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Dysregulation of GSK3 β -Target Proteins in Skin Fibroblasts of Myotonic Dystrophy Type 1 (DM1) Patients

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Abstract. Myotonic dystrophy type 1 (DM1) is the most common monogenetic muscular disorder of adulthood. This multisystemic disease is caused by CTG repeat expansion in the 3'-untranslated region of the DM1 protein kinase gene called *DMPK*. *DMPK* encodes a myosin kinase expressed in skeletal muscle cells and other cellular populations such as smooth muscle cells, neurons and fibroblasts. The resultant expanded (CUG)_n RNA transcripts sequester RNA binding factors leading to ubiquitous and persistent splicing deregulation. The accumulation of mutant CUG repeats is linked to increased activity of glycogen synthase kinase 3 beta (GSK3 β), a highly conserved and ubiquitous serine/threonine kinase with functions in pathways regulating inflammation, metabolism, oncogenesis, neurogenesis and myogenesis. As GSK3 β -inhibition ameliorates defects in myogenesis, muscle strength and myotonia in a DM1 mouse model, this kinase represents a key player of DM1 pathobiochemistry and constitutes a promising therapeutic target. To better characterise DM1 patients, and monitor treatment responses, we aimed to define a set of robust disease and severity markers linked to GSK3 β by unbiased proteomic profiling utilizing fibroblasts derived from DM1 patients with low (80–150) and high (2600–3600) CTG-repeats. Apart from GSK3 β increase, we identified dysregulation of nine proteins (CAPN1, CTNBN1, CTPS1, DNMT1, HDAC2, HNRNP3, MAP2K2, NR3C1, VDAC2) modulated by GSK3 β . *In silico*-based expression studies confirmed expression in neuronal and skeletal muscle cells and revealed a relatively elevated abundance in fibroblasts. The potential impact of each marker in the myopathology of DM1 is discussed based on respective function to inform potential uses as severity markers or for monitoring GSK3 β inhibitor treatment responses.

Keywords: GSK3 β , fibroblast proteomics, CTPS1, CAPN1, HDAC2, CTNBN1

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INTRODUCTION

Myotonic dystrophy type 1 (DM1; MIM: 160900) is the most common monogenetic muscular disorder of adulthood with an estimated worldwide prevalence of 5/100,000, ranging from 0.5 in Taiwan up to 18.1/100,000 in Croatia [1]. In addition, DM1 is highly prevalent in Canada (Saguenay-Lac-Saint-Jean) where its carrier rate reaches 1/550 [2, 3]. The disease is characterised by progressive muscle atrophy and weakness, often combined with myotonia, fatigue, respiratory insufficiency, and speech and swallowing difficulties [4, 5]. DM1 represents a multisystemic disorder and affects multiple organs including the central nervous system, heart, eye, skin, gastrointestinal and reproductive tracts, as well as the endocrine and immune systems [5]. Traditionally, DM1 is categorized according to phenotype and age of onset. However, this classification into congenital, juvenile, childhood, adult and late onset is complicated by significant intrafamilial and interfamilial variability [5, 6].

DM1 is an autosomal dominant disorder, caused by a CTG repeat expansion [(CTG) n] in the 3'-untranslated region of the DM1 protein kinase gene (*DMPK*). *DMPK* is located at position 19q13.32 and encodes a myosin kinase expressed in skeletal muscle [7]. Expanded alleles display microsatellite instability towards expansion in germline cells (causing genetic anticipation) and somatic cells (contributing to disease progression and phenotype variability [8–11]). The resultant expanded (CUG) n RNA transcripts cause the DM1 phenotype by sequestering RNA binding factors and leading to ubiquitous and persistent splicing deregulation [5, 12–17]. This toxic (CUG) n RNA effect is mediated by gain of function of combined CUGBP1-like family member 1 (CELF1) and loss of function of muscleblind like splicing regulator (MBNL) due to clustering of both proteins [15–17]. Several aberrant splicing events have been linked to muscular [15, 16, 18–21], cardiac [15, 20, 22, 23], neurocognitive [24, 25] and endocrine [15, 16, 26–28] features of DM1.

Glycogen synthase kinase 3 beta (GSK3 β) activity is significantly increased in the skeletal muscles of DM1 patients [29]. This increase in GSK3 β activity is linked to the accumulation of mutant CUG repeats and, by promoting the phosphorylation of cyclin D3 at T283, leading to degradation and resultant in muscle weakness. GSK3 β is a highly conserved and ubiquitous serine/threonine kinase with an important pleiotropic role in pathways regulating inflammation,

metabolism, oncogenesis, neurogenesis and myogenesis [29–34]. Pharmacological inhibition of GSK3 β ameliorates defects in myogenesis, muscle strength and myotonia in DM1 mice (HSA^{LR}) [29] further supporting the emergence of GSK3 β as a novel and promising therapeutic target for DM1 [35]. To better characterise DM1 patients, and monitor treatment responses, it will be necessary to define a set of robust disease and severity markers.

PATIENTS, MATERIAL AND METHODS

In this study, we aimed to identify DM1 protein markers reflecting activation of pathways, such as GSK3 β overactivation, in patient-derived fibroblasts, a suitable cell model to study the molecular etiology of neuromuscular diseases [36]. Proteomic profiling was performed on fibroblasts derived from DM1-patients with a mild/late onset [(CTG) 80–150] and a severe/congenital [(CTG) 2600–3600] DM1 phenotype. Obtained data were filtered for dysregulated proteins that are known to be modulated by GSK3 β or involved in GSK3 β -dependent processes.

Ethical considerations

This study has been ethically approved as a sub-study as part of OPTIMISTIC (reference: NRES Committee North East - Sunderland 13.NE.0342) and PHENODM1 (reference: NRES Committee North East – Tyne & Wear South 15.NE.0178). Patients were consented and recruited at Newcastle upon Tyne NHS Foundation Trust and all procedures leading to these results complied with the Good Clinical Practices and the Declaration of Helsinki. For skin biopsies studies, informed consent was obtained from all patients.

Fibroblasts and cell culture

Fibroblasts were isolated from fresh donor skin biopsies following standardised EuroBioBank protocols (www.eurobiobank.org/biobanking-sops): biopsies were washed with sterile phosphate-buffered saline (PBS) and digested at 37°C for 15 minutes with 2.5% trypsin (Thermo Fisher Scientific) and a further 90 minutes with 0.5% collagenase (Type IV, Sigma-Aldrich). Fibroblasts were proliferated in Ham's F-10-Complete Medium (Thermo Fisher Scientific) supplemented with 20% foetal bovine serum (FBS, SeraLab – Bioreclamation IVT), 2% penicillin-streptomycin (Thermo Fisher Scientific), 1%

Table 1

Clinical findings for the six DM1 patients who provided fibroblasts for the proteomic profiling study. MIRS: Muscular impairment rating scale. Repeat expansions refer to DNA extracted from fibroblasts

Patient/sample	CTG-repeats	Age at disease onset [years]	Age at time of study (skin biopsy)	Main DM symptoms (in addition to muscle weakness)	MIRS
1	84	34	50	Conduction defect, excessive daytime sleepiness	III
2	94	45	55	Excessive day-time sleepiness	II
3	140	49	58	Conduction defect, cataracts	IV
4	2431	9	43	Conduction defect, cataracts, breathing difficulties, excessive day-time sleepiness	IV
5	2683	16	35	Excessive day-time sleepiness, breathing difficulties	III
6	3187	25	60	Excessive day-time sleepiness, conduction defect, breathing difficulties, cataracts	V

135 GlutaMAX™ (Thermo Fisher Scientific) and 1% fun-
 136 gonzone (Thermo Fisher Scientific). Once fibroblast
 137 cells attained sufficient confluency, they were frozen
 138 and stored long-term in liquid nitrogen. For fur-
 139 ther experiments including proteomic profiling, cells
 140 were cultured as described above until a confluence
 141 of 70%, harvested by scraping from culture flasks,
 142 washed twice with ice-cold PBS and cell pellets were
 143 snap-frozen in liquid nitrogen and stored at -80°C
 144 until further processing.

145 In total 12 fibroblast samples were included in our
 146 proteomic study: 3 DM1 fibroblast lines from patients
 147 with early onset, severe disease and CTG-repeat
 148 expansions of more than 2000 (patients 4–6:2431,
 149 2683 and 3187 CTG-repeats, respectively), 3 DM1
 150 fibroblast lines from patients with late onset, mild
 151 disease and CTG-repeat expansions between 80 and
 152 150 CTG-repeats (patients 1–3:84, 94 and 140 CTG-
 153 repeats, respectively), and a total of 6 sex- and age-
 154 matched controls (three controls per patient-group).
 155 DM1-patient derived cell lines were obtained from
 156 the “MRC Centre Neuromuscular Biobank” (New-
 157 castle upon Tyne, UK) [37]. All patients were adults
 158 when the skin biopsies were taken. Clinical data for
 159 the individual patients are listed in Table 1.

160 *Unbiased label-free LC-MS/MS and data* 161 *analysis*

162 The following reagents were used for LC-MS/MS:
 163 Ammonium hydrogen carbonate (NH₄HCO₃), anhy-
 164 drous magnesium chloride (MgCl₂), guanidine hyd-
 165 rochloride (GuHCl), iodoacetamide (IAA), and urea
 166 (Sigma-Aldrich, Steinheim, Germany), tris base (Ap-
 167 plichemBiochemica, Darmstadt, Germany), sodium
 168 dodecyl sulfate (SDS) (Carl Roth, Karlsruhe, Ger-
 169 many), dithiothreitol (DTT), EDTA-free protease in-
 170 hibitor (Complete Mini) tablets (Roche Diagnostics,

Mannheim, Germany), NaCl, CaCl₂ (Merck, Darm-
 171 stadt, Germany), sequencing grade modified trypsin
 172 (Promega, Madison, WI USA), Benzonase® Nucle-
 173 ase (Novagen), a bicinchoninic acid assay (BCA)
 174 kit (Thermo Fisher Scientific, Dreieich, Germany).
 175 All chemicals for ultra-pure HPLC solvents such
 176 as formic acid (FA), trifluoroacetic acid (TFA) and
 177 acetonitrile (ACN) were obtained from Biosolve,
 178 Valkenswaard, The Netherlands.
 179

180 *Cell lysis, sample clean-up and proteolysis*

181 Cell pellets were lysed in 100 μ L of lysis buffer
 182 (50 mM Tris-HCl (pH 7.8), 150 mM NaCl, 1 % SDS,
 183 and Complete Mini) using a manual glass grinder.
 184 Extracts were centrifuged at 6000 g for 5 min at 4°C
 185 to separate cell debris from the protein lysate and
 186 protein concentration was determined by BCA assay
 187 (according to the manufacturer’s protocol). Cysteines
 188 were reduced via the addition of 10 mM DTT fol-
 189 lowed by an immediate incubation at 56°C for 30 min.
 190 Alkylation of free thiol groups with 30 mM IAA was
 191 performed at room temperature (RT) in the dark for
 192 30 min.

193 Sample preparation was performed using filter-
 194 aided sample preparation (FASP) [38, 39] with some
 195 minor changes: 100 μ g of protein lysate was diluted
 196 10-fold with freshly prepared 8 M urea/100 mM Tris-
 197 HCl (pH 8.5) buffer and placed on a PALL microsep
 198 centrifugal device (30 KDa cut off) and centrifuged
 199 at 13,500 g at RT for 20 min (all the following cen-
 200 trifugation steps were performed under the same
 201 conditions). Three washing steps were carried out
 202 with 100 μ L of 8 M urea/100 mM Tris-HCl (pH 8.5).
 203 For buffer exchange, the device was washed three
 204 times with 100 μ L of 50 mM NH₄HCO₃ (pH 7.8).
 205 The digestion buffer (final volume of 100 μ L) com-
 206 posed of trypsin (1:25 w/w, protease to substrate),
 207 0.2 M GuHCl and 2 mM CaCl₂ in 50 mM NH₄HCO₃

(pH 7.8), was added to the concentrated proteins and samples incubated at 37°C for 14 h. The resulting tryptic peptides were recovered by centrifugation with 50 μ L of 50 mM NH₄HCO₃ followed by 50 μ L of ultra-pure water. Peptides were acidified (pH < 3 by addition of 10 % TFA (v/v)). All digests were quality controlled as described previously [40].

LC-MS/MS analysis

1 μ g of each sample was analyzed using an Ultimate 3000 nano RSLC system coupled to an Orbitrap Fusion Lumos mass spectrometer (both Thermo Scientific) in a randomized order to minimize systematic errors. Peptides were preconcentrated on a 100 μ m \times 2 cm C18 trapping column for 10 min using 0.1 % TFA (v/v) at a flow rate of 20 μ L/min followed by separation on a 75 μ m \times 50 cm C18 main column (both Pepmap, Thermo Scientific) with a 95 min LC gradient ranging from 3–35 % of 84 % ACN, 0.1 % FA (v/v) at a flow rate of 250 nL/min. MS survey scans were acquired in the Orbitrap from 300 to 1500 m/z at a resolution of 120,000 using the polysiloxane ion at m/z 445.12002 as lock mass [41], an automatic gain control target value of 2.0×10^5 and maximum injection times of 50 ms. TopSm most intense signals were selected for fragmentation by higher-energy collisional dissociation with an energy of 30 % and MS/MS spectra were acquired in the Ion-trap using an automatic gain control target value of 2.0×10^3 ions, a maximum injection time of 300 ms, a dynamic exclusion of 15 s and an isolation window of 1.2 (m/z).

Label free data analysis

Data analysis of the acquired label free MS data was performed using the Progenesis LC-MS software from Nonlinear Dynamics (Newcastle upon Tyne, U.K.). Raw MS data was aligned by Progenesis, which automatically selected one of the LC-MS files as reference. After automatic peak picking, only features within retention time and m/z windows from 0–95 min and 300–1500 m/z , with charge states + 2, + 3, and + 4 were considered for peptide statistics and analysis of variance (ANOVA) and MS/MS spectra were exported as peak lists. Peak lists were searched against a concatenated target/decoy version of the human Uniprot database (downloaded on 22.07.2015 containing 20273 target sequences) using Mascot 2.4 (Matrix Science, Boston, MA, USA), MS-GF+ (beta,v10282), X!Tandem (X!Tandem Vengeance, 2015.12.15.2) and MyriMatch (2.2.140) with the help of searchGUI 3.2.5 [42]. Trypsin was selected as the

enzyme, with a maximum of two missed cleavages, carbamidomethylation of cysteine was set as fixed and oxidation of methionine was selected as variable modification. MS and MS/MS tolerances were set to 10 p.p.m and 0.5 Da, respectively.

To obtain a peptide-spectrum match and to maximize the number of identified peptides and proteins at a given quality, we used PeptideShaker software 1.4.0 (<http://code.google.com/p/peptide-shaker/>) [43]. Combined search results were filtered at a false discovery rate of 1% on the peptide and protein level and exported using the PeptideShaker features that allow direct re-import of the quality-controlled data into Progenesis. Peptide sequences containing oxidized methionine were excluded from further analysis. Only proteins quantified with unique peptides were exported. For each protein, average of the normalized abundances obtained from Progenesis was calculated to determine the ratios between the patient and control cells. Only proteins which were (i) commonly quantified in all the replicates with (ii) a minimum of one unique peptide, (iii) an ANOVA p -value of ≤ 0.05 (Progenesis) and (iv) an average ratio $\log_2 \leq 5.8$ or ≥ -0.73 for the low repeats (comparison 1) and an average ratio $\log_2 \leq 5.6$ or ≥ -2.3 for the high repeats (comparison 2) were considered as significantly regulated.

“STRING” (Search Tool for the Retrieval of Interacting Genes/Proteins; www.string-db.org) enables the delineation of direct and functionally related protein-protein interactions. Thus, to identify functional interdependences of proteins with altered abundances in diseased cells and tissues, we applied this *in silico* tool to decipher proteins interacting with the ten marker proteins identified by our proteomic profiling of DM1-patient derived skin fibroblasts.

The Genotype-Tissue Expression (GTEx; www.gtexportal.org) project, representing a comprehensive public resource to study tissue-specific gene expression, was used to address expression of GSK3 β and identified markers in DM1 vulnerable tissues including different brain areas, peripheral nervous system, skeletal and cardiac muscle and skin/cultured fibroblasts. The data used for the analyses described in this manuscript were obtained from the GTEx Portal on 20/05.2020 dbGaP accession number phs000424.vN.pN on 12/10/2012.

Immunohistochemistry on murine muscles

Immunological staining of CAPN1 was performed utilizing cardiac and tibialis anterior muscle derived

from DMSXL mice (a suitable mouse model of DM1) [44] and wildtype littermates aged two and four months (two animals per group) as described previously [45]. For that purpose, a primary anti-Calpain 1 polyclonal antibody (Invitrogen: #PA5-86056) was used.

RESULTS

Expression of GSK3β in fibroblasts derived from patients with DM1

Proteomic profiles of fibroblasts derived from DM1 patients (early and adult disease onset; Table 1) and respective controls were generated. First, we analysed the proteomic signature for GSK3β within the whole protein extracts obtained from DM1-patient derived fibroblasts. This analysis revealed an increase in GSK3β expression in fibroblasts derived from DM1-patients of both groups; and this effect was more pronounced in cells derived from patients mildly affected by DM1 showing a 2.80-fold increase compared to a 1.62-fold increase in fibroblasts derived from severely affected DM1-patients (severe vs mild = 0.58, p-ANOVA = 0.110, statistically not significant; Table 2 & 3; Fig. 1A).

Identification of dysregulated proteins related to GSK3β

Next, we screened these profiles for detection of one of the ~100 proteins known to be modulated by GSK3β or involved in GSK3β-dependent processes, allowing the identification of 25 dysregulated proteins [31, 30]: 9 of these dysregulated proteins were quantified with a statistically significant p-ANOVA (≤ 0.05) in both patient groups (Table 2 & Fig. 1A) and 16 with a statistically significant p-ANOVA (≤ 0.05) in only one of the defined groups (Supplementary Table 1). Out of the nine proteins, four are decreased (DNMT1, VDAC2, HNRNPH3 & CTNNB1) and five (MAP2K2, HDAC2, NR3C1, CTPS1 & CAPN1) are increased in both patient groups as compared to controls (Table 2 & Fig. 1A). To further define a disease marker as a marker of severity, the statistical significance of the change in fold-regulation for each protein between mild and severe patient groups was taken into account. This resulted in the definition of three severity markers (Table 2): HDAC2 is 2.42-fold upregulated in cells derived from mild patients, and 4.86-fold increased in severe patients (p-ANOVA 0.005). Similarly, CTPS1 is 1.99-fold

Table 2
Dysregulation of GSK3β and associated proteins in DM1-patient derived fibroblasts

Accession number	Protein name	Gene symbol	p-ANOVA mild group	p-ANOVA severe group	Fold of regul. mild group	Fold of regul. severe group	Disease marker	Severity marker
P49841	Glycogen synthase kinase-3 beta	GSK3B	0.044	0.030	2.80	1.62	x	
P26358	DNA (cytosine-5)-methyltransferase 1	DNMT1	0.036	0.001	0.18	0.27	x	
P35222	Catenin beta-1	CTNNB1	0.012	0.010	0.35	0.77	x	
P31942	Heterogeneous nuclear ribonucleoprotein H3	HNRNPH3	0.013	0.004	0.37	0.48	x	
P45880	Voltage-dependent anion-selective channel protein 2	VDAC2	0.042	0.001	0.55	0.59	x	
P07384	Calpain-1 catalytic subunit	CAPN1	0.007	0.000	1.39	1.71		x
P17812	CTP synthase 1	CTPS1	0.033	0.005	1.99	2.55		x
P04150	Glucocorticoid receptor	NR3C1	0.037	0.001	2.02	2.01	x	
Q92769	Histone deacetylase 2	HDAC2	0.042	0.008	2.42	4.86		x
P36507	Dual specificity mitogen-activated protein kinase 2	MAP2K2	0.034	0.002	4.77	4.10	x	

Table 3

Abundance of each GSK3 β -related protein identified in the proteomics of DM1 patient-derived fibroblasts, as compared to expression levels of GSK3 β

Gene symbol	Average intensities			Ratio of each protein compared to GSK3 β		
	Severegroup	Mild group	Controls	Severegroup	Mild group	Controls
<i>GSK3B</i>	1.14E+06	2.55E+06	9.10E+05	1.00	1.00	1.00
<i>DNMT1</i>	4.40E+05	3.38E+05	1.74E+06	0.39	0.13	1.92
<i>CTNNB1</i>	4.79E+06	5.53E+06	1.09E+07	4.19	2.17	12.00
<i>HNRNPH3</i>	7.71E+06	6.27E+06	1.65E+07	6.75	2.46	18.15
<i>VDAC2</i>	2.15E+07	3.02E+07	4.53E+07	18.84	11.87	49.81
<i>CAPN1</i>	5.82E+06	7.99E+06	4.57E+06	5.10	3.14	5.03
<i>CTPS1</i>	2.11E+06	2.58E+06	1.06E+06	1.85	1.01	1.16
<i>NR3C1</i>	1.27E+06	1.49E+06	6.73E+05	1.11	0.58	0.74
<i>HDAC2</i>	1.59E+06	3.70E+06	4.03E+06	1.39	1.45	4.43
<i>MAP2K2</i>	7.08E+05	5.61E+05	1.45E+05	0.62	0.22	0.16

355 increased in mild patient fibroblasts and 2.55-fold in
 356 cells of the severe patient group (p-ANOVA 0.04).
 357 Moreover, CAPN1 is 1.39-fold upregulated in cells
 358 derived from mild patients, and 1.71-fold increased
 359 in severe patients (p-ANOVA 0.002).

360 *Change in expression of dysregulated proteins in* 361 *relation to GSK3 β*

362 To investigate potential stoichiometric changes
 363 between GSK3 β and the nine substrates dysregulated
 364 in DM1-patient derived fibroblasts, we compared the
 365 abundance of each protein with GSK3 β expres-
 366 sion. This comparison revealed decreased ratios for
 367 DNMT1, MAP2K2, VDAC2, HDAC2, HNRNPH3
 368 and CTNNB1 in both patient groups compared to
 369 controls. For NR3C1 and CTPS1 decreased ratios
 370 were observed in the mild group and increased ra-
 371 tios in the severe DM1-patient group. For CAPN1,
 372 decreased ratios were identified by comparing control
 373 and mild patient group, but ratios remained almost
 374 unchanged between the controls and the group of
 375 severely affected DM1 patients. All protein inten-
 376 sities and calculated ratios to GSK3 β are shown in
 377 Fig. 1B and listed in Table 3.

378 *Protein network analysis*

379 “STRING” protein-network analyses revealed a
 380 predicted functional interaction of GSK3 β , MAP
 381 2K2, CTNNB1, HDAC2, NR3C1, DNMT1& VD-
 382 AC2 (Fig. 1C). Expanding the interaction network
 383 to include first level interactors of our dysregulated
 384 proteins revealed a further predicted interplay of 13
 385 proteins, with the addition of CDH1, FYN, CTNNA1,
 386 APC and BCL9 as well as connecting CAPN1 to
 387 the functional network (Fig. 1C). Considering second

level binding partners, a functional interplay of 18
 proteins was delineated, with the addition of AXIN1,
 FER, AKT1, AKT3 and CTNNBIP1 (Fig. 1C).

391 *In silico tissue expression analysis of* 392 *dysregulated proteins*

393 To address tissue expression of the identified dys-
 394 regulated proteins, the GTEx database was used, with
 395 a focus on DM1-associated tissues, such as different
 396 areas of the central nervous system (amygdala, ante-
 397 rior cingulate cortex, frontal cortex, basal ganglia,
 398 cerebellum and cerebellar hemisphere, hippocampus,
 399 hypothalamus, substantia nigra and spinal cord), tib-
 400 ial nerve, skeletal and cardiac muscle as well as skin
 401 and cultured fibroblasts. Of note, among the different
 402 brain areas, all proteins show highest abundance in
 403 the cerebellum, including the cerebellar hemisphere.
 404 For CAPN1 and MAP2K2 increased abundance was
 405 also observed in cortex (Fig. 2). In addition, the nine
 406 markers and GSK3 β are highly expressed in the tib-
 407 ial nerve, skin and cultured fibroblasts. Compared to
 408 the other tissues addressed, moderate abundance in
 409 skeletal muscle was observed for GSK3 β , VDAC2,
 410 CAPN1, CTPS1, NR3C1 and MAP2K2 (Fig. 2).

411 *Calpain-1 expression analysis in murine muscle*

412 To verify our proteomic findings, expression CA-
 413 PN1 as a paradigmatic protein was studied in cardiac
 414 and tibialis anterior muscle derived from DMSXL
 415 mice and wildtype littermates (two and four months
 416 of age). For both tissues elevated sarcoplasmic CA
 417 PN1 level were observed at two and four months of
 age, respectively (Fig. 3).

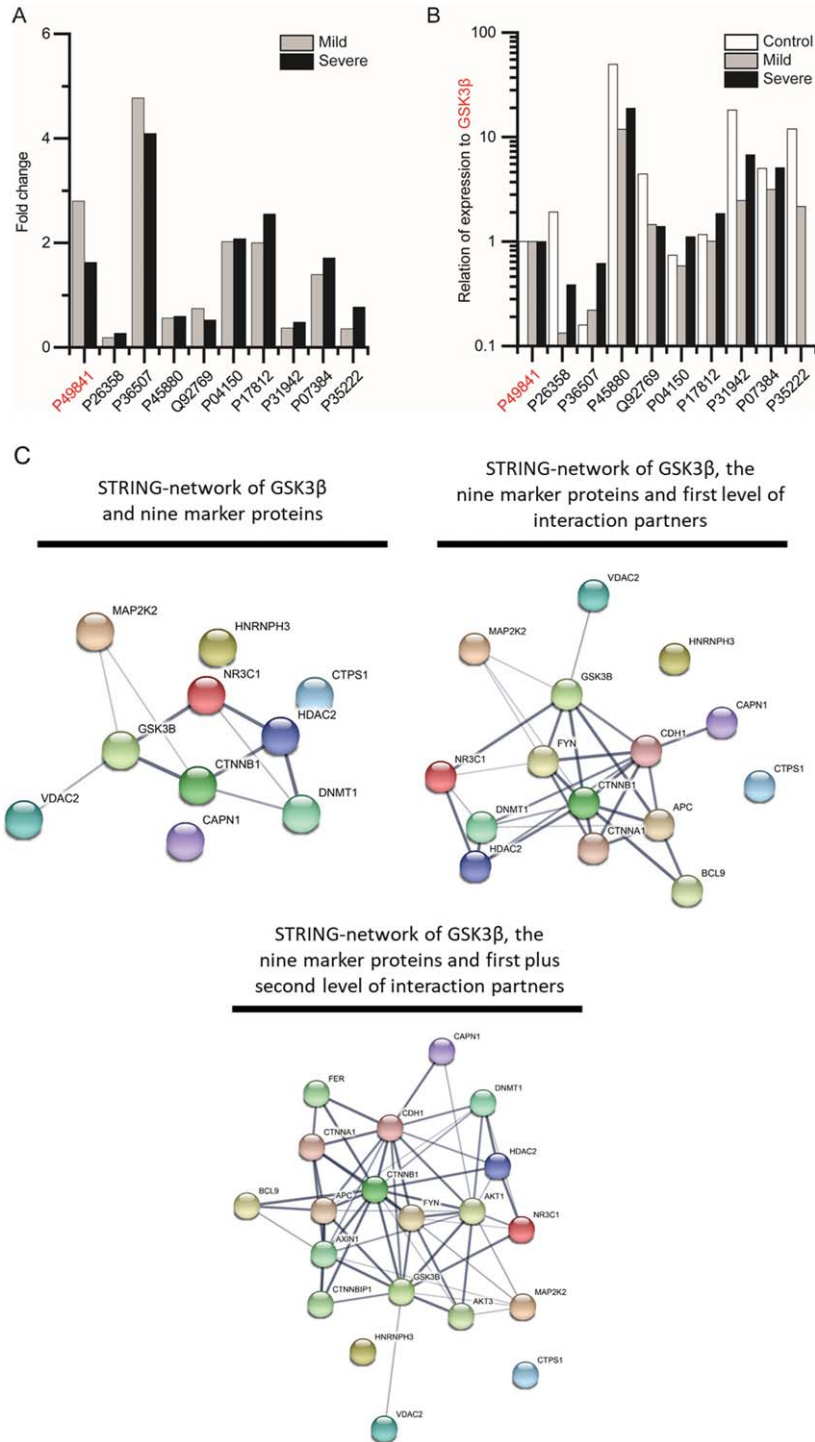


Fig. 1. Dysregulation of GSK3 β and related proteins identified by unbiased proteomic profiling of DM1-patient derived skin fibroblasts (mildly and severely affected cases). (A) Altered relative expression of GSK3 β (P49841; highlighted in red) and nine proteins known to be modulated by its function, thus serving as GSK3 β -dependent cellular markers of DM1. (B) Changes in abundance of proteins expressed as a ratio with GSK3 β expression in fibroblasts derived from mild and severely affected DM1 patients compared to controls. (C) STRING-based protein interaction networks reflecting interaction network of the nine markers identified, including first and second level binding partners. Thickness of the lines between the nodes refers to the confidence of interaction.

DISCUSSION

Proteomic profiling is a powerful tool for the unbiased identification of proteins contributing to neuromuscular disease pathology in cells and tissues derived from patients [46, 47]. Fibroblasts are known to be affected in DM1 [48], therefore, we performed LC-MS/MS-based analysis of the protein signature of DM1 patient-derived fibroblasts to identify proteins that may be markers of DM1 presence or severity.

Nearly 100 proteins are proposed to be substrates for GSK3 β , highlighting the role of this kinase as a fundamental regulator of many cellular processes [31–49]. Given that GSK3 β -overactivation is found in DM1-patient cells and tissues [29] and that inhibition of this overactivation represents a promising treatment strategy in DM1, we analysed GSK3 β abundances in DM1-patient derived cells. This revealed an increase in GSK3 β in both mild and severely affected patients. This effect was more pronounced in cells derived from mildly affected DM1-patients, thus in fibroblasts GSK3 β protein level does not correlate with CTG-repeat expansion (and the resultant disease-severity classification). However, given that GSK3 β -overactivation is modulated by post-translational modification, studies of GSK3 β -phosphorylation are needed to draw a conclusion regarding a correlation of GSK3 β -activation and CTG-repeat expansion/disease severity in DM1-fibroblasts.

After filtering out proteomic data for proteins modulated by GSK3 β , we were left with nine proteins that may be potential markers of DM1 disease or severity. To the best of our knowledge, none of the nine proteins has been described as a DM1 blood biomarker thus far. *In silico*-based interaction studies suggested a down-stream impact on pathways perturbed in DM1, such as AKT-signalling (AKT1 & AKT3) [50]. The known ubiquitous expression of the nine cellular markers hints toward a potential overall involvement in DM1-pathophysiology and moreover suggests that further functional studies or pre-clinical intervention concepts can be performed in DM1-patient derived skin fibroblasts. This postulate is further supported by the proven high expression in human cultured skin fibroblasts as exemplified by or *in silico*-based expression studies. A comparison of their relative abundance with GSK3 β levels showed many proteins had expression that varied with severity. This finding suggests a pathophysiological shift of GSK3 β , and some of its substrates, that may allow their use for tracking progression of disease or

response to GSK3 β inhibitor therapy. The relevance of each GSK3 β -related dysregulated protein to DM1 pathophysiology is discussed in subsequent sections:

DNA (cytosine-5)-methyltransferase 1 (DNMT1; decreased in DM1 fibroblasts): DNA (cytosine-5)-methyltransferase 1, encoded by the *DNMT1* gene, maintains DNA methylation profiles after DNA replication and cell division [51]. Koh and colleagues linked DNMT1 to GSK3 β in fibroblasts: they found that when GSK3 β is inactivated by phosphorylation, DNMT1 levels are elevated by TGF-beta1 [52]. Recent evidence shows that DNMT11 is required for correct myogenesis, a process impaired in DM1, leading to defective muscle regeneration and to muscle weakness and wasting [53, 54]. Studies performed in mice with muscle-specific deletion of *Dnmt1* revealed a reduced ability of myoblasts to differentiate [55]. *Dnmt1*-knock down in HT1080 cells, a human fibrosarcoma cell line carrying a copy of a human HPRT minigene inactivated by a (CAG)₉₅ repeat tract, destabilizes CAG-repeats, suggesting a fundamental role of *Dnmt1* in CAG-repeat instability [56].

DNMT1 expression is more pronounced in “young” human fibroblasts than in passage-aged, and DNMT11-knockdown induces a senescence phenotype in “young” fibroblasts [57]. The premature aging in DM1 patients [58] could link to the decreased DNMT1 protein level in DM1 patient derived fibroblasts (Table 1), presumably caused by GSK3 β -overactivation.

Catenin beta-1 (CTNNB1; decreased in DM1 fibroblasts): is a key component of the canonical Wnt signaling pathway controlling physiological processes like embryonic development, cellular proliferation and differentiation [59]. In the absence of Wnt ligands, β -catenin is part of a cytoplasmic complex also containing GSK3 β . This complex facilitates the phosphorylation and ubiquitination of CTNNB1, thus controlling its activation and proteasome-mediated degradation. In skeletal muscle, Wnt/ β -catenin signalling plays a central role in myogenesis, differentiation and myotube hypertrophy [60–62]. Consequently, CTNNB1 is considered a promising target in diseases such as cachexia or muscular dystrophies, characterized by muscle wasting. A chronic overactivation of Wnt pathways was found in skeletal muscles of DMD-patients, driving the activation of TGF β 2 and thus promoting fibrosis [63]. Of note, exogenous inhibition of the Wnt pathway was shown to reduce fibrosis in dystrophic muscles of the *mdx* mouse model [64]. In the disease etiology of DM1, CTNNB1-upregulation (via the Wnt signaling

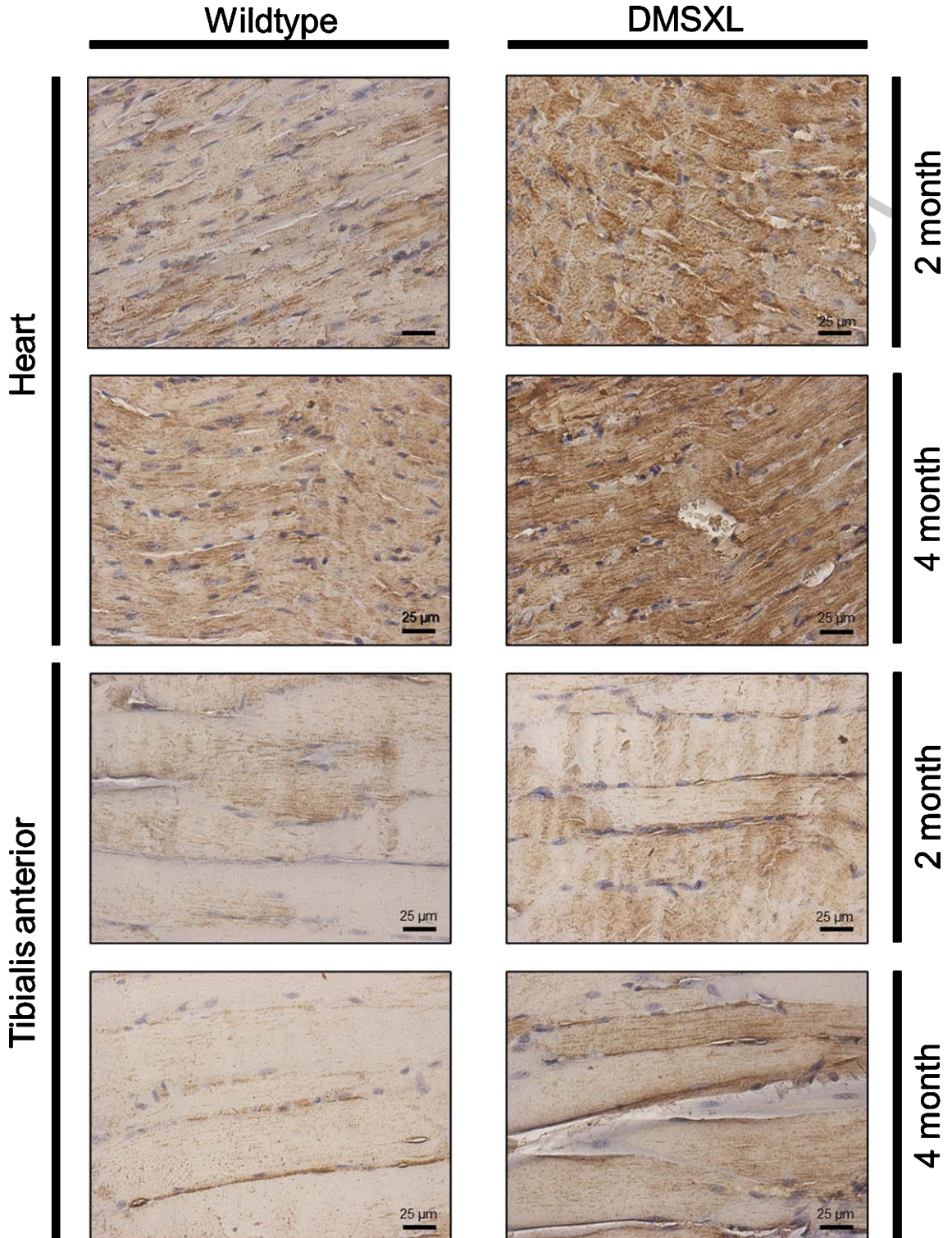


Fig. 3. Immunohistochemistry-based analysis of CAPN1 expression in cardiac and tibialis anterior muscle derived from DMSXL mice. Increased immunoreactivity of CAPN1 is detected in cardiac and tibialis anterior muscle (longitudinal sections) of diseased animals compared to controls at the age of two and four months.

521 pathway) was linked to increased cancer risk [65].
522 We identified decreased CTNFB1-levels in fibroblasts of both mild and severely affected patients, and
523 in accordance with the expression level of GSK3 β ,
524 this effect is more pronounced in mild patients rather
525 than severe.
526

527 Heterogeneous nuclear ribonucleoprotein H3(hn
528 RNPH3; decreased in DM1 fibroblasts): is an RNA
529 binding protein involved in splicing, in particular by
530 participating in early heat shock-induced splicing
531 arrest [66]. Inhibition of GSK3 β controls cellular
532 levels of hnRNPH3, thus showing a functional inter-
533 dependence of both proteins [67]. hnRNPH3 binds
534 to splice regulator muscleblind 1 variants (MBNL1
535 CUG) that co-localize with CUG foci in DM1-pat-
536 ient-derived muscle cells [68]. Furthermore, hnRN
537 PH3 levels are increased on average 3-fold in DM1
538 myoblasts [68], however, this is in contradiction with
539 the decreased level observed in our study. This might
540 be related to cell-type specific protein-composition
541 of these foci, perhaps in turn influenced by cell type
542 specific expression patterns of the related proteins.
543

544 Voltage-dependent anion-selective channel protein
545 2 (VDAC2; decreased in DM1 fibroblasts): local-
546 izes to the mitochondrial outer membrane where it
547 constitutes a channel for the membrane transport
548 of various substrates [69]. GSK3 β is a major posi-
549 tive regulator of mitochondrial permeability during
550 oxidative stress, a trigger of apoptosis, and interacts
551 with VDAC2 [70]. Apart from its role in regulating
552 the flux across the mitochondrial outer membrane,
553 VDAC2 has a fundamental function in sequestering
554 the proapoptotic protein BAK in the mitochondrial
555 outer membrane and maintaining it in the inactive
556 state [71].

557 Mitochondrial dysfunction and impaired oxida-
558 tive metabolism are known pathophysiological me-
559 chanisms in DM1 [72, 73]. Impaired mitochondrial
560 bioenergetics, accompanied by complex 1-dysfun-
561 ction and decreased VDAC2 expression, were de-
562 scribed in quadriceps and gastrocnemius muscle of
563 *mdx* mice, an animal model for Duchenne muscular
564 dystrophy [74]. Hence, one might assume that the
565 VDAC2-decrease in DM1 contributes to mitochon-
566 drial dysfunction and the initiation of apoptosis.

567 Calpain-1 catalytic subunit (CAPN1; increased in
568 DM1 fibroblasts): also called μ -calpain, belongs to
569 a family of Ca²⁺-dependent cysteine proteases [75].
570 It is expressed ubiquitously as a heterodimer [76].
571 Calpains are involved in several physiological func-
572 tions such as cytoskeletal remodeling [77], signal
transduction [78], apoptosis [79], cell cycle [80],

573 regulation of gene expression [81] and long-term
574 potentiation [82]. Truncation and activation of GS
575 K3 β is modulated by CAPN1 [83] and recent evi-
576 dence showed that GSK3 β phosphorylates desmin
577 filaments (an essential event for myofibril destruc-
578 tion) in a CAPN1 dependent manner [84]. In skeletal
579 muscle, increased calpain activation was described
580 in atrophic conditions following disuse, denervation,
581 glucocorticoid treatment and sepsis [85–87] as well
582 as in necrotic fibers from *mdx* mice [88]. Moreover,
583 elevated CAPN1 levels were found in sera samples
584 of young DMD patients not yet treated with glu-
585 cocorticoids, who also present with high levels of
586 GSK3 β that decline with aging [89]. Further evi-
587 dence for a significant role of CAPN1 in muscle
588 fibre integrity is given by *in vivo* studies: reduced
589 muscle necrosis and, consequently, a significantly
590 decreased regeneration was observed in *mdx* mice
591 crossed with transgenic animals overexpressing cal-
592 pastatin, a CAPN1-inhibitor [90]. Based on these
593 findings, one might suppose that increased CAPN1
594 levels may be linked to the dystrophic conditions
595 characterizing DM1. Indeed, immunohistochemical
596 studies of CAPN1 on cardiac and tibialis anterior
597 muscles derived from DMSXL mice, a suitable DM1
598 mouse model, revealed an increase compared to
599 wildtype littermates in both tissues. This molecular
600 finding suggests an applicability of our proteomic
601 findings on tissues of major vulnerability in DM1
602 such as cardiac and skeletal muscle.

603 CTP synthase 1 (CTPS1; increased in DM1 fibrob-
604 lasts): is responsible for *de novo* synthesis of CTP
605 (cytidine triphosphate) and biosynthesis of phospho-
606 lipids, nucleic acids and in sustaining the proliferation
607 of activated lymphocytes [91–93]. GSK3 β is a known
608 regulator of CTPS1 activity [94]. We hypothesize that
609 the increased level of CTPS1 is linked to the altera-
610 tions in lipid composition or metabolism that can
611 occur during skeletal and cardiac myopathies [95].
612 With regards to the insulin resistance in the cardiac
613 and skeletal muscles of DM1 patients and animal
614 models (see below), it is worth noting that this not
615 only results in increased circulating glucose but
616 also lipid levels [96]. Thus, an interdependent co-
617 regulation of both CTPS1 and NR3C1 in a GSK3 β -
618 dependent manner might be plausible.

619 Glucocorticoid receptor (NR3C1; increased in
620 DM1 fibroblasts): is a member of the superfamily
621 of nuclear receptors (acting as modulators of gene
622 transcription) that, in the absence of glucocorticoid/
623 cortisol, localizes to the cytosol in a chaperone-
624 containing multiprotein complex [97]. GSK3 β is

involved in ligand-dependent activation of transcription and cellular localization of this receptor [98]. A study performed in a large cohort of DM1 patients revealed alterations in glucocorticoid metabolism with an increased reactivation of cortisone to cortisol, mainly in male patients [99]. An excess of glucocorticoids leads to insulin resistance, which is also present in 5–17% of DM1 cases [100, 101]. In skeletal muscle, insulin resistance leads to impaired glucose uptake into the muscle fibers where it is required as an energy source. Due to insulin resistance and subsequent lipid metabolism alterations, DM1 patients can present with cardiac and vascular defects [101]. Therefore, increased NR3C1 might serve as a marker for the presence of DM1.

Histone deacetylase 2 (HDAC2; increased in DM1 fibroblasts): is a chromatin modifying enzyme that prevents gene-transcription by removal of acetyl groups from histones and deacetylates proteins such as transcription factors and those involved in the control of cell growth, differentiation and apoptosis [102]. HDAC2 expression and function is known to be controlled by GSK3 β [103, 104]. In the context of DM1 pathophysiology it is important to note that two small molecule HDAC inhibitors, ISOX and vorinostat, increase MBNL1 expression, partially rescuing aberrant splicing in DM1 patient-derived fibroblasts [105]. HDAC2 might represent a marker protein in DM1-patient derived fibroblasts that plays a direct role in DM1 pathophysiology.

Dual specificity mitogen-activated protein kinase kinase 2 (MAP2K2; increased in DM1 fibroblasts): also known as MEK2, is a ubiquitously expressed protein that catalyses the phosphorylation of tyrosine and threonine in target proteins participating in the RAS–RAF–MEK–ERK signal transduction cascade. Consequently, via this cascade, MEK2 is involved in the regulation of a variety of processes including apoptosis, cell cycle progression, cell migration, differentiation, metabolism, and proliferation [106]. It also modulates phosphorylation and thus function of GSK3 β via ERK [34]. Of particular interest for the muscle vulnerability of DM1, the MEK–ERK pathway is important in muscle cell differentiation [107]. Sustained activation of this pathway presumably also includes MEK2, as found in DM1 myoblasts, and can be considered as a contributor to the abnormal myoblast differentiation observed [108]. Moreover, RAS-ERK pathway activation is known to regulate alternative splicing [109], the main pathophysiological process in DM1. One of the most common symptoms in DM1, myotonia, is based on aberrant

splicing of the skeletal muscle chloride channel (*CLCN1*) transcript [110, 111] and RAS pathway inhibitors correct this alternative splicing, alleviating some DM1 features in mice [112].

Although a transfer of the relevance of the proteomic data acquired from human skin fibroblasts could be demonstrated for CAPN1 in skeletal muscle utilizing a DM1 mouse model, further biochemical investigations would be needed to draw the same conclusion for the other marker proteins highlighted in this study. Along this line, confirmational studies on fibroblasts derived from further DM1 patients would be needed to substantiate the robustness of the proposed cellular marker proteins.

CONCLUSIONS

Overall, in this study we have identified nine proteins associated with GSK3 β that may provide much-needed biomarkers for DM1 disease identification using easily obtained patient fibroblasts. Furthermore, we have also identified three proteins (CTPS1, CAPN1 and HDAC2) that increase with severity of the disease, providing potential biomarkers that can be used in categorizing patients, monitoring disease progression and response to treatment. Validation of such biomarkers and their hypothesized roles in DM1 and GSK3 β -associated pathophysiology will be required in future studies. Given that recent clinical trials in DM1 are based on GSK3 β -inhibition [113], as the proteins defined in our study, due to their interaction with GSK3 β , may serve as suitable markers to monitor success of the tested treatment concepts.

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CONFLICTS OF INTEREST/COMPETING INTERESTS

All authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

AR, DH and HL designed the study. NN collected skin biopsies and provided clinical information. DH performed proteomic studies. AH performed data analysis and *in silico* studies. VG, EOC, USS, HL & AR drafted the manuscript. GG provided the tissue of the DM1 mouse model. All authors declare that the work described has not been published before and is also not under consideration for publication anywhere else. The final manuscript-draft has been approved by all co-authors.

SUPPLEMENTARY MATERIAL

The supplementary material is available in the electronic version of this article: <https://dx.doi.org/10.3233/JND-200558>.

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