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### Citation

Kanhai, K. M. S., Goulooze, S. C., Stevens, J., Hay, J. L., Dent, G., Verma, A., ... Groeneveld, G. J. (2016). Quantifying Beta-Galactosylceramide Kinetics in Cerebrospinal Fluid of Healthy Subjects Using Deuterium Labeling. *Cts-Clinical And Translational Science*, 9(6), 321-327. doi:10.1111/cts.12424

Version: Not Applicable (or Unknown)

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**Note:** To cite this publication please use the final published version (if applicable).

ARTICLE

# Quantifying Beta-Galactosylceramide Kinetics in Cerebrospinal Fluid of Healthy Subjects Using Deuterium Labeling

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Therapeutics promoting myelin synthesis may enhance recovery in demyelinating diseases, such as multiple sclerosis. However, no suitable method exists to quantify myelination. The turnover of galactosylceramide (myelin component) is indicative of myelination in mice, but its turnover has not been determined in humans. Here, six healthy subjects consumed 120 mL 70% D<sub>2</sub>O daily for 70 days to label galactosylceramide. We then used mass spectrometry and compartmental modeling to quantify the turnover rate of galactosylceramide in cerebrospinal fluid. Maximum deuterium enrichment of body water ranged from 1.5–3.9%, whereas that of galactosylceramide was much lower: 0.05–0.14%. This suggests a slow turnover rate, which was confirmed by the model-estimated galactosylceramide turnover rate of 0.00168 day<sup>-1</sup>, which corresponds to a half-life of 413 days. Additional studies in patients with multiple sclerosis are needed to investigate whether galactosylceramide turnover could be used as an outcome measure in clinical trials with remyelination therapies.

*Clin Transl Sci* (2016) 9, 321–327; doi:10.1111/cts.12424; published online on 15 October 2016.

## Study Highlights

### WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

✓ Human myelin turnover and its breakdown product  $\beta$ -GalC has not been measured before, it has only been estimated based on animal turnover. Imaging methods are not yet accurate enough to determine changes in myelin turnover.

### WHAT QUESTION DID THIS STUDY ADDRESS?

✓ Can we calculate the myelin breakdown product  $\beta$ -GalC turnover in healthy volunteers by labeling it with deuterium?

### WHAT THIS STUDY ADDS TO OUR KNOWLEDGE

✓ We describe a method that is capable of quantifying the  $\beta$ -GalC in humans and may be accurate enough to measure change in turnover.

### HOW THIS MIGHT CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE

✓ This method may be able to show effect of remyelinating therapeutic interventions.

Multiple sclerosis is a highly prevalent inflammatory disease that affects the central nervous system (CNS), causing progressive disability. Multiple sclerosis is characterized by a loss of myelin combined with incomplete remyelination, leading to a progressive demyelination of nerves in the CNS. Current treatments for multiple sclerosis are designed to inhibit the inflammatory component of the disease.<sup>1</sup> However, increasing the remyelination process is another promising strategy, which may improve patient outcome.<sup>2</sup> Enhancing remyelination may also be beneficial for other demyelinating diseases, such as neuromyelitis optica, Krabbe disease, and metachromatic leucodystrophy.

Because of interest in remyelinating therapies, there is a need for methodologies that quantify remyelination in a clinical setting. Unfortunately, no such methodology currently exists; the use of several imaging techniques has been explored, but no single technique is sufficiently sensitive, specific for myelin, and correlated to clinical outcome.<sup>3</sup>

An alternative way to assess myelin kinetics would be to quantify the turnover rate of a relevant myelin component. Different components of myelin have a different turnover rates.<sup>4,5</sup> Although there are no lipids that are absolutely specific for myelin, galactosylceramide (a cerebroside, also known as galactosylcerebroside) is the most typical of myelin.<sup>5</sup> The half-replacement time of galactosylceramide is 94–250 days in adult mice, but has never been determined in humans.<sup>4</sup> In mice, the turnover of galactosylceramide has been reported to be a good measure of myelination, whereas several other myelin components correlated poorly with myelination.<sup>6</sup> By quantifying the turnover of galactosylceramide in humans, a measure for (altered) myelin kinetics might be obtained.

The ability to monitor demyelination and remyelination is essential to determine possible therapeutic efficacy of interventions that enhance remyelination. The imaging techniques that are currently being used to try to quantify this

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Received 9 May 2016; accepted 8 September 2016; published online on 15 October 2016. doi:10.1111/cts.12424

process are, however, not sufficiently sensitive or specific.<sup>3</sup>

The myelin sheath that surrounds most axons in the CNS is a spiral structure that arises from an extension of the plasma membrane of oligodendrocytes. The thickness of the myelin sheath is dynamic and may be modulated.<sup>7</sup> Myelin is composed of 40% water and 60% dry mass. The dry mass is composed of 70% lipids and 30% proteins; this high concentration of lipids facilitates the rapid conduction of action potentials along the nerve fiber. In the adult brain, the various components of myelin have specific turnover rates.<sup>5</sup> The most specific myelin degradation product is  $\beta$ -D-galactosylceramide (24:1;  $\beta$ -GalC).<sup>5</sup>

Stable isotope labeling is commonly used to study the *in vivo* kinetics of biomolecules.<sup>8–11</sup> This method relies on the labeling of a precursor and the subsequent quantification of the labeled target molecule over time. The stable isotope can be a direct precursor (e.g., a labeled amino acid for incorporation into proteins) or a more upstream precursor (e.g., deuterated water [D<sub>2</sub>O] or labeled glucose). Animal studies have shown that deuterium levels up to 15% are generally without harmful effects.<sup>8,9,12</sup> In humans, continuous administration of D<sub>2</sub>O administration over several months—resulting in deuterium levels of 1–2% in body water—did not result in adverse effects.<sup>13,14</sup>

After the stable isotope has been administered, mass spectrometry (MS) can be used to measure labeling of the target molecule. This technique separates molecules based on differences in mass (due to different isotope compositions), but not chemical structure. The resulting data can then be interpreted using several methods, for example, mass isotopomer distribution analysis, or compartmental modeling of both the precursor and target molecule.<sup>15</sup>

Here, we used a 70-day deuterium labeling protocol, combined with MS and compartmental modeling, to estimate the turnover rate of  $\beta$ -GalC (24:1) in healthy human subjects.

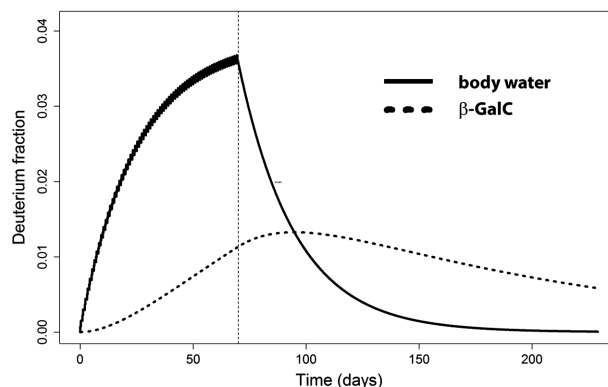
## MATERIALS AND METHODS

### Prestudy simulation

A prestudy simulation of the expected deuterium enrichment of body water and  $\beta$ -GalC was performed to support the study design. Assuming a daily body water clearance of 2 L and a total body water volume of 50 L, the half-life of deuterium in body water was set to a conservative estimate of 17 days.<sup>4,16</sup> Because the turnover rate of  $\beta$ -GalC in humans is unknown, we used the reported half-life for young adult mice of 90 days.<sup>4</sup> After simulations of different study designs, a 70-day labeling period with daily consumption of 120 mL of 70% D<sub>2</sub>O was selected (**Figure 1**). With this dosing regimen, the peak deuterium fraction in the total body water was estimated to reach 0.037 at day 70; after the dosing regimen ended, this fraction returned to baseline by day 200 (i.e., 130 days after the last D<sub>2</sub>O dose). The peak deuterium fraction in  $\beta$ -GalC was expected to reach 0.013 at approximately 30 days after the last D<sub>2</sub>O dose.

### Study design

During the study, the six healthy subjects were instructed to consume 120 mL of 70% D<sub>2</sub>O daily for 70 days. Weekly urine samples were collected and used to measure the percentage of D<sub>2</sub>O in the subject's total body water. The subjects



**Figure 1** Prestudy simulation of the deuterium enrichment of total body water and  $\beta$ -D-galactosylceramide ( $\beta$ -GalC) after 70 days of consuming a daily dose of 120 mL of 70% deuterated water (D<sub>2</sub>O). The vertical dashed line indicates day 70.

also underwent four lumbar punctures (LPs) for cerebrospinal fluid (CSF) collection. Blood was collected for safety measurements (routine hematology and chemistry). The LPs and venous punctures were performed 35, 70, 94, and 167 days after the start of the study. Because initial results indicated slower kinetics of  $\beta$ -GalC than expected, the study protocol was amended to include an additional long-term LP. This LP was taken at approximately 1.5 (subjects 3–6) or 2.0 (subjects 1 and 2) years after the start of the study.

### Subjects

Healthy male or female subjects between 18 and 70 years old with a body mass index of 18–30 kg/m<sup>2</sup> were to be enrolled after having given written informed consent. The subjects underwent a full medical screening, including medical history, a physical examination, blood chemistry, hematology and virology (hepatitis B, hepatitis C, and human immunodeficiency virus), urinalysis, and electrocardiogram to assess eligibility. Key exclusion criteria were: contraindications for LP, clinical significant abnormalities during screening, and regular user of any illicit drugs or history of drug abuse or a positive drug screen at screening.

### Study approval

The study was approved by the Medical Ethics Committee of the BEBO Foundation (Assen, The Netherlands). The study was conducted according to the Dutch Act on Medical Research Involving Human Subjects, and in compliance with Good Clinical Practice and the Declaration of Helsinki.

### Measurement of deuterium enrichment in the total body water

D<sub>2</sub>O content in total body water was calculated using a derivatization reaction with a ketone followed by gas chromatography/mass spectroscopy analysis. The deuterium fraction in the urine sample was calculated using the ratio of deuterated to nondeuterated forms. Measuring D<sub>2</sub>O in urine established it as reliable marker of body water D<sub>2</sub>O, and was used in other studies.<sup>8,11,17</sup> Urine was used because its collection is least invasive for the participating subjects.

### Measurement of deuterium enrichment of $\beta$ -GalC

$\beta$ -GalC was extracted from the CSF using a chloroform-methanol mixture. After isolation and subsequent evaporation of the organic layer, an aliquot of the reconstituted sample was injected into a high-performance liquid chromatography system (Shimadzu Nexera LC30; 's Hertogenbosch, The Netherlands) equipped with a Kinetic C18 column (100  $\times$  3.0 mm, 2.6  $\mu$ m; Phenomenex, Utrecht, The Netherlands) that was kept at ambient temperature. A gradient elution using 5% acetonitrile/95% water (v/v), 1% formic acid/5% acetonitrile/94% water (v/v/v), and acetonitrile as mobile phase was used to separate the  $\beta$ -GalC from matrix components and was delivered at a flow rate of 0.8 mL/min into the electrospray ionization chamber of the MS. Quantification was achieved with MS–MS detection in positive ion mode using an AB SCIEX Triple Quad 5500 LC-MS/MS (Nieuwerkerk aan den IJssel, The Netherlands) equipped with a Turboionspray interface at 650°C. The ion spray voltage was set at 5,500 V. The source parameters: curtain gas ion source gas 1 and 2, and collision gas were set at 30, 40, 60, and 6 psi, respectively. The compound parameters: the declustering potential, collision energy, entrance potential, and collision cell exit potential were 121, 129, 10, and 15 V for  $\beta$ -GalC. Detection of the ions was carried out in the multiple-reaction monitoring mode, by monitoring the transition pairs of 810.6  $\rightarrow$  630.6 amu (M0), m/z 811.6  $\rightarrow$  631.6 (M1), m/z 812.6  $\rightarrow$  632.6 (M2), m/z 813.6  $\rightarrow$  633.6 (M3), m/z 814.6  $\rightarrow$  634.6 (M4), m/z 815.6  $\rightarrow$  635.6 (M5), m/z 816.6  $\rightarrow$  636.6 (M6), ... and m/z 819.6  $\rightarrow$  639.6 (M9), where  $M_i$  is the  $\beta$ -GalC mass isotopomer with  $i$  additional neutrons compared with the mass isotopomer without any heavy isotope (M0). Quadrupoles Q1 and Q3 were set on unit resolution. The analysis data obtained were processed by Analyst software version 1.5.2.

The isotopomer data of  $\beta$ -GalC were used to calculate the change in average number of additional neutrons (the replacement of a hydrogen atom by a deuterium atom adds one neutron to  $\beta$ -GalC). This was then divided by the total number of hydrogen atoms in  $\beta$ -GalC (78) to yield the change from baseline of the average deuterium fraction of the hydrogen atoms in  $\beta$ -GalC.<sup>18</sup>

### Compartmental model

A compartmental model that describes the turnover of body water (precursor) and  $\beta$ -GalC (product) was fit to the data using the nonlinear mixed effects modeling program NONMEM version 7.3.0.<sup>19</sup> The first-order conditional estimation with interaction method was used to estimate typical parameter values and their interindividual variability. The interindividual variability was only included in the model when it significantly ( $p < 0.05$ ) improved the model fit (minus two times the log likelihood reported by NONMEM).

The body water turnover was characterized with two structural parameters: the daily water input/output and the size of the total body water pool. A theoretical estimate of subjects' total body water—based on their sex, height, weight, and age—was used as a covariate for daily water input/output.<sup>20</sup> This model described the deuterium enrichment of body water over time.

**Table 1** Subject characteristics

Characteristic	Mean (range) or number (%)
Age, y	28.2 (19–67)
Sex	
Male	2 (33)
Female	4 (67)
Weight, kg	67.5 (56.8–87.5)
Height, m	1.75 (1.64–1.90)
BMI	21.9 (19.3–25.3)

BMI, body mass index.

The production of  $\beta$ -GalC from the (indirect) precursor body water was characterized with two structural parameters: fraction of  $\beta$ -GalC with a fast turnover, and the turnover rate of that fraction. The turnover rate of the remaining fraction of  $\beta$ -GalC (with a slow turnover) was assumed to have a negligible impact on the  $\beta$ -GalC deuterium enrichment in this study, and was therefore not estimated (i.e., assumed to be zero).

## RESULTS

### Clinical study

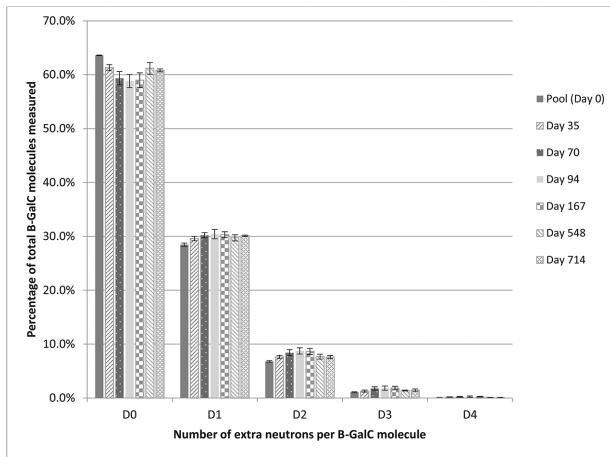
Six healthy adult subjects were enrolled in the study; their demographics are summarized in **Table 1**. All subjects completed the study, and all of the reported adverse events were mild and transient. Most frequent reported adverse events were headache (14%) and nasopharyngitis (10%). No post-dural puncture headache occurred. All subjects appeared to reach steady-state deuterium enrichment in body water during the 70-day labeling period. There was considerable interindividual variability in the extent of body water enrichment, with maximum values ranging from 0.015–0.039. Body water enrichment decreased rapidly after the last  $D_2O$  dose: to an average of 16.3% of maximum levels on day 93.

The maximum deuterium enrichment of  $\beta$ -GalC was about 25-fold lower than that of body water and ranged from 0.0005–0.0014. The enrichment of  $\beta$ -GalC decreased slowly: at day 167, the  $\beta$ -GalC enrichment was still high (92% of maximum enrichment on average). Even 1.5–2.0 years after the start of the study, a measurable  $\beta$ -GalC enrichment above baseline was still present in all subjects (average 41% of maximum enrichment; see **Figure 2**).

### Compartmental model output

Interindividual variability was only estimated for daily water input/output; for the other structural parameters, it did not result in a significantly improved model fit. The interindividual differences in deuterium enrichment of  $\beta$ -GalC were attributed exclusively to differences in deuterium enrichment of body water (precursor). The compartmental model was used to fit the data, resulting in an adequate characterization of the deuterium enrichment of both body water and  $\beta$ -GalC (**Figures 3 and 4**).

The final model's parameter estimates and variability are summarized in **Table 2**. All parameters were estimated with good precision (i.e., low standard errors). The typical values for the daily water input/output (3.25 L) and the size of the total body water pool (35.5 L) correspond to a turnover rate constant of 0.092 or a body water half-life of 7.6 days. The



**Figure 2** The isotopomers of  $\beta$ -D-galactosylceramide ( $\beta$ -GalC) and how the relative quantity of each isotopomer changes over time during and after 10 weeks of deuterated water ( $D_2O$ ) labeling. D0 implies that the  $\beta$ -GalC molecule has no extra neutron, whereas D1, D2, D3, and D4 means that the  $\beta$ -GalC molecule has 1, 2, 3, and 4 extra neutrons, as determined by mass spectrometry. In the baseline sample (pool, day 0) the D1, D2, D3, and D4 isotopomers are also present, which is due to natural occurrence of stable radioisotopes in food substances (primarily C13).

**Table 2** Parameter estimates derived from the final model

	Estimate	RSE (%) <sup>a</sup>	IIV <sup>b</sup>
DWI, L·day <sup>-1</sup>	3.25	6.55	15.5
BW, L	35.5	3.24	NE
$K_{\beta\text{-GALC}}$ , day <sup>-1</sup>	0.00168	8.81	NE
$S_{\beta\text{-GALC}}$	0.379	9.66	NE
Additive error <sub>body water</sub> <sup>c</sup>	0.00130	20.5	NA
Additive error <sub><math>\beta\text{GalC}</math></sub> <sup>c</sup>	0.000116	19.7	NA

BW, body weight; DWI, daily water intake; NA, not applicable; NE, not estimated; RSE, relative standard error.

<sup>a</sup>RSE, calculated from NONMEM's reported standard error.

<sup>b</sup>Coefficient of variation (%) of interindividual variability.

<sup>c</sup>Residual variability expressed as SD.

estimated  $\beta$ -GalC turnover rate constant (of the fast fraction) is much lower: 0.00168, which corresponds to a half-life of 413 days. This slow  $\beta$ -GalC turnover is consistent with its relatively low, but long-lasting deuterium enrichment.

## DISCUSSION

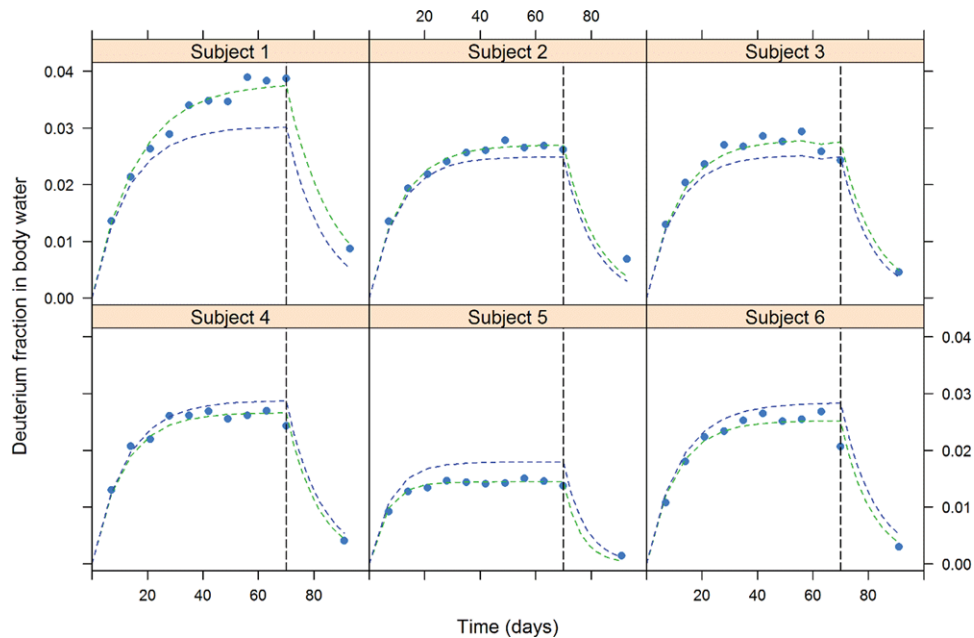
Here, we report that orally administered deuterated water can be used to label and track the metabolism of  $\beta$ -GalC in human subjects, and these data can be used to estimate the turnover rate for this myelin breakdown product. The low, but long-lasting enrichment shows that  $\beta$ -GalC has a slow turnover. We used compartmental modeling to characterize this turnover, as it allowed us to account for differences in deuterium enrichment of body water between subjects and within subjects over time. With this empirical model, we estimated a half-life of 413 (352–499) days for  $\beta$ -GalC in CSF. Importantly, the turnover of  $\beta$ -GalC has been reported for mice,<sup>4</sup> but this has never been reported in human subjects.

An estimated fraction of  $\beta$ -GalC with a fast turnover was included in the model to accurately describe the  $\beta$ -GalC

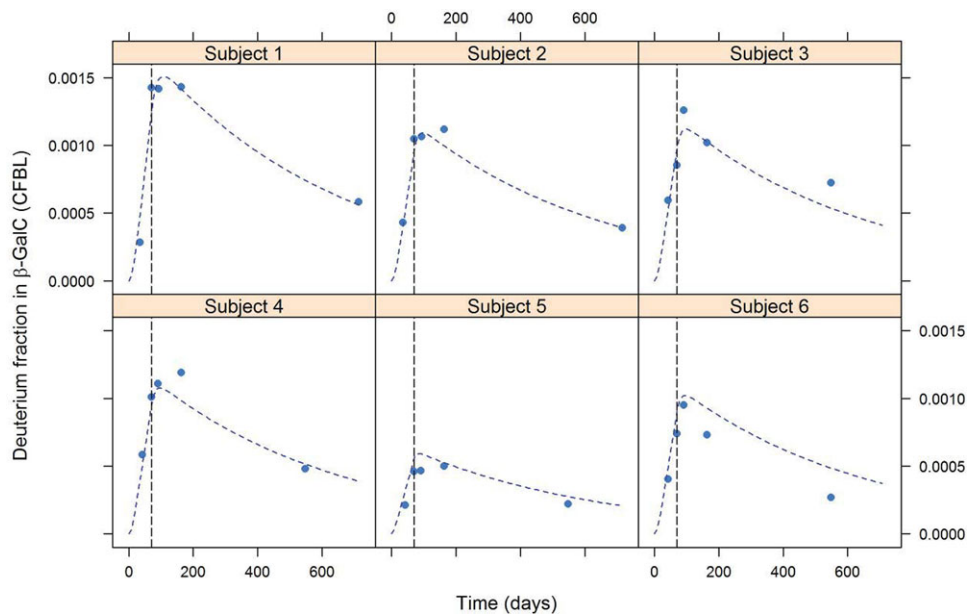
enrichment data. Although this factor does not have a direct physiological meaning, one possible interpretation would be that  $\beta$ -GalC in CSF is produced from two metabolic compartments. One compartment would represent myelin with a shorter half-life (413 days, as estimated by the model). The other compartment would turnover even more slowly, to the extent that the amount of deuterium enrichment present in its breakdown products after a 70-day labeling period is negligible. In this interpretation of the empirical model, the estimated fraction of  $\beta$ -GalC with a fast turnover then represents the fraction of  $\beta$ -GalC in the CSF that originates from the compartment with a “fast” turnover. This combination of fast and slow turnover seems physiologically plausible, as it has been described for various myelin components in animals: cholesterol, galactosylceramide, sulfatide, and phospholipids.<sup>4,21</sup> One of several possible interpretations of these data is that there is a more stable metabolic pool consisting of inner layers of myelin that may be less accessible for metabolic turnover and that some of the newly formed myelin remains in outer layers and stays accessible for whatever mechanisms are involved in catabolism, thus accounting for the rapid turnover of this pool.<sup>5</sup>

The thickness of the myelin sheath can be modified by oligodendrocytes, and oligodendrocyte turnover contributes to myelin remodeling. However, we expect the effect of oligodendrocyte turnover to be minimal as myelination has been shown to be considerably more dynamic than oligodendrocyte generation in human white matter.<sup>7</sup> Different myelin dynamics in different parts of the brain are not taken into account in our study as we estimated the average  $\beta$ -GalC turnover.

It is important to realize that the turnover of  $\beta$ -GalC does not necessarily represent the turnover of myelin. Two scenarios are possible: either the myelin turnover is slower than the  $\beta$ -GalC turnover and therefore rate limiting, or the  $\beta$ -GalC turnover is slower than myelin turnover and therefore rate-limiting.  $\beta$ -GalC kinetics would only be a suitable marker of myelin turnover if the myelin turnover is slower and therefore the rate limiting step. If  $\beta$ -GalC kinetics are rate limiting, it would not be suitable as a marker of myelin kinetics. We believe it is far more likely, however, that myelin turnover is rate limiting. First of all, a previous study in mice demonstrated that labeling of  $\beta$ -GalC with  $D_2O$  provided a good proxy marker for myelin turnover.<sup>6</sup> Second, most metabolic processes in the human body have a much faster turnover than 413 days<sup>21–23</sup> and it is therefore far more probable that the  $\beta$ -GalC turnover that we measure is this slow because  $\beta$ -GalC is attached to the myelin surrounding nerves, and nerves are known to have an extremely slow turnover.<sup>22</sup> Conversely it is unlikely that  $\beta$ -GalC removal from the CSF compartment would be rate limiting and, therefore, influence the slow turnover of  $\beta$ -GalC that we report here. CSF proteins in general have a residence time in the CSF of hours to days, maybe some of weeks, but not of months to years.<sup>6,23–25</sup> An average adult human produces approximately 500 mL of CSF per day, which, with an average CSF volume of 150 mL, will lead to replacement of all circulating CSF more than three times daily. It would be hard to imagine how any molecule could reside in the CSF for months without being removed. It is therefore far more likely that if we measure deuterated



**Figure 3** Individual body water deuterium enrichment profiles and model predictions over time during and following 70 days of oral deuterated water ( $D_2O$ ) administration. In each panel, the individual observations are represented as blue dots, the population predictions are represented as a dashed blue line, and the individual prediction is represented as a dashed green line. The vertical dashed line indicates day 70.



**Figure 4** Individual baseline-corrected  $\beta$ -D-galactosylceramide ( $\beta$ -GalC) deuterium enrichment profiles and model predictions during and following 70 days of oral deuterated water ( $D_2O$ ) administration. In each panel, the observations are represented as blue circles, and the model prediction is shown as a dashed blue line. The vertical dashed line indicates day 70. Baseline samples were healthy volunteer cerebrospinal fluid (CSF) samples from a biobank. CFBL, change from baseline.

$\beta$ -GalC in the CSF over a very long period of up to 1.5 years, that there is a continuous source of shedding of  $\beta$ -GalC into the CSF from a reservoir, which we know from the literature to be myelin in the CNS.<sup>26</sup> In conclusion, although the turnover of  $\beta$ -GalC does not necessarily represent the turnover of myelin,<sup>6</sup>  $\beta$ -GalC found in the CSF is most likely produced

by the breakdown of myelin in the CNS, and  $\beta$ -GalC CSF kinetics therefore likely reflect myelin turnover.

A measurement of the turnover rate of myelin in mice of various ages revealed that the turnover rate ranged from 94–250 days.<sup>4</sup> This rate differs considerably from the rate that we observed in healthy human subjects. However, such a

difference in the rate of a metabolic process between humans and mice is not unexpected.<sup>27–29</sup> For example, previous studies reported that the overall turnover rate for proteins in mice is nearly 10-fold higher in mice than in humans.<sup>22</sup> The rate of cholic acid synthesis is 9.3-fold higher in mice than in humans, and the rate of cholesterol synthesis is 16-fold higher in mice. These differences are consistent with our observed difference between mice and humans with respect to the apparent turnover rate of myelin. Although it is likely that the turnover of myelin is correlated with age, the relatively low number of subjects in our study precluded the possibility of drawing any meaningful conclusions with respect to the relationship between age and turnover rate.

Here, we used compartmental modeling to accurately describe deuterium enrichment in body water and labeling of  $\beta$ -GalC during and after chronic ingestion of deuterium. Our use of compartmental modeling enabled us to interpret the isotope labeling data in the absence of steady-state labeling of any precursor. This was an important feature of our study, as the deuterium fraction in body water did not reach steady-state levels for a significant part of the study duration. It is also important to note that the model and its estimated parameters are empirical. As body water is not an immediate precursor of  $\beta$ -GalC, the apparent myelin turnover rate constant should not be interpreted as a measure for  $\beta$ -GalC turnover. Instead, it is a single rate constant for the entire biochemical pathway between body water and  $\beta$ -GalC in CSF. As this pathway includes the synthesis and degradation of myelin, the estimated turnover rate constant of  $\beta$ -GalC may, however, be suitable as a biomarker for the kinetics of myelin. Interestingly, the model did not improve upon including interindividual variability in the myelin kinetics; correcting for the individual differences in body water turnover seemed to explain most of the interindividual variability seen in the deuterium enrichment of  $\beta$ -GalC as well. This suggests that the variability in myelin kinetics in healthy human subjects is modest, although confirmation of this would require a study with more than six subjects.

The method that we describe here, in which we measured the deuterium enrichment of  $\beta$ -GalC in the CSF of healthy human subjects, may be used to test novel therapeutic interventions designed to enhance remyelination. Currently, the demyelination and remyelination process is measured with a magnetic resonance imaging scan. A recent review evaluated imaging modalities that may be better suited to measuring myelin content *in vivo*, including magnetization transfer ratio, restricted proton fraction *f* (from quantitative magnetization transfer measurements), myelin water fraction, diffusion tensor imaging, and positron emission tomography imaging.<sup>3</sup> Unfortunately, no individual modality provides sufficient sensitivity or specificity for myelin, nor can any individual method provide a suitable correlation with clinical features.<sup>3</sup> Positron emission tomography imaging using [<sup>11</sup>C]PIB and [<sup>11</sup>C]MeDAS are promising imaging modalities. However, further studies are needed in order to validate their use, particularly with respect to the study of multiple sclerosis in human patients.<sup>3</sup> We therefore suggest that stable isotope labeling using D<sub>2</sub>O may be a more useful method for measuring demyelination and remyelination. Drugs that can increase remyelination are currently being developed. There-

fore, a method for quantifying remyelination is essential in order to determine the efficacy of such drugs. In this respect, we suggest that our method for measuring myelin kinetics may provide superior results compared with the imaging modalities that are currently available.

In a proof-of-concept study with a compound that is expected to enhance remyelination, it may not be necessary to follow-up subjects for the same 1.5–2.0-year period of this study. This is because peak  $\beta$ -GalC enrichment is probably more indicative of myelination than the elimination phase of  $\beta$ -GalC. Therefore, there would be no need to measure long-term elimination of deuterium from  $\beta$ -GalC. This could shorten the study duration to as little as 15 weeks: 10 weeks of deuterium labeling and 5 weeks of washout to allow  $\beta$ -GalC to reach peak enrichment. Five weeks is approximately five times the calculated half-life of D<sub>2</sub>O disappearance from body water. In the absence of ongoing D<sub>2</sub>O labeling, deuterium-enrichment of the  $\beta$ -GalC cannot further increase when D<sub>2</sub>O has been washed-out from body water.

In this study, we developed a safe methodology to measure the turnover rate of the myelin component  $\beta$ -GalC in humans, which is likely indicative of myelination. Additional studies in patients with multiple sclerosis are needed to validate this method as an outcome measure in clinical trials with remyelination therapies.

The ability to accurately measure the rate of myelin formation and breakdown may be used to measure pharmacological effects of novel drugs designed to increase remyelination, thereby facilitating the development of a more effective treatment for patients with multiple sclerosis or other demyelinating diseases/disorders.

**Acknowledgments.** The authors thank the volunteers who participated in the clinical study.

**Author Contributions.** K.M.S.K. and G.J.G. wrote the manuscript. K.M.S.K., J.S., G.D., A.V., J.C.C., and G.J.G. designed the research. K.M.S.K., J.L.H., A.C., and G.J.G. performed the research. S.C.G., J.S., T.H., T.dB., and H.M. analyzed the data. T.dB. and H.M. contributed new reagents/analytical tools.

**Conflict of Interest.** G.D., A.V., and J.C.C. are employees of Biogen.

**Source of Funding.** Funding for this study was provided by Biogen

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