disease progression in DMD\textsuperscript{98,230} and sensitivity to GC treatment\textsuperscript{95,103}. Two of the most robust of the MR biomarkers for tracking DMD disease progression, are vastus lateralis MRI T\textsubscript{2} (VL T\textsubscript{2}) and MRS fat fraction (VL FF)\textsuperscript{104,228}. As fat is a large contributor to increased T\textsubscript{2} values in boys with DMD these values are often commutable. Creatine was significantly correlated similarly with both VL T\textsubscript{2} (r = 0.42, Figure 5-3) and VL FF, suggesting that urine creatine increases with disease progression. Importantly, we discovered that there was not a boy in our study with a creatine/creatinine ratio less than 1 that had lost ambulation, which may be a useful prognostic gauge of disease progression.
Furthermore, serum MMP-9 levels have been reported as a sensitive biomarker for DMD. Using MMP-9 as a guidepost, we compared the urine citrate as potential biomarker for DMD and intervention to that of serum MMP-9. Serum MMP-9 levels demonstrated competency to differentiate DMD subjects vs. controls, but citrate (AUC=0.80, p=0.0014) resolved steroid status better than MMP-9 (AUC = 0.69 p=0.15). As GC steroids are the current gold standard of treatment for DMD, this is an important proof-of-concept finding that urine metabolites may be sensitive to assess therapeutic efficacy. In addition there are other promising serum and urine markers for DMD in the literature including, proteins revealed by the SOMAscan proteomics study, urine titin fragments and GDF8. Further studies are needed to investigate and compare each surrogate marker to citrate and other urine metabolites in differentiating steroid status in DMD and possibly other therapeutic interventions.

Surrogate biomarkers need to be compared with clinical meaningful outcomes used in trials, such as the ability to walk with DMD. Creatine, but not citrate, was correlated with both the 6MWD and the 10-m W/R, however, DMD subjects not taking steroids did generally show a trend for worse function scores at higher citrate levels (Figure 5-5). By determining association to functional tests, we have found that urine metabolites also have associations to clinical meaningful measures such as muscle function. In all, these data suggest that creatine levels reflect disease progression (prognostic) and citrate levels reflect treatment by GCs (predictive).

Correlation matrices for control, DMD+GC, and DMD-GC subjects were created to visualize global associations of urine metabolites and MR parameters.
These correlation matrices not only revealed differences between the metabolic profiles of DMD subjects to controls, but also importantly between DMD+GC and DMD-GC subjects. The largest differences between groups were observed correlations between amino acids and TCA cycle intermediates in the DMD-GC group as compared to controls. Due to the amounts of proteolysis and damage in muscle, this was expected. Unexpectedly, arginine and aspartate did not follow the same trends, showing insignificant correlation coefficients when compared to amino acids. However, when compared to MR parameters (vastus lateralis FF and T2), arginine showed strong negative correlations, opposite of aspartate, which showed positive correlations. When we compare the DMD-GC group to the DMD+GC group, correlations to MR parameters are reduced, with aspartate showing strong positive correlations to amino acids. The GC interaction in the DMD data suggest that metabolic pathways, including arginine and aspartate, may be associated with the physiologic axis by which GCs are working. Transcriptomic analysis by Morrison-Nozik et al. revealed regulation of proteins in KEGG pathways involving arginine, lysine, and other amino acids shown by our data to be affected by the KLF15–GC axis, which is related to steroid treatment\textsuperscript{65}. In the DMD population, this KLF15-GC axis ameliorated the disease severity, potentially by working through these biochemical pathways.

We found that metabolomics networks are altered in DMD subjects that are on and off GC treatment. As an example of the changing correlations via intervention of GC, we used MetaboNetwork\textsuperscript{233}, to show the network of metabolites correlated to disease progression for both the DMD-GC and DMD+GC separately.
For both steroid statuses, metabolites were correlated with VL FF, an MR biomarker for DMD disease progression. In each network, analyzed metabolites were colored corresponding with the correlation with VL FF (Figure 5-6, bottom color bar). We observed a clear difference in metabolite correlations with VL FF depending on steroid status. The metabolite network of the DMD+GC group shows strong positive correlations with malate, fumarate, ornithine and proline, but negative correlations with arginine and glycine. Conversely, the metabolic network of the DMD-GC group contains mostly modest positive correlations with VL FF, but lysine, demonstrates a large negative correlation. Interestingly, many of the metabolites changing between GC statuses are also within the KEGG pathways affected by the KLF12-GC axis. Further studies are needed to potentially determine exact mechanisms by which these metabolites are regulated with and without GC intervention in the DMD population over time.

**Conclusion**

In conclusion, we found metabolites in the urine that both increase to disease progression and the use of GC treatment in DMD. We found that urine creatine levels were increased in DMD and associated with disease progression. Urine creatine was also correlated with VL MRI T₂ and clinically meaningful outcome measurements, suggesting that urine metabolome may be a simple and a non-invasive tool to measure DMD disease progression. Citrate levels in DMD boys were elevated not using GC, but returned to control levels in boys under GC treatment. When compared to other serum biomarkers for DMD, urine citrate was more sensitive to the detection of GC therapy than serum MMP-9 levels in this DMD population. The correlation matrix of metabolites and MRI measurements revealed
insight into potential mechanisms to perform further investigations. Overall, these data suggest that a simple measurement of urine metabolites can differentiate DMD from healthy, unaffected controls and may be useful in future clinical trials as a noninvasive biomarker to determine DMD progression and potential therapeutic efficacy.
Figures

Figure 5-1. Urine creatine and 1- and 3-methylhistidine levels in DMD and in control. (A) 1D $^1$H NMR urine spectra from all subjects showing creatine and creatinine region (3.90-4.06 ppm). Creatine was quantified in by (B) normalizing to creatinine levels (DMD = 38, Control = 7). (C) Serum CK was increased in DMD (n = 30) relative to controls (n = 4). (D) Urine creatine values from (B) were plotted against serum CK levels from (C), demonstrating no correlation (D). Muscle associated metabolites 1-methylhistidine (E) and 3-methylhistidine (F), both normalized to creatinine, significantly increase in the DMD relative to controls (n = 11), but with no significance between steroid groups, DMD-GC (n = 9) and DMD+GC (n = 26). Panels B and C used unpaired t-test with Bonferroni correction while panel D was compared using a Pearson correlation. Black dotted lines represent the 95% confidence intervals in panel D. Panels E and F used a one-way ANOVA with a Bonferroni correction. Significance values of (***) refer to p <0.0001 and (**) to p< 0.01.
Figure 5-2. TCA cycle metabolite levels in DMD and control urine. Quantification of each TCA cycle metabolite was determined by targeted MS and normalized to creatinine. Citrate and alpha-ketoglutarate were found to be increased in DMD-GC in the urine, but not in the DMD+GC group. Lactate was increased in all DMD groups significantly as compared to controls. Glutamine was only significant in DMD-SN group as compared to controls. All other comparisons were found to be not significant (NS). All panels contain Controls (n = 11), DMD-GC (n = 9) and DMD+GC (n = 26) compared using a one-way ANOVA with a Bonferroni correction. Significance values of p <0.001 (**), p < 0.01 (**), and p <0.05 (*) are indicated.
Figure 5-3. Relationship between urine creatine levels to the vastus lateralis MRI $T_2$. Urine normalized creatine values are plotted against vastus lateralis MRI $T_2$ relaxation times. The association of DMD subjects ($n = 38$) was compared using a Pearson correlation and was found to have a correlation coefficient ($r$-value) of 0.42 and $p$-value of 0.008. Control subjects ($n = 7$) showed no significant correlations.
Figure 5-4. ROC curves comparing citrate and serum protein MMP-9 levels in DMD-GC and DMD+GC. Receiver operator characteristic (ROC) curves were used to differentiate DMD+GC from DMD-GC. (A) Citrate had a larger AUC with 0.80 (p=0.0014) as compared to (B) MMP-9 (p=0.15) with an AUC of 0.69. Panel A used ROC curves to compare DMD-GC (n = 9) to DMD+GC (n = 26) citrate levels where panel B used ROC curves to compared DMD-GC (n = 6) to DMD+GC (n = 40) MMP-9 levels.
Figure 5-5. Relationship between urine creatine and citrate to functional measures in DMD. Normalized urine creatine values show significant correlations with the 6 minute walk test (A) and the 10 meter walk/run (C). Normalized citrate does not show significant correlations to either the 6MWT (B) or the 10-meter walk/run (D). Creatine values from subjects with DMD (n = 38) were compared to functional tests using a Pearson correlation. Citrate levels from DMD-SN (n = 9) and DMD-GC (n= 26) were also compared using Pearson correlation.
Figure 5-6. Correlation matrix of metabolites and MR parameters from control, DMD-GC and DMD+GC subjects. Global matrix correlations were made for Controls (left, n = 11), DMD+GC (middle, n = 26), and DMD-GC (right, n = 9). Pearson correlations of metabolites and MR parameters can be found on the left/lower portion of the matrix, where the right/upper portion represents p-values associated to the corresponding correlations. Each correlation is colored according to the Spearman correlation coefficient (r) values scale below the matrices and each p-value less than or equal to 0.05 is colored according to the barscale to the right of the. P-values greater than 0.05 are represented as white squares. The following are abbreviations of the muscles analyzed by MR: vastus lateralis (VL); biceps femoris long head (BFLH); gracilis (GRA); medial gastrocnemius (MG); peroneal (PER); soleus (SOL); tibialis anterior (TA); tibialis posterior (TP).
CHAPTER 6
CONCLUSIONS AND FUTURE DIRECTIONS

In this dissertation I studied the metabolic profile of both dystrophic mice and humans. In that process I developed workflows to automate QC and demonstrated the utility of the addition of $^{13}$C spectra for better metabolite annotation and multivariate analysis of complex mixtures. When applied to the DMD mouse model, $mdx$, we found lipid saturation changes in the serum of $mdx$ mice. These lipid alterations were found to correlate not only to alterations in the tissue but also with MRI $T_2$, an objective marker. Further, I found metabolic alterations in boys with DMD could be measured in urine associating to MRI and clinically meaningful outcomes. These are promising findings but only scratch the surface of the complexity metabolomics brings to the field of DMD.

**Methods for furthering the metabolomics field**

As metabolomics is in its infancy, we are in a unique position to help shape the future of the field. Metabolomics has not reached acceptance as other –omics have. The adoption of genomics, transcriptomics and proteomics have taken time and years of research to learn the best ways to analyze and interpret the data; still, people pioneer forward for new and exciting techniques and methods. Metabolomics has progressed quickly. Instrumentation technology is enhancing but we still do not appropriately handle the data coming from current technology. In the field, there is still ambiguity and inconsistencies in approaches to 1) handle and prepare samples, 2) acquire data 3) standardize and QC data and 4) store data. Fortunately, each of these areas of interest are being addressed, but there is not a universal form to date. At the onset of this project, I
used current standards of QC which fell short of the rigor necessary to appropriately understand the data.

I had used manual, subjective tests throughout an experiment appropriate for NMR experiments meant to check structure or purity. However, these methods discount sensitivities the metabolomics approach was able to detect. I used the entire spectra, and compared the variation of all spectra to help understand covariance of all metabolites. The workflow I created not only automates the manual tests but also determines if metabolites change over time and evaluates instrumentation and biological fluctuation. Although a small contribution, I think by suggesting one approach to QC, that involves more than a PCA plot, it can start a conversation and build on these ideas to improve the quality of future data. There are many ways variation can be introduced into an experiment and it is important to the interpretation of data that we know from where it generates. I hope QC workflow/algorithms become incorporated at the NMR workstation. Recently, we worked to begin implementation of our workflow at a NMR work station at the University of Georgia as a first step to QC in real time. Of course there are still additions we would like to make to the current workflow. Primarily, we would like to create a way to determine an acceptable baseline in an automated algorithm. This is still one characteristic of the NMR spectrum that remains a subjective assessment. Also, it would be beneficial if the Metabolomics Workbench (a metabolomics data repository) provided a dataset of standards that experimental samples can be compared as means to define acceptable data quality. Until these basics are established, it will be difficult to explore the full extent of metabolomics.
Another large problem facing metabolomics is the ability to confidently and unambiguously identify and quantify metabolites. There are multiple ways people have attempted to automate or improve these methods. In Chapter 3, we suggest the use of $^{13}\text{C}$ is one way to add confidence in identification and multivariate analysis. As explained in Chapter 1, the Brüschweiler lab has attempted to streamline identification with 2D spectra and database searching\textsuperscript{144}. We built on this by creating a graphical interface to overlay 2D NMR standard spectra over a 2D experimental spectrum. It then calculates the percentage of overlap between the standard and the experimental as a means to quantify the match. Others have taken more probabilistic approaches using Bayesian analyses to statistically model peak locations and quantifications. Of course, the standardization of data collection could help streamline many ways that these methods could work. For example, the Wishart Lab has an online application that uses the Bayesian algorithm to match an experimental spectrum to a synthetic spectrum made from metabolites expected to be in urine. The algorithm then attempts to determine the dilution and match the peaks\textsuperscript{251}. Unfortunately, the protocol for sample preparation and data collection that is required for this online application to work deviates from the more established protocols. Again, our contributions to this were minor but if annotation and quantification could be more common place then there could be a tremendous opportunity. Ideally, leaders in the field envision metabolomics could be as common as a routine checkup at an annual doctor’s appointment or urine immediately analyzed once in the toilet.

Although it seems far-fetched, this seems to be the anticipated future for metabolomics. Metabolomics has a strong argument to be more involved in
precision medicine and integrating with other –omics data will be very powerful. In my experiences, metabolites are much more stable from person to person than has been suggested. When metabolites are significantly altered it is often due to an actual problem. This presents a new problem because the small alterations we will likely be testing for may not be determined by multivariate statistics, the most common initial analysis of metabolomics, because the variation may be too small. Overall, for metabolomics to grow into a regular role in the clinic and medical decisions there must be standardization in the field and new analysis and quantification techniques.

**Lipid saturation in DMD**

When investigating the metabolomics of the serum in the *mdx* mouse the lipids were of great interest. The role of lipids is still unclear in DMD. It is known that lipids infiltrate healthy muscle but the mechanism is not characterized. From ImagingDMD, we know there is increased MRI $T_2$ which is contributed to by inflammation, lipid and damage in the muscle. Water $T_2$, taken from the spectroscopy of the same muscle, does not increase to the same degree as the MRI $T_2$. This suggests vast changes in the MRI $T_2$ are not primarily due to the water (inflammation and damage) but instead the fat. This is confirmed when fat fraction has also been seen to associate more closely to MRI $T_2$ than spectroscopy $T_2$. This suggests further that fat is a major contributor to disease progression as both MRI $T_2$ and fat fraction are the most robust MR markers for disease progression. Ultimately, the fact there are systemic lipid saturation alterations suggest it is not merely a case that lipids are just accumulating in the muscle, but it might be the type of lipid accumulating in the muscle. There is a
need for further studies to characterize the lipid pathways that are dominant in the liver and muscle. Also, we have begun to use spectroscopy from the boys in ImagingDMD to calculate the saturation of the lipids. It will be interesting to see if 1) the lipid is high or low in saturation, 2) if the saturation changes over disease progression and 3) compare the serum lipids in boys with and without DMD.

It also must be discussed that these are studies from the mdx mouse model. The mdx model, as previously stated, is not an ideal model as it does not recapitulate the human condition exactly. I think this is a common and ongoing problem, not only in the metabolomics field but in translational science overall. However, I do not think that this should discount this model. I am merely suggesting we should be aware and skeptical when we interpret data, make realistic expectations for how well it will translate to human and take necessary precautions when we do.

It would be ideal to take these differences into account and use multiple models to differentiate the metabolic profile for different aspects of a disease. For example, the use of the utrophin and dystrophin double knock out is not as genetically relevant but better resembles the phenotype in humans. The DB-2/mdx could have been used to understand the metabolic profile once fibrosis infiltrates the muscle. The mdx at 24 weeks was meant to be a mild state of disease but acted more as a correction model. Although, we did not intend for that, the findings were very clear that by 24 weeks the metabolites had reverted to control levels once the membrane became more stable. From a physiologic perspective, the metabolism is renormalizing but the disease continues once the
mice age. Further studies are needed to determine the metabolic changes occurring once the mice become sick again at an older age.

**Urine metabolites as biomarkers in DMD**

The purpose of this study was to determine if urine metabolites were a viable resource to investigate the metabolic changes occurring in DMD. There had been limited studies investigating a small cohort of urine metabolites all relating to muscle breakdown, including creatine, creatinine and methylhistidine isomers. The correlation matrices (Figure 5-6) revealed the entire metabolic pathways were altered in DMD from control. Even more interestingly, the metabolic pathways were quite different with those DMD-GC as compared to DMD+GC. This is an obvious transformation that needs to be researched further. Of course we do not need citrate or any marker to identify if the boys that are taking GCs or not. However, it’s an important proof of concept that we can use a metabolite to identify if a boy is taking a treatment or not. Further, the mechanism by which GCs ameliorate DMD is still unclear. These metabolic profiles can give potential insight as to pathways to investigate the effect of GCs.

Further research is necessary for many aspects of this disease. Primarily, additional test studies need to be used to verify these findings, preferably from different labs. Most importantly, longitudinal samples of these boys need to be investigated for changes in individuals over the disease progression. As they are all in the ImagingDMD study, their metabolic profile progression can then be compared to their function and MRI changes as well. We have recently run 231 urine NMR samples from 90 individuals containing annual points for up to 4 years. This next study will be interesting to try new analysis such as k-means
clustering methods to attempt to separate boys with different metabolic progression rates in an objective manner. We also intend to see the metabolic profile changes with the initiation of GCs in a small subset of individuals.

**Conclusion**

In conclusion, these experiments progress scientific knowledge. The workflows address the disparities within the metabolomics field in both the quality of data and the identification of metabolites in a system. Metabolomics of *mdx* serum brought new insight into lipid saturation in DMD and potentially highlight the need for characterization of the mechanism of such a large and detrimental part of the disease. The urine and serum metabolomes in DMD have revealed the ability to be promising surrogate markers and potentially a tool to investigate unknown aspects of the disease and its progression. These finding are promising and can hopefully be used to investigate the many questions that still revolve around DMD. Finally, I can say that I am happy to have been a part of such a deserving cause.
APPENDIX A
URINE SAMPLE PREPARATION AND DATA ACQUISITION STANDARD
OPERATION PROCEDURES

1. MATERIALS

1.1. CHEMICALS AND REAGENTS

- KH$_2$PO$_4$, Potassium dihydrogen phosphate (Fisher Scientific)
- NaN$_3$, Sodium azide (United States Biochemical)
- KOH, Potassium hydroxide (Fisher Scientific)
- HCL, Hydrochloric acid (Fisher Scientific)
- D$_2$O, Deuterium oxide, 99.9 atom % D (Cambridge Isotope Laboratories, Inc.)
- DSS-D6, Sodium 2,2-dimethyl-2-silapentane-5-sulfonate, 98 atom % D (Cambridge Isotope Laboratories, Inc.)
- Pooled quality control (QC) urine (Ethanol, drug and nicotine free human urine female, Golden West Biologicals, Inc.)

1.2. Equipments

- Calibrated micropipettes (100 μl, 200 μl, and 1000 μl)
- Pippette tips
- 1.5 ml eppendorf tubes
- 5 mm SampleJet NMR tubes from Bruker
- 100ml volumetric flask
- Eppendorf centrifuge
- Analytical weighing balance
- Vortex mixer
- pH meter
- Labels

2. PROCEDURE

2.1. PHOSPHATE BUFFER PREPARATION (1.5 M KH$_2$PO$_4$ buffer)

- Dissolve 20.4 g of the KH$_2$PO$_4$ in 80 ml of D$_2$O
- Dissolve 24.9 mg of DSS-D6 (1/9 of 1mM DSS when mixed with sample) and 13 mg of NaN$_3$ in 10 ml of D$_2$O
- Mix the solutions thoroughly using sonication
- Check the pH of the solution and adjust it to pH=7.4, the solution should be clear after pH adjusted
- Use a 100 ml volumetric flask and adjust the volume with D$_2$O and mix well
- Recheck the pH
- Store the buffer at 4°C

2.2. GENERAL GUIDELINES
• Frozen samples should be thawed in 4°C cold room or on ice
• Check for the minimum volume of all the samples received
• For 48 samples or less use two blanks (NMR buffer containing DSS), one at the beginning and one at the end
• For more then 48 samples use three blanks, at the beginning, middle, and the end of the set
• Total number of QC samples is 10% of study samples. One QC after the first blank at the beginning and one before the last blank at the end of the run. The rest of the QC samples are randomized with the client samples using excel randomizer

2.3. SAMPLE PREPARATION FOR NMR

NOTE: Samples should be randomized before preparation

1. Thaw samples at 4°C cold room or on ice
2. Centrifuge each sample at 12,000 g for 5 min at 4°C
3. Add 60 µl of NMR buffer with 540 µl of an aliquot of samples and QC samples in a 1.5 ml eppendorf tube
4. Vortex each sample for 2 min
5. Transfer 590 µl of supernatant into 5 mm NMR tube
6. Cap the NMR tubes with Bruker caps without holes or use POM balls to close the tube caps
7. Keep NMR samples on ice and transfer to NMR bay to conduct NMR, if any short delay keep them in 4°C refrigerator

3. REFERENCES:
CHEMICALS AND REAGENTS

- NaH$_2$PO$_4$ (Fisher Scientific)
- Na$_2$HPO$_4$ (Fisher Scientific)
- HCl (Fisher Scientific)
- NaOH (Fisher Scientific)
- D$_2$O, DLM-4-100, 99.9% D (Cambridge Isotope Laboratories, Inc.)
- Sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS-D6)(Cambridge Isotope Laboratories, Inc.)
- Pooled Normal Human Red Cross plasma, Quality Control (QC)

MATERIALS

- Calibrated micropipettes (100 μl, 200 μl, and 1000 μl), Eppendorf
- 1.5 ml eppendorf tubes from Fisher Scientific
- 5 mm SampleJet NMR tubes from Bruker
- 100 ml volumetric flask

INSTRUMENTATION

- Eppendorf 5415C centrifuge
- CentriVap Concentrator, Labconco
- Analytical balance, Mettler Toledo (XPE105)
- Vortex Mixer
- Avance III 600 MHz Bruker NMR instrument, 5mm CryoProbe

REAGENT SETUP

- Dissolve 928.6 mg anhydrous NaH$_2$PO$_4$ and 320.9 mg of Na$_2$HPO$_4$ in 80 ml D$_2$O in volumetric flask
- Add 333.3 μl of 01.M DSS-D6 stock solution to make 1/3 mM concentration
- Add more D$_2$O, but do not bring the volume to 100 ml and adjust the pH to 7.0
- Bring volume to 100 ml with D$_2$O
- Check the pH again

PROCEDURE

General guidelines

- All sample preparation has to be done at 4°C cold room or on ice.
- For 48 samples or less use two blanks (NMR buffer containing DSS), one at the beginning and one at the end of the run
- For more then 48 samples use three blanks: beginning, middle, and at the end of the run
• Use two plasma/serum QC samples: one QC plasma/serum after the first blank at the beginning and one before the last blank at the end of the run
• Total number of QC plasma samples is 10% of study samples and are randomized with the client samples using excel randomizer

Plasma and QC plasma extraction.

1. Thaw samples at 4°C cold room
2. Plasma/serum and QC plasma/serum is mixed with 100% cold methanol 1:2 ratios (v/v) in a 1.5 ml eppendorf tube on ice (in general 300 µl plasma/serum with 600 µl methanol)
3. Vortex the mix and incubated in – 20°C for 20 min
4. Centrifuge at 16,000 rcf for 30 min to pellet proteins
5. Transfer supernatant into new labeled 1.5 ml eppendorf tube and dry it using CentriVap concentrator
6. Store the dry pellet into -80°C if NMR is not ready right after

NMR samples preparation
8. Thaw samples by transferring them on ice
9. For 5mm NMR tube resuspend the pellet in 600 µl of NMR buffer
10. Vortex each sample until dried sample is completely dissolved
11. Short centrifuge for 10 seconds
12. Transfer 590 µl into 5 mm NMR tube
13. Keep NMR samples on ice and transfer to NMR instrument to acquire data, if any short delay keep them in 4°C refrigerator
Standard Operating Procedure for High-Resolution Magic-Angle Spinning NMR

University of Florida, SECIM

**Bolded** words are topspin commands

Setting the Magic Angle:

1. Turn on the BCU II cooling unit to setting 2 for trying to regulate at 4 degrees. This should allow it to get cold enough for 4 degrees but not ice over. If either it cannot make it to 4 degrees OR is  icing over make adjustments accordingly to higher or lower settings.

2. Open Topspin 3.2

3. There should be a temperature on the bottom. Double click in that box and this will open the temperature screen in Topspin. Set temperature to 4 degrees. (This may take a while if it was at room temperature.)

4. Still in the temperature screen (tab labeled T) check the Target gas flow and if not already change to 200 lph. Insert the KBr into the probe using the On the MAS II unit touching the insert button and follow directions on the screen. Once the sample is properly inserted then change the Target Gas flow back to 800-1200 in Topspin. (Check gas flow Standby gas flow should be around 200-500 lph, Target gas flow is around 800 to 1200 lph while not changing a sample)

5. Either manually or using the automatic screen using the buttons on the MAS II unit set the spin rate to 6000 Hz. (The baring pressure (BP) and the drive pressure (DP) range but try to keep as low as possible.)

6. Wait for the temperature and spinning to become stable before moving on.

7. While waiting for KBr to be at temp and spinning correctly, create a new KBr experiment by typing edc into the command line of Topspin and make new experiment. (For SECIM make file under the BR2_RSV_1_KBr file as a new experiment.)

8. Use edhead to define the probe and check that the correct slice is being used. If not already selected, define the probe as 4mm HR-MAS 1H/2H-13C Z-GRD.

9. To match and tune type in wobb and proceed to match and tune on carbon (red/big silver) *(Don’t need proton for the KBr experiment)*

10. Type zg and enter to start experiment, efp to process, phase apks (apk)

11. You should get a spectrum with a large middle peak and peaks of decaying size on both sides. The middle peak Full Width of the Half Max (FWHM) should be around 125 - 145 Hz then check the 5th peak over and that should be around 10% larger than the middle peak.

12. If peaks are not spanning the entire spectral width then the magic angle is not optimized.

13. To optimize the magic angle - type gs opening a window showing the free induction decay (FID). The FID should have prominent spikes. Turn the gold knob on the probe until the peaks protruding from the FID are as prominent as long as possible.
14. Redo steps 10 and 11 until the magic angle is optimized.

Making the Quality Control sample:
We are using a 100mM creatine in 90% ddH₂O /10% D₂O solution in a 2% agarose gel.
1. Make the 100mM creatine solution (To make a 1L stock solution)
   a. In a 1L graduated cylinder add 100 mL of D₂O and 800 mL of ddH₂O.
   b. Measure out 13.114 g of creatine (check FW on label, should be 131.14 daltons)
   c. Add creatine to the graduated cylinder and use the last 100 mL of ddH₂O to clean out the weigh boat so all creatine is transferred. Make sure that it is an exact 1L.
   d. Add a stirring rod and heat and stir until clear.
   e. In case of making dilutions, be sure to also make a solution of 90% ddH₂O /10% D₂O to add to dilution to maintain the 10%D₂O throughout.
2. For a 2% weight/volume (g/ml) agarose gel. Measure out 25ml of the creatine solution in a graduated cylinder and add in a beaker. Then add 0.5g agarose to the creatine solution. Stir
3. Heat in microwave for 30 sec stir for about 1 min then microwave for another 30 sec. If need be continue until clear and all agarose should be melted.
4. Add to glass petri dish, label and store at 4 degree Celsius.
5. After this has a gel consistency, use a 4mm capillary tube to remove a portion of the gel through the capillary. Remove this portion from the capillary tube directly into the rotor and add 30 – 50 uL of D₂O and close rotor as usual.
6. Then bring to NMR and run acquisition. (see Acquiring Spectra section)

Making a sample:
1. Weigh and record the weight of the HR-MAS rotor.
2. Weigh and record the weight of 20-30 mg of tissue. 10-20 mg is acceptable if only lower amounts of tissue are obtained. If tissue needs to be cut, be sure to keep the sample on ice when doing so. If tissue is muscle, we recommend de-tendon the tissue while on ice.
3. While the tissue is on an empty agar plate kept on ice, add 30 μL of D₂O to tissue.
4. Place the tissue into rotor, then add the D₂O that is left over into the rotor as well.
5. Weigh and record the weight of the rotor with the tissue in it. Wet weight can now be obtained by subtraction from original rotor weight.
6. Be sure to keep sample on ice as often as possible and bring to NMR and run acquisitions. (see Acquiring Spectra section)
7. After data collection, remove sample and place back on ice.
8. Weigh and record an Eppendorf tube.
9. Extract tissue from rotor, place tissue in Eppendorf tube weigh and record.
10. Store at -80 degrees Celsius.
11. Clean rotor using water. Be sure that rotor is dry before using again. Because of this, it is best to use a rotation of at least 3 rotors so that one can be washed, one can dry, and another can be used to run a sample.

Putting sample into HR-MAS and regulate temp and spin:
1. If there is a rotor already in the NMR, spinning and at temperature, go to the MAS II unit and touch the stop button on the touch pad. The spinning will begin to slow down and the temperature will become unregulated.
2. It will not stop entirely until the Target gas flow is changed to 200 lph. Once it stops press eject on the MAS II unit and a burst of air will release the sample. Go up the stairs and remove the sample an turn off the air by pressing stop if it does not do this automatically.
3. While everything is still stopped drop the rotor with the next sample in it into the probe so that the cap with fins on it is at the top. Then using the MAS II unit touching the insert button and follow directions on the screen. Once the sample is properly inserted then change the Target Gas flow back to 800-1200 in Topspin. (Check gas flow Standby gas flow should be around 200-500 lph, Target gas flow is around 800 to 1200 lph while not changing a sample)
4. Either manually or using the automatic screen using the buttons on the MAS II unit set the spin rate to 6000 Hz. (The baring pressure (BP) and the drive pressure (DP) range but try to keep as low as possible.)
5. Wait for the temperature and spinning to become stable before moving on.
6. While waiting, create folders for the experiments on the sample. (If for SECIM: follow the following folder structure: BR2_initials_#of times you ran NMR_pulse sequence (all lowercase). If these have already been created for the run of samples pull a former spectra from that main folder you wish to repeat into the working screen and create a new experiment folder by typing edc and changing the expno to the next sequential number)

Acquiring spectra:
1. Lock on D2O. This can be done by either typing lock into the command line or by pushing the “Lock on” button on the BSMS screen. Wait until the lock signal has equilibrated to move on.
2. Use the command wobb to match and tune the sample this time using on the proton channel (yellow M and T knobs on probe)
3. Acquire ‘zg’ spectra by typing zg and enter to start experiment, efp to process, phase apks (apk). Use this spectrum to calibrate 90-degree pulse. To automatically calibrate the 90 degree pulse, use the command pulse cal to on each sample (preferred). To check the number it returns, multiply it by 4 to estimate the 360 degree pulse which should create the best null. The us the command paropt and follow the directions. Recommended to run enough experiments to test about 5us seconds before and after the calculated time. This should allow you to see the negative peaks minimize to a null and become positive again. The null should be the 360 degree pulse, divide by 4 and compare to the time calibrated by pulse cal.
4. Acquire a ‘zgpr’ spectrum to check water suppression and shims by typing zg and enter to start experiment, efp to process, phase apks (apk). If shims are not good type gs and click on the half FID and half spectra button. This will change the view to a processed spectrum. To phase click on the checks and use the mc and pk buttons to phase. Then you can phase to make the shim of the metabolites with singlets having minimal line broadening. Check the FWHM of a singlet. This should be around 3 Hz.

5. If samples contain a lot of water and it is not possible to evaluate shims based on this, run a ‘noesypr1d’ pulse sequence with a low number of scans by typing zg and enter to start experiment, efp to process, phase apks (apk).

6. If the water suppression is not ideal, adjust radio frequency carrier frequency offset value (o1) also on the gs page. Use the command gs and open the tab offset. Click either to increase the o1 by 1 in one direction or the other. Make the area of the FID as low as possible. The area can be found at the bottom of the screen.

7. Set up sequence of experiments. All experiments should have the same 90 degree pulse as the P1 and the same o1 that was calibrated in the previous steps. Also be sure each experiment has the appropriate receiver gain (RG). To check use rga and this will automatically be calculated for you.
   a. ‘zg’ with 1 scan
   b. ‘zgpr’ with 4 scans
   c. ‘noesypr1d’ with 128 scans
   d. ‘cpmgpr1d’ with 128 scans
   e. ‘zg’ with 1 scan. This is to check for degradation in the sample.
   f. ‘jresgprqf’ with 4 scans.

Note: This protocol is modified from Beckonert et al. High-resolution magic-angle-spinning NMR spectroscopy for metabolic profiling of intact tissue. Nature Protocols. 5, 1019-1032. (2010).
% QC NMR Workflow
% Brittany Lee-McMullen
% UF, 2015

%% This Workflow is comprised of three sections. 1. Load data and process,
% 2. Data preparation for QC and 3. QC tests and flagging. If you have
% fully processed data (aligned and normalized) begin by loading in you
% data and begin at Section 2 (many examples of how to do this are provided below, but
% worse case
% just load in by the clicking the 'import data' button in matlab).
% If beginning from NMRpipe processed spectra or Topspin processed
% NMR files START HERE! This first part is getting the data ready by
% removing water and unwanted areas, baseline correction, aligned and
% normalized.

%% Section 1. Getting your data processed
%% Step 1. Load in spectra. Here I have showed two ways to do this, but any
% .csv file can be added and formatted correctly to do this.
%% Step 1a. Load in data processed in NMRpipe. This will load in all processed
% .ft files that are in the current working directory. This will output your
% data in a variable called spectr
loadallft

%% Step 1b. Create variables for data processed file from Topspin. This is an example
% of the
% in Topspin File format ending in the processed 1.
path_1D{1}='../data/SampleFileName/Sample.fid/pdata/1';
path_1D{2}='../data/SampleFileName/Sample.fid/pdata/1';
path_1D{3}='../data/SampleFileName/Sample.fid/pdata/1';
path_1D{4}='../data/SampleFileName/Sample.fid/pdata/1';
path_1D{5}='../data/SampleFileName/Sample.fid/pdata/1';

%% Step 1c. Load in Topspin data only. This will then output the variable spectra and
% and now all data will be the same from here on out.
spectra=Load1D(path_1D,'bruker');

%% Step 2. Reference the spectra
[spectra] = ref_spectra(spectra,0.01)

%% Step 3. Create the sample matrix, chemical shift vector and the sample id vector
[X,ppm,XTitles]=Setup1D(spectra);
%% Step 4. Remove water and ends
% First plot to check the chemical shift range that you want to remove
plotr(ppm,X)

%% Actual removal
XR=remove_region(X,ppm,4.75,5.15);
[XR1,ppm]=remove_ends(XR,ppm,-0.2,10.0);

%% Step 5. Baseline correction -This function allows you to visualize a red
% line that will show the zero new zero line and where it will touch the
% spectra correction that it is currently making. This is good to check and make
% sure that the baseline correction is not over correcting. To make changes
% adjust the last two inputs until you are confident in the baseline
% correction
[A,S]=showBaseline(XR, ppm, 9e7, 1e5);

%% 5b. By running this here it will take the parameters A and S from showBaseline
% and will apply them to adjust the entire matrix of spectra
XNew=CorrectBl(XR1, A, S);

%% Visualize to check new baseline
plotr(ppm,XNew)

%% Step 6. Align spectra
% Disclaimer - there are many options so feel free to 'edit guide_align'
% or 'edit star_align' to see all options that are available. Hint: before
% going through alignment 'plotr(ppm,XR1)' and find peaks that need
% alignment and see which alignment method works best
XAL=guide_align1D(XNew,ppm,'correlation','CCOW');

%% Step 6 (optional but recommended before moving on) Visualize to make sure
% that the alignment is correct
figure
hold on
plotr(ppm,XAL);
hold off

%% Step 7. Normalize your data. There are also many options here so to see
% all options 'edit normalize' where there is more information. You may
% also want to check and make sure before and after you normalize to make
% sure that it is working.
% % check normalization before
normcheck(XAL)

%% Actual normalization
XALN=normalize(XAL2,ppm,'PQN');
%% Check the normalization afterwards
normcheck(XALN)

%% Step 7 (optional) Visualize your fully figure
hold on
plotr(ppm,XALN)
hold off

%% Section 2: This next section will help get the data into groups that you will use % in the QC. This will include getting the sample titles in different forms % and separating your disease vs controls vs QC samples into different % matrices.

%% Step 8. Making the identity vector, Yvec
% Disclaimer- for groups2.m to work you need to fix or make sure that the groups % do not start with same letter otherwise is made into the same group
[Yvec]= group2('Core2_sheet.xlsx')

%% Or you can make one by hand where a number is a code for a group %example: 1=pooled 0=blanks 2=diseased 3=controls
[Yvec]=[1 1 1 0 0 ]

%% Step 9. Making your flagging vectors. You will need one list of your % sample names that is a string for labeling we call this Xtitles. You will % need another for flagging (XTitles) which will remove the '_1D' from the % name and will create a vector of numbers.
Xtitles=XTitles

%% removes anything but numbers and changes string to number
for i = 1:length(XTitles)
    s = XTitles{i};
    XTitles(i) = {s(1:end-3)};
end
XTitles=cellfun(@str2num, XTitles)

%% Step 10. Makes the different matrix groups
% Reminder i used these from example above: 0=blanks 1=pooled 2=diseased
3=controls
X_blanks=XALN(Yvec==0,:);
X_QC=XALN(Yvec==1,:);
X_diseased=XALN(Yvec==2,:);
X_controls=XALN(Yvec==3,:);

%% Makes the different XTitles vector
X_blanks_XTitles=XTitles(Yvec==0,:);
X_QC_XTitles=XTitles(Yvec==1,);
X_diseased_XTitles=XTitles(Yvec==2,);
X Controls_XTitles=XTitles(Yvec==3,);

%% Makes the string array for labeling
X_blanks_Xtitles=Xtitles(Yvec==0,);
X_QC_Xtitles=Xtitles(Yvec==1,);
X_diseased_Xtitles=Xtitles(Yvec==2,);
X Controls_Xtitles=Xtitles(Yvec==3,);

%% Peakpicking for BA plots - be sure to optimize the threshold for peak
% for your own data

[peaks_x_blanks,shifts_x_blanks]=Peakpick1D(X_blanks,ppm,'mean',0.25);
[peaks_qc,shifts_qc]=Peakpick1D(X_QC,ppm,'mean',0.25);
[peaks_diseased,shifts_diseased]=Peakpick1D(X_diseased,ppm,'mean',0.25);
[peaks_con,shifts_con]=Peakpick1D(X_control,ppm,'mean',0.25);

%% Section 3: The QC workflow. This is comprised of multiple tests that will
% result in flagging of data points either at your discretion or by an
% automated threshold in the script (still in the works.. )

%% Instrumental variation - Here we will plot temperature, 90 degree pulse times
% and the FWHM for shimming values to check for instrumental variation. For
% this section you will need to get these parameters from the raw data.
% ** if not SECIM data - easiest way is on terminal to grep and create a .csv file for
each
% parameter. I have provided examples for the ones I will show you.

% ex >> grep '$TE=' ./file_containing_all_raw_data/*/acqus temp_data.csv

% ** if data comes from the SECIM workflow and has the 'Project_name'_methods will
% have all of these parameters. Go into the methods folder and run the
% following:
load(''Study_ID_pulseSequence'_methods')

%%
temperature=horzcat(XTitles,(ismember(XTitles,'Study_ID_pulseSequence_methods'.TE)));
pulse=horzcat(XTitles,(ismember(XTitles,'Study_ID_pulseSequence_methods'.P1)));

%% 11. First just look at spectrum and flag any obvious spectra that stand out.
% Use whichLine and highlightLine to help identify specific spectra to
% flag.
% % plot it (can also look at the normalized spectra.. just replace XAL with XALN)
figure
hold on
plotr(ppm,XAL)
hold off

whichLine() % only labels the spectrum by index

this will highlight and label (sampleID given) the spectrum that is selected
highlightLine(Xtitles)

flag (name the flags to describe and that you will remember)
odd_spectra=[2];
flag_odd_spectra=horzcat(XTitles,(ismember(XTitles,odd_spectra)));

12. Distribution of data - A nice overview of your data. Displays histogram
% and box plots of log-fold change vs. median for all features in each spectrum.
% Dilution / normalization effects are often visible as distributions not centered at 0.
normcheck(XAL)

create a flag vector of those that are questionable data
% where 1=abnormal and 0=normal
% Need to fix.. this gives index instead of xtitles, but SECIM will be the
% same either way??
distribution=[];
flag_distribution=horzcat(XTitles,(ismember(XTitles,distribution)));

13. Temperature variations
flag_temperature=autoflag_reg
figure
hold on
scatter(temperature(:,1),temperature(:,2))
text(temperature(:,1),temperature(:,2),Xtitles)
hold off
% create a flag vector of those that are questionable data
temperature_variations=[];
flag_temperature=horzcat(XTitles,(ismember(XTitles,temperature_variations)));

14. 90 degree pulse variations per group
flag_pulse=autoflag(pulse(:,2),Yvec,XTitles,Xtitles);

15. 90 degree pulse variations over time
flag_temperature=autoflag_reg
figure
hold on
scatter(pulse(:,1),pulse(:,2))
text(pulse(:,1),pulse(:,2),Xtitles)
hold off
%% flag by hand
temperature_variations=[];
flag_temperature=horzcat(XTitles,(ismember(XTitles,temperature_variations)));

%% 16. QC of shimming - Full width of the half max of the internal standard
[FWHM_DSS] = horzcat(XTitles,fwhm(XAL,ppm,-0.04,0.04));
flag_FWHM=autoflag(FWHM_DSS(:,2),Yvec,XTitles,Xtitles);

% this should not change with normalization.
%% Now for intra-sample variations
% 17. Integration of internal standard
DSS_integration=horzcat(XTitles,IntegralPeak_roi(XAL,ppm,-0.04,0.04));
flag_DSS_integration=autoflag(DSS_integration(:,2),Yvec,XTitles,Xtitles);

%% This can be re-done with the normalized data as well. This should give % and idea of how well the normalization worked as well.
norm_DSS_integration=horzcat(XTitles,IntegralPeak_roi(XAL,ppm,-0.04,0.04));
flag_norm_DSS_integration=autoflag(norm_DSS_integration(:,2),Yvec,XTitles,Xtitles);

%% 18. If there are any metabolites that could potentially be changing over % time (for example are there sugars or lactate?)use the following to check:
% use the following to quantify a metabolite of interest
metabolite=horzcat(XTitles,IntegralPeak_roi(XALN,ppm,ppm1,ppm2));
% THIS IS STILL BEING WRITTEN
flag_temperature=autoflag_reg(metabolite(:,2),XTitles,Xtitles);

%% 19. Inter-sample variations - Bland Altman plots are the avg against the % difference between two samples. This will create multiple flags 1) slope % flag will show if there is a linear pattern, 2) the residuals are plotted % and points that are 2 std dev from standardized residuals will be flagged % for each sample comparison 3) The flags for each data point will be % summed and a final vector will be given for data points that are flagged % the most.

% It is recommended to use peak picked data from a spectra for this as the % noise can make it very difficult to interpret. Also, it is recommned to % compare spectra of the same group. The autoBA script will take all % possible combinations within the data set provided, will ouput the flags % and figures.

%% CURRENLY STILL WRITING THE FULLY AUTOMATED VERSION!!
% so must do each group by hand. repeat the following for all groups:
autoBA(X_blanks,X_blanks_titles)

%% flag by hand
ba_blanks=[];
flag_blanks=horzcat(XTitles_blanks,(ismember(XTitles_blanks,ba_blanks)));

## Table E-1: Composition and summary results for the synthetic metabolite mixtures used in this study.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Target Concentration(^a) Per Group [mM]</th>
<th>Database IDs(^b/) False correlations(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>D-Trehalose</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Malate</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>L-Aspartic Acid</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>D-Fructose</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>L-Valine</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>L-Methionine (Met)</td>
<td>2.0</td>
<td>1.0</td>
</tr>
<tr>
<td>L-Proline (Pro)</td>
<td>3.0</td>
<td>1.5</td>
</tr>
<tr>
<td>L-Glutamate (Glu)</td>
<td>5.0</td>
<td>2.0</td>
</tr>
<tr>
<td>D-Glucose (Glc)</td>
<td>2.5</td>
<td>1.5</td>
</tr>
<tr>
<td>L-Isoleucine (Ile)</td>
<td>3.0</td>
<td>1.0</td>
</tr>
<tr>
<td>L-Arginine (Arg)</td>
<td>2.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Nicotinamide (NAM)</td>
<td>2.0</td>
<td>3.5</td>
</tr>
<tr>
<td>L-Carnitine (Car)</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>L-Lysine (Lys)</td>
<td>1.0</td>
<td>3.5</td>
</tr>
<tr>
<td>L-Tryptophan (Trp)</td>
<td>1.5</td>
<td>2.5</td>
</tr>
</tbody>
</table>

**Compounds Identified in BMRB:** 15 / 20    7 / 20

---

\(a\) Compounds were transferred to NMR tubes individually to simulate typical between-sample variation, as described in the Methods.

\(b\) A database ID is yes (“Y”) when a COLMAR query search of the BMRB returns the known compound in the top 10 matches. Otherwise it is no (“N”).

\(c\) False correlations are peaks that were in the peak list generated from the correlation maps but not part of the identified compound.

\(d\) Results obtained using \(^{13}\text{C}\) and the entire workflow outlined in this paper.

\(e\) Results obtained by only using \(^{1}\text{H}-^{1}\text{H}\) STOCSY data and no \(^{13}\text{C}\) data.


42. Involvement of TRPV2 and SOCE in calcium influx disorder in DMD primary human myotubes with a specific contribution of α1-syntrophin and PLC/PKC in SOCE regulation. 304, C881–94 (2013).

43. Read-through compound 13 restores dystrophin expression and improves muscle function in the mdx mouse model for Duchenne muscular dystrophy. 21, 4007–4020 (2012).


45. THE 6-minute walk test and other endpoints in Duchenne muscular dystrophy: Longitudinal natural history observations over 48 weeks from a multicenter study. (2013). doi:10.1002/mus.23902


47. The 6-minute walk test and other clinical endpoints in Duchenne muscular dystrophy: Reliability, concurrent validity, and minimal clinically important differences from a multicenter study. (2013). doi:10.1002/mus.23905


52. A prostaglandin D2 metabolite is elevated in the urine of Duchenne muscular dystrophy patients and increases further from 8 years old. 423, 10–14 (2013).

53. Temporal changes in magnetic resonance imaging in the mdx mouse. 6, 262 (2013).


85. Changes in muscle T2 and tissue damage after downhill running in mdx mice. 43, 878–886 (2011).

86. The 6-minute walk test and person-reported outcomes in boys with duchenne muscular dystrophy and typically developing controls: longitudinal comparisons and clinically-meaningful changes over one year. 5, (2013).


103. Arpan, I. *et al.* Examination of effects of corticosteroids on skeletal muscles of boys with DMD using MRI and MRS. *Neurology* *83*, 974–980 (2014).


135. Mass-spectrometry-based metabolomics: limitations and recommendations for future progress with particular focus on nutrition research. (2009).


BIOGRAPHICAL SKETCH

Brittany Lee was born and raised in Lakewood, Colorado. She attended both primary and secondary school at Bear Creek Elementary and Bear Creek High School, respectively. Brittany enjoyed an eclectic combination of extracurricular activates, including making pizzas, acting, and soccer. She attended the University of Louisiana at Monroe for college on a soccer scholarship and majored in chemistry with minors in mathematics and biology. After college, Brittany taught high school Chemistry and Biology at St. Frederick’s High School in Monroe, Louisiana for two years. During this time, she was involved in the community and was able to coach the women’s soccer team at St. Frederick’s as well. Brittany was then accepted and attended graduate school at the University of Florida in the Interdisciplinary Program for Biomedical Sciences in the College of Medicine under the mentorship of Dr.’s Glenn Walter and Arthur Edison. She feels lucky for the mentorship and guidance she received and hopes to make them proud in her future endeavors.