Relationship between markers of disease activity and progression in skeletal muscle of GNE myopathy patients using quantitative nuclear magnetic resonance imaging and $^{31}$P nuclear magnetic resonance spectroscopy

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Background: Quantitative nuclear magnetic resonance imaging (NMRI) is an objective and precise outcome measure for evaluating disease progression in neuromuscular disorders. We aimed to investigate predictive ‘disease activity’ NMRI indices, including water T$_2$ and $^{31}$P NMRS (NMRS), and its relation to NMRI markers of ‘disease progression’, such as the changes in fat fraction ($\Delta$Fat%) and contractile cross-sectional area ($\Delta$cCSA), in GNE myopathy (GNEM) patients.

Methods: NMRI was performed on a 3T clinical scanner, at baseline and at a 1-year interval, in 10 GNEM patients and 29 age-matched controls. Dixon-based fat-water imaging and water T$_2$ mapping were acquired in legs and thighs, and in the dominant forearm. $^{31}$P NMRS was performed at the level of quadriceps and hamstring. Water T$_2$ and $^{31}$P NMRS indices were determined for all muscle groups and visits. Correlations were performed with ‘disease progression’ indices $\Delta$Fat%, $\Delta$cCSA and the muscle fat transformation rate ($R_{\text{muscle transf}}$).

Results: In quadriceps, known to be relatively preserved in GNEM, water T$_2$ at baseline was significantly higher compared to controls, and correlated strongly with the one-year evolution of Fat% and cCSA and $R_{\text{muscle transf}}$. Various $^{31}$P NMRS indices showed significant differences in quadriceps and hamstring compared to controls and correlations existed between these indices and Fat%, cCSA and $R_{\text{muscle transf}}$.

Conclusions: This study demonstrates that disease activity indices such as water T$_2$ and $^{31}$P NMRS may predict disease progression in skeletal muscles of GNEM patients, and suggests that these measures may be considered to be valuable surrogate endpoints in the assessment of GNEM disease progression.

Keywords: GNE myopathy (GNEM); quantitative nuclear magnetic resonance imaging (quantitative NMRI); $^{31}$P nuclear magnetic resonance spectroscopy ($^{31}$P NMRS); water T$_2$; skeletal muscle

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**Introduction**

GNE myopathy (GNEM) is a rare, autosomal recessive, progressive neuromuscular disorder, caused by mutations in the UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase gene (abbreviated as GNE), which is a bifunctional enzyme of the sialic acid biosynthetic pathway (1). Sialic acids play an important role in maintaining membrane stability (2), and a decrease in sialic acid production will result in a reduced sialylation, which is the incorporation of sialic acid in glycoproteins and glycolipids, found in large numbers in cellular membranes (3). Reduced levels of sialic acid were observed in a mouse model expressing mutated GNE (4) that develop a pathology resembling a rimmed vacuolar myopathy (4) and named after the autophagic byproducts called ‘rimmed vacuoles’ (5). The exact mechanisms leading to these aggregates are still unknown. Although some degree of inflammation is a common feature in (sporadic) inclusion body myositis (3), it is usually not observed in GNEM. GNEM is characterized by an onset typically in the third decade of life, with initial distal lower limb muscle atrophy as a clinical presentation and a gradual spreading to more proximal muscles and upper limb (5).

Qualitative T₂-weighted nuclear magnetic resonance imaging (NMRI) revealed fatty infiltrations in all leg (6), thigh (6,7), gluteus (6,8) and forearm (9) muscles. The disorder is known for the relative preservation of the quadriceps (QUAD), yet affected by fatty replacement in the later stages of the disease (3). Currently, quantitative NMRI is being used more and more in the evaluation of the disease of neuromuscular disorders (10) such as Duchenne muscular dystrophy (DMD) (11-13), Becker muscular dystrophy (BMD) (14), facioscapulohumeral muscular dystrophy (FSHD) (15), limb-girdle muscular dystrophy type 2I (16), late-onset Pompe disease (17) and inclusion body myositis (18,19). A study in healthy volunteers has also illustrated changes in water T₂ values with ageing (30). Quantitative muscle water T₂ evaluations allow to mutually compare patients and/or to assess the changes within patients in longitudinal studies.

The purpose of the current work was to assess changes of water T₂ and ⁴⁰P NMR spectroscopy (NMRS) indices in a one-year longitudinal study of GNEM patients. We investigated the predictive value of these ‘disease activity’ indices, by determining their relationship with parameters reflecting the disease progression (i.e., change in Fat% and cCSA), and, hence, to assess whether these indices prove to constitute surrogate endpoints in the longitudinal evaluation of GNEM.

**Methods**

**Study population**

The NMR protocol was performed at baseline and at a 1-year interval in 10 GNEM patients (mean age, 47±15 years; range, 25–73 years; 5 male, 3 non-ambulant), as previously described (19), and 29 age-matched healthy control subjects (mean age, 48±14 years; range, 18–70 years; 17 male). The patients were included from the French cohort of the Ultragenyx GNE-Myopathy Disease
Monitoring Program (GNEM-DMP UX001-CL401), participating in the ClinBio GNE study. GNEM diagnosis was genetically confirmed. Healthy control subjects were scanned as part of a methodology NMRI/S protocol approved by the local ethics committee (CPP-Ile de France VI–Groupe Hospitalier Pitié-Salpêtrière, ID RCB: 2012-A01689-34) and informed consent was obtained from all controls and patients.

NMR acquisitions

NMR data were acquired on a 3-T clinical system (Trio until December 2014 or PrismaFit from January 2015, Siemens Healthineers, Erlangen, Germany). For quantitative NMRI, the system’s body coil for radiofrequency transmission was used with two 18-channel phase array surface coils (Siemens) covering both legs and thighs or a 4-phase array surface coil (Siemens) covering the dominant forearm, combined with a 32-channel spine coil (Siemens) for signal collection. Additionally, for $^{31}$P NMRS experiments, a dual-tuned $^{31}$P/$^1$H transmit/receive surface coil (RAPID Biomedical GmbH, Rimpar, Germany) was placed over the thigh (centered at mid-femur), once on the anterior side, once on the posterior side, interrogating the QUAD and the hamstring (HSTR) muscles, respectively. We have described subject position in detail in a recently published work (19).

The NMR protocol began with a whole-body (3-point) Dixon acquisition (i.e., multiple 3D gradient echo sequences for quantitative water-fat imaging across the entire body), employing a methodology described in an earlier publication by some of the co-authors of this work (31). The objective of the whole-body Dixon measurement was to determine which of the segments demonstrated an overall Fat% ≥60%, which was determined as the upper limit for performing further quantitative NMRI and $^{31}$P NMRS.

Indeed, the actual quantitative NMRI protocol in this study comprised the acquisition of 3-point Dixon and water $T_1$ mapping NMRI sequences at the level of the legs, the thighs and the dominant forearm, separately. Details of the 3-point Dixon NMRI sequence were described in a separate publication in GNEM (19). For water $T_1$ mapping, a multi-slice multi-echo (MSME) sequence was employed, covering 3 slices in the forearm and 9 slices in legs and thighs (10 mm slice thickness) and the following acquisition parameters: a train of 17 equidistant echoes (8.7 to 147.9 ms for the forearm; 9.5 to 161.5 ms for the legs/thighs); TR = 4,000 ms (forearm) or 3,000 ms (legs/thighs); TR parameters: a train of 17 equidistant echoes (8.7 to 147.9 ms)

Using the Dixon images, Fat% values (expressed in percentage) were computed as the ratio between the fat signal and the sum of the water and fat signals. The Fat% value per muscle group was determined as a weighted

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average across five central slices (corresponding to the five central slices acquired with MSME, see further on in the text). The values for CSA were calculated in the same muscle groups on the same slices on the co-registered MSME images. The CSA value per muscle group was a mean value across these slices. The contractile CSA (cCSA), defined as the lean muscle CSA corresponding to the muscle volume fraction containing the contractile apparatus, was determined as follows:

\[
cCSA = CSA \times \left[1 - (0.01 \times \text{Fat\%})\right]
\]

Indices of disease progression were the Fat\% change after 1 year (ΔFat\%, in %), the muscle transformation rate \((R_{\text{muscle trans}}, \text{ in year}^{-1})\), the (absolute) change in cCSA (ΔcCSA, in mm\(^2\)), and the relative change in cCSA (ΔcCSA\(_{\text{rel}}, \text{ in } \%\)); and were calculated as follows (with baseline and year-1 abbreviated as BL and Y1):

\[Δ\text{Fat}\% = \text{Fat\%}_{Y1} - \text{Fat\%}_{BL}\]

\[R_{\text{muscle trans}} = \frac{Δ\text{Fat}\%}{100 - \text{Fat\%}_{BL}}\]

\[ΔcCSA = cCSA_{Y1} - cCSA_{BL}\]

\[ΔcCSA_{\text{rel}} = \frac{cCSA_{Y1} - cCSA_{BL}}{cCSA_{BL}} \times 100\]

For assessment of water T\(_2\), ROIs delineated the interior of the muscle avoiding fasciae and blood vessels (33). ROIs for water T\(_2\) were drawn FIB, in the individual muscles of EXT\_LEG, TRIC, QUAD and HSTR, as well as in the muscle groups EXT\_FOREARM and FLEX (because of the difficulty to distinguish the different muscles in these groups). The MSME images were processed based on a tri-exponential fitting procedure (33), which is a model that takes into account both water and fat components in the muscle tissue. Water T\(_2\) values were determined as a mean value within the drawn muscle ROIs, averaged across the five centrally acquired slices (Figure 1). The acquisition of a T\(_2\) map enabled voxel sorting by eliminating those voxels having a T\(_2\) value outside the prescribed boundaries (i.e., lower than 80% or higher than 120% of the nominal flip angle). The threshold for abnormal water T\(_2\) was set at two standard deviations above mean muscle water T\(_2\) in healthy volunteers, which is approximately 39 ms on the system used in this work (33). Water T\(_2\) values of individual muscles were pooled, weighted by the size of the ROI, to compare with all other measured NMR indices which were all determined on a muscle group level.

\(^{31}\text{P} \text{ NMRS data were processed as previously described (11), using AMARES (34) in jMRUI (35) for the deconvolution of the two inorganic phosphate (P) resonances, and Topspin (Bruker Medical GMbG, Ettlingen, Germany) for the determination of PME (phosphomonoesters) and PDE (phosphodiester). The following resonances were quantified: PME, P\(_{\text{i,b}}\) (alkaline inorganic phosphate), P\(_{\text{i,a}}\) (cytosolic inorganic phosphate), P\(_{\text{i,tot}}\) = P\(_{\text{i,b}}\) + P\(_{\text{i,a}}\) (total inorganic phosphate), PDE, PCR (phosphocreatine), γATP, αATP, NAD(H) (nicotinamide adenine dinucleotide) and βATP (adenosine triphosphate) (11). Prior knowledge was imposed on signal amplitude, line width, phases and line shape (11). All metabolites were expressed as metabolite ratios, corrected for partial saturation. The (weighted) pH\(_L\) value was calculated based on the chemical shift difference between PCr and the P\(_r\) resonances (36). The intramuscular Mg\(^{2+}\) concentration was calculated based on the chemical shift difference between αATP and βATP (37,38). In Figure 1, two examples of \(^{31}\text{P} \text{ NMR spectra are given for patients with a different disease progression.}

### Statistical analysis

Statistical analyses were conducted using SPSS software version 22 (SPSS, Chicago, IL, USA). Given the small number of patients, non-parametric tests were performed, including the Mann-Whitney test for comparing control and patient values and the Wilcoxon test for comparing sides and 1-year differences. Ambulant and non-ambulant patients were pooled for all analyses. The Spearman-rank correlation test was used for investigating the relationship between variables reflecting ‘disease activity’ (water T\(_2\) and \(^{31}\text{P} \text{ NMRs}) and the earlier assessed indices of disease progression (19). Water T\(_2\) values and \(^{31}\text{P} \text{ NMRs} indices used for correlation analyses are presented as the average value between the baseline and year-1 visit. The level of statistical significance was corrected for multiple comparisons and set at P<0.007 and at P<0.02, for NMRI and \(^{31}\text{P} \text{ NMRs parameters, respectively.}

The standardized response mean (SRM) is defined as the mean change over 1 year divided by the standard deviation of this change (39). An SRM ≥0.8 is considered to reflect a high responsiveness to change (39). For variables that are known to be less integrative but are, nevertheless, significantly different between the pathological and normal state, the standard SRM calculation is less pertinent. Therefore, we introduced an additional variable for the disease activity indices, which we further will call the ‘standardized difference mean’ (SDM). The SDM is defined...
as the sum of the mean change over 1 year and the mean difference between the patient and control group, divided by the pooled standard deviation of the 1-year change. Similar as for the SRM, an SDM \( \geq 0.8 \) is considered to reflect a strong measurable difference from normality.

**Results**

**Data overview**

Quantitative NMRI data were obtained in the dominant forearm and the thigh of all ten GNEM patients. In four of the ten patients, no quantitative NMRI data were obtained in the leg due to overall Fat% values that were higher than 60%, as assessed by the whole-body Dixon measurement at the beginning of the NMR protocol. Three of these four patients were non-ambulant.

\( ^{31} \)P NMRS measurements were performed at the level of the QUAD in all ten GNEM patients. In four of the ten patients (including the three non-ambulant subjects), \( ^{31} \)P NMRS was not obtained in the HSTR, due to very high Fat% values in HSTR muscles. A fourth patient felt discomfort and the NMR protocol was ended before the \( ^{31} \)P NMRS measurement in the HSTR.

‘Disease progression’ NMR indices in patients

As can be observed from Table 1, changes in Fat% were significant for the QUAD. No other significant changes

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**Figure 1** MSME-based baseline water T\(_2\) maps (A,E), Dixon-based baseline (B,F), year-1 Fat% maps (C,G) and baseline QUAD \( ^{31} \)P NMR spectra (D,H) of two GNE myopathy patients. Values for water T\(_2\), Fat%\(_{BL}\), Fat%\(_{Y1}\) and \( \Delta \)Fat% in QUAD are given. In the first patient, water T\(_2\) was normal (A), Fat%\(_{BL}\) was relatively low (B), \( \Delta \)Fat% was the lowest of all patients (B,C), and PCr/\( \gamma \)ATP and pH\(_w\) values were normal (D). In the second patient, water T\(_2\) was abnormal (E), Fat%\(_{BL}\) was intermediate (F), \( \Delta \)Fat% was the highest of all patients (F,G), and PCr/\( \gamma \)ATP and pH\(_w\) values were significantly decreased and increased (H), respectively, as compared to controls. Notice that HSTR muscles shows much higher fatty depositions in the first and much less in the second patient. ATP, adenosine triphosphate; Fat%\(_{BL}\), baseline fat fraction; Fat%\(_{Y1}\), year-1 fat fraction; MSME, multi-slice multi-echo; NAD(H), nicotinamide adenine dinucleotide; PCr, phosphocreatine; PDE, phosphodiesters; P\(_{i,a}\), cytosolic inorganic phosphate; P\(_{i,b}\), alkaline inorganic phosphate; PME, phosphomonoesters; T\(_{2w,av}\), average water T\(_2\); value; \( \Delta \)Fat%, fat fraction difference between baseline and year-1 visit.
were observed.

**Changes in ‘disease activity’ NMR indices between patients and controls**

No significant age differences were found between patients and controls (P=0.292). There were no differences between left and right sides for water $T_2$ values (P>0.05 for all muscles).

Compared to controls, significantly higher water $T_2$ values (**Figure 2**) were only found in QUAD and HSTR muscles of GNEM patients, as depicted in **Figure 2**. SDM values for water $T_2$ were high for QUAD, FIB and forearm muscles. No significant differences between muscles were observed for the average water $T_2$ values in both patients and controls (P>0.05).

Additionally, anomalies in various $^{31}$P NMRS indices were observed in QUAD and HSTR as compared to controls (**Figure 3**). Corresponding SDM values for all depicted $^{31}$P NMRS indices were all higher than 0.8.

Significant differences between QUAD and HSTR were only found for pH in the healthy controls (7.08±0.03 vs. 7.05±0.02, P=0.003) and for PCr/γATP (5.56±0.39 vs. 4.89±0.33, P=0.016) in GNEM patients.

**Changes in ‘disease activity’ NMR indices in patients after 1 year**

Water $T_2$ did not change after one year of follow-up. The SRM value in FLEX_FOREARM reached 0.8 but water $T_2$ changes were non-significant (37.3±2.6 to 39.5±2.0 ms, P=0.022).

A few $^{31}$P NMRS indices changed significantly over the course of one year, such as increases in pH$_w$ (7.08±0.03 vs. 7.10±0.03, P=0.012, SRM =1.6) and PME/γATP (0.27±0.04 vs. 0.33±0.07, P=0.014, SRM =1.0) in QUAD. SRM reached 0.8 in HSTR for PME/γATP but changes were, however, non-significant after 1 year.

**Correlations between ‘disease activity’ and ‘disease progression’ NMR indices**

All correlation coefficients and corresponding P-values can be found in **Table 2**.

In QUAD, ΔFat% correlated significantly with water $T_2$ (**Figure 4**). This significance was even more pronounced when correlating water $T_2$ with the R$_{\text{muscle,transf}}$ index, as seen in **Figure 4B**. Water $T_2$ values in QUAD seemed to be correlated (albeit non-significant) with the relative ΔcCSA.

### Table 1: Quantitative NMRI indices of disease progression ΔFat% and ΔcCSA (expressed as a mean and standard deviation)

<table>
<thead>
<tr>
<th>Muscle group</th>
<th>ΔFat% (%)</th>
<th>ΔcCSA (cm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FIB</td>
<td>2.8±3.4</td>
<td>−0.3±0.3</td>
</tr>
<tr>
<td>EXT</td>
<td>4.9±3.5</td>
<td>−0.5±0.4</td>
</tr>
<tr>
<td>TRIC</td>
<td>1.5±3.7</td>
<td>−1.3±1.2</td>
</tr>
<tr>
<td>Thigh</td>
<td></td>
<td></td>
</tr>
<tr>
<td>QUAD</td>
<td>3.1±2.1*</td>
<td>−2.4±2.8</td>
</tr>
<tr>
<td>HSTR</td>
<td>0.2±2.4</td>
<td>−1.0±1.1</td>
</tr>
<tr>
<td>Forearm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EXT</td>
<td>0.5±3.0</td>
<td>−0.4±0.5</td>
</tr>
<tr>
<td>FLEX</td>
<td>0.5±2.7</td>
<td>−0.5±0.7</td>
</tr>
</tbody>
</table>

ΔFat%, Fat% change between baseline and year-1 (in %); ΔcCSA, absolute change in contractile cross-sectional area change between baseline and year-1 (in mm$^2$); EXT, extensor; FIB, fibularis; FLEX, flexor; HSTR, hamstring; QUAD, quadriceps; TRIC, triceps surae. Significance level: *, P<0.007.

**Figure 2** Box-and-whisker plots of water $T_2$ at baseline. The horizontal line represents an empirically determined threshold water $T_2$ value at 39 ms (i.e., the mean value plus twice the standard deviation of water $T_2$ in normal muscle) (31). SRM and SDM values ≥0.8 are indicated in bold. EXT, extensor; FIB, fibularis; FLEX, flexor; HSTR, hamstring; QUAD, quadriceps; SDM, standardized difference mean; SRM, standardized response mean; TRIC, triceps surae. Significance level: *P<0.007.
Figure 3 Box-and-whisker plots of pH_w (A), [Mg^{2+}] (B), PDE/γATP (C) and PME/γATP (D) at baseline in QUAD and HSTR. SRM and SDM values ≥0.8 are indicated in bold. ATP, adenosine triphosphate; HSTR, hamstring; [Mg^{2+}], intramuscular magnesium concentration (in mM); pH_w, weighted pH; PDE, phosphomonoesters; PME, phosphomonoesters; QUAD, quadriceps; SDM, standardized difference mean; SRM, standardized response mean; significance level: *P<0.02.

values, as illustrated in Figure 4D. Additionally, significant correlations were found between PCr/γATP and ΔcCSA and ΔcCSA_rel in QUAD (Figure 5).

Discussion

This study demonstrates that water \( T_2 \) and \(^{31}\)P NMRS have a predictive value on the progression of disease in skeletal muscles as established by fat-water separation-based quantitative NMRI in GNEM patients.

‘Disease progression’: ΔFat% and ΔcCSA

The extent of muscle destruction in GNEM, as reflected by the increased fatty infiltration and decreased lean muscle tissue as compared to controls, was especially evident in the thigh (QUAD and HSTR) and the extensor compartment of the leg, with a relative sparing of the QUAD muscle group as compared to the other muscles (19), confirming observations from earlier reports (3). Disease progression in GNEM, illustrated by both an increase in fatty depositions (ΔFat%) and a decrease in lean muscle tissue (ΔcCSA), was also seen in these three muscle groups (although only significant changes were seen for Fat% in QUAD). In general, all leg muscles in GNEM patients were heavily fatty infiltrated, confirming the distal-to-proximal disease evolution known in GNEM (40). Disease progression indices were handled in detail in a separate publication (19).
Figure 4 All ‘disease progression’ NMRI indices as a function of water $T_2$ in QUAD. Every point represents 1 patient. The water $T_2$ value represents the average value of baseline and year 1 visits (the analysis with baseline water $T_2$ values resulted in the same outcome for $\rho$ and $P$ values). See Table 2 for Spearman correlation coefficients ($\rho$) and corresponding $P$ values. $\Delta$Fat%, fat fraction change between baseline and year-1 (in %); $\Delta$cCSA, absolute change in contractile cross-sectional area change between baseline and year-1 (in mm$^2$); $\Delta$cCSA$_{rel}$, relative change in contractile cross-sectional area change between baseline and year-1 (in %); $R_{\text{muscle\_transf}}$, muscle transformation rate (year$^{-1}$). Significance level: *$P<0.007$.

‘Disease activity’: water $T_2$

An increase in water $T_2$ might be related to different phenomena such as inflammation, myocytic lesions, cell necrosis or edema, and can therefore be regarded as a non-specific NMR biomarker reflecting ‘disease activity’ (10). Whereas the abovementioned variables, Fat% and cCSA, are more integrators of the global disease progression over time, water $T_2$ reflects a more instantaneous phenomenon.

The significantly increased water $T_2$, as found in this study in 25% of all QUAD and HSTR muscles, might be perceived as surprising, as it is known that inflammation is generally absent in GNEM (3). A similar apparent paradox has been observed in late-onset Pompe disease patients (17) where the increased water $T_2$ could not be associated to an inflammatory cause, from which it is hypothesized that the abnormal water $T_2$ values rather reflect myocytic lesions, possibly related to the rupture of lysosomes. In the patients assessed in our study, a significantly increased water $T_2$ was only found in the thigh. This could be due to a lack of statistical power since leg data were not acquired in four of the ten patients due to very high Fat% levels (19). EXT_ FOREARM muscles revealed to have an SDM similar as found in thigh, illustrating its potential as a muscle where water $T_2$ might be significantly different between healthy and pathological muscle tissue. GNEM patients did not show any significant change in water $T_2$ over a 1-year time period, which does not rule out fluctuations between these two time points. Altogether, these data indicate that the well-preserved QUAD is the most appropriate muscle group to assess disease evolution through water $T_2$ measurements in GNEM.

‘Disease activity’: $^{31}$P NMRS

Although inter-patient differences in $^{31}$P NMRS indices were apparent, significant differences as compared to controls were found for only a few of these parameters. One of the prominent differences were the elevated levels of PDE, found in QUAD and HSTR, which reflects an...
Table 2 Correlations between predictive ‘disease activity’ and ‘disease progression’ NMR indices (the Spearman-rank correlation coefficient ρ is given with the P value between brackets; n indicates the number of patients)

<table>
<thead>
<tr>
<th>Muscle group</th>
<th>n</th>
<th>ΔFat% (%)</th>
<th>R_{muscle_transf} (year⁻¹)</th>
<th>ΔcCSA (mm²)</th>
<th>ΔcCSA_rel (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water T₂ (ms)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FIB (leg)</td>
<td>6</td>
<td>-0.37 (0.468)</td>
<td>-0.26 (0.623)</td>
<td>0.77 (0.072)</td>
<td>0.26 (0.623)</td>
</tr>
<tr>
<td>EXT (leg)</td>
<td>6</td>
<td>0.54 (0.266)</td>
<td>0.20 (0.0704)</td>
<td>-0.89 (0.019)</td>
<td>-0.66 (0.156)</td>
</tr>
<tr>
<td>TRIC (leg)</td>
<td>6</td>
<td>-0.37 (0.468)</td>
<td>-0.31 (0.544)</td>
<td>0.09 (0.872)</td>
<td>0.37 (0.468)</td>
</tr>
<tr>
<td>QUAD (thigh)</td>
<td>10</td>
<td>0.79 (0.006)</td>
<td>0.81 (0.005)</td>
<td>-0.55 (0.098)</td>
<td>-0.67 (0.033)</td>
</tr>
<tr>
<td>HSTR (thigh)</td>
<td>10</td>
<td>-0.27 (0.446)</td>
<td>-0.19 (0.603)</td>
<td>0.58 (0.200)</td>
<td>-0.03 (0.934)</td>
</tr>
<tr>
<td>EXT (forearm)</td>
<td>10</td>
<td>0.51 (0.130)</td>
<td>0.53 (0.119)</td>
<td>-0.31 (0.379)</td>
<td>-0.44 (0.199)</td>
</tr>
<tr>
<td>FLEX (forearm)</td>
<td>10</td>
<td>0.10 (0.783)</td>
<td>0.10 (0.783)</td>
<td>-0.54 (0.109)</td>
<td>-0.38 (0.285)</td>
</tr>
<tr>
<td>pH_w QUAD</td>
<td>10</td>
<td>0.09 (0.803)</td>
<td>0.10 (0.777)</td>
<td>-0.03 (0.934)</td>
<td>0.01 (0.987)</td>
</tr>
<tr>
<td>HSTR</td>
<td>6</td>
<td>-0.03 (0.957)</td>
<td>0.20 (0.704)</td>
<td>0.09 (0.872)</td>
<td>-0.66 (0.156)</td>
</tr>
<tr>
<td>[Mg²⁺] (mM) QUAD</td>
<td>10</td>
<td>-0.18 (0.627)</td>
<td>-0.22 (0.533)</td>
<td>0.71 (0.022)</td>
<td>0.53 (0.117)</td>
</tr>
<tr>
<td>HSTR</td>
<td>6</td>
<td>-0.03 (0.957)</td>
<td>-0.31 (0.544)</td>
<td>-0.49 (0.329)</td>
<td>0.37 (0.468)</td>
</tr>
<tr>
<td>PDE/γATP QUAD</td>
<td>10</td>
<td>-0.19 (0.603)</td>
<td>-0.25 (0.489)</td>
<td>0.18 (0.627)</td>
<td>0.30 (0.405)</td>
</tr>
<tr>
<td>HSTR</td>
<td>6</td>
<td>-0.37 (0.468)</td>
<td>-0.49 (0.329)</td>
<td>0.54 (0.266)</td>
<td>0.60 (0.208)</td>
</tr>
<tr>
<td>PME/γATP QUAD</td>
<td>10</td>
<td>0.59 (0.074)</td>
<td>0.60 (0.067)</td>
<td>-0.10 (0.777)</td>
<td>-0.24 (0.511)</td>
</tr>
<tr>
<td>HSTR</td>
<td>6</td>
<td>0.31 (0.544)</td>
<td>0.54 (0.266)</td>
<td>0.09 (0.872)</td>
<td>-0.77 (0.072)</td>
</tr>
<tr>
<td>PCR/γATP QUAD</td>
<td>10</td>
<td>-0.58 (0.082)</td>
<td>-0.53 (0.117)</td>
<td>0.73 (0.016)</td>
<td>0.78 (0.008)</td>
</tr>
<tr>
<td>HSTR</td>
<td>6</td>
<td>-0.83 (0.042)</td>
<td>-0.88 (0.019)</td>
<td>0.09 (0.872)</td>
<td>0.60 (0.208)</td>
</tr>
<tr>
<td>P_i,tot/ATP QUAD</td>
<td>10</td>
<td>-0.02 (0.960)</td>
<td>0.07 (0.855)</td>
<td>-0.19 (0.603)</td>
<td>-0.16 (0.651)</td>
</tr>
<tr>
<td>HSTR</td>
<td>6</td>
<td>-0.49 (0.329)</td>
<td>-0.20 (0.704)</td>
<td>0.09 (0.872)</td>
<td>-0.09 (0.872)</td>
</tr>
<tr>
<td>P_i,tot/PCr QUAD</td>
<td>10</td>
<td>0.43 (0.214)</td>
<td>0.50 (0.138)</td>
<td>-0.65 (0.043)</td>
<td>-0.61 (0.060)</td>
</tr>
<tr>
<td>HSTR</td>
<td>6</td>
<td>-0.43 (0.397)</td>
<td>-0.09 (0.872)</td>
<td>-0.26 (0.623)</td>
<td>-0.49 (0.329)</td>
</tr>
<tr>
<td>P_i,b/P_i,tot QUAD</td>
<td>10</td>
<td>0.22 (0.533)</td>
<td>0.21 (0.556)</td>
<td>-0.14 (0.701)</td>
<td>-0.25 (0.489)</td>
</tr>
<tr>
<td>HSTR</td>
<td>6</td>
<td>-0.26 (0.623)</td>
<td>-0.43 (0.397)</td>
<td>-0.09 (0.872)</td>
<td>-0.03 (0.957)</td>
</tr>
</tbody>
</table>

The water T₂ value and the ³¹P NMR indices represent the average value of baseline and year 1 visits (the analysis with baseline water T₂ values resulted in the same outcome for ρ and P values). ΔFat%, Fat% change between baseline and year-1 (in %); ΔcCSA, absolute change in contractile cross-sectional area change between baseline and year-1 (in mm²); ΔcCSA_rel, relative change in contractile cross-sectional area change between baseline and year-1 (in %); ATP, adenosine triphosphate; EXT, extensor; FIB, fibularis; FLEX, flexor; HSTR, hamstring; PDE, phosphodiesters; PCr, phosphocreatine; P_i,b, alkaline inorganic phosphate; P_i,tot, total inorganic phosphate; PME, phosphomonoesters; QUAD, quadriceps; [Mg²⁺], intramuscular magnesium concentration (in mM); pH_w, weighted pH; R_{muscle_transf}, muscle transformation rate (year⁻¹). Significance levels: P<0.007 (NMRI) and P<0.02 (³¹P NMRS).
increased turnover of phospholipids, indicating a metabolic disturbance at the level of the sarcolemmal membrane (41). Both in BMD and DMD, PDE was found to be an early marker during the disease, as changes were observed prior to any other NMR-detectable changes such as Fat% (12,14). When comparing our results to those of the BMD study where the patient age range (i.e., 20–60 years) was similar to the one in this work (i.e., 25–73 years), we can, however, not assess the possibility of PDE as an early-phase biomarker of the disease, since the relatively spared QUAD demonstrated already low to moderate fat replacement (i.e., between 4.3% and 29.7%) (19). Additionally, it is known that PDE correlates positively with age (42,43), making age a confounding factor for further interpretation. Also, an elevated pH value was found in the HSTR and QUAD (at least at year 1), when comparing to the controls. An alkaline pH has shown to be a biomarker for dystrophic muscle, as illustrated in several studies already, with a normal pH in non-fat infiltrated DMD patients (12) compared to an alkaline pH when muscle began to show fatty replacement (11,12,20). An elevated pH reflects an increase in the second more alkaline P pool (P_{2}), which is hypothesized to originate from suffering myocytes or from an expanded interstitial space, as was demonstrated in dystrophic muscle (11,20). Increased PDE, as a marker of membrane disturbance, could result from abnormal or insufficient glycosylation/sialylation of important transmembrane glycoproteins, such as the voltage-gated sodium channels or α-dystroglycan as part of the dystrophin-glycoprotein complex (44). Dysfunction of these proteins could result in a reduced mechanostability of the sarcolemma, causing leaky membranes and a disturbed ionic homeostasis with implications on the value of pH. Further studies are required to investigate the dynamics of pH and PDE before the occurrence of fatty depositions in the muscle of GNEM patients. Finally, a significantly decreased intramuscular Mg^{2+} content, as was observed in both QUAD and HSTR, is also an interesting finding, knowing that Mg^{2+} is an essential and one of the most effective metal ions with respect to the activity of N-acetylmannosamine kinase activity, being...
one of the two GNE-coded enzymes (45,46). Despite the difference in Fat% between QUAD and HSTR (4.3–29.7% and 8.1–86.4%, respectively) (19), only the PCr/γATP ratio was shown to differ significantly between the two muscle groups. This illustrates that the very different extent of fatty infiltration in these muscle groups (at least up until a certain threshold) seemed to have, on average, little effect on the outcome of these parameters, whereas this correlation has been observed earlier in FSHD (47) and DMD (11). Significant changes coupled to high SRM values were only found for pH, PME/γATP and PCr/γATP in QUAD. This doesn’t rule out, nevertheless, the use of Mg2+ and PDE/γATP as biomarkers that are potentially subjective to treatment effects, since they show, just as pH and PME/γATP, a high degree of discriminative power between patients and controls (SDM ≥0.8) in both QUAD and HSTR.

**Relationship between ‘disease activity’ and ‘disease progression’**

We demonstrated that QUAD muscles of GNEM patients with abnormal water T2 experienced a faster disease progression, as seen with most variables reflecting ‘disease progression’. A similar relationship was observed in late-onset Pompe disease patients (17) where an elevated water T2 implied a doubling of the fatty infiltration rate. Along the same line, convincing evidence have been repeatedly produced in FSHD, a disease where bursts of toxic gene expression and inflammation in a particular muscle result in its rapid destruction. Several studies have shown that muscle hyperintensities in STIR imaging or muscle water T2 increases in quantitative maps precede muscle fatty replacement in T1-weighted images or in fat fraction maps (48-50). Correlations between water T2 and disease progression indices were less clear in other lower limb muscle groups. This illustrates that the very different extent of fatty infiltration in these muscle groups (at least up until a certain threshold) seemed to have, on average, little effect on the outcome of these parameters, whereas this correlation has been observed earlier in FSHD (47) and DMD (11). Significant changes coupled to high SRM values were only found for pH, PME/γATP and PCr/γATP in QUAD. This doesn’t rule out, nevertheless, the use of Mg2+ and PDE/γATP as biomarkers that are potentially subjective to treatment effects, since they show, just as pH and PME/γATP, a high degree of discriminative power between patients and controls (SDM ≥0.8) in both QUAD and HSTR.

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**Methodological aspects**

The major methodological issue in this study was the combination of the limited amount of patients, which is due to the very low prevalence of the disease (estimated 1/1,000,000 worldwide) (3) and the heterogeneous phenotype inherent to the disease, emphasizing even more the need for objective outcome measures. Because the
ambulant and non-ambulant cohorts were too small for a paired ambulant versus non-ambulant analysis, data were pooled. Despite the low number of patient data, however, we were able to detect significant changes and correlations in skeletal muscle over a 1-year period using quantitative NMRI and $^{31}$P NMRS. Nevertheless, more data is needed to confirm and verify correlations between ‘disease prediction’ and ‘disease progression’ NMR indices, since above a certain threshold of fatty infiltration (approximately 50%), water $T_2$ assessment becomes unreliable (33) and $^{31}$P NMRS does not reach a sufficient signal-to-noise level due to lack of viable muscle tissue, as was, for example, the case for HSTR in this study.

**Conclusions**

To summarize, this study revealed that, in GNEM, water $T_2$ and $^{31}$P NMRS are complementary NMR indices to the standard Fat% assessment for evaluating muscle changes during the course of the disease. More specifically, the QUAD, which is relatively preserved in GNEM, in patients with higher water $T_2$ values showed a faster disease progression. The results obtained in this study confirm the ability of the use of water $T_2$ and $^{31}$P NMRS as possible surrogate endpoints in longitudinal studies of neuromuscular disorders. Future steps could be the implementation of this quantitative NMR protocol in a larger patient cohort as well as in clinical trials before and after a suitable treatment.

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**Footnote**

**Conflicts of Interest:** All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/qims-20-39). AB reports personal fees from Ultragenyx pharmaceutical, during the conduct of the study; LS reports grants and personal fees from Avexis, grants and personal fees from Biogen, grants and personal fees from Roche, personal fees from Cytokinetics, personal fees from Sarepta, personal fees from Biophytis, personal fees from Pfizer, personal fees from Catabasis, personal fees from Lupin, grants and personal fees from Dynacure, personal fees from Audentes, outside the submitted work; PGC reports personal fees from Santhera, personal fees from Sanofi, personal fees from Sarepta, outside the submitted work. The authors have no other conflicts of interest to declare.

**Ethical Statement:** Healthy control subjects were scanned as part of a methodology NMRI/S protocol approved by the local ethics committee (CPP-Ile de France VI – Groupe Hospitalier Pitié-Salpêtrière, ID RCB: 2012-A01689-34) and informed consent was obtained from all controls and patients.

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