Accurate Quantitation of Plasma Globotriaosylsphingosine (lyso-Gb$_3$) in Healthy Individuals and Fabry Patients by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

Rick Hamler[1], Nastry Brignol[1], Robert E. Boyd[1], Daniel G. Bichet[2], Dominique P. Germain[3], Roberto Giugliani[4], Derralyn A. Hughes[5], Raphael Schiffmann[6], William R. Wilcox[7], Hadis N. Williams[1], Julie Yu[1], Jay Barth[1], Jeff Castelli[1], Kenneth J. Valenzano[1], and Elfrida R. Benjamin[1]

[1]Amicus Therapeutics, Cranbury, NJ, USA; [2]Hôpital du Sacré-Coeur, Montréal, Quebéc, H4J1C5, Canada; [3]Hôpital Raymond Poincaré (AP-HP), University of Versailles – St. Quentin en Yvelines (UVSQ), Garches, France; [4]Medical Genetics Service, HCPA/UFRGS Porto Allegre, Brazil; [5]Royal Free Campus, University College London, London, UK; [6]Baylor Research Institute, Dallas, TX; [7]Department of Human Genetics, Emory University, Atlanta, GA

Introduction

Fabry disease is a lysosomal storage disorder caused by mutations in the gene (GLA) that encodes α-galactosidase A (α-Gal A), and is characterized by pathological accumulation of globotriaosylceramide (GL-3) and its deacylated form, globotriaosylsphingosine (lyso-Gb$_3$). Lyso-Gb$_3$ is an important indicator of Fabry disease, and warrants further evaluation as a marker of disease severity and progression. An LC-MS/MS method was developed and used to measure plasma lyso-Gb$_3$ levels in healthy individuals and Fabry patients enrolled in the ATTRACT Phase 3 clinical study (AT1001-012).

Normal human plasma contains significant endogenous levels of lyso-Gb$_3$, which causes high background interference and negatively impacts the quantitative accuracy of the method at low lyso-Gb$_3$ levels. A novel, stable isotope-labeled internal standard (SIL-IS), $^{13}C_6$-lyso-Gb$_3$, was synthesized and enabled accurate quantitation of lyso-Gb$_3$ in plasma from both healthy individuals and Fabry patients using solvent calibration standards.
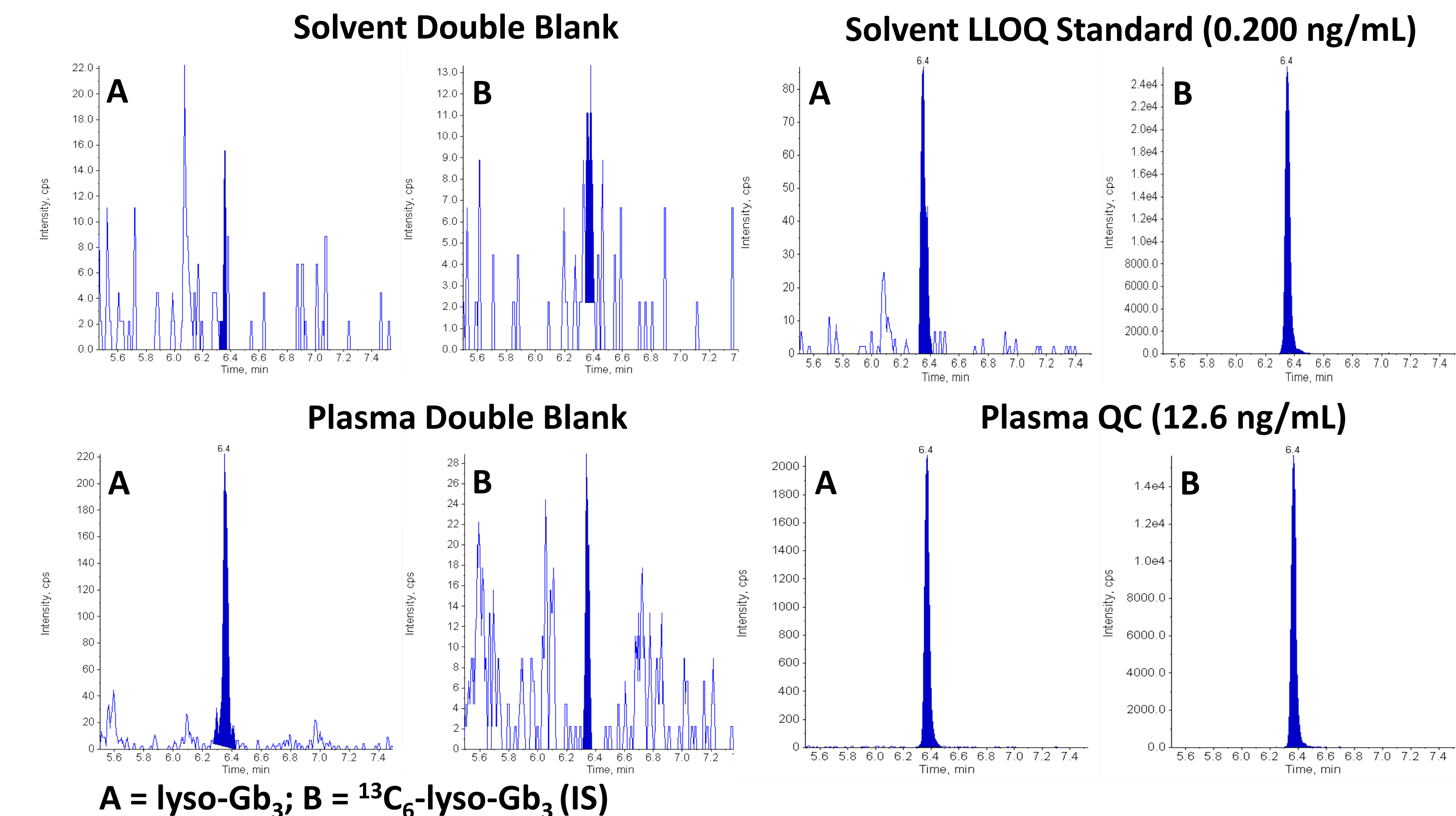
1. Lyso-Gb$_3$ LC-MS/MS Experimental Method

Sample Preparation

- Plasma Volume: 50 µL
- Internal Standard: $^{13}C_6$-lyso-Gb$_3$
- Extraction: Solid Phase (Waters Oasis MCX)

LC-MS/MS Conditions

- LC-System: Shimadzu LC-20AD Pumps
- LC Column: Halo HILIC; 4.6 x 75 mm (Advanced Materials Technology)
- MP A: 5 mM ammonium formate in acetonitrile/water: 5/95 + 0.5% formic acid
- MP B: 5 mM ammonium formate in acetonitrile/water: 95/5 + 0.5% formic acid
- Flowrate: 0.550 mL/min
- Elution: Gradient
- Autosampler System: Shimadzu SIL-30AC
- Injection volume: 5-10 µL
- Mass Spectrometer: 4000 QTRAP, AB Sciex
- Ionization: Positive Ion Electrospray
- MRM transitions: m/z 787 > 282 (lyso-Gb$_3$) and m/z 793 > 282 ($^{13}C_6$-lyso-Gb$_3$)
- Regression Type: Linear, 1/x^2 weighting
- Solvent Calibration Standards Assay Range: 0.200 -0200 ng/mL (0.254 – 254 nM)

Solvent Double Blank   **Solvent LLOQ Standard (0.200 ng/mL)**

Plasma Double Blank   **Plasma QC (12.6 ng/mL)**

A = lyso-Gb$_3$; B = $^{13}C_6$-lyso-Gb$_3$ (IS)

2. Lyso-Gb$_3$ LC-MS/MS Method Validation Results

Previously published LC-MS/MS methods have reported endogenous lyso-Gb$_3$ plasma levels from healthy individuals by extrapolation below the lowest limit of quantitation (LLOQ) of the assay range. A method was analytically validated (non-GLP) at Amicus Therapeutics, using both solvent and plasma calibration standards and quality control (QC) samples. The slopes for the solvent and plasma calibration curves matched well in all validation runs. The SIL-IS corrects for plasma matrix effects and enables the use of solvent standards and accurate quantitation in plasma from 0.200 - 200 ng/mL. The endogenous lyso-Gb$_3$ concentration of the pooled plasma used to prepare plasma standards and QCs was determined in the three core runs to be 0.590 ng/mL and was added to the nominal spike-in concentrations of lyso Gb$_3$ for the purpose of run accuracy acceptance criteria.
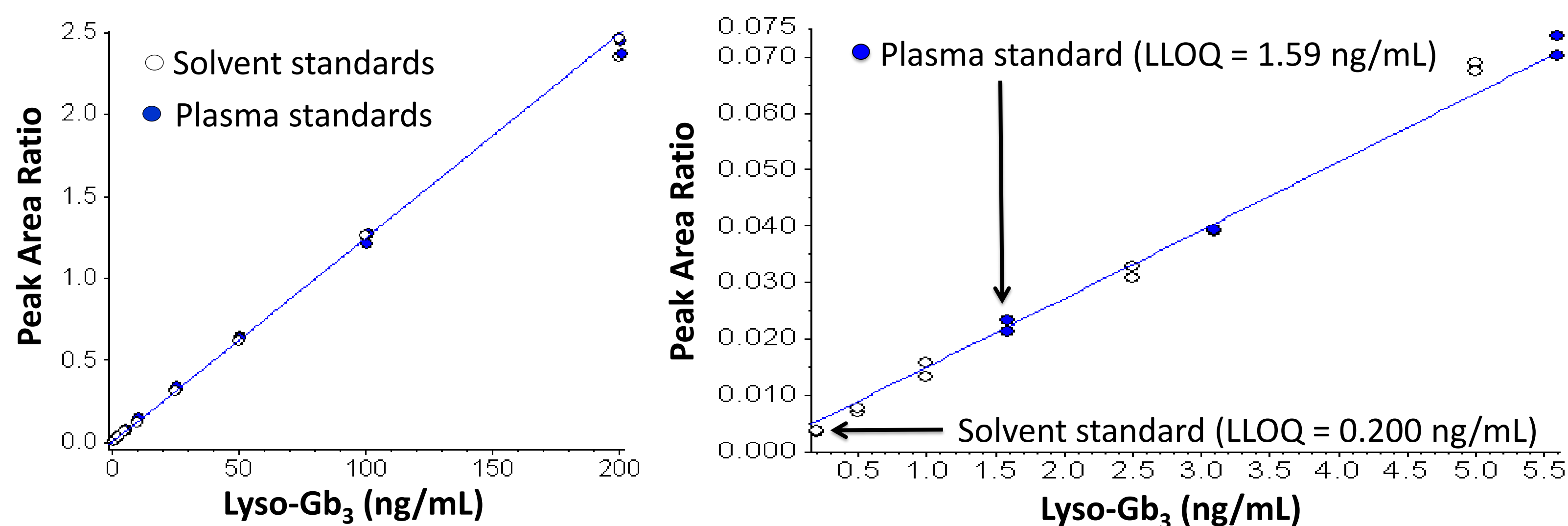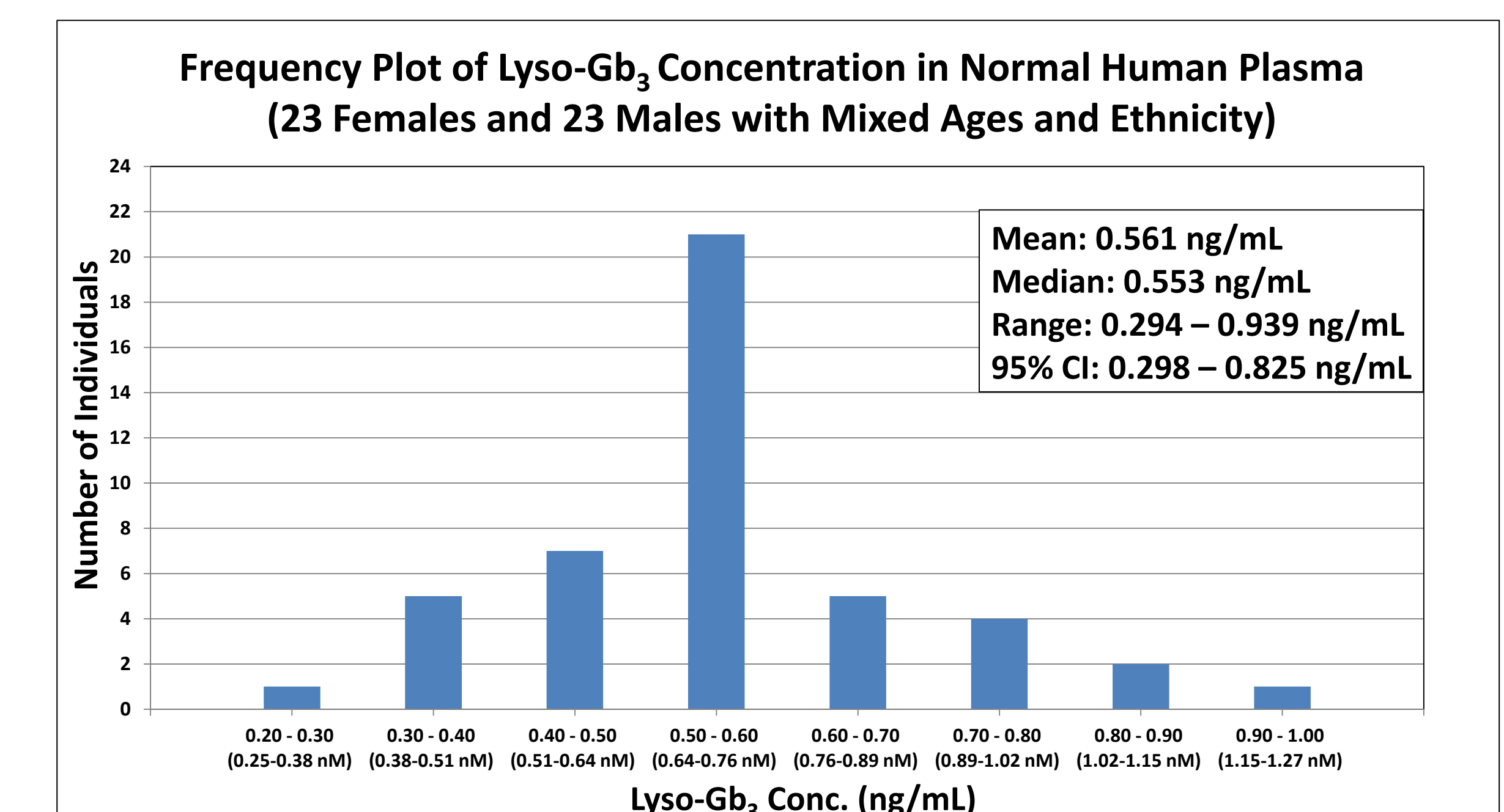
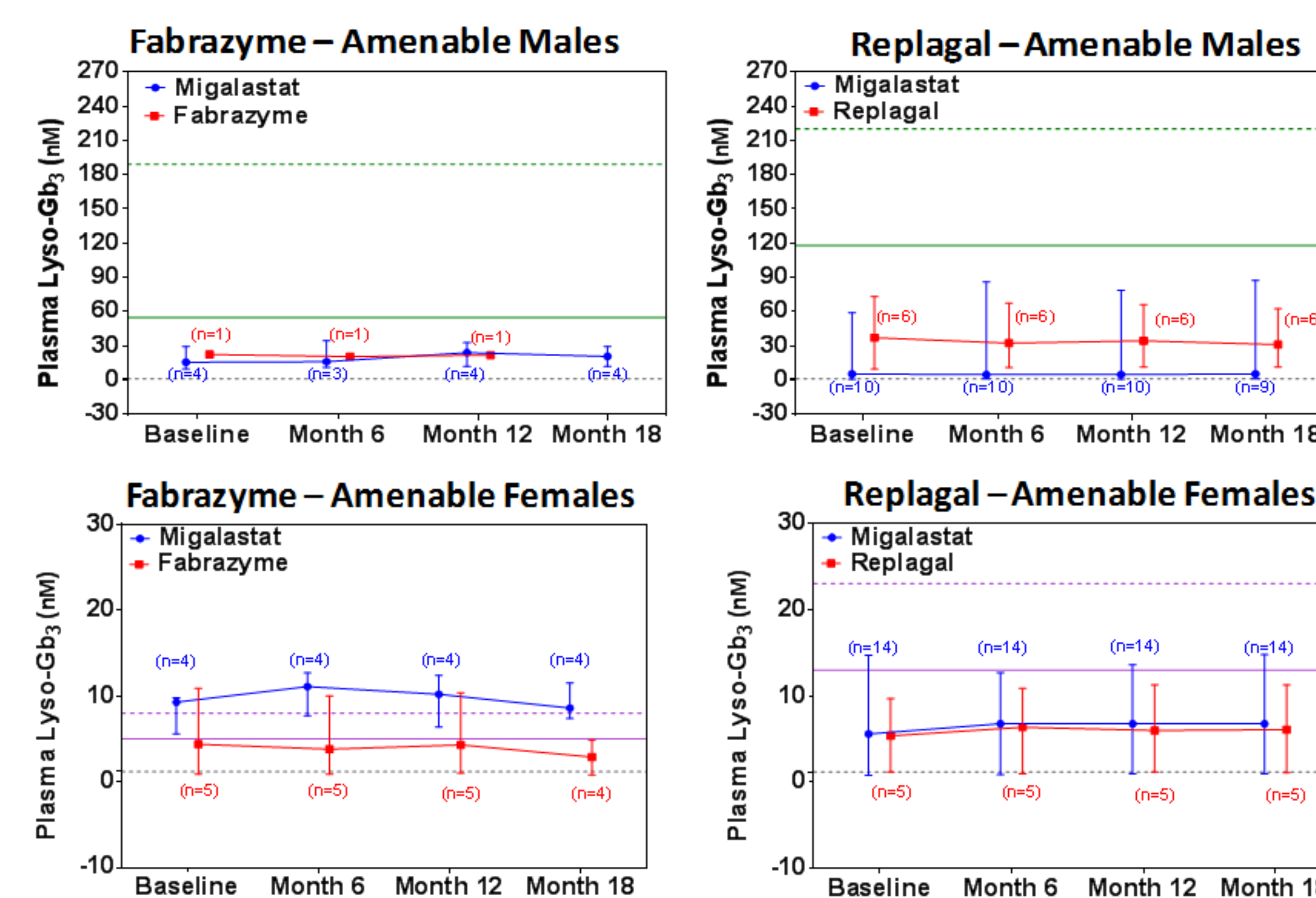| Human Plasma Lyso-Gb$_3$ Method Validation Results	
Solvent QC Intra-assay Accuracy	% Diff ranged from -19.0 to 6.5%
Solvent QC Inter-assay Accuracy	% Diff ranged from -13.4 to -0.7%
Solvent QC Intra-assay Precision	% CV ranged from 2.4 to 21.8%
Solvent QC Inter-assay Precision	% CV ranged from 5.7 to 16.1%
Plasma QC Intra-assay Accuracy	% Diff ranged from -3.1 to 11.1%
Plasma QC Inter-assay Accuracy	% Diff ranged from 1.9 to 4.2%
Plasma QC Intra-assay Precision	% CV ranged from 1.3 to 7.1%
Plasma QC Inter-assay Precision	% CV ranged from 4.4 to 8.4%
Plasma Room Temperature Stability	7 hours
Plasma 37°C Stability	4 hours
Plasma Freeze-Thaw Stability	4 cycles
Plasma Recovery	71%
Plasma Matrix Effect	129%
Dilution Linearity	5-fold and 10-fold (500 ng/mL)
Parallelism	Acceptable with incurred samples
Reinjection Reproducibility	96 hours at room temperature
Migalastat Interference (200 µM)	Acceptable
Iohexol Interference (4000 µg/mL)	Acceptable

3. Normal Plasma Reference Range

Plasma lyso-Gb$_3$ levels from 46 healthy individuals were measured to establish a normal human plasma reference range. Plasma was obtained from a diverse group of individuals, with regards to gender, age and ethnicity. Each individual plasma lot was analyzed in duplicate and the mean of the two values reported.

Frequency Plot of Lyso-Gb$_3$ Concentration in Normal Human Plasma (23 Females and 23 Males with Mixed Ages and Ethnicity)

Mean: 0.561 ng/mL
Median: 0.553 ng/mL
Range: 0.294 – 0.939 ng/mL
95% CI: 0.298 – 0.825 ng/mL

4. ATTRACT Phase 3 Clinical Study (AT1001-012)

- Plasma lyso-Gb$_3$ levels were measured in Fabry patients enrolled in the ATTRACT (AT1001-012) Phase 3 clinical study
- All patients received enzyme replacement therapy (ERT; Fabrazyme, agalsidase beta; Genzyme, Cambridge, MA or Replagal, agalsidase alfa; Shire, Cambridge, MA) for at least 12 months prior to entering the study
- Subjects were randomized to either continue with ERT or switch to oral migalastat treatment (150 mg QOD)
- Plasma samples were collected at months 0 (baseline) 6, 12, and 18, and lyso-Gb$_3$ levels were measured
- Of the 60 subjects in study AT1001-012, 56 had amenable mutations, and 4 had non-amenable mutations, based on the GLP HEK assay, the assay used to identify α-Gal A mutations responsive to migalastat treatment (i.e. amenable)
- In subjects with amenable mutations, the plasma lyso-Gb$_3$ levels remained low and stable for up to 18 months following the switch from ERT to migalastat, comparable to that seen with subjects who remained on ERT
- In two male subjects with non-amenable mutations, plasma lyso-Gb$_3$ levels increased following the switch from ERT to migalastat, as compared to two subjects (one male and one female) who remained on ERT

- In subjects with amenable mutations, plasma lyso-Gb$_3$ levels were further grouped by prior ERT treatment (i.e., Fabrazyme or Replagal) and gender
- Lyso-Gb$_3$ levels remained low and stable for up to 18-months in both males and females following their switch from ERT (Fabrazyme or Replagal) to oral migalastat

Amenable
Non-Amenable

Fabrazyme – Amenable Males
Replagal – Amenable Males
Fabrazyme – Amenable Females
Replagal – Amenable Females

- Data points are median values, error bars represent the range
- Blue dotted line represents upper range of the normal reference range
- Dotted and solid lines (green for males and magenta for females) represent the baseline and median values, respectively, for a cohort of classic males and females after 1 year of ERT (Fabrazyme or Replagal) treatment (van Breemen, Rombach et al. 2011)
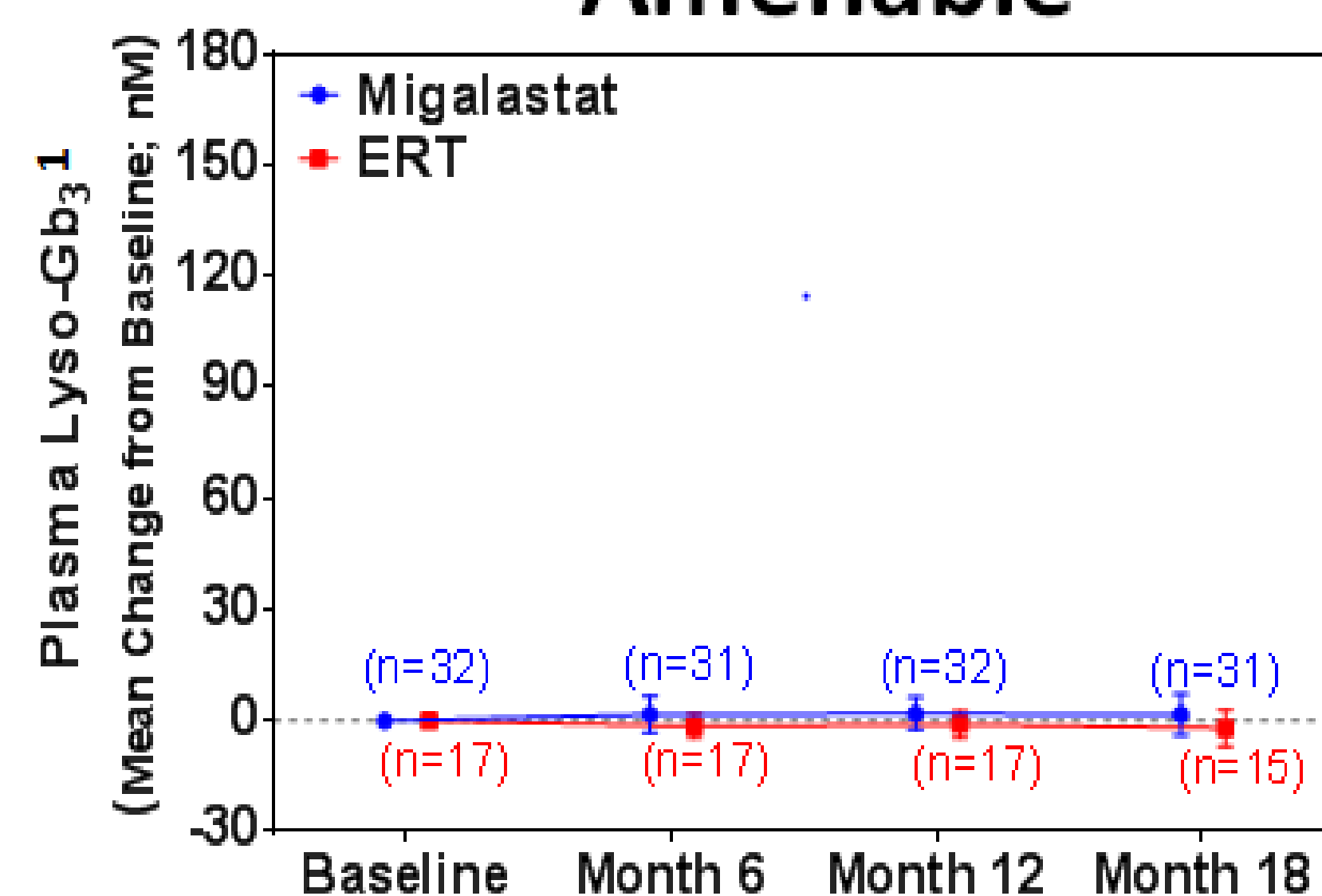
Conclusion

An LC-MS/MS method was analytically validated (non-GLP) at Amicus Therapeutics for the quantitation of lyso-Gb$_3$ in human plasma. A novel stable isotope-labeled internal standard, $^{13}C_6$-lyso-Gb$_3$, was synthesized and enabled the use of solvent calibration standards and the accurate quantitation of plasma lyso-Gb$_3$ levels in the concentration range 0.200 – 200 ng/mL.

The method was used to measure lyso-Gb$_3$ levels in plasma from 46 healthy individuals to establish a normal human plasma reference range.

Using this validated assay, plasma lyso-Gb$_3$ levels were measured in Fabry subjects enrolled in the ATTRACT Phase 3 clinical study (AT1001-012). In subjects with amenable mutations, lyso-Gb$_3$ levels remained low and stable for up to 18 months following their switch from ERT (Fabrazyme or Replagal) to oral migalastat.

Amicus Therapeutics