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Longitudinal biomarker evaluation in Fabry disease patients receiving lentivirus-mediated gene therapy

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Abstract

Aim: In 2016, a team of Canadian researchers initiated the world's first gene therapy clinical trial for Fabry disease. The study, aiming to determine the safety and toxicity of lentivirus α-galactosidase A transduced autologous CD34+ cells in adult males with Fabry disease (n = 5), was conducted at three Canadian centers. The objective of the present work was to evaluate a profile of Fabry disease-related biomarkers in five participants during a 5-year surveillance period, as part of the evaluation of this novel therapy.

Methods: Sixteen globotriaosylceramide (Gb_3) isoforms and eight globotriaosylsphingosine (lyso-Gb_3) analogues were measured by LC-MS/MS in plasma and urine at numerous time points before and after gene therapy. Plasma and peripheral blood leukocyte α-galactosidase A activity were also measured.

Results: Levels of urine and plasma lyso-Gb_3 and analogues, and urine Gb_3 were lower after gene therapy and while treated with enzyme replacement therapy (ERT) compared to baseline (before gene therapy) in participants treated with ERT only. Three participants chose to cease ERT treatment at some point after gene therapy. Two of these patients had lower levels of urine and plasma lyso-Gb_3 and analogues compared to baseline, whereas one participant had higher levels of these biomarkers compared to baseline. An increase in urine Gb_3 was observed when ERT treatment ceased after gene therapy ($P < 0.05$) in all three participants.

Conclusion: The evaluation of this complete glycosphingolipid biomarker profile was useful in monitoring the biochemical response to gene therapy in this cohort of five patients with Fabry disease.

Keywords: Fabry disease, biomarkers, gene therapy, globotriaosylceramide, globotriaosylsphingosine, analogues

INTRODUCTION

Fabry disease (OMIM #301500) is a genetic disorder caused by a mutation in the *GLA* gene located on the X chromosome (position Xq22)[1]. This leads to a deficiency of the lysosomal enzyme α-galactosidase A (EC3.2.1.22), which is involved in the catabolism of glycosphingolipids with terminal α-galactosyl moieties, and to their subsequent systemic accumulation[2]. It is noteworthy to mention that even though Fabry disease is an X-linked disorder, women may also be affected, and sometimes as severely as men[3]. The disease typically progresses gradually, starting in childhood or adolescence, with acroparesthesia, pain crises, hypohidrosis, gastrointestinal problems, proteinuria, and angiokeratomas[4,5]. In adulthood, a multisystemic disorder is on-going where cardiac (left ventricular hypertrophy, myocardial fibrosis, heart failure, arrythmias), renal (proteinuria, low glomerular filtration rate, end-stage renal failure) and neurological (ischemic lesions, transient ischemic attacks, stroke) involvement are also observed[6-9]. Life expectancy of untreated patients with Fabry disease is shortened by 20 years for men and 10 years for women[9], and quality of life is severely impaired. Treatments aimed at restoring enzyme activity are presently approved for Fabry disease. Enzyme replacement therapy (ERT) was approved in 2001 and consists of intravenous infusions of recombinant α-galactosidase A every two weeks, either with agalsidase alfa (Replagal™, Takeda Pharmaceuticals, 0.2 mg/kg body weight) or agalsidase beta (Fabrazyme®, Sanofi, 1.0 mg/kg body weight)[10,11]. Pegunigalsidase alfa (now branded Elfabrio, Protalix Biotherapeutics and Chiesi Global Rare Diseases, 1 mg/kg body weight) has been approved by the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) to treat adults with Fabry disease[12,13] in 2023. The pharmacological chaperone migalastat (Galafold™, Amicus Therapeutics, 123 mg capsule) was approved in 2016. This medication is taken orally every other day and can be prescribed to patients having mutations amenable to increasing endogenous residual enzyme activity by stabilizing mutant forms of α-galactosidase A in the endoplasmic reticulum and assisting proper trafficking to the lysosome[14].

Research for novel Fabry disease therapies is on-going. A promising avenue is the *ex vivo* gene therapy approach, where hematopoietic stem cells are harvested from the patient, engineered to express α-galactosidase A, and then returned to the patient for autologous engraftment[15]. In July 2016, a team of Canadian researchers initiated the world's first gene therapy clinical trial for Fabry disease (NCT02800070), which was completed in February 2024 (the FACTs project)[16]. The objective of this study was to determine the safety and toxicity of lentivirus α-galactosidase A transduced CD34+ cells in adult males with Fabry disease. Recruitment occurred in three Canadian centers (Calgary, Alberta; Halifax, Nova Scotia; Toronto,

Ontario). Among other outcomes, biomarker analyses are needed as part of the evaluation of this new therapy. A biomarker is a measurable characteristic (or parameter) indicating changes in biological processes, either normal or pathogenic. Biomarkers are thus useful for assessing the response to a therapeutic intervention, studying disease progression, and improving patient outcomes[17,18]. The analysis of globotriaosylceramide (Gb_3) as a useful biomarker played a key role in the approval of ERT treatments for Fabry disease. The approval followed two short-term randomized, placebo-controlled, double-blind studies[19]. It was shown that ERT cleared microvascular endothelial Gb_3 deposits from the kidneys, heart, and skin, and that plasma Gb_3 levels reflected the clearance of these deposits[19-21]. Aerts *et al.* suggested that circulating globotriaosylsphingosine (lyso-Gb_3), the deacylated form of Gb_3, could also be a useful biomarker to monitor Fabry disease[22]. More recently, Auray-Blais *et al.* performed metabolomic studies using time-of-flight mass spectrometry and discovered the presence of lyso-Gb_3 analogues[23,24] and Gb_3 isoforms and analogues[25,26] in urine and plasma of Fabry patients. As confirmed by tandem mass spectrometry and exact mass measurements, analogues and isoforms have modifications on their sphingosine and fatty acid moieties, respectively. Some analogues were associated with the left ventricular mass index (LVMI) and the Mainz Severity Score Index (MSSI) in patients with a late-onset IVS4+919G>A cardiac variant mutation in a Taiwanese cohort[27]. Urine lyso-Gb_3 analogues were also found to be more elevated than lyso-Gb_3 itself[28].

The objective of the present work was to evaluate this panel of Fabry disease biomarkers measured as part of the Canadian gene therapy clinical trial[16]. Gb_3, lyso-Gb_3, and their isoforms and analogues were measured in plasma and urine for all five Fabry participants at numerous time points as part of a surveillance and monitoring period of 5 years. The chemical structures of the biomarkers under study are shown in Figure 1. Enzyme activity levels of α-galactosidase A were also measured in plasma and peripheral blood leukocytes for the same duration. To our knowledge, this is the first time that a complete biochemical profile for biomarker evaluation has been performed as part of a gene therapy clinical trial for Fabry disease.

METHODS

Ethics approval

The biomarker study was conducted in agreement with the Declaration of Helsinki and approved by the Institutional Review Board at the Faculty of Medicine and Health Sciences at the Centre intégré universitaire de santé et de services sociaux de l'Estrie-Centre hospitalier universitaire de Sherbrooke (CIUSSS de l'Estrie-CHUS), where the biomarker analyses were performed (Project #2017-1575), authorization obtained on 14 November 2016). The trial (NCT02800070) was approved by Health Canada on 26 April 2016, and conducted in compliance with local institutional and/or university Human Experimentation Committee requirements. Research Ethics Board (REB) approval was provided by University Health Network REB, Alberta Health Services REB, Capital Health Services REB, Hamilton Integrated Research Ethics and the Medical College of Wisconsin Institutional Review Board. Informed consent was obtained from all subjects involved in the study.

Reagents

The internal standard (IS) of lyso-Gb_3 and related analogues (N-glycinated lyso-Gb_3, or lyso-Gb_3-Gly) was synthesized in-house as previously described[29]. Lyso-Gb_3, [Gb_3(d18:1)(C17:0)], [Gb_3(d18:1)(C18:0)D_3], and [Gb_3(d18:1)(C23:0)] were bought from Matreya LLC (State College, PA, USA). HPLC-grade acetonitrile, and methyl tert-butyl ether (MTBE) were obtained from MilliporeSigma (Darmstadt, Germany). Formic acid (99+% purity) was from Acros Organics (Morris Plains, NJ, USA), while A.C.S. grade o-phosphoric acid (85% purity), ammonium hydroxide (29%), and Optima LC/MS grade water and methanol were from Fisher Scientific (Fair Lawn, NJ, USA).

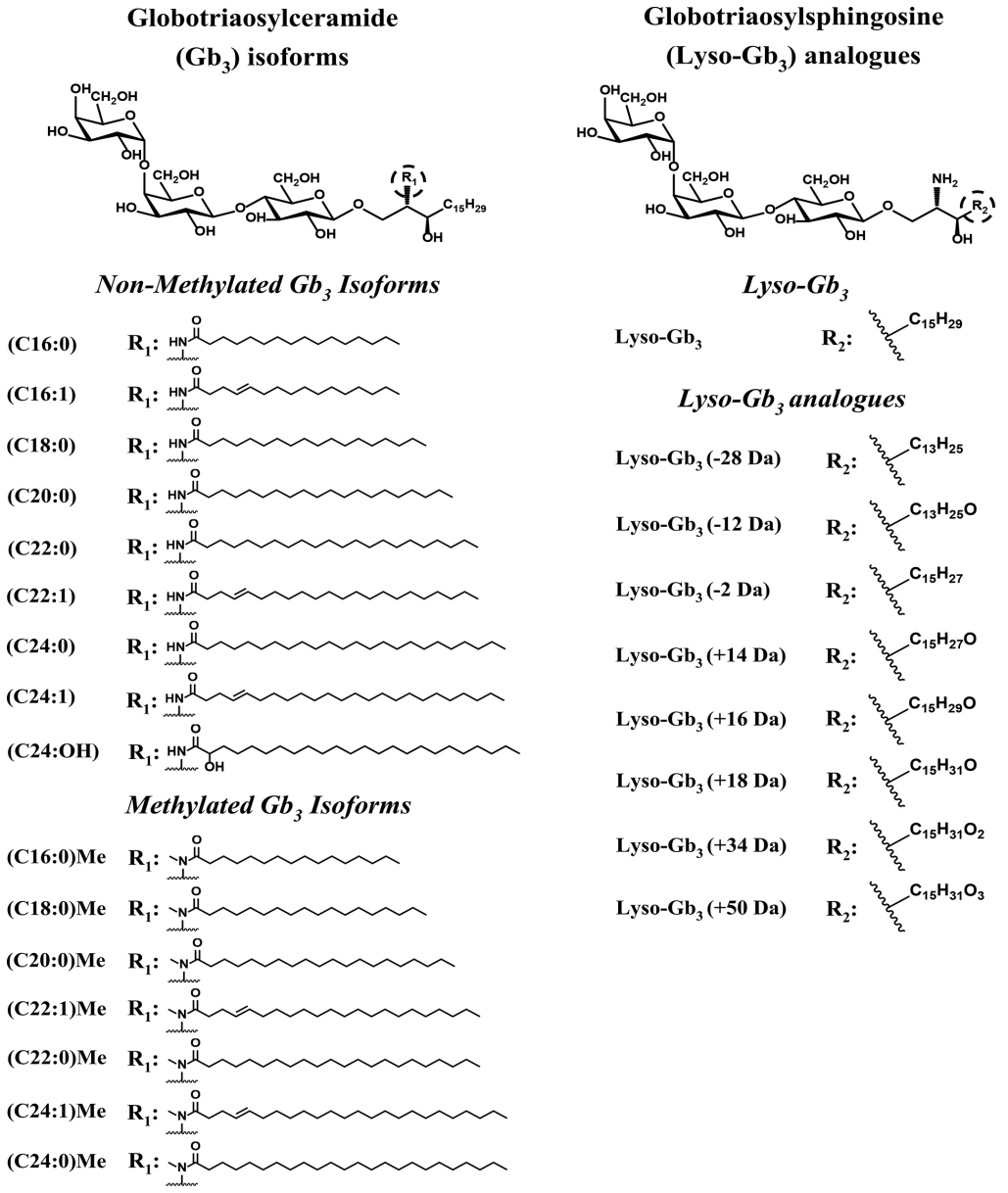

Figure 1. Chemical structures of globotriaosylceramide (Gb_3) isoforms, globotriaosylsphingosine (lyso-Gb_3), and related analogues. The exact positions of the hydroxyl group on the fatty acid of Gb_3(C24:OH), and of the double bond on the fatty acids of Gb_3(C16:1), Gb_3(C22:1), Gb_3(C24:1), Gb_3(C22:1)Me, and Gb_3(C24:1)Me isoforms are unknown and might differ from the position displayed in the figure.

Study participants

Biomarkers were evaluated in all five participants from the Fabry disease gene therapy clinical trial (NCT02800070). Eligibility criteria at baseline were previously described in detail[16]. Briefly, recruitment was restricted to men aged 18 to 50 years with a confirmed classical Fabry disease phenotype (< 7.0% or absent α-galactosidase A activity in plasma or leukocytes) on ERT for at least 6 months and with adequate organ function. Fabry symptoms at baseline and treatment phase outcomes were previously described[16]. *GLA* mutations and age at transplant were the following: Participant #1: p.Gln321Arg, 48 years; Participant #2: p.Ser345Pro, 39 years; Participant #3: p.Ala143Pro, 40 years; Participant #4: p.Ala143Pro, 37 years; Participant #5: p.Tyr134Ser, 30 years.

Conduct of the study and interventions

This gene therapy clinical trial was subdivided into five distinct phases that were previously described[16]. Briefly, ERT was stopped for a minimum of 30 days before gene therapy. Levels of α-galactosidase A activity were then assessed to establish baseline measurements. During the mobilization phase, peripheral blood CD34+ hematopoietic stem and progenitor cells (HSPCs) were mobilized at the regional stem cell center using filgrastim (Participants #1 and #5) or a combination of filgrastim and plerixafor (Participants #2, #3, and #4) as mobilizing agents, followed by leukapheresis. CD34+ selected cells were transduced with lentiviral α-galactosidase A and cryopreserved while waiting for the certificate of analysis approval to be obtained from Health Canada (approximately 30 days). The day before infusion of autologous CD34+ transduced cells, participants received melphalan IV (100 mg/m^2). Safety review of participant data and a return to ERT were performed 1 month post-transplant, except for Participant #3, who chose not to restart ERT. ERT for the other participants was paused after at least 6 months post-transplant if certain clinical endpoints were reached and patients consented to this change in procedure. Participants were followed for a period of 5 years. Urine and plasma specimens were collected for biomarker evaluation at different time points as part of this follow-up period. Figure 2 shows the timeline for each participant, including the urine and plasma specimen collection time points, the periods where the participants were treated with ERT, and the type of ERT treatment. Participant #1 was treated with Replagal up to one month before transplant, then with Fabrazyme from 1 to 18 months post-transplant, and then ERT treatment was stopped. Participants #2 and #5 were treated with Fabrazyme up to one month before transplant and resumed Fabrazyme at 1 month post-transplant until the end of the 5-year follow-up. Participant #3 was treated with Replagal up to one month before transplant, and never resumed ERT after transplant. Finally, participant #4 was treated with Fabrazyme up to one month before transplant, and then with Fabrazyme from 1 to 7 months post-transplant when ERT treatment was stopped.

Sample collection

For each time point, 4 milliliters (mL) of blood was collected in a lavender (potassium EDTA) vacutainer blood collection tube and 15 mL of urine was collected in a urine specimen container. Immediately after collection, blood specimens were centrifuged for 10 min at 2 000× *g* (room temperature), and the plasma fraction was transferred into a polypropylene tube. Urine and plasma specimens were kept frozen (-20 °C) until biomarker quantitation in Sherbrooke.

Biomarker analyses

Methylated and non-methylated Gb_3 isoforms in urine

Regarding the analysis of Gb_3 isoforms in urine, we used a quantitative assay that was devised according to the metabolomic study published by our group[26]. This urine assay was devised on a newer generation of mass spectrometer that allowed the analysis of more molecules simultaneously without compromising the sensitivity and reproducibility of the results. It is noteworthy to mention that after performing urinary metabolomic studies with a time-of-flight mass spectrometry system, we discovered different groups of Gb_3 biomarkers[25,26] for Fabry disease. Among these groups, we detected methylated and non-methylated Gb_3 isoforms. The main difference between these two groups is that methylated Gb_3 isoforms (n = 7) have a chemical structure comprising different fatty-acid chains where the methylation is on the nitrogen atom [see Figure 1]. The exact position of the methyl group was determined by mass spectrometry[26]. In fact, we found that the isoform Gb_3(d18:1)(C22:0)Me had fragments at m/z 296.3 on the methylated sphingosine, and at m/z 354.4 on the methylated fatty acid. These studies thus confirmed that the methylation process is on the nitrogen atom shared by the sphingosine and the fatty acid fragments[26]. Moreover, it has been reported in the literature that methyltransferases catalyze methylation for different biological processes[30-33], thus confirming these methylated molecules.

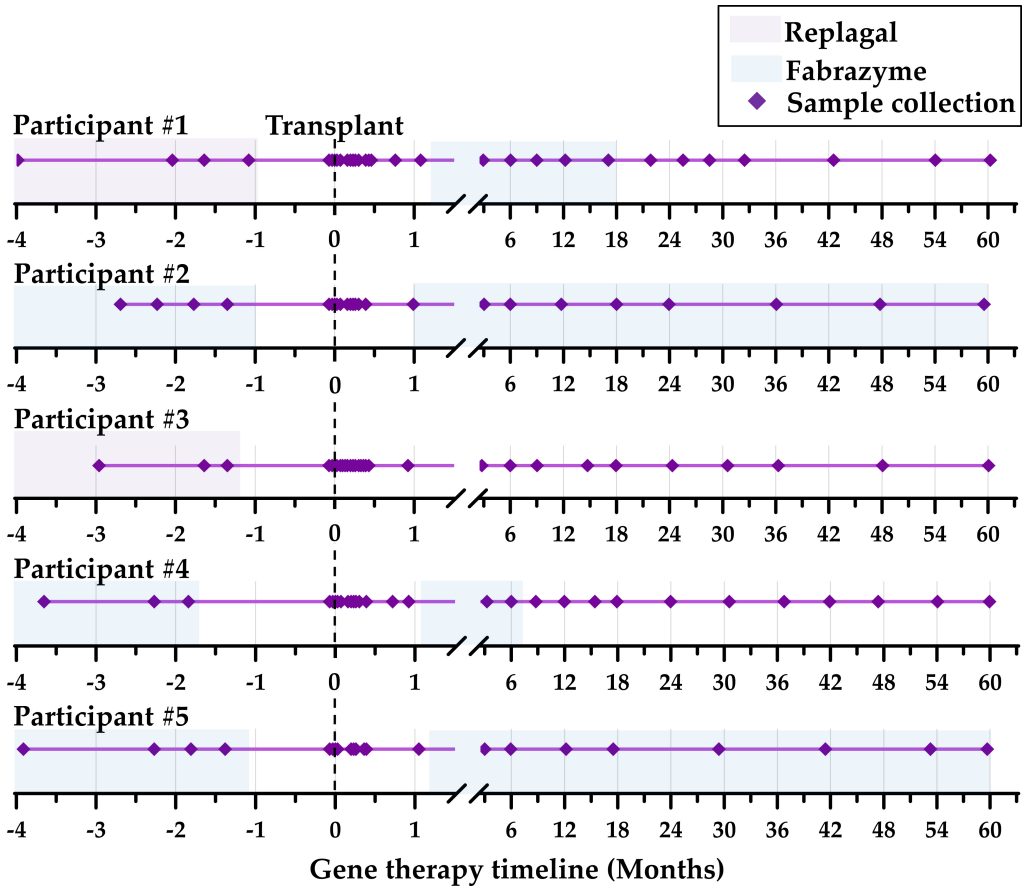

Figure 2. Urine and plasma specimen collection time points according to the types of treatment for 5 Fabry patients who received gene therapy.

Seven methylated Gb_3 isoforms [Gb_3(d18:1)(C16:0)Me; Gb_3 (d18:1)(C18:0)Me; Gb_3(d18:1)(C20:0)Me; Gb_3(d18:1)(C22:1)Me; Gb_3(d18:1)(C22:0)Me; Gb_3(d18:1)(C24:1)Me; and Gb_3 (d18:1)(C24:0)Me], and eight non-methylated Gb_3 isoforms [Gb_3(d18:1)(C16:0); Gb_3(d18:1)(C18:0); Gb_3(d18:1)(C20:0); Gb_3(d18:1)(C22:1); Gb_3(d18:1)(C22:0); Gb_3(d18:1)(C24:1); Gb_3(d18:1)(C24:0); Gb_3(d18:1)(C24:OH)] were analyzed in urine specimens. Tandem mass spectrometry multiple reaction monitoring (MRM) transitions are shown in Supplementary Table 1. The samples were purified by liquid-liquid extraction with MTBE and analyzed by ultra-high-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) as previously described[34,35]. A standard of Gb_3(d18:1)(C17:0) was used to prepare the calibration curve, and a standard of deuterated Gb_3(d18:1)(C18:0)D_3 was used as the IS. Results were normalized to creatinine. The reference values were ≤ 0.7 nmol/mmol creatinine for total methylated Gb_3 and ≤ 6.5 nmol/mmol creatinine for total non-methylated Gb_3.

Total Gb_3 in plasma

Regarding the selection of plasma Gb_3 isoforms, we used an assay that was already devised and validated in our laboratory, thus readily available, when the gene therapy clinical trial started. This specific assay was devised for the quantification of the most abundant Gb_3 isoforms in plasma.

Six Gb_3 isoforms [Gb_3(d18:1)(C16:1); Gb_3(d18:1)(C16:0); Gb_3(d18:1)(C18:0); Gb_3(d18:1)(C22:0); Gb_3(d18:1)(C24:1); and Gb_3(d18:1)(C24:0)] were analyzed in plasma specimens. Tandem mass spectrometry

MRM transitions are shown in Supplementary Table 1. The samples were purified by liquid-liquid extraction and analyzed by high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS). A standard of Gb_3(d18:1)(C17:0) was used as a calibrator for isoforms Gb_3(d18:1)(C16:1), Gb_3(d18:1)(C16:0), and Gb_3(d18:1)(C18:0), while a standard of Gb_3(d18:1)(C23:0) was used as a calibrator for isoforms Gb_3(d18:1)(C22:0), Gb_3(d18:1)(C24:1), and Gb_3(d18:1)(C24:0). Gb_3(d18:1)(C18:0)D3 was used as the IS. The reference value was ≤ 4961 nmol/L for total Gb_3.

Lyso-Gb_3 and related analogues in urine and in plasma

Two previous metabolomic studies[23,24] led us to detect a total of 8 analogues of lyso-Gb_3 in urine and plasma specimens of patients with Fabry disease. Lyso-Gb_3 (-12) and lyso-Gb_3 (+14) were only detected in urine specimens, while lyso-Gb_3 (+18) was only detected in plasma specimens. It is noteworthy to mention that the abundance of these analogues varies significantly between these two matrices, as shown in Supplementary Figures 1 and 2.

Lyso-Gb_3 and related analogues in urine

Lyso-Gb_3 and seven related analogues (lyso-Gb_3 -28 Da; lyso-Gb_3 -12 Da; lyso-Gb_3 -2 Da; lyso-Gb_3 +14 Da; lyso-Gb_3 +16 Da; lyso-Gb_3 +34 Da; and lyso-Gb_3 +50 Da) were analyzed in urine by UHPLC-MS/MS following a solid phase extraction (SPE) purification procedure as previously described[29,36]. Tandem mass spectrometry MRM transitions are shown in Supplementary Table 1. Standards of lyso-Gb_3 and lyso-Gb_3-Gly were used for calibration and IS, respectively. Results were normalized to creatinine. The reference values for lyso-Gb_3, lyso-Gb_3 -28 Da, lyso-Gb_3 -12 Da, lyso-Gb_3 -2 Da, and lyso-Gb_3 +14 Da were "not detected", while reference values for lyso-Gb_3 +16 Da, lyso-Gb_3 +34 Da, and lyso-Gb_3 +50 Da were ≤ 25, ≤ 20, and ≤ 85 pmol/mmol creatinine, respectively.

Lyso-Gb_3 and related analogues in plasma

Lyso-Gb_3 and six related analogues (lyso-Gb_3 -28 Da; lyso-Gb_3 -2 Da; lyso-Gb_3 +16 Da; lyso-Gb_3 +18 Da; lyso-Gb_3 +34 Da; and lyso-Gb_3 +50 Da) were analyzed in plasma specimens by UHPLC-MS/MS following a SPE purification procedure as previously described[37]. Tandem mass spectrometry MRM transitions are shown in Supplementary Table 1. Standards of lyso-Gb_3 and lyso-Gb_3-Gly were used for calibration and IS, respectively. The reference values were "not detected", except for lyso-Gb_3 (≤ 2.4 nM), lyso-Gb_3 -2 Da (≤ 0.9 nM), and lyso-Gb_3 +34 Da (≤ 0.3 nM).

α-galactosidase a enzyme assay

The α-galactosidase A assay uses a fluorescent artificial substrate 4-methylumbelliferyl-alpha-D-galactopyranoside based on the method previously described by Desnick *et al.*[38]. The normal range for α-galactosidase a enzyme activity was 5.1-9.2 nmol/hr/mL in plasma and 24-56 nmol/hr/mg protein in leukocytes.

Statistical analyses

Statistical analyses and longitudinal follow-up graphs were generated using GraphPad Prism 9.5.1. (GraphPad Software, San Diego, CA, USA). Each biomarker was evaluated at two different periods: Enzyme Replacement Therapy-Gene Therapy (ERT-GT) and only Gene Therapy (GT) compared to the baseline period. The baseline value (BV) was defined as the mean of the biomarker values obtained at each time point in the baseline period, which is the pre-GT period when the participant was treated with ERT (-4 to -1 month). The ERT-GT period included the time points collected when ERT treatment was resumed at 1 month post-GT and was useful for comparing ERT treatment alone with ERT + gene therapy. The GT period included the time points collected when ERT treatment was paused after a minimum of 6 months

post-GT and was useful for comparing ERT treatment alone with gene therapy alone. Mean deltas and 95% confidence intervals (CI) were calculated for each participant during ERT-GT and GT periods.

Deltas were defined as the difference between BV and biomarker values obtained at each time point collected during a specific period, expressed as a percentage of BV. Despite a small sample, normality of the population distribution was assumed, and therefore CI were based on a Student's distribution. *P*-values were obtained using a two-sided one-sample Student's t-test to conclude for an elevation, reduction, or stability (mean delta compared to the theoretical value of 0). For all analyses, statistical significance was established at $P < 0.05$.

RESULTS

The complete biomarker study dataset, including the individual measurements of the different Gb_3 isoforms and lyso-Gb_3 analogues, is available in Supplementary Table 2. The longitudinal follow-ups of individual lyso-Gb_3 analogue measurements are shown for urine and plasma in Supplementary Figures 1 and 2, respectively. The longitudinal follow-ups of individual Gb_3 isoforms are shown for plasma in Supplementary Figure 3 and for urine in Supplementary Figure 4 (non-methylated isoforms) and Supplementary Figure 5 (methylated Gb_3 isoforms). For clarity purposes and considering that their longitudinal follow-ups tended to vary in a similar fashion, lyso-Gb_3 analogues were reported as a total value in the following sections. For the same reasons, Gb_3 isoforms were also reported as a total value.

Mean deltas from baseline values (BV) for biomarkers

The mean deltas from baseline values (BV) for biomarkers are shown in Figure 3 for all Fabry participants.

Longitudinal biomarker follow-up for 5 Fabry patients

Supplementary Figure 6 shows combined longitudinal results for all 5 patients for each biomarker under study.

The longitudinal follow-ups of the biomarkers under study over the gene therapy timeline, where transplant is day 0, are shown in Figure 4 (Participant 1), Figure 5 (Participant 2), Figure 6 (Participant 3), Figure 7 (Participant 4), and Figure 8 (Participant 5) and expressed as the %difference from the screening period, except for the enzyme activity measured in leukocytes and plasma which is expressed as nmol/hr/mg protein, and nmol/hr/mL, respectively. The period from 1-month pre-GT to 1-month post-GT will not be discussed, considering the medical procedures involved during this period which might have introduced more variability in the biomarker measurements.

Participant #1

Urine and plasma lyso-Gb_3 and total analogues show similar profiles [Figure 4A-D]. As expected, the lowest levels were observed during the ERT-GT period [Figure 3], when participant #1 was treated with both Fabrazyme and gene therapy ($n = 5$ time points). During this period, mean urine lyso-Gb_3 levels were at -30.0% ± 35.2% ($P = 0.077$), and mean urine analogues were at -38.8% ± 17.6% ($P < 0.050$) compared to BV (ERT only, $n = 4$ time points). On the other hand, mean plasma lyso-Gb_3 levels were at -46.7% ± 7.6% ($P < 0.001$), and mean plasma analogues were at -55.6% ± 9.6% ($P < 0.001$) for the same period. It is noteworthy to mention that participant #1 changed his ERT treatment between the baseline period (Replagal) and the ERT-GT period (Fabrazyme) to standardize post-transplant ERT according to the formal clinical protocol. The biomarker levels might thus be affected by this treatment switch, considering the different treatment regimens, especially the higher dosage of Fabrazyme (1.0 mg/kg body weight) compared to Replagal (0.2 mg/kg body weight). After Fabrazyme was stopped (GT period, $n = 7$ time points), the

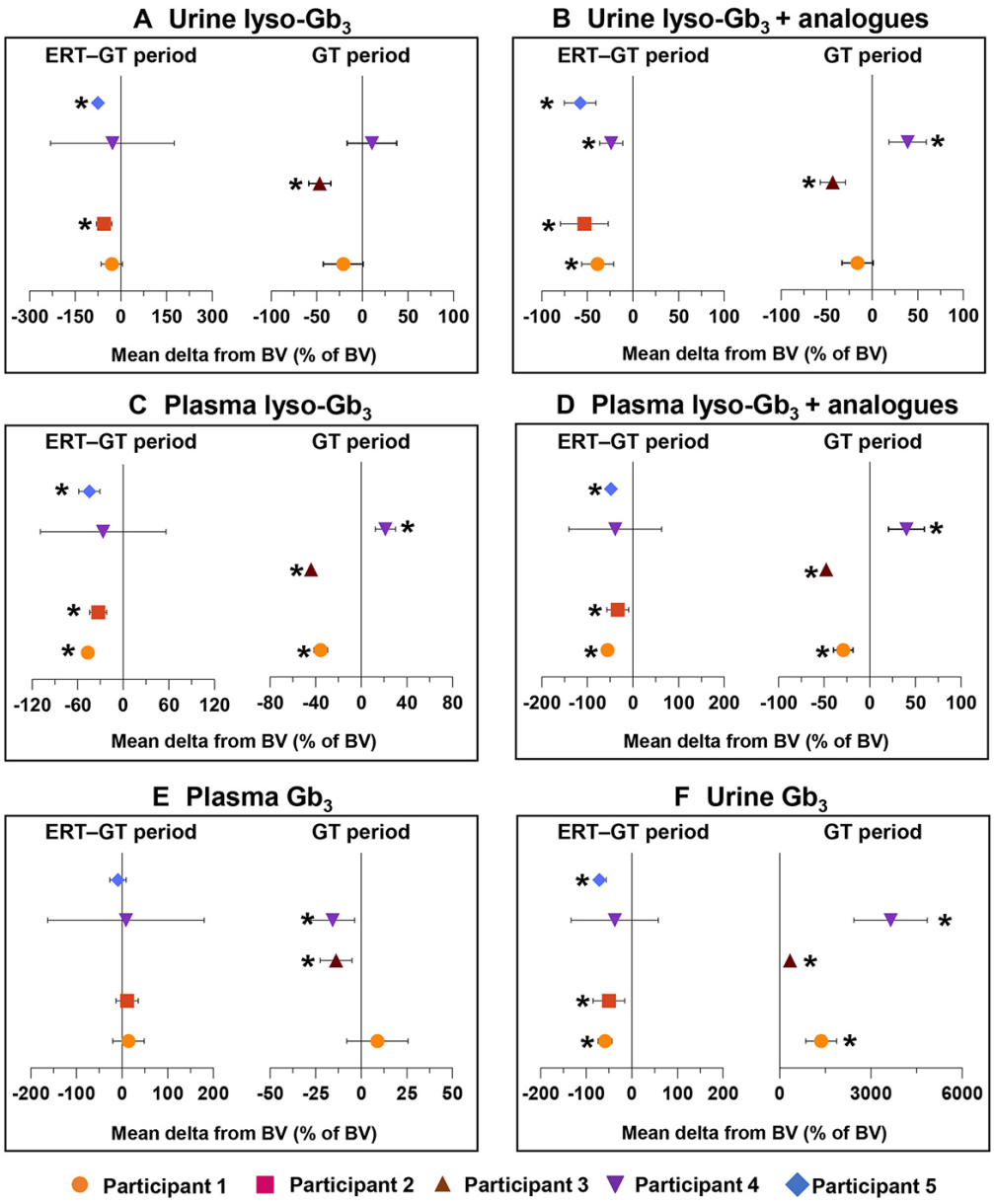

Figure 3. Mean deltas from baseline value and 95% confidence intervals (CI) in the periods after transplant where participants were treated with enzyme replacement therapy (ERT-GT period) or not (GT period). The baseline value (BV) was defined as the mean of the biomarker values obtained at each time point in the baseline period, which is the pre-GT period when the participant was treated with ERT (-4 to -1 month). *P value < 0.05).

biomarker levels remained generally stable from 21 to 60 months post-GT (except for an outlier at 31 months post-GT for urine lyso-Gb_3) and tended to be slightly higher compared to the ERT-GT period, but lower than the BV. The mean levels compared to the BV were the following: urine lyso-Gb_3: -21.0% ± 21.7% (P = 0.056); urine analogues: -16.1% ± 17.1% (P = 0.060); plasma lyso-Gb_3: -35.6% ± 6.1% (P < 0.001); plasma analogues: -29.1% ± 10.7% (P < 0.001). Plasma Gb_3 levels [Figure 4E] were more variable and a clear correlation with treatment status could not be observed. However, plasma Gb_3 levels did not increase significantly in the GT period and the %difference since screening remained in the negatives without ERT, except at 42 months post-GT, where it was +12%. The lowest levels of urine Gb_3 [Figure 4F]

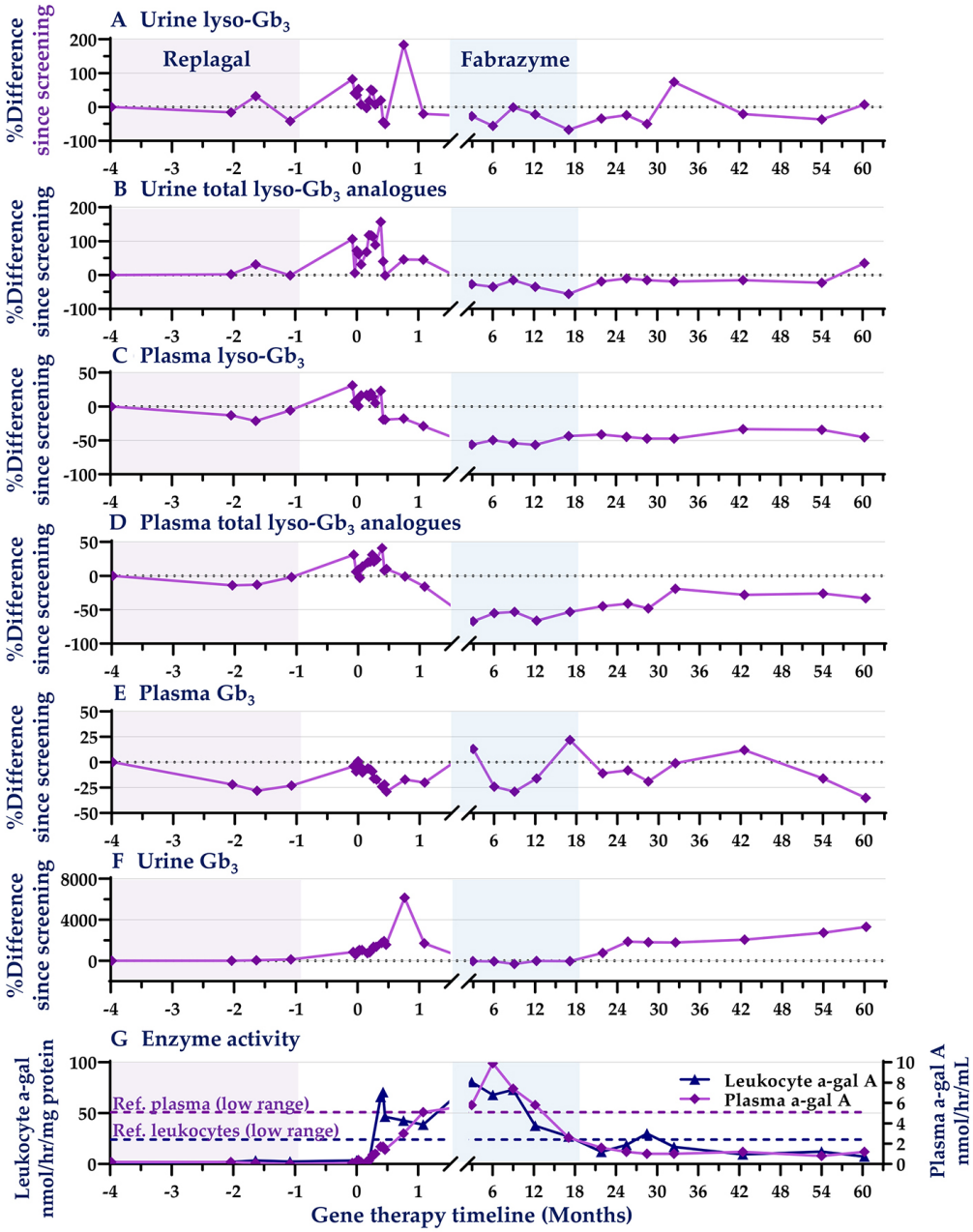

Figure 4. Longitudinal biomarker follow-up of Fabry gene therapy trial participant #1.

were observed during the ERT-GT period, where mean levels were at -58.8% ± 15.3% ($P < 0.001$). In the GT period, urine Gb_3 gradually increased from 21 to 60 months post-GT, and mean levels were at +1,363.0% ± 506.1% ($P < 0.001$).

Plasma and leukocyte α-galactosidase A activities are shown in Figure 4G. The plasma activity was 0.2 nmol/hr/min at screening for participant #1. After gene therapy, plasma α-galactosidase A activity gradually increased until 6 months post-GT, and then gradually lowered from 6 to 24 months post-GT. Stable levels were observed from 24 to 60 months post-GT (median [min-max]: 1.1 [0.8-1.2] nmol/hr/min). Leukocyte α-galactosidase A activity was at 0.9 nmol/hr/mg protein at screening. After gene therapy,

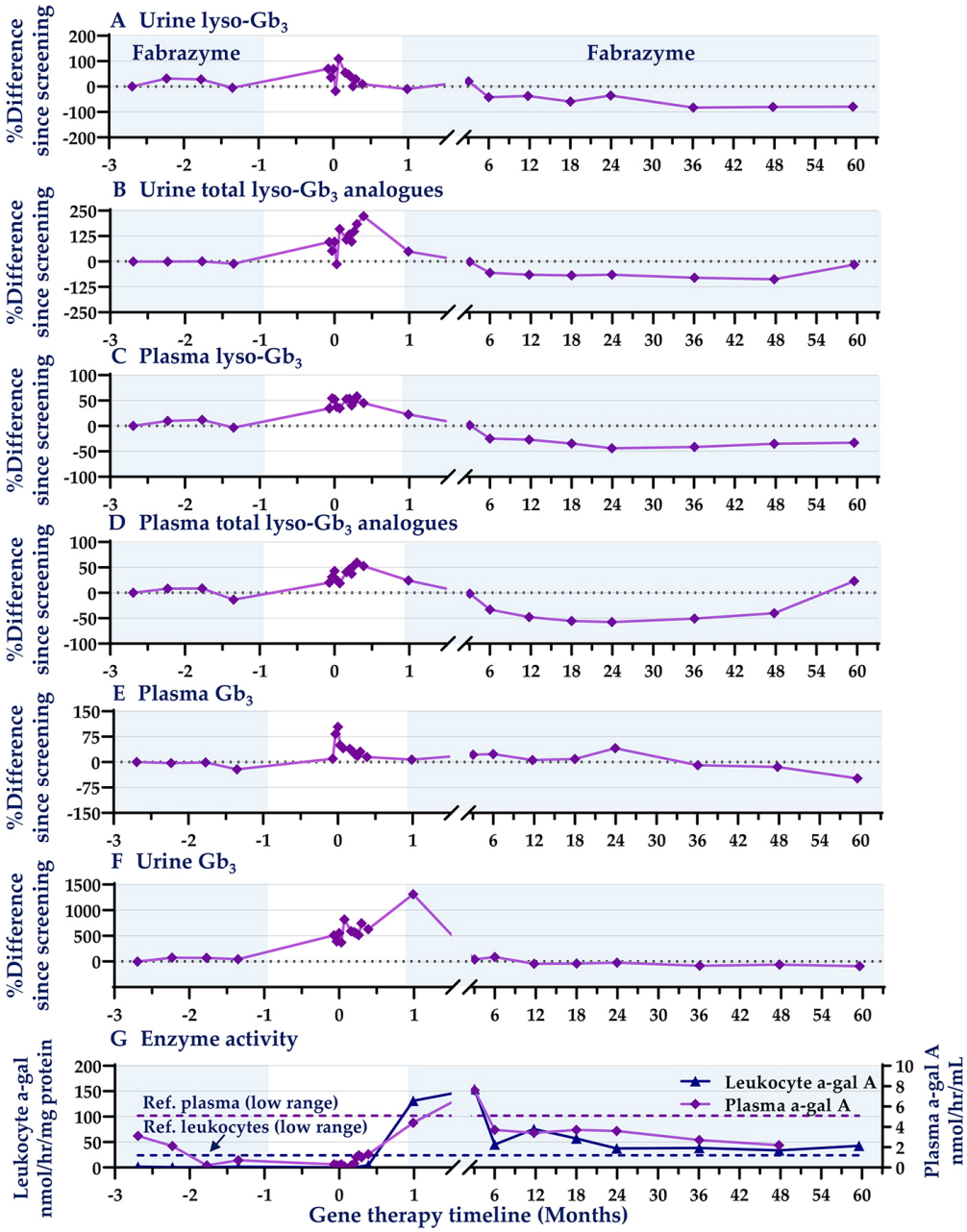

Figure 5. Longitudinal biomarker follow-up of Fabry gene therapy trial participant #2.

leukocyte α-galactosidase A activity increased and reached a maximum at 3 months post-GT, and then gradually lowered from 3 to 21 months post-GT. Stable levels were observed from months 24 to 60 post-GT: 14.5 [7.3-30.0] nmol/hr/mg protein.

Participant #2

Urine and plasma lyso-Gb_3 and total analogues show similar profiles [Figure 5A-D]. As expected, the lowest levels were observed during the ERT-GT period [Figure 3], when participant #2 was treated with both Fabrazyme and gene therapy (1-60 months post-GT, n = 9 time points). During this period, mean urine

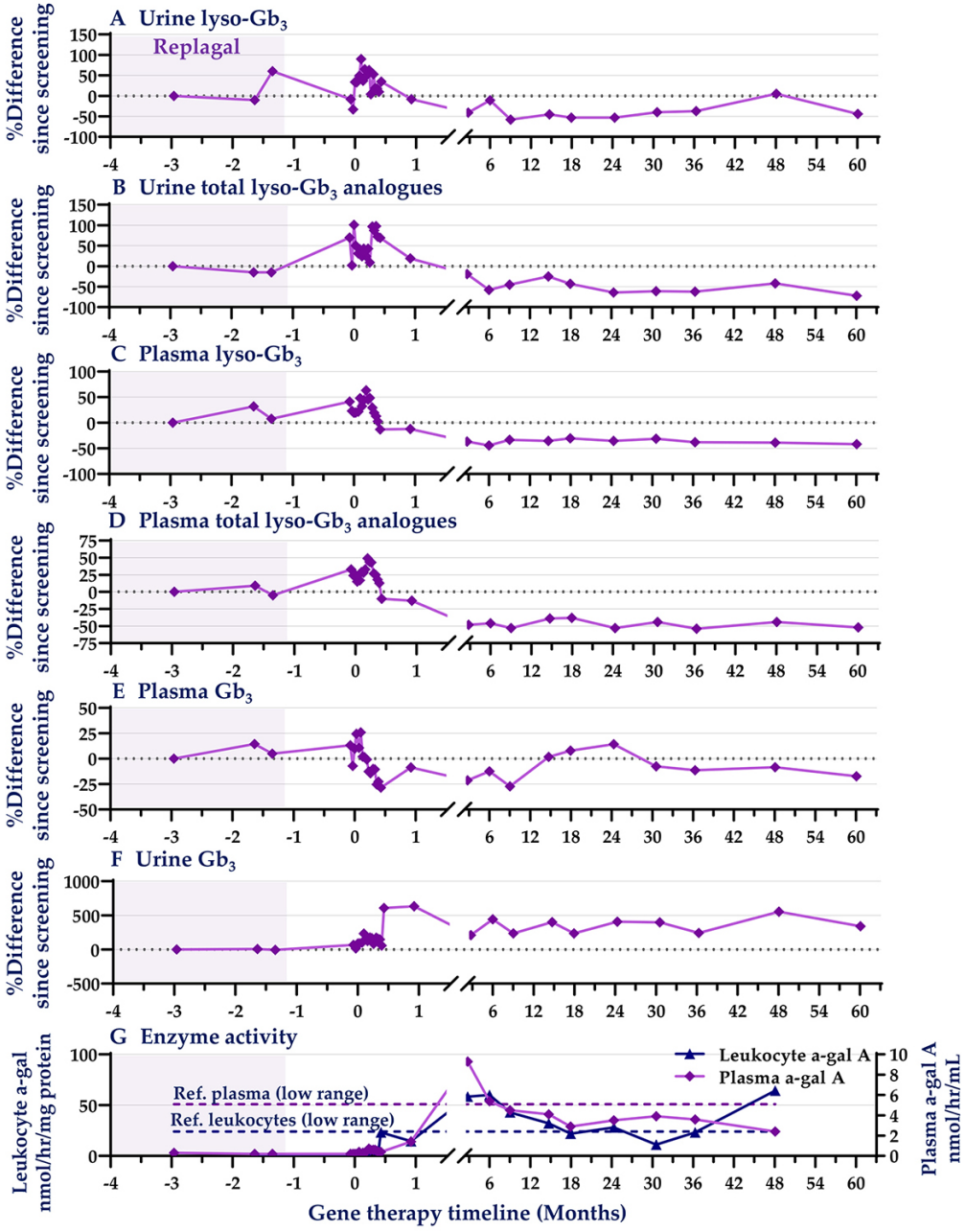

Figure 6. Longitudinal biomarker follow-up of Fabry gene therapy trial participant #3.

lyso-Gb_3 levels were at -55.5% ± 25.4% ($P < 0.050$), and mean urine analogues were at -53.3% ± 26.1% ($P < 0.050$) compared to BV (ERT only, $n = 4$ time points). Urine and plasma analogues augmented at 60 month post-GT, but this would need to be confirmed with an additional time point. On the other hand, mean plasma lyso-Gb_3 levels were at -33.0% ± 11.2% ($P < 0.001$), and mean plasma analogues were at -33.5% ± 24.0% ($P < 0.050$) for the same period. Participant #2 remained on ERT for the five-year surveillance period. Plasma Gb_3 levels [Figure 5E] remained stable after gene therapy (+11.0% ± 24.5%, $P = 0.324$). The lowest levels of urine Gb_3 [Figure 5F] were observed during the ERT-GT period, with mean levels at -50.5% ± 34.9% ($P < 0.050$).

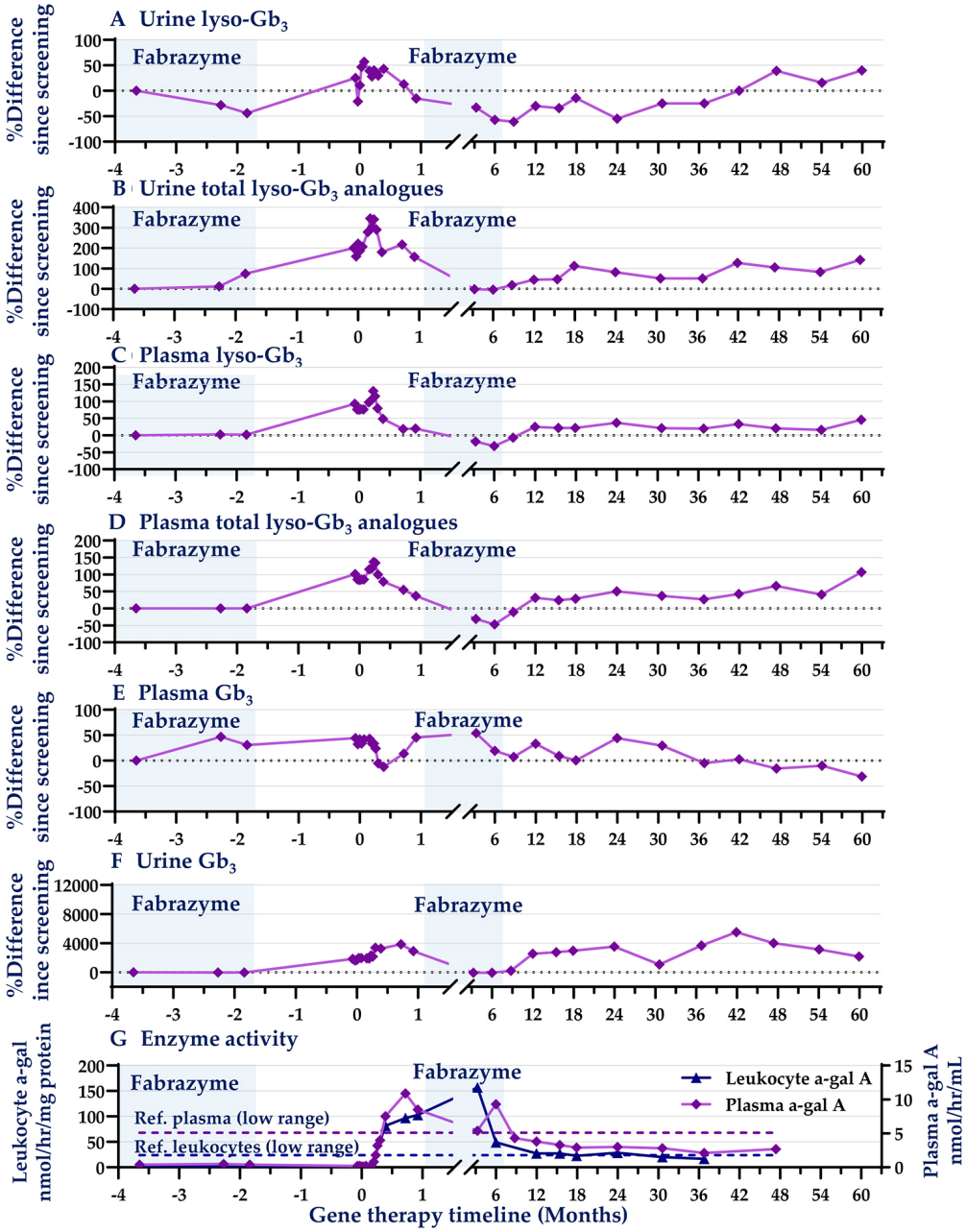

Figure 7. Longitudinal biomarker follow-up of Fabry gene therapy trial participant #4.

Plasma and leukocyte α-galactosidase A activities are shown in Figure 5G. The plasma activity was 3.1 nmol/hr/min at screening for participant #2. After gene therapy, plasma α-galactosidase A activity gradually increased and reached a maximum at 3 months post-GT (7.6 nmol/hr/min), then slowly decreased from 6 to 48 months post-GT (median [min-max]: 3.5 [2.2-3.7] nmol/hr/min). Leukocyte α-galactosidase A activity was at 1.6 nmol/hr/mg protein at screening. After gene therapy, leukocyte α-galactosidase A activity increased and reached a maximum at 3 months post-GT (154.0 nmol/hr/mg), then slowly decreased from 3 to 24 months post-GT. Stable levels were observed from months 24 to 60 post-GT: 38.0 [33.8-42.4] nmol/hr/mg protein.

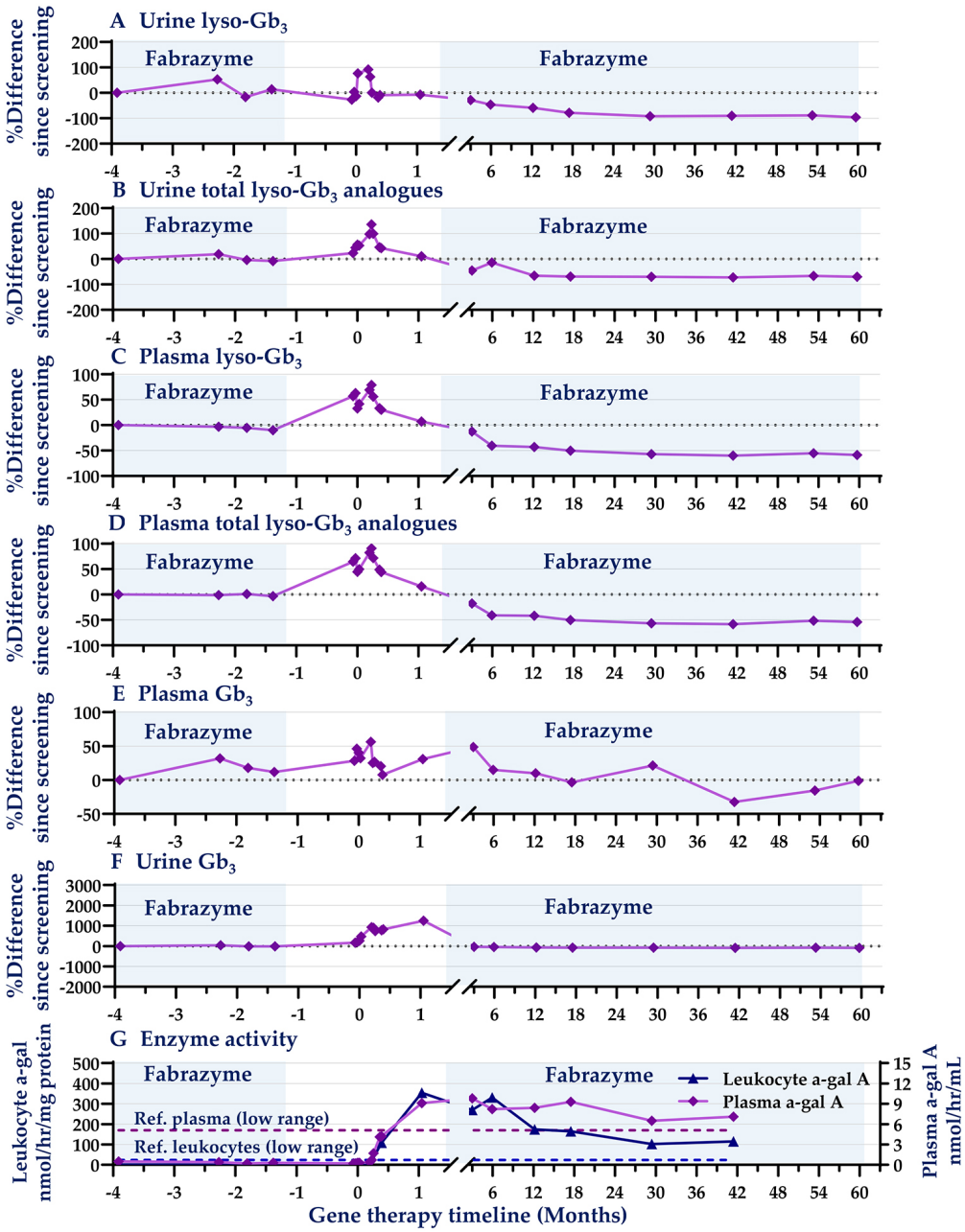

Figure 8. Longitudinal biomarker follow-up of Fabry gene therapy trial participant #5.

Participant #3

Urine and plasma lyso-Gb_3 and total analogues show similar profiles [Figure 6A-D]. It is noteworthy to mention that participant #3 chose not to resume ERT treatment after gene therapy [Figure 3]. During the period from 1-60 months post-GT (GT period, n = 10 time points), mean urine lyso-Gb_3 levels were at -46.7% ± 12.1% ($P < 0.001$), and mean urine analogues were at -43.3% ± 13.8% ($P < 0.001$) compared to BV (ERT only, n = 3 time points). On the other hand, mean plasma lyso-Gb_3 levels were at -44.0% ± 2.8% ($P < 0.001$), and mean plasma analogues were at -47.9% ± 4.2% ($P < 0.001$) for the same period. Plasma Gb_3 levels [Figure 6E] were more variable, but overall, mean Gb_3 plasma levels were lower after gene therapy at

-13.8% ± 8.7% ($P < 0.050$). Urine Gb_3 levels [Figure 6F] increased during the GT period, and mean levels were at +343.3% ± 80.4% ($P < 0.001$).

Plasma and leukocyte α-galactosidase A activities are shown in Figure 6G. The plasma activity was 0.3 nmol/hr/min at screening for participant #3. After gene therapy, plasma α-galactosidase A activity gradually increased and reached a maximum at 3 months post-GT (9.3 nmol/hr/min), and then slowly decreased from 6 to 48 months post-GT (median [min-max]: 3.8 [2.4-5.4] nmol/hr/min). Leukocyte α-galactosidase A activity was at 0.4 nmol/hr/mg protein at screening. After gene therapy, leukocyte α-galactosidase A activity increased and reached a maximum at 6 months post-GT (60.0 nmol/hr/mg), and then slowly decreased from 9 to 30 months post-GT: 28.0 [11.1-43.0] nmol/hr/mg protein. From 30 to 48 months post-GT, values increased to reach 64.4 nmol/hr/mg.

Participant #4

Urine and plasma lyso-Gb_3 and total analogues show similar profiles [Figure 7A-D]. As expected, the lowest levels were observed during the ERT-GT period, when participant #4 was treated with both Fabrazyme and gene therapy (3-6 months post-GT, $n = 2$ time points) [Figure 3]. During this period, mean urine lyso-Gb_3 levels were at -28.0% ± 203.3% ($P = 0.331$), and mean urine analogues were at -24.0% ± 12.7% ($P < 0.050$) compared to BV (ERT only, $n = 3$ time points). On the other hand, mean plasma lyso-Gb_3 levels were at -26.5% ± 82.6% ($P = 0.153$), and mean plasma analogues were at -39.0% ± 101.6% ($P = 0.129$) for the same period. After Fabrazyme was stopped (GT period, $n = 11$ time points), the biomarker levels tended to increase from 9 to 60 months post-GT. The mean levels compared to BV were the following: urine lyso-Gb_3: +10.5% ± 27.1% ($P = 0.409$); urine analogues: +38.8% ± 20.5% ($P < 0.050$); plasma lyso-Gb_3: +21.2% ± 8.8% ($P < 0.001$); plasma analogues: +40.1% ± 19.7% ($P < 0.001$). Plasma Gb_3 levels [Figure 7E] were more variable and a clear correlation with treatment status could not be observed. However, plasma Gb_3 levels seemed stable during the ERT-GT period (+8.5% ± 171.5%, $P = 0.642$) and lower during the GT period (-15.6% ± 12.0%, $P < 0.050$), compared to BV. Urine Gb_3 [Figure 7F] levels were stable during the ERT-GT period with a mean delta from BV at -37.5% ± 95.3% ($P = 0.126$). During the GT period, urine Gb_3 gradually increased with a mean delta from BV at +3,648.0% ± 1,207.0% ($P < 0.001$).

Plasma and leukocyte α-galactosidase A activities are shown in Figure 7G. The plasma activity was 0.4 nmol/hr/min at screening for participant #4. After gene therapy, plasma α-galactosidase A activity gradually increased until day 22 post-GT, and then gradually decreased from day 22 to 18 months post-GT. Stable levels were observed from 18 to 47 months post-GT (median [min-max]: 2.8 [2.1-3.0] nmol/hr/min). Leukocyte α-galactosidase A activity was 2.0 nmol/hr/mg protein at screening. After gene therapy, leukocyte α-galactosidase A activity increased and reached a maximum at 3 months post-GT, and then gradually lowered from 3 to 36 months post-GT, with a value of 16.7 nmol/hr/mg protein at 36 months post-GT.

Participant #5

Urine and plasma lyso-Gb_3 and total analogues show similar profiles [Figure 8A-D]. As expected, the lowest levels were observed during the ERT-GT period, when participant #5 was treated with both Fabrazyme and gene therapy (3-60 months post-GT, $n = 8$ time points) [Figure 3]. During this period, mean urine lyso-Gb_3 levels were at -75.5% ± 18.7% ($P < 0.001$), and mean urine analogues were at -57.9% ± 17.3% ($P < 0.001$) compared to BV (ERT only, $n = 4$ time points). On the other hand, mean plasma lyso-Gb_3 levels were at -44.6% ± 13.9% ($P < 0.001$), and mean plasma analogues were at -48.3% ± 7.3% ($P < 0.001$) for the same period. Participant #5 never discontinued ERT. Plasma Gb_3 levels [Figure 8E] remained stable after gene therapy (-8.8% ± 17.9%, $P = 0.287$). The lowest levels of urine Gb_3 [Figure 8F] were observed during the ERT-GT period, where the mean delta from BV was at -71.0% ± 14.5% ($P < 0.001$).

Plasma and leukocyte α-galactosidase A activities are shown in Figure 8G. The plasma activity was 0.5 nmol/hr/min at screening for participant #5. After gene therapy, plasma α-galactosidase A activity gradually increased and reached a maximum at 3 months post-GT (9.8 nmol/hr/min), then slightly decreased to reach 7.1 nmol/hr/min at 41 months post-GT. Leukocyte α-galactosidase A activity was 1.6 nmol/hr/mg protein at screening. After gene therapy, leukocyte α-galactosidase A activity increased and reached a maximum at 1 month post-GT (353.9 nmol/hr/mg), then slowly decreased to reach 115.4 nmol/hr/mg at 41 months post-GT.

DISCUSSION

A longitudinal biomarker follow-up was performed in a cohort of five participants who were treated as part of the world's first gene therapy clinical trial for Fabry disease at three Canadian centers. Figure 3 summarizes the biomarker results obtained as part of this study. It is noteworthy to mention that, considering that the participants under study had classical mutations, most biomarkers remained above normal reference values, even after treatment with ERT and/or gene therapy (see Supplementary Table 2). Supplementary Figure 1 shows that the analogues are more elevated than lyso-Gb_3 itself in urine specimens. We would like to emphasize that the evaluation of the complete profile of lyso-Gb_3 and analogues is particularly important in this matrix.

The levels of urine and plasma lyso-Gb_3 and analogues, as well as urine Gb_3, were lower compared to baseline (ERT alone) when participants were treated with both ERT and gene therapy (Participant #1: $P < 0.050$, except for urine lyso-Gb_3; Participant #2: $P < 0.050$; Participant #3: Data not available since this participant never resumed ERT after gene therapy; Participant #4: Not statistically significant, except for urine lyso-Gb_3 analogues at $P < 0.050$; Participant #5: $P < 0.050$). Plasma Gb_3 levels remained stable when participants were treated with ERT + gene therapy, compared to ERT alone ($P > 0.050$).

Biomarker levels were also evaluated in three participants who discontinued ERT treatment at some point after gene therapy (Participant #1, Participant #3, and Participant #4). When ERT treatment was stopped post-GT, the levels of urine and plasma lyso-Gb_3 and analogues remained lower than baseline (ERT alone) for Participant #1 (not statistically significant for urine lyso-Gb_3 and analogues, $P < 0.050$ for plasma lyso-Gb_3 and analogues) and Participant #3 ($P < 0.050$). For Participant #4, urine and plasma lyso-Gb_3 and analogues were either stable (urine lyso-Gb_3, $P > 0.050$), or elevated compared to baseline values while treated with ERT alone ($P \leq 0.050$). This is consistent with the fact that the diminution of biomarker levels was less important for this participant when treated with both ERT and gene therapy. Plasma Gb_3 values remained stable ($P > 0.050$) for Participants #1 and #4, while a statistically significant diminution was observed for Participant #3 (-12.9% ± 9.6%, $P < 0.050$). In all three participants who stopped ERT treatment post-GT, an important augmentation of urine Gb_3 was observed compared to baseline values (Participant #1: +1,363.0% ± 506.1%, $P < 0.001$; Participant #3: +343.9% ± 91.6%, $P < 0.001$; Participant #4: +3,648.0% ± 1,207.0%, $P < 0.001$).

The reason for the augmentation of urine Gb_3 in these participants is unclear. We do know from previous studies that Gb_3 accumulates in virtually all renal cell types, with particularly important accumulations in podocytes and distal tubular epithelial cells[39]. The exfoliation of kidney tubular cells contributes to the presence of Gb_3 in voided urine specimens[40]. Urine Gb_3 might also originate from the shedding of podocytes or from the leakage of circulating Gb_3 through renal glomeruli[41]. It would thus be plausible that the augmentation of urine Gb_3 reflects an increased accumulation of Gb_3 in different renal cell types following treatment switch from ERT to gene therapy in the present study. In fact, it was previously shown that Gb_3 was cleared or decreased in several renal cell types following the administration of ERT for a period

of 11 months[39]. A potential increased accumulation of Gb_3 in podocytes would also exacerbate their detachment into the urine (podocyturia)[42,43]. This phenomenon would be in accordance with an increased measured level of urine Gb_3. Nevertheless, the prognostic value of urine Gb_3 as a biomarker for kidney disease progression is debatable. Shiga *et al.* found higher levels of urine Gb_3 in patients with Fabry disease showing renal events (proteinuria, elevated serum creatinine, or decreased eGFR) and suggested closely monitoring patients without any renal symptoms but with high levels of urinary Gb_3 to prevent kidney function deterioration[44]. On the other hand, other researchers did not find any correlations between urinary Gb_3 levels and renal function parameters such as albuminuria, eGFR, serum creatinine, and proteinuria[45,46]. Regarding the participants of the present study, the increased levels of urine Gb_3 were not associated with a decrease in eGFR. Nevertheless, this finding warrants further investigations and close monitoring of renal function with known predictors of kidney disease progression (proteinuria, albuminuria, and serum creatinine) in the participants who stopped ERT after gene therapy treatment. In fact, the clinical outcomes at 5 years for the five participants from this gene therapy clinical trial and their relationship with biomarker changes are currently under evaluation and will provide more answers regarding these increased urinary Gb_3 levels and kidney disease status.

Nowak *et al.* have found that serum lyso-Gb_3 levels were associated with kidney function, renal replacement therapy, cardiomyopathy, and stroke/transient ischemic attacks and that it was a significant risk factor associated with important clinical events[47,48]. Considering that lyso-Gb_3 levels tend to increase with disease severity[27,47-49], it can be hypothesized that the stability or decrease of this biomarker and its analogues in plasma and urine after discontinuation of ERT is important and shows the benefits and success of gene therapy treatment. In fact, toxic effects of lyso-Gb_3 exposure were previously reported and include alteration of pathways involved in protein translation and folding[50], induction of necroptosis via autophagy[51], release promotion of secondary mediators of glomerular injury[52], and sensitization of peripheral nociceptive neurons[53]. Moreover, van der Veen *et al.* recently showed that, in untreated patients with Fabry disease, plasma lyso-Gb_3 eventually reached a stable level during childhood and that this level was associated with the course of disease progression[54]. Ouyang *et al.* also found that lyso-Gb_3 measurements could improve the prediction of kidney disease progression in patients with Fabry disease[55]. Our group showed the association of lyso-Gb_3 and some related analogues with the LVMI and MSSI in patients with a late-onset IVS4+919G>A cardiac variant mutation in Taiwan[27]. It was previously shown that serum lyso-Gb_3 and lyso-Gb_3 analogues varied according to the type of mutations and related phenotype[56,57]. A group of experts recently recommended routine monitoring of plasma lyso-Gb_3 at baseline and every 6 to 12 months as part of treatment initiation/switch for longitudinal level evaluations, considering that the reduction in lyso-Gb_3 levels seems important[58]. Participants #1 and #3 had lower levels of plasma and urine lyso-Gb_3 and analogues when treated with gene therapy compared to previous levels under ERT treatment alone. It was not the case for participant #4, for whom an increase was observed after ERT treatment discontinuation. Considering the lowest levels of urine and plasma lyso-Gb_3 and analogues obtained under both ERT and gene therapy treatments, it might be hypothesized that the combination of these two treatments gives the best outcomes in patients affected with Fabry disease. This observation warrants further investigation.

The strength of this study is the analysis of a unique and extensive profile of sixteen Gb_3 isoforms in urine, six Gb_3 isoforms in plasma, six lyso-Gb_3 analogues in plasma, and seven lyso-Gb_3 analogues in the urine of participants. These mass spectrometry analyses were performed using validated methodologies for maximum accuracy and precision. This complete profile of Fabry disease-related glycosphingolipids was never reported before in adults with Fabry disease as part of gene therapy treatment. One limitation of this study was the low number of participants included in the research. The variability in ERT treatments before and after gene therapy for each participant was also a confounding variable. As shown in Figure 2, some

participants were treated with Fabrazyme, and others with Replagal at baseline. This also implies different dosages between these two ERT treatments. Participant #1 was on Replagal before gene therapy and resumed ERT with Fabrazyme, while Participant #2 never stopped ERT after gene therapy, and Participant #3 never resumed ERT after gene therapy. It would be easier to draw definite conclusions regarding the effect of treatment on biomarker levels with a more homogeneous group of participants. Another limitation is that all patients were treated with ERT at baseline. For this reason, it was not possible to evaluate the effect of gene therapy on biomarker levels in naïve participants. Future studies should address these concerns and include more participants, including participants who were untreated at baseline, to confirm the biomarker results obtained as part of this study.

In summary, high sensitivity and specificity mass spectrometry assays enabled the analysis of a complete biochemical profile of Fabry disease-related biomarkers in urine and plasma, leading to better monitoring and follow-up of these treated patients as part of a precision medicine approach. The biochemical profile also helped learn more about the complex pathophysiology involved in Fabry patients. Future perspectives will focus on correlation studies between these biomarkers and the clinical manifestations of the disease.

DECLARATIONS

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Validation, formal analysis, investigation: Auray-Blais C, Martineau T, Lavoie P
Data curation: Auray-Blais C, Martineau T, Lavoie P, Medin JA
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Writing-review: Auray-Blais C, Lavoie P, Martineau T
Supervision, project administration, technical and material support: Auray-Blais C
Editing: Auray-Blais C, Lavoie P, Martineau T, Rupar CA, Barber DL, Keating A, Foley R, Khan A, West ML, Medin JA
All authors have read and agreed to the published version of the manuscript.

Availability of data and materials

Mass spectrometry data supporting these findings are provided in Supplementary Material and are stored into secured data repositories at the Faculty of Medicine and Health Sciences at the Université de Sherbrooke, Sherbrooke, Quebec. All enzyme activity results were stored in Dr T Rupar's office in London, ON.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

The biomarker study was conducted in agreement with the Declaration of Helsinki and approved by the Institutional Review Board at the Faculty of Medicine and Health Sciences at the Centre intégré universitaire de santé et de services sociaux de l'Estrie-Centre hospitalier universitaire de Sherbrooke (CIUSSS de l'Estrie-CHUS), where the biomarker analyses were performed (Project #2017-1575), authorization obtained on November 14th, 2016). The trial (NCT02800070) was approved by Health Canada on April 26th, 2016, and conducted in compliance with local institutional and/or university Human Experimentation Committee requirements. Research Ethics Board (REB) approval was provided by University Health Network REB, Alberta Health Services REB, Capital Health Services REB, Hamilton Integrated Research Ethics, and the Medical College of Wisconsin Institutional Review Board. Informed consent was obtained from all subjects involved in the study.

Consent for publication

Not applicable.

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