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PATHOGENIC MITOCHONDRIAL DNA MUTATIONS IN PROTEIN-CODING GENES

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Mitochondria are cellular organelles whose major function is to produce the energy molecule adenosine triphosphate (ATP) for all cellular activities. There are estimated to be over 1000 proteins localized to mitochondria, the vast majority of which are encoded by nuclear genes, with only 13 encoded by the mitochondrial genome. Hence, most mitochondrial disorders will be inherited in a mendelian fashion. For example, deficiency of SURF1, an assembly protein for cytochrome c oxidase (COX; complex IV of the electron transport chain), is a common cause of Leigh syndrome, and DNA polymerase gamma deficiency causes Alpers syndrome, both demonstrating autosomal-recessive inheritance. Other inheritance patterns, such as an autosomal-dominant–type chronic progressive external ophthalmoplegia, can also be found. In contrast, mitochondrial DNA (mtDNA) mutations, in general, exhibit matrilineal inheritance, although de novo and somatic mutations have been reported. Multiple copies of mtDNA, usually hundreds to thousands, are present per cell. mtDNA exhibits a high mutation rate, due to the lack of protective histone proteins, inefficient DNA repair mechanism, and its close vicinity to where reactive oxygen species are produced. Because mtDNA is present in multiple copies, a pathologic mtDNA mutation often results in heteroplasmy, meaning the coexistence of the wild-type and mutant mtDNA molecules. The degree of mutant heteroplasmy can vary between 0% and 100%. The clinical expression of mtDNA mutations is extremely heterogeneous, depending on the percentage of mutant loads in affected tissues, the energy demand of the tissue, and the type of mutation.

As a group, mitochondrial respiratory chain disorders represent a dual-genome complex disease

Abbreviations: ATP, adenosine triphosphate; BSN, bilateral striatal necrosis; COX, cytochrome c oxidase; CPEO, chronic progressive external ophthalmoplegia; ESOC, epilepsy, stroke-like episodes, optic atrophy, and cognitive decline; HCM, hypertrophic cardiomyopathy; LEYI, LHON and dystonia; LHON, Leber hereditary optic neuropathy; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes; MM, mitochondrial myopathy; MCI, multiple cerebral infarction; MW, muscle weakness; NAION, non-arteritic ischemic optic neuropathy; ND, NADH dehydrogenase; NIDDM, non–insulin-dependent diabetes mellitus; PD, Parkinson disease; PEM, progressive encephalomyelopathy; RC, respiratory chain; RP, retinitis pigmentosa; SIDS, sudden infant death syndrome; SNHL, sensorineural hearing loss; WPW, Wolff–Parkinson–White syndrome

Key words: mitochondrial mRNA mutations; mtDNA mutations; mtDNA mutations in protein coding regions; pathogenic mtDNA mutations

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that is genetically and clinically heterogeneous. In general, mitochondrial disorders caused by mutations in nuclear-encoded genes are severe and typically of infantile onset.34,61,70,96 By contrast, primary mtDNA mutations result in diseases that are usually milder and of later onset, although many examples of severe, infantile-onset mtDNA-related disease are known.82,98,114 Due to the clinical and diagnostic complexity of mitochondrial disorders, establishing a definitive diagnosis is often challenging. If a patient develops a well-documented mitochondrial “syndrome,” a screening approach to detect common mtDNA mutations is often utilized as the first step toward diagnosis.56 Nevertheless, in most patients with clinical features of a mitochondrial disorder, it is usually not possible to determine whether the pathogenic mutation is in the nuclear or mitochondrial DNA. If the family history is suggestive of maternal inheritance, screening for previously unidentified mutations by various mutation detection methods or direct sequencing of the mtDNA can be performed. However, because mtDNA is highly polymorphic, the pathogenic significance of a detected sequence alteration needs to be determined using a series of criteria before a diagnosis can be established with confidence.

The mitochondrial genome is a 16.6-kb double-stranded circular DNA. It encodes 37 genes, including 13 proteins, 22 transfer RNAs, and 2 ribosomal RNAs. The 13 mtDNA-encoded proteins are all subunits of the mitochondrial respiratory chain (RC). The mitochondrial RC is composed of five multiheteromeric enzyme complexes (I, II, III, IV, and V) embedded in the mitochondrial inner membrane.

At least 86 protein subunits are involved in the respiratory chain, including 7, 1, 3, and 2 mtDNA-encoded subunits of complexes I, III, IV, and V, respectively. It is noteworthy that the structural components of complex II are encoded exclusively by nuclear genes. Thus, protein-encoding genes of mtDNA are entirely devoted to energy metabolism. These protein subunits are assembled together with prosthetic groups and metal-containing reactive centers by assembly factors and chaperone proteins that are encoded by nuclear genes.74,76,95 This review focuses on the molecular genetics and pathogenicity of clinically significant mutations in mtDNA-encoded protein subunit genes.

**CRITERIA FOR THE ASSESSMENT OF THE PATHOGENICITY OF mtDNA ALTERATIONS IN PROTEIN-CODING GENES**

Over 200 mtDNA point mutations have been reported to be associated with a wide variety of human diseases (Wallace and Lott, 2006: http://www.mitomap.org; accessed on December 1, 2006).112 Excluding somatic mtDNA mutations in cancer cells, 124 mutations have been found in protein-encoding genes and 120 mutations in tRNA genes (Wallace and Lott, 2006: http://www.mitomap.org; accessed on December 1, 2006). Secondary LHON mutations, homoplasmic missense variants at evolutionarily non-conserved amino acid residues, or variants leading to substitutions within the same amino acid classes are not included in this review. Mutations were evaluated for their pathogenicity based on the scoring criteria proposed by Mitchell et al.,67 with

### Table 1. Scoring criteria for pathogenicity of mutations in mitochondrial protein coding genes (modified from of Mitchell et al.67).

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Conditions</th>
<th>Maximum score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biochemical defects</td>
<td>Demonstrated in affected tissues</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Demonstrated in multiple tissues</td>
<td>2</td>
</tr>
<tr>
<td>Functional studies</td>
<td>Single-fiber PCR or cybrid studies, or other methods, such as western blot, enzyme kinetics, immunochemistry</td>
<td>7 or 4</td>
</tr>
<tr>
<td>Multiple reports of the mutation in unrelated patients with disease</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Heteroplasm</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Segregation of variant with disease within a family</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Evolutionary conservation* in organisms</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>1 for each variant seen in a vertebrate listed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 for variation in each of the four adjacent residues</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 for each of the adjacent residues if variant is from different amino acid classes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total score</td>
<td></td>
<td>40</td>
</tr>
</tbody>
</table>

*Evolutionary conservation was evaluated by comparing the amino acid sequences from yeast (Saccharomyces cerevisiae), sea urchin (Strongylocentrotus purpuratus), fruit fly (Drosophila melanogaster), frog (Xenopus laevis), mouse (Mus musculus), cow (Bos taurus), and human (Homo sapiens).
slight modification (Table 1). Briefly, a measurable biochemical defect (complex activity or blue native gel) is demonstrated in affected tissues (8 points). If the defect is present in multiple tissues, 2 more points are added. Seven points are given if the functional deficiency is revealed by single-fiber polymerase chain reaction or transmi tochondrial cybrid studies. If single-fiber or cybrid studies are lacking, but other methods such as Western blot, enzyme kinetics, or immunocytochemistry were performed to demonstrate pathogenicity, a partial score of 4 is given (Table 1). If a candidate pathogenic mutation has been reported on two or more occasions by independent groups, 5 additional points are given. Three original standard criteria are also included: heteroplasmy (5 points); segregation with the disease within a family (3 points); and evolutionary conservation (maximum 10 points). To evaluate evolutionary conservation, the amino acid sequences from yeast (Saccharomyces cerevisiae), sea urchin (Stronglylocentrotus purpuratus), fruit fly (Drosophila melanogaster), frog (Xenopus laevis), mouse (Mus musculus), cow (Bos taurus), and human (Homo sapiens) were compared. From the maximum of 10 points, 1 point is deducted for each variant seen in a vertebrate, 1 point off for variation in each of the four adjacent residues, and another point off for each of the four adjacent residues if the variant is from a different amino acid class (Table 1). Mutations with scores between 30 and 40 are definitely pathogenic, 20–29 are probably pathogenic, and 10–19 and 0–9, respectively, are possibly and unlikely to be pathogenic. For any criterion, if an experiment or relevant information is missing, it will score zero for that criterion. This scoring system was applied to every single mutation listed in Table 2. Because not all the biochemical or cybrid studies are performed for every mutation, the scores listed in Table 2 probably represent the minimum scores.

### CLINICAL PHENOTYPES ASSOCIATED WITH COMPLEX I MITOCHONDRIAL GENE MUTATIONS

Complex I, which contains 46 protein subunits, is the largest of the mitochondrial respiratory chain enzyme complexes. Seven of these subunits are encoded by the mitochondrial genome. Complex I, a NADH dehydrogenase, catalyzes the oxidation of NADH to NAD⁺ using coenzyme Q, a lipidoidal quinone, as the electron shuttle to complex III. Deficiencies of mtDNA-encoded complex I subunits cause a wide range of clinical phenotypes. The most common are the Leber hereditary optic neuropathy (LHON) mutations, 11778G>A, 3460G>A, and 14484T>C in MTND4, MTND1, and MTND6 subunit genes, respectively.¹¹¹ Most of the putative LHON pathogenic mutations and variants that are associated with LHON are located in the ND1 subunit. Recently, several complex I mtDNA mutations causing infantile mitochondrial encephalopathies, such as Leigh syndrome, lethal infantile mitochondrial disease, and MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes) have also been reported (Table 2).

LHON mutations are usually homoplasmic and inherited. However, the mutations causing more severe infantile mitochondriopathies are usually heteroplasmic. Although approximately 80% of the MELAS cases are due to a single mutation, 3243A>G in the tRNA⁹⁰⁴Leu(UUR) gene, a number of mutations in complex I genes, most notably the mutations in MTND1 and MTND5, have been reported to cause the clinical syndrome of MELAS as well. A number of mutations in MTND1, MTND3, MTND4, MTND5, and MTND6 also cause dystonia or Leigh syndrome (Table 2). MTND genes harbor most mtDNA mutations that arise in protein-encoding genes. This is partly due to the large number of ND subunit genes. All but one are missense mutations. In fact, the only nonsense mutation in mtDNA-encoded complex I subunits reported to date was in a patient presenting with exercise intolerance, which is not the major clinical feature of complex I deficiency. The patient had a somatic mutation, 11832G>A (W358X in MTND1), in the post-mitotic muscle tissue. This is the first and only reported isolated myopathy case caused by mutation in complex I subunits.⁷

According to the Mitomap database (http://www.mitomap.org), about half of the mutations in complex I mtDNA are LHON mutations. These have occurred in multiple unrelated families and are usually homoplasmic. The mutation 11777C>A (R340S, MTND1), affecting the identical amino acid altered by the most common LHON 11778G>A mutation, was reported in a 67-year-old man with late-onset Leigh syndrome.²⁸ This case underscores the fact that mtDNA abnormalities can cause a wide spectrum of clinical phenotypes and should be considered in older patients with late-onset encephalopathy. A few homoplasmic missense alterations may be associated with Alzheimer or Parkinson disease, such as 3397A>G, 5460G>A, and 5460G>T.¹³,⁶⁷,⁸⁷,⁹⁰ In addition, numerous missense alterations in mtDNA-encoded complex I subunit genes are associated with LHON, called secondary LHON mutations. These variants by themselves do not cause diseases but may act synergistically in the presence of other disease-causing primary mutations.¹¹¹,¹¹² These include
**Table 2. Mutations in protein-coding regions.**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Changes</th>
<th>Amino acid change</th>
<th>Het/</th>
<th>Inherited/sporadic</th>
<th>Disease</th>
<th>Evolutionary conservation*</th>
<th>Score</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTND1</td>
<td>3308T-&gt;C</td>
<td>M1T het/hom</td>
<td>I</td>
<td>BSN, MELAS, SIDS</td>
<td>associated with SIDS</td>
<td>L/F/L/L/M/M/M</td>
<td>5</td>
<td>72, 81</td>
</tr>
<tr>
<td></td>
<td>3308T-&gt;G</td>
<td>M1X het/hom</td>
<td>I</td>
<td></td>
<td></td>
<td>L/F/L/L/M/M/M</td>
<td>28</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>3460G-&gt;A</td>
<td>A52T het/hom</td>
<td>I</td>
<td>LHON</td>
<td>A/G/A/G/A/A</td>
<td>36</td>
<td>2, 67</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3644T-&gt;C</td>
<td>V113A het/hom</td>
<td>I</td>
<td>Bipolar disorder, MCI, ptosis, MW, NIDDM, MELAS</td>
<td>V/V/V/V/V/V</td>
<td>20</td>
<td>69</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3697G-&gt;A</td>
<td>G131S het</td>
<td>I</td>
<td>MELAS</td>
<td>A/G/G/G/G/G</td>
<td>38</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3735G-&gt;A</td>
<td>E143K het/hom</td>
<td>I</td>
<td>LHON</td>
<td>E/E/E/E/E/E</td>
<td>30</td>
<td>1, 07</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3796A-&gt;G</td>
<td>A164T het</td>
<td>I</td>
<td>Adult-onset dystonia</td>
<td>S/Y/Y/T/T/T</td>
<td>13</td>
<td>67, 93</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3949T-&gt;C</td>
<td>Y215H het</td>
<td>D</td>
<td>MELAS</td>
<td>Y/Y/Y/Y/Y/Y</td>
<td>32</td>
<td>50, 67</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4171C-&gt;A</td>
<td>L289M het/hom</td>
<td>I</td>
<td>LHON</td>
<td>C/T/A/L/L/L</td>
<td>17</td>
<td>49, 67</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4681T-&gt;C</td>
<td>L71P het</td>
<td>D</td>
<td>Leish syndrome</td>
<td>L/M/L/L/L/L</td>
<td>28</td>
<td>1, 04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5244G-&gt;A</td>
<td>G259S het</td>
<td>I</td>
<td>LHON</td>
<td>A/G/G/G/G/G</td>
<td>9</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10198&gt;C</td>
<td>S34P het</td>
<td>I/D</td>
<td>Leigh</td>
<td>T/S/L/A/T/S</td>
<td>33</td>
<td>52, 65</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10191&gt;T</td>
<td>S45P het</td>
<td>D</td>
<td>ESOC/Leigh-like</td>
<td>R/N/S/G/S/G</td>
<td>32</td>
<td>52, 65</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10197&gt;A</td>
<td>A477 het</td>
<td>I</td>
<td>Leigh, dystonia</td>
<td>A/A/A/A/A/A</td>
<td>40</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>MTND4</td>
<td>1068T&gt;C</td>
<td>V65A hom</td>
<td>I</td>
<td>LHON</td>
<td>V/T/V/V/V/V</td>
<td>31</td>
<td>2, 12, 14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11232&gt;T</td>
<td>L158P het</td>
<td>ND</td>
<td>CPEO</td>
<td>L/L/L/V/L/L</td>
<td>26</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11696&gt;C</td>
<td>V313I het</td>
<td>I</td>
<td>LHON</td>
<td>L/L/Y/M/T/T/T</td>
<td>16</td>
<td>67, 117</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11777&gt;C</td>
<td>R403S het</td>
<td>I</td>
<td>Leigh</td>
<td>R/R/R/R/R/R</td>
<td>30</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11778&gt;A</td>
<td>R394I het/hom</td>
<td>I</td>
<td>LHON</td>
<td>R/R/R/R/R/R</td>
<td>37</td>
<td>67, 117</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11863&gt;C</td>
<td>W358X het</td>
<td>S</td>
<td>Exercise intolerance</td>
<td>Y/Y/W/W/W/W</td>
<td>35</td>
<td>67, 67</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11994&gt;C</td>
<td>T4121 hom</td>
<td>I</td>
<td>Low sperm motility</td>
<td>T/W/L/W/S/Q</td>
<td>9</td>
<td>89</td>
<td></td>
</tr>
<tr>
<td>MTND5</td>
<td>12706&gt;T</td>
<td>F124L het</td>
<td>I/D</td>
<td>Leigh</td>
<td>F/F/F/F/F/F/F</td>
<td>30</td>
<td>52, 65</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12782&gt;G</td>
<td>I469S het</td>
<td>I</td>
<td>LHON</td>
<td>I/F/I/I/I/I</td>
<td>21</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12948&gt;T</td>
<td>A171V het</td>
<td>I</td>
<td>LHON</td>
<td>A/A/A/A/A/A</td>
<td>16</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13042&gt;A</td>
<td>A236T het</td>
<td>I</td>
<td>LHON-like</td>
<td>A/A/A/A/A/A</td>
<td>26</td>
<td>108</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13054&gt;C</td>
<td>M237L het</td>
<td>ND</td>
<td>MELAS/LHON/Leigh</td>
<td>M/M/M/M/M/M/M</td>
<td>23</td>
<td>57, 67</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13084&gt;A</td>
<td>S250C het</td>
<td>I</td>
<td>MELAS/Leigh</td>
<td>A/S/S/S/S/S</td>
<td>26</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13514&gt;A</td>
<td>D303G het</td>
<td>I/D</td>
<td>MELAS</td>
<td>D/D/D/D/D/D</td>
<td>37</td>
<td>52, 83</td>
<td></td>
</tr>
<tr>
<td>MTND6</td>
<td>14453&gt;A</td>
<td>A74V het</td>
<td>ND</td>
<td>MELAS</td>
<td>F/S/A/A/A/A</td>
<td>19</td>
<td>80</td>
<td></td>
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<tr>
<td></td>
<td>14459&gt;G</td>
<td>A72V het/hom</td>
<td>I</td>
<td>LDY/Leigh</td>
<td>S/A/S/A/A/A</td>
<td>37</td>
<td>37, 111</td>
<td></td>
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<tr>
<td></td>
<td>14482&gt;A</td>
<td>M64I het</td>
<td>I</td>
<td>LHON</td>
<td>A/L/L/L/L/M</td>
<td>10</td>
<td>58, 106</td>
<td></td>
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<td></td>
<td>14483&gt;C</td>
<td>M64R het</td>
<td>I</td>
<td>LHON</td>
<td>A/L/L/L/L/M</td>
<td>10</td>
<td>58, 106</td>
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<td></td>
<td>14484&gt;C</td>
<td>M64V het</td>
<td>I</td>
<td>LHON</td>
<td>A/L/L/L/L/M</td>
<td>37</td>
<td>12, 111, 112</td>
<td></td>
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<tr>
<td></td>
<td>14487&gt;C</td>
<td>M63V het</td>
<td>I</td>
<td>Dystonia/Leigh</td>
<td>M/M/M/M/M/M</td>
<td>39</td>
<td>52</td>
<td></td>
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<tr>
<td></td>
<td>14495&gt;A</td>
<td>L60S het</td>
<td>I</td>
<td>LHON</td>
<td>M/L/L/L/L/L</td>
<td>17</td>
<td>19</td>
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<tr>
<td></td>
<td>14498&gt;C</td>
<td>Y59C het/hom</td>
<td>I</td>
<td>LHON</td>
<td>Y/Y/Y/Y/Y/Y</td>
<td>17</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14596&gt;T</td>
<td>128M het/hom</td>
<td>I</td>
<td>LHON</td>
<td>M/Y/F/L/I/I</td>
<td>19</td>
<td>27</td>
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**MTCYB**

<table>
<thead>
<tr>
<th>Changes</th>
<th>Frameshift</th>
<th>het</th>
<th>D</th>
<th>PD/MELAS</th>
<th>Truncated protein</th>
<th>26</th>
<th>25</th>
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<tbody>
<tr>
<td></td>
<td>14846&gt;G</td>
<td>G43S het</td>
<td>S</td>
<td>Exercise intolerance</td>
<td>G/G/G/G/G/G</td>
<td>27</td>
<td>4, 35</td>
</tr>
<tr>
<td></td>
<td>15059&gt;G</td>
<td>A105X het</td>
<td>S</td>
<td>Mitochondrial myopathy</td>
<td>G/G/G/G/G/G</td>
<td>27</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>15084&gt;A</td>
<td>W113X het</td>
<td>S</td>
<td>Exercise intolerance</td>
<td>W/W/W/W/W/W/W</td>
<td>27</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>15100&gt;A</td>
<td>W135X het</td>
<td>S</td>
<td>Exercise intolerance</td>
<td>W/W/W/W/W/W/W</td>
<td>29</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>15168&gt;G</td>
<td>W141X het</td>
<td>S</td>
<td>Exercise intolerance</td>
<td>W/W/W/W/W/W/W</td>
<td>27</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>15242&gt;A</td>
<td>G166X het</td>
<td>D</td>
<td>Mitochondrial</td>
<td>G/G/G/G/G/G</td>
<td>30</td>
<td>48</td>
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<tr>
<td></td>
<td>15243&gt;A</td>
<td>G166E het</td>
<td>D</td>
<td>Hypertrophic cardiomyopathy</td>
<td>G/G/G/G/G</td>
<td>31</td>
<td>109</td>
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<tr>
<td></td>
<td>15257&gt;A</td>
<td>D171N hom</td>
<td>I</td>
<td>LHON</td>
<td>S/D/D/D/D/D</td>
<td>30</td>
<td>12, 111, 112</td>
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<tr>
<td></td>
<td>15497&gt;A</td>
<td>G251S hom</td>
<td>I</td>
<td>Paracrystalline inclusions with exercise intolerance</td>
<td>G/N/G/G/G/G</td>
<td>33</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>15498&gt;A</td>
<td>G251D hom</td>
<td>D</td>
<td>Exercise intolerance</td>
<td>G/N/G/G/G/G</td>
<td>23</td>
<td>6, 8</td>
</tr>
<tr>
<td></td>
<td>15579&gt;A</td>
<td>Y278C het</td>
<td>D</td>
<td>Multiple system disorder, exercise intolerance</td>
<td>Y/Y/Y/Y/Y/Y</td>
<td>25</td>
<td>113</td>
</tr>
<tr>
<td></td>
<td>15615&gt;A</td>
<td>G290D het</td>
<td>S</td>
<td>Exercise intolerance; antimycin resistance</td>
<td>G/G/G/G/G/G</td>
<td>35</td>
<td>11, 32</td>
</tr>
<tr>
<td></td>
<td>15699&gt;C</td>
<td>R318P het</td>
<td>D</td>
<td>SNHL, migraine, muscle weakness</td>
<td>K/R/Y/R/R/R/R</td>
<td>29</td>
<td>10</td>
</tr>
</tbody>
</table>

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282 Pathogenic DNA Mutations

MUSCLE & NERVE September 2007
This table lists the primary pathogenic mutations in the mRNA coding region. The LHON secondary mutations and mutations that involve conserved aminoacid changes are not listed. Somatic mutations in cancers or cell lines are also not listed. Pathogenicity scores were assigned according to the modified criteria of Mitchell et al.67 (described in Table 1). The maximum score is 40. Definite pathogenic, 30–40; probable, 20–29; possible, 10–19; unlikely, 0–9. For references changes are not listed. Somatic mutations in cancers or cell lines are also not listed. Pathogenicity scores were assigned according to the modified criteria of Table 1).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
<th>Het/ hom</th>
<th>Inherited/sporadic</th>
<th>Disease</th>
<th>Evolutionary conservation* (y/u/d/f/m/b/h) Score Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTCO1</td>
<td>6020del5</td>
<td>AELGQ&gt; AGPAX</td>
<td>het D Motor neuron disease</td>
<td>Highly conserved</td>
<td>30 22</td>
<td></td>
</tr>
<tr>
<td>6489C&gt;A</td>
<td>L196I</td>
<td>Het I seizures</td>
<td>L/L/L/L/L/L</td>
<td>29 110</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6708G&gt;A</td>
<td>G269X</td>
<td>Het S MM and rhabdomyolysis</td>
<td>G/G/G/G/G/G</td>
<td>34 51</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6721T&gt;C</td>
<td>M273T</td>
<td>Het D Acquired idiopathic sideroblastic anemia</td>
<td>M/L/MM/M/MM</td>
<td>27 36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6742T&gt;C</td>
<td>L280T</td>
<td>Het D Acquired idiopathic sideroblastic anemia</td>
<td>I/I/I/I/I</td>
<td>27 36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6930G&gt;A</td>
<td>G343X</td>
<td>Het D Multisystem disorder</td>
<td>A/G/G/G/G/G</td>
<td>35 15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7443A&gt;G</td>
<td>X&gt;GQXX</td>
<td>Hom I Deaf</td>
<td>Not conserved</td>
<td>20 73</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7444C&gt;A</td>
<td>X&gt;KQXX</td>
<td>Hom I LHON, SNHL, deaf</td>
<td>Not conserved</td>
<td>20 73, 116</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7445A&gt;C</td>
<td>X&gt;SQXX</td>
<td>Het I deaf</td>
<td>Not conserved</td>
<td>15 73</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7587T&gt;C</td>
<td>M1T</td>
<td>Het I Mitochondrial encephalopathy</td>
<td>M/M/M/M/M/M</td>
<td>35 21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTCO2</td>
<td>7671T&gt;A</td>
<td>M29K</td>
<td>Het D Mitochondrial myopathy</td>
<td>V/I/L/MM/MM</td>
<td>32 79</td>
<td></td>
</tr>
<tr>
<td>7706G&gt;A</td>
<td>A41T</td>
<td>Het I Alpers–Huttenlocher-like</td>
<td>I/G/I/I/I/A</td>
<td>27 105</td>
<td></td>
<td></td>
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<tr>
<td>7896G&gt;A</td>
<td>W104X</td>
<td>Het D Multisystem disorder</td>
<td>W/W/W/W/W/W/W</td>
<td>34 16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7970G&gt;C</td>
<td>E129X</td>
<td>Het I Encephalopathy, multisystem disorder</td>
<td>E/S/E/E/K/K/E</td>
<td>20 42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7989T&gt;C</td>
<td>L135P</td>
<td>Het S Rhabdomyolysis, exercise intolerance</td>
<td>L/L/L/L/L/L</td>
<td>30 66</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8042del2</td>
<td>155X</td>
<td>Het I Severe lactic acidosis, liver failure, neonatal death</td>
<td>Truncated protein, missing one third of C-terminal</td>
<td>28 114</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTCO3</td>
<td>9379G&gt;A</td>
<td>W58X</td>
<td>Het S Progressive myopathy, exercise intolerance, lactic acidosis</td>
<td>F/W/W/W/W/W/W/W</td>
<td>26 41</td>
<td></td>
</tr>
<tr>
<td>9438G&gt;A</td>
<td>G78S</td>
<td>Hom I LHON secondary myoglobinuria</td>
<td>N/G/G/G/G/G</td>
<td>12 63, 71</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9457del10S</td>
<td>DeFAGFF</td>
<td>Het D Leigh-like</td>
<td>Highly conserved</td>
<td>32 47</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9537insC</td>
<td>QLG&gt;PIX</td>
<td>Het/hom</td>
<td>Leigh-like</td>
<td>30 102</td>
<td></td>
<td></td>
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<tr>
<td>9738G&gt;T</td>
<td>A178S</td>
<td>Het I LHON</td>
<td>A/A/A/A/A/A</td>
<td>17 44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9952G&gt;A</td>
<td>W249X</td>
<td>Het E Encephalopathy, exercise intolerance</td>
<td>W/W/W/W/W/W/W</td>
<td>30 40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9957C&gt;T</td>
<td>F251L</td>
<td>Het I PEM, MELAS, NAON</td>
<td>F/F/F/F/F/F/F</td>
<td>27 1, 52</td>
<td></td>
<td></td>
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<tr>
<td>MTATP6</td>
<td>8851T&gt;C</td>
<td>W109R</td>
<td>Het/hom I/D BSN</td>
<td>W/W/W/W/W/W/W</td>
<td>30 26</td>
<td></td>
</tr>
<tr>
<td>8993T&gt;C</td>
<td>L156P</td>
<td>Het I/D NARP/Leigh</td>
<td>L/L/L/L/L/L</td>
<td>40 112</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8993T&gt;G</td>
<td>L156R</td>
<td>Het I/D NARP/Leigh</td>
<td>L/L/L/L/L/L</td>
<td>40 112</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9176T&gt;C</td>
<td>L217P</td>
<td>Het/hom I BSN/Leigh</td>
<td>L/L/L/L/L/L</td>
<td>35 31, 59</td>
<td></td>
<td></td>
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<tr>
<td>9176T&gt;G</td>
<td>L217R</td>
<td>Het I Leigh</td>
<td>L/L/L/L/L/L</td>
<td>37 18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9185T&gt;C</td>
<td>L220P</td>
<td>Het I Leigh</td>
<td>G/F/L/L/L/L</td>
<td>22 68</td>
<td></td>
<td></td>
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<tr>
<td>9205delTA</td>
<td>X226M</td>
<td>Het I Seizures, lactic acidemia</td>
<td>Continue CO3 reading frame</td>
<td>21 101</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

This table lists the primary pathogenic mutations in the mRNA coding region. The LHON secondary mutations and mutations that involve conserved amino acid changes are not listed. Somatic mutations in cancers or cell lines are also not listed. Pathogenicity scores were assigned according to the modified criteria of Mitchell et al.67 (described in Table 1). The maximum score is 40. Definite pathogenic, 30–40; probable, 20–29; possible, 10–19; unlikely, 0–9. For references changes are not listed. Somatic mutations in cancers or cell lines are also not listed. Pathogenicity scores were assigned according to the modified criteria of Table 1).
some very common single-nucleotide polymorphisms (mtSNPs), such as 3394T>C, 4136A>G, 4160T>C, 4216T>C, 4917A>G, and 5244G>A. These homoplasmic missense variants have pathogenic scores of less than 9.67 and are not considered disease causative by themselves. Thus, they are not listed in Table 2.

The majority of the remaining mutations in complex I are mostly heteroplasmic and are responsible for Leigh and MELAS syndromes (Table 2).24,28,30,52,57,63,80,97 These include two of the most recent reports: a sporadic mutation 4681T>C (L71P) and an inherited mutation 10197G>A (A47T) in MTND3 and MTND2, respectively.85,104 In addition, complex I mtDNA mutations may cause LHON/MELAS (3376G>A)9 or LHON/MELAS/Leigh (13045A>C) overlap syndromes.27 Furthermore, 3398T>C and 3398T>G (M1T and M1X, respectively, in the ND1 gene) were reported to play a role in sudden infant death syndrome,72 and 3644T>C (V113A, ND1) has been associated with bipolar disorder.69

The initiation methionine of the MTND1 gene is not conserved throughout evolution. The 3398T>C (M1T, ND1), having a pathogenicity score of 5, appears unlikely to be a primary mutation based on the criteria just described (Table 1). Both 3398T>C and 3398T>G mutations may cause the initiation site to shift to the next in-frame methionine residue, two amino acids downstream. However, the 3398T>G mutation has a probable pathogenicity score of 28. This could be due to the presence of the stop codon, which may mediate degradation of the mutant mRNA. Among the 15 mRNAs encoded by mtDNA, only two, ND2 and COII, contain an evolutionarily conserved initiation methionine. An initiation codon mutation in MTND2 gene has not been reported. The only mitochondrial mRNA initiation codon mutation that is definitely pathogenic is 7587T>C in the MTCO2 gene (Table 2, see also mutations in complex IV in what follows).21

Several recurrent de novo complex I mtDNA mutations causing infantile mitochondrial encephalopathy or Leigh-like diseases have been reported.52,65 Some heteroplasmic mutations, namely 10158T>C, 10191T>C (S34P and S45P, respectively, in ND3), 12706T>G, 13513G>A, 13514A>G (F124L, D393N, and D393G in ND5), and 14487T>C (M63V in ND6), have occurred either in an inherited manner or sporadically in unrelated affected probands.52,65,85 The 14487T>C mutation occurs at a highly conserved region around a mutation hot spot where mutations 14482C>A and 14482C>G (both M64I) and 14484T>C (M64V) have been reported in many LHON cases.52,58,106,111,112 However, sporadic heteroplasmic 14487T>C mutations cause dystonia and Leigh-like disease instead of LHON.52

The mtDNA 13513G>A (D393N, ND5) mutation was found to be a frequent cause of MELAS20,23,75,78,84 and Leigh-like syndromes.29 Some patients with the 13513G>A mutation may also have cardiac conduction abnormalities similar to Wolff–Parkinson–White (WPW) syndrome.83,97 Interestingly, a recurrent de novo mutation, 13514A>G, changing the same aspartic acid 393 to glycine also has been reported to cause MELAS syndrome in at least three patients.23,52,83

In addition to LHON, homoplasmic complex I mtDNA mutations can cause a heterogeneous clinical spectrum ranging from asymptomatic to severe multisystem disorders, including encephalopathy. Most remarkable is the homoplasmic 14459G>A (A72V in ND6) mutation that can variably manifest as LHON, dystonia, Leigh-like disease, and clinically unaffected, all within the same family.37,45,99 Such variable clinical features among members of a single family are unlikely due to the presence of different secondary mtDNA mutations among family members because they are from the same maternal lineage. It is likely that other nuclear modifier genes and environmental factors are involved in the pathogenesis of the 14459G>A mutation.

**EXERCISE INTOLERANCE IS THE MAJOR CLINICAL FEATURE OF MUTATIONS IN MITOCHONDRIAL CYTOCHROME b GENE**

Complex III (ubiquinol cytochrome c reductase), forming the central part of the respiratory chain and catalyzing the transfer of electrons from ubiquinol to cytochrome c, consists of 11 protein subunits. Cytochrome c, iron–sulfur protein, and cytochrome b carry the prosthetic groups.86 Among these, cytochrome b is the only protein subunit encoded by the mitochondrial genome. Complex III deficiency caused by mutations in the cytochrome b gene is characterized by exercise intolerance, episodic myoglobinuria, hypertrophic cardiomyopathy, WPW syndrome, and mitochondrial encephalopathy (Table 2).6 Mutations in mitochondrial cytochrome b gene are mostly sporadic and de novo or somatic and heteroplasmic.

Exercise intolerance is a common clinical symptom associated with mtDNA mutations in multisystem mitochondrial disorders. Establishing that exercise intolerance may be the sole manifestation of mitochondrial dysfunction in patients with sporadic disease was difficult until recent reports identified
several somatic mutations in the cytochrome b gene (Table 2).\textsuperscript{6} Almost all patients with \textit{MTCYB} mutations had severe, progressive exercise intolerance beginning at various ages. There was no ophthalmoplegia or other associated neurologic abnormalities. All patients had lactic acidosis and isolated complex III deficiency and mitochondrial myopathy with ragged-red fibers in their muscle biopsies. The mutations are nonsense mutations and small deletions that produce truncated protein polypeptides or missense mutations at evolutionarily highly conserved amino acids at the ubiquinone binding sites essential for ubihydroquinone oxidation and ubiquinone reduction.\textsuperscript{6} Several patients were reported to have myoglobinuria with progressive exercise intolerance.\textsuperscript{4,6}

Although exercise intolerance is the predominant clinical manifestation of the patients with mutations in the cytochrome b gene, heterogeneous phenotypes have also been observed. Hypertrophic cardiomyopathy was found in at least two patients with 15243G\textsuperscript{H11022}A (G166E) and 15498G\textsuperscript{H11022}A (G251D), respectively.\textsuperscript{6,109} Interestingly, mutation 15242G\textsuperscript{H11022}A, changing a glycine at position 166 to a stop codon instead of a missense substitution, was found in a patient with encephalomyopathy in addition to severe exercise intolerance.\textsuperscript{109} Although it is not entirely clear why mutations in the same amino acid would give rise to a different clinical phenotype,\textsuperscript{109} it is conceivable that a truncated protein may have a more profound effect than a mutant protein containing a missense mutation.

Multisystem disorders associated with missense mutations in the cytochrome b gene have also been reported. For example, Wibrand et al. reported a patient who harbored the 15579A\textsuperscript{G} (Y278C) mutation and exhibited deafness, mental retardation, retinitis pigmentosa, cataracts, growth retardation, and epilepsy, in addition to severe exercise intolerance.\textsuperscript{113} Other examples include a patient with the 14849T\textsuperscript{C} (S35P) mutation who suffered from septo-optic dysplasia, retinitis pigmentosa, exercise intolerance, hypertrophic cardiomyopathy, and rhabdomyolysis;\textsuperscript{88} another patient with the 15699G\textsuperscript{C} (R318P) mutation presenting with migraine and bilateral sensorineural deafness, in addition to muscle weakness;\textsuperscript{10} and an unusual parkinsonism/MELAS overlap syndrome in a young boy with a 4-bp deletion at nt14787.\textsuperscript{25}

In general, the phenotypes of the patients with cytochrome b mutations are relatively similar in comparison to the greater clinical heterogeneity in patients with complex I or complex IV mutations. Approximately 60% of patients with cytochrome b mutations have isolated exercise intolerance, 30% have multisystemic features in addition to encephalopathy and myopathy, and 10% have hypertrophic cardiomyopathy (Table 2). Some patients also have myoglobinuria.\textsuperscript{4,6} Noteworthy is the high mutation rate in cytochrome b gene, about two to three times higher than the mutation rate per kilobasepair in other protein-coding genes (Table 3). About half of the mutations in the mtDNA cytochrome b gene are nonsense mutations producing truncated proteins. The remaining missense mutations are all involved in changes at evolutionarily highly conserved amino acid residues. The high frequency of de novo and somatic mutations (17 of 20, 85%) in this gene is also consistent with the large number of polymorphisms observed in normal individuals.\textsuperscript{3} Furthermore, the majority (15 of 20, 75%) of the pathogenic mutations are involved in the transition of adenine for guanine, suggesting somatic oxidative DNA damage may be the cause of the mutations.

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|c|}
\hline
Complex & Size & Number & No. of definite & Frequency of & & & & \\
& (bp) & of mutations & and probable mutations & nonsense, frameshift, & Definite & Probable & Possible & Unlikely \\
& & & (per kb) & or deletion mutations & & & & \\
\hline
I (NDs) & 6350 & 41 & 4.1 & 1/41 = 0.024 & 18 & 8 & 12 & 3 \\
III (cyt b) & 1141 & 20 & 17.5 & 9/20 = 0.45 & 7 & 13 & 0 & 0 \\
IV (CO1, 2, 3) & 3010 & 22 & 6.6 & 11/22 = 0.50 & 12 & 8 & 2 & 0 \\
V (ATP6, ATP8) & 841 & 8 & 10 & 0 & 5 & 3 & 0 & 0 \\
Total & & 91 & & & 42 & 32 & 14 & 3 \\
\hline
\end{tabular}
\caption{Frequencies of pathogenic mutations in mtDNA-encoded complexes.}
\end{table}

\textit{ND}, NADH dehydrogenase; \textit{CO}, cytochrome c oxidase.
DIVERSE CLINICAL SPECTRUM IS ASSOCIATED WITH MUTATIONS IN mtDNA ENCODING COMPLEX IV SUBUNITS

Cytochrome $c$ oxidase (COX), also referred to as complex IV, catalyzes the last step in the electron transport chain, resulting in the reduction of molecular oxygen to water.\(^{17}\) Human complex IV consists of 13 protein subunits, of which subunits I, II, and III (COI, COII, and COIII) form the catalytic core of the enzyme where the two heme A moieties ($a$ and $a_3$) and two copper centers (CuA and CuB) are involved in the redox reaction.\(^{17,103}\) Subunits COI, COII, and COIII are encoded by the mitochondrial genome, whereas the remaining 10 subunits are encoded by the nuclear genome. In addition to its catalytic role, the largest subunit, COI, acts as a scaffold protein.\(^{103}\) It contains 12 transmembrane helices that surround two heme groups and one CuB site. The N-terminal helix-hairpin of COII is anchored to the mitochondrial inner membrane with its C-terminal hydrophilic domain protruding into the intermembrane space. This domain contains the CuA center with several charged amino acids; it serves as the docking site for cytochrome $c$ and provides a hydrophilic passage for water molecules.\(^{17,103}\)

A role for COIII in proton pumping has been suggested, but deletion mutants of COIII lead to accumulation of assembly intermediates of COX.\(^{38}\) Additional experiments have shown that the COIII subunit does not play a direct role in energy conversion by COX, but rather is involved in COX assembly and stabilization of the entire complex.\(^{39,77}\)

In general, mutations in nuclear genes involved in complex IV assembly cause severe infantile Leigh syndrome.\(^{30,92}\) The clinical presentations of patients with mutations in mtDNA-encoded COX subunits are arguably the most variable among the phenotypes of RC complex deficiencies caused by mutations in mtDNA-encoded genes (Table 4). The clinical features vary from the myopathy and exercise intolerance\(^{41,42,46,47,51,79,92}\) typical of cytochrome $b$ deficiency to severe, early-onset multisystem disease or Leigh syndrome, more typical of complex I and complex V deficiency (Table 4).\(^{15,16,21,114}\)

Although primary respiratory chain dysfunction is a rare cause of rhabdomyolysis, among the 21 mutations in mtDNA complex IV subunit coding regions (excluding the mutation at the termination codon of COI), 5 (5920G$\rightarrow$H11022, 6708G$\rightarrow$H11022, 7989T$\rightarrow$H11022C, 9487del15, and 9789T$\rightarrow$H11022C) cause rhabdomyolysis and recurrent myoglobinuria in addition to myopathy and exercise intolerance (Table 2).\(^{42,46,47,51,66}\) The age of onset in this group is 15–33 years. The mutations are sporadic and occur in muscle tissue. The molecular defects so far reported with rhabdomyolysis are mostly localized to mtDNA-encoded complex IV subunits, except for a couple in the mitochondrial cytochrome $b$ gene.\(^{4,6}\) Mitochondrial myopathy without rhabdomyolysis also occurs. Rahman et al. reported a 14-year-old boy with proximal myopathy and lactic acidosis who carried the missense mutation 7671T$\rightarrow$H11022A (M29K) in COII.\(^{79}\) In this patient, single-fiber analysis showed a good correlation between mutant load and COX activity. Higher mutant loads were found in COX-negative muscle fibers than in COX-positive fibers. Immunohistochemistry and immunoblot analyses showed severe reduction in COII staining.\(^{79}\) Horvath et al. reported a heteroplasmic mutation, 9379G$\rightarrow$H11022A (W58X), generating a premature stop codon in the COIII subunit, in a 6-year-old boy who presented with a relatively mild, slowly progressive myopathy with exercise intolerance, lactic acidosis, growth delay, numerous ragged-red fibers, lipidosis, and severe COX deficiency in skeletal muscle.\(^{41}\) Although this was a nonsense mutation truncating $\geq 80\%$ of the protein and the mutant load in skeletal muscle was relatively high ($93\%$), this sporadic somatic mutation was absent in the patient’s blood or hair follicles, and the patient had only mild myopathy.\(^{41}\)

Encephalomyopathy and multisystem disorders are also clinical features of complex IV defici-
A novel heteroplasmic 7587T>C (MIT in COII) mutation was found in a family with gait ataxia. This was an inherited case of mitochondrial COX subunit mutation, where an affected mother transmitted the mutation to her son who was similarly affected in early childhood and became wheelchair bound by age 25 years. In addition to progressive gait abnormality, he had bilateral optic atrophy, pigmentary retinopathy and mild muscle wasting, and was severely impaired cognitively.

The 7587T>C mutation in the MTCO2 gene is the only mitochondrial mRNA initiation codon mutation that is definitely pathogenic.21 The mutant load was present at a higher level in muscle from the affected son (91%) than in muscle from his mother (67%). Single muscle-fiber analysis revealed a good correlation between the levels of mutant load and COX activity. In COX-positive fibers, the mutant load was 17%–52%, whereas in COX-negative fibers the wild-type mtDNA was undetectable.21 Because the mutation changes the initiation methionine of COII to threonine, the initiation of translation could occur either at the next initiation site (AUG), which is out of frame and would yield a five-residue polypeptide, or at the next in-frame initiation site (AUA) 16 codons downstream, which would yield an amino-terminally truncated protein, COII. Mitochondrial protein synthesis and immunoblotting analysis did not detect any COII protein, consistent with the absence of translation of mutant COII mRNA. Furthermore, COII mRNA levels were substantially lower in the patient’s fibroblasts, presumably due to the instability of the mutant transcript leading to the absence of translation.

Encephalopathy and exercise intolerance was also reported in a woman with disease onset at age 21 years. She had the first reported nonsense mutation in mtDNA, 9952G>A (W249X, COIII). As mentioned previously, the COIII subunit does not play a direct role in the electron transfer reaction of COX, but is involved in the assembly and stabilization of the entire complex. This hypothesis was supported by the finding of a near-homoplasmic frameshift mutation, 9537insC, in the MTCO3 gene, in a girl with severe lactic acidosis and Leigh-like lesions of the putamina. Studies suggest that in the absence of COIII there is a reduction in the amount of correctly assembled complex IV; however, the residual complex exhibits a similar Km to the wild-type complex. Thus, mutations in the MTCO3 gene cause a quantitative defect of COX holoprotein, rather than qualitative abnormality of the catalytic activity of the COIII-defective enzyme. This explains why all mutations in the MTCO3 gene cause a relatively mild clinical phenotype, despite the severity of the mutations.

Multisystem disorders have been observed in patients with mutations in mitochondrial COX subunit genes. These include 6930G>A (G170X, COI) in a woman with cataracts, sensorineural hearing loss, myoclonic epilepsy, cerebellar ataxia, optic atrophy, and muscle weakness; 7970G>T (E129X, COII) in a man with cataracts, sensorineural hearing loss, myopathy, ataxia, cardiac arrhythmia, depression, and short stature; 7896G>A (W104X, COII) in a 1-year-old patient with multisystem disorder, and two reports of 9957T>C (F251L, COIII) in patients with MELAS, progressive encephalomyopathy, and non-arteritic ischemic optic neuropathy.

Two patients with mutations in the MTCO2 gene, 7706G>A (A41T in COI) and 8042delAT (155X), were reported to have liver dysfunction. Both patients had onset of disease in infancy. The patient with a heteroplasmic 7706G>A mutation had liver pathology and a disease phenotype similar to that reported for Alpers–Huttenlocher syndrome. However, the nuclear gene, DNA polymerase gamma, responsible for Alpers syndrome, was not investigated. Notably, the mutated amino acid was not evolutionarily conserved and the phenotype was more severe than the missense substitution (alanine to threonine) would have predicted. The patient died at 6 years of age of an unexpected cardiac arrest. A second patient with an 8042delAT mutation died of severe lactic acidosis and liver failure at 10 days of life. He had a much more severe clinical course than could be explained by the level of mutant loads (20% in skeletal muscle). Another case was a girl with a 6489C>A mutation (L196I) in COI. The patient had severe seizures starting at age 3 years. At the age of 5 years, hepatic failure occurred after 2 months of valproate therapy, which resulted in a therapeutic switch to lamotrigine. The patient died of intractable seizures and respiratory failure at the age of 17 years. The leucine-to-isoleucine change occurs at a highly conserved position 196, but it involves amino acids from the same class and the substitution would be predicted to cause little effect. Furthermore, the mutant heteroplasmic was also present in >90% in unaffected relatives. Thus, the primary pathogenic role of the mutation did not appear to be convincing. These three cases remind us of the consideration of nuclear genes, when severe clinical features are more consistent with autosomal-recessive disorders. Analysis of the recently discovered nuclear genes, POLG1, DGUOK, and MPV17, which are responsible for the infantile hepatocerebral form of mtDNA depletion syn-
drome, may be helpful in understanding the molecular etiology of these cases.

Although COX deficiency primarily affects muscle and the nervous system, Gattermann et al. described the identification of two heteroplasmic missense mutations in the MTCO1 gene in two adult patients, a 58-year-old woman and a 68-year-old man with acquired idiopathic sideroblastic anemia. These two mutations, 6721T>C (M273T) and 6742T>C (I280T), are located in the seventh transmembrane helix of COI, in the immediate vicinity of the heme-a3-CuB center. It has been speculated that this metal redox center is involved in the reduction of ferric iron to ferrous iron, which is essential to the last step of mitochondrial heme biosynthesis.

In addition to the heterogeneous clinical phenotype just described, mutations in the termination codon of COI, nt7443–7445, are one of the major causes of maternally inherited sensorineural hearing impairment. Four mutations have been reported at this termination codon, 7443A→G (514X>GQKX), 7444G→A (514X>QKQX), 7445A→C (X>SQKX), and 7445A→G (X>X). Three of these mutations, resulting in the extension of three amino acids at the end of COX subunit I, are probably not the main cause of the disease, because the last few amino acids in this protein are not conserved in other organisms. In fact, in some organisms, the C-terminal is three amino acids longer than the human sequence. Several reports support the hypothesis that the effect of these mutations is at the level of processing and stabilization of the precursor tRNA\textsuperscript{ser(UCN)}, which is adjacent to the COI subunit gene and encoded by the light strand of mtDNA. The mutations at the 3’ end of COI, overlapping with the 3’ end of the precursor tRNA\textsuperscript{ser(UCN)}, are predicted to affect the secondary structure at the 3’ tRNA cleavage site for processing by tRNase\textsuperscript{ZL}. Studies using mitochondrial hybrids containing homoplasmic 7445A→G mutant have demonstrated a decrease in the steady-state level of tRNA\textsuperscript{ser(UCN)} and aminoacylation of tRNA\textsuperscript{ser(UCN)}. Furthermore, there was a reduction of ND6 mRNA, which is transcribed in the same RNA precursor as that of tRNA\textsuperscript{ser(UCN)}. Unlike other mutations in COX subunits, these mutations are all homoplasmic in patients. Although the mutation is at the termination codon of the COI gene, pathogenesis likely reflects altered processing of the tRNA\textsuperscript{ser(UCN)} precursor rather than the translational product of the COI gene.

The majority (14 of 22, 64%) of the mutations in mitochondrial-encoded COX subunit genes are sporadic and heteroplasmic, found in the affected tissues of the proband and not in any of the matrilineal relatives. As shown in Table 3, 50% of the mutations in complex IV mtDNA-encoded subunit genes are nonsense or frameshift mutations that result in defective truncated proteins lacking function, compared to only 2.4% of complex I mtDNA subunits being nonsense mutations (Table 3). The missense mutations in mtDNA COX subunit genes are all located in evolutionarily highly conserved regions.

Remarkably, the three mitochondrial COX subunits are the most highly conserved among all the 13 mtDNA-encoded polypeptides in terms of structure and function throughout evolution due to their roles as the redox center, the binding pocket for the prosthetic groups, and the passage for water molecules. About 29–47% of the amino acids in COXI, II, and III subunits are invariable from yeast to human, compared to 1.7–22.3% in mtDNA complex I subunits, and 3% and 15% for ATP6 and ATP8 genes (Table 5). Cytochrome b is also highly conserved, with 32.4% of the amino acids unchanged throughout evolution (Table 5).

### Table 5. Percentage of amino acids residues conserved throughout evolution in mitochondrial-encoded mRNA genes.

<table>
<thead>
<tr>
<th>Complex subunit</th>
<th>Total number of amino acids</th>
<th>Number of amino acids conserved from yeast to human</th>
<th>% conserved amino acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND1</td>
<td>318</td>
<td>71</td>
<td>22.3</td>
</tr>
<tr>
<td>ND2</td>
<td>347</td>
<td>6</td>
<td>1.7</td>
</tr>
<tr>
<td>ND3</td>
<td>115</td>
<td>20</td>
<td>17.5</td>
</tr>
<tr>
<td>ND4</td>
<td>459</td>
<td>58</td>
<td>12.6</td>
</tr>
<tr>
<td>ND4L</td>
<td>98</td>
<td>6</td>
<td>6.1</td>
</tr>
<tr>
<td>ND5</td>
<td>603</td>
<td>81</td>
<td>13.4</td>
</tr>
<tr>
<td>ND6</td>
<td>174</td>
<td>6</td>
<td>3.4</td>
</tr>
<tr>
<td>ND13</td>
<td>380</td>
<td>123</td>
<td>32.4</td>
</tr>
<tr>
<td>ND8</td>
<td>513</td>
<td>242</td>
<td>47.2</td>
</tr>
<tr>
<td>ND3</td>
<td>227</td>
<td>65</td>
<td>28.6</td>
</tr>
<tr>
<td>ND4</td>
<td>260</td>
<td>80</td>
<td>30.8</td>
</tr>
<tr>
<td>ATP6, ATP8</td>
<td>226</td>
<td>34</td>
<td>15.0</td>
</tr>
<tr>
<td>ATP8</td>
<td>68</td>
<td>2</td>
<td>2.9</td>
</tr>
</tbody>
</table>

**Leigh Syndrome is the Most Prevalent Clinical Presentation in Patients with Mutations in Mitochondrial Complex V Subunits**

There are 13 protein subunits in complex V, the ATP synthase. Two of these, ATP6 and ATP8, are encoded by the mitochondrial genome. These two genes overlap by 46 nucleotides at the 3’ end of the MTATP8 gene and the 5’ end of the ATP6 gene. The precursor transcript is a bi-cistronic mRNA unit (RNA14) encoding ATPase 8 and ATPase 6 with two different reading frames. Because these two subunits are in-
volved in ATP synthesis, most of the mutant proteins appear to affect the brain predominantly. The most common mutations are 8993T>C (L156P) and 8993T>G (L156R), which are heteroplasmic and cause a continuous clinical spectrum of retinitis pigmentosa, NARP (neuropathy, ataxia, retinitis pigmentosa), and Leigh syndrome. In general, the disease is more severe in patients with the 8993T>G change than those with the 8993T>C change. Several other heteroplasmic mutations, 8851T>C (L222P), 9176T>G (L217P), 9185T>C (L220P), and 9191T>C (L222P), have also been reported. Interestingly, with the exception of 8851T>C, all of these mutations involve leucine-to-proline or leucine-to-arginine substitutions, and the clinical phenotype is consistently Leigh syndrome. The 8851T>C mutation, changing a highly conserved hydrophobic tryptophan residue to a basic amino acid arginine, has been found in patients with bilateral striatal necrosis. All mutations occur at an evolutionarily highly conserved amino acid residue. A very unusual frameshift deletion of two basepairs (9205delTA, X226M), which removes the termination codon for the bi-cistronic RNA unit encoding ATP8 and ATP6, RNA14, and extends to the COIII subunit reading frame, was identified in a patient with seizures and lactic acidosis. The removal of the termination codon for MTATP6 brings MTCO3 immediately in frame. Accurate processing still occurs, but the steady-state level of RNA14 is markedly reduced. It was found that the majority of mutated RNA14 terminated with short poly(A) and deadenylation occurred rapidly. In the absence of translation termination codons, release factors cannot bind, the mitoribosome proceeds through the poly(A) tail, and the transcript is recognized as aberrant and rapidly degraded.

CONCLUSIONS

This review has applied a scoring system to evaluate the pathogenicity of reported mutations in protein-encoding genes of the mitochondrial genome. Known secondary LHON mutations have not been included.

Based on the scoring system described in Table 1, all 91 mutations listed in Table 2 were scored. Among them, 42 scored definite, 32 probable, 14 possible, and 3 unlikely pathogenic (Table 3). Interestingly, the mutations that score possible or unlikely for their pathogenicity are all homoplasmic LHON-associated mutations except 11994C>T, which is related to low sperm motility. All except one are in complex I. If only definite and probable pathogenic mutations are considered, the MTCTB gene has the highest mutation rate per kilobase, 17.5, compared to 10, 6.6, and 3.8 for complex V, IV, and I genes, respectively. A total of 74 mutations have a score of 20 and above. Among them, 10 mutations occur as homoplasmic or homoplasmic/heteroplasmic. Disease expression of these homoplasmic mutations is apparently modulated by nuclear-encoded tissue-specific factors. The majority (64 of 74, 86%) of the pathogenic mutations are heteroplasmic. The frequency of nonsense, frameshift, and deletion mutations is highest in the COX subunit genes at 50%, compared to 45% in the MTCTB gene, 2.4% in the complex I subunit genes, and 0% in complex V subunits. Thus, although the complex I genes occupy about 40% of the entire mitochondrial genome, they have the lowest rates of pathogenic mutations per kilobase. These differences in frequency and type of mutations may reflect inherent properties of the sequence composition of these genome regions or a bias of ascertainment in the ensuing clinical phenotypes.

As shown in Table 4, mutations in complex IV subunits cause the most diverse clinical phenotype, which varies from myopathy/exercise intolerance to multisystem disorders. Although patients with mutations in mitochondrial encoded complex I subunit genes also have diverse clinical phenotypes, it is predominantly these patients who have LHON and encephalopathy with multisystem disorders. Mutations in the MTCTB gene typically cause myopathy and exercise intolerance, whereas mutations in MTATP6 usually cause Leigh syndrome.

A review of the evolutionarily conserved amino acid residues in each mtDNA-encoded gene suggests that complex IV subunits are the most evolutionarily conserved proteins, followed by the cytochrome b subunit of complex III (Table 5). Thus, it is conceivable that mutations in these subunits are less tolerated, consistent with the high frequency of somatic heteroplasmic mutations. In contrast, complex I subunits are the least conserved and have higher tolerance for high heteroplasmic or homoplasmic missense alterations, resulting in a high frequency of mutations in complex I subunit genes categorized as possible or unlikely pathogenic.

Both heteroplasmic and homoplasmic mutations can be pathogenic, although the majority are heteroplasmic. Most of the mutations in the MTCTB gene are sporadic or somatic (17 of 20, 85%) (Table 6). The majority (14 of 22, 64%) of mutations in COX subunit genes are also sporadic (Table 6). Thus, for the molecular or biochemical diagnosis of an isolated mitochondrial myopathy case, muscle tissue,
tRNAser(UCN) gene rather than translation of the post-transcriptional processing of the adjacent stop codon of the mRNA and is rapidly degraded. The mutations at the for the binding of the translation release factors. However, a transcript without a termination codon into the same reading frame. Mutations in both initiation and termination codons have been found. The amino acid sequences at the N-terminal region in most of the mitochondrial encoded protein subunits are not highly conserved. Two initiation codon mutations, 7587T>C (M1T) in the MTCO2 gene and 3308T>G (M1X) in the MTND1 gene, have been reported. Mutation at the initiation codon leads to a rapid decay of the mutant transcript. Two termination codon changes have been reported. The 9205delTA in the MTATP6 gene brings MTCO3 gene and 3308T>G (M1X) in the MTND1 gene, have been reported. Two termination codon changes, 7443A>G, 7444G>A, 7445A>C, and 7445A>G, affect the post-transcriptional processing of the adjacent tRNA\[^{\text{UCN}}\] gene rather than translation of the mRNA of the MTCO1 gene. Finally, mutations in the MTCO3 gene do not alter the catalytic activity of complex IV, but instead reduce the amount of the correctly assembled COX subunits.

In conclusion, application of the scoring system allows for assessment of the pathogenic significance of a mutation in mRNA coding regions. Although the clinical spectrum of patients with mtDNA mutations in mRNA coding regions is heterogeneous, mutations in each different complex may be associated with a predominance of certain disease syndromes. However, a strict genotype–phenotype correlation cannot be applied. Although the molecular and genetic characteristics of the mutations differ in different complexes, the specific mechanisms that account for this variability remain to be established.

### Table 6. Inherited and sporadic mutations.

<table>
<thead>
<tr>
<th>Complexes</th>
<th>Predominant clinical features</th>
<th>Total number of mutations</th>
<th>Hom or het/hom* (LHON mutations)</th>
<th>Inherited or inherited/de novo(^{\dagger})</th>
<th>De novo or maternal germ cells</th>
<th>Somatic in post-mitotic tissues</th>
<th>Family members not available for the determination of inheritance</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>LHON, Leigh, multisystem</td>
<td>41</td>
<td>16 (12)</td>
<td>32</td>
<td>4</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>III</td>
<td>Myopathy, exercise intolerance</td>
<td>20</td>
<td>2 (2)</td>
<td>2 (LHON)</td>
<td>7</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>IV</td>
<td>Myopathy, encephalomyopathy, multisystem</td>
<td>22</td>
<td>3 (2)</td>
<td>8</td>
<td>8</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>V</td>
<td>Leigh</td>
<td>8</td>
<td>3 (0)</td>
<td>7</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>91</td>
<td>24</td>
<td>48</td>
<td>20</td>
<td>17</td>
<td>6</td>
</tr>
</tbody>
</table>

*Mutations occur in homoplasmy (hom) or both heteroplasmy (het) and homoplasmy (hom).\(^\dagger\)Mutations occur as either inherited or sporadic.

### REFERENCES


Myotonic dystrophies, the most common type of adult-onset muscular dystrophy, are dominantly inherited disorders characterized by muscle weakness and atrophy, myotonia, and cataracts, together with involvement of a number of different organs, including the brain. Myotonic dystrophy type 1 (DM1) is caused by an expanded (CTG)n repeat (from 37 to several thousands) within the noncoding 3’ untranslated region of the myotonic dystrophy protein kinase (DMPK) gene\(^9,35,61\) on chromosome 19q35. Myotonic dystrophy type 2 (DM2) is caused by an expanded CCTG repeat (from 75 to 11,000 repeats) in the first intron of the zinc finger protein 9 (ZNF9) gene on chromosome 3q21.25,60,95,96

Fascinating aspects of the myotonic dystrophies are the multisystem involvement and the way that two unrelated genes cause strikingly similar yet distinct phenotypes. Both DM1 and DM2 manifest signs of myotonia, muscle weakness, and early-onset cataracts. In addition, there is often testicular atrophy, frontal balding, insulin resistance, and cardiac conduction defects, as well as occasionally dilated cardiomyopathy.\(^41,70,97\) There is, however, one key clinical difference between DM1 and DM2: the degree of cognitive impairment. The DM1 locus presents a severe congenital form of mental retardation, which is not present in DM2. In addition, whereas personality changes, visual-spatial defects, and behavioral problems have been described mainly in the juvenile and adult-onset forms of DM1, it is only in the last few years that there have been reports of cognitive and behavioral deficits in DM2, and these may be captured only by specific neuropsychological tests.

Considering these results and the absence of a congenital form, there is still controversy as to the clinical significance of the cognitive and behavioral abnormalities reported in patients with DM2.

Recent advances in molecular genetics in vitro and using mouse models have shed light on the pathophysiology of myotonic dystrophies.\(^8,17,44,54,59,63,64,83,89,116–119\) The current model is that expression of RNA transcripts containing pathogenic repeat lengths produces defects in alternative splicing of multiple RNAs by sequestering specific repeat-binding proteins, ultimately leading to the expression of splice products that are developmentally inappropriate for a particular tissue. This provides a fascinating rationale for many of the multisystem features of DM1 and
The occurrence of mental retardation in the congenital form and behavioral abnormalities in the juvenile form is unquestionable. In such patients the brain is undoubtedly involved primarily in the disease process.

Congenital DM1. Congenital DM1 is associated with (CTG)n expansions greater than 1,000. It occurs in the offspring of mothers affected with myotonic dystrophy. Congenital myotonic dystrophy is the most severe presentation of DM1, not only from a muscle and respiratory perspective, but also because of the degree of cognitive involvement. In contrast with the classic adult form, neuromuscular involvement and arrhythmias are not a major concern. Myotonia is not even present at birth and cardiovascular involvement is not a common problem.  

Onset is in the prenatal period. In almost half of the pregnancies reported, fetal movements are reduced and polyhydramnios occurs. A small percentage of premature births occur. After birth, one prominent sign is delay in motor milestones. Hypotonia results in a floppy baby and immobility. Failure to thrive is due to an inability to suck that affects almost all patients as a result of bilateral facial and jaw muscle weakness. This is typically present at birth, giving rise to a characteristic facial appearance: the newborn baby keeps the mouth open with a tented upper lip and a high-arched palate. Respiratory distress may be the presenting symptom in up to 50% of patients. This is the result of different factors including intercostal and diaphragmatic muscle weakness, pulmonary immaturity, aspiration pneumonia, and failure of respiratory control. In nearly half of the reported cases talipes is present, most often bilateral and in the equinovarus position. Contractures may be present and athrogyrosis multiplex may be an associated finding. Other dysmorphic features and organ malformations may be present. There is no clinical or electromyographic (EMG) myotonia. Tendon reflexes are usually absent.

As time passes muscle symptoms improve but the delayed motor development becomes prominent, closely paralleling the degree of hypotonia. In those patients who survive the early weeks of life the prognosis is one of steady improvement throughout early childhood. Almost all children eventually become able to walk independently.

Mental retardation is observed in a number of patients and does not parallel motor retardation. Intelligence quotient (IQ) levels varying between 40 and 80 are present in 50%-90% of patients. Regardless, patients are able to take care of themselves throughout adult life with no evidence of a gross deterioration in intellectual function. Speech development is delayed because of hypotonia and the weakness of facial and jaw muscles as well as by delayed psychomotor learning abilities. Behavioral abnormalities such as hyperactivity attention deficit, autistic behavior, and difficulty in social relationships with peers and family members are frequently reported in the preschool age group. Neuroimaging studies in these patients reveal ventricular dilatation in more than 50% of patients, which is present at birth, thus supporting a prenatal origin for the mental retardation.

The congenital form differs from the classic form not only clinically, but also from a biomolecular view. Specifically, there are several distinct features in congenital DM. The first is aberrant methylation at the DM1 locus. Dinucleotide CpGs in the region of the CTG repeat are aberrantly methylated only in congenital DM. The second is that increased levels of DMPK transcripts have been found.
in the congenital form, whereas in the adult form these levels are typically decreased.\textsuperscript{24,104} Congenital myotonic dystrophy is only transmitted by affected mothers and there is a suggestion of maternal imprinting; paternally transmitted cases do not occur even if the CTG repeats are similar in size.\textsuperscript{13}

**Childhood and Juvenile Onset.** In DM1 a childhood or juvenile onset implies a different clinical presentation than in the classic adult form. In the juvenile form, CNS symptoms predominate over muscle symptoms.

Unlike the congenital form, transmission is maternal or paternal. Symptoms are present in childhood before the age of 10, and there are usually no prenatal signs. Motor symptoms are typically mild, and motor development is usually normal or delayed only slightly. There may be some degree of distal weakness and atrophy as in the classic adult form, as well as mild signs of clinical myotonia.\textsuperscript{40,41} The major features, however, are behavioral and there is usually a paucity of neuromuscular or cardiorespiratory symptoms. Learning disabilities and difficulties in relationships with peers generally become evident during school age. Cognitive impairment has been reported with IQ below normal for age.\textsuperscript{31,115,125} Children may require teacher assistance and manifest autistic behavior, lack of interest, and inhibition that link to the adult-onset dysexecutive syndrome. Attentional deficit hyperactive disorder is frequent and is often diagnosed in preschool children, before evolving into anxiety disorders in childhood and young adulthood. Excessive daytime sleepiness and fatigue may be present in this form as in the classic adult form,\textsuperscript{93,113} suggesting that complaints of fatigue and somnolence should be investigated by polysomnography to look for sleep apnea syndrome and periodic limb movements.

Conventional neuroimaging is often normal but quantitative studies on a large number of patients are lacking. There may be ventricular dilatation and sulcal enlargement but, unlike the congenital form, these are not prominent. The white mater hyperintense lesions that are seen in the adult form are infrequent.\textsuperscript{27,41}

**Adult-Onset DM1.** More than 50\% of patients with classic DM1 are referred because of excessive daytime sleepiness\textsuperscript{55,90,102} or fatigue.\textsuperscript{19} Patients usually minimize their symptoms, do not keep outpatient appointments, and seem unconcerned about their health. The general clinical impression in most cases is one of apathy, decreased emotional participation, and psychomotor delay.

The result of neuropsychological tests provides some evidence for this. Neuropathology and neuroimaging studies provide further support for the clinical impression of brain involvement in the classic adult form of DM1 (Table 1).

**Neuropsychological Evidence.** **Global Intelligence.**

Patients may give the impression of having reduced overall intelligence because of the delay in motor responses related to muscle impairment, the facial expression resulting from bilateral ptosis and facial muscle weakness, and the lack of initiative that may predominate during an examination. Scores on overall IQ as measured by the Wechsler Adult Intelligence Scale (WAIS) are within the normal range in patients with DM1,\textsuperscript{11,15,20,87,91,122,132,134} although lower than age- and education-matched controls. In a subset of patients with moderately severe DM1 (CTG range 500–700), we have found reduced IQ values in one-third of patients irrespective of the degree of muscle disability.

On Mini Mental State Examination (MMSE), scores are also within the normal range, although lower than age- and education-matched controls.\textsuperscript{71,72,78}

These studies, although interesting, are limited by methodological restrictions and especially by the variability in disability range and the molecular status of the patients considered. Whether measurements of global intelligence correlate with CTG size is a controversial issue. Some authors have concluded that CTG size correlates with IQ or MMSE scores and thus may be of predictive value,\textsuperscript{23,47,65,88,101,122} but others have not found any correlation.\textsuperscript{71,72,78}

**Visual-Spatial Deficits.** Visual-spatial impairment is appreciated when patients with DM1 are asked to perform everyday tasks requiring the assembly of elements (e.g., dice, puzzles, or similar things), to draw two-dimensional (2D) or three-dimensional (3D) figures, or to make a spontaneous drawing of a single figure. Patients may then admit to having decreased ability to draw 2D or 3D shapes and figures or to join up elements of a figure to form a complete figure. There may be impairment in spatial orientation as shown by map reading, target reaching on a specific trajectory through the quickest route, or constructional ability. Solving maze-like problems may constitute a limitation. In the workplace, visual-spatial impairment may translate into a certain difficulty in aligning numbers in the correct rows and putting items in appropriate columns. From a neuropsychological perspective, visual-spatial deficits are detected when patients are asked to copy a complex figure while maintaining its correct intersections and angles (Rey copy and recall). There is
general agreement that patients with DM1 present deficits in visual-spatial performance\textsuperscript{14,57,71,125} because their scores on neuropsychological tests are significantly lower than in age- and education-matched controls. Modoni et al.\textsuperscript{78} strengthened this conclusion in a recent study performed on a larger sample of patients stratified by CTG size and demonstrated that, despite small expansions, visual-spatial deficits characterize the neuropsychological profile of adult patients with DM1 in contrast with the more general intellectual impairment of patients with congenital forms.

\textbf{Attentional Deficits.} Several investigators have described attention deficits assessed by neuropsychological test scores as below the normal range compared to normal controls.\textsuperscript{78,153} In our experience, a significant difference was observed in test scores for attention ability between patients with moderately severe DM1 and controls.\textsuperscript{72}

\textbf{Verbal Fluency.} Weakness of facial, jaw, and palatal muscles, often in combination, results in a speech disturbance that may be a concern for the patient and family owing to the resulting difficulties in communication. Additional factors may also aggravate speech production, such as tongue and jaw muscle myotonia and structural malformations such as jaw subluxation and malocclusion, which are present more frequently than in the general population. Despite these peripheral causes of speech dysfunction, language function seems to be normal in patients with adult DM1. Normal performance on verbal function tests in patients with DM1 using the Token Test and the Controlled Association Letters and Categories Test has been described.\textsuperscript{71,72}

\textbf{Behavior. Dysexecutive Syndrome.} Reduced initiative and inactivity are to be expected in any chronic muscle disease. However, clinical experience suggests that this is a prominent and consistent finding in patients with DM1 as compared to patients with other muscular dystrophies and similar or even worse muscle weakness and functional limitations. Frontal lobe motor areas and the prefrontal cortex are involved in strategic planning tasks. Earlier reports had documented a significant impairment of frontal lobe function,\textsuperscript{14,24} as is typical of a dysexecutive syndrome. Several authors tried to correlate neuropsychological and neuroimaging findings.\textsuperscript{6,24,27,28,45,62,101} Meola et al.\textsuperscript{71,72} confirmed a selective impairment on tests of frontal lobe function in DM1 and suggested that this type of deficit does not correlate with cortical atrophy and white matter hyperintense lesions, but might be associated with fronto-temporal lobe hypoperfusion on PET studies.

Although interesting, these studies were limited by the lack of quantitative magnetic resonance imaging (MRI) studies. Other studies have confirmed a selective involvement of frontal lobe function.\textsuperscript{15,36}

\textbf{Apathy.} Apathetic temperaments are often reported by family members of patients with DM1. It is another aspect of the frontal dysexecutive syndrome reported above. Rubinsztein et al.\textsuperscript{102} applied a specific rating scale for apathy and demonstrated that apathy was significantly greater in patients with DM1 than in normal controls or patients with Charcot-Marie-Tooth disease. This was not related to the degree of muscle impairment or the duration of illness.

\textbf{Anxiety.} There are conflicting data as to the occurrence of anxiety in DM1,\textsuperscript{10,20,22} although there are suggestions that DSM-IVR Axis I disorders are frequent in DM1 as in the general population.\textsuperscript{41} Meola et al.\textsuperscript{71} demonstrated that anxiety was not a feature of a subset of patients with DM1 subjected to a battery of neuropsychological tests and psychiatric interviews. They concluded that the abnormal performance found after specific tests for frontal lobe function could not be attributed to anxiety or similar affective disturbances.

\textbf{Depression.} Depression does not seem to be increased in patients with myotonic dystrophy compared to other patients with muscular dystrophies,\textsuperscript{29} although some reports describe a higher frequency in DM1.\textsuperscript{22} Meola et al.\textsuperscript{71} investigated patients with DM1 and did not find a higher frequency of depression.

\textbf{Personality Patterns.} Whether patients with DM1 have specific personality patterns is debatable.\textsuperscript{7,12,26,33,72} It is common clinical experience that patients with DM1 have characteristic personalities: they tend to be either obsessive in their health-related care, continuously consulting their referring physician, or avoidant and passive in their attitudes toward health care. In an initial study by Bird et al.,\textsuperscript{7} one third of a small series of patients had prominent abnormalities, but these were considered to be the natural consequence of their motor and cognitive impairment. In a later study, Delaporte\textsuperscript{26} described a specific personality pattern in patients with DM1. Obsessive-compulsive, avoidant, and passive-aggressive behavior were prominent in these patients. More recently, Meola et al.\textsuperscript{71} confirmed these results and suggested that there may be a homogeneous avoidant personality profile in patients with DM1. In a minority of patients tested by Delaporte\textsuperscript{26} but in none of those tested by Meola et al.,\textsuperscript{71} the scores reached a pathological level for personality disorder. Winblad et al.\textsuperscript{135} applied the Temperament and
Character Inventory to patients with DM1 and compared the results with those of patients with other neuromuscular disorders, again emphasizing that their DM1 patients had a deviant personality regarding temperament and character; signs of personality disorder were found in 20% of patients. In all of these studies the personality profile did not correlate with the degree of muscle impairment or CTG expansion size.

**Emotion.** Winblad et al.\(^{131}\) demonstrated that facial emotion recognition is also impaired in patients with DM1, the findings correlating with CTG size. This impairment correlated only mildly with scores on tests of frontal lobe function, suggesting that mechanisms other than cognitive ability are involved.

**Somnolence.** Excessive daytime sleepiness is a prominent complaint made by partners and relatives of patients with DM1,\(^{55,90,102}\) but the patients seem to minimize the problem for unclear reasons that may relate to their global cognitive and behavioral profile. This attitude may explain why the Epworth Sleep Scale often underestimates excessive daytime sleepiness in patients with DM1.\(^{36}\) In patients with end-stage disease, degeneration of oropharyngeal, intercostals, and diaphragm muscles may lead to obstructive sleep apnea and nocturnal alveolar hypventilation.\(^{18,19}\) There is evidence that excessive daytime sleepiness in DM1 is not the result of sleep apnea\(^{124}\) but may result from direct involvement of bulbar neurons in the reticular formation of the brainstem.\(^{81,82}\) Broughton et al.\(^{10}\) concluded that cognitive impairment cannot be attributed to a secondary effect of nocturnal sleep apnea or sleep disturbance in patients with DM1, but probably represents a direct effect of CNS lesions. It is interesting to consider that hereditary canine narcolepsy is caused by mutations in the hypocretin receptor 2 (HcrtR2) gene that induces aberrant splicing of the HcrtR2 pre-mRNA, resulting in a truncated receptor.\(^{56}\) Recent reports have demonstrated low levels of Hcrt1 in some patients with DM1\(^{68}\) but how these findings and whether receptor abnormalities relate to excessive daytime sleepiness in DM1 is still unclear.

**Fatigue.** Fatigue is a prominent complaint in patients with DM1. The degree of inactivity in patients with DM1 may go beyond the degree of objective muscle weakness. Kalkman et al.\(^{49}\) assessed the prevalence of fatigue in patients with DM1 as compared to patients affected by muscular dystrophies with a similar degree of muscle impairment but no cognitive involvement (such as facioscapular muscular dystrophy and hereditary motor and sensory neuropathy type I). They concluded that patients with

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**Table 1. Summary of cognitive, behavioral, and neuropsychological evidence for brain involvement in DM1 and DM2.**

<table>
<thead>
<tr>
<th></th>
<th>DM1</th>
<th>DM2</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td><strong>Clinical presentations</strong></td>
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<td></td>
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<tr>
<td>Congenital onset</td>
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<td>–</td>
<td>27, 40, 41, 103, 107, 113, 120, 129</td>
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<tr>
<td>Juvenile onset</td>
<td>+</td>
<td>–</td>
<td>40, 93, 113, 125</td>
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<tr>
<td>Adult onset</td>
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<td>+</td>
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<tr>
<td><strong>Signs and symptoms of CNS involvement</strong></td>
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<td>General intelligence</td>
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<td>IQ</td>
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<td>MMSE</td>
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<td>–</td>
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<tr>
<td>Visual spatial deficits</td>
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<tr>
<td>Attentional deficits</td>
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<td>+</td>
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<tr>
<td>Verbal fluency</td>
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<td><strong>Behavior</strong></td>
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<td>+</td>
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<td>Dependent</td>
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<td>–</td>
<td>12</td>
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<td><strong>Emotion</strong></td>
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<td></td>
<td></td>
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<tr>
<td>Facial emotion recognition deficit</td>
<td>+</td>
<td>?</td>
<td>131</td>
</tr>
<tr>
<td>Excessive daytime sleepiness</td>
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<td>+</td>
<td>10, 18, 19, 55, 56, 90,102, 124</td>
</tr>
<tr>
<td>Fatiguability</td>
<td>+</td>
<td>+/-</td>
<td>49</td>
</tr>
</tbody>
</table>
myotonic dystrophy had significantly higher scores of severe fatigue, reported more problems with concentration, and had more difficulty in initiating and planning than the other two groups. Factors related to cognitive abnormalities may play a more prominent role in patients with DM1.

Neuropathological Evidence. Cell Loss. Ono et al.\(^81\) reported cell loss in specific areas of the brain at postmortem of patients with DM1, such as in the dorsal raphe nucleus, superior central nucleus, dorsal and ventral medullary nuclei, and subtrigeminal medullary nucleus. Cell loss was more prominent in patients suffering from excessive daytime sleepiness and hypoventilation so that primary degeneration of specific brainstem nuclei was considered a possible neuropathological correlate of the clinical findings. Other authors\(^77\) have also reported neuronal loss in the superficial layer of the frontal, parietal, and occipital cortex as well as in the substantia nigra and locus ceruleus. The patient described by Mizukami et al.\(^77\) had extrapyramidal traits as well as behavioral abnormalities such as hallucinations, indiffrence, mental slowness, and visual cognitive impairment, so that in this case also the neuropathological changes correlate with the clinical findings. These and other observations\(^81,99\) suggest that, in the brains of patients with DM1, cell loss of specific areas may occur and contribute to the cognitive and behavioral abnormalities observed.

Neuronal Intranuclear Inclusions. Neuronal eosinophilic inclusion bodies have been described in early studies\(^80\) in a relatively large proportion of the thalamic nuclei (up to 30%) of patients with DM1, similar to findings in primary progressive neurodegenerative disorders. These observations were confirmed by other authors,\(^86\) although their clinical significance is still unclear. Not only the thalamus but also the substantia nigra\(^80\) and caudate nucleus\(^86\) may be involved. More recently, immunostaining of the inclusions has demonstrated that they are composed of ubiquitin and microtubule-associated proteins, thus creating the neuropathological substrate for including myotonic dystrophies amongst the neurodegenerative disorders.

Strong support for the hypothesis of neurodegeneration has come from the work of Thornton and colleagues,\(^48\) who demonstrated in postmortem brain slices from patients with DM1 that mutant RNA accumulates as nuclear foci in specific brain areas where muscleblind proteins are also sequestered, leading to deregulated alternative splicing in neurons of specific gene proteins including tau (exons 2 and 10), amyloid precursor protein, APP (exon 7), and N-methyl-D-aspartate receptor 1, NMDAR1 (exon 5). The distribution of ribonuclear inclusions was wide, involving all sectors of the hippocampus, dentate gyrus, thalamus, substantia nigra, and the brainstem tegmentum. The only exception was the cerebellar cortex, where localization was minimal. RNA foci were also detected in the subcortical white matter and the corpus callosum. Whereas in other neurodegenerative disorders characterized by inclusion bodies the significance of these is still uncertain, the fact that ribonuclear inclusions in muscle are directly involved in disease pathogenesis raises the possibility that CNS symptoms may also be the result of alternative splicing of specific brain protein mRNAs.

Neurofibrillary tangles of the type seen in Alzheimer’s disease and other neurodegenerative disorders have been demonstrated in DM1.\(^85,126,135\) The main constituent of neurofibrillary tangles is pathologic tau proteins, which are usually hyperphosphorylated and insoluble, phosphatase-resistant, and aggregated. Vermersch et al.\(^126\) have demonstrated specific tau variants in the brains of patients with DM1 in the hippocampus, entorhinal cortex, and most of the temporal areas. In contrast to the situation in Alzheimer’s disease, tau precipitates are not linked to amyloid deposits. In light of the abnormal posttranscriptional control of tau protein demonstrated in recent studies of postmortem brain slices from patients with DM1, and in vitro studies on transgenic mice,\(^58,83,109,110\) it is conceivable that abnormal splicing gives rise to abnormal tau variants that precipitate in specific brain areas and constitute the neurofibrillary tangles described. In vitro studies have demonstrated that expanded CUG repeats disturb tau phosphorylation in a specific cell line.\(^42\) Whether splicing alteration of tau transcripts involves various factors needs further investigation.\(^57\) Whether the effects of this spliceopathy on tau transcripts alone account for the neurodegenerative aspects of patients with DM1 requires further in-depth molecular evidence.

Cerebrospinal Fluid Findings. No specific cerebrospinal fluid findings have been reported in patients with DM1. One report documented abnormal protein content in a subset of patients with DM1,\(^43\) the significance of which is unclear. More recently, Martínez-Rodriguez et al.\(^68\) reported decreased orexin-A levels in the cerebrospinal fluid of 3 of 9 patients with DM1 affected by excessive daytime sleepiness, thus suggesting analogies with the mechanisms involved in narcolepsy.
Neuroimaging Evidence.  

CT Scans. The most frequent finding is hyperostosis frontalis interna together with a more diffuse hyperostosis in the calvarium. Avrahami et al. found significant hyperostosis in 17 of 24 patients studied and concluded that this was independent of endocrine abnormalities such as increased growth hormone secretion or abnormal calcium metabolism, but rather might be the result of brain atrophy; endocrine abnormalities have not been excluded by other authors.

Conventional MRI Studies. Several reports have documented cortical atrophy and white matter hyperintense lesions in patients with DM1. Fiorelli et al. documented an increased frequency of subarachnoid cysts in DM1. Cortical atrophy is generally more prominent in the frontal and temporal lobes, and white matter hyperintense lesions are usually diffuse in both hemispheres, single or confluent, and often asymmetric in appearance. There has been inconsistency in interpreting these white matter hyperintense lesions and no general agreement as to their clinical relevance. Some authors do not find a clinical correlate to the neuroimaging profile, whereas others find a correlation between neuropsychological data and the distribution and severity of white matter abnormalities. We found no correlation between the degree of cerebral involvement on traditional MRI studies and cognitive and behavioral profiles. Other authors suggest an evolution of white matter abnormalities during the disease.

Quantitative MRI Studies. Kassubek et al. confirmed and extended the initial reports with traditional MRI, demonstrating that cortical brain atrophy occurs in both diseases but to a minor degree in DM2 compared to DM1. Voxel-based morphometry has been used by several authors to map cortical and subcortical gray matter atrophy in DM1 even where there were no or minimal abnormalities on traditional MRI studies, suggesting that these abnormalities play a role in the wide range of CNS symptoms described in DM1. Ota et al. demonstrated microstructural changes in fractional anisotropy and diffusivity in the corpus callosum subregions connecting to cortical areas, especially motor ones.

PET Studies. PET studies have demonstrated hypoperfusion of frontal and temporal lobes in patients with DM1 and to a minor degree in patients with DM2. This regional hypoperfusion correlated with the frontal dysexecutive syndrome demonstrated on neuropsychological testing. Fiorelli et al. demonstrated that cortical glucose utilization decreased in 20% of patients with DM1 using 2-fluoro-2-deoxy-D glucose (FDG) and dynamic PET.

Brain MR Spectroscopy. Neurochemical alterations observed with proton magnetic spectroscopy have been documented in patients with DM1 and these were correlated with CTG size.

BRAIN INVOLVEMENT IN DM2

The adult presentation of DM2 usually occurs in the 3rd to 6th decade, later than the classic adult form of DM1. There is remarkable clinical heterogeneity in DM2 and high serum CK levels may be the sole manifestation of the disease, but, in general, two main clinical pictures of DM2 emerge. Homozygosity for the DM2 expansion does not seem to alter the disease phenotype as compared with the heterozygous state. There is the proximal myotonic myopathy (PROMM) phenotype characterized by onset in the 3rd or 4th decade, proximal muscle weakness (usually mild to moderate), mostly affecting the pelvic girdle with little or no muscle atrophy, and normal or increased deep tendon reflexes associated with mild clinical myotonia. Early-onset iridocyclitis posterior lens cataracts are usually present. In addition to this classic form, there may be a distinct clinical presentation of DM2, the proximal myotonic dystrophy phenotype (PDM), first described by Udd et al. In this form, onset occurs at a later age and is characterized by remarkable proximal muscle atrophy and weakness, often mimicking progressive spinal muscular atrophy. Clinical myotonia may be absent and early-onset cataracts are not as frequent as in PROMM. In both presentations, brain symptoms are not prominent. However, there is some evidence from neuropsychological test scores, psychiatric interviews, and neuroimaging data that brain involvement may be present in DM2 as in the adult form of DM1, although to a minor degree.

Neuropsychological Evidence. Cognition. The main neuropsychological evidence of cognitive impair-
ment in DM2 comes from the studies of Meola et al., although these are limited by small numbers of patients. In general, IQ and MMSE scores were in the normal range, whereas neuropsychological scores were below normal for age- and education-matched controls on tests for visual-spatial performance and attention, a situation similar to that in DM1 patients, but milder. Regarding verbal fluency, patients with DM2, like those with DM1, do not display lexical impairment on clinical examination and, in agreement with the clinical impression, have normal verbal fluency. This is in contrast with findings of Gaul et al.,

**Behavior.** Evidence for some degree of behavioral abnormalities in DM2, similar to those described in DM1, comes from studies on a small number of patients. As in patients with DM1, the main clinical finding suggesting behavioral abnormalities comes from tests of frontal lobe function, suggesting a dysexecutive syndrome. As in DM1, abnormal scores on tests of frontal lobe function did not correlate with cortical atrophy and white matter hyperintense lesions but rather were associated with fronto-temporal lobe hypoperfusion as documented by PET studies. These studies were limited by the lack of quantitative MRI studies. There was no evidence in these studies that anxiety or depression were a characteristic finding of patients with DM2. Meola et al. found a homogeneous avoidant personality profile in patients with DM2 as in DM1.

**Somnolence.** The degree of this complaint and frequency in DM2 has not been investigated in detail. In our experience, there is a subset of patients in whom this complaint is prominent and cases of central sleep apnea have been demonstrated, but less frequently in DM2 than DM1.

**Fatigue.** There are no reports on the prevalence and relevance of fatigue in patients with DM2, but this does not seem to be a prominent feature in these patients except for those with severe lower-limb muscle weakness or muscle pain and locking.

**Neuropathological Evidence.** There is limited neuropathological data for DM2. Maurage et al. suggested that a similar brain tau pathology is found in DM2 as in DM1, but further studies and evidence at a molecular level are needed.

**Neuroimaging Evidence. Conventional and Quantitative MRI Studies.** The first reports of CNS involvement on neuroimaging in DM2 came from Hund et al. using conventional MRI studies. They described a subset of patients with diffuse and confluent white matter hyperintense lesions similar to those found in CADASIL. There was no apparent link to neuropsychological abnormalities, but these were not explored in detail in the initial description. Other studies followed, demonstrating that brain atrophy and white matter hyperintense lesions are found in DM2 just as in DM1, but the severity of white matter involvement described by Hund et al. remained an isolated finding. These initial studies were limited by the lack of quantitative MRI studies. More recently, Kassubek et al. confirmed and extended the initial reports with traditional MRI in DM2, demonstrating that cortical brain atrophy occurs in both diseases but to a lesser degree in DM2 than DM1.

**Brain MR Spectroscopy.** Recent studies suggest that, although structural abnormalities may occur in patients with DM1 and DM2, changes in cerebral metabolites can differentiate these disease groups. Further studies are needed to clarify whether this difference accounts for involvement of different neuropathological pathways in the two disorders.

**PET Studies.** PET studies have demonstrated mild hypoperfusion of the frontal and temporal lobes in patients with DM2 as in patients with DM1. Sansone et al. studied one DM2 patient with camptocormia using PET. Mesotemporal glucose was reduced but the DOPA pathway was normal, suggesting that the extrapyramidal signs encountered in this patient involved different pathways and pathomechanisms than those of Parkinson’s disease, and emphasize the clinical heterogeneity of DM2.

**MULTIORGAN INVOLVEMENT AND COGNITIVE FUNCTION**

Cardiac conduction arrhythmias and cardiomyopathy together with respiratory insufficiency frequently present in these patients and contribute to the general hypoperfusion and hypoxia of brain structures, aggravating the cognitive and behavioral features described.

Insulin resistance may play a role in aggravating brain function. Brain metabolism accounts for 50% of total body glucose utilization. The brain depends on glucose as an energy substrate, with most brain insulin coming from the pancreas, whence it is taken up by the brain via a receptor-based carrier similar to those demonstrated in muscle. Animal models of type 2 diabetes associated with insulin resistance show reduced insulin brain uptake and content. There are suggestions that brain insulin receptors may become less sensitive to insulin, and this could...
reduce synaptic plasticity. In addition, there is some indication from animal models that reduced sensitivity to insulin in the brain, as observed in aging, decreases the clearance of a beta amyloid, thus increasing amyloid toxicity. These fascinating observations are limited to animal models of type 2 diabetes, and caution is needed in interpreting these findings; however, these preliminary suggestions may provide some indirect speculative evidence that glucose metabolism abnormalities such as those observed in DM might influence brain symptoms.

**CLINICAL RELEVANCE OF BRAIN INVOLVEMENT IN DM**

Although cognition in terms of overall intelligence may not be impaired, lack of initiative, inactivity, and apathetic attitudes constituting the cardinal feature of the dysexecutive syndrome are all characteristic of DM patients. In clinical practice this represents a limitation because patients may be passive in their health-related care, may miss outpatient visits, and may not attach any importance to certain symptoms. Family members, relatives, and possibly even employers need to be aware of these aspects because inactivity may be related to CNS dysfunction rather than muscle disability.

Excessive daytime sleepiness is a prominent feature that may interfere with daily and work-related activities. This is often underestimated by patients and should be sought by asking family members and relatives. Deviant personalities are another characteristic of patients with DM1 and DM2. Obsessive-compulsive, passive, or avoidant traits may dominate the clinical picture and limit patients’ relationships and everyday activities. This knowledge is important to clinicians and relatives in order to improve the approach to these patients, who may require more time and persuasion if they are to overcome their obsessive, passive, or avoidant personality traits.

Although no specific therapeutic agents are currently available to influence CNS symptoms, and while awaiting drugs that may revert abnormal splicing of brain gene proteins or replace MBNL depletion, awareness of the frequent occurrence of CNS symptoms even in the adult form of myotonic dystrophy is helpful. It may help physicians, family members, and employers to understand why, despite mild muscle disability, patients with DM may not function as well as expected by their muscle strength and status. These aspects should be taken into account when planning a clinical trial. Measures of cognitive and behavioral involvement should be included to assess the efficacy of treatment. If the target is muscle, strength may improve, but if inactivity related to the dysexecutive syndrome is unaffected the results of a treatment may be underestimated.

As to the psychosocial aspects, Prevost et al. have said that predictive testing for DM1 was perceived as a change for the worse by many DM1 carriers, suggesting that despite the apparently apathetic attitudes and reduced ability to capture emotion by facial expression, the disease represents an emotional burden for these patients.

In general, patients and relatives should be reassured about the degree of brain involvement. If patients with the congenital form survive the initial phases of respiratory distress, the outcome in general is one of gradual improvement, especially with adequate family and psychosocial support.

There are suggestions that health-related quality of life assessed by short form 36 item health status survey (SF-36) is severely impaired in patients with DM1 and that it is negatively influenced by the severity and duration of the disease as well as by specific cognitive deficits such as visual-spatial and verbal-abstract reasoning. Emphasis on these findings may target therapeutic strategies that could improve the quality of life for these patients.

There are limited studies on the progression of cognitive decline over time and whether the brain abnormalities described so far in patients with DM1, and to a lesser degree in patients with DM2, culminate in a dementia syndrome is yet to be demonstrated. We have followed a small group of patients with DM1 and DM2 over a mean follow-up period of about 8 years, and observed that there was worsening of neuropsychological test scores over time, but no extension to additional areas of cognition or interference with everyday activities. These observations, although they relate to only a small group of patients, may have important prognostic implications.

Finally, we can conclude that signs of CNS involvement (inactivity, decreased initiative, memory deficits, and visual-spatial abnormalities) together with multiple organ degeneration contribute, at least in part, to premature aging in patients with myotonic dystrophies so that DM1 has, in fact, been considered a possible segmental progeroid syndrome. The exact mechanisms of premature aging in DM1, at least, may in part be related to increasing (CTG)n repeat lengths with age. Future treatments that revert abnormal brain gene protein splicing may maintain proper protein processing and folding, thus representing a partial antidote to...
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with adult-form of myotonic dystrophy type 1 and no or minimal MRI abnormalities. J Neurol 2006;253:1471–1477.


ABSTRACT: A simple and reliable method for recording sensory nerve action potentials (SNAPs) of the medial plantar nerve is described. Medial plantar SNAPs were recorded by placing surface electrodes on the sole. Ring electrodes were used for orthodromic stimulation at the big toe. Sixty-four healthy subjects ranging in age from 13 to 81 years were examined to establish normal values. Mean amplitude for the medial plantar SNAP was 4.7 ± 2.8 μV and mean maximum conduction velocity was 43.5 ± 6.4 m/s. The normal values for amplitude obtained for the medial plantar SNAPs were higher than those obtained by the method of Guiloff and Sherratt, and the sensitivity of our method for diagnosis of early sensory neuropathy was relatively higher. The method should therefore be useful in the diagnosis of early sensory neuropathy.

SIMPLE AND NOVEL METHOD TO MEASURE DISTAL SENSORY NERVE CONDUCTION OF THE MEDIAL PLANTAR NERVE

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The medial plantar nerve is a distal branch of the posterior tibial nerve, the sensory territory of which includes the medial aspect of the sole and the medial three and a half digits. It also gives motor branches to the abductor hallucis muscle, the five short toe flexors, and the first lumbrical muscle. The value of medial plantar sensory nerve conduction studies (NCS) in the diagnosis of peripheral neuropathy was established by Guiloff and Sherratt. However, their method of recording sensory nerve action potentials (SNAPs) at the ankle and stimulating at the big toe gives small potentials (mean amplitude, 2.3 μV; range, 0–8.1 μV). Their method has therefore rarely been used in routine practice for the diagnosis of peripheral neuropathy. Ponsford reported that stimulation of the medial plantar nerve on the sole and recording at the ankle is a more reliable method because it gives higher amplitude responses (mean, 9.94 μV; range, 2.0–30.0 μV). Although better results were obtained using this method, only the proximal portion of the nerve above the sole could be tested. This method cannot be used to evaluate lesions of the distal segment. Furthermore, the responses obtained with this method may be mixed sensory and motor nerve action potentials. Since the stimulation point of the medial plantar nerve is close to the motor branches, it is technically difficult to eliminate the possible stimulation of motor branches.

We describe a new method for recording medial plantar SNAPs with surface electrodes placed on the sole and stimulation of the big toe with ring electrodes. We also describe six cases in which early sensory neuropathy was diagnosed using this new method.

METHODS AND MATERIALS

This study was approved by our institutional ethics committee.

Methods. Subjects lay in a supine position on a couch with their legs supported on pillows. Right or left feet were examined randomly. The skin of the foot was cleaned with alcohol. All tests were performed using a Neuropack 8 EMG machine (Nihon Kohden, Tokyo, Japan).

Abbreviations: ATR, Achilles tendon reflex; EMG, electromyography; NCS, nerve conduction study; NCV, nerve conduction velocity; SNAPs, sensory nerve action potentials

Key words: distal nerve conduction; early sensory neuropathy; medial plantar nerve; sensory nerve action potentials; SNAPs

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Medial plantar SNAPs were recorded orthodromically by placing a surface active electrode on the sole at a distance of 8 cm from the base of the big toe along the line from the mid-point of the big toe to the mid-point of the heel (Fig. 1). The reference electrode was positioned 3 cm proximal to the active electrode. The surface electrodes were round silver discs, 1.0 cm in diameter. Ring electrodes were used on the big toe to stimulate the medial plantar nerve. A ground electrode was placed over the medial plantar surface between the stimulating and recording electrodes. Stimuli were 0.1 ms in duration and were rectangular electrical pulses given at a rate of 1 Hz. Supramaximal stimulation was assured by increasing the stimulus intensity by 25%–30% beyond the intensity at which an SNAP was observed on each stimulus. When it was difficult to record the SNAP with a single stimulus due to the low signal-to-noise ratio, the stimulus intensity was increased until it led to contamination by muscle action potentials. To assure reliability in recording relatively small SNAPs, 30–100 stimuli were averaged. When no recognizable potential was noted after 100 stimuli had been averaged, we considered that no potential was present. To assure reproducibility, at least two recordings for each SNAP were made. The high- and low-frequency filter settings were 2 kHz and 20 Hz, respectively. Skin temperature was measured at the medial plantar surface and kept at 30–32°C.

Amplitude of the response was measured from peak to peak. Latency was measured from the onset of the stimulus to the beginning of the major negative deflection for maximum nerve conduction velocity (NCV) and to the peak of the major negative deflection for negative-peak NCV. Response duration was measured from the beginning of the major negative deflection to the baseline return of the last component. The SNAPs were recorded at 1 μV/div for ease of measurement using a sweep speed of 3 ms/div.

Studies were regarded as abnormal when the NCVs were more than 2 SDs below normal mean values and when the durations of SNAPs were more than 2 SDs above normal mean values. When the SNAP amplitude was lower than the lowest value in the normal range, it was considered abnormal.

Control Subjects. Sixty-four healthy subjects (34 men, 30 women; ages 13–81 years) were examined. All participants signed informed consent prior to evaluation and had normal sural nerve conduction studies as a requirement criterion. Individuals with a previous history of lumbar laminectomy, foot trauma, peripheral neuropathy, diabetes mellitus, or alcoholism were excluded. To compare the findings with those obtained by a previously reported technique, we applied the method of Guiloff and Sherratt (Guiloff’s method) to the same nerves, following the previously reported procedure.6 Statistical analysis using paired t-tests was carried out to compare values obtained by the different methods. P-values < 0.05 were considered statistically significant.

Patients. To evaluate the clinical usefulness of the new method, medial plantar SNAPs were recorded in nine patients with diabetic neuropathy (seven men, two women; aged 40–77 years) in whom no abnormality was found on routine NCS. A diagnosis of diabetic neuropathy was made when the diabetic patient had (1) complaints of dysesthesia in the distal lower extremities; (2) one or more objective clinical signs, including impairment of pinprick sense, impairment of vibration sense, and diminished or absent Achilles tendon reflex (ATR); and (3) no previous history of other diseases known to induce peripheral neuropathy. Routine NCS included motor and sensory NCS of the median and ulnar nerves, motor NCS of the posterior tibial nerve, sensory NCS of the sural nerve, and F-wave studies of the median and posterior tibial nerves. A diagnosis of impaired vibration sense was made according to a previously reported study.4 To compare the diagnostic sensitivity of our technique with previously reported ones, we also performed NCS using Guiloff’s method and Ponsford’s method in the same nerves.6,21
RESULTS

Control Subjects. Medial plantar SNAP usually consisted of a negative wave, sometimes preceded or followed by a positive wave. The SNAPs obtained by our method (Table 1) were always higher in amplitude and shorter in duration than those obtained by Guiloff’s method. SNAPs were not obtained by our method in only 1 of the 64 control subjects (who was age 63 years), whereas SNAPs were not obtained by Guiloff’s method in four control subjects (ages 58, 63, 68, and 75 years).

The mean amplitudes of SNAPs obtained by using our method and Guiloff’s method were 4.7 ± 2.8 μV and 2.6 ± 1.7 μV, respectively, which were significantly different (P < 0.001). The mean amplitude of SNAPs obtained by our method was higher than that of SNAPs obtained by Guiloff’s method in each age decade. There was a statistically significant correlation between age and amplitude for the SNAPs (both methods: P < 0.001). However, a higher correlation was found with our method than with that of Guiloff (our method: R² = 0.565, Guiloff’s method: R² = 0.461).

Mean maximum NCVs obtained by the present and Guiloff’s methods were 43.5 ± 6.4 m/s and 45.9 ± 5.4 m/s, respectively. Mean negative-peak NCVs with the two methods were 33.3 ± 4.2 m/s and 39.1 ± 4.2 m/s, respectively. There was a significant difference in maximum NCV (P = 0.046) and negative-peak NCV (P < 0.001) when the methods were compared. NCV obtained by Guiloff’s method was faster than that obtained by our method. There was a statistically significant correlation between age and decrease of maximum NCV (our method: P < 0.001, Guiloff’s method: P = 0.003), especially in persons older than 70 years, whereas this was not apparent in the negative-peak NCV in Guiloff’s method. Mean duration of the SNAPs obtained using our method and Guiloff’s method were 2.0 ± 0.3 ms and 2.5 ± 0.3 ms, respectively (P < 0.001). There was no statistically significant correlation between age and SNAP duration as obtained by either method.

Patients. The clinical features and medial plantar NCS results in the nine diabetic patients are summarized in Table 2. All of the patients had impairment

<table>
<thead>
<tr>
<th>Case</th>
<th>Age</th>
<th>Sex</th>
<th>Signs</th>
<th>Present method</th>
<th>Guiloff’s method</th>
<th>Ponsford’s method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pinprick</td>
<td>Amplitude (µV)</td>
<td>NCV (m/s)</td>
<td>Amplitude (µV)</td>
</tr>
<tr>
<td>1</td>
<td>40</td>
<td>M</td>
<td>Abnormal</td>
<td>2.6</td>
<td>32.8</td>
<td>1.4</td>
</tr>
<tr>
<td>2</td>
<td>42</td>
<td>M</td>
<td>Abnormal</td>
<td>1.8</td>
<td>30.0</td>
<td>No potential</td>
</tr>
<tr>
<td>3</td>
<td>52</td>
<td>F</td>
<td>Normal</td>
<td>1.4</td>
<td>21.9</td>
<td>0.4</td>
</tr>
<tr>
<td>4</td>
<td>53</td>
<td>M</td>
<td>Abnormal</td>
<td>0.4</td>
<td>27.5</td>
<td>No potential</td>
</tr>
<tr>
<td>5</td>
<td>52</td>
<td>M</td>
<td>Normal</td>
<td>0.0</td>
<td>30.6</td>
<td>1.0</td>
</tr>
</tbody>
</table>

ATR, Achilles reflex test; NCS, nerve conduction study; NCV, nerve conduction velocity. Abnormal values are in bold.

Table 1. Normal values for medial plantar SNAPs in 64 healthy subjects.

<table>
<thead>
<tr>
<th>Age group (yrs)</th>
<th>Subjects</th>
<th>SNAP amplitude (µV)</th>
<th>Maximum NCV (m/s)</th>
<th>Negative-peak NCV (m/s)</th>
<th>SNAP duration (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10–19</td>
<td>7</td>
<td>8.9 ± 3.6</td>
<td>6.0–15.0</td>
<td>45.2 ± 6.8</td>
<td>36.5–56.7</td>
</tr>
<tr>
<td>20–29</td>
<td>12</td>
<td>6.5 ± 2.0</td>
<td>4.0–10.0</td>
<td>45.4 ± 7.1</td>
<td>38.6–62.2</td>
</tr>
<tr>
<td>30–39</td>
<td>11</td>
<td>5.2 ± 1.6</td>
<td>3.0–8.0</td>
<td>45.7 ± 6.2</td>
<td>33.3–54.4</td>
</tr>
<tr>
<td>40–49</td>
<td>11</td>
<td>4.1 ± 1.6</td>
<td>1.3–6.0</td>
<td>43.0 ± 5.1</td>
<td>37.0–54.2</td>
</tr>
<tr>
<td>50–59</td>
<td>10</td>
<td>3.1 ± 0.9</td>
<td>1.7–5.0</td>
<td>43.0 ± 5.9</td>
<td>35.8–55.6</td>
</tr>
<tr>
<td>60–69</td>
<td>6</td>
<td>2.0 ± 1.7</td>
<td>0.0–4.0</td>
<td>44.1 ± 7.2</td>
<td>37.0–52.3</td>
</tr>
<tr>
<td>≥ 70</td>
<td>7</td>
<td>1.7 ± 0.5</td>
<td>1.0–2.4</td>
<td>36.4 ± 2.9</td>
<td>32.5–40.4</td>
</tr>
<tr>
<td>Total</td>
<td>64</td>
<td>4.7 ± 2.8</td>
<td>0.0–15.0</td>
<td>43.5 ± 6.4</td>
<td>32.5–62.2</td>
</tr>
</tbody>
</table>
of pinprick sense on the sole, seven patients had diminished or absent ATR, and three patients had decreased vibration sense on the medial malleolus. Diagnosis of sensory neuropathy was confirmed electrophysiologically in six patients (cases 1, 2, 3, 4, 5, and 7) by our method and in three patients (cases 2, 3, and 4) by Guiloff’s method. However, diagnosis was confirmed in only one patient by Ponsford’s method (case 5). The amplitude of SNAPs obtained by our method was below the lowest value of the normal range in three patients (cases 3, 4, and 5), and maximum NCV obtained by our method was below the slowest value of the normal range in five patients (cases 1, 2, 3, 5, and 7). SNAPs were not obtained by Guiloff’s method in four patients (cases 2, 4, 5, and 7), in whom they were recorded by our method.

Medial plantar SNAPs obtained by the three different methods in a typical case (case 7) are shown in Figure 2. Although SNAPs were not obtained by Guiloff’s method in this patient, this is not regarded as abnormal, as SNAPs may be absent in a normal subject of this patient’s age (60s) with this technique. SNAPs obtained by Ponsford’s method were normal. However, markedly slowed NCV was found by our method in the same nerve. Thus, it was concluded that the sensitivity of our method for diagnosis of early diabetic neuropathy is higher than that of Guiloff’s or Ponsford’s methods.

DISCUSSION

The results obtained in control subjects confirmed that our technique is a simple and reliable method for measuring medial plantar SNAPs. The results obtained in patients support the conclusion that the present technique is a sensitive diagnostic test for early sensory neuropathy.

For recording SNAPs of the medial plantar nerve, surface recording electrodes were placed at the ankle and stimulation electrodes at the big toe in early studies. In 1966, Mavor and Atcheson recorded SNAPs of the medial plantar nerve by the orthodromic method using a signal-averaging technique. Similar methods with slight modifications were used in later studies. For example, in 1977, Guiloff and Sherratt reported that mean amplitude of SNAPs obtained by using a similar method was 2.3 ± 1.4 µV and that SNAPs were absent in 3 of 69 healthy subjects. This method was accepted as the standard method for testing medial plantar conduction, but the SNAPs obtained were of low amplitude. Since it was difficult to obtain them consistently, this method was rarely used for routine clinical studies.

Other investigators used the near-nerve needle and signal averaging technique for the orthodromic method of medial plantar sensory NCS. Needle electrodes were used instead of surface electrodes for recording SNAPs, with an active recording needle electrode inserted close to the nerve at the medial malleolus; stimulating electrodes were placed at the big toe. In 1971, Behse and Buchthal reported that mean amplitude of SNAPs obtained by using the near-nerve needle technique was 3.6 ± 2.6 µV in 23 healthy subjects ages 15–30 years, and 1.6 ± 1.8 µV in 10 healthy subjects 40–65 years of age. In 1969, Ertekin reported that mean amplitude of SNAPs obtained by using the near-nerve needle technique was only 1.3 ± 0.4 µV in eight healthy subjects and that SNAPs were absent in 25% of their cases. Although it has been established that the near-nerve needle technique is superior to the surface electrode method in sensory NCS, the amplitude of medial plantar SNAPs recorded by using the near-nerve needle technique is low.

In 1984 Oh et al. reported the near-nerve needle technique in NCS of the digital and interdigital nerves of the foot. This technique has the advantage of obtaining sensory nerve conduction in six branches derived from the medial and lateral plantar nerves. The technique was useful for the diagno-
sis of tarsal tunnel syndrome, \(^{15}\) medial plantar neuropathy, \(^{17}\) lateral plantar neuropathy, \(^{16}\) distal sensory neuropathy, \(^{18}\) and early stage of diabetic sensory neuropathy. \(^{20}\) It revealed a “definite neuropathy” pattern (abnormalities in more than three of six tested nerves) in 57.1% of the patients who had diabetic sensory neuropathy with normal routine NCS. \(^{20}\)

The relatively high mean amplitude of SNAPs (4.7 ± 2.8 \(\mu V\)) obtained in normal subjects by our method suggests that it is superior to previous techniques for recording medial plantar SNAPs. The values were, for example, 44.7% higher than that of SNAPs obtained by Guiloff’s method. In addition, medial plantar SNAPs were obtained using our method in all but 1 of 64 healthy subjects, whereas SNAPs were not obtained in four subjects using Guiloff’s method. The main reason for this is probably the difference in measurement distance between the recording and stimulating sites (8 cm vs. 17–20 cm in Guiloff’s method), with the longer distance likely causing the lower amplitude of SNAPs due to temporal dispersion. In the present study, reliable results were obtained using our method in all subjects under 60 years of age.

Our method also has the advantage of more accurate measurement of conduction distance. Since the conduction distance is a straight line that is only an approximation of the actual course of the nerve, the measurement of conduction distance by our method is more accurate than previous techniques in which conduction distance was measured with a flexible measuring tape or caliper, and did not provide a true measure of its length of a nerve in a three-dimensional structure such as the foot. \(^{14}\)

In our study, marked slowing of NCV was observed in persons older than 70 years. Our method showed that SNAP may not be obtainable in individuals older than 60 years, as discussed above. These results may have been caused by subclinical neuropathy of the medial plantar nerves in older persons due to the wear-and-tear phenomenon affecting the foot. Similar findings with aging phenomenon in plantar nerve conduction have been reported previously. \(^{9,14}\)

In contrast to our method, the stimulator has been placed on the sole for measurement of more proximal conduction. \(^{3,8,21,22}\) The mean response amplitude obtained by Ponsford \(^{21}\) using an orthodromic method with the stimulator just proximal to the first metatarsophalangeal joint was 9.94 ± 3.5 \(\mu V\) in 100 healthy subjects, which is twofold higher than that obtained by our method. Two factors may explain Ponsford’s results: more sensory fibers are stimulated and mixed nerves may be stimulated. Saeed and Gates \(^{22}\) suggested that the SNAPs obtained by the proximal conduction method are mixed compound action potentials, rather than pure SNAPs, because the stimulation point of the nerve is close to the motor branches. Thus, this method does not measure pure SNAPs. In any event, since this proximal conduction method tests the proximal and not the distal portion of the nerve, it is less sensitive for the diagnosis of distal neuropathy. Recently, Ifergane et al. \(^{7}\) reported that distal medial plantar neuropathy was not detected by this proximal conduction method. Similarly, in our study, medial plantar SNAPs elicited by stimulating the sole did not predict the existence of early diabetic sensory neuropathy in most patients, whereas markedly reduced SNAPs or slow sensory NCV was found using our new method in six of nine patients (67%). As common polyneuropathies have a predilection for distal sensory fibers, it is important to evaluate those fibers when such a neuropathy is suspected.

REFERENCES

ABSTRACT: Intramuscular pressure (IMP) has been used to estimate muscle stress indirectly. However, the ability of this technique to estimate muscle stress under dynamic conditions is poorly characterized. Therefore, the purpose of this study was to determine the extent to which IMP is a valid surrogate for muscle stress during dynamic contractions. IMP and muscle stress were compared under steady-state isotonic conditions and during complex dynamic length changes. During concentric contractions the shape of the IMP–velocity curve mimicked the basic shape of the force–velocity curve but with much higher variability. For eccentric contractions, a precipitous drop in IMP was observed despite increased muscle stress. The dissociation between muscle stress and IMP during dynamic contractions was partially explained by sensor movement. When the muscle was not moving, IMP explained 89% ± 5% of the variance in muscle force. However, when transducer movement occurred the linear relationship between IMP and stress was no longer observed. These findings demonstrate the difficulty in interpreting IMP under dynamic conditions when sensor movement occurs. They also illustrate the need to control transducer movement if muscle stress is to be inferred from IMP measurements such as might be desired during clinical gait testing.


RELATIONSHIP BETWEEN MUSCLE STRESS AND INTRAMUSCULAR PRESSURE DURING DYNAMIC MUSCLE CONTRACTIONS

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The fact that skeletal muscles generate significant intramuscular pressure (IMP) during active contraction was established by the eminent physiologist A.V. Hill in the 1940s. Since that time, IMP has been studied in order to understand normal muscle function and the etiology of such pathological states as compartment syndromes (for review, see Hargens et al.). Although there is evidence in the literature that IMP is a good predictor of relative isometric joint torque in humans and relative passive and active isometric stress in animal models, the extent to which such a relationship is maintained during dynamic muscle movement is not known. For IMP to serve a useful role in understanding in vivo human muscle function, it must provide unique information that reflects muscle force under all conditions, not just the precisely controlled conditions that may be created in a laboratory setting. There is reason to believe that IMP might not accurately reflect muscle force under all conditions since previous work using a very small pressure transducer showed that pressure was a much better surrogate for predicting isometric muscle force at longer muscle lengths during both active contraction and passive load bearing. It was postulated that long muscle lengths secured the transducer during contraction. Anecdotal observations indicated that, at shorter muscle lengths, transducer movement resulted in aberrant pressure readings.

This article includes Supplementary Material available via the Internet at http://www.interscience.wiley.com/jpages/0148-639X/suppmat/ 

Abbreviations: FSO, full-scale output; IMP, intramuscular pressure; NIST, National Institute of Standards and Technology; PCSA, physiological cross-sectional area; TA, tibialis anterior 

Key words: fluid mechanics; force–velocity relationship; interstitial fluid pressure; intramuscular pressure; isotonic contraction

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Normal joint movements involve complex dynamic muscle length changes. In vivo, these contractions have been shown to vary in terms of strain magnitude and strain rate even within the same muscle. As mentioned, muscle length changes may be accompanied by transducer movement that may affect the pressure–stress relationship. To date, the pressure–stress relationship has not been explicitly studied under conditions of either laboratory controlled isotonic contraction or during more complex in vivo muscle movements. Since the muscle force–velocity relationship is well understood during isotonic shortening and lengthening, dynamic isotonic experiments provide the ideal opportunity to probe the pressure–stress relationship under dynamic conditions. Our objective was to determine the extent to which IMP serves as a force surrogate during dynamic contraction conditions. This relationship was investigated under both steady-state isotonic and during dynamic contraction conditions. This relationship was investigated under both steady-state isotonic conditions as well as during dynamic length changes that included eccentric contractions.

MATERIALS AND METHODS

The experimental model used was the tibialis anterior (TA) muscle of the New Zealand White rabbit (mass, 2.5 kg ± 0.5; n = 10). This model was selected primarily for the accessibility of the TA, its 3° pennation angle, and parallel fiber arrangement. Since it is believed that fiber curvature may affect IMP, we chose the TA, as any curvature even during shortening is negligible. The muscle size also permitted pressure transducer insertion without significant muscle trauma, as repeated insertions resulted in no change in contractile force. All experimental procedures adhered to the guidelines set forth by the National Institutes of Health.

Anesthesia was induced with 4% halothane and maintained on 2% halothane (2 L/min). Heart rate and oxygen saturation were monitored (VetOx; Heska Co., Fort Collins, Colorado) throughout the test duration and anesthesia was adjusted as needed. A midline incision was made from the ankle to the mid-thigh. Fascia was removed in order to minimize confounding effects of fascial restriction on IMP, thus exposing the entire TA muscle. The leg was immobilized using 3.2 mm Steinmann pins placed in the mid-tibia and distal femur and secured to a custom jig. A cuff electrode was placed around the exposed peroneal nerve for direct muscle activation (Pulsar 6Bp Stimulator; FHC Inc., Bowdoinham, Maine). The TA was released at the retinaculum and attached to a servomotor (Cambridge Model 310B; Aurora Scientific Inc., Ontario, Canada) aligned with the force-generating axis of the motor. A 360-μm diameter fiber optic pressure sensor (Luna Innovations Inc., Blacksburg, Virginia) was inserted via an 18-gauge angiocatheter in line with the force-generating axis of the fibers and at the thickest proximal portion of the muscle. The pressure transducer was adjusted to provide a zero volt output (defined as zero mmHg) after insertion into the muscle. (Absolute pressure readings obtained immediately after transducer insertion ranged from approximately +5 to −5 mmHg.) Sensor calibration was performed by comparison with a National Institute of Standards and Technology (NIST) traceable pressure sensor. The microsensor had an accuracy, repeatability, and linearity better than 2% full-scale output (FSO) and hysteresis slightly higher than 4.5% FSO. The TA force–velocity relationship was created by repeated activation of the muscle at 60 Hz over a 650-ms period with a 2-min rest interval interpolated between each contraction to avoid complications of fatigue. Muscle fiber length (Lₐ) was calculated from muscle length for each subject using the rabbit TA fiber length-to-muscle length ratio of 0.67. For concentric contractions, muscle length was first set to L₀ + 5%Lₐ. After the muscle activation, length was held constant for 200 ms, during which time isometric force was generated. Then, length was decreased by 10% Lₐ at a selected velocity (Fig. 1A) and the TA was again held at a constant length, permitting re-development of isometric tension at the new, shorter length. Shortening velocity was increased in 0.5 Lₐ/s increments ranging from 0–5.5 Lₐ/s to generate the force–velocity relationship for concentric contractions. For the eccentric contractions, length was first set to L₀–5%Lₐ and the timing of the concentric protocol duplicated except that positive length ramps of 1, 2, and 3 Lₐ/s were applied (Fig. 1B). The smaller velocity increment and range compared to concentric contractions were chosen based on the observation that muscle stress is relatively insensitive to lengthening velocity and that repetitive eccentric contractions cause muscle injury in this system. The experiment ended with two contractions that combined both shortening and lengthening. Specifically, a 10% shortening contraction at 1 Lₐ/s (Pₒ) was followed by an isometric contraction for 200 ms at the new length (Pₒ) and then a 10% lengthening contraction at 1 Lₐ/s that returned the muscle to its original length (Pₑ) for a second isometric contraction of 200 ms duration (Pₒₑ). Next, a 10% lengthening contraction at 1 Lₐ/s (Pₑ) was followed by an isometric contraction for 200 ms at the new length (Pₒ), and then a 10% shortening contraction at 1
Lf/s that returned the muscle to its original length (PC) for a second isometric contraction of 200 ms duration (PO2) (Fig. 1C). The order of these contraction paradigms was randomized. Length, tension, pressure, and temperature were recorded for each contraction using a data acquisition board (610E series; National Instruments, Austin, Texas) in the LabView environment (National Instruments) acquiring data at 4,000 Hz.

Tension records were converted to stress by dividing tension by the muscle’s calculated physiological cross-sectional area (PCSA), using the equation described by Sacks and Roy. Stress and IMP values were averaged across 10 animals for each velocity and data are presented graphically as mean ± SEM.

A high-speed video system (OmniSpeed, Model LR400; Speedvision Technologies, San Diego, California) was used to measure transducer position relative to the muscle surface. The video system was placed above and orthogonal to the long axis of the muscle. The muscle surface was marked with a small hair and the transducer tip was easily visualized just beneath the muscle surface because of its white tip (see online supplemental video). Video data were acquired at 400 frames/s for ~1.5 s, exported to AVI format, and manually analyzed frame-by-frame using the NIH ImageJ package (ImageJ, a public domain image analysis program freely available at http://rsb.info.nih.gov/ij/index.html). Final spatial resolution provided by the optical system (70 mm focal length, 1:3.5 macro lens) was 8 μm/pixel after exporting to the digital format.

RESULTS

The TA muscle stress–velocity curve had the classic shape of a rectangular hyperbola which was characterized by a Vmax of 5.5 ± 0.33 Lf/s and a P0 of 230 ± 23 kPa (Fig. 2A) which compares favorably with literature values for fast mammalian muscle. The shape of the IMP–velocity curve roughly mimicked

![Figure 1](image1.png)

**FIGURE 1.** Length changes imposed on the isolated rabbit tibialis anterior muscle during isotonic testing. Muscle length is plotted as a function of time and is approximated by motor position. Muscle velocity is depicted graphically by decreasing dash length as velocity increases and small numbers refer to velocity in units of Lf/s. (A) Shortening ramps ranging from 0.5 to 3 Lf/s are shown. In practice, velocities up to 5 Lf/s were used but are not discernable graphically on this time base. (B) Lengthening ramps ranged from 1 to 3 Lf/s. (C) Combined shortening then lengthening (solid line) and lengthening then shortening (dashed line) protocol described in the text. Bar represents the timing of the 650 ms nerve stimulation period. Note different calibration bars for isotonic experiments (A,B) compared to combined experiments (C).

![Figure 2](image2.png)

**FIGURE 2.** (A) Relationship between muscle stress and velocity for lengthening (negative velocities) or shortening (positive velocities). These data follow the classic force–velocity relationship described in the literature. (B) Relationship between intramuscular pressure (IMP) and velocity across all velocities tested. Each symbol represents mean ± SEM for 10 animals. Note the increased variability of the IMP records compared to stress. Note also that IMP decreases with lengthening in spite of the fact that stress increases.
the shape of the force–velocity curve for concentric contractions but with much higher variability (Fig. 2B). The corresponding points on the IMP–velocity curve demonstrated a maximum IMP (I₀) at P₀ of 25 ± 8.4 mmHg and, at Vₘₐₓ IMP decreased to 4.9 ± 11.8 mmHg. These IMP values are generally lower than those recorded in human subjects during muscle contraction.²⁶

During eccentric contractions (negative velocities), muscle stress was higher compared to all values recorded during concentric contraction and relatively constant as previously demonstrated,¹⁸ with active stress ranging from 291 ± 22 kPa to 323 ± 28 kPa across velocities (Fig. 2A). However, in contrast to concentric contractions, where pressure and stress covaried, for the eccentric portion of the IMP–velocity curve a precipitous decline in pressure relative to I₀ was observed (Fig. 2B). Furthermore, in some cases negative pressures were even recorded during eccentric contractions.

The steady-state IMP–velocity relationship roughly mimicked the form of the muscle force–velocity relationship during concentric contractions, but when the experimental paradigm was changed to a more complex form, the covariation previously noted was completely lost. These complex contraction paradigms demonstrated a profound yet repeatable effect of history. For purposes of discussion, the forces at various points during the protocol were defined as: P₀, the initial isometric force, Pₑ, the force developed during eccentric contraction, P₀₁, the isometric force developed after the first length change, Pₑ, the force developed during eccentric contraction, and P₀₂, the isometric force developed at the end of this series of length changes when the muscle returned to its original length. The pressures corresponding to these timepoints were defined as I₀, Iₑ, I₀₁, Iₑ, and I₀₂.

For the combined protocol that began with shortening (Fig. 3A), muscle stress behaved as expected based on classic muscle mechanics¹⁷,¹⁸: Pₑ was lower than P₀, P₀₁ recovered to a level slightly less than P₀ due to shortening onto the ascending limb of the length–tension curve, Pₑ was higher than P₀₁ due to the eccentric contraction, and P₀₂ was slightly higher than Pₑ presumably due to force enhancement after stretching onto the descending limb of the length–tension curve.⁹,¹⁰ These stresses were significantly different from one another as revealed by one-way ANOVA and post-hoc Fisher’s tests (P < 0.0001). Values for IMP at each of these points displayed reproducible yet unexpected values compared to stress. For example, whereas Iₑ decreased with Pₑ as seen during the steady-state force–velocity experiment (Fig. 2B), I₀₁ was greater than I₀ in spite of the fact that P₀₁ was lower than P₀. Further, whereas Pₑ was significantly greater than P₀ due to eccentric contraction, Iₑ was slightly lower than I₀₁. Thus, a clear dissociation between stress and IMP was measured for complex contractions that began with shortening.

For the combined protocol that began with lengthening, a similar dissociation between IMP and stress was observed, but the nature of the change was different even compared to the protocol that began with shortening. In other words, the combined lengthening/shortening and shortening/lengthening protocols demonstrated a history effect within a contraction combination and also an order effect between combinations. Thus, Iₑ decreased (Fig. 3B) even though Pₑ increased, but then I₀₁ decreased relative to I₀ at the new, longer muscle length even though P₀₁ increased. Then, while Pₑ decreased significantly in the subsequent concentric contraction, Iₑ increased slightly, unlike the steady state force–velocity results presented above (Fig. 2). Thus, a clear dissociation between stress and IMP was measured for complex contractions that began with lengthening. To demonstrate this dissociation ana-
lytically, we subjected the data set to a two-way ANOVA using contraction number and order (shortening first or lengthening first) as the grouping variables. This analysis demonstrated significant main effects of contraction number and order as expected \((P < 0.0001)\) and, most important, a highly significant interaction term between order and contraction number \((P < 0.001)\).

Real-time measurement of transducer position revealed that not only did significant movement occur, but this movement was related to the usefulness of the IMP signal itself. Tip movement followed muscle length change (Fig. 4). Thus, the onset of transducer tip movement corresponded with the timing of muscle shortening in all trials and the cessation of tip movement lagged only slightly behind the cessation of muscle shortening. Quantitative analysis of the predictability of muscle force based on IMP demonstrated that, when the muscle was not moving, IMP explained \(89\% \pm 0.5\%\) of the variance in muscle force \((i.e., \rho = 0.89)\). However, when attempting to predict muscle force from IMP either during movement \((P_e)\) or in during the isometric period after movement \((P_{01})\), correlation coefficients were negative, suggesting that the relationship between IMP and stress was no longer valid (Fig. 5).

**DISCUSSION**

The purpose of this study was to investigate the relationship between muscle stress and muscle IMP measured with a small solid-state transducer during dynamic contractions. This is an important problem since IMP is often used in the diagnosis and investigation of neuromuscular disorders.\(^{14}\) These experiments were motivated, in part, by the concern that transducer movement appeared to be associated with variable pressure recordings.\(^{7}\) Even under isometric conditions, IMP was more variable compared to muscle stress and, during these conditions, transducers occasionally “squeezed out” of the contracting muscle.

The current study exploits the fundamental and well-established relationship between muscle stress and isotonic force established in the 1930s for whole muscle\(^{15,18}\) and subsequently confirmed at the level of the single cell to be a fundamental property of the sarcomere.\(^{8}\) For all conditions investigated in the current study we obtained the expected results for muscle stress: stress decreased with increasing contraction velocity (Fig. 2A), was relatively independent of lengthening velocity (Fig. 2A), and achieved the appropriate steady-state level during and after dynamic length change, either lengthening or shortening (Fig. 5). However, the behavior of IMP during dynamic muscle contraction yielded unexpected results. First, dynamic IMP during isotonic contraction was highly variable (Fig. 2B). This was not due to uncontrolled muscle length fluctuations since muscle length and motor movement were tightly coupled and the compliance of this experimental system was only \(0.3 \mu m/g\),\(^{21}\) which would not permit the small length changes to occur that could modulate force even at the high forces generated in this study \((<2,000g)\).

Transducer placement is known to affect intramuscular pressure both along and across a skeletal...
Cadaveric studies have demonstrated that tourniquets produce increased pressures in deeper tissues that juxtaposed bones and increased pressures at the margins of the tourniquet itself where shear strains appear to be the highest during muscle contraction. We created a much less complex but more controlled experimental model by releasing the rabbit TA from surrounding fascia and skin, essentially creating a “floating” muscle that was free to expand during contraction. Muscle lateral expansion is known to occur during isometric contraction but this would not result in pressure development in our model since the muscle boundary was unconstrained. We attempted to place the transducer tip in the same position for each experiment (see Materials and Methods) but acknowledge that some variation in placement could result in different absolute pressures. We believe that such small absolute pressure offsets would affect the baseline pressure level but not the dynamic behavior of the pressure record during contraction. We were also unable to decrease pressure variability by normalizing dynamic pressure to either resting pressure, peak pressure, or average pressure. We thus conclude that the erratic pressure records were accurate depictions of the IMP of the muscle during dynamic contraction.

It should be noted that such an experiment, where pressure and force are correlated in real time, has never been performed previously, at least to our knowledge. Previous so-called “dynamic” studies performed in humans compared such general parameters as ground reaction force or joint moment with IMP measured at one point in time and concluded that IMP was an accurate surrogate of muscle force. There are several problems with this approach. First, ground reaction forces are notoriously complex in origin and reflect gross parameters such as body mass, body inertia, joint kinematics, and step length, in addition to muscle activation. Thus, it is not surprising that ground reaction forces would correlate grossly with IMP since almost any muscle activation would increase IMP compared to rest and appear to create a valid correlation with changing gait parameters. Additionally, since joint moment results from the simultaneous action of many muscles, a joint moment–IMP correlation does not provide a rigorous test of the hypothesis that muscle stress and IMP are causally related. We thus reject previous assertions of the correlation between IMP and muscle force.

Transducer movement represented, in part, the basis for the measured pressure variability and is illustrated by our real-time measurement of transducer position during dynamic contraction (Fig. 5; see online sample video). During the video, the transducer tip is clearly seen moving relative to the muscle surface. Since the movements are relatively small (<1 mm, Fig. 4) compared to either fiber length ~60 mm, or PCSA ≈100 mm², the transducer is extremely sensitive to the micromechanical muscle environment. Forced muscle lengthening appears to create a small vacuum between the transducer and the muscle surface, which could explain the variability in pressure measurements.
ducer tip and the muscle such that IMP decreases with stretch even though stress increases greatly. This may explain the decrease in pressure during isotonic lengthening (Fig. 2B) and a lengthening/shortening or shortening/lengthening maneuver (Fig. 3). The fact that a large history effect was observed (Fig. 3) also supports this argument. Presumably, small transducer movements occur after either shortening or lengthening and this creates a new IMP-stress relationship (see examples in Fig. 5). This effect was demonstrated explicitly for a dynamic condition in which transducer tip position was measured in real time. The IMP-stress relationship was always most highly correlated for the case when the transducer was not moving and the absolute relationship itself changed after movement, even if the muscle was once again contracting isometrically. This is the likely explanation for the IMP decrease even though stress increased with lengthening and then pressure decreased relative to the initial value at the new, longer muscle length even though stress had increased (Fig. 3B). Transducer tip movement appears to be due to actual muscle movement rather than storage of elastic strain energy since, even after muscle stimulation ceased, the transducer tip did not move back to the starting position (Fig. 4) but simply appeared to track muscle length.

It should be noted that these studies do not negate the important clinical studies that demonstrate the relationship between resting IMP and muscle pathology (i.e., compartment syndrome) since under measurement conditions used clinically, transducer movement is nearly zero due to the passive condition of the muscle and the additional anchoring of the transducer that may be provided by surrounding fascia and skin.

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ABSTRACT: The aim of this study was to develop a reliable, sensitive, quantitative measure of grip myotonia and strength and to determine whether CTG repeat length is correlated with grip myotonia and with muscle strength in myotonic dystrophy type 1 (DM1). Three maximum voluntary isometric contractions (MVICs) of the finger flexors (i.e., handgrip) were recorded on 2 successive days using a computerized handgrip myometer in 29 genetically confirmed DM1 patients and 17 normals. An automated computer program calculated MVIC peak force (PF) and relaxation times (RTs) along the declining (relaxation) phase of the force recordings at 90%, 75%, 50%, 10%, and 5% of PF. Patients also underwent quantitative strength testing (QST) manual muscle testing (MMT). The patients had longer grip RTs and lower PFs than normals. RT (90% to 5%) was above the normal mean +2.5 SD in 25 (86%) patients. In DM1, prolongation of RT was mainly in the terminal (50% to 5%), rather than the initial (90% to 50%) phase of relaxation. PFs and RTs for each patient were reproducible on consecutive days. RTs were positively correlated with leukocyte CTG repeat length, whereas measures of muscle strength, such as PF, QST, and MMT, were negatively correlated with repeat length. We conclude that computerized handgrip myometry provides a sensitive, reliable measure of myotonia and strength in DM1 and offers a method to assess natural history and response to treatment.


COMPUTERIZED HAND GRIP MYOMETRY RELIABLY MEASURES MYOTONIA AND MUSCLE STRENGTH IN MYOTONIC DYSTROPHY (DM1)

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Myotonic dystrophy type 1 (DM1) is a multisystem, dominantly inherited disease caused by an unstable CTG repeat expansion in the 3′ nontranslated region of the DM1 gene on chromosome 19.1,4,9,13 Myotonia, weakness of facial and distal limb muscles, and cataracts are core clinical findings.7,19,20 Grip myotonia and weakness of long flexors of the fingers are often the earliest clinical signs of skeletal muscle involvement in patients.20,24 Myotonia is typically elicited at the bedside by percussion of intrinsic hand muscles (thenar) and forearm wrist or finger extensor muscles or by observing the rate of relaxation of voluntarily (isotonically) contracted muscles, such as the finger flexors (making a tight fist) or the orbicularis oculi muscles (closing the eyes tightly). The presence of myotonia can be confirmed with needle electromyographic testing, but neither bedside examination nor electromyographic testing allows precise quantitation of myotonia. The method we report utilizes a simple, commercially available handgrip myometer to measure the peak forces of maximum voluntary isometric muscle contractions (MVIC) and applies a readily usable computer program to quantify the rate of relaxation of forearm finger flexors.

There have been few reported methods to quantify myotonia precisely. Previous investigations of
Hand Grip Myometry in DM1

paramyotonia congenita associated with periodic paralysis\textsuperscript{23} and myotonia fluctuans, another nondystrophic sodium-channel disorder,\textsuperscript{25} utilized a similar method of quantitating handgrip force and myotonia compared to one used in the present study. However, one key difference is that neither of these past investigations used standardized sitting and arm positioning. Instead, the patients in those studies had to place their arms in a trough to permit simultaneous immersion of the forearm and hand in water while performing isometric contractions of the long flexors of the fingers. To our knowledge, the only previous attempt to carry out quantitative measurements of isometric contraction force and handgrip myotonia using standardized sitting and forearm positioning in patients with DM1 was that of Torres et al.\textsuperscript{28} Their results showed greater variability than ideal for a clinical investigative technique. However, the method of positional standardization we report takes further steps to assure consistency and reduced adventitious movement by introducing a secure, adjustable frame to stabilize the wrist and finger. Aside from improved positional standardization, we developed automated computer software that objectively analyzes the recorded force curves and removes some of the potential human error inherent in the previous methodologies.\textsuperscript{21,25,28} Furthermore, we report methodological reliability on same-day and different-day testing of handgrip strength and myotonia in DM1 patients.

We previously reported the use of electrical stimulation of the first dorsal interosseus muscle (FDI) via the ulnar nerve at the wrist as a quantitative method to assess myotonia.\textsuperscript{11} This method, like the present technique, involves an evaluation of myotonia in muscles that develop myotonia in the early stages of DM1.\textsuperscript{7,20} The advantages of studying evoked muscle contractions are that this approach requires less time to instruct and coach the patient, the muscles responding to the stimuli are relatively well defined, and control by the central nervous system is not necessary to initiate contraction and relaxation of the muscles. One undesirable aspect of this methodology is that electrically evoked muscle contractions, using single or repetitive nerve stimulation, causes mild or moderate pain in some patients and may discourage their participation in studies that require serial assessments of myotonia. Grip myometry, by contrast, is not painful and requires less equipment for setup, making it more feasible for multicenter treatment trials that require serial evaluations of the same patients over many months. Our group recently used grip myometry to quantitate “warm up” of grip myotonia over the course of six repeated maximum voluntary isometric contractions (MVICs) in DM1.\textsuperscript{12} The present investigation focuses on the relaxation time (RT) and peak force (PF) of the initial MVIC in a series of six contractions, and specifically determines its sensitivity and reproducibility as a quantitative method to measure grip myotonia and grip strength. We believe this methodology will enhance future studies on the natural history of myotonia and weakness in DM1 and will help determine efficacy of new therapies in this disease.

MATERIALS AND METHODS

Study Participants. All DM1 patients who participated in this investigation were recruited through the University of Rochester Neuromuscular Disease Center. The 29 patients enrolled in this study were part of a larger cohort of patients recruited to participate in a trial to assess the efficacy of mexiletine as an antymyotonia treatment in DM1. All measurements used for analysis in this report were collected at the baseline (predrug) visit of that therapeutic trial. Patients were eligible if they were between the ages of 18 and 80 years and had sufficient finger flexor strength to grasp the handle of the dynamometer used to measure myotonia. All patients satisfied standard clinical criteria for the presence of myotonia (action myotonia exceeding 3 s following maximal hand grip), or percussion myotonia in finger extensor or thenar muscles, and satisfied clinical criteria previously published for DM1.\textsuperscript{6} They also met the molecular genetic diagnostic classification of having an abnormal expansion of the CTG repeat in the myotonic dystrophy type 1 gene (DM1 gene), using standard leukocyte DNA analysis ([CTG]n repeat size in DM1 gene >100 repeats). Patients were ineligible if they were taking a medication known to affect myotonia or had coexisting neuromuscular disease or other serious medical illness. Normal volunteers (n = 17) were free of acute medical problems, medications known to affect muscle contraction, or neuromuscular disease. An effort was made to select normal volunteers who were the same sex as, and of comparable age to, the DM1 patients. All study participants gave informed consent, and all study procedures received approval from the Institutional Review Board.

Measurements of Myotonia. All DM1 patients were admitted to our institutional General Clinical Research Center (GCRC) for a 2-day visit. The measurements of myotonia took place in the morning (06:30–08:30) following an overnight fast. We de-
fined myotonia to be any delay in RT greater than 2.5 standard deviations (SD) above the mean RT in the normal group. The same testing procedures were performed on 17 normal volunteers in the morning following an overnight fast, but only on one day.

Study participants sat in a modified, adjustable chair that permitted standardized body positioning. Seat and arm heights were established for each subject to support and maintain a consistent angle for the shoulder, elbow, and wrist joints. The arm was adducted, the forearm was in a neutral position, and the wrist was extended to permit comfortable grasping of the handles of the dynamometer. Testing was performed on the right arm of all study participants. Forearm and hand placement were standardized for each participant by a labeled pegboard fixed to the armrest of the chair. Subjects placed their forearms on the pegboard and the tester inserted 4-inch pegs around the forearm to limit movement during each trial and to permit reproducible positioning of the arm and forearm for each testing session. The tester fixed the forearm firmly to the armrest with two inelastic straps. Individualized chair settings to adjust for the leg length and height of the torso were also the same for each session to assure identical positioning of the arm rests.

A strain gauge measured the force of contraction exerted on the handle of a handgrip dynamometer (Jamar Dynamometer; Asimow Engineering, Los Angeles, California), which was connected to a force transducer. When squeezed, the dynamometer produced an analog signal that was sent to an analog-to-digital converter and subsequently to a computer that graphically displayed the signal in real time on a monitor. Participants observed their force curves during each trial. This immediate visual feedback helped them maintain relatively constant levels of contraction force for the 3-s interval. The computer stored the force curves for analysis and customized software (QMA v. 3.4; QMA Systems, Computer Source, Atlanta, Georgia) calculated rates of contraction and relaxation.

For the purpose of analysis, we calculated RTs from only the first of the six squeezes of each trial. We reported force in kg units rather than converting the force to Newtons. The computer software calculated contraction and relaxation times as follows: Peaks were identified by marking the points on the force curves where the maximum positive and minimum negative slopes occurred. Between these two points the plateau of the peak force was identified and the force was averaged over the plateau to yield the average PF for each contraction. There was a relatively flat low level of force recorded when the test participants had their hand on the dynamometer while they were completely relaxed. This baseline force was close to but not always exactly 0 kg. Therefore, we corrected the peak-average force by subtracting the baseline force level, which the software estimated by taking the minimum force values observed during the trial and adding 0.2 kg to account for digital “noise.” RTs (seconds) were then calculated at set percentage declines in force (90%, 75%, 50%, 10%, and 5%) from the PF value.

For the purpose of analysis, we calculated RTs from 90% of PF rather than 100% because there were often small spikes in the peak force during maximum contractions. These brief, elevated forces did not represent the true sustainable value for the MVIC force. Similarly, on the tail end of each contraction curve the force did not always return exactly to baseline before the next contraction. It was unreliable to compute RT values based on a return to a precise baseline. Rather, 10% or 5% of peak force represented the most useful limits closest to baseline. Therefore, we used the following percentage declines in peak force to calculate RT intervals: 90%–75%, 90%–50%, 90%–10%, 90%–5%, and 50%–5%.
Measurements of Limb Strength. Clinical evaluators performed maximum voluntary isometric contraction testing (MVICT) on both days to assess limb muscle weakness. This included quantitative strength testing (QST) and manual muscle testing (MMT). The QST system (Quantitative Muscle Assessment; Computer Source) utilized an adjustable cuff attached to the arm or an inelastic strap attached to the leg, both of which were connected to a force transducer with a load of 0.5–250 kg. The maximum force generated by the patient was recorded for each trial and the maximum over two trials was used as the final measurement for each muscle. Six muscle groups were tested bilaterally (elbow flexors and extensors, knee flexors and extensors, ankle flexor, and hand grip muscles) for a total of 12 muscle groups. We calculated a global QST strength score based on SDs from previously established normative data adjusted for age and height as previously described.3,23

MMT was performed on 26 muscle groups (right and left shoulder abductors, elbow flexors, wrist flexors, wrist extensors, hip flexors, knee extensors, ankle dorsiflexors, hip extensors, hip abductors, knee flexors, ankle plantar flexors, elbow extensors, plus neck extensor and neck flexor muscles). The muscles were tested in specific positions including sitting, supine, prone, and side lying and each graded on a modification of the Medical Research Council scale (0 to 5) to construct a composite MMT Whole-Body Strength score as previously reported.5,17

Statistical Analysis. We averaged the three RT measurements and PF measurements for each individual on each day of testing. Using these values, we performed scatterplot analyses, calculated mean values for the entire group of patients and normal volunteers, and used standard t-test analysis to compare RTs in the DM1 patients to RTs in the normal volunteer group.

We analyzed variation in PF and RT in two ways: (1) between the three trials of the same day 1 testing session (same-day intertrial variation) in both patients and normals, and (2) between day 1 and day 2 of testing (day-to-day variation) in patients (normal volunteers were only tested on one day). Calculation of same-day intertrial variation in PF and RT consisted of computing the SD from the three trials on day 1 and expressing this as a percentage of the mean PF or RT (coefficient of variation). Calculation of day-to-day variation in PF and RT consisted of determining the mean of the three trials on day 1, the mean of the three trials on day 2, and then computing the percentage change between the two mean values: [(maximum value – minimum value) / maximum value] × 100. In addition, we plotted mean RT from day 1 against mean RT from day 2 and calculated the correlation coefficient for this relationship. The same plot and statistical analyses were performed for day 1 and day 2 mean PF values.

To investigate the hypothesized relationship between the genetic lesion and clinical severity, we calculated correlation coefficients for the DM1 gene CTG repeat size in leukocyte DNA compared to RTs (severity of myotonia), grip strength, and body strength (QST and MMT) for the group of DM1 patients. The mean values combining all six trials (three on day 1 and three on day 2) for relaxation time and for peak MVIC force were used for this comparison.

RESULTS

Study Participants. Mean age (normal, 40.9 years; DM1, 45.9), the range of ages (normal, 21–62 years; DM1, 23–62), hand temperature ranges (normal, 25–33°C; DM1, 27–33°C), and gender (normal, 47% female; DM1, 38%) did not differ between the 17 normal volunteers and 29 DM1 patients. All patients had experienced disease symptoms for at least several years (range, 5–41 years). DNA analysis of the size of the CTG repeat of the DM1 gene in patients showed a mean of 563 repeats with a range of 169–1731 repeats (the upper limit for normal is 37 repeats).

Myotonia and Muscle Strength. Inspection of the MVIC force curves showed dramatic differences between DM1 patients and normal volunteers. Aside from a marked difference in overall strength, there was a prominent difference in the shape of the grip force relaxation curves, especially the later phases of relaxation following each contraction (Fig. 1). For normals, the relaxation curve was an almost vertical decline from maximum force back to baseline. For the DM1 group, there was a distinct tail at the end of each relaxation phase in which the final return to baseline (or near baseline) was prolonged. This was most apparent in the first contraction of each trial. We used results from the initial contractions for analysis, since the tail tended to shorten with each of the subsequent contractions, thus demonstrating the warm-up phenomenon.12

The contribution to the duration of myotonia made by the marked delay in the late phase of MVIC relaxation was apparent from comparison of the RT values obtained in the different portions of the total relaxation phase. Handgrip RT values from 90% to
5% of PF, which encompassed the longest relaxation time interval that was analyzed, were longer in DM1 patients than normal volunteers, being above the normal upper limit in 25 patients (86%, Fig. 2A).
The early phases of relaxation—90% to 75% (normal mean, 0.10 s; DM1 mean, 0.24 s; \( P/H11021 \) \( 0.01 \)) and 90% to 50% (normal mean, 0.19 s; DM1 mean, 0.46 s; \( P/H11021 \) \( 0.001 \))—were only slightly prolonged in DM1 patients compared to normals. Only 9 DM1 patients (31%) were above the upper limit of normal for the 90% to 75% RT interval and only 12 (41%) were above the upper limit of normal for 90% to 50% RT (Fig. 2B). In contrast, the late phase of muscle relaxation (50% to 5% RT; normal mean, 0.18 s; DM1 mean, 1.96 s; \( P/H11021 \) \( 0.0001 \)) was markedly prolonged in the patients compared to normals (Fig. 2C), with 28 of 29 patients (97%) above the upper limit of normal. The primary influence that the late phase delay in relaxation exerted was also apparent from analysis of the RT values that encompassed the early portion of the relaxation phase, and only a portion of the late phase. For example, 90% to 10% RT values (normal mean, 0.33 s; DM1 mean, 1.77 s; \( P < 0.0001 \)) were more prolonged than the 90% to 50% RTs, but less prolonged than the 90% to 5% (normal mean, 0.37 s; DM1 mean, 2.42 s; \( P < 0.0001 \)) or the 50% to 5% RT values.

The same-day intertrial variation in handgrip PF and RT during MVIC testing was determined from comparisons between the three sequential trials performed on day 1 of testing. Same-day intertrial variation in the 90% to 5% RT [normal mean coefficient of variation (COV), 23.1%; DM1 mean COV, 33.2%; \( P < 0.08 \)], 50% to 5% RT (normal mean COV, 18.7%; DM1 mean COV, 41.2%; \( P < 0.001 \)), and PF values (normal mean COV, 5.4%; DM1 mean COV, 10.7%; \( P < 0.004 \)), expressed as percentage of mean COV were all greater in DM1 patients than normals. For each of the DM1 patients and normals, variation in handgrip RT was greater than that of PF. There was no correlation between the COV for RT and that of PF in DM1, indicating that intertrial variation of RT is independent from that of PF.

In the DM1 patients, day-to-day variation in handgrip PF (mean, 11.5%) was significantly less than that of the 90% to 5% RT value (mean, 22.6%, \( P < 0.01 \)) and of the 50% to 5% RT (mean, 26.1%, \( P < 0.01 \)), similar to the findings in the analysis of same-day intertrial variation. In addition, the day-to-day test–retest results in our study demonstrated that the mean PF (Fig. 3A), 90% to 5% RT (Fig. 3B) and 50% to 5% RT (Fig. 3C) over the three trials on day 1 were highly correlated with the mean PF, 90% to 5% and 50% to 5% RT values, respectively, over the three trials on day 2. The correlation was higher for PF values (\( r = 0.96 \)) than for 90% to 5% RT (\( r = 0.77 \)) or 50% to 5% RT values (\( r = 0.76 \)), but all three correlations were highly significant (\( P < 0.0001 \)).

**Muscle Strength, Myotonia, and CTG Repeat Size.** Total muscle strength as measured by QST was below average for DM1 patients in comparison to QST normative data (mean z-score: \(-4.81\), range \(-10.32\)
to \(-1.88\). The mean MMT Whole-Body Strength Score for the DM1 group was 3.9 (range: 2.7–4.8). The PF of handgrip was lower \((P < 0.0001)\) in the DM1 group (10.4 kg) than normal volunteers (42.5 kg). In the DM1 group, handgrip myotonia, as determined by 90% to 5% RT values, was positively correlated with leukocyte DNA CTG repeat length in the DM1 gene \((r = 0.52, P < 0.01)\), whereas measures of muscle strength, such as, grip PF \((r = -0.42, P < 0.03)\), MMT \((r = -0.37, P < 0.05)\), and QST \((r = -0.42, P < 0.03)\) showed a significant negative correlation with CTG repeat length.

**DISCUSSION**

Forearm and intrinsic hand muscles are among the earliest muscle groups to be affected by myotonia in patients with DM1. Therefore, clinical assessments of myotonia in DM1 patients often focus on observation of the thenar muscles and forearm wrist or finger extensor muscles following percussion, or on estimating the rate of relaxation of the finger flexor muscles following a tight squeeze of the examiner’s finger, i.e., MVIC. Although useful and practical in the clinical setting, more objective and sensitive myotonia measurement is required for thorough study of disease progression and pathology, as well as for studying the outcomes of potential treatments. The method we report uses components of the standard bedside evaluation of handgrip myotonia while producing objective, reproducible PF and RT in patients with DM1. This study establishes the reliability of same-day and different-day testing of both handgrip strength and myotonia in patients with DM1.

To our knowledge, Torres et al. are the only other group that have attempted a similar method of measuring handgrip myotonia in patients with DM1 using standardized testing position. Our methodology utilizes a more stringent approach to maintaining consistent, controlled body and forearm positioning than theirs, which showed a wide variation in RTs (myotonia) in repeated measurements of maximum isometric grip strength in individual patients. We observed variation in RTs in repeated testing of the same individuals in our study, but the variation in our DM1 patients was less than that observed by Torres et al. The greater variability in serial measurements of handgrip myotonia in that earlier study probably resulted primarily from greater adventitious movements of the hand, fingers, and wrist during muscle relaxation; an adjustable frame to stabilize the wrist and finger position was not used.

Another key difference between that study and ours is the method for measuring PF and RT. Torres et al. measured MVIC force of handgrip, total and half RTs (i.e., 100% to 0% PF and 100% to 50% PF), and fatigue time before and after administration of acetazolamide in 18 control subjects and 10 patients with myotonic dystrophy (clinical criteria in the article were those for DM1). They labeled the time-force values at 100% PF, 50% PF, and 0% PF manually from hardcopies of the force curves. Our method used automated computer software to mark precisely and objectively preset percentages of handgrip PF. The software calculated the handgrip PF by averaging the force during the 3 s of sustained isometric contraction. This lessened the possibility of misinterpreting nonsustained force spikes that sometimes occur during each 3-s contraction. In addition, to reduce variability and to improve accuracy, we programmed our software to use the 90% and 5% PF levels for the onset and terminus of muscle relaxation rather than 100% and 0% of PF values used by Torres et al. The point of absolute highest force during each MVIC does not necessarily occur immediately prior to the start of relaxation, and therefore cannot be used to time the beginning of RT. Thus, the time at 90% PF has proved to be a more reliable point to begin each measurement of RT than either the absolute peak in handgrip force or the calculated 100% PF of handgrip. Likewise, using the 5% PF value as the terminal timepoint for RT measurement largely eliminates the greater error introduced by trying to place cursors along a slowly declining force trace as it approaches the zero kilogram level.

To assess the reproducibility of our methodology, we calculated day-to-day and same-day intertrial variability of handgrip RT and PF. The variability of these measurements allows determination of clinically significant changes in strength and myotonia over time and provides a basis for designing future trials of treatment in DM1. The day-to-day test–retest results for each patient demonstrated that the mean RT and PF over the three trials on day 1 were highly correlated with the mean RT and PF over the three trials on day 2. The correlation was higher for PF \((r = 0.96)\) than for 90% to 5% RT \((r = 0.77)\) or 50% to 5% RT \((r = 0.76)\), but all three correlations were highly significant \((P < 0.0001)\).

Although mean RT was reproducible over two consecutive days, there was considerable same-day, intertrial variation in handgrip myotonia (trials separated in time by 10 min during which the patient remained in the apparatus). Intertrial variation was greatest in the terminal portion of muscle relaxation.
(50% to 5% RT) and was significantly more pronounced in DM1 patients than normal controls. In both patients and controls, the same-day intertrial variation of RT exceeded that of PF and was independent of PF. The variation in RT was also independent of the warm-up phenomenon or the effect of preceding exercise, as there was no systematic decline in RT between one trial and the next. This suggests that our 10-min intertrial rest period is adequate to minimize warm-up of myotonia between trials.

The causes for the variation in handgrip RTs and PF values in DM1 patients in our study were probably multifactorial. Variation in forearm circulation during MVICs and technical factors, such as minor changes in hand positioning, may have contributed to intertrial variation in RTs. However, we believe that the variation in RT was due primarily to dynamic physiologic, metabolic phenomena; that is, that myotonia in DM1 fluctuates to some degree from minute to minute even in the overnight fasted state, perhaps in response to minute-to-minute fluctuations in the extracellular concentration of potassium or other factors (e.g., hormones). Interestingly, in our previous study of evoked myotonia of the FDI muscle after ulnar nerve stimulation, we also found that intertrial variability in evoked RTs was greater in DM1 patients than normal controls.11 The fact that we obtained the same result in a different muscle (FDI rather than forearm finger flexors) activated under different conditions (by nerve stimulation rather than voluntary contraction) supports the hypothesis that variability in intertrial myotonia is on a physiological rather than technological basis. The practical importance of recognizing the existence of intertrial variability in handgrip RT in DM1 patients is that an accurate assessment of myotonia requires calculating an average of several trials.

Nearly all our DM1 patients (97%) demonstrated a prolonged terminal phase of handgrip RT (50% to 5%), whereas only 41% of patients demonstrated prolonged RT in the early portion of handgrip relaxation (90% to 50%). We observed a similar pattern of delayed relaxation in the FDI muscle with evoked myotonia.11 This common feature of the relaxation curve in different symptomatic muscles in patients with DM1 underscores the importance of including the terminal portion of muscle relaxation in quantitative measurements of myotonia. We do not have a clear explanation of why myotonia is more evident in the terminal compared to the initial portion of the handgrip relaxation curve. We speculate that this is due in part to the passive elastic properties of muscle in series with the contractile element26,27 and that there may be two populations of muscle fibers that relax at different rates.11 Further research is necessary to resolve this question.

Our technique of handgrip myometry to measure myotonia is best suited to quantitate myotonia in patients with grip myotonia on clinical examination. Although reproducible and easy to implement, one limitation of the method is in its sensitivity to detect milder degrees of myotonia. Despite the presence of readily elicited percussion myotonia (an entry criteria for the present study), prolonged handgrip RT (greater than 2.5 SDs above normal) was not demonstrable in all patients using the current method of handgrip myometry. Four patients (14%), all having relatively smaller CTG repeat expansions in the DM1 gene (196, 221, 243, and 496 repeats), had 90% to 5% RT values within normal limits. However, all but one of these four patients (a 42-year-old woman with 221 repeats in the DM1 gene) had a prolonged handgrip RT based on analysis of the terminal portion of muscle relaxation phase. This finding emphasizes again the importance of measuring the final portion of the muscle force relaxation curve in the quantitative assessment of myotonia.

Our results demonstrate a positive correlation between the severity of handgrip myotonia (RT) and the size of the CTG repeat expansion in DM1 gene isolated from leukocyte DNA. Our data also shows that handgrip PF, along with QST and MMT, have a negative correlation with the CTG repeat length. These findings agree with our evoked myotonia study,11 along with previous reports, which demonstrate that CTG repeat length in leukocyte DNA from symptomatic DM1 patients correlates with disease severity.7,22 At a more basic level, this observation is also consistent with recent investigations in a mouse model of DM1 that demonstrate a causative relationship between the size of the CTG repeat expansion and myotonia.14–16,18 However, the relationship between the size of the CTG repeat expansion in the nuclei of a given skeletal muscle and the severity of muscle wasting and weakness is still not certain, a point made by a recent study in which no clear correlation was found between the average CTG repeat size isolated from different muscles and the severity of weakness in those muscles.8 Still, our findings that an increase in handgrip RT and decrease in handgrip PF both correlate with leukocyte CTG repeat length indicate that our method provides measurements that are relevant to the DM1 disease process.

The findings in this study support the view that computerized analysis of handgrip RT and PF pro-
vides a reproducible, sensitive, and quantitative measure of grip myotonia and strength, respectively. We propose that our method can yield reliable endpoints to assess natural history and evaluate muscle relaxation and strength in therapeutic trials in DMD provided that: (1) patients are carefully coached to carry out the voluntary isometric handgrip contractions following standardized positioning and technique; (2) handgrip RT values are averaged from three or more force recordings; and (3) the terminal portion of muscle relaxation (50% to 5% MVIC force) is targeted for analysis.

The authors thank the late Professor Kenneth Ricker for valuable advice and recommendations about isometric grip testing to measure myotonia. Ms. Linda Fisher for help in designing the frame-pegboard in this study, and Ms. Karen Berk for help in preparing the article. This research was supported by the Food and Drug Administration (FD-R-001662), by NIH/NIAMS (grant RO1 AR44069A), by The Saunders Foundation, and by a General Clinical Research Center grant (5 MO1 RR00044) from the National Center for Research Resources, NIH.

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ABSTRACT: We employed physiogenomic analyses to investigate the relationship between myalgia and selected polymorphisms in serotonergic genes, based on their involvement with pain perception and transduction of nociceptive stimuli. We screened 195 hypercholesterolemic, statin-treated patients, all of whom received either atorvastatin, simvastatin, or pravastatin. Patients were classified as having no myalgia, probable myalgia, or definite myalgia, and assigned a myalgia score of 0, 0.5, or 1, respectively. Fourteen single nucleotide polymorphisms (SNPs) were selected from candidates within the 5-HT receptor gene families (HTR1D, 2A, 2C, 3A, 3B, 5A, 6, 7) and the serotonin transporter gene (SLC6A4). SNPs in the HTR3B and HTR7 genes, rs2276307 and rs1935349, respectively, were significantly associated with the myalgia score. Individual differences in pain perception and nociception related to specific serotonergic gene variants may affect the development of myalgia in statin-treated patients.

PHYSIOGENOMIC ASSOCIATION OF STATIN-RELATED MYALGIA TO SEROTONIN RECEPTORS

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The 3-hydroxy-3-methylglutaryl–coenzyme A (HMG-CoA) reductase inhibitors or statins are the most commonly prescribed drugs worldwide because of their ability to reduce atherosclerotic vascular events.41 Statins are generally well tolerated but can produce a variety of adverse drug reactions (ADRs). Most of these ADRs are myopathic, ranging from myalgia with or without elevations in serum creatine kinase (CK) to clinically important rhabdomyolysis.6,8,17,47–49 However, neuropathic ADRs, including changes in cognition, mood, or behavior,16 and autoimmune ADRs, including myositis,13 are increasingly being recognized. The prevalence of statin-related myopathic complaints has increased with the frequency of statin use, and their incidence has increased with the use of higher doses.

Various mechanisms have been proposed for statin-induced myopathy including altered pharmacokinetics due to drug metabolism or drug–drug interactions,9,54 physicochemical properties of the drugs, effects on metabolic end products such as coenzyme Q10,5 and interference with metabolic pathways regulating muscle repair.51 Statin use may also unmask asymptomatic metabolic myopathies.18,32,53 Effects on cellular regulatory proteins causing activation of molecular pathways, ultimately leading to apoptosis, have also been proposed.47 However, none satisfactorily explains the heterogeneity of the adverse effects and the range of clinical symptomatology.

It is also not clear why some patients experience asymptomatic serum CK elevations during statin therapy, whereas other patients experience myopathic symptoms such as myalgia without serologic...
Physiogenomics of Statin Myalgia MUSCLE & NERVE September 2007

We used physiogenomics to determine whether polymorphisms in serotonergic genes affected the report of statin-related myalgia. Physiogenomics is a medical application of sensitivity analysis, an engineering discipline. The physiogenomic approach has been successfully applied to cardiovascular and neuropsychiatric studies of drugs and diet. Sensitivity analysis is the study of the relationship between the input and the output of a model and, more specifically, utilizing systems theory, it analyzes how variation of the input leads to changes in output quantities. Physiogenomics utilizes the variability in genes as input, measured by single nucleotide polymorphisms (SNPs), and determines how the SNP frequency among individuals relates to the variability in physiologic characteristics, the output. Using this approach, we have previously shown that serum CK levels during statin therapy are associated with genes affecting vascular smooth muscle and raised the novel hypothesis that statins may affect vascular function. The present report suggests that statin-related myalgia may be related to certain serotonin receptors.

MATERIALS AND METHODS

Patient Enrollment. Patients treated with statins for at least 1 month were recruited from Hartford Hospital clinics. They provided written informed consent to participate in the study, which was approved by the Hartford Hospital institutional review board. All patients were recruited and entered into the study by one investigator (A.W.). Subjects were recruited if they were on atorvastatin, simvastatin, or pravastatin, and excluded if they were on other statins or multiple lipid-lowering medications. Valid genotype data were obtained for 195 patients (78 women, 117 men), of whom 82% were Caucasian by self-report. The mean age was 68 ± 13 years. Statin name and dose were obtained by self-report. There were a total of 107 patients on atorvastatin (45 on 10 mg, 29 on 20 mg, 18 on 40 mg, 2 on 80 mg, and 13 on an unspecified dose), 69 patients on simvastatin (1 on 5 mg, 7 on 10 mg, 21 on 20 mg, 28 on 40 mg, 1 on 60 mg, 9 on 80 mg, and 2 on an unspecified dose), and 19 patients on pravastatin (5 on 20 mg, 7 on 40 mg, and 7 on 80 mg).

The patients were interviewed and assessed by a qualified investigator as to whether they suffered from myalgia or "muscle pain" (definite myalgia, 39 patients) or not (no myalgia, 144 patients). For some patients, myalgia symptoms were likely but assessed as ambiguous (probable myalgia, 12 patients). A myalgia score of 1 was assigned to patients with definite myalgia when any of the following criteria could be established from the patient interview: (1) muscle pain began concurrently with the initiation of statin therapy; (2) muscle pains coincided with an increase in statin dosing; (3) muscle pains resolved when the inducing statin was switched to another statin; or (4) muscle pains resolved when statin therapy was discontinued altogether. A myalgia score of 0.5 was assigned to patients with probable myalgia if they complained of muscle pain, but the underlying etiology was not clear or possibly resulted from a comorbidity unrelated to statin therapy. A myalgia score of 0 was assigned to patients with no myalgia, which occurred when there was complete denial of any muscle pain by the patient.

Laboratory Analysis. Blood was either collected prospectively or retrieved from routine clinical analysis. Samples were collected into tubes containing either ethylene-diamine tetraacetic acid (EDTA) or citrate for DNA extraction. The blood was centrifuged, and the plasma was assayed within 2 days for total CK activity using an analyzer (Cobas Integra; Roche Diagnostics, Indianapolis, Indiana). The normal reference range was <200 U/L for men and <140 U/L for women. The DNA was extracted from leukocytes in 1 ml of whole blood using a DNA isolation kit (Puregene Gentra; Qiagen, Valencia, California).

Gene Selection and Genotyping Technology. Nine candidate genes were selected for their role in serotonergic neurotransmission, which has been widely implicated in pain detection and processing in the brain, spinal cord, and peripheral tissues. The genes are listed in Table 1 with brief descriptions of their function in the footnote. Genotyping was performed using the Illumina BeadArray platform and the GoldenGate assay (Table 1).

Data Analysis. Covariates between the myalgia score and SNPs were analyzed using stepwise, multiple linear regression. Age, gender, race, statin, and dose were not significantly associated with the myalgia score. To test for association, a linear regression model was constructed including the SNP genotype.
SNP genotype was coded quantitatively as a numerical variable indicating the number of minor alleles: 0 for major homozygotes; 1 for heterozygotes; and 2 for minor homozygotes. The $F$-statistic $P$-value for the SNP variable was used to evaluate the significance of association (Table 2).

### Statistical Analysis

The endpoint in this study has a discrete, non-normal distribution, so a linear regression test based on the $F$-distribution is not automatically valid. To establish validity, we performed an independent calculation of the $P$-values using permutation testing. 12,22 Permutation testing requires extensive computation, but the resulting $P$-values are non-parametric, that is, they are valid regardless of the endpoint distribution. The agreement between permutation $P$-values and those from the $F$-distribution was very good ($R^2 = 98\%$, RMSD = 0.055), and statistical analysis was performed.

<table>
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<tr>
<th>Gene</th>
<th>SNP</th>
<th>Chromosome</th>
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<th>Minor MAF</th>
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Table 1. Genes and SNPs analyzed for associations with myalgia.

Table 2. Genes and SNPs analyzed for associations with myalgia.

Shown are gene function, HUGO gene symbol, and dbSNP identifier for each locus. Also shown is the minor allele frequency (MAF) as determined in our study population, the allele frequency in the CEPH population (Utah residents of central European ancestry) of the HapMap project, the $P$-value of association to the myalgia score (MScore), and the $P$-value of association to the serum creatine kinase activity for comparison. The coefficient indicates the average increase (or decrease) in the myalgia score for each copy of the minor allele. The SNPs in boldface type were significantly associated with myalgia score.
the ranking of all SNPs is identical under both, with the exception of rs659734, which is not significantly associated and changes in $P$-value from 0.43 to 0.5, and in rank from 8 to 10. Because they are based on a Monte Carlo simulation, the permutation $P$-values have a stochastic component and vary slightly from simulation to simulation. For this reason, we report here the more reproducible values from the $F$-distribution.

As an alternative test for significance, we performed a chi-square test on the contingency table linking genotype with myalgia outcome. We found the same two SNPs to be significant, although less so ($P = 0.017$ and 0.03). This is likely due to the fact that a contingency table does not assume a dose–response relationship between allele and phenotype, which makes it the less sensitive test if such a relationship is present. We also performed a logistic regression on a binary endpoint of no myalgia vs. probable or definite myalgia. Again, the $P$-values are significant ($P = 0.01$ and 0.02), but not as strong as those from the linear regression. This is explained by the loss of power inherent in the loss of distinction between probable and definite myalgia. To account for the multiple testing of 14 SNPs, we calculated adjusted $P$-values using Benjamini and Hochberg’s false discovery rate (FDR) procedure.$^{10,11,32}$

RESULTS

The distribution of the myalgia phenotypes in the study population is shown in Figure 1. It features a trough between the definite phenotypes to account for probable myalgia. There was no significant difference in the proportion of myalgia patients among the patients treated with atorvastatin, simvastatin, and pravastatin.

Table 2 shows the results of the SNP association tests. Only $HTR3B$:rs2276307 and $HTR7$:rs1935349 were significantly associated. The $R^2$ values, which indicate the proportion of the observed variation explained by each SNP, were 4% and 3%, respectively. The coefficients were 0.14 and 0.13, meaning that each allele leads to an increase of the myalgia score of 14%. The false discovery rates under multiple testing were 8% and 17%, respectively. Table 2 also indicates the allele frequency for all SNPs as observed in our study population. These agree remarkably well with the ones reported by the HapMap project for the CEPH population of Caucasians, except that $HTR6$:rs1805054 is not available in the HapMap.

No SNP was significantly associated with serum CK activity (Table 2). We previously reported SNPs to be significantly associated with statin-related CK activity for the angiotensin II type 1 receptor ($AGTR1$) and nitric oxide synthase 3 ($NOS3$) genes.$^{37}$ Neither gene was associated with the myalgia score.

Figure 2 shows physiogenomic representations of myalgia severity by depicting for each gene the SNP frequency in the subpopulation with no myalgia compared to those in the subpopulations with probable and definite myalgia. For SNPs with a strong association, the marker frequency will be significantly different between myalgia scores of 0 and 1. Conversely, if a marker is neutral, the frequency will be independent of myalgia score and the plot will be essentially flat. For example, the first panel in Figure 2 shows the plot for SNP rs2276307 of the $HTR3B$ gene. The frequency of the minor allele is <20% in subjects with myalgia scores of 0, whereas it approaches 35% in subjects with myalgia scores of 1 and has an intermediate frequency in subjects with myalgia scores of 0.5. This finding indicates a strong association between the $HTR3B$ marker and myalgia. As the frequency of the minor allele is higher in patients with definite myalgia, $HTR3B$ SNP rs2276307 is considered a risk marker for statin-related myalgia.

DISCUSSION

Statin-related muscle toxicity may consist of statin myopathy, myalgia (muscle complaint without serum CK elevation), myositis, or rhabdomyolysis.$^{30}$ Of these, myalgia is the most common and adversely
affects quality of life and compliance with these medications.

The myalgia rate of 20% in the present study is higher than the myopathy rates of 10% demonstrated in most published studies.4,20,50 Myalgia per se has rarely been examined in statin clinical trials.48 Industry-sponsored trials have relied on serum CK elevations to document statin myopathy. In contrast, we inquired directly about muscle pain. We believe that this approach elicits muscle discomfort–related complaints from patients more frequently than other approaches and is also a strategy that uncovers subtle symptoms. Other strategies might also have reduced the rate of myopathy in industry trials. High-risk patients were often not recruited. Some large trials, such as the Heart Protection Study,21 used a "run-in" period before the study to exclude patients intolerant of or non-compliant with the medications.4,20,50

We examined the relationship between serotonin metabolism and statin myalgia because the serotonergic system has been implicated in clinical syndromes with muscle pain and tenderness, such as certain rheumatic diseases.24,28 Serotonin has also been implicated in other neurological disorders including migraine2 and epilepsy3 in addition to its well-known role in psychiatric conditions.35,44 Various lines of evidence implicate the serotonin receptors in nociception.1,14,33,43 We found a statistically significant relationship between myalgia and two SNPs (rs2276307 and rs1935349) in genes HTR3B and HTR7, which encode serotonin receptors. These results suggest that gene polymorphisms producing individual differences in pain perception may have an important role in patients’ reports of muscle pain.

Previous pharmacogenetic studies of statins have concerned mostly cholesterol- and muscle-related genes.27,55 We have examined the possibility that SNPs in genes expressed in neurological pathways affect the incidence of statin myalgia. We found associations between genes involved in serotonergic function and statin myalgia. Consequently, it is possible that "statin myopathy" may be a constellation of independent syndromes with varying innate predispositions in the population and diverse physiological mechanisms encompassing various gene pathways.

The present study has various limitations. We focused only on potential class-wide effects. The subjects studied were a diverse group of patients who were treated with different statins for differing periods of time. Only 40 patients reported definite statin myalgia. Myalgia is necessarily subjective as there are no objective measurement instruments to quantify this condition. We examined only statistically significant associations that may not reflect cause and effect. It is also possible that the significant SNPs are in linkage disequilibrium with other genes responsible for statin myopathy. Further research is ongoing to substantiate these findings in additional patient cohorts and to discover new drug-specific associations using genome-wide arrays.
As more physiological pathways and eventually the entire genome are screened, a multi-gene model can be developed where an individual's configuration of various significant SNPs can reliably predict the probability of statin-related adverse events for each patient. Generalized clinical use of such diagnostics for DNA-guided medical management may help to improve statin tolerability and safety as these drugs are deployed ever more widely in treatment and prevention of many cardiovascular disorders.

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ABSTRACT: Corticotrophin-releasing factor 2 receptor (CRF2R) agonists prevent muscle atrophy due to immobilization, denervation, and corticosteroid-induced muscle atrophy in wildtype mice. We hypothesized that a CRF2R agonist will increase skeletal muscle mass in mdx mice. Mdx (C57BL/10ScSn-Dmd<sup>mdx</sup>) and wildtype (C57BL/6) mice were divided into four groups: sedentary placebo, sedentary CRF2R agonist, exercised placebo, and exercised CRF2R agonist. Mice exercised on a treadmill twice weekly for 30 min (8–12 m/min, 8 weeks). Muscle and heart weights, serum creatine kinase, and γ-glutamyltransferase activities were measured. The CRF2R agonist increased extensor digitorum longus and soleus muscle weights (P < 0.05) in wildtype and mdx mice. Sedentary mdx CRF2R and exercised mdx placebo mice had lower serum creatine kinase activity than sedentary mdx placebo mice. CRF2R-treated mice had decreased heart weights compared to placebo-treated mice. We conclude that CRF2R agonists should be further evaluated as a potential therapy for dystrophinopathies.


EFFECTS OF A CRF2R AGONIST AND EXERCISE ON mdx AND WILDTYPE SKELETAL MUSCLE

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The mdx mouse contains a nonsense mutation in the dystrophin gene that leads to absent dystrophin expression in the sarcolemma. Although these mice have evidence of muscle necrosis, elevated serum creatine kinase (CK) activity, and myofibrillar macrophage accumulation, mdx skeletal muscle undergoes cycles of degeneration and regeneration, with mice living to ~2 years of age and muscle weakness being mild relative to the human condition, Duchenne muscular dystrophy. Consequently, researchers have investigated better methods to characterize and induce contractile dysfunction and damage in mdx mice. Forced treadmill running, especially combined with episodes of eccentric exercise, induces muscle damage in mdx mice. Exercised mdx mice may therefore be a useful tool in the evaluation of potential therapeutic interventions such as drugs and nutraceuticals.

Corticotrophin-releasing factors are a family of naturally occurring peptides that bind to a corticotrophin-releasing factor receptor (CRFR). These peptides aid in many functions including the coordination of endocrine, autonomic, behavioral, and immune responses to stress. There are two CRFRs; corticotrophin-releasing factor 1 receptor (CRF1R) is found predominately in the brain, whereas corticotrophin-releasing factor 2 receptor (CRF2R) is expressed ubiquitously throughout all tissues. The CRF2R protein is localized in neuromuscular and myotendinous junctions as well as in endomysium and perimysium surrounding muscle fibers in rats, mice, and humans; however, expression was not observed in myocytes. Blood vessels have also been shown to express the CRF2R protein.

Recently, it was shown that sauvagine, a nonselective CRFR agonist, completely inhibited muscle atrophy in casted CRF1R knockout mice. However, the same treatment with noncasted mice had no significant effect on muscle mass. Amino acids
were substituted in sauvagine and were tested in vivo in wildtype, CRF1R knockout, and CRF2R knockout mice, in both control and casted conditions. Several CRF2R-selective sauvagine analogs were found to be more effective and potent than sauvagine in preventing atrophy due to casting.17

We hypothesized that the CRF2R agonist would cause an increase in muscle mass in both mdx and wildtype mice. Our objective in this study was to determine whether the CRF2R agonist would have a beneficial effect on mdx mice as measured by serum CK activity, an indirect marker of muscle damage, as well as functional capacity measured by motor performance and voluntary wheel-running capacity. Since previous researchers have demonstrated treadmill exercise as a valuable method for testing potential therapeutic compounds,13 moderate intensity exercise was used to screen the therapeutic potential of the CRF2R agonist.

MATERIALS AND METHODS

Animals. Both mdx (C57BL/10ScSn-Dmdmdx) and wildtype (C57BL/6) mice were purchased from Jackson Laboratory (Bar Harbor, Maine) at ~4 weeks of age. Animals were housed in micro-isolator cages and maintained on a 12/12-h light/dark cycle at ~22°C in our institutional animal facility in accordance with national guidelines and under the approval of our institutional review board. Mice were acclimatized to the facility, cages, and a standard rodent diet (2016 Global Rodent Diet; Harlan-Teklad, Madison, Wisconsin) for a 1-week period prior to initiation of the experiment. Following the adaptation period, animals were randomly assigned to treatment groups, ensuring that body mass was similar between all groups. Body weight and food intake were monitored and recorded weekly for 8 weeks.

Experimental Groups. Mice were assigned to one of eight treatment groups: wildtype sedentary placebo, wildtype sedentary CRF2R, mdx sedentary placebo, mdx sedentary CRF2R, wildtype exercise placebo, wildtype exercise CRF2R, mdx exercise placebo, and mdx exercise CRF2R. The CRF2R selective agonist (PG-873637) was synthesized at Procter & Gamble Pharmaceuticals (Mason, Ohio) as described previously.17,22,23 The substances were administered using osmotic minipumps (Alzet, Cupertino, California) containing either the CRF2R agonist or placebo (0.9% saline, 0.2% Tween 80). The osmotic pump was inserted subcutaneously while the mouse was under gaseous anesthesia (isoflorane). Lidocaine was applied to the implantation site to act as an analgesic. Stitches were then used to close the incision. Osmotic pumps were replaced every 28 days. The old pump was removed and a new pump filled with the CRF2R agonist or placebo was inserted.

Exercise. Mice assigned to the sedentary group did not undergo treadmill running and were allowed to carry out their normal in-cage daily activities. Mice assigned to the exercise group ran twice weekly for 30 min on a 6-lane variable speed treadmill (Eco 3/6 Treadmill; Columbus Instruments, Columbus, Ohio) starting at a speed of 8 m/min including a 5-min warm-up at 6 m/min. The exercise intensity was increased by 1 m/min at weekly intervals until an intensity of 12 m/min was reached; for the final 6 weeks of the study, exercise intensity was set at 12 m/min.

Motor Performance. Motor performance of all mice was assessed twice per week using a Rotarod apparatus (EzRod; Accuscan Instruments, Columbus, Ohio). Mice were placed on a rotating rod that increased to 20 rpm over a 15-s interval and given three trials to a maximum of 180 s for each trial. The point in time at which the mouse fell off the rotating rod was used as a measure of competency of this task. The best of the three trials was used as motor performance for the given testing day. During the week of pump reimplantation, mice were tested only once to allow for recovery.

Voluntary Wheel-Running. Daily activity was estimated each week by placing mice in a cage that monitors voluntary wheel-running (Mouse Single Activity Wheel System 80820; Lafayette Instruments, Lafayette, Indiana). The total distance run in 24 h was used as a measure of voluntary activity to determine whether exercise or the drug induced changes in voluntary running capacity.

Muscle Tissue. Upon completion of the 8-week intervention, animals were anesthetized using pentobarbital (55 mg/kg) and muscle tissue was excised. Extensor digitorum longus, soleus, tibialis anterior, and quadriceps were weighed following excision and were immediately quenched in liquid nitrogen. A piece of the quadriceps was removed and fixed in 10% formalin. White and red gastrocnemius muscles were snap-frozen for protein content analysis; the heart was removed and weighed.

Histology. Following fixation in formalin, the piece of quadriceps was embedded in paraffin and then sectioned and stained with hematoxylin and eosin by
the Pathology Department at McMaster University Medical Centre. The slides were then examined by a pathologist under a light microscope for fibrosis and adipose tissue. Three pictures for each specimen were obtained using a digital camera system (SPOT; Diagnostic Instrument, Sterling Heights, Michigan) mounted on a white light microscope (Olympus Bx60; Carsen Group, Markham, Ontario). The total number of muscle fibers and internally nucleated fibers were counted and the results were expressed as the ratio of internally nucleated fibers to total fibers.

**Protein Content.** White and red gastrocnemius muscles were homogenized on ice in a glass-Teflon homogenizer 1:25 (wt/vol) in buffer containing 50 mM potassium phosphate, 1 mM EDTA, 0.5 mM DTT, 1.15% KCl, and protease inhibitors at pH 7.4. The homogenates were centrifuged at 750 g for 10 min at 4°C. Protein content was assessed using the Lowry method as previously described. 

Briefly, 10 μl of sample was added to 990 μl of ultrapure water. Lowry reagent (Sigma-Aldrich, Oakville, Ontario) was dissolved in 40 ml of ultrapure water. Lowry reagent (1 ml) was added to each sample and mixed well using a vortex followed by 10 min of incubation. Folin (500 μl, diluted 1:6) was added to each sample and mixed using a vortex. Following 20 min of incubation, samples were read at 750 nm using a spectrophotometer (Cary 300 Bio UV-Visible Spectrophotometer; Varian, Palo Alto, California). Results were expressed as mg per g wet weight of muscle.

**Blood Sampling.** Whole blood was taken by cardiac puncture upon completion of the study. Blood was collected in untreated 1.5-ml polyethylene tubes, allowed to clot, and then spun at 1200 g for 10 min. Serum was removed and stored at −80°C. A commercially available kit (Diagnostic Chemicals, Charlottetown, Prince Edward Island) was used to measure γ-glutamyltransferase (GGT) activity. GGT activity was assessed by measuring the kinetic production of p-nitroaniline from glycylglycine and L-γ-glutamyl-p-nitroanilide. Briefly, 1 ml of buffer was added to 5 μl of serum. Samples were incubated for 3 min and then read spectrophotometrically (Cary 300 Bio UV-Visible Spectrophotometer; Varian) at 405 nm at 37°C for 5 min. The GGT and CK activities were expressed as U/L.

**Statistical Analysis.** A software package (Statistica v. 5.0; Tulsa, Oklahoma) was used to perform a three-way ANOVA, with Tukey’s HSD for unequal sample size used post hoc. The results are expressed as mean ± SEM and statistical significance was established at P < 0.05.

**RESULTS**

**Muscle Weight.** Extensor digitorum longus was heavier in CRF2R-treated than placebo-treated mice (P < 0.05) (Fig. 1A), whereas the soleus was heavier in mdx than wildtype mice (P < 0.05). Similarly, CRF2R agonist-treated mice had a heavier soleus than placebo-treated mice (P < 0.001) (Fig. 1B). There was no effect of CRF2R agonist treatment on the tibialis anterior. There was a three-way interaction (genotype, training, and drug, P < 0.05) in
quadriceps muscle; however, there were no significant differences between groups with post-hoc testing. The heart muscle was found to be smaller in CRF2R-treated mice than placebo-treated mice \((P < 0.001)\) (Fig. 1C).

Body Weight and Food Intake. Both mdx and wild-type mice gained body weight over the 8 weeks of the study \((P < 0.01\) for all weeks). Mdx sedentary CRF2R mice were heavier than mdx sedentary placebo mice \((P < 0.05)\). There was a complex interaction between genotype and week with mdx mice being heavier than wildtype mice starting at week 6 \((P < 0.01)\). Similarly, there was also a two-way interaction between drug and week, with CRF2R-treated mice being heavier than placebo-treated mice starting at week 4 \((P < 0.01)\).

Mdx mice consumed 7% more food than wild-type mice \((P < 0.01)\) with no effect of the drug intervention, and all mice ingested more food over the duration of the study after week 1 \((P < 0.001)\).

Motor and Exercise Performance. All exercised groups had higher motor performance than sedentary mice at week 3 testing day 1 of the study \((71.3 \pm 9.8 \text{ s and } 42.1 \pm 6.1 \text{ s}, P < 0.05)\). There were no significant differences between groups in voluntary wheel-running activity.

Histology. Hematoxylin and eosin slides of the quadriceps were randomly screened for the presence of fibrosis, adipose tissue, and internalized nuclei. There was no evidence of adipose tissue, with minimal fibrosis only in mdx samples. Differences were not observed between groups. The ratio of internalized nuclei to total number of fibers was higher in mdx than wildtype mice \((0.480 \pm 0.05 \text{ and } 0.166 \pm 0.06, \text{ respectively}, P < 0.001)\) with no differences between groups.

Protein Content. In the white gastrocnemius, there was a trend for mdx mice to have lower protein content compared to wildtype mice \((80.4 \pm 1.4 \text{ and } 84.8 \pm 1.8 \text{ mg/g wet weight, respectively}, P = 0.051)\). In the red gastrocnemius, CRF2R agonist-treated mice had higher protein content than placebo-treated mice \((81.8 \pm 1.2 \text{ and } 77.6 \pm 1.3 \text{ mg/g wet weight, respectively}, P < 0.05)\).

Enzyme Activities. There were no significant differences between treatment groups in GGT activity. Serum CK activity was found to be higher in mdx sedentary placebo mice than in all other groups, with the exception of mdx exercised CRF2R mice \((P < 0.001)\) (Fig. 2). There was a trend for mdx exercised CRF2R-treated mice to have lower CK activity than mdx sedentary placebo mice \((P = 0.055)\).

DISCUSSION

The three main findings of this study are: (1) administration of a CRF2R agonist resulted in an increase in muscle weight in both mdx and wildtype mice; (2) mdx mice that were exercised or given a CRF2R agonist had lower serum CK activity; and (3) both mdx and wildtype mice treated with the CRF2R agonist had lower heart weights.

Since the peptide used in this study was a sauvgine analog, an increase in muscle mass was predicted. Increased weight of the extensor digitorum longus and soleus was seen in CRF2R-treated mice regardless of genotype. In contrast, there was no significant effect of the CRF2R agonist on the quadriceps muscle. Since fibrosis and adipose tissue were not observed in the quadriceps muscle, the increase in muscle weight is likely due to an increase in muscle mass. It should be noted that urocortin II had a more potent effect on white than red denervated and casted muscle.13 However, the CRF2R agonist appeared to have a more significant effect on red than white muscle. Similarly, the quadriceps did not increase muscle weight in response to drug administration. It is possible that lower-leg muscles are more active and are therefore more responsive to CRF2R agonist treatment. Alternatively, mice have a greater proportion of white than red muscle fibers, and since the quadriceps is a mixed muscle, there may be a lesser effect on this muscle group given the higher proportion of white muscle fibers. Although there was no effect of the CRF2R agonist or exercise on Rotarod performance, this is an indirect measure of motor performance and is influenced by a variety of factors such as balance, coordination, and strength and is therefore not directly related to the specific muscles examined. Since aerobic exercise
does not significantly increase muscle weight, it is not surprising that exercise had no additional effect on any of the muscle weights when combined with CRF2R treatment.

The CRF2R in muscle modulates the stress response resulting from activation of the CRF1R. Stimulation of the CRF1R activates the hypothalamus–pituitary–adrenal axis resulting in the production of corticosteroids, which have a catabolic effect on skeletal muscle. CRF2R activation appears to limit the loss of skeletal muscle mass resulting from stress-induced corticosteroid production. Several lines of evidence support this hypothesis. First, corticosteroids upregulate expression of the glucocorticoid response element in the urocortin II gene promoter. Second, activation of the CRF2R in mice inhibits dexamethasone-induced skeletal muscle atrophy. Third, removal of the adrenal glands results in maintenance of muscle mass when denervated rats are treated with sauvagine. Given that CRF2R agonists appear to limit loss of skeletal muscle mass due to dexamethasone-induced muscle atrophy, there is great potential for their use in patients afflicted with muscular dystrophy. Corticosteroids such as prednisone and dexamethasone are a mainstay of therapy for Duchenne muscular dystrophy patients, but have detrimental side effects such as muscle atrophy, osteopenia, hypertension, attenuation of growth, and increased fat mass. The possibility of a combination therapy of steroids and a CRF2R agonist may offer a superior alternative, providing the benefits of steroid therapy with the CRF2R agonist counteracting muscle atrophy associated with this treatment.

The observation that serum CK activity was lower in CRF2R agonist–treated mice and was not different than wildtype placebo-treated mice is both novel and of potential clinical benefit. Others have previously reported a reciprocal relationship between a higher muscle weight and a lower CK activity in mdx mice. Furthermore, the mdx/myostatin knockout mouse had higher muscle mass and strength with lesser cellular infiltration and fibrosis than untreated mice. Similarly, when myostatin was suppressed using intraperitoneal injections of blocking antibodies for 3 months, an increase in body weight, muscle mass, muscle size, absolute muscle strength, and motor performance was observed. There was also a significant decrease in muscle degeneration and serum CK. Collectively, these findings support our observations and the concept that further hypertrophy is associated with histological and phenotypic outcomes that would predict clinical efficacy, in spite of the mild hypertrophy seen in the mdx muscle. Although some investigators have reported that exercise leads to greater muscle damage and weakness in mdx mice, others have reported that voluntary running for 1 year and even 5 weeks of treadmill running at 15 m/min followed by 5 weeks at 23 m/min did not induce necrosis, fibrosis, nor inflammatory cell infiltration. Recently, in our laboratory it was also found that following 8 weeks of low-intensity exercise (9.5 m/min), exercised mdx mice did not have significantly different serum CK activity and motor performance than sedentary mdx mice, indicating that treadmill running had no detrimental effects. The finding that exercised mdx CRF2R mice tended to have lower CK activity than sedentary placebo mdx mice indicates that exercise provided no additional benefit when combined with CRF2R agonist treatment. This observation may suggest that similar pathways are activated in response to both the CRF2R agonist and exercise, possibly explaining the lack of a combined effect.

The final finding of the study was that mice, regardless of genotype, have a lower heart weight when treated with the CRF2R agonist. Urocortin II and III have been described as efficacious vasodilators that induce vasodilation via the p38 mitogen-activated protein kinase and protein kinase A signaling cascades. Urocortin I induces coronary vasodilation, and urocortin II increases coronary blood flow and reduces systemic blood pressure. Since urocortin II binds to the CRF2R, similar effects could be hypothesized using other CRF2R agonists, such as the one used in our study. It is therefore possible that the CRF2R agonist decreases systemic blood pressure, resulting in decreased heart weight due to reduced afterload on the heart. The progressive dilated cardiomyopathy that typically occurs in dystrophinopathies is usually preceded by a hypertrophic phase. Among affected boys, 30% will have detectable cardiac abnormalities by the age of 14, and 50% by the age of 18, as will all individuals who live into their adult years. The early appearance of cardiomyopathy typically has a negative influence on lifespan of Duchenne muscular dystrophy patients, as heart complications are often the cause of death. Cardiomyopathy also occurs in other muscular dystrophies, such as limb-girdle muscular dystrophy. Given that the CRF2R agonist was found to decrease heart weight as well as decrease serum CK activity, this drug may have applications not only for muscle damage, but also for the cardiomyopathy associated with all muscular dystrophies.

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ABSTRACT: Myostatin is a transforming growth factor-β family member that acts as a negative regulator of skeletal muscle growth. In mice, genetic disruption of the myostatin gene leads to a marked increase in body weight and muscle mass. Similarly, pharmacological interference with myostatin in vivo in mdx knockout mice results in a functional improvement of the dystrophic phenotype. Consequently, myostatin is an important therapeutic target for treatment of diseases associated with muscle wasting. To construct a therapeutic DNA vaccine against myostatin, we coupled the foreign, immunodominant T-helper epitope of tetanus toxin to the N terminus of myostatin, and BALB/c mice were immunized with the recombinant vector. Sera from vaccinated mice showed the presence of specific antibodies against the recombinant protein. In addition, body weight, muscle mass, and grip endurance of vaccinated mice were significantly increased. Our study provides a novel, pharmacological strategy for treatment of diseases associated with muscle wasting.

MYOSTATIN DNA VACCINE INCREASES SKELETAL MUSCLE MASS AND ENDURANCE IN MICE

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Myostatin, formerly known as growth and differentiation factor-8 (GDF-8), is a member of the transforming growth factor-beta family that plays an essential role in regulating skeletal muscle growth.1,11,15 Myostatin is a negative regulator of skeletal muscle mass and its sequence has been highly conserved through evolution.15 Mutations in the myostatin gene lead to dramatic increases in skeletal muscle mass and muscle strength in mice and to less fat accumulation than in wild-type littermates.6,8,9,22 In humans, mutations in the myostatin gene are correlated with excess muscle mass, whereas expression of myostatin is increased in the setting of muscle loss, including chronic illnesses, infection with human immunodeficiency virus, and during aging.5,7,20

Systematic administration of exogenous myostatin to adult mice is sufficient to induce severe muscle and fat loss, similar to human cachexia syndromes.26 Conversely, myostatin antagonists, such as monoclonal antibodies specific to myostatin and follistatin, as well as activin type II receptor antagonists, can significantly increase skeletal muscle mass. Given the highly conserved role of myostatin among animals, improved methods for inhibiting myostatin activity could have important implications not only for human therapeutics, but also other areas such as agriculture.10

Although myostatin antagonists are obvious candidates for intervention, obtaining sufficient quantities of purified myostatin antagonist proteins can be costly and time-consuming. By contrast, DNA vaccines are stable, inexpensive, safe, and easy to produce in large quantities and have high levels of purity.2,4,13 Moreover, DNA vaccines stimulate a full spectrum of immune responses, including cytotoxic T lymphocytes generally not induced by protein vaccines, and generate exceptionally long-lasting immune responses.3,24

We describe the development of a myostatin-specific DNA vaccine. The pVAC1-cms plasmid is a DNA vaccine vector specifically designed to stimulate a humoral immune response, using the rhesus monkey elongation factor 1-alpha gene promoter to achieve high levels of expression in skeletal muscle cells and antigen-presenting cells. Expression levels are further increased by the addition of the SV40 enhancer, which heightens the ability of the plasmid...
to be transported into the nucleus. DNA encoding a fusion protein between the T-helper epitope of tetanus toxin (TT) and the mature myostatin peptide was cloned into pVAC1-cms vector (pVAC-TTMs). The immunogenicity of the recombinant DNA vaccine was examined in BALB/c mice. Sera were analyzed from vaccinated mice containing specific antibodies to the recombinant protein. The muscle and function of the mice were evaluated as well.

**MATERIALS AND METHODS**

A DNA fragment encoding the TT epitope (QYIKANSKFIGITEL),12 followed by the N terminus of mature myostatin (encoding amino acid residues 267–375, GenBank Accession No. 014793), was synthesized by Shenggong Biotechnology (Shanghai, China). This fragment was subcloned into the EcoRI and BamHI sites of pVAC1-cms (Invitrogen, Carlsbad, California). The recombinant plasmid (pVAC-TTMs) was confirmed by sequencing.

Plasmid pVAC1-cms and pVAC-TTMs were transformed into expression-competent Escherichia coli harboring the DH5 lysogen by heat shock. The transformation mixture (10 μl) was added to Luria–Bertani (LB) medium (10 ml) containing 100 μg/ml zeocin (Invitrogen) and incubated overnight at 37°C with vigorous shaking. This culture was used to inoculate 200 ml of prewarmed LB medium containing 100 μg/ml zeocin for 24 h at 37°C. Cells were harvested by centrifugation at 5000 × g for 20 min and the plasmids were extracted and purified as described elsewhere.20

Male BALB/c mice (2–3 weeks old) were purchased from the National Rodent Laboratory Animal Resource (Shanghai, China) and housed in a room controlled for temperature (22 ± 2°C) and humidity (60 ± 5%) and regulated to provide alternating 12-h periods of light and darkness. Mice were allocated to two groups (n = 6 for each group) and injected intramuscularly (IM) with 50 μg each of pVAC1-cms or pVAC-TTMs. Mice were vaccinated on days 0, 14, and 28, then boosted (IM) on day 42, and killed on day 67 for serum and tissue analysis. Serum total cholesterol, triglycerides, creatine kinase, and antibody titer were analyzed. At the same time, the hind limb was photographed. The abdominal fat pad, a portion of the quadriceps (rectus femoris), and the gastrocnemius were dissected and weighed.

Detection of Expression of Plasmid-Encoded TT-Ms in Mice by Western Blot. Tissues kept at −80°C from quadriceps muscle of different groups were homogenized in 10–20 vol of a buffer containing 1% sodium dodecylsulfate, 100 mM Tris–HCl (pH 6.8), 1 mM phenylmethylsulfonylfluoride, and 0.1 mM β-mercaptoethanol. The supernatant obtained after centrifugation of homogenized tissue at 15,000 × g for 25 min (4°C) was designated the homogenate, and the same amounts of supernatant quadriceps muscle (20 μl) from the mice vaccinated with pVAC-TTMs and pVAC1-cms and purified B7H1 (control protein from our laboratory) were loaded for sodium dodecylsulfate–polyacrylamide gel electrophoresis (15% acrylamide). The proteins were then electrophoretically transferred (80 V, 2 h) onto nitrocellulose (NC) membranes (Bio-Rad Instruments, Hercules, California). The NC membranes were blocked using phosphate-buffered saline (PBS) containing 2% bovine serum albumin (BSA) and 0.05% Tween-20 (PBST) at 4°C overnight. The NC membranes were then incubated with either anti-TT antibody at 1:500 dilution (Abcam, Cambridge, Massachusetts) or anti–GDF-8 antibody at 1:250 dilution (Bethyl, Montgomery, Texas) for 2 h at room temperature. Membranes were washed three times for 10 min in PBST and incubated with alkaline phosphatase–conjugated goat anti-mouse IgG (Life Technologies, Carlsbad, California) at 1:1000 dilution and goat anti-rabbit IgG (Life Technologies) at 1:1000 dilution in 2% BSA-PBST for 2 h at room temperature. Finally, the membranes were washed as described previously, and developed by adding 10 ml of alkaline phosphatase developer (Life Technologies). The reaction was stopped by rinsing the NC membranes with de-ionized water.

**Enzyme-Linked Immunosorbent Assay.** Enzyme-linked immunoassay (ELISA) plates were coated with 100 ng of purified myostatin protein and incubated overnight at 4°C. The plates were washed three times with PBS containing 5% Tween-20. Serially diluted mouse sera (100 μl) were added to each well and assayed in duplicate after blocking as described earlier, and then incubated for 1 h at 37°C. After washing, horseradish peroxidase–conjugated goat anti-mouse IgG (diluted 1:1000) was added to each well (100 μl per well) and incubated for 1 h at 37°C. The reactions were then visualized with o-phenylenediamine dihydrochloride and H₂O₂ and stopped by adding 100 μl of 2 M H₂SO₄. The plates were read at OD₄₅₀ using a Bio-Rad ELISA reader.

**Competitive Inhibition Assay.** Ninety-six-well plates were coated with 100 ng of purified myostatin protein and incubated overnight at 4°C. Plates were then washed three times with PBS containing 5%...
Tween-20 and blocked with BSA for 30 min at 37°C. Anti–GDF-8 polyclonal antibody (100 ng/well) was preincubated with serially diluted sera from mice treated with pVAC-TTMs, pVAC1-cms (negative control), or B7H1 (control antiserum from our laboratory) protein for 30 min at 37°C before addition to myostatin-coated wells. After washing, bound GDF-8 antibody was detected by incubation with horseradish peroxidase–conjugated secondary antibody. The reactions were visualized as described earlier. Inhibition rate was defined as $1 - \frac{OD_{490} (pVAC-TTMs)}{OD_{490} (pVAC1cms)} \times 100\%$.

**Grip Test.** Grip tests were conducted as described by Peled-Kamar et al. with some modifications. Mice were allowed to grip and hang from a 2-mm horizontal tight-rope, 80 cm above the ground, but the measurement of grip time was different from the method of Peled-Kamar et al. The tails of mice were immobilized when the forelimbs of the mice gripped the rope. The time that elapsed until the forelimbs loosened and the mice fell to the ground was measured. The mice were studied three times each on two different days, and measurements were averaged.

**Morphometric Analysis.** The gastrocnemius muscles were dissected rapidly and freed of fat and connective tissue. Muscle tissues were weighed and fixed with 4% polyaldehyde for 24 h. Serial 8–10-μm transverse sections, made with a cryostat, were mounted on silanized slides (Dako, Tokyo, Japan). Cross-sectional areas were measured at quadricep midportions following staining with hematoxylin–eosin.

**Statistical Analysis.** Statistical analysis of the data was performed with the Student’s unpaired $t$-test. Results are expressed as the mean ± SD. Differences were considered statistically significant at $P < 0.05$.

**RESULTS**

The average body weights for each group of mice were measured and found to increase during the course of the study. The mice vaccinated with pVAC-TTMs gained more weight than control mice vaccinated with pVAC1-cms (Fig. 1A). Photographs of the bodies and hindlimbs showed that muscle tissues of the mice vaccinated with pVAC-TTMs were obviously larger than those of control mice. Measurements for average mass of the quadriceps and gastrocnemius muscles from mice immunized with pVAC-TTMs were 31.4% and 13.9% greater, respectively, compared with the control group (Fig. 1B). Abdominal fat pad weights, however, did not differ between the two groups (Fig. 1C).

To confirm the expression of recombinant plasmid in muscle cells in vaccinated mice, we performed Western blot with two antibodies: anti–GDF-8 antibody and anti-TT antibody. The expression of myostatin in skeletal muscle cells could be detected by anti-TT antibody (Fig. 2). The results confirm the expression of recombinant protein TT-Ms in muscle cells.

We performed morphometric analyses of the quadriceps muscles from the two groups (Fig. 3A) to determine whether the increase in muscle mass in the mice vaccinated with pVAC-TTMs was due to hypertrophy or hyperplasia. A significant increase in whole-muscle cross-sectional area in mice immu-
nized with pVAC-TTMs was observed, as compared with control mice (Fig. 3B). No significant difference was found in the number of muscle fibers per quadricep between the two groups (Fig. 3C), suggesting increased muscle mass was due to hypertrophy rather than hyperplasia.

We calculated the product of time and body weight for each animal (Fig. 4A). The average grip time/body weight was about 36.5% greater in the mice vaccinated with pVAC-TTMs than in control mice (Fig. 4B). The results show that the grip endurance of the mice vaccinated with pVAC-TTMs was significantly greater than that of control mice.

The immunogenicity of pVAC-TTMs was evaluated in BALB/c mice by ELISA. Sera from pVAC-TTMs–vaccinated mice contained specific antibodies against myostatin protein (Fig. 5). The ability of antibodies induced by pVAC-TTMs to bind myostatin was examined by ELISA in a competitive assay with a polyclonal GDF-8 antibody. Sera from pVAC-TTMs–vaccinated mice were able to reduce binding of the anti–GDF-8 antibody to myostatin (Fig. 6). The inhibition rates were 62.2% at 1:5 dilution and 30.0% at 1:40 dilution, respectively, suggesting that sera from pVAC-TTMs–vaccinated mice are able to interfere with myostatin in vitro.

Serum levels of total protein, albumin, globulin, urea nitrogen, glucose, cholesterol, triglycerides, and creatine kinase in pVAC-TTMs–vaccinated mice and control mice were not significantly different.
DISCUSSION

Myostatin is highly conserved, both in sequence and in function, across animal species. The amino-acid sequences of mouse and human myostatin are identical. Our results indicate that specific antibodies to the recombinant myostatin vaccine were effectively induced and the titers could inhibit myostatin in mice. Importantly, significant increases in body weight and skeletal muscle mass were observed following DNA vaccination. The effect of the myostatin DNA vaccine was comparable with other non–DNA vaccine methods of myostatin interference. For example, a recent study demonstrated that the body weights of mice treated with a neutralizing monoclonal antibody to myostatin increased by 10.0%. Similarly, mice expressing a dominant-negative myostatin variant showed an increase of up to 35.0% in skeletal muscle mass. Our results suggest that a myostatin DNA vaccine could promote an increase in skeletal muscle mass.

Interestingly, there was no significant difference in abdominal fat pad weights and level of triglycerides and cholesterol in blood in vaccinated mice in our study. These results differ from the reported myostatin knockout phenotype, in which fat accumulation was decreased. This discrepancy could be due to differences in loss of myostatin activity during development and during adulthood. Our results suggest that the increase in body weight is caused by the increase in muscle mass but not in fat weight.

Although the muscle mass of mice immunized with the myostatin DNA vaccine was found to be increased significantly relative to the control group,
this is not proof of increased muscle function. We therefore quantified functional improvement and found an increase in forelimb grip endurance in the mice immunized with the myostatin DNA vaccine. The increase in muscle endurance was proportional to the increase in muscle mass in the mice immunized with the myostatin DNA vaccine. However, more accurate methods than the grip test are necessary to determine the precise effect of myostatin DNA vaccine on muscle strength.

Our study has provided physiological evidence of functional improvement in muscle growth caused by myostatin inhibition in vivo. Myostatin activates the ubiquitin proteolytic system through a nuclear factor–kappaB (NF-κB)-independent, FoxO1-dependent mechanism. The central nervous system (CNS) and peripheral blood flow may play an important role in the activation and performance of myostatin, but it is unclear whether the inhibition of myostatin caused by myostatin DNA vaccine is related to them.

The manner in which muscle mass is increased is important. We found that the number of muscle fibers from mice immunized with the myostatin DNA vaccine did not increase but the muscle fibers were hypertrophic. By contrast, in mice expressing dominant-negative myostatin, muscle fibers showed both hypertrophy and hyperplasia.

The physiological changes observed in the mice immunized with myostatin DNA vaccine were not accompanied by obvious detrimental changes in serum biochemistry, suggesting that vaccine-mediated inhibition of myostatin can increase skeletal muscle size without major side-effects. The functional enhancement of muscle by myostatin DNA vaccine thus provides a novel, pharmacological strategy for the treatment of diseases associated with muscle wasting. However, further safety studies and studies of the long-term effects of induced autoimmunity are required. The present study has provided the foundation for future work on this exciting and promising new strategy.

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ABSTRACT: This investigation analyzes the temporal characteristics of maximal depolarization times for three waveforms: end-plate spikes, fibrillation potentials, and positive sharp waves (PSWs) to provide support for the electrode initiation hypothesis of PSW induction. The maximal depolarization times for PSWs are documented to comprise two distinct populations conforming to relatively short and comparatively longer maximal depolarization times. Those PSWs with short maximal depolarization times were found to be equivalent to end-plate spike maximal depolarization times, whereas those with longer times were comparable to fibrillation potentials. The PSW group with shorter maximal depolarization times was encountered more frequently. The combination of two distinct groups of PSWs with respective times comparable to end-plate spikes and fibrillation potentials supports the hypothesis that the majority of PSWs originate at the recording electrode during insertion, whereas a smaller population of PSWs arises as propagating fibrillation potentials that block at the recording electrode. Subcutaneous compared to intramuscular recordings from denervated muscle document that the recording electrode is necessary to both record and produce PSWs. Hence, this study confirms the proposed hypothesis that the majority of observed PSWs represent a suprathreshold single muscle-fiber discharge induced by, and originating in close proximity to, a perielectrode crushed membrane that then propagate away from the electrode; a smaller population of PSWs conform to that of a blocked fibrillation potential.


POSITIVE SHARP WAVE ORIGIN: EVIDENCE SUPPORTING THE ELECTRODE INITIATION HYPOTHESIS

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A previous investigation has posited a complete revision of the manner in which positive sharp waves (PSWs) are generated. Specifically, instead of conceptualizing the PSW as a blocked fibrillation potential (FP), the majority of detected PSWs were postulated to initiate at, and subsequently propagate away from, the recording electrode. Further, a second comparatively smaller population of PSWs was theorized to conform to the previous blocked FP hypothesis. Hence, there are conjectured to be two populations of PSWs generated by different mechanisms, but related in configurational etiology through the so-called “crush membrane hypothesis.”

The aforementioned revised theory of PSW generation makes several verifiable predictions. Specifically, a waveform that initiates at the recording electrode should have a shorter maximal depolarization time than a distant propagating action potential. This is because the action potential coincident with, or adjacent to, the recording electrode does not propagate and, therefore, the temporal domain associated with its volume-conducted initial wave of depolarization is absent. The second potentially verifiable prediction is the supposition of two separate populations of PSWs based on their respective times to maximal depolarization. Third, the shorter-duration PSW population should be detected in greater numbers than the population with longer maximal depolarization times. Fourth, the needle electrode–muscle membrane interaction is necessary for simultaneously producing and detecting PSWs.

In this investigation, these predictions were examined by measuring the time of waveform onset to maximum depolarization for three waveforms: end-plate spikes (EPSs); fibrillation potentials; and positive sharp waves.
tive sharp waves. End-plate spikes clearly represent single muscle-fiber discharges that originate at, or in close proximity to, the recording electrode, whereas FPs (triphasic, initially positive waveforms) are potentials that typically originate away from, and propagate toward, the recording electrode. The time-course from potential onset to maximal depolarization for each of these waveforms with known “propagation” characteristics was compared to the maximal depolarization times for PSWs so as to better define their origin. Additionally, two distinct populations of PSWs with different maximal depolarization times were sought. Finally, subcutaneous compared to intramuscular electromyographic recordings in patients with denervated muscles were compared to assess the recording electrode’s influence on the waveform configurations derived from spontaneously depolarizing muscle membranes.

**MATERIALS AND METHODS**

The present investigation was approved by our human experimentation institutional review board.

**Simulation Data: Single Muscle-Fiber Simulation.** A computer program designed to simulate the extracellular waveform characteristics associated with a propagating single muscle-fiber action potential on a finite length of muscle fiber was used to define maximal depolarization times for EPSs and propagating waveforms. This particular program utilized the following well-accepted volume-conductor principles: core conductor model; anisotropic homogeneous volume conductor; finite single muscle-fiber length with fiber terminations simulating a musculotendinous junction; single muscle-fiber diameter of 50 μm; muscle-fiber conduction velocity of 3.7 m/s; and an ability to record the extracellular voltage field distribution about the simulated single muscle fiber at any radial location along the fiber’s length.

The single muscle fiber was modeled to be 30 mm in length with an end-plate located in the middle of the fiber and both muscle-fiber terminations simulating a musculotendinous junction. A recording electrode position was designated 25 mm radially from the fiber’s surface and sequentially positioned in 0.2-mm increments from the end-plate region (0 mm) to the fiber’s termination (15 mm), with a waveform observed at each of these locations. The time from waveform onset to maximal peak depolarization was measured. For the EPS, this time corresponded to the initial negative baseline departure to the maximal negative peak, and for the propagating triphasic single muscle discharge the measured time corresponded to the waveform’s initial onset (positive baseline departure) to maximal negative spike following the initial positive deflection (Fig. 1).

**Clinical Data: Waveforms.** Our electrophysiologic data base contained individual waveforms stored within our instrument’s hard drive in 20-second ep-
Each epoch consisted of individual waveforms comprising essentially all of the common and unusual potentials recorded during a needle electromyographic examination and subsequently categorized according to previously defined criteria. Three distinct types of waveforms were extracted from the database for analysis in this investigation; biphasic initially negative EPSs; triphasic, initially positive FPs; and biphasic, PSWs.

Each waveform trace was recalled from the instrument’s hard drive and displayed on the electrophysiologic instrument in a rastered mode with 10 traces per screen. Distinct waveforms were identified by visual inspection and reviewed over multiple 100-ms traces so that each waveform was accepted for analysis only once. In order to ensure accurate waveform parameter assessment, at least 10 ms of a flat baseline preceding each potential was required prior to analyzing any of the waveforms’ temporal parameters.

Waveform onset to maximal depolarization was measured for each of the three potentials noted previously. For the EPS, this time frame extended from the potential’s initial negative departure from baseline to maximal negative spike magnitude (Fig. 1). Fibrillation potential time to maximal depolarization was defined as extending from initial baseline departure in the positive direction to the subsequent negative spike’s peak. Finally, PSW maximal depolarization time was assessed as initial baseline departure to maximal positive peak magnitude.

End-plate spikes were recorded from individuals without evidence of electrophysiologic abnormalities during routine needle electromyographic assessment. Positive sharp waves and FPs were recorded only from persons with various lesions resulting in denervation from peripheral nerve axonal damage. None of the FPs or PSWs analyzed were derived from individuals with myogenic disorders.

### Subcutaneous vs. Intramuscular Electromyographic Recordings

During the routine needle electromyographic assessment of 5 patients with peripheral nerve lesions to the sciatic/peroneal nerve resulting in muscle-fiber denervation of the tibialis anterior muscle, a monopolar needle electrode was initially inserted just beneath the skin surface over the tibialis anterior muscle and the instrument’s amplifier immediately activated. The needle electrode was verified to be located in the subcutaneous tissue overlying the muscle, ensuring the complete absence of insertional activity during slight needle movements. Approximately 2 minutes of recording time was saved to the instrument’s hard drive for future analysis.

### Instrumentation

An electromyographic instrument (Synergy; Viasys Healthcare, Madison, Wisconsin) was utilized to collect and display each waveform trace. All of the waveforms were recorded, displayed, and analyzed at the following instrument settings: sweep, 10 ms/division; sensitivity, 100 μV/division; low-frequency filter, 10 Hz; and high-frequency filter, 10,000 Hz. A monopolar needle electrode with a surface reference and ground electrode located adjacent to the skin insertion site was used to record all of the clinical waveforms analyzed in this study.

### Statistical Analysis

The spontaneous single muscle-fiber data represented three separate and unrelated waveforms obtained from multiple individuals. As a result, statistical consultation suggested the most appropriate approach would be that of a distributional analysis. Specifically, particular attention was given to the data’s distribution as to how well it conformed to a normal distribution utilizing the kernel density plot methodology. This type of analysis was suggested to yield the clearest assessment as to whether more than one population of PSWs could be detected. In conjunction with our institutional statistician, the Stata software package (StataCorp., College Station, Texas) was used to perform all of the statistical analysis on the clinically derived data. Further, a robust analysis of variance (ANOVA) and a post hoc Games–Howell analysis were utilized to define the various standard comparative metrics between each population.

### RESULTS

#### Single Muscle-Fiber Simulation

Analysis of individual waveforms of time to maximal depolarization of single muscle-fiber waveforms revealed a linear relationship between the time to maximal depolarization and distance of action potential propagation (Fig. 1). Maximal depolarization time for potentials recorded in close proximity to the end-plate zone approximated 1 ms, whereas those discharges recorded at between 5 mm and 7 mm approached 2.5 ms (Fig. 1). The transition between an end-plate spike and triphasic configuration occurred between 0.4 and 0.6 mm from the site of action potential initiation (Fig. 1). These findings documented that
those recorded waveforms with relatively short times to maximal depolarization arise in close proximity to the recording electrode whereas those with comparatively longer times originate at some distant location and propagate toward the recording electrode.

Clinical Data: End-Plate Spikes. Statistical analysis of the data’s size (number of waveform observations) revealed that a significance level of $P < 0.01$ was most appropriate for all statistical evaluations performed on the various data sets in this investigation.

A total of 304 EPS waveforms were analyzed. Mean maximal depolarization time was 1.3 ms ($\pm 0.4$ ms; 0.5–2.6 ms). In particular, the data were clearly distributed normally with a skewness of 0.3 and kurtosis of 3.2 at a statistical level of $P > 0.01$ (Fig. 2A).

Fibrillation Potentials. The FPs totaled 378 individual waveforms. Mean maximal depolarization time was 1.8 ms ($\pm 0.5$; 0.7–3.4 ms). Of note, the data distribution was normally distributed with a skewness of 0.2 and kurtosis of 2.7 at $P > 0.01$ (Fig. 2B). The respective probabilities for skewness and kurtosis were calculated to be 0.2 and 0.3; ($P > 0.01$), thereby supporting the conclusion that the fibrillation maximal depolarization time data were distributed normally.

Positive Sharp Waves. There were 358 PSWs for analysis. The mean time to maximal depolarization for all PSWs was 1.6 ms ($\pm 0.5$; 0.7–3.7 ms). Analysis revealed a unique data distribution (Fig. 2C), with a bimodal time to maximal depolarization distribution, the first peak approximating 1.3 ms and the second approaching 2.1 ms. Visual inspection of this bimodal curve clearly demonstrated a non-normal data distribution with supportive quantitative data of skewness (1.1) and kurtosis (4.3) at associated probabilities of $P < 0.001$.

A robust ANOVA of the group means revealed that they were all significantly different from each other ($P < 0.001$). Subsequently, a post-hoc Games–Howell test demonstrated that the pairwise comparison for EPS vs. PSW, EPS vs. FP, and PSW vs. FP group means were all significantly different ($P < 0.001$).

The kernel density graphic representation for the PSW data strongly suggested there were two subpopulations of PSWs because of the observed bimodal distribution (larger number of waveforms designated PSW A, and smaller population designated PSW B; Fig. 2C). Unfortunately, because of a limited number of variables to describe the PSW (maximal depolarization time only), with no way to distinguish between them by configuration (both are biphasic, initially positive), there was no straightforward manner in which to attempt a well-delineated separation for these two presumed PSW subpopulations, thereby requiring a number of assumptions. The data from this investigation clearly substantiate the belief that maximal depolarization times for both EPSs and FPs had normal distributions. Hence, we assumed that those PSWs comprising the smaller delayed subpopulation would also have a normal distribution. Further, based on the graphical data (Fig. 2C), we chose 1.9 ms to represent the PSW B subpopulation’s median, and this also happened to represent the 95th percentile of data for EPS maximal depolarization times. Therefore, in utilizing the 1.9-ms criterion, a data set was constructed for the PSW B population by extracting those values from the total PSW data set so as to construct a distribution utilizing data >1.9 ms as a guide to form a relatively symmetrical distribution about this median (Fig. 2E).

Once the PSW B data set, consisting of 139 total maximal depolarization values, was extracted from
the total PSW data set, the remaining values comprised a population referred to as PSW A (219 data values). The PSW B data set revealed a mean maximal depolarization time of 1.9 ms ($0.5; 1.0–3.7$ ms). The remaining data comprising the PSW A group demonstrated a mean maximal depolarization time of 1.4 ms ($0.3; 0.7–2.5$ ms). This group was tested and found to be distributed normally at a level of $P < 0.01$. The EPS, FP, and two PSW subsets (PSW A and PSW B) were evaluated with a robust ANOVA and the results ($P < 0.001$) permitted a post hoc Games–Howell analysis for all data set comparisons. Specifically, comparisons revealed a statistically significant difference at $P < 0.001$ for EPS vs. PSW B, EPS vs. FP, PSW A vs. FP, and PSW A vs. PSW B. However, this analysis demonstrated no significant difference between the EPS vs. PSW A data ($P > 0.01$) and the PSW B vs. FP data at $P > 0.01$.

**Subcutaneous vs. Intramuscular Recordings.** The comparison recordings revealed a number of findings. It was certainly possible to easily record spontaneously discharging single muscle fibers from the subcutaneously located electrode. The waveforms were of small amplitude but were sufficiently large to be clearly discerned above the background noise (Fig. 3A). All observed spontaneously discharging waveforms displayed the primary configuration of a negative spike representing FPs. At no time in any patient were PSWs observed with the subcutaneously located electrode. Upon redirecting the needle electrode into the muscle tissue, numerous FPs and PSWs were easily detected (Fig. 3B). This limited number of observations was designed primarily as an observational assessment to verify a previous preliminary study utilizing a similar methodology, but was quite relevant to this investigation’s primary hypothesis of the needle interacting with the muscle fiber to produce a PSW. No attempt was made to quantify the absolute numbers of various potentials observed. Recording the intramuscularly detected waveforms with a low-frequency filter of 500 Hz compared to 10 Hz without altering the high-frequency filter (10,000 Hz) revealed that the primary positive spike component of the PSW remained obvious and could continue to be observed clearly (Fig. 3C and D).

**DISCUSSION**

A reconceptualization of PSW origin from that of exclusively a blocked FP to an initiation at the recording electrode should be able to be verified. Simulation data utilizing a well-accepted volume core-conductor model objectively demonstrates that the
time to maximal depolarization is directly related to the distance between the recording electrode and site of action potential initiation. This finding is certainly to be anticipated given that waveforms initiating at the recording electrode immediately depolarize and reach peak depolarizations quickly (Fig. 1). However, those waveforms that require propagation reveal their initial depolarizing current as a positive deflection from the baseline in advance of their corresponding negative sink’s arrival at the electrode. Hence, the temporal aspects of maximal depolarization times vary depending upon the distance between the recording electrode and waveform initiation site. It is to be noted that additional variables may contribute to this temporal domain and include muscle-fiber conduction velocity, sodium activation time, sodium current density, volume-conductor effects, and very likely other factors. Therefore, some biologic variability among individual muscle fibers can be anticipated, with faster conducting fibers and those with comparatively short sodium activation times likely to have shorter maximal depolarization times than those fibers further from the recording electrode with slower conduction velocities and longer sodium activation times. It is not possible to control for these factors while recording from muscle fibers in subjects. Perhaps the best approach is to investigate respective group means of large sample sizes and employ the most feasible statistical methodologies to arrive at the best generalizations possible.

A logical waveform to consider initially is the EPS. It is certainly well accepted that biphasic, initially negative EPSs have particular characteristics permitting relatively easy identification. Further, it is accepted that these waveforms display their particular configurations (biphasic, initially negative waveform onset) specifically because they originate coincident with, or in very close proximity to the recording electrode. Data derived from this study demonstrate that the maximal EPS depolarization times are normally distributed, suggesting a rather uniform population and hence the most appropriate statistical methodologies from which to compare other waveforms (Fig. 2A).

A large number of triphasic, initially positive FPs were assessed. Data analysis also documented a normal distribution of maximal depolarization times for these potentials (Fig. 2B). Their mean maximal depolarization time of 1.8 ms exceeded the corresponding end-plate depolarization time (1.3 ms) at a statistically significant level, verifying the theoretical supposition of longer maximal depolarization times for propagating waveforms. Volume-conductor theory clearly states that triphasic, initially positive waveforms initiate at some distance from, and then propagate toward, the recording electrode. The most likely explanation for the shorter FP maximal depolarization times relates to the electrode coincidentally located in close proximity to this potential’s site of origin, but far enough away for the electrode to be located in a region acting as a positive source current. In other words, for those relatively shorter FP depolarization times, the electrode was located just far enough from the FP’s origin to be in a region of source current for the potential’s approaching negative sink, but still relatively close to the electrode, resulting in a comparatively short maximal depolarization time. Similarly, a number of EPSs had maximal depolarization times that may be considered relatively long, approaching 2.6 ms. According to volume-conductor theory, the waveform’s initial negative deflection confirms that the electrode was within the action potential’s negative sink. This negative sink is not a finite point but has a spatial expanse. Longer EPS depolarization times may occur for some muscle fibers when the electrode is located at the periphery of the end-plate zone, thereby requiring more time to reach the fiber’s threshold value. Additionally, single-fiber jitter studies have documented considerable variation in times required to reach the muscle membrane’s threshold value between different end-plates.

We documented a bifid PSW distribution, with one population appearing larger in number than the other (Fig. 3C). A statistical analysis with reasonable assumptions permitted a separation of these two PSW populations. Those PSWs with the greatest number (PSW A: 61%) had shorter maximal depolarization times (1.4 ms) than the smaller population of PSWs (PSW B: 39%) with longer depolarization times (1.9 ms), and were not significantly different from the EPS maximal depolarization times (1.3 ms). These findings correlate with the previously noted predictions for PSW origin postulated from insertional spike activity, and all of these strongly support the notion that the majority of clinically observed PSWs originate in close proximity to the recording electrode. First, there are indeed two populations of PSWs. Second, the PSWs with the largest population, and hence most commonly detected, had the shortest maximal depolarization times. Third, the most common PSW maximal depo-
larization times were not significantly different from EPS maximal depolarization times.

Additionally, a second distinct subset of PSWs appeared to originate at some distance from the recording electrode, most likely as an FP, to subsequently block at the electrode. Both suppositions require the recording electrode to adversely affect action potential initiation/propagation in order to both generate and record PSWs.

The comparison of subcutaneous and intra-muscular recordings in denervated muscle clearly demonstrates that the recording electrode’s presence within the muscle tissue is crucial for the detection of PSWs. All recorded observations from this portion of the investigation documented that, unless the needle electrode is positioned within the affected muscle, only FPs are observed (Fig. 3A and B).2 The position of the needle recording electrode is responsible for both the detection and production of PSWs.6,7 Additionally, this is the likely explanation for the failure of basic studies utilizing microelectrodes and intracellular electrodes in denervated muscle to detect PSWs.8,10,11,14 Therefore, the hypothesis that a PSW is the result of an interaction between the recording electrode’s mass effect and muscle membrane can be substantiated. Further, this finding clearly documents that attempts to trivialize the complexity of FP and PSW configuration as merely a tissue-filtering effect are simply erroneous. In particular, elevating the low-frequency filter in an attempt to mimic proposed tissue-filtering effects to convert a PSW into an FP simply cannot convert a 3–5-ms FP into a ≥20-ms PSW.8 Quite the contrary, as demonstrated (Fig. 3C and D), both FPs and PSWs shorten considerably in duration, as would be anticipated from basic filter effects, and do not appreciably alter their respective configurations. Similarly, an electrode simultaneously recording both PSWs and FPs with short rise-times, thereby defining the discharging muscle fibers to be in close proximity to the recording electrode, would not detect such strikingly disparate-appearing waveform configurations if tissue filtering were the sole explanation. Specifically, it is unlikely that such dramatically different tissue characteristics, which were postulated but not documented,5 are operational over such short distances. The difference between PSWs and FPs, therefore, appears to be related primarily to the presence of a particular needle-induced membrane deformation as opposed to other ill-defined causes. It is appropriate, however, to be concerned about the connective tissue and fat layers acting to “filter-out” the low-frequency component of the PSW for subcutaneous electrode recordings, thereby either rendering PSWs completely absent or altering their configuration substantially so as to be unrecognizable. However, comparative recordings at low frequencies of 10 Hz and 500 Hz demonstrated that both FPs and PSWs were reduced in amplitude and shortened in duration, but the primary configuration of each waveform remained easily definable (Fig. 3C and D). Additionally, PSWs and FPs are of similar amplitude and hence both should be detected from simply a voltage magnitude perspective. Therefore, the failure to detect PSWs with the subcutaneous electrode is a real effect and not an artifact of the recording distance or tissue-filtering effects, thereby supporting the conclusion that the electrode itself is responsible for, and required for generation of a PSW.

A compelling understanding of PSW generation is now possible. In short, a PSW represents a foci of depolarization, originating from a single muscle fiber with an unstable resting membrane potential, immediately adjacent to a perielectrode-induced region of membrane crush, which then propagates away from the electrode. A second category of PSW appears to represent the more traditionally held concept of a blocked FP at the aforementioned region of perielectrode crush membrane deformation. Either type of PSW may transition into an FP if the crush region dissipates secondary to membrane accommodation.6,7 Similarly, an FP may transition to a PSW if the mass effect of the electrode induces a crush region over time.

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ABSTRACT: Performance in high-intensity exercise is dependent on the ability to activate motor units. The main aim of this study was to test the hypothesis that adult men and women (age 19–27 years) are able to maintain higher levels of voluntary activation (VA) in knee extensor muscles than boys and girls (age 12–14 years). The volunteers \( (n = 7 \text{ in each group}) \) performed three 5-s maximal voluntary contractions (MVCs) and a continuous 2-min MVC. The VA and fatigue of the muscles was assessed by applying 250-ms 100-Hz test tetani (TT100Hz). During brief MVCs girls showed lower VA than women, but the difference between boys and men was not significant. During the 2-min MVC, VA in boys and girls was more depressed than in adults. The end-exercise values of the relative TT100Hz torque correlated with the average VA during the exercise. Thus, the results of the study support the hypothesis that children are more susceptible to central fatigue than adults. This should be taken into account when evaluating results of fitness tests that require high levels of motor unit activation.


CHILDREN ARE MORE SUSCEPTIBLE TO CENTRAL FATIGUE THAN ADULTS

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Exercise-induced fatigue can be assessed by measuring a decrease in muscle force-generating capacity. It occurs due to limitations in skeletal muscles themselves or in the nervous system. The terms peripheral fatigue and central fatigue have been used to discriminate between these two possible sites of muscle fatigue. It is now clear that peripheral fatigue occurs during many types of muscle exercise, but increasing evidence indicates that central fatigue is common as well.1,12,15,25,27

It is still not quite clear how age affects resistance to muscle fatigue. Children often demonstrate lower fatigue rates and faster recovery than adults.9,14,19 Fatigue studies are usually based on measurements of muscle force or power during high-intensity exercise, and the ability to activate skeletal muscles might be of importance in this regard.2,12,17 Assessments of muscle force reserve using electrical stimulation suggest that adults achieve 85%–100% activation of skeletal muscles during maximal voluntary contractions (MVCs).12,15 The findings regarding voluntary activation in children are somewhat contradictory. Early studies showed an almost complete activation of motor units in the triceps surae and quadriceps muscles during MVC.4,8 However, the results of these studies could have been affected by difficulties in assessing muscle force reserve due to low amplitude of interpolated twitches. A recent study using interpolated tetani showed that 8–12-year-old children have a significant deficit in voluntary activation of the quadriceps muscle.24 Thus, there is a need to reinvestigate and compare voluntary activation of children and adults. This would help to clarify whether the superior fatigue resistance of children is due to lower voluntary activation of skeletal muscles during exercise.

The ability to activate skeletal muscles deteriorates during strenuous exercise, but it is largely unknown whether children and adults differ in susceptibility to such central fatigue.12,15,19,23 In view of these uncertainties, the main aim of this study was to compare the ability to activate knee extensor mus-
cles in adults and 13–14-year-old children. The study also aimed to investigate whether children and adults differed in their ability to maintain voluntary activation of skeletal muscles during exercise.

There are two popular indices of muscle voluntary activation.\textsuperscript{3,7,12} The voluntary activation index (VA) can be calculated as the ratio of extra torque generated by electrical stimulation during MVC to the torque when the same stimulation is applied to relaxed muscles. The central activation ratio (CAR) is assessed as the ratio of MVC torque to the total torque of MVC with superimposed electrical stimulation. The VA may be more sensitive than CAR in detecting central fatigue.\textsuperscript{7} However, it is not clear whether the same applies to individuals of different gender and age. Thus, both indices of voluntary activation were used in assessment of muscle voluntary activation in this study.

**Materials and Methods**

**Volunteers.** We studied 7 men (age 22.2 ± 0.9 years, height, 1.78 ± 0.02 m, mass, 72.3 ± 4.9 kg), 7 women (20.8 ± 0.5 years, 1.70 ± 0.02 m, 61.5 ± 2.3 kg), 7 boys (13.9 ± 0.3 years, 1.66 ± 0.05 m, 54.0 ± 3.8 kg), and 7 girls (13.6 ± 0.2 years, 1.64 ± 0.04 m, 47.7 ± 3.0 kg) in this study, which was approved by the local Ethics Committee. Written informed consent was obtained from the adults, children’s parents, and children. The adults were students of physical education, while the children were recruited from a local school. All participants took part in classes of physical activity two or three times per week and could be considered physically active. However, none of the volunteers specialized in any form of sports training.

**Isometric Torque and Electrical Stimulation.** The isometric torque of knee extensor muscles was measured using an isokinetic dynamometer (System 3; Biodex Medical Systems, Shirley, New York). The subjects sat upright in the dynamometer chair with the knee joint positioned at a 120° angle. The equipment and procedure for electrical stimulation were essentially the same as previously described.\textsuperscript{20,21} Direct muscle stimulation was applied using two carbonized rubber electrodes, covered with a thin layer of electrode gel (ECG-EEG Gel; Medigel, Modi’in, Israel). One of the electrodes (6 × 11 cm) was placed transversely across the width of the proximal portion of the quadriceps femoris. Another electrode (6 × 20 cm) covered the distal portion of the muscle above the patella. A standard electrical stimulator (MG 440; Medicor, Budapest, Hungary) was used. The electrical stimulation was delivered in square-wave pulses, 0.5 ms in duration. The tolerance of volunteers to electrical stimulation was assessed on a separate occasion, and only participants who showed good compliance with the procedure were recruited for the study. The intensity of electrical stimulation was selected individually by applying single stimuli to the tested muscles. During this procedure the current was increased until no increment in single twitch torque could be detected by an additional 10% increase in current strength.

**Experimental Procedures.** The volunteer was positioned in the dynamometer chair and the stimulating electrodes were placed on the right leg. Single stimuli were applied every 30 s at a progressively higher intensity until the required current was reached. Four to five stimuli were usually delivered. After a 5-min rest period the force-generating capacity of the quadriceps muscle was assessed by applying 1-s trains of electrical stimuli at 1, 10, 20, 50, and 100 Hz. Approximately 2–3 s were needed to change the stimulation frequency. After a 5-min rest, three 5-s MVCs were obtained with a 2-min rest between them. At ~3 s of MVC, a 250-ms test train of stimuli at 100 Hz (TT100Hz) was superimposed on the voluntary contraction. The same TT100Hz was repeated 1–2 s after the MVC. These TT100Hz contractions were used to assess the voluntary activation of knee extensors. A 5-min rest period was allowed after the third MVC, and the experiment was terminated by a 2-min MVC. The TT100Hz was superimposed on the contraction at approximately 3, 14, 29, 44, 59, 74, 89, 104, and 119 s. At approximately 30, 60, 90, and 120 s the knee extensors were relaxed for 2–3 s and TT100Hz was delivered as after the brief MVCs in the first part of the experiment.

Examples of typical torque curves with these TT100Hz contractions are presented in Figure 1. The amplitude of the superimposed tetani was calculated from the baseline, estimated as the average torque over 1 s, just before the stimulation. The superimposed TT100Hz produced measurable torque increments in all subjects. The sensitivity of the Biodex System 3 in torque measurements is ±1.36 Nm. In one particular instance, the superimposed TT100Hz induced a torque increment of just 1.5 Nm. However, in the majority of cases the torque increments were well above this level. The amplitude of superimposed TT100Hz was 23.4 ± 3.2 Nm in the 5-s MVCs when maximal level of voluntary activation was achieved, and 41.8 ± 5.3 Nm during the 2-min MVC.
For the voluntary activation index (VA), the TT100Hz torque of the relaxed muscles was used as the control torque and the following formula was applied:

\[
\text{Voluntary activation index (\%)} = \frac{100 - \text{superimposed TT100Hz torque}}{\text{control TT100Hz torque}} \times 100
\]

In addition to VA, the central activation ratio (CAR) was calculated. The CAR is the ratio of the maximal voluntary torque to the peak torque generated with additional TT100Hz superimposed on an MVC.3,7

Statistics. Descriptive data are presented as means ± SEM. The effects of age, sex, and time of measurement were assessed using repeated measures analyses of variances (ANOVA). If significant effects were found, post-hoc testing was performed, applying paired \(t\) tests with a Bonferroni correction for multiple comparisons. Pearson’s correlation coefficient was calculated to investigate the association between the variables. Statistical significance of all tests was set at \(P < 0.05\).

RESULTS

Force-Generating Capacity and Voluntary Activation. Data on force-generating capacity and voluntary activation of knee extensors are presented in Figure 2. In general, knee extension torque increased \((P < 0.0001)\) with the frequency of electrical stimulation, although differences between stimulation at 50 Hz and 100 Hz were not significant. The effectiveness of electrical stimulation was similar in different groups of volunteers, since 100-Hz tetani generated similar percentages of MVC torque (80.3 ± 6.1, 69.2 ± 5.7, 77.1 ± 8.7 and 76.8 ± 8.9% for men, women, boys, and girls, respectively). The ANOVA showed that the torque at 50 Hz and 100 Hz, as well as MVC, depended \((P < 0.05)\) on the age and gender of the volunteers. Men were stronger than women \((P < 0.01)\), although the difference between boys and...
girls was not significant. Both adult groups generated more torque than the respective groups of children \((P < 0.05)\).

In general, CAR values were higher than those of VA, but the findings were similar for both indices. The VA and CAR did not depend on gender, but adults tended to achieve higher values of these indices than children \((P < 0.05)\). However, only girls showed a lower \((P < 0.05)\) VA and CAR than the respective adult group.

**Torque in 2-min MVC.** Data on knee extension torque during 2-min MVC are presented in Figure 3. At the beginning of the contraction, volunteers produced \(\sim 90\%\) of their best MVC, but the torque decreased \((P < 0.0001)\) during the prolonged MVC in all studied groups. Men generated the highest torque \((P < 0.01)\) during the first 30 s of exercise. However, differences between the groups were not significant after this time. At the end of the 2-min contraction, percentage decreases in torque were similar between groups.

**Muscle Force-Generating Capacity in 2-min MVC.** Data on the control TT100Hz torque, as measured during the short breaks in the MVC, are presented in Figure 4. The torque tended to increase during the first 30 s of MVC, but this increase was not significant. Afterwards, however, TT100Hz torque decreased progressively \((P < 0.0001)\) in all groups. The ANOVA analyses showed a significant effect of age \((P < 0.05)\) but not gender on the relative decrease in TT100Hz torque from 30 s to 2-min MVC. At the end of exercise the control TT100Hz torque was depressed more in adults than children \((P < 0.05)\). This difference was significant \((P < 0.05)\) both for males and females.

**Muscle Activation in 2-min MVC.** Data on CAR and VA of knee extensor muscles are presented in Figure 5. CAR was calculated every 15 s, whereas values of VA were obtained every 30 s. As in the case of the short MVCs, the values for CAR were higher than for VA. The CAR showed a gradual decline during the first 30 s of the exercise in all studied groups \((P < 0.05)\). Compared to the values achieved in brief MVCs, VA was also depressed \((P < 0.05)\) after the 30-s MVC. The CAR and, particularly, VA, tended to decrease further. However, only VA in boys and girls showed a significant drop \((P < 0.05)\) between 30 s
and 2 min of MVC. Age, but not gender, had an effect on CAR and VA \((P < 0.01)\) during the 2-min contraction. Values of CAR and VA did not differ significantly between boys and girls, but they were consistently lower \((P < 0.05)\) than those in adults when assessed after 30 s of exercise. This difference between adults and children was well maintained during the 2-min contraction.

Data on the association between changes in voluntary activation (as measured by CAR and VA) and muscle force-generating capacity (as measured by the control TT100Hz torque) are presented in Figure 6. Using the pooled data of all volunteers, the end-exercise values of the TT100Hz torque showed a significant correlation with the average CAR and VA during 2-min MVC \((r = -0.57, P < 0.01\) and \(r = -0.58, P < 0.01\), respectively).

DISCUSSION

The main aim of this study was to compare the ability to activate skeletal muscles in children and adults. Children tended to show lower voluntary activation during brief MVCs. The difference between children and adults became clear when the 2-min MVC was studied, as children showed a larger decrease in voluntary activation during the exercise. At the same time, children experienced a significantly smaller depression in muscle force-generating capacity, as tested by TT100Hz. The end-exercise values of the TT100Hz torque correlated with the level of voluntary activation during exercise. Thus, the less severe peripheral fatigue in children compared to adults could be due to a lower degree of motor unit activation during the 2-min MVC.

Irrespective of age and gender, all volunteers showed a similar relative decrease in MVC torque at the end of the 2-min contraction. These torque changes were also similar to those measured previously in ankle dorsiflexors during a continuous MVC.\(^\text{16}\) It is often reported that children demonstrate lower levels of fatigue and faster recovery than adults.\(^\text{10,14}\) In this study, the differences between children and adults were less clear when assessed by changes in MVC torque. There is some evidence that strength-matched women demonstrate higher fatigue resistance than men.\(^\text{11}\) We did not attempt to match the tested individuals by strength. It is also possible that the absence of clear differences between the studied groups was due to the specificity of the continuous MVC, although more evidence is needed to support this contention.

The assessment of muscle fatigue during voluntary contractions is complicated due to the interac-

![Figure 5](image.png)  
**FIGURE 5.** Central activation ratio (CAR) and voluntary activation (VA) of the quadriceps muscle during the 2-min maximal voluntary contraction (MVC) of knee extensors in men, women, boys, and girls. The values at time point 0 are the highest values achieved in the experiments with brief \((-5 \text{ s})\) MVCs. Values are means \(\pm\) SEM.

![Figure 6](image.png)  
**FIGURE 6.** Association between the end-exercise values of control TT100Hz torque and the average values of central activation ratio (A) and voluntary activation (B) during 2-min MVC.
tion between the ability to activate skeletal muscles and muscle force-generating capacity. Muscle electrical stimulation can be used to separate these two factors. In our experiments the decrease in the control TT100Hz torque can be used as an indicator of peripheral fatigue. At the beginning of exercise the TT100Hz torque tended to increase in all subjects, perhaps due to myosin light chain phosphorylation, which increases the rate of force generation and potentiates the amplitude of twitch contractions in exercising muscles.13,18 Twitch potentiation peaks after ~20s MVC, whereas fatigue prevails afterwards.10,13 In our study the decrease in the control TT100Hz torque after MVC for 2 min s was less marked in children than adults. Thus, peripheral fatigue was lower in children.

Discussions of the ability to exercise at high intensity often focus on muscle properties.17,18 A lower glycolytic rate, which results in less marked lactate accumulation in skeletal muscles, is given as the major reason for low power output, high fatigue resistance, and fast recovery in children compared to adults.10,18 However, the role of lactate as a mediator of muscle fatigue has been questioned.1 Interestingly, children also show a lower ratio of force to muscle cross-sectional area than adults.14 Specific tension could be higher in skeletal muscles of adults compared to children, but there is no convincing evidence to support this claim. It is more likely that the ability to activate skeletal muscles plays an important role. The results of our study suggest that children experience a significant impairment in voluntary activation of skeletal muscles when high-intensity exercise is continued for longer periods of time. In our study the voluntary activation of skeletal muscles was already impaired after MVC for 30 s. Thus, it is likely that the lower level of peripheral fatigue in children was at least partially due to the larger impairment in activation of high-threshold motor units. Indeed, depression of muscle force-generating capacity, as judged by the control TT100Hz torque, correlated with the voluntary activation of skeletal muscles during exercise.

Relatively few adults can achieve full activation of their skeletal muscles during exercise.2,12,15 Our results agree with these observations. The findings on voluntary activation of skeletal muscles in children are more controversial. Applying single twitches as interpolated contractions during MVC, two earlier studies showed an almost complete activation of plantar flexor and knee extensor muscles in children and adolescent boys.4,8 However, assessments using single twitches can overestimate VA due to a low signal-to-noise ratio.5,15 We used interpolated tetani instead of twitches. As expected, the values of VA were lower in our study than in the two earlier studies of children that used single twitches.1,8 However, our values of VA for boys were slightly higher than those reported in another study using similar methods (~85% vs. ~68%, respectively).24 This could be due to the fact that the boys were more mature in our study (average age of 13.9 years compared to 10.5 years, respectively).

Continuous MVC induces central fatigue in adults.7,16 Our results extend these observations and suggest that children are more susceptible to central fatigue than adults. It is unlikely that the observed differences between children and adults were related to methodological problems. Conditions of electrical stimulation did not differ between the age groups as 100-Hz tetani generated similar percentages of MVC torque. The TT100Hz induced sufficiently large responses that could be reliably measured in these experiments. This is particularly important during the prolonged MVCs, which are characterized by a significant decline in muscle force-generating capacity.7,15,16 Isometric contractions can induce low-frequency fatigue, which is associated with a large depression of single twitch amplitude.20,21 Thus, application of 100-Hz trains is likely to give more reliable results than single twitch contractions.15

Two indices of muscle activation were used in this study, and both produced similar results. It has been suggested that VA is more sensitive in the evaluation of central fatigue when compared to CAR.7 Indeed, VA decreased to a lower level than CAR by the end of the 2-min MVC in our study. However, CAR showed significant differences between children and adults as well. Our data for VA and CAR show that the major change in ability to activate quadriceps muscle occurs during the first 30 s of the continuous MVC. This could be related to the decrease in motor unit firing rates that occurs in the initial stage of the continuous MVC.5,12 However, the VA values of adults and children, in particular, decreased further between 30 s and 2 min of MVC. The underlying cause of this change is not clear. It appears that central fatigue shows a more gradual development during low-intensity isometric contractions than in our experiments with MVC.23 Different mechanisms might operate to cause central fatigue in different types of exercise, and it is not clear whether the results of our study can be extended to other types of exercise. There are also uncertainties in applying the results of this study to other muscles. In our study, central fatigue was already significant after 30 s of MVC, whereas voluntary activation of the triceps su-
rae muscle becomes impaired only after a 90-s MVC. The quadriceps muscle shows lower values of VA than several other human muscles when tested by brief MVCs. It is possible that the voluntary activation of the quadriceps muscle can be maintained at a near-maximum level for shorter periods of time than in other muscles.

In summary, the results of this study show that children maintain a lower level of muscle voluntary activation than adults when exposed to continuous high-intensity exercise. Thus, it is likely that the lower power output and faster recovery rate often observed in children could be related to some extent to impaired voluntary activation of skeletal muscles in children.

We thank Dr. Derek Scott for critical review of the manuscript.

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ABSTRACT: It is important to have strong predictors of outcome in traumatic neuropathies so that appropriate management can be instituted early. Our objective in this study was to evaluate the prognostic value of electrodiagnostic studies in traumatic radial neuropathy. In this retrospective study, 33 of 67 subjects with traumatic radial neuropathy met the inclusion criteria. Good outcome was defined as grade 3 or higher strength on the Medical Research Council scale in wrist extensors. Compound muscle action potential (CMAP) responses from extensor indicis proprius (EIP) predicted prognosis: 92% of subjects with a recordable CMAP had a good outcome; and 65% of those with an absent response had a good outcome. Recruitment in brachioradialis was also predictive: 92% of those with full, central, or reduced recruitment had a good outcome; 67% of those with discrete recruitment had a good outcome; and only 33% of those with absent recruitment had a good outcome. Studies performed more than 3 months after injury produced more prognostic certainty than those performed earlier. We conclude that electrodiagnostic studies produce useful prognostic information in traumatic radial neuropathy. It is noteworthy, however, that 65% of subjects with an absent radial CMAP (suggesting complete or nearly complete axon loss) still have a good outcome.


PROGNOSTIC VALUES OF ELECTRODIAGNOSTIC STUDIES IN TRAUMATIC RADIAL NEUROPATHY

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The radial nerve is the most commonly injured peripheral nerve. These injuries most frequently result from motor vehicle accidents, followed by penetrating injury, falls, and industrial accidents. It has been reported that 2%–16% of humeral fractures result in radial nerve injury. It has been regular practice for surgeons to explore open injuries, whereas radial nerve lesions associated with closed injuries (i.e., without associated laceration or skin penetration) are usually closely observed for at least 3 months before any surgical intervention is considered.

The initial evaluation of a patient with a suspected radial nerve injury includes a physical examination, imaging, and electrodiagnostic studies. Electrodiagnostic studies are usually not performed in the first few weeks after injury to allow time for electrophysiological changes to occur. For motor and sensory conduction studies, it will take up to 10 days for wallerian degeneration to occur and thus for a decline in response amplitude distal to the site of the lesion. After day 10, nerve conduction studies can help to differentiate between neurapraxia and axonotmesis based on amplitude of the compound muscle action potential (CMAP). On needle electromyography (EMG), it can take 3–4 weeks for fibrillation potentials to appear.

The use of CMAP amplitudes for prognosticating recovery in traumatic radial nerve injuries has not been well defined. Typically, clinicians have extrapolated from studies of the facial nerve in Bell’s palsy to determine prognosis for recovery in limb-nerve trauma. The practice with facial nerve lesions has been inter-side comparison of the CMAP amplitude, expressed as a percentage of the nonaffected side. Of particular interest, these studies were performed at regular intervals within the first 2 weeks from the date of injury. Fisch reported that patients with an evoked response amplitude of at least 10% that of the nonaffected side had a good chance for recovery, whereas those with responses at less than 10% had a poor prognosis for recovery. Another study showed that 25% or greater amplitude, rather than 10%, signified a good progno-
sis for recovery. It is unknown whether the facial-nerve data can be extrapolated to nerves in the limbs.

The objectives of this study were to: (1) determine the relationship between early CMAP amplitude and later motor recovery after traumatic radial neuropathy; (2) examine whether needle EMG provides prognostic information; and (3) determine whether the time between injury and electrodiagnostic testing influences the prognostic value of testing.

METHODS

All 67 patients examined at the Harborview Medical Center EMG laboratory over an 11-year period (1993–2004) for possible radial nerve injury were identified for this study. Harborview is the only Level 1 trauma center in a five-state area of the Pacific Northwest. These patients were referred from either primary or other specialty services for nerve conduction study and EMG evaluation for radial nerve injury, which usually consisted clinically of decreased sensation in the radial distribution or weakness in elbow, wrist, or finger extension. Data were collected by retrospective chart review of the clinical information and review of the records of the EMG laboratory for the electrophysiological findings. Retrospective chart review was approved by the University of Washington Institutional Review Board.

The inclusion criteria included the following: (1) radial nerve injury secondary to trauma; (2) evaluation to include a motor conduction study of the radial nerve (all were surface electrode recordings over the extensor indicis proprius); and (3) adequate follow-up to measure outcome. Exclusion criteria included: (1) failure to have a radial motor nerve conduction study (17 cases); (2) inadequate follow-up (16 cases); or (3) nontraumatic radial nerve injuries (1 case of radial tunnel syndrome). Of the available 67 cases, 33 met the inclusion criteria. A radial nerve diagnosis was made by either the examining neurologist or physiatrist at the time of the study based on the clinical and electrophysiological examination. Patients had their electrophysiological studies performed from 2 weeks up to 8 months after the time of injury.

Electrophysiological data analyzed included EMG recruitment in brachioradialis and CMAP amplitude recorded from surface electrodes over the extensor indicis proprius (EIP). Recruitment was rated as full, central (reduced numbers of motor unit potentials firing slowly), reduced (reduced numbers of motor unit potentials firing rapidly), discrete (isolated rapidly firing motor unit potentials with maximal contraction), or none. For radial motor conduction studies, the active electrode was placed over the EIP, 2 cm proximal to the ulnar styloid, and the reference electrode was placed over the ulnar styloid. Stimulation occurred in the mid-forearm, just radial to the ulna. Sensory responses were recorded variously over the hand dorsum at 14 cm (19 cases) or 10 cm (2 cases), and from the thumb at 10 cm (12 cases).

The follow-up examinations were collected from chart/computer review of patients’ follow-up records and were performed by treating physicians from 2 months up to 3 years postinjury.

The primary outcome variable was strength in wrist and finger extensors of at least grade 3 on the Medical Research Council (MRC) scale. The degree of recovery was based on data collected from review of follow-up examinations performed by either the patient’s primary or specialist physician. Recovery was noted to be good if the patient had at least grade 3 on the manual muscle examination in most muscle groups supplied by the radial nerve. Partial recovery was defined as at least grade 3 strength in the wrist extensors and 0–2 strength in the finger extensors. Poor recovery was defined as having 0–2 strength in the majority of radial-innervated muscles or the need for tendon transfer procedures.

Statistical analysis was performed using the chi-square test with Yates correction as well as Fisher’s exact test. These tests were chosen based on the small sample size.

RESULTS

The age of patients included in this study ranged from 16 to 58 years. Of the 33 patients, 19 were men and 14 were women. Recovery rates did not vary with age or gender. Injuries were to the right upper extremity in 21 cases, and left left upper extremity in 12. The causes of injuries, in descending order of frequency, included motor vehicle accidents with upper limb fractures, gunshot wounds, and falls. The location of the radial nerve injury was distal to the branch to the triceps muscle in 16 cases (with normal recruitment and no fibrillation potentials in the triceps), more proximal with the triceps affected in 13 cases, and not tested in 4 cases. The recovery rate was the same for both distal and proximal injuries.

The radial CMAP was studied in all 33 cases. The contralateral side was examined in only 2 cases, and was not analyzed.

Based on reports in the facial nerve literature that a CMAP amplitude of 10% or greater predicts a good prognosis, we set our CMAP amplitude cut-off at 0.5 mV, which is 10% of a reported reference value of 5 mV. Due to the small number of cases, we
also analyzed results using a cut-off amplitude of 1.0 mV. With amplitudes of ≥1.0 mV, 83% of patients had partial to full recovery. The recovery rate was similar at 86% for those with small-amplitude responses (<1.0 mV), and 65% for those with an absent radial CMAP response (Table 1; differences not significant). When the CMAP response was absent, only 60% had good recovery, defined as better than grade 2 strength in the finger extensors.

Radial sensory responses were also tested in all 33 cases. Responses were present in 10 cases, with full recovery occurring in 7 (70%) and partial recovery in 2 cases. Sensory responses were absent in 23 cases, with full recovery occurring in 65% (differences not significant). All patients with sensory response amplitudes of >2 μV had partial or full recovery.

The role of needle EMG in determining prognosis of radial nerve injury was also evaluated. The brachioradialis muscle was chosen because it is typically the first radial-innervated muscle after the spiral groove (the anconeous is typically supplied by the same branch that supplies the medial head of the triceps). Of note, only 27 of the 33 patients in this study had an EMG examination of the brachioradialis. Table 2 shows the total percentage of patients with partial to full recovery when segregated by the level of motor unit potential (MUP) recruitment in the brachioradialis. Regardless of CMAP response, 92% of patients had partial to full recovery when there was reduced, central, or full recruitment of motor units at any test time [95% confidence interval (CI), 0.59–1.00]. When the MUP recruitment was discrete or absent, regardless of the CMAP, only 47% of patients had partial to full recovery (95% CI, 0.22–0.73).

The EIP was also tested in 23 cases. MUP recruitment was reduced, central, or full in only 4 cases, with partial to full recovery occurring in all of them. MUP recruitment was discrete or absent in 19 cases, with recovery occurring in 47% (differences not significant).

Abnormal spontaneous activity was sought in the brachioradialis in 27 cases, with the finding of fibrillation potentials in 21 subjects. The presence of fibrillation potentials was not very helpful for determining prognosis. Fibrillations were scored as 0–2+ in 19 cases, with recovery occurring in 79%, and as 3+ or 4+ in 8 cases, with recovery occurring in 38% (difference not significant).

We were able to achieve greater separation between prognostic groups by using measures of recruitment in the brachioradialis and the radial motor response in combination. For subjects with full, central, or reduced MUP recruitment in the brachioradialis, the presence or absence of a radial CMAP did not contribute as a predictor; 92% of this group had full or partial recovery [positive predictive value (PPV) = 0.92; 95% CI, 0.76–1.00]. For those with discrete or no MUP recruitment, the radial CMAP demonstrated a trend toward separation between two recovery groups. Among those with a preserved CMAP, 75% recovered (PPV = 0.75; 95% CI, 0.15–1.00); among those with no detectable CMAP, only 36% recovered (PPV = 0.36; 95% CI, 0.08–0.65; Fig. 1). Only the difference between the best (92%) and worst (36%) prognostic groups was statistically significant.

### Table 1. Recovery based on radial CMAP amplitude.

<table>
<thead>
<tr>
<th>CMAP amplitude (mV)</th>
<th>No. of patients</th>
<th>Percent with partial or full recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥1</td>
<td>5</td>
<td>83</td>
</tr>
<tr>
<td>0.5–1</td>
<td>2 1 1</td>
<td>75</td>
</tr>
<tr>
<td>&lt;0.5</td>
<td>3 0</td>
<td>100</td>
</tr>
<tr>
<td>Absent</td>
<td>12</td>
<td>65</td>
</tr>
</tbody>
</table>

### Table 2. Recovery based on motor unit potential recruitment in brachioradialis.

<table>
<thead>
<tr>
<th>Brachioradialis recruitment</th>
<th>No. of patients CMAP present</th>
<th>Percent with partial or full recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full or central</td>
<td>1 4</td>
<td>100</td>
</tr>
<tr>
<td>Reduced</td>
<td>4 3</td>
<td>86</td>
</tr>
<tr>
<td>Discrete</td>
<td>3 3</td>
<td>67</td>
</tr>
<tr>
<td>None</td>
<td>1 8</td>
<td>33</td>
</tr>
</tbody>
</table>

**FIGURE 1.** Percentage of subjects recovering based on MUP recruitment and CMAP amplitude. Criteria 1—MUP recruitment full, central, or reduced, and CMAP present or absent. Criteria 2—MUP recruitment discrete or none; CMAP present. Criteria 3—MUP recruitment discrete or none; CMAP absent.
We examined whether the results of studies performed within 3 months after injury would be different at predicting prognosis than those performed after 3–6 months. Figure 2 shows the percentage of patients with partial to full recovery, segregated into these two intervals, depending upon the electrodiagnostic findings. There was greater separation of prognostic groups with later testing.

Although 5 patients had subsequent tendon transfers, and 1 additional patient currently has this planned, none had nerve exploration or nerve grafting.

DISCUSSION

To date, electromyographers have typically extrapolated evidence from the facial nerve literature to determine prognosis of other nerve injuries. The rule that CMAP amplitudes at less than 10% of the normal side are associated with a poor prognosis has been used to determine prognosis in other peripheral nerve injuries. In our study, it is of interest to note that 60% of patients had full recovery even when the CMAP was absent, which paints a better picture for recovery of wrist extension.

Although 5 patients had subsequent tendon transfers, and 1 additional patient currently has this planned, none had nerve exploration or nerve grafting.

There are a number of possible reasons that prognosis is different for the radial than for the facial nerve. First, the facial nerve is not as well protected by muscles and other soft tissue in the face as are nerves in the extremities. Thus, the neural tubes may be more intact in the radial than facial nerve. Second, it is possible that fewer axons are needed to produce satisfactory results in wrist and finger extension compared to complex muscles of facial expression. Finally, it is possible that a good outcome for the wrist extensors does not necessarily predict a good outcome in finger extension.

Our data demonstrate that the time between injury and testing has a marked influence on the prognostic value of these studies. In studies done between 3 and 6 months after injury, an absence of both radial CMAP and brachioradialis MUP recruitment predicts a poor prognosis, whereas either the presence of a radial CMAP or reduced, central, or full MUP recruitment can always be associated with partial to full recovery. The data at 0–3 months is of less certain predictive value but earlier data may be more useful in clinical decision-making.

Like other retrospective studies, this study is limited by its small sample size and the difficulty with controlling factors, such as the interval between injury and electrodiagnostic studies, the time to follow-up examinations, the subjective nature of manual muscle testing by different testers, the skill of the electromyographers, and the test protocol. This investigation has, however, suggested the need for future studies of other peripheral nerves prospectively to clarify the contributions to prognostication of electrodiagnostic studies.

REFERENCES

ABSTRACT: Measurements of T2 relaxation times in tissues have provided a unique, noninvasive method to investigate the microenvironment of water molecules in vivo. As more clinical imaging is performed at higher field strengths, tissue relaxation times need to be reassessed in order to optimize tissue contrast. The purpose of this study was to investigate the water proton T2 relaxation time in human median nerve at 7 T. High-resolution images of the wrist were obtained using a home-built dedicated microstrip coil. Gradient echo images provided a good anatomical delineation of the wrist structure, with a clear definition of the median nerve, tendons, bone, and connective tissue within the wrist in an acquisition time of 2 min. Measurements of the T2 relaxation time were performed with a spin echo imaging sequence. The T2 relaxation time of the median nerve was 18.3 ± 1.9 ms, which is significantly shorter than the T2 measured in previous studies performed at 1.5 T and 3 T. Further, the T2 relaxation time of the median nerve is shorter than the T2 relaxation time of other tissues, such as brain tissue, at the same field strength. Since the T2 relaxation time of water protons is sensitive to the water microenvironment, relaxation measurements and, in general, a more quantitative magnetic resonance imaging approach might help in detecting and investigating diseases of peripheral nervous system, such as compressive and inflammatory neuropathies, in humans.

MAGNETIC RESONANCE IMAGING AND T2 RELAXOMETRY OF HUMAN MEDIAN NERVE AT 7 TESLA

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Tissue characterization by T1 and T2 relaxation times has been one of the first topics of interest in magnetic resonance (MR) investigations of biological systems, both in vivo and in vitro studies. The knowledge of tissue T1 and T2 relaxation times is essential in designing MR protocols, from the basic protocols aimed to obtain anatomical delineation of tissue structures to the more complex protocols used to investigate tissue function. Currently, the T1 and T2 relaxation times of water protons of most tissues, such as brain,21,32 muscle,18,21 pelvic and abdominal tissue,7 breast tissue,50 and cartilage26,28 are well known at the common clinical magnetic field strengths of 1.5 and 3 Tesla. For some tissues, such as brain and muscle, the T1 and T2 relaxation times of water protons have also been measured at magnetic field strengths higher than 3 T.10,23,24,27 The need to determine experimentally the relaxation times stems from the fact that no theory can satisfactorily predict, from a quantitative point of view, the values of tissues water proton relaxation times or their dependence on magnetic field strength,4 despite the fact that, in pure liquids, successful relaxation theories were developed decades ago.3 For tissue water in vivo
there is a major difficulty in modeling the water molecule motion, one of the major factors affecting the T1 and T2, due to the complex microenvironment which results in a number of additional interactions that are absent in pure liquids, such as interactions between water molecules and membranes or macromolecules.

Surprisingly, in human studies one type of tissue that has received very little attention from the relaxometry point of view is the peripheral nervous system (PNS). Indeed, very little is known about the relaxation times of the PNS in humans, despite the growing application of MR neurography in clinical settings.\textsuperscript{6,12,13,19,20,25} Since the beginning of MR neurography,\textsuperscript{14} the majority of the studies on the PNS have been focused on the median nerve. This nerve is medium sized (~2–3 mm cross-sectional diameter), compared to the other nerves in the human body, and it is quite superficial in the wrist region (distance from the skin 5–10 mm), so it can be imaged with high signal-to-noise ratio (SNR) by using a surface coil. As such, it represents a good model for MR investigation of peripheral nerves.

For a proper MR characterization of median nerve, T1 and T2 relaxation times need to be assessed. The T1 relaxation time of median nerve in vivo has been measured at the field strength of 3 T\textsuperscript{17}; the T2 relaxation has been investigated at field strengths of 1.5 T and 3 T.\textsuperscript{5,17} The relaxation behavior of water protons in the median nerve—a relatively long T1 (~1,400 ms) and a short T2 (~35 ms), at 3 T—is consistent with the highly oriented and densely packed structure of the peripheral nerves, which consist of fascicles of individual fibers enclosed by connective tissue sheaths. At field strengths higher than 3 T, only qualitative T1- and T2-weighted imaging has been performed.\textsuperscript{1,11} As more clinical imaging is performed at higher field strengths, tissue relaxation times need to be assessed in order to optimize tissue contrast. The purpose of this study was to investigate the T2 relaxation time of water protons in human median nerve at 7 T.

**MATERIALS AND METHODS**

**Microstrip Coil.** A dedicated transmit/receive radiofrequency (RF) microstrip coil (50 × 70 mm) was built in order to acquire MR images of the wrist with high SNR. The microstrip coil consisted of a copper strip taped onto a 7-mm thick polytetrafluoroethylene (Teflon) layer. In order to enable fine tuning and matching, the original design\textsuperscript{34} of the RF microstrip coil was modified by adding a network of two capacitors which served as a tuning and matching network.\textsuperscript{16} In a previous study, it was shown that the sensitivity of this microstrip coil was higher than that of a small birdcage coil up to a distance of ~2 cm, with a 2-fold increase in sensitivity for distances of 5–10 mm.\textsuperscript{16} Since the distance of the median nerve from the skin at the level of the wrist is within this range, the microstrip coil was preferred over the birdcage coil for the current study. Mechanical adaptations to the cradle were made to position and fix the wrist onto the microstrip coil. The coil was tested for safety on a phantom which properly mimicked the wrist load. The protocol used in the current study resulted in an increase in temperature of less than 0.1°C, which is well below the FDA (Food and Drug Administration) limit of 1°C.\textsuperscript{22} Further details about the microstrip coil can be found elsewhere.\textsuperscript{16}

**MRI Experiments.** MRI experiments were performed on a 7T/200 mm horizontal-bore MR magnet interfaced to a SMIS console and equipped with a gradient insert with a gradient strength of 150 mT/m and a rise time of 150 μs. The subjects scanned were MR researchers, familiar with the general risks associated with high-field MRI, and they provided informed consent without a formal review board process.

The microstrip coil, placed over the wrist, acted as a transmitter/receiver. The arm was aligned in the direction of the static magnetic field B\textsubscript{0} within the magnet bore. As a result, the median nerve was oriented at an angle of ~0° with B\textsubscript{0}. After tuning and matching of the coil, multislice gradient echo images were acquired for anatomical localization of the median nerve. Imaging parameters were: repetition time (TR) of 500 ms, echo time (TE) of 10 ms, image matrix size of 256 × 256, receiver bandwidth of 100 kHz, field of view (FOV) of 70 mm × 70 mm, slice thickness (SLT) of 2 mm, and one signal average per phase-encoding step.

T2 relaxation times were measured with a Hahn spin echo imaging sequence (TR = 1,500 ms, FOV of 70 × 70 mm, SLT of 2 mm, image matrix size of 256 × 256, receiver bandwidth of 100 kHz, and one signal average per phase-encoding step) in four healthy men with a mean age of 36 ± 5 years. Measurements were performed on the right wrist in three subjects and on the right and left wrist in one subject. From pilot MRI experiments on the median nerve (unpublished observations), the nerve appeared to have a short T2 relaxation time. Thus, we acquired spin echo images at five TEs (12, 16, 20, 24, 28 ms) with an increment in TE of only 4 ms. It should be noticed that the spatial resolution (in-plane resolution of 273 × 273 μm and a slice thick-
ness of 2 mm) employed here for quantitative measurements of the median nerve T2 relaxation time is lower than spatial resolution achieved in previous investigations\(^{11,31}\) of median nerve at high field (in-plane resolution of 195 × 195 μm and a slice thickness of 2 mm\(^{11}\)), where, however, only qualitative imaging (T2*-weighted imaging) was performed.\(^{11}\)

**Data Analysis.** For each subject, a region of interest (ROI)—which included the median nerve—was drawn on the spin echo images. The ROI was carefully chosen to include only those voxels well within the median nerve. The relaxation decay curves were analyzed with the software package Prism (GraphPad Software, San Diego, California). For each subject, the mean T2 was calculated by fitting the signal intensity to a monoeponential function and the coefficient of determination (R\(^2\)), which is a measure of the goodness of the fit, was assessed.\(^{16}\) Data are expressed as mean value ± standard deviation.

**RESULTS**

High spatial resolution gradient echo axial images (in-plane resolution of 273 × 273 μm and a slice thickness of 2 mm, acquisition time of 2 min) provided a good anatomical delineation of the wrist, with a clear definition of the tendons, median nerve, bone, and connective tissue within the wrist (Fig. 1, top). Figure 1, bottom, illustrates in more detail the location of the median nerve within the wrist. Tendons and bone marrow were characterized by a low signal intensity, whereas the median nerve and the connective tissue, which encapsulates the tendons and the median nerve, had a moderate signal intensity. The SNR of the tissue structures in the carpal tunnel was assessed. For the gradient echo images, the SNR of the median nerve was equal to 23 ± 3; the connective tissue had an SNR of 22 ± 3, comparable to that of the median nerve, whereas tendons (next to the median nerve) had an extremely low SNR (3 ± 1).

In the spin echo images (Fig. 2), the signal intensity of the tissues within the wrist displayed the same characteristics as in the gradient echo images, with the notable exception of bone marrow, which, due to its long transverse relaxation time T2, appeared with high signal intensity. The microscopic static inhomogeneities within bone marrow resulted in short T2* values, which caused significant signal loss from marrow in the gradient echo images. The dephasing induced by the microscopic static inhomogeneities, however, is refocused in the spin echo images. Tendons were readily identified as regions of very low signal intensity, suggesting very short T2 values for this tissue, in addition to the very short T2* observed in the gradient echo images. The quantitative analysis of SNR in the structures of interest yielded the values of 29 ± 3 (median nerve), 28 ± 4 (connective tissue), 3 ± 1 (tendons) for the shortest echo time.

In all subjects, the nerve diameter was on the order of 2–4 mm in the images acquired at the level of wrist. Given the in-plane resolution of 273 × 273 μm, the number of voxels included in ROI for determining the T2 calculation was in all cases greater than 40. One example of T2 relaxation decay curve is given in Figure 3. The fit of the T2 decay data yielded T2 values of 19.1 ± 1.5 ms (R\(^2\) = 0.989). The average T2 relaxation time averaged over the five nerves was 18.3 ± 1.9 ms, with average R\(^2\) of 0.979.
DISCUSSION

MRI of the PNS presents a few challenges since nerves have approximately a cylindrical structure with radius in the range of ~1–30 mm. The largest nerves, such as the sciatic nerve, lie distant from the body surface and therefore cannot be properly imaged with surface coils. In addition, due to their tissue structure, nerves are characterized by T2 values shorter than many other tissues. In this study, the median nerve was chosen as a model system for investigation of the T2 relaxation time of water protons in PNS, since this nerve is of medium size (2–3 mm) and relatively superficial, allowing high SNR imaging with a surface coil.

The T2 relaxation time of median nerve at the field strength of 7 T was found to be relatively short, when compared with the T2 relaxation time of brain (T2 ~50–70 ms, in human visual cortex\(^27\)) at the same field strength. This finding is consistent with the fact that the tissue in PNS is characterized by a structure that is considerably different than that of the central nervous system (CNS). Peripheral nerves consist of fascicles of individual nerve fibers enclosed by connective tissue sheaths. Each nerve fiber is surrounded by a loose connective tissue, the endoneurium. A large number of nerve fibers are enclosed in robust connective tissue, the perineurium, to form a nerve fascicle. All nerve fascicles are then surrounded by a connective tissue sheath, the epineurium. In such an environment, the molecular rotational motion of water molecules is most probably highly impeded and the long value of rotational correlation time results in a short T2. The values of T2 measured in this study were obtained from ROIs that included the entire cross-section of the median nerve, and thus are representative of the T2 values of nerve fibers, endoneurium, perineurium, and epineurium.

The T2 of median nerve measured in the current study is significantly shorter than the value measured at a field strength of 1.5 T (T2 ~50 ms\(^2\)) and 3 T (T2 ~35 ms\(^3\)). This is consistent with the functional dependence of the T2 relaxation times on the magnetic field strength, that is, a decrease of T2 with the magnetic field strength, which has already been observed in the CNS\(^27\) and in muscle tissue.\(^10\) Although the decrease in T2 with increasing \(B_0\) is a well-established fact, it should be stressed here that the in vivo environment of water molecules in tissues is far too complex to be adequately modeled by current relaxation theories. The presence of multiple compartments and of complex interactions between water protons and their surroundings—membranes, macromolecules, paramagnetic centers—as well as exchange mechanisms, can be modeled in a quantitative way only to a certain degree. As a result, the values of tissues relaxation times need to be determined from experiments at each magnetic field strength. At a field strength of 1.5 T, Chappell et al.\(^5\)

![FIGURE 2. Spin-echo axial MR image of the wrist at 7 T. (A) Spin-echo image at TE = 12 ms (TR = 1500 ms, in-plane resolution = 273 × 273 μm). (B) Close-up of the carpal tunnel region indicated by the rectangular box of A.](image)

![FIGURE 3. Semi-log plot of the Hahn echo signal amplitudes from an ROI within the median nerve. The line indicates the monoeponential fit of the T2 relaxation data. The T2 relaxation time was 19.1 ± 1.5 (\(R^2 = 0.989\)). Square symbols represent the baseline noise values.](image)
showed changes in signal intensity and in T2 relaxation time with orientation of the median nerve relative to the B₀ static magnetic field, i.e., the magic angle effect. It has been long known that tissues with highly oriented and densely packed structures, such as the collagen fibers in cartilage and tendons, display an orientational dependence of the T2 relaxation time.¹¹,¹² The dipole–dipole interaction between spins is proportional to (3Cos²Θ – 1), where Θ is the angle between the B₀ magnetic field and the fiber direction. When fibers are oriented 54.7° (the magic angle) relative to B₀ magnetic field, the dipole–dipole contribution to the transverse relaxation vanishes and the tissue displays a longer T2 relaxation time. No investigation of the magic angle effect for the median nerve was performed in the current study. It should be noted also that at a magnetic field strength of 1.5 T, as at 7 T, the T2 of nerve is significantly shorter than the T2 of the CNS (T2 of white/gray matter in the range of 80–100 ms vs. T2 of median nerve in the 50–60 ms range). This result can be ascribed to the substantial structural difference between the CNS tissue and the PNS tissue mentioned in the previous paragraph.

Qualitative MRI has proven to be sensitive for detecting lesions and monitoring their changes over time in PNS disorders.¹²,¹³,¹⁹ To date, the MR assessment of peripheral nerve pathology has relied exclusively on qualitative imaging, mostly on observing abnormal signal intensities in T2-weighted imaging. However, quantitative T2 measurements as presented here have the potential to allow longitudinal studies, providing additional information about the course of the neuropathy over time, and make comparisons between individual patients possible. A quantitative approach is also crucial when monitoring the changes in whole nerve structure due to changing neurobiology and neurochemistry of a PNS disorder. A number of PNS disorders cause a changing neurobiology and neurochemistry of a tissue that might reflect important structural properties of nerves. Improvements in hardware and MR techniques will hopefully lead to tissue characterization in terms of multiple relaxation components, as already observed in animal studies.²,⁸,⁹

REFERENCES


ABSTRACT: Loss of muscle mass and limitations in activity have been reported in persons infected with human immunodeficiency virus (HIV), even those who are otherwise asymptomatic. The extent to which factors other than muscle atrophy impair muscle performance has not been addressed in depth. The purpose of this study was to determine the extent of neuromuscular activation of the knee extensors and ankle dorsiflexors of 27 men infected with HIV receiving antiretroviral therapy and its relationship to muscle performance. The central activation ratio (CAR) was determined using superimposed electrical stimulation during maximum voluntary contractions. In addition to force and power measurements, muscle cross-sectional area and composition was evaluated using computed tomography. Aerobic capacity was determined from treadmill exercise testing. Eleven of the subjects had an impaired ability to activate the knee extensors (CAR = 0.72 ± 0.12) that was associated with weakness and decreased specific force. The reduced central activation was not associated with muscle area, body composition, aerobic capacity, CD4 count, or medication regimen. Those individuals with low central activation had higher HIV-1 viral loads and were more likely to have a history of AIDS-defining illness. These results suggest the possibility of a different mechanism contributing to muscle impairment in the current treatment era that is associated with impairment of central motor function rather than atrophy. Further investigation is warranted in a larger, more diverse population before more definitive claims are made.


CENTRAL ACTIVATION, MUSCLE PERFORMANCE, AND PHYSICAL FUNCTION IN MEN INFECTED WITH HUMAN IMMUNODEFICIENCY VIRUS

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Loss of skeletal muscle mass has a significant impact on functional performance, independent function, and associated quality of life in persons infected with the human immunodeficiency virus (HIV).1,36 Since highly active antiretroviral therapy (HAART) became the standard of care for HIV infection in 1995, the incidence of wasting (involuntary weight loss >10% ideal body weight) has declined, although it is still common.29,49 In the era of HAART, recent work indicates that HIV-associated weight loss is primarily due to fat atrophy, which accounts for approximately two-thirds of the weight loss, while loss of lean body mass accounts for the other third.18,29,49 Given the wide-ranging physiological consequences of HIV infection and HAART, mechanisms beyond reduction in muscle mass likely contribute to impaired muscle function.3,13 Such additional factors may account for the limitations in vigorous activity that have been reported even in asymptomatic persons infected with HIV.11 It is reductions in the ability to generate force and power that lead to decreases in physical performance and loss of function, rather than loss of muscle per se.26,32 Identifying the factors beyond the reduction of muscle mass that contribute to impaired muscle function is required for optimizing...
the design of rehabilitation strategies to improve muscle strength and functional capacity in this chronically ill patient population.

One factor that can reduce muscle force production in the absence of muscle atrophy is an impairment of central activation (i.e., the ability to activate the available muscle mass). Central activation failure has been shown to contribute significantly to muscle weakness in other clinical populations, e.g., in persons with osteoarthritis, cerebral palsy, or previous knee arthroplasty, and to negatively influence the relationship between muscle strength and physical function. The role of central activation in muscle performance in persons with HIV has not been evaluated, but such an impairment would be expected to lead to decreased specific force (muscle force per unit cross-sectional area) and potentially limit physical function in persons with HIV, even in the absence of muscle atrophy. Persons with HIV receiving HAART have reduced aerobic capacity, likely due to impaired oxygen extraction/utilization by the working muscle, and when oxidative metabolism is compromised, as with circulatory occlusion, central activation is impaired. There is also the possibility that the central nervous system (CNS) effects of HIV may lead to central activation impairments. Given these observations, it is possible that persons with HIV are particularly likely to exhibit losses of central activation.

In the present study we evaluated middle-aged men infected with HIV who were on HAART. We measured the cross-sectional area, muscle composition, strength, and neuromuscular activation of two muscle groups (knee extensors and ankle dorsiflexors), as physiological changes in muscle associated with systemic processes such as disease or aging can be muscle-specific. In addition, we measured the subjects’ aerobic function and functional status. We hypothesized that specific force in these subjects would be associated with muscle composition, as well as the level of central activation. We further hypothesized that reductions in central activation would be related to aerobic capacity.

MATERIALS AND METHODS

Subjects. We recruited 30 community-dwelling patients infected with HIV from the Baltimore VA Medical Center HIV Clinic. All subjects underwent a comprehensive history and physical examination, including blood sampling and electrocardiogram. Subjects had been prescribed HAART for ≥6 months prior to the start of the study. Additional exclusion criteria included any acquired immunodeficiency syndrome (AIDS)-defining illness within the previous 6 months, anabolic steroid use or growth hormone replacement, and major comorbidities that would preclude functional testing. Specifically, patients with the following conditions were excluded: myocardial infarction, unstable angina, uncontrolled hypertension (systolic pressure >180 or diastolic pressure >105 mmHg), poorly controlled diabetes mellitus (fasting blood sugar >200 mg/dl, glycosylated hemoglobin level >10%), peripheral vascular disease leading to claudication, endstage liver disease (cirrhosis or decompensated liver disease), renal dialysis, severe anemia (hematocrit <25%), severe pulmonary disease-related disability, other neurological impairments, or use of home oxygen. The study was approved by the Institutional Review Board of the University of Maryland. All subjects provided written informed consent.

History of AIDS-defining illnesses and HAART therapy were obtained by self-report and confirmed by review of the patient’s electronic medical record in the VA Computer Patient Record System. Blood sampling, including a complete blood count and chemistry panel, was performed on the day of enrollment. Immunological/virological measures included CD4 cell count (cells/mm³) and HIV viral load (RNA copies/ml) (Amplicor; Roche Applied Science, Indianapolis, Indiana), a polymerase chain reaction (PCR) assay for the quantification of HIV type 1 (HIV-1).

Functional Status. For the 6-min walk distance (6MWD), subjects were instructed to walk quickly but comfortably on an even surface between two cones. The distance walked in 6 min is considered a valid marker of functional status.

Peak Oxygen Consumption. Subjects performed a graded exercise test (GXT) on a treadmill using a modified Bruce protocol and exercised to voluntary exhaustion as described previously. Open circuit spirometry was used to measure oxygen consumption (VO₂), CO₂ production, and minute ventilation breath-by-breath with a SensorMedics Vmax 29C series metabolic cart. Continuous 12-lead electrocardiogram was recorded throughout the test. Peak oxygen consumption (VO₂peak) and respiratory exchange ratio (RER) were determined from the data averaged over 20-s intervals. In addition, subjects’ peak work rates were determined from body mass and treadmill incline and speed, as described elsewhere.

Muscle Imaging. Two 5-mm computed tomography (CT) scans (Siemens Somatom Sensation 16,
Malvern, Pennsylvania) of the lower extremities were taken, one at the midpoint between the anterior superior iliac spine and center of the patella, and the other at the midpoint between the tibial tuberosity and medial malleolus. Cross-sectional areas (CSAs) of each muscle group from the right lower extremity were determined by using Medical Imaging Process Analysis and Visualization software (MIPAV, v. 2.7.47; Center for Information Technology, National Institutes of Health, Bethesda, Maryland) to analyze CT scans of the thigh and lower leg. Muscle attenuation of the knee extensor and dorsiflexor muscle groups was also assessed using MIPAV, expressed in Hounsfield units and used as an index of intramuscular fat.16

**Muscle Performance Testing.** The ankle dorsiflexors and knee extensors of each subject were tested using a KinCom dynamometer (Chattecx, Chattanooga, Tennessee). For both muscle groups the dynamometer was configured and gravity-corrected according to the manufacturer’s guidelines. The order in which the muscle groups were tested was randomly determined for each subject. For the dorsiflexors, a pair of stimulating electrodes was placed roughly longitudinally along the peroneal nerve, just posterior to and ~1 cm distal to the fibular head, taped in place, and further stabilized with a foam pad strapped over the electrodes. EMG recording electrodes (Grass Instruments, Warwick, Rhode Island) were placed as previously described.37 Subjects were positioned supine with the KinCom configured for dorsiflexion testing according to the manufacturer’s guidelines. Once the electrodes were in place, subjects lay supine with the trunk supported and foot in the KinCom’s dorsiflexion apparatus at a fixed angle of ~120° to the shank. The knee was kept at ~0° flexion and the pelvis, thigh, and foot were secured with nonelastic straps.

For the knee extensors, subjects were tested sitting with the knee in 90° of flexion and the transducer secured around the lower leg ~2 cm superior to the lateral malleolus of the ankle. Large (~4 × 5 in) stimulating electrodes were placed over the rectus femoris and vastus medialis motor points as previously described5 and the EMG recording electrodes were placed over the quadriceps as described by McComas et al.30 Subjects were secured to the KinCom with nonelastic straps across the waist and chest.

Electrical stimulation was delivered via an S48 constant-voltage stimulator delivered through an SIU8T stimulation isolation unit (Grass Instruments). Stimulation intensity was determined by monitoring the amplitude of the compound muscle action potential (CMAP) and isometric twitch force in response to single pulses (400 μs for the dorsiflexors and 600 μs for the knee extensors). Intensity was increased incrementally until an increase in voltage produced no increase in CMAP amplitude or peak twitch force. The voltage for central activation testing was set ~10% greater than that which produced a maximum response from the muscle.

The dorsiflexor and knee extensor muscles were tested using similar protocols. Subjects performed three maximum voluntary isometric contractions (MVCs), each separated by 2 min or more of rest. Subjects were verbally encouraged to produce a maximum effort during each of the attempts. During the final MVC, a supramaximal, 100-ms, 50-Hz (100-Hz for the knee extensors) stimulation train was delivered to the muscle to measure central activation. Following the isometric testing, dynamic testing was performed to determine peak power. Dynamic testing involved switching the KinCom to isotonic mode, which allowed subjects to produce force against a fixed load. This load was set at 30% of the subjects’ MVC because a force–velocity model predicts that maximum power is produced at approximately this load.20 Subjects performed three maximum-effort dynamic contractions, each separated by rest for 2 min. Again, subjects were verbally encouraged to give their best effort. The knee extensors were tested over a 60° range of motion from 90°~30° of knee flexion. The ankle dorsiflexors were tested over a 40° range of motion from 120°~80° of ankle plantarflexion.

All force responses were sampled at 500 Hz with force, velocity, and angle data recorded using customized software. The highest peak force value recorded from either the first two MVCs or during the third MVC prior to delivery of the supramaximal electrical stimulation was used to indicate the maximum voluntary force generated by the subjects. We report the MVC force measure as torque (Newtons × lever arm length in meters). Central activation was determined by calculating a central activation ratio (CAR) as previously described.22,37 The CAR equals the maximum voluntary force produced divided by the force produced following the burst during the third MVC. In the absence of any increase in force, CAR equals 1, and any value less than 1 indicates a failure of central activation.22,42

The relationship between the CAR and %MVC is curvilinear rather than linear.40,41 Stackhouse and colleagues have determined that the %MVC/CAR relationship is accurately described ($r^2 = 0.98$) by a second-order polynomial.39,40,43 We used the mea-
sured CAR and the published quadratic equation to generate a corrected CAR value for each subject, and used this as our measure of central activation impairment. Power was calculated as the product of force and velocity during each of the three dynamic contractions. The peak power was recorded for each subject. Each subject’s MVC torque and peak power were divided by the CSA of the respective muscles to give a measure of specific torque and specific power.

Data Analysis. Statistical analyses were performed using SAS software (SAS Institute Inc., Cary, North Carolina) with the threshold for statistical significance at P ≤ 0.05. Except as noted, data are presented as means ± SD. Linear regression analyses were performed to determine the strengths of the relationships among the variables of interest. We divided the subjects into two groups post-hoc, based on the quadriceps femoris CAR data (see Fig. 1), and comparisons between these two groups (low and normal CAR) were evaluated by unpaired t-tests or Wilcoxon tests for nonparametric data (e.g., CD4 count, log_{10} viral load). Chi-square tests were used for frequency count data. A viral load below the level of assay detection (<50 copies/ml) was classified as nondetectable. Linear regression analyses were used to investigate the relationship between quadriceps torque and CAR and muscle CSA within the low and high CAR groups. Finally, stepwise regression analyses were used to investigate the amount of quadriceps MVC torque variability explained by the combination of CAR and muscle CSA for all subjects as well as the low and normal CAR groups.

RESULTS

Subject Characteristics, Functional Status, and VO2peak Testing. Two subjects who disclosed non-compliance with prescribed HAART at the study exit interview and one transgender subject were excluded. The data presented are from the remaining 27 subjects, of whom 93% were African American, and all of whom were male. The subjects were 48.7 ± 6.5 years of age. Average weight, height, body mass index (BMI), quadriceps muscle attenuation, 6MWD, VO2peak, maximum heart rate, and RER are summarized in Table 1, along with the peak work rate achieved during the GXT. The subjects’ mean CD4 count was 408 ± 293 cells/mm³ and the viral load was 2.18 ± 0.94 log copies/ml (Table 2). All of the subjects were on a nucleoside reverse transcriptase inhibitor–based regimen with 82% receiving a protease inhibitor as a third agent (Table 2).

Muscle Imaging. The average CSA of the knee extensors was 71.6 ± 13.0 cm² and muscle attenuation expressed in Hounsfield units was 56.0 ± 5.5. The CSA of the ankle dorsiflexors was 11.8 ± 2.7 cm² and the average muscle attenuation in this group was 73.3 ± 11.3 Hounsfield units.

Muscle Performance. For the knee extensors, mean MVC torque was 211 ± 54 Nm, mean peak power was 442 ± 160 watts, and mean CAR was 0.88 ± 0.16. The average specific torque of the knee extensors was 2.9 ± 0.6 Nm/cm² and the specific power was 6.1 ± 1.8 watts/cm². Regression analysis showed a moderately strong relationship between quadriceps muscle torque and CAR ($R^2 = 0.371, P < 0.001$, Fig. 1A) and torque and CSA of the muscle ($R^2 = 0.249, P = 0.01$, Fig. 1B). No significant relationship existed between CAR and knee extensor CSA (Fig. 1C). There was, however, a significant, though moderately strong, relationship between knee extensor specific torque and CAR ($R^2 = 0.396, P = 0.001$, Fig. 1D). A stepwise regression model was used to explore the amount of variability in quadriceps MVC torque that was explained by the CAR and muscle CSA. The model selected CAR as the variable that accounted for most of the variability in knee extensor MVC torque ($R^2 = 0.371, P = 0.001$), and adding

![Figure 1](image-url)
CSA to the model accounted for more of the variability in knee extensor torque, improving both the fit and significance of the relationship ($R^2 = 0.582$, $P < 0.001$).

All subjects had a CAR of 1 in the ankle dorsiflexors. The average MVC torque of this muscle group was 39.2 ± 8.8 Nm and the average peak power was 102 ± 33 watts. Specific torque and power were 3.54 ± 0.93 Nm/cm² and 9.1 ± 3.3 watts/cm², respectively.

**Normal and Low CAR Groups.** Inspection of the quadriceps femoris muscle CAR data revealed two groups of subjects: a normal CAR group ($n = 16$) with CAR values of 1 or only slightly lower (0.99 ± 0.01) and a low CAR group ($n = 11$) with significant impairment in central activation as indicated by CAR values of 0.89 or lower (0.72 ± 0.12) (Fig. 1A). The normal CAR group produced greater MVC torques (233 ± 53 vs. 181 ± 59 Nm, $P = 0.01$, Fig. 2A) but not peak powers (471 ± 182 vs. 402 ± 119 watts, Fig. 2D) than the low CAR group. There was no difference between the CSAs of the quadriceps femoris muscles of the normal and low CAR groups (71.8 ± 13.6 vs. 71.3 ± 12.7 cm², Fig. 2B). Consequently, the low CAR group had a reduced specific torque as compared to the normal CAR group (2.5 ± 0.6 vs. 3.2 ± 0.5 Nm/cm², $P = 0.001$, Fig. 2C), although there was no difference in specific power (5.4 ± 1.5 vs. 6.6 ± 1.8). Dorsiflexor MVC torque (low: 38.56 ± 9.54; normal: 39.87 ± 8.38), CSA (low: 11.45 ± 3.48; normal: 11.77 ± 2.17), and specific torque (low: 3.41 ± 0.86; normal: 3.59 ± 0.99) were not different across groups.

The low and normal CAR groups did not differ with regard to most of our measures other than CAR (Tables 1 and 2). During the GXT, the low CAR group achieved similar peak VO₂ and maximum heart rate values to those of the normal CAR group. Fewer of the low CAR subjects, however, achieved an RER of ≥1.1 (3/11 vs. 11/16; chi-square $P = 0.01$).

### Table 1. Physical characteristics and performance.

<table>
<thead>
<tr>
<th>Variables</th>
<th>All subjects ($n = 27$)</th>
<th>Low CAR group ($n = 11$)</th>
<th>Normal CAR group ($n = 16$)</th>
<th>$P$-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>48.7 ± 6.5</td>
<td>48.1 ± 6.8</td>
<td>49.1 ± 6.4</td>
<td>0.70</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>75.7 ± 14.9</td>
<td>72.8 ± 11.3</td>
<td>77.6 ± 16.9</td>
<td>0.44</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.77 ± .08</td>
<td>1.76 ± .08</td>
<td>1.78 ± .08</td>
<td>0.41</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.2 ± 4.1</td>
<td>24.1 ± 4.4</td>
<td>24.2 ± 4.0</td>
<td>0.92</td>
</tr>
<tr>
<td>Quadriceps femoris muscle attenuation (Hounsfield units)</td>
<td>56.0 ± 5.5</td>
<td>57.0 ± 4.8</td>
<td>55.6 ± 6.0</td>
<td>0.51</td>
</tr>
<tr>
<td>VO₂peak (mL/Kg/min)</td>
<td>22.6 ± 5.0</td>
<td>21.4 ± 4.4</td>
<td>23.4 ± 5.4</td>
<td>0.30</td>
</tr>
<tr>
<td>Maximum heart rate (beats/min)</td>
<td>142 ± 20</td>
<td>148 ± 18</td>
<td>134 ± 21</td>
<td>0.09</td>
</tr>
<tr>
<td>RER</td>
<td>1.08 ± 0.10</td>
<td>1.02 ± 0.1</td>
<td>1.12 ± 0.08</td>
<td>0.01</td>
</tr>
<tr>
<td>Treadmill max work rate (W)</td>
<td>125 ± 46</td>
<td>98 ± 31</td>
<td>143 ± 46</td>
<td>0.01</td>
</tr>
<tr>
<td>6MWD (m)</td>
<td>593 ± 73</td>
<td>584 ± 83</td>
<td>599 ± 67</td>
<td>0.61</td>
</tr>
</tbody>
</table>

Data presented as means ± SD.

*Comparison of low and normal CAR group with unpaired t-tests.

### Table 2. HIV blood tests and history.

<table>
<thead>
<tr>
<th>Variables</th>
<th>All subjects ($n = 27$)</th>
<th>Low CAR group ($n = 11$)</th>
<th>Normal CAR group ($n = 16$)</th>
<th>$P$-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4 count (cells/mm³) median (range)</td>
<td>398 (18–1401)</td>
<td>426 (101–950)</td>
<td>359 (18–1401)</td>
<td>0.7</td>
</tr>
<tr>
<td>HIV-1 viral load (log copies/ml) median (range)</td>
<td>1.7 (1.7–4.6)</td>
<td>1.9 (1.7–4.6)</td>
<td>1.7 (1.7–4.5)</td>
<td>0.03</td>
</tr>
<tr>
<td>Detectable viral load,† &gt;50 copies/ml, n (%)</td>
<td>4 (14.8)</td>
<td>3 (27.3)</td>
<td>1 (6.3)</td>
<td>0.2</td>
</tr>
<tr>
<td>History of AIDS-defining illness, n (%)</td>
<td>13 (48.1)</td>
<td>8 (72.7)</td>
<td>5 (31.3)</td>
<td>0.04</td>
</tr>
<tr>
<td>Cumulative months HAART in prior 6 months</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NRTI, median (range)</td>
<td>10.3 (3–20.1)</td>
<td>8.4 (3–17.8)</td>
<td>11.0 (4–20.1)</td>
<td>0.4</td>
</tr>
<tr>
<td>PI, median (range)</td>
<td>5.9 (0–16.4)</td>
<td>6.9 (0–11.4)</td>
<td>5.7 (0–16.4)</td>
<td>0.9</td>
</tr>
<tr>
<td>NNRTI, median (range)</td>
<td>0 (0–4.9)</td>
<td>0 (0–4.9)</td>
<td>0 (0–4.8)</td>
<td>0.7</td>
</tr>
<tr>
<td>Thymidine analog</td>
<td>0 (0–5.9)</td>
<td>0 (0–5.9)</td>
<td>0 (0–5.7)</td>
<td>0.4</td>
</tr>
</tbody>
</table>

NRTI, nucleoside reverse transcriptase inhibitor; PI, protease inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor

*Comparison of low and normal CAR group with chi-square, Fisher’s Exact or Wilcoxon rank-sum test

†Lower limit of PCR assay, 50 c/ml, used to calculate log value for nondetectable values.
Interestingly, the low CAR group also attained lower peak work rates (Table 1), despite achieving comparable VO₂peak values. There was no significant difference in type or dose of the different HAART medications or cumulative drug class exposure between the low and normal CAR groups in the prior 6 months (Table 2). The low CAR group, however, did have a higher viral load than the normal CAR group (2.54 ± 1.12 vs. 1.93 ± 0.73 log copies/ml, \( P = 0.03 \)), which reflects the finding that the viral load was suppressed in 15 of 16 subjects in the normal CAR group versus 8 of 11 in the low CAR group. A greater proportion of the low CAR group had a history of AIDS-defining illness compared to the normal CAR group (Table 2), but there was no difference in current immune status as measured by CD4 cell count.

For the low CAR group, regression analyses revealed a moderately strong and significant relationship between the quadriceps MVC torque and CAR (\( R^2 = 0.599, P = 0.005 \), Fig. 3A), but not quadriceps CSA (Fig. 3B). Conversely, for the normal CAR group there was a significant relationship between quadriceps MVC torque and CSA (\( R^2 = 0.667, P < 0.001 \), Fig. 3D), with a trend toward significance for a weak relationship between torque and CAR (\( R^2 = 0.197; P = 0.055 \), Fig. 3C). The same stepwise regression procedure was used for all subjects to evaluate the relationship between torque and the combination of CAR, and CSA was employed with the low and normal CAR groups. For the low CAR group, torque was most closely related to CAR and adding CSA did not improve the fit or significance of the model. Conversely, for the normal CAR group, torque was most closely related to CSA and adding CAR only marginally improved the fit (\( R^2 = 0.681, P = 0.002 \)).

**DISCUSSION**

The principal finding of this study was that nearly half (~40%) of subjects with HIV on HAART had an impaired ability to activate their knee extensor muscles that was associated with weakness and decreased specific force, although no deficits in the central activation of the dorsiflexor muscles were observed. The low CAR subjects had higher viral loads and were more likely to have a history of AIDS-defining illness. The inability of the low CAR group to fully activate their knee extensors was unrelated to muscle size or composition, or to VO₂peak. However, this group did exhibit what might be termed an “aerobic inefficiency” in that they achieved significantly lower work rates than the normal CAR group, while achieving comparable VO₂peak values.

When examined as a whole, the subjects studied had an average CAR that was between those of healthy elderly subjects and healthy younger subjects, as reported by Stevens et al.\(^4\) Given that our subjects were, for the most part, middle-aged, this
was not surprising. Furthermore, the subjects’ knee extensor MVCs were comparable to those predicted by the equations of Lindle et al.\textsuperscript{28} based on subject age (observed, 619 ± 167 N; predicted, 605 ± 30 N). In contrast, the observed VO\textsubscript{2peak} values were substantially lower than established, age-predicted norms (observed, 22.6 ± 5.0 ml/kg/min; predicted, 36.1 ± 2.9 ml/kg/min).\textsuperscript{15} Similar impairments in oxygen consumption have been observed previously in HAART-treated individuals with HIV.\textsuperscript{7,8} The subjects in this study also appeared to have a decreased functional status based on their 6MWDs, which were less than the values predicted based on their BMI and age\textsuperscript{12} (observed, 593 ± 73; predicted, 666 ± 52).

Inspection of the CAR data suggested that our subjects could be divided into two groups, based on their ability to activate their knee extensors (Fig. 1A). In effect, we had two subpopulations, one with normal CAR values in the knee extensors and the other with markedly lower values. Indicative of the concept of two subpopulations, the results of the multivariate regression model incorporating both CAR and CSA produced a much stronger association with MVC torque than either variable alone. This model utilized the major factors for both the low and normal CAR groups (CAR and CSA, respectively), producing the more robust fit.

Subsequent comparisons of the low and normal CAR groups revealed that the low CAR group was weaker, despite comparable knee extensor muscle CSA, resulting in reduced specific force (Fig. 2). Our index of muscle attenuation was similar in both groups (Table 1), suggesting that the lower specific forces were not the result of increased intramuscular fat and less contractile tissue in the low CAR group. When we calculated the age-predicted MVC forces for the two groups as we did for the entire subject pool (see above), the low-CAR group was below its predicted values (observed, 549 ± 115 N; predicted, 608 ± 30 N), whereas the normal CAR group was actually stronger than predicted (observed, 701 ± 174 N; predicted, 602 ± 30 N). The study that provided the equations for these predictions\textsuperscript{28} did not include CSA, and so it is possible that our subjects had larger average muscle areas, which would account for the higher MVC values in the normal CAR group. The impaired activation in the low CAR group would explain why the MVC forces in this group were lower than predicted, despite having CSAs similar to the normal CAR group.

Decreased central activation contributing to weakness has been reported in other patient populations. For example, inhibition of quadriceps activation has been reported in orthopedic conditions of the knee, including osteoarthritis\textsuperscript{14,27,43} and joint replacement surgery.\textsuperscript{31,43} Pain has been shown to account for at least some of the central activation failure in these populations.\textsuperscript{43} Although we did not screen for knee pain during our testing of the quadriceps muscles, no subject reported pain or appeared to have knee discomfort during or after the burst test. Kent-Braun and colleagues\textsuperscript{25,33} have shown central activation impairment of the ankle dorsiflexors in patients with either multiple sclerosis or amyotrophic lateral sclerosis, likely mediated by upper motor neuron dysfunction associated with these diseases. Interestingly, a clinical syndrome mimicking amyotrophic lateral sclerosis in many respects has been reported, although infrequently, in patients infected with HIV.\textsuperscript{7,17}

The inability of the subjects in the low CAR group to activate fully their quadriceps muscle was not related to our measure of functional performance (6MWD). However, the 6MWD test does not place a significant demand on the force-generating capacity of the knee extensors, and is more likely to be influenced by aerobic capacity than maximum force-producing ability. Consistent with this position, both the low and normal CAR groups failed to achieve their predicted 6MWD and had VO\textsubscript{2peak} values substantially lower than established norms, which were calculated as described above. It is possible that if we had chosen a more demanding task as a measure of functional performance, such as a timed stair climb, we would have observed a decreased functional performance associated with the low CAR.

Although the low and normal groups did not differ with regard to VO\textsubscript{2peak}, the mean RER of the low CAR group was less than that of the normal CAR group, indicative of the fact that fewer of the subjects in the low CAR group reached an RER of 1.1 or more. In isolation, these data might be taken to indicate that the low CAR group was simply poorly motivated and that this lack of drive accounted for the low CAR values. Had the low CAR group exhibited comparable maximum work rates to those of the normal CAR group, while not attaining the higher RERs, we would be inclined to accept this interpretation. However, the low CAR group actually achieved lower maximum work rates than the normal group, despite producing comparable VO\textsubscript{2peak} values at exhaustion. Essentially, the low CAR subjects had to utilize comparable aerobic metabolism to achieve less work, an observation that we feel makes it unlikely that the subjects were simply not trying. Perhaps this finding is related to the impaired
Although HIV is not thought to routinely infect motor neurons, it is well established that the virus does enter the CNS and infect glial cells, and can lead to neurological impairment via inflammation, neurotoxic viral proteins, or both. Because of the blood–brain barrier, both the infection and the inflammation may be difficult to treat. Although our findings are consistent with an infection-mediated as opposed to a HAART-mediated impairment of central motor function, the present study is limited by a small homogeneous patient sample and less than 1 year of HAART data. Further research is clearly needed to establish any sort of definitive link. Nevertheless, it appears that a significant proportion of individuals with HIV (40% in the present study) may have muscle activation deficits that are not related to medication regimen or muscle atrophy.

We examined the dorsiflexors as well as the knee extensors and observed no central activation deficits in this muscle group, as all subjects appeared to be able to maximally activate their ankle dorsiflexor muscles (i.e., CAR = 1). Although it is not unusual to see uniformly high CAR values in this muscle group,10,24,38 even in elderly individuals,24,25 we were surprised by the lack of impairment in the low CAR group, given the marked reductions observed in knee extensor activation. One potential concern is that dysfunction of the peripheral nervous system may be present. Our assessment of CAR uses electrical stimulation to activate muscles via their peripheral nerves. Detection of central activation failure could be hindered in the distal dorsiflexor muscles if they are affected by some peripheral nerve disorder to a greater extent than the proximal knee extensors. Several peripheral neuropathic syndromes have been described in patients with HIV, including distal symmetric polyneuropathy, inflammatory demyelinating polyneuropathy, and multineuropathy multiplex.13,46,50 Distal symmetric polyneuropathy is the most common neurological disorder associated with HIV infection and has greater impact distally than proximally because longer motor neurons are more affected than shorter ones.13 Unfortunately, the subjects did not undergo neurological examination to determine whether it was present.

Overall, the results of this study indicate that impaired central activation may be a problem in a significant proportion of individuals with HIV, although perhaps not in all muscle groups. The subjects with impaired central activation were weaker than those without impairment, but their muscles were of similar sizes. Consequently, impaired central activation was associated with reduced specific force, although mechanisms other than the loss of central activation may well have been at work. Contrary to our hypothesis of a link between aerobic capacity and CAR, the two groups did not differ with regard to VO₂peak, although some form of aerobic impairment may have been present in the low CAR group. These subjects attained similar VO₂peak values to
the normal CAR group, while performing at lower work rates. In these subjects the central activation deficit appeared to be more closely linked to viral loads and past history of AIDS-defining illnesses than to HAART medications, suggesting that this impairment is related to the progression of the disease itself. While such a mechanism would be consistent with existing reports of impaired motor function in HIV-positive individuals, further research is needed to examine this possibility.

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IDENTIFICATION OF DE NOVO BSCL2 SER90LEU MUTATION IN A KOREAN FAMILY WITH SILVER SYNDROME AND DISTAL HEREDITARY MOTOR NEUROPATHY

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Distal hereditary motor neuropathy (dHMN) or distal spinal muscular atrophy (OMIM 182960) is divided into seven subtypes and is a heterogeneous group of disorders characterized by the almost exclusive degeneration of motor fibers, mainly in the distal part of the limbs.4 dHMN type V is characterized by predominant wasting and weakness of the small hand muscles that can occur unilaterally and is sometimes confined to the thenar and first dorsal interosseus muscles.3,4,10 Silver syndrome (OMIM 270685) belongs to the group of autosomal-dominant, complicated hereditary spastic paraparesis (HSP) and is characterized by spasticity of the lower limbs. The condition is often accompanied by marked weakness and wasting of the small hand muscles.8 The locus of this disease (designated SPG17) has been mapped to the chromosome region 11q12-14.7 Recently, heterozygous mutations in the Berardinelli–Seip congenital lipodystrophy (BSCL2) gene were identified in SPG17 families and families with dHMN type V.11 We report a Korean family with the BSCL2 mutation showing variable expression within the family. We are unaware of any previous reports of genetically confirmed cases from Asia.

MATERIALS AND METHODS

Patients. A 52-year-old woman (II-2; Fig. 1) presented with walking difficulties and frequent falling beginning at around the age of 14 years. At age 15 she had noticed stiffness in both hands, and she developed symmetrical wasting and weakness of both hands thereafter. At age 49, distal muscle weakness and atrophy in her lower legs, an equinovarus foot and ankle deformity, and intrinsic muscle atrophy in both feet and hands were observed. Physical examination at that time revealed hyperreflexia at the knees, hyporeflexia at the ankles, and grade 1 spasticity (Ashworth scale) in the knee extensors and hip adductors. Babinski sign was not noted but absence...
of this pathologic sign might have been due to marked atrophy and weakness of the lower-limb and the foot intrinsic muscles. She could not perform palmar abduction of the thumb and opposition of the fingers. However, strength of the forearm muscles was relatively preserved and of the wrist flexors and extensors was normal. There were no paresthesias, sensory loss, or sphincter disturbances. Nerve conduction study (NCS) showed normal or slightly decreased conduction velocities without blocking or temporal dispersion on proximal stimulation, as well as normal sensory nerve action potentials (SNAPs) in the median and ulnar nerves bilaterally and the left tibial nerve. SNAPs were not evoked in either of the superficial peroneal nerves and the left sural nerve; in the right sural nerve the SNAP was 5 μV in amplitude. The motor nerve conduction velocities were 41.5–51.5 m/s in the median and ulnar nerves and 39.7 m/s in the left tibial motor nerve. However, the amplitudes of the compound muscle action potentials (CMAPs) were reduced markedly in those nerves, ranging from 0.2–0.5 mV in the median and ulnar nerves and 0.3 mV in the left tibial nerve. Concentric needle electromyography (EMG) revealed abnormal spontaneous activity, abundant polyphasic motor unit action potentials (MUAPs), and MUAPs with high amplitude and long duration in the left first dorsal interosseus, abductor pollicis brevis, tibialis anterior, and medial gastrocnemius muscles. The recruitment of the MUAPs was also reduced.

Her 42-year-old younger sister (II-8) was affected in a similar manner and showed difficulties in walking and intrinsic hand-muscle weakness as well as atrophy leading to impaired opposition in the fingers of both hands. Active dorsiflexion of ankles was impossible and a mild pes cavus foot deformity was observed.

The 16-year-old son (III-2) of the proband showed gait abnormalities at age 9 and foot deformities since then. Both foot deformities progressed and the patient underwent surgery for severe spastic equinovarus deformities at the age of 12. At age 12 he showed marked distal muscle weakness and atrophy in the lower extremities, weakness and atrophy of the intrinsic hand muscles that was milder than those of his mother, no sensory impairment, increased tendon reflexes at the knees with grade 1 spasticity (Ashworth scale) in the knee extensors, decreased Achilles tendon reflexes, Babinski signs, and a waddling gait. Opposition of the thumb and little finger was weak. The findings on NCS were similar to those of his mother. No other family members appeared to be affected.

**Genetic Analysis.** After obtaining informed consent, sequence analysis of all the coding exons and their flanking introns of the BSCL2 gene was performed by using appropriate primers designed by the authors (available upon request).

**RESULTS**

A previously reported missense mutation (c.269C>T; Ser90Leu) in the BSCL2 gene was identified in the proband, her younger sister, and the second son. Her parents and the first son were healthy and did not carry the mutation (Fig. 1). Her paternity was confirmed using polymorphic markers. Considering that her parents did not have the Ser90Leu mutation and that two of nine offspring carried the same mutation, a possibility of germline mosaicism was suspected but could

**FIGURE 1.** (A) Pedigree of the family showing the clinical features of Silver syndrome and dHMN type V. Circle, female; square, male; black symbol, affected; diagonal line, deceased. (B) Direct sequencing of the BSCL2 gene revealed a heterozygous c.269C>T (Ser90Leu) mutation (arrow).
not be confirmed. The other family members were unavailable for genetic analysis.

**DISCUSSION**

dHMN type V and Silver syndrome share the unusual characteristic of prominent hand-muscle weakness and wasting that occur early in the course of the disease and a linkage region, which suggests that the two diseases may be allelic.\(^1,7,11,12\) Windpassinger et al.\(^{11}\) reported that heterozygous missense mutations in *BSCL2* lead to dHMN type V and Silver syndrome, which are an extreme phenotype with a similar genetic etiology. They determined that the Ser90Leu mutation affects the consensus sequence for N-glycosylation in the *BSCL2* encoded protein, seipin, which can lead to misfolding and subsequent aggregation of the mutated seipin. Both conditions are genetically heterogeneous\(^7,11\) and *BSCL2* mutations have been reported to show incomplete penetrance and variable clinical expression.\(^9,11\) In a collaborative study of one Belgian, one Italian, one Brazilian, two English, and 14 Austrian families with either the dHMN type V or Silver syndrome phenotype, two heterozygous mutations (N88S, S90L) in the *BSCL2* gene were demonstrated to be responsible for the phenotypic expression of these disorders.\(^6,11\) Thus far, only two mutations in the *BSCL2* gene have been identified in patients with dHMN, Silver syndrome, and HSP.\(^5,11\)

The range of phenotypic manifestations in carriers of the *BSCL2* mutations vary from asymptomatic cases to severe disease expression in patients showing dHMN, Charcot–Marie–Tooth disease, and HSP phenotype.\(^2\) In our Korean family with the *BSCL2* mutation, we found a somewhat different phenotypic expression pattern within the family. The clinical patterns in our family included presentation with lower-limb and hand-muscle involvement early in the disease course as well as the presence of Babinski signs with nonprogressive mild spastic paraparesis that resembles classic Silver syndrome and dHMN type V.

We are unaware of other reports of a *BSCL2* mutation in the Asian population and the clinical details in this family reconfirm the wide phenotypic spectrum and variable expression of *BSCL2* gene mutations. The Ser90Leu mutation has also been reported in two families from Brazil and Belgium.\(^{11}\) Considering that this mutation can arise in different ethnic populations, Ser90Leu might be a mutational hotspot or a recurrent mutation. However, the testing of additional individuals with a broad spectrum of clinical features of dHMN and Silver syndrome and having various geographic ancestries will be needed.

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ABSTRACT: The progression of Duchenne muscular dystrophy (DMD) is, in part, due to satellite cell senescence driven by high replicative pressure as these muscle stem cells repeatedly divide and fuse to damaged muscle fibers. We hypothesize that telomere shortening in satellite cells underlies their senescence. To test this hypothesis, we evaluated the diaphragm and a leg muscle from dystrophic mice of various ages for telomere dynamics. We found 30% telomere shortening in tibialis anterior muscles from 600-day-old mdx mice relative to age-matched wildtype mice. We also found a more severe shortening of telomere length in diaphragm muscles of old mdx mice. In those muscles, telomeres were shortened by ~15% and 40% in 100- and 600-day-old mdx mice, respectively. These findings indicate that satellite cells undergo telomere erosion, which may contribute to the inability of these cells to perpetually repair DMD muscle.

**TELOMERE SHORTENING IN DIAPHRAGM AND TIBIALIS ANTERIOR MUSCLES OF AGED mdx MICE**

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Duchenne muscular dystrophy (DMD) is the most common childhood muscular dystrophy. It is caused by a mutation in the dystrophin gene whose protein product assists in muscle plasma membrane stability. In the absence of dystrophin, progressive muscle weakness occurs and ultimately death in about the third decade.

Histologically, the disease has recurrent cycles of muscle-fiber injury followed by regeneration, but eventually fibrosis predominates. Although satellite cells, the regenerative cells of muscle, repair damaged fibers initially, their proliferative capacity is ultimately reduced. Reduction of satellite cell proliferation may be due to replicative aging as a consequence of replacing damaged, dystrophin-deficient fibers. Replicative aging is accompanied by telomere shortening leading to cellular senescence as the cells reach their limit of divisions, i.e., the Hayflick limit. Although telomere length in skeletal muscle could serve as a surrogate marker of cell turnover because a significant portion of nuclei (33%) in DMD muscle are of satellite cell origin, it is not clear whether telomere shortening occurs in DMD muscle fibers.

Oexle and Kohlschutter found that DMD patients did not show significant telomere shortening in quadriceps muscles when compared to controls, suggesting that impaired differentiation contributed more to DMD progression. Conversely, Decary et al. reported that satellite cells of human muscle have a limited proliferative capacity that decreased with and correlated to short telomeres. A later study by the same group showed that telomere length was significantly shorter in quadriceps muscles isolated from children with DMD. This telomere shortening was thought to cause premature senescence of satellite cells due to exhaustive replicative pressure. Thus, the occurrence of telomere shortening in human DMD muscle is contentious.

The goal of our work was to evaluate skeletal muscle telomere dynamics in a DMD mouse model, mdx mice. We evaluated tibialis anterior, an important leg muscle for ambulation, and diaphragm, a unique skeletal muscle because its chronic use leads to severe injury in mdx mice and therefore is subject
to more rapid regeneration relative to intermittently used leg muscles. Thus, we hypothesized that telomere shortening with age would be greater in diaphragm than tibialis anterior muscle of mdx mice.

**MATERIALS AND METHODS**

**Animals.** Wildtype (C57BL/6) and mdx mice were obtained from our colony at Virginia Polytechnic Institute. At a given age (~20, 40, 80, 100, 240, or 600 days), mice were anesthetized (2 mg xylazine and 20 mg ketamine / 100 g body mass, i.p.) and tissues were harvested and flash-frozen in liquid nitrogen. Tissues were sent on dry ice to the University of Minnesota. All procedures were approved by our institutional animal care committee.

**Telomere Analysis.** DNA was prepared using reagents and protocol from Gentra Systems (Minneapolis, MN). Briefly, tissues were ground into a fine powder using a chilled mortar and pestle, digested overnight at 55°C by proteinase K (Sigma, St. Louis, Missouri), and contaminating protein was removed by precipitation and centrifugation. DNA was precipitated with isopropyl alcohol, briefly air-dried, and resolubilized in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Telomere length was assessed as described previously. DNA (1 μg) was digested with HinfIII and RsaI, separated on 0.5% agarose gel, and vacuum transferred to positively-charged nylon membrane. Telomere DNA was detected using a digoxigenin 3' and 5' end-labeled probe, (TTAGGG)3 (TelotAGGG Telomere Length Assay; Roche, Mannheim, Germany). Average telomere length (in kb) was determined using Telometric, an NIH Image software macro. Relative muscle telomere length was calculated by dividing the mdx average telomere length by that of its age-matched wildtype control (n = 4–6 pairs per age per muscle). Within an age, mdx and wildtype groups were compared by t-tests and differences were considered significant when P < 0.05. Telomere lengths of liver, blood, and fibroblast expansions were periodically compared between mdx and age-matched wildtype mice.

**RESULTS**

Telomere length in diaphragm muscle was not different between mdx and wildtype mice at young ages (Fig. 1), e.g., average length was 10.68 and 10.69 kb, respectively, at 20 days of age. Telomere shortening was evident in diaphragm muscles from older mdx mice. Shortening was significant at age 100 days, when telomeres were ~15% shorter in mdx than age-matched wildtype mice (Fig. 1B). By age 600 days, mdx mice had lost 40% of telomere length relative to wildtype mice (4.9 vs. 8.2 kb).

No shortening was found in the tibialis anterior muscles of younger mdx mice (e.g., 9.5 vs. 9.9 kb at 20 days). Telomere shortening in tibialis anterior muscle of mdx mice was significant only at 600 days (6.4 vs. 9.4 kb; Fig. 1).
Telomere lengths were periodically analyzed in liver, blood, and fibroblast expansions as controls. Figure 1A shows identically long telomeres from fibroblast outgrowth cultures from age-matched diaphragms, indicating that muscle fibroblasts are probably not a significant contributor to telomere shortening in mdx muscle. Within liver or blood, no differences in telomere length were detected between age-matched mdx and wildtype mice, demonstrating the specificity of the disease to muscle in mdx mice.

DISCUSSION

We found that telomere shortening occurs in mdx mouse muscle, in accord with Decary et al., who showed shorter telomeres in quadriceps muscle of children with DMD. In addition, we found greater shortening in mdx diaphragm compared with tibialis anterior, likely because the diaphragm is continuously working and is therefore subject to an earlier onset of degeneration–regeneration than leg muscles, which are used intermittently. The progression of telomere shortening correlates with the time line of disease progression in the mdx mouse, which develops a more severe dystrophic phenotype with age.1,15

Skeletal muscle regeneration relies on satellite cells. The regenerative capacity of satellite cells decreases with the progression of muscular dystrophy3,10,20 and with age, which is accompanied by telomere shortening.13 Likewise, athletes with exercise-associated fatigue have shorter muscle telomere lengths, presumably due to increased satellite-cell proliferation.1 These observations support the contention that short satellite-cell telomeres may contribute to dwindling regeneration in the context of injury-prone, dystrophin-deficient muscle.

A major difference between human DMD and mdx mice is that the life span of this mouse is not appreciably reduced, whereas that of a DMD patient is only ~30% of normal. Telomeres of mice are much longer than that of humans (20–40 kb vs. 10–15 kb) and this “telomere reserve” may be a factor contributing to the more robust regenerative capacity and corresponding milder phenotype seen in the mdx mouse relative to the DMD patient. Mice lacking telomerase do not show a phenotype until 4–6 generations,10 further demonstrating how extra-long telomeres may be protective for mouse muscle. In fact, the loss of a single telomere may be more important than the average telomere length as a contributor to DNA instability/senescence.17 In addition, human and mouse cells regulate telomeres in somewhat different manners, such that telomere erosion by itself is probably not the exclusive mechanism leading to senescence.18 Lastly, although we cannot entirely exclude the possibility that non-muscle cells contributed to telomere loss, our analysis of such cells suggest that this was minimal.

We conclude that significant telomere shortening occurs in dystrophic muscle and may contribute to satellite-cell senescence. Although the effects of this are mild in the mdx mouse, telomere shortening could potentially account for the declining regenerative ability of human DMD muscle as the disease progresses.

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ABSTRACT: We investigated the use of human muscle-derived cells (hMDCs) for the treatment of stress urinary incontinence (SUI) in a nude rat model. hMDCs were isolated from adult skeletal muscle. Three groups of six animals consisting of controls, animals undergoing sciatic nerve transection (SNT) with periurethral sham-injection, and SNT with hMDCs (1 × 10^6 cells/20 μl saline) were utilized. Leak point pressure (LPP) was measured 4 weeks following injection. Bilateral SNT resulted in a significantly lower LPP that was significantly higher following hMDCs than sham injection. The results demonstrate the efficacy of human muscle cell therapy alone in improving physiologic outcomes in an animal model of SUI.


HUMAN MUSCLE-DERIVED CELL INJECTION IN A RAT MODEL OF STRESS URINARY INCONTINENCE

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Stress urinary incontinence (SUI) is a common condition with involuntary leakage of urine on exertion, sneezing, or coughing.1 Its etiology is multifactorial, involving functional impairment of muscle and associated nerves that may occur as a result of advancing age, hormonal status, and pelvic floor damage resulting from childbirth. The use of periurethral injectables is a minimally invasive treatment option.4 However, included among the disadvantages is the need for multiple injections due to loss of the long-term bulking effect as a result of degradation, reabsorption, or migration.

We have previously proposed an alternative treatment using skeletal muscle–derived cells (MDCs) as an injectable therapy for SUI. Such cells may not only act to generate tissue with bulking properties but also have the potential to improve sphincter function by remodeling the damaged tissue.5,8 The objective of this study was to investigate the potential of human MDCs (hMDCs) for the treatment of SUI in a rat model.

Abbreviations: H&E, hematoxylin–eosin; hMDC, human muscle-derived cells; LPP, leak point pressure; SNT, sciatic nerve transection; SUI, stress urinary incontinence

Key words: incontinence; muscle; skeletal muscle; stem cell; stress urinary incontinence; urethra

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MATERIALS AND METHODS

Six-week-old female, athymic nude rats (Hsd:RH-rnu, Harlan Laboratory, Indianapolis, Indiana) were used. Nude rats were used in this study to decrease adverse host immune reactions to human origin cells. Protocols were approved by the Animal Research Care Committee of Children’s Hospital of Pittsburgh. SUI was created through bilateral transection of the sciatic nerve.8 Rats were given isoflurane anesthesia (2 L/min) and bilateral vertical dorsal incisions were performed over the ischiorectal fossa. The sciatic nerve on each side was identified and 2 mm of those trunks were excised distal to its origin from the vertebral column, but proximal to the branching of the pudendal nerve.

The hMDCs were isolated from rectus muscle (∼250 mg) of a single donor, provided by Cook MyoSite (Pittsburgh, Pennsylvania). Culture expansion was carried out in an antibiotic-free medium supplemented with 10% fetal bovine serum. Flow cytometric analysis of the hMDC suspensions was performed to evaluate myogenic content through antibody labeling of CD56 expression (BD Pharmingen, San Jose, California).

Seven days following denervation, under isoflurane anesthesia (2 L/min), a low midline incision was made. Cryopreserved hMDCs or sham suspensions were thawed with an equal volume of saline. A 3/10-ml
insulin syringe was used to inject either 10 μl (5 × 10^5 cells) of hMDC suspension or sham aliquot into each lateral wall of the mid-urethra with microscopic guidance. Nondenervated, noninjected, age-matched animals served as controls.

Leak point pressure (LPP) measurements were performed 4 weeks after injections. Under urethane anesthesia (1.2 g/kg s.c.), a midline abdominal incision was made and the ureters were ligated. A transvesical catheter (PE-90) was inserted into the dome of the bladder and the abdomen was closed. A three-way stopcock was connected to the transvesical tube to monitor the bladder pressure during cystometry (continuous infusion of normal saline at 0.04 ml/min). The voided volume bladder capacity and maximal voiding pressure were monitored. After cystometry, all rats underwent T9 spinal cord transection to eliminate spontaneous bladder activity and were mounted on a tilt table in the vertical position. Intravesical pressure was controlled by connecting a large 50-ml syringe to the bladder catheter. The syringe reservoir was mounted on a metered vertical pole for controlled height adjustment. Intravesical pressure was increased in steps of 1–3 cm H2O from zero upward until visual identification of leakage; this pressure was identified as the LPP. Three consecutive readings were obtained, averaged for each animal, and presented as a single LPP.

Immediately following the LPP measurement, the entire urethra–bladder complex was removed. The tissues were snap-frozen using 2-methylbutane precooled in liquid nitrogen. Cryosections of the urethra were labeled with hematoxylin–eosin (H&E) for general histology, and also immunofluorescently labeled with human specific anti-lamins A/C antibody (Novocastra, Newcastle upon Tyne, UK) to follow the fate of the injected hMDC.

Data are presented as means ± SE. Overall comparisons between groups were performed using a one-way analysis of variance (Tukey’s multiple comparison test). A P-value of less than 0.05 was accepted as significant.

RESULTS

The injected hMDC suspensions contained 87.7% myogenic (CD56-positive) cells; the remainder of the

FIGURE 1. The proximal urethral sphincter of the denervated rats (A) was atrophic at 4 weeks compared with control (B). Images taken with 10× objectives. C: Immunofluorescent labeling with a human-specific nuclear antibody (lamins A/C) reveals the presence of human nuclei (red) incorporated within the striated sphincter muscle layer in hMDC-injected tissues (100×; arrows point to individual nuclei).
cell suspension was fibroblastic. No serious adverse effects were observed in any rat in any group. No difference in any cystometric parameter was observed between the control, sham, and hMDC-injected groups. Denervation of the urethra resulted in a decrease in LPP from the control to sham groups (43.4 ± 0.6 to 27.8 ± 0.7 cmH2O, respectively; P < 0.05). LPP was restored following hMDC injection (35.7 ± 2.0 cm H2O) when compared to the sham group (P < 0.05); however, at the 4-week timepoint this level of restoration remained less than control (P < 0.05).

In the denervated rats the proximal urethral sphincter was atrophic at 4 weeks compared with control (Fig. 1A,B). Tissues from the hMDC-injected group showed clear positive labeling of numerous human nuclei incorporated within the external (striated) sphincter muscle (Fig. 1C).

**DISCUSSION**

Our study demonstrated the feasibility of using human cell therapy for urological application; the human MDCs were harvested from a clinically obtainable sized muscle biopsy and improved physiologic outcomes for up to 4 weeks in an immunocompromised rat model of SUI.

Previous attempts to treat SUI with autologous cells have utilized fat cells and chondrocytes, but both were limited to a purely bulking effect and suffered long-term decreases in effectiveness. MDC is a muscle progenitor cell that has also shown the capacity to differentiate into bone, cartilage, nerve, and endothelium. MDCs have been successfully isolated from mouse, rat, and human and thus far have been utilized in therapies for both cardiac and myopathetic diseases with limited success. In a long-term study, Lee et al. showed that MDCs could persist for longer than 12 weeks when injected into the urethra, in contrast to fat, which loses mass due to inflammation and necrosis.

We have previously shown that periurethral injection of MDCs improved the LPP in a denervated female rat model of SUI and in rats that modeled the intrinsic sphincter deficiency that follows radical prostatectomy. In contrast to fibroblasts, which act to increase the LPP by bulking effects, MDCs significantly increase the LPP by improving urethral muscle strip contractility without causing deleterious obstruction, as is seen with fibroblast injection.

In conclusion, treatment with hMDCs led to restoration of LPP to near-normal levels in an experimental model of SUI in the rat. Based on histological and physiological findings we hypothesize that the injected hMDCs differentiated into new muscle fibers and prevented periurethral muscle atrophy.

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**REFERENCES**

CASE OF THE MONTH

ABSTRACT: We report a patient with myasthenia gravis who had neurogenic muscle atrophy in association with external ophthalmoplegia and weakness of the upper limbs. Neurogenic changes in the limb muscles were found on needle electromyography and histological studies. Symptoms improved and atrophy of the limbs diminished after intravenous immunoglobulin and oral corticosteroid therapy. We concluded that functional interruption of the neuromuscular junction caused the neurogenic muscle atrophy and that this was relieved by appropriate therapy.


MYASTHENIA GRAVIS PRESENTING WITH UNUSUAL NEUROGENIC MUSCLE ATROPHY

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Myasthenia gravis (MG) is characterized by weakness and muscle fatigue caused by failure of neuromuscular transmission due to loss of acetylcholine receptors at the postsynaptic membrane. Although muscle atrophy may occur in as many as 10% of patients with MG,13,14 severe atrophy is rare in the early course of the disease. Furthermore, improvement of muscle atrophy associated with MG has only been reported previously in one patient.6 Herein we report an unusual patient with associated neurogenic muscle atrophy and discuss the features on single-fiber electromyography (SFEMG) and histological studies of muscle biopsy samples, as well as the response to treatment.

CASE REPORT

A 28-year-old Japanese man presented to our hospital in June 2000 complaining of bilateral ptosis, diplopia, and muscle weakness of both hands and forearms. His gestational period, delivery, and developmental milestones had all been normal and he was healthy until the age of 27, at which time (March 1999) he experienced diplopia and weakness in the left arm. Bilateral ptosis and weakness in his right arm appeared in June 1999 and gradually progressed. In September he had diplopia, bilateral ptosis, weakness of both upper extremities, and mild dysphagia. His symptoms initially showed some improvement, but then remained unchanged until his presentation to us. The family history was unremarkable.

On examination, the patient was a tall, slender, and intelligent man, and general physical findings were unremarkable. He showed moderate bilateral ptosis, severe bilateral gaze limitation in all directions, and moderate symmetrical weakness of the orbicularis oculi muscles. He also had symmetrical weakness and atrophy, chiefly in the muscles innervated by both ulnar nerves. His strength was grade 3 on the Medical Research Council (MRC) scale in the abductor digiti minimi, dorsal interossei, and flexor carpi ulnaris on both sides, grade 4 in the sternocleidomastoid, deltoid, biceps brachii, triceps, flexor carpi radialis, extensor carpi radialis, extensor carpi ulnaris, extensor digitorum, flexor digitorum superficialis, flexor digitorum profundus, flexor pollicis brevis, abductor pollicis brevis, and iliopsoas on both sides, and grade 5 in other muscles on both sides. Strength tests revealed no increase in fatigability as maneuvers were repeated. There was no fasciculation, sensory abnormalities, or ataxia, the deep tendon reflexes were generally hypoactive, and no pathological reflexes were elicited. The patient...
stood without a Gowers’ sign and walked normally. Although ptosis showed some variation over the day, other symptoms did not fluctuate from day to day. An intravenous injection of edrophonium chloride increased grasp power unequivocally, but did not improve ophthalmoplegia or ptosis.

Urinalysis, stool examination, electrocardiogram and chest, abdominal, and cervical X-rays were all normal. Chest computed tomography (CT) and 201Tl single-photon emission CT did not reveal a thymoma. Orbital magnetic resonance imaging (MRI) revealed atrophy of all the external ocular muscles. Serum creatine kinase (CK) was 185 U/L (normal 55–306 U/L), and other blood chemistry parameters, including electrolytes and thyroid hormones, were within the normal range. In the serum, anti–acetylcholine receptor (AChR) antibodies were elevated at 1.8 nM (normal <0.37 nM). Anti–skeletal muscle antibodies, antineuronal nuclear antibody type 1, antiganglioside antibodies (anti-GD1a, anti-GD1b, anti-GQ1b, anti-GM2, anti-fucosyl-GM1, anti-GalNAc-GD1a, and anti-sulfoglucuronyl paragloboside antibodies), and other autoantibodies were all negative. Antiganglioside antibodies were examined using two different methods: an enzyme-linked immunosorbent assay and a thin layer chromatography immunoblot method using glycolipids from the brain, spinal cord, and peripheral nerve as antigens. The level of anti–muscle-specific receptor tyrosine kinase (anti-MuSK) antibody was 0.01 nM (normal range: <0.05 nM). Cerebrospinal fluid protein values and cell count were within the normal ranges.

Electrophysiological Studies. Motor nerve conduction studies revealed normal motor nerve conduction velocities, normal distal latencies without conduction block in the median, ulnar, posterior tibial, and deep peroneal nerves bilaterally. F-responses were all normal in those nerves. The amplitude of the compound muscle action potential recorded from the left abductor digiti minimi was mildly reduced at 5.7 mV (control mean ± SD in our laboratory: 8.94 ± 3.07 mV, n = 50). Sensory nerve conduction studies were all normal in the median, ulnar, and sural nerves, bilaterally. Repetitive nerve stimulation at 3 and 5 Hz, with recording from the orbicularis oculi, trapezius, abductor pollicis brevis, and abductor digiti minimi, demonstrated decremental responses (Fig. 1A). Repetitive nerve stimulation at 50 Hz revealed no incremental response in the abductor digiti minimi. SFEMG recorded from the right extensor digitorum communis showed increased jitter in 17 of 31 recorded fiber pairs, with the mean consecutive difference in abnormal pairs ranging from 64.3 to 145.9 μs (upper normal limit of 55 μs, which is close to the median ± 3 SD: 56 μs)20 (Fig. 1C). Significant blocking was not observed. Fiber density was 1.23 (normal, 1.49 ± 0.16).20 Needle EMG was performed in the right biceps brachii, triceps, flexor carpi ulnaris, flexor carpi radialis, first dorsal interosseous, abductor pollicis brevis, and flexor digitorum profundus (III, IV), and the left tibialis anterior and posterior. Needle EMG revealed normal insertional activity and motor unit potentials (MUPs) of normal amplitude and duration (mostly triphasic) recorded by monopolar electrodes in the muscles examined. Profuse fibrillation potentials and positive sharp waves (PSWs) were present in

![FIGURE 1. (A,B) Ulnar compound muscle action potential of the abductor digiti minimi muscle evoked by repetitive stimulation at 3 Hz, showing a mild decremental response (A), which was improved by intravenous injection of 10 mg of edrophonium chloride (B). (C) Sample recording from single-fiber electromyography demonstrating increased jitter, as judged by criteria described by Stålberg et al.20 The tracing is a composite of 64 superimposed consecutive potentials obtained from the extensor digitorum communis muscle. The mean consecutive difference was 74.0 μs.](image-url)
right triceps, flexor carpi ulnaris, first dorsal interosseous, and flexor digitorum profundus (III, IV), and the left tibialis anterior, and posterior. The number of MUPs was decreased markedly in the right triceps, flexor carpi ulnaris, and flexor digitorum profundus (III, IV). However, reinnervation changes, such as high-amplitude or long-duration MUPs, were not detected.

**Histological Studies.** The left biceps brachii muscle was biopsied and the muscle showed no apparent atrophy. Hematoxylin and eosin (H&E) staining showed muscle-fiber diameters ranging pathologically from 10–120 μm. Atrophic fibers were seen in small groups (Fig. 2a). There was no increase in the number of central nuclei, degenerative or regenerative fibers, inflammatory cell infiltrations, or lymphorrhage; blood vessels, connective tissue, and peripheral nerves in the section appeared normal. Treatment with nicotinamide adenine dinucleotide-tetrazolium reductase (NADH-TR) revealed the presence of some targetoid fibers (Fig. 2b). Judging by myofibrillar adenosine 5-triphosphatase (ATPase) reactions, the normal checkerboard pattern of muscle fiber was preserved (Fig. 2c). The atrophic fibers were composed of both type I and type II fibers. Histochemical and immunohistochemical stains of the motor endplates revealed a decrease in reactivity to AChR compared to that in the control, with deposits of C3. Electron microscopic observation of the motor endplates showed simplification of the synaptic folds.

**Clinical Course.** Although the patient had several atypical clinical features, we believed that a myasthenic neuromuscular transmission deficit played an important role in causing his symptoms. Since we did not detect a thymoma, we began treatment with double-filtration plasmapheresis (DFPP); however, daily DFPP for 3 consecutive days did not relieve the symptoms. We then tried intravenous immunoglobulin therapy (IVIg); two courses of 0.4 mg/kg IVIg daily for 5 consecutive days with a 2-day interval between courses improved the strength bilaterally of the triceps, wrist extensors, and abductor digiti minimi, but ptosis and gaze limitation remained. Thereafter, the patient underwent thymectomy, but neither hyperplasia nor malignant findings of the thymus were found. Corticosteroid therapy was initiated after thymectomy, with an alternate-day dose of oral prednisolone that was gradually increased from 20 to 80 mg. One month after the beginning of corticosteroid therapy his grip power had increased from 15 to 20 kg. Bilateral ptosis disappeared and eye movement showed a remarkable improvement.

![Figure 2](image-url)
Binding anti-AChR antibodies decreased to 0.8 nM. However, orbital MRI in April 2001 indicated that atrophy of the external ocular muscles had not improved dramatically. Nevertheless, his symptoms continued to improve gradually and the alternate-day dose of prednisolone was decreased to 14 mg in December 2003. At that time, although mild bilateral ophthalmoplegia remained, weakness and atrophy of the upper limbs and ptosis had diminished. In January 2005, bilateral ophthalmoplegia progressed and the alternate-day dose of prednisolone was increased to 60 mg. His symptoms again improved gradually and the alternate-day dose of prednisolone was decreased to 45 mg in January 2006. Throughout the clinical course, atrophy of the upper limb has never recurred.

DISCUSSION

We diagnosed this case as MG for the following reasons: (1) decremental responses on repetitive nerve stimulation and increased jitter on SFEMG; (2) a positive edrophonium test; (3) elevation of binding anti-AChR antibodies; and (4) biopsy findings including decreased AChR sites, deposition of C3, and simplification of the synaptic folds at motor endplates. However, we note that an absence of marked fatigability, a lack of daily fluctuations of ophthalmoplegia and limb weakness, unresponsiveness to DFPP, and the presence of severe atrophy of limb muscles from an early stage of the disease are uncommon as clinical features of MG.

Muscle atrophy in the limbs is particularly important with regard to diagnosis and pathology. We speculate that the muscle atrophy in our patient was caused by neurogenic changes for the following reasons: (1) atrophy localized chiefly in muscles innervated by the ulnar nerve; (2) spontaneous activity such as fibrillation potentials or PSW, polyphasic MUPs at low volition, and a reduction in the number of MUPs at maximal volition were observed on needle EMG; and (3) changes of grouped atrophy were present in biopsied muscles. Additionally, we were unable to detect reinnervation changes such as high-amplitude MUPs, except for some polyphasic MUPs.

Although profuse fibrillations and PSWs can be seen in some myopathies, such as polymyositis, our patient presented with distal-dominant limb weakness and normal serum CK; furthermore, he lacked other features of myopathy in electrophysiological and histological studies. MG and muscle atrophy have been associated with polyneuropathy or amyotrophic lateral sclerosis in some patients. However, the normal nerve conduction and improvement of muscle atrophy in our patient makes an association with such disorders unlikely. Kinoshita et al. reported four patients with similarities to our case, and described their disorder as myasthenic neuromyopathy. These patients were diagnosed with myasthenia gravis due to daily fluctuations of weakness and a positive edrophonium test; they also had muscle atrophy and neurogenic changes on needle EMG and histological studies. However, myopathic features such as proximal-dominant muscle weakness and atrophy were present and elevation of serum CK was observed, in contrast to our case.

The adult form of MG was originally divided into five classifications and in 1958 the Osserman group V classification was used to indicate generalized myasthenia with muscle atrophy. Among 282 patients, 17 showed muscle atrophy; among these 17 patients only 4 demonstrated muscle atrophy within 2 years from symptom onset. Thereafter, several reports indicated that a subset of MG patients had muscle atrophy and neurogenic changes on biopsy. In 1973, Oosterhuis and Bethlem reported that 14 of 148 patients with generalized MG had muscle atrophy and that 8 of 10 biopsies from atrophic muscles showed neurogenic changes. In 1982 Barbieri et al. found fibrillation in 17% of 131 patients with generalized MG. Since the 1980s few reports have been published on neurogenic muscular atrophy in MG; perhaps because advances in treatment for MG have decreased the development of atrophy. However, several recent articles have reported muscle atrophy in seronegative MG patients with anti-MuSK antibody. Bulbar palsy is one of the clinical characteristics of seronegative MG patients and some of the cases include muscle atrophy of the face and the tongue. Several hypotheses have been advanced, including (1) refractoriness to conventional therapy that causes disuse atrophy of muscles over a long clinical course, (2) the action of anti-MuSK antibodies themselves, and (3) the effects of corticosteroids on muscle; however, the pathogenetic mechanism remains unclear. Regarding the present case, other mechanisms may have caused muscle atrophy because of the absence of anti-MuSK antibodies and the different clinical features compared to MG patients with anti-MuSK antibody.

The mechanism underlying neurogenic muscular atrophy in seropositive MG is also unclear. Brownell et al. found that the terminal intramuscular nerves in atrophied muscles of the tongue in patients with MG showed an excessive degree of ramification and branching. Based on this observa-
tion, it was speculated that functional interruption at the neuromuscular junction might result in denervation atrophy at a later stage, and that findings in terminal nerves might reflect compensation for breakdown of normal neuromuscular interactions. Bickerstaff et al. reported other pathological changes in MG patients, such as terminal expansion of endplates and abnormalities of synaptic membranes, and described one patient with MG who presented with both terminal expansion of endplates and polyphasic MUPs on needle EMG. Our patient also showed abnormalities of synaptic membranes in electron microscopic observation of motor endplates. Bickerstaff et al. did not mention whether the patient showed muscle atrophy, but these pathological findings could also be relevant to muscle atrophy. Finally, Oosterhuis et al. proposed that muscle atrophy in MG might be caused by permanent denervation of a number of muscle fibers at the level of the motor endplate. These reports suggest that a myasthenic neuromuscular transmission deficit might sometimes be the cause of muscle atrophy.

Lymphorrhage has also been associated with muscle atrophy. However, it is unknown whether cellular infiltration causes muscle atrophies or is a secondary, nonspecific response following muscle fiber destruction initially mediated by antibody and complement deposition; therefore, the true relevance of lymphorrhage in muscle atrophy has not been established. Moreover, there have been no reports of lymphorrhage in biopsied muscles from patients with changes on needle EMG that suggested neurogenic changes. We did not detect any lymphocytic infiltration in biopsied muscle specimens in the present case; however, it remains possible that lymphorrhage would be seen if atrophied muscles or a larger number of sections of muscle specimens were examined.

Despite the presence of active denervation potentials, needle EMG failed to detect reinnervation changes and SFEMG indicated normal fiber density in our patient. Although small groups of atrophic fibers and some targetoid fibers were present, histochemical studies did not indicate fiber-type grouping. These findings suggest that the neurogenic muscle atrophy in this case included an active denervation process but a limited ability for reinnervation. The muscle atrophy of the upper limbs improved after IVIg and oral corticosteroid therapy, indicating that functional interruption of the neuromuscular junction mediated by an immunological mechanism can lead to neurogenic muscle atrophy.

To our knowledge, there has only been one report of improvement of muscle atrophy associated with MG; one of 10 myasthenic patients with muscle atrophy showed improvement of tongue atrophy after corticosteroid therapy. Therefore, correct diagnosis of unusual muscle atrophy in MG may be important because application of appropriate therapies is sometimes effective in such instances.

We thank Dr. Toshiro Yoshimura, Dr. Hirokazu Shiraiishi, and Dr. Masakatsu Motomura, of the Graduate School of Biomedical Sciences, Nagasaki University, for carrying out immunohistochemical stains of the motor endplates and measurement of anti-MuSK antibody.

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CASE OF THE MONTH

ABSTRACT: Satoyoshi syndrome is a rare postnatal disorder with muscle spasms, alopecia, and diarrhea of unknown etiology. Nutritional deficiency seems to influence lifespan. We present a patient with this syndrome having a unique “mesh-like” mucosal change radiographically and white granules endoscopically in the gastrointestinal tract. A common antibody against brain, stomach, and duodenal tissue, according to Western blot analysis, was detected in the sera of two patients with this syndrome. These findings suggest that Satoyoshi syndrome is a systemic autoimmune disease involving the nervous, endocrine, and gastrointestinal systems.

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SATOYOSHI SYNDROME HAS ANTIBODY AGAINST BRAIN AND GASTROINTESTINAL TISSUE

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Satoyoshi syndrome is a rare postnatal disorder of unknown etiology characterized by progressive, painful, and intermittent muscle spasms of the whole body, and by alopecia and diarrhea.13,14 Malabsorption, amenorrhea, and skeletal abnormalities are also frequent.5,8 Satoyoshi and Yamada first reported this disorder in Japan in 1967,14 and it was called generalized “komura–gaeri” disease (“komura,” meaning calf, and “gaeri,” meaning spasm). To our knowledge, 52 cases, including the present case, have been reported worldwide. In most cases, the disease starts in the first or second decade of life. The mean age of onset is 10.6 years (range, 4–36 years). Patients are usually small in stature from nutritional deficiency and have a shorter lifespan attributed to this deficiency. Satoyoshi syndrome is known to occur in association with autoimmune diseases such as myasthenia gravis,12 idiopathic thrombocytopenia,15 or nephritis. The dramatic effect of immunotherapy with glucocorticoids,5,10,11,15 intravenous immunoglobulins,2 and tacrolimus7 on muscle spasms, alopecia, and gastrointestinal symptoms suggests that Satoyoshi syndrome is an autoimmune disease.

Although the gastrointestinal problem is a major determinant of outcome, this aspect has not been the subject of detailed study. We present a patient with Satoyoshi syndrome in whom gastrointestinal imaging studies and duodenal biopsy disclosed abnormalities unique to this syndrome.

CASE REPORT

A 17-year-old Japanese woman was admitted for diagnostic evaluation of painful intermittent muscle spasms, alopecia, diarrhea, and short stature. She was born to healthy, non-consanguineous parents. Family and prenatal histories were unremarkable. At the age of 2 years, she developed atopic dermatitis followed by bronchial asthma a few years later. Her growth and psychomotor development were normal until the age of 8 years, when she was diagnosed with pituitary dwarfism. Although she received growth hormone (GH) supplementation therapy, it was not effective. At the age of 10 years, she noted progressive loss of eyebrows and scalp hair and painful involuntary muscle contractions of the extremities. Within a year she had completely lost her eyebrows and scalp hair. Since the age of 15 years she has had recurrent diarrhea and abdominal spasms. The spasms became increasingly severe and spread throughout her body, which caused her to move slowly to avoid precipitating cramps.

Abbreviations: EMG, electromyography; GH, growth hormone; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Key words: alopecia; antibody; autoimmune disease; diarrhea; muscle cramp; Satoyoshi syndrome
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Physical examination revealed a girl of short stature with a height of 128 cm and weight of 24 kg (weight appropriate for height). Other characteristics were as follows: body mass index, 14.6; blood pressure, 95/58 mm Hg; pulse, regular at 72 beats per minute; and body temperature, 36.2°C. Her mental status was normal. Muscle spasms were not accompanied by myokymia and fasciculations. We did not find any other neurological abnormality. She had not begun to have menstrual periods or to develop secondary sexual characteristics. Bowel sounds were slightly hyperactive. She had clubbed fingers. Urination occurred five or six times daily, and bowel movement occurred four to seven times daily.

Pertinent laboratory findings were as follows: hemoglobin, 8.5 g/dl; platelet count, 400,000/µl; total protein, 5.2 g/dl (normal, 6.7–8.3); total cholesterol, 98 (128–219) mg/dl; triglyceride, 79 (30–149) mg/dl; potassium, 3.0 (3.6–4.9) mEq/L; Na and Cl, normal; iron, 10 (40–158) µg/dl; ferritin, 1 (1.0–124.7) ng/ml; copper, 72 (80–130) µg/dl; calcium, 5.7 (8.7–10.3) mg/dl; magnesium, 0.6 (1.7–2.6) mg/dl; zinc, 39 (39–135) mg/dl; fasting blood sugar, 88 mg/dl; vitamin A, 366 (431–1041) ng/ml; vitamin E, 0.43 (0.75–1.41) mg/dl; 1α-25-vitamin D, 85 (20–60) pg/ml; and creatine kinase, 454 (45–163) U/L. Serum free T3 and T4, thyroid-stimulating hormone, follicle-stimulating hormone, luteinizing hormone, estradiol, cortisol, immunoglobulin (Ig)M, IgG, IgA, total complement activity, and C3 and C4 were normal. Serum IgE was elevated at 2387 (3–304) U/ml. Anti-RNP, anti-Sm, anti–SS-A, anti–SS-B, and rheumatoid factor were negative. Acetylcholine receptor antibody was negative. D-Xylose absorption test was 1.9 g (4.1–8.2 g) per 5 hours. Serum lactate was not elevated. All abnormal findings were thought to be caused by malabsorption due to severe diarrhea. Nerve conduction studies of median, ulnar, tibial, peroneal, and sural nerve were normal. Needle electromyography (EMG) of anterior tibial, biceps brachii, first dorsal interossei, rectus femoris, and paraspinal (10th thoracic level) muscles showed no abnormality.

**Radiographic Study of Intestine.** A double-contrast barium study of the duodenum revealed mild luminal dilation and decrease of Kerckring’s folds. Fine granular changes were observed in the upper part of the second portion of the duodenum and relatively large, granular, “mesh-like” mucosal changes were present in the lower part of the second and third portions. Unclear contour and flocculation of barium, suggesting malabsorption syndrome, were also observed. These radiographic changes were attributed to chronic inflammatory damage (Fig. 1A, B).

**Endoscopic Study of Upper Gastrointestinal Tract.** Almost the entire mucosa of the stomach was atrophic, and multiple ulcer scars were observed, mainly in the body. Small white granules, speculated to be a type of secretion, were observed from the first to the second portion of the duodenum (Fig. 1C). Histopathology of the first portion of the duodenum showed mucosal infiltration with inflammatory cells (Fig. 1D). *Helicobacter pylori* was not present.

**Western Blot.** To determine whether antibody against tissues other than brain were present in the patient’s serum, we purchased human brain, spinal cord, stomach, duodenum, and uterine tissue lysates (Clontech, Mountain View, California) and performed Western blot analysis. Lysates were analyzed on 10% polyacrylamide gels by sodium dodecyl-sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically to nitrocellulose membranes at 150 mA for 1 h. The membranes were incubated with the patient’s serum and 10 control sera (normal controls and patients with cerebrovascular dementia, essential tremor, hemifacial spasm, diabetic neuropathy, and headache), followed by sheep anti-human IgG coupled with horseradish peroxidase (Amersham Pharmacia Biotech, Piscataway, New Jersey). Peroxidase activity was visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

After incubation with control sera, no band was detected for human brain, spinal cord, stomach, duodenum, or uterine tissue lysates. In contrast, incubation with the patient’s serum showed a band at the 90-kDa position for brain, stomach, and duodenum tissue lysates (Fig. 2) but not for spinal cord and uterine tissue lysates. We also examined the serum of another patient with typical Satoyoshi syndrome at our hospital (a 36-year-old woman with generalized painful muscle cramps, primary amenorrhea, alopecia, and diarrhea). Serum from this patient also reacted against a 90-kDa protein in the same tissue lysates as in the first patient (Fig. 2).

**DISCUSSION**

Our study revealed abnormalities of the gastrointestinal tract that are not found in other neurological diseases with muscle spasm such as stiff-person syndrome or Issacs’ syndrome.1 The double-contrast barium study revealed a decrease of the circular...
folds, mild luminal dilation, and relatively large granular, “mesh-like” mucosal changes, which have not previously been reported in Satoyoshi syndrome or in other inflammatory bowel diseases. Endoscopic study also revealed disseminated white granules in the duodenum and diffuse mucosal atrophy as well.

FIGURE 1. Radiographic and endoscopic findings. (A, B) Fine granular change in the upper part of the second portion of the duodenum and relatively large, granular, mesh-like mucosal change (arrow) in the lower part of the second portion. (C) Small white granules were observed at the duodenal bulb. (D) Histopathological findings of the first portion of the duodenum show invasion of inflammatory cells in the mucosal layer.

FIGURE 2. Western blot. After incubation with patient’s sera, a band was detected at the 90-kDa position for human brain, human stomach, and human duodenum lysates. The band was not detected for human uterus and human spinal cord lysates. No band was detected after incubation with normal control sera (St, stomach lysate; U, uterine tissue lysate; D, duodenal lysate; S, spinal cord lysate; B, brain lysate).
as multiple ulcer scars in the stomach, as was also described in the original report\(^9\) and is thus an important characteristic of Satoyoshi syndrome.

Several laboratory findings were abnormal in our case secondary to malabsorption. However, obvious gastrointestinal symptoms are found in only half of the patients with Satoyoshi syndrome and often appear some years after the onset of muscle spasms. Endo et al. reported that a 13-year-old girl with Satoyoshi syndrome had no gastrointestinal symptoms but had an asymptomatic gastric ulcer.\(^7\) We therefore recommend that a thorough investigation of the gastrointestinal system be performed in suspected cases of Satoyoshi syndrome, even in those without relevant symptoms.

Antinuclear antibody,\(^2,15\) acetylcholine receptor antibodies,\(^7\) antiglutamic acid decarboxylase antibodies,\(^4\) and antibodies to brain\(^6\) have already been reported in this disease, leading to speculation of its autoimmune nature. However, the specific antibody that accounts for the pathogenesis of this syndrome has not been reported. In 2001, Endo et al.\(^6\) reported the presence of autoantibody against normal brain tissue in a patient with Satoyoshi syndrome. Our Western blot detected an antibody at the 90-kDa position against not only human brain but also stomach and duodenum in the sera of two patients with Satoyoshi syndrome, suggesting that the unique appearance of stomach and duodenum are due to a common autoimmune pathogenesis.

Despite the amenorrhea often seen in Satoyoshi syndrome, the same antibody was not detected against the uterus. We suspect that amenorrhea may be secondary to nutritional deficiency.

Our findings, which include an absence of family history, the coexistence of various autoimmune disorders, and a dramatic efficacy of immunotherapy for all symptoms, suggest that Satoyoshi syndrome is a systemic autoimmune disease.

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LETTER TO THE EDITOR

COMPARISON OF CLINICAL METHODS FOR FASCICULATION DETECTION IN AMYOTROPHIC LATERAL SCLEROSIS

Fasciculations can be an important diagnostic clue in amyotrophic lateral sclerosis (ALS). Although surface electromyography (sEMG) detects more fasciculations than needle EMG and the clinical examination,² the optimal technique for clinical detection is unknown. We compared four clinical methods of identifying fasciculations, with simultaneous sEMG recordings, in ALS patients.

Twenty-six patients (6 women, 20 men), aged 49–86 years (mean 65), were prospectively recruited and consented. Twenty-five patients fulfilled the El Escorial criteria for probable or definite ALS, with one meeting the criteria for possible ALS.¹ Our institutional review board approved this study.

sEMG testing was performed on the arm with the greater clinical or needle EMG abnormality or selected at random when there was no apparent difference between the two sides. Two 5-mm tin-disc surface electrodes were taped on the skin in the middle third of the forearm with a 10-cm interelectrode distance, midway between the palpable medial and lateral borders of the extensor and flexor muscles. Data were recorded on a Viking 4-channel EMG machine (Nicolet, Madison, Wisconsin) with the sensitivity set to 100 or 200 µV/division based on the size of the fasciculations. Sweep speed was 1 s/division. All waveforms were recorded on paper with the audio amplifier turned off.

A fasciculation was identified as a single spike discharge clearly distinguished from the baseline and distinct from motor unit potentials (MUPs) with sEMG fasciculation count reproducibility confirmed independently by the authors. When one MUP was firing, fasciculations were still easily distinguished and counted. When the density of voluntary MUPs precluded reliable identification of fasciculation potentials for periods of 30 s or less, that part of the tracing was disregarded and the sEMG fasciculation count for the remaining period of time was extrapolated to the full 3 min.

While simultaneously recording sEMG, fasciculations were counted, in 3-min periods, by four methods in sequence: (1) observation of the extensor muscles of the forearm; (2) observation of the flexor muscles of the repositioned forearm; (3) observation of finger twitches of any type; and (4) simultaneous palpation of the flexor and extensor muscles of the forearm by wrapping both hands fully around the forearm with the thumbs on the flexor muscles and fingers on the extensor muscles.

A fasciculation was counted for each single, isolated twitch observed or palpated. No method of provoking fasciculations was employed. Two subjects were excluded when voluntary MUPs could not be eliminated.

None of the clinical methods was superior at detecting the proportion of fasciculations recorded relative to sEMG [analysis of variance (ANOVA) P < 0.320, F = 2.699]. Observation of forearm extensors detected 28.2% of sEMG fasciculations; forearm flexors, 37.5%; finger twitches, 19.6%; and simultaneous palpation of flexor and extensor forearm muscles, 34.8%. Palpation, on average, recorded the most fasciculations of the four clinical methods (P = 0.001).

No clinical method detected fasciculations in every patient, (considered to be at least five within 30 s). Although all patients demonstrated fasciculations on sEMG, seven patients had not recognized their fasciculations in themselves. Fasciculations were detected by palpation in 95% of the patients, by observing finger twitches in 68%, by observing forearm extensors muscles in 64%, and by observing finger flexor muscles in 55%. All methods detected the fasciculations in 11 patients, and in only one patient were the fasciculations not detected by any clinical method. Chi-square analysis showed palpation to be the superior clinical method (P = 0.01).

This study demonstrates the low sensitivity of clinical detection of fasciculations, whereas sEMG
detected fasciculations in all ALS patients. Previous studies in normal subjects have shown no correlation between handedness or age and fasciculation frequency. A particular limitation in this study was patients with spasticity and inability to relax their muscles, making them particularly prone to ongoing MUPs, so that the distinction between a fasciculation and a volitional MUP was difficult.

We conclude that in patients with suspected ALS, when fasciculations are not identified on routine clinical examination, muscle palpation, a sensitive method for fasciculation detection, should be performed. Rare patients, in whom fasciculations remain undetected, should be considered for sEMG to assist in the diagnosis of ALS.

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