PMP22 messenger RNA levels in skin biopsies: testing the effectiveness of a Charcot–Marie–Tooth 1A biomarker

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Charcot–Marie–Tooth disease type 1A (CMT1A) is associated with increased gene dosage for PMP22. Therapeutic approaches are currently aiming at correcting PMP22 over-expression. It is unknown whether PMP22 can be used as a biological marker of disease progression and therapy efficacy. We performed quantitative real-time polymerase chain reaction on skin biopsies of 45 patients with CMT1A, obtained at study entry and after 24-months of treatment either with ascorbic acid or placebo. Data of a subgroup of patients were also compared with matched healthy subjects. Finally, we analysed PMP22 messenger RNA levels in sural nerve biopsies. We did not find significant differences in the levels of any known PMP22 transcripts in treated or untreated patients with CMT1A, thus confirming that ascorbic acid does not impact on the molecular features of CMT1A. Most importantly, we did not observe any correlation between PMP22 messenger RNA levels and the different clinical and electrophysiological outcome measures, underscoring the weakness of PMP22 to mirror the phenotypic variability of patients with CMT1A. We did not find increased PMP22 messenger RNA levels in skin and sural nerve biopsies of patients with CMT1A compared with relative controls. In conclusion, this study shows that ascorbic acid does not impact on PMP22 transcriptional regulation and PMP22 is not a suitable biomarker for CMT1A.
Keywords: PMP22; ascorbic acid; CMT1A; biological marker

Introduction

Charcot–Marie–Tooth disease type 1A (CMT1A) neuropathy is the most prevalent genetic neurological disorder with no pharmacological treatment available (Schenone et al., 2011). CMT1A is characterized by demyelination and reduced nerve conduction velocity and is associated with a 1.5 Mb duplication on chromosome 17p11.2. After the recognition of the CMT1A duplication in the majority of CMT1 families the PMP22 gene was mapped within the CMT1A region (Lupski et al., 1991). PMP22 is a minor component of the peripheral myelin sheath and the observation that genetic alterations in the PMP22 gene lead to various forms of myelination deficiencies, suggests that the protein plays a crucial role in peripheral myelination (Suter, 2004). The phenotype in patients with CMT1A results from abnormal PMP22 gene dosage, as suggested by multiple lines of independent experimental evidence including: (i) quantitative PMP22 messenger RNA and protein studies in the peripheral nerves from patients with the duplication rearrangement (Yoshikawa et al., 1994; Vallat et al., 1996); (ii) the fact that smaller duplications containing the PMP22 gene within the rearrangement interval still cause neuropathy (Palau et al., 1993); and (iii) the observation that rodent models that over-express PMP22 recapitulate the CMT1A phenotype (Fledrich et al., 2012). Indeed, strategies aimed at normalizing the PMP22 gene dosage may provide therapeutic approaches as demonstrated in rodent models by treatment with different molecules, such as a progesterone receptor inhibitor or ascorbic acid (Sereda et al., 2003; Passage et al., 2004). As a consequence of these promising preclinical results, different human trials with ascorbic acid have been performed. None of them succeeded in improving the clinical features of CMT1A, even when high dosage of ascorbic acid was used and large population of patients considered (Pareyson et al., 2011; Lewis et al., 2013). At present, reducing production of PMP22 is considered the best way to positively impact on the disease (Jang et al., 2012). Indeed, outcome measures sensitive to change are needed in CMT1A, either to detect intervention efficacy and to monitor disease evolution and worsening. PMP22 gene has three known splicing variants regulated by alternatively used promoters, defining their tissue-specific expression. These PMP22 transcripts encode the same polypeptide but differ in the sequence of their 5' untranslated regions (Suter et al., 1994). We assessed PMP22 messenger RNA levels for all these splicing variants in skin biopsies of a subset of patients enrolled in the Italian ascorbic acid trial (CMT-TRIAAL; Pareyson et al., 2011) to see whether ascorbic acid was efficacious in reducing PMP22 expression and if PMP22 levels change over time. We also correlated, for each patient, their molecular data with clinical outcome measures. Moreover, PMP22 messenger RNA levels in skin and sural nerve biopsies of patients with CMT1A were compared with those of control subjects to assess the usefulness of this measure as a biological marker of CMT1A.

Materials and methods

Human samples

Skin biopsies from 45 patients with clinical and genetic diagnosis of CMT1A, enrolled in the CMT-TRIAAL were studied (Pareyson et al., 2006, 2011). Skin biopsies were obtained in consenting patients with CMT1A before (Baseline, Time 0: T0) and after 24 months (T24) of ascorbic acid or placebo treatment, immediately frozen in liquid nitrogen and stored at −80 °C until processing. Moreover, skin biopsies of six healthy subjects, matched by gender and age with six patients with CMT1A, were collected and analysed. The protocol was approved by the Institutional Review Board at every site, and patients gave written informed consent before the biopsy procedure (Supplementary material). Sural nerve biopsies from five patients with CMT1A and five unaffected subjects who underwent nerve biopsy for suspected peripheral neuropathy were also used.

Real-time quantitative polymerase chain reaction

Real-time quantitative PCR was performed on 500 ng of total RNA from human skin and sural nerve biopsies by the LightCycler 480 System using the Probes Master Kit and following the manufacturer’s instructions (Roche Applied Science). Primers and probes were designed using the Universal Probe Library Assay Design Centre for real-time quantitative PCR (Roche) (Supplementary Table 1, Supplementary Fig. 3 and Supplementary Material).

Assessment of clinical and paraclinical endpoints

Clinical data from each patient involved in this study were obtained and correlated to the molecular findings. In particular, patients were evaluated at T0 and T24 with the following outcome measures: (i) Charcot–Marie–Tooth Neuropathy Score and CMT Examination Score; (ii) maximal voluntary isometric contraction as determined with a hand-held myometer (Cit Technics), for distal arm (hand grip, large three-point pinch) and leg (foot dorsiflexion and plantar flexion) movements; (iii) compound muscle action potential amplitudes (sum of compound muscle action potential amplitudes of ulnar, median, and peroneal nerves), and other electrophysiological parameters (median and ulnar motor conduction velocity; sensory conduction velocity and sensory action potential amplitude of the ulnar nerve).

Statistical analysis

Summary statistics for each splicing variant (mean, median, standard deviation, and median) were tabulated according to treatment and Charcot–Marie–Tooth Neuropathy Score value groups. Twenty-four-month changes for all splicing variants were box-plotted against treatment group. Based on the results of the Shapiro-Wilk normality test, between-groups differences were tested either by the two-sample Wilcoxon test or the unpaired t-test, as appropriate. Correlation between splicing variant measurements with main clinical outcomes and age were estimated by the Spearman’s correlation coefficient. All tests
were two-tailed and considered statistically significant at the 5% level. Statistical analyses were done using SAS 9.2 (Supplementary material).

Results

Real-time quantitative PCR performed on a human tissue panel to analyse expression levels of \( \text{PMP22} \) transcript variants confirmed the nerve specificity of variant 1 as compared to the more ubiquitous variant 2, and clearly showed that \( \text{PMP22} \) messenger RNA is also well represented in the skin and poorly expressed in fibroblasts, the major cell component of this tissue (Supplementary Figs 1 and 2 and Supplementary Material).

Ascorbic acid does not decrease \( \text{PMP22} \) messenger RNA levels in skin biopsies

Real-time quantitative PCR analysis was performed on skin biopsies (baseline and after ascorbic acid treatment) from 45 patients with CMT1A. These patients were divided in two groups, according to treatment, that were comparable for number, age, and gender: (i) the placebo group, represented by 22 subjects, 13 females and nine males, mean age: 40 ± 11 years, range 18–59 years; and (ii) the ascorbic acid group, with 23 subjects, 13 females and 10 males, mean age: 39 ± 12 years, range 20–60 years. At baseline, the expression levels of \( \text{PMP22} \) messenger RNA did not differ among the two groups of patients, neither when we analysed the nerve-specific transcriptional variant 1 \((P = 0.240)\), nor when we quantified the splicing variants 2 \((P = 0.465)\) and 3 \((P = 0.676)\) (Table 1). Notably, we found no difference between \( \text{PMP22} \) messenger RNA expression at \( T_0 \) and \( T_{24} \) both in the ascorbic acid group and in the placebo arm (the lowest, still not significant \( P \)-value was \( P = 0.127 \) for variant 2), indicating that there was no evidence of downregulation of \( \text{PMP22} \) by ascorbic acid (Fig. 1).

**PMP22 messenger RNA levels do not correlate with Charcot–Marie–Tooth Neuropathy Score and the other clinical outcome measures**

Ascorbic acid supplementation was unable to ameliorate CMT1A neuropathy (Pareyson et al., 2011) and failed to downregulate \( \text{PMP22} \) messenger RNA. Because in this study we had the opportunity to evaluate a high number of patients with CMT1A, well characterized from both the clinical and molecular point of view, we correlated messenger RNA levels of \( \text{PMP22} \) splicing variants with the main clinical outcome measures and with age. The results for each single \( \text{PMP22} \) transcript variant showed no significant correlation with any of the different outcome measures at baseline and at 24 months \((P > 0.181)\). Those regarding the nerve-specific \( \text{PMP22} \) variant 1 are shown in Tables 1, 2 and Fig. 2. There was no correlation between \( \text{PMP22} \) messenger RNA levels and any of

<table>
<thead>
<tr>
<th>Table 1 Univariate analysis and summary statistics of ( \text{PMP22} ) splicing variants by treatment and severity (Charcot–Marie–Tooth Neuropathy Score levels at baseline)</th>
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<tr>
<td><strong>PMP22 Variant</strong></td>
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<tr>
<td><strong>VAR 1</strong></td>
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<tr>
<td>Severity</td>
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<td>Severity</td>
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<td>Overall</td>
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CMT1A patients were grouped based on treatment (ascorbic acid and placebo) and on disease severity according to Charcot–Marie–Tooth Neuropathy Score values (mild = 0–10, moderate = 11–20, severe = 21–36) and \( \text{PMP22} \) messenger RNA levels analysed before treatment. None of the three \( \text{PMP22} \) transcriptional variants differed across the groups. The baseline levels were comparable for the two treatment groups, and they did not correlate with disease severity.

*Two-sample, two-sided Wilcoxon test or unpaired \( t \)-test as appropriate.

\( n \) = number of patients, SD = standard deviation, AA = ascorbic acid.

\( \text{PMP22} \) messenger RNA levels are expressed as relative fluorescent units (RFU).
the clinical and electrophysiological measures used in the trial, including Charcot–Marie–Tooth Neuropathy Score, myometry, motor and sensory potential amplitudes, and nerve conduction velocities. Expression levels of PMP22 did not correlate with disease severity even when the population was subdivided into categories according to Charcot–Marie–Tooth Neuropathy Score values (0–10 for mild, 11–20 for moderate, and 21–36 for severe disease) (Table 1). These results demonstrate not only that PMP22 messenger RNA levels do not follow the natural history of CMT1A neuropathy as already known, but also that they are not reliable to distinguish the less affected from the more severe patients. Lack of correlation with age further confirms the poor biological sensitivity of this molecular measure to follow the disease progression (Table 2).

**PMP22 messenger RNA levels are not increased in skin and sural nerve biopsies of patients with Charcot–Marie–Tooth disease type 1A compared with control subjects**

We compared levels of PMP22 transcript variants in a subgroup of our patients with an equivalent population of healthy subjects, carefully matched for age and gender. None of the PMP22 splicing variants was increased in skin biopsies of six patients with CMT1A as compared to healthy control subjects (Fig. 3A–C). To strengthen this issue, we performed the same analysis on sural nerve biopsies of five patients with CMT1A and compared PMP22 messenger RNA levels with those of five subjects with no changes at nerve biopsies that were performed to investigate the presence of a possible neuropathy. None of the patients with CMT1A expressed higher levels of PMP22 transcript variants as compared to the controls (Fig. 3D–F).

**Table 2 Correlations of PMP22 splicing variant 1 at baseline with age and the main clinical and electrophysiological outcome measures**

<table>
<thead>
<tr>
<th>Outcome Measure</th>
<th>Spearman’s rho</th>
<th>P-value</th>
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<tr>
<td>CMT Neuropathy Score</td>
<td>−0.05</td>
<td>0.736</td>
</tr>
<tr>
<td>CMT Examination Score</td>
<td>−0.10</td>
<td>0.505</td>
</tr>
<tr>
<td>Myometry, foot dorsiflexion</td>
<td>−0.03</td>
<td>0.865</td>
</tr>
<tr>
<td>Myometry, hand grip</td>
<td>0.20</td>
<td>0.181</td>
</tr>
<tr>
<td>Age</td>
<td>0.03</td>
<td>0.858</td>
</tr>
<tr>
<td>CMAP amplitude</td>
<td>−0.08</td>
<td>0.587</td>
</tr>
<tr>
<td>Median motor conduction velocity</td>
<td>−0.02</td>
<td>0.868</td>
</tr>
<tr>
<td>Ulnar motor conduction velocity</td>
<td>0.01</td>
<td>0.928</td>
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<tr>
<td>Ulnar nerve sensory action potential amplitude</td>
<td>−0.02</td>
<td>0.900</td>
</tr>
<tr>
<td>Sensory conduction velocity</td>
<td>−0.00</td>
<td>0.955</td>
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</table>

Molecular data did not show any correlation with either the severity of patients with CMT1A or age. CMAP amplitude = sum of compound muscle action potential amplitude of ulnar, median and peroneal nerves.
Discussion

Ascorbic acid does not impact on PMP22 messenger RNA levels in patients with Charcot–Marie–Tooth disease type 1A

CMT1A is a slowly progressive disease, in which responsive markers to monitor the natural history of the disease and to assess the efficacy of therapeutic treatments are still lacking (Bouhy and Timmerman, 2013). In the CMT-TRIAAL study, we assumed PMP22 transcriptional levels as a potentially sensitive-to-change biological marker able to detect the molecular effects of ascorbic acid, based on the promising results obtained in experimental settings (Passage et al., 2004) and on the fact that levels of PMP22 messenger RNA are currently considered a biomarker of CMT1A (Jang et al., 2012). Besides failing to improve CMT1A phenotype, ascorbic acid was unable to influence PMP22 messenger RNA levels, fully confirming recent findings obtained with high doses of this molecule (Lewis et al., 2013). It has to be considered that the molecular mechanisms involving ascorbic acid in CMT1A are far more complex than expected. In particular, it has been shown that ascorbic acid is able to lower PMP22 messenger RNA expression by negatively interfering with the activity of adenylate cyclase, the enzyme responsible for intracellular cyclic adenosine monophosphate (cAMP) synthesis: cAMP stimulates PMP22 transcription by acting on its minimal promoter and ascorbic acid counteracts this pathway (Saberan-Djoneidi et al., 2000). Nevertheless, controversial data exist concerning the effects of cAMP on myelination. Indeed, Schwann cell differentiation and myelination require elevated intracellular cAMP levels, whereas low concentrations induce cell proliferation (Arthur-Farraj et al., 2011). Moreover, we recently found decreased cAMP levels in primary Schwann cell cultures from CMT1A rats (Nobbio et al., 2014). Thus, lowering cAMP levels in CMT1A may even be detrimental for myelination. Different mechanisms, other than cAMP inhibition, must be advocated to justify the effects of ascorbic acid treatment in CMT1A experimental models and, conversely, explain the lack of efficacy in human patients.

PMP22 messenger RNA levels do not mirror the clinical phenotype of patients with Charcot–Marie–Tooth disease type 1A

PMP22 messenger RNA and protein levels are highly variable among patients with CMT1A. Most importantly, no correlation was found between neurological disabilities and the level of expression of PMP22 protein or messenger RNA in previous small series of patients with CMT1A (Hanemann et al., 1994; Katona et al., 2009). Interestingly, the observation of a more severe phenotype in patients homozygous for the CMT1A duplication...
co-exist with the description of homozygous patients for the CMT1A duplication developing a milder neuropathy (Lupski et al., 1991; LeGuern et al., 1997). Clinical variability has been also described in two pairs of identical twins with CMT1A duplication (Garcia et al., 1995). For the first time, expression of PMP22 has been tested in a large population of patients with CMT1A and compared to clinical and instrumental evaluations. Our results confirm that PMP22 messenger RNA levels are extremely variable and show that they do not correlate with the Charcot–Marie–Tooth Neuropathy Score or with other relevant outcome measures used in clinical practice and trials (Katona et al., 2009; Lewis et al., 2013). Thus, it is highly questionable whether PMP22 messenger RNA levels in skin may be considered a biological marker for CMT1A neuropathy, suitable to measure disease progression and response to treatment.

PMP22 messenger RNA levels do not discriminate patients with Charcot–Marie–Tooth disease type 1A from healthy subjects

PMP22 messenger RNA is commonly considered over-expressed in CMT1A neuropathy (Yoshikawa et al., 1994). Nevertheless, controversial data concerning its expression do exist (Hanemann et al., 1994; Katona et al., 2009). At present, the function of PMP22 has yet to be defined. We know that PMP22 is involved in cell cycle regulation, it has a role at cell junction complexes in peripheral nerve, it is highly expressed by myelinating Schwann cells and is strongly upregulated in parallel with the initiation of myelination (Suter, 2004). Why should we expect an increase of PMP22 levels in a disease in which myelin is lost? Indeed, an accumulation of PMP22 in onion bulbs and Schwann cells of adult patients with CMT1A has been reported without a clear over-expression of the protein (Nishimura et al., 1996). Even when semiquantitative, ultrastructural methods have been used, results were controversial (Vallat et al., 1996; Li et al., 2005; Katona et al., 2009). Lack of evidence for increased PMP22 messenger RNA and protein levels might be due to the age of patients analysed. In fact, as CMT1A is slowly progressive, it could be hypothesized that PMP22 expression is higher in children with CMT1A as the density of myelinated fibres is not as affected as later in life. In contrast, electrophysiological and morphological data show clear deficiencies early in childhood with an almost negligible worsening over time (Gabreels-Festen et al., 1992; Garcia et al., 1998), and high-dose ascorbic acid treatment of CMT1A was not efficacious even in children (Burns et al., 2009). Our findings that PMP22 messenger RNA levels do not correlate with age strengthen this observation.

Thus, the ultimate molecular mechanisms underlying CMT1A phenotype are still unclear. We then speculate that as PMP22 is subjected to genetic rearrangements some of which are upstream to the coding region (Weterman et al., 2010) and to both transcriptional (Suter et al., 1994; Saberan-Djoneidi et al., 2000; Weterman et al., 2010) and post-transcriptional modifications (Verrier et al., 2009), the dynamic regulation of PMP22 should be the ideal target of a molecular therapy. PMP22 steady state levels change over time, most probably as a consequence of unclear epigenetic factors that could be abnormally expressed themselves in CMT1A. Thus, in our opinion, we still lack the right molecular target of CMT1A.

Conclusions

Our results show that PMP22 messenger RNA levels are extremely variable, inconsistent with clinical outcome measures and poorly sensitive to be used for diagnostic and prognostic purposes. This observation and the lack of ascorbic acid impact on CMT1A explain the negative results of ours and other previous studies. As in CMT1A, it is still largely unknown how PMP22 genetic overloading reflects on the clinical phenotype, more studies are needed to disclose the molecular mechanisms that have to be targeted to cure the disease (Bouhy and Timmerman, 2013).

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Supplementary material

Supplementary material is available at Brain online.

Appendix 1

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