Validation of an LC-MS/MS Assay To Measure Arginine, α-k-δ-GVA, Argininic Acid, Guanidinoacetic Acid, and Nα Acetyl-L-Arginine Concentrations in ARG1-D Patients

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1 INTRODUCTION

Arginase 1 deficiency (ARG1-D) is a rare, progressive, multisystem, autosomal recessive, serious disease with early mortality and high unmet medical need.[1,2] This disorder of arginine metabolism causes elevated plasma levels of arginine (Arg) and arginine-derived metabolites such as α-k-δ-GVA (GVA), Argininic Acid (ArgA), Guanidinoacetic Acid (GAA), and Nα Acetyl-L-Arginine (NAArg), otherwise known as guanidino compounds (GCs). Pegzilarginase is an engineered, PEGylated, cobalt-substituted human arginase 1, currently in development for the treatment of patients with ARG1-D. To support clinical development, an LC-MS/MS method was developed and validated to measure the concentration of these biomarkers in plasma.

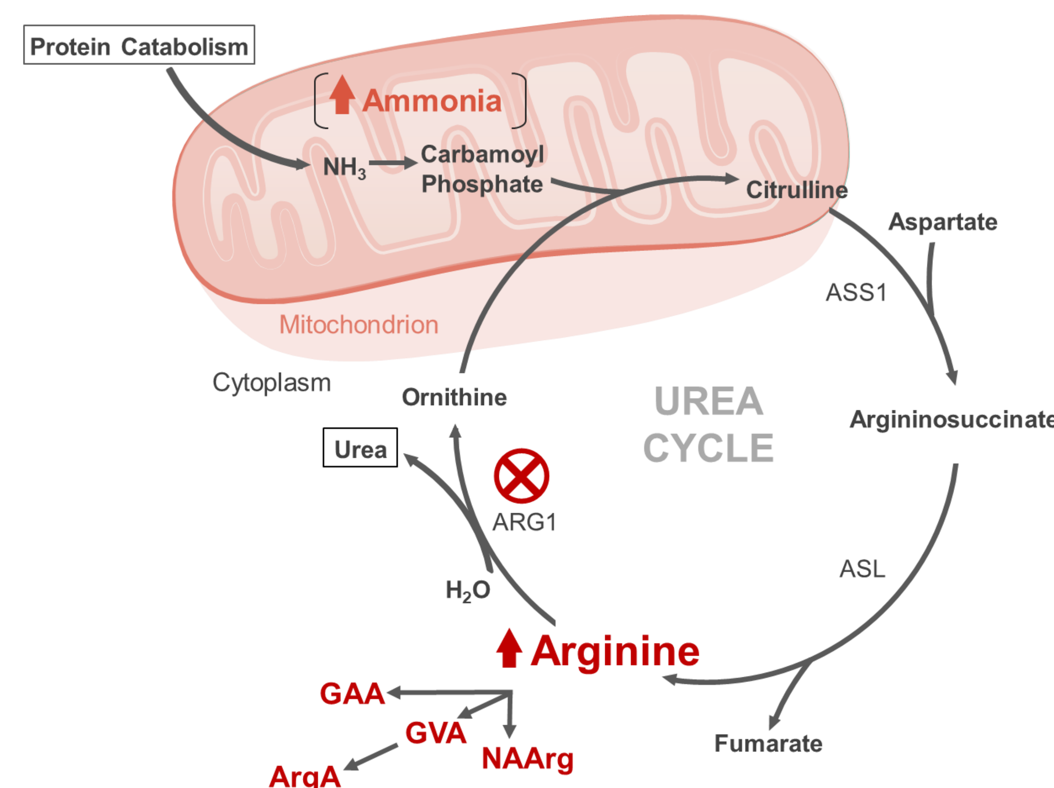

Figure 1: Metabolic effects of ARG1-D
Enzymes: ASL = argininosuccinate lyase; ASS1 = argininosuccinate synthase 1; ARG1=arginase 1
Arginine-derived metabolites: ArgA, GVA, GAA, NAArg

2 METHODS

Treatment of the collected blood samples with an inhibitor was required to halt further pegzilarginase-mediated enzymatic activity following sample collection, therefore human blood was collected into a chilled tube containing the pegzilarginase inhibitor [N^{ω}-Hydroxy-nor-L-arginine (nor-NOHA)]), K_2EDTA, and mannitol. Prior to extraction, plasma samples were treated with glacial acetic acid (to further inactivate the enzyme), thawed, and mixed well. Since the analytes are endogenous biomarkers, calibration standards were prepared in phosphate buffered saline (1X PBS), while quality control samples (QCs) were prepared in stabilized plasma containing pegzilarginase. A QC.0, with no added analyte, is used to correct for endogenous content of Arg and each GC QC in matrix.

	Arginine Method	Guanidino Method
Sample Aliquot Volume	25 µL	50 µL
Extraction Temperature	Wet Ice	
Internal Standard (IS) Solution	75:25:0.5 (v/v/v) ACN:MeOH:HCl (Containing arginine-$^{13}C_6$ and ornithine-d7)	1N HCl (Containing GVA-$^{13}C_6$, ArgA-$^{13}C_6$, NAArg-$^{13}C_6$, and GAA–$^{13}C_2$)
Reconstitution Solution	10 mM Ammonium Acetate in Water	No Reconstitution Step for the Guanidino Method
Extraction Steps	Precipitate with IS, Filter through Phenomenex® Phree Phospholipid Removal Plate, Evaporate at 50°C, Reconstitute, Mix, Centrifuge	Precipitate with IS, Add chilled 10% TCA, Mix, Centrifuge, Transfer Supernatant
Extracted Sample Storage	4°C	
UHPLC	Shimadzu Nexera®	
Mobile Phase A	10 mM Ammonium Acetate in MQ	80:20:0.2 (v/v/v) MeOH:MQ:FA
Mobile Phase B	5 mM Ammonium Acetate in 10:90 (v/v) MQ:ACN	70:30 (v/v) 100 mM Ammonium Formate:MeOH
Column	Agilent Zorbax Solvent Saver RRHT SB-CN, 3.0 x 100 mm, 1.8 µm particle size	Imtakt Intrada Amino Acid 50 x 2 mm
Column Temperature	50°C	40°C
MS	Applied Biosystems/MDS Sciex API 5500™	
Ionization Mode	ESI Positive	
IS Voltage	5500 v	
MS Temperature	600°C	550°C

Table 1: Arginine, ArgA, GVA, GAA, and NAArg plasma extraction and UHPLC-MS/MS analysis details.

3 RESULTS

An LC-MS/MS method for the analysis of Arg and GCs was successfully developed and validated over the calibration ranges: Arg 1.00 to 1,000 µM, GVA, ArgA, NAArg 0.030 to 10.0 µM, and GAA 0.300 to 100 µM. Acidified plasma stability was maintained for ≥3 freeze/thaw cycles and > 350 days for long term storage at -70°C.

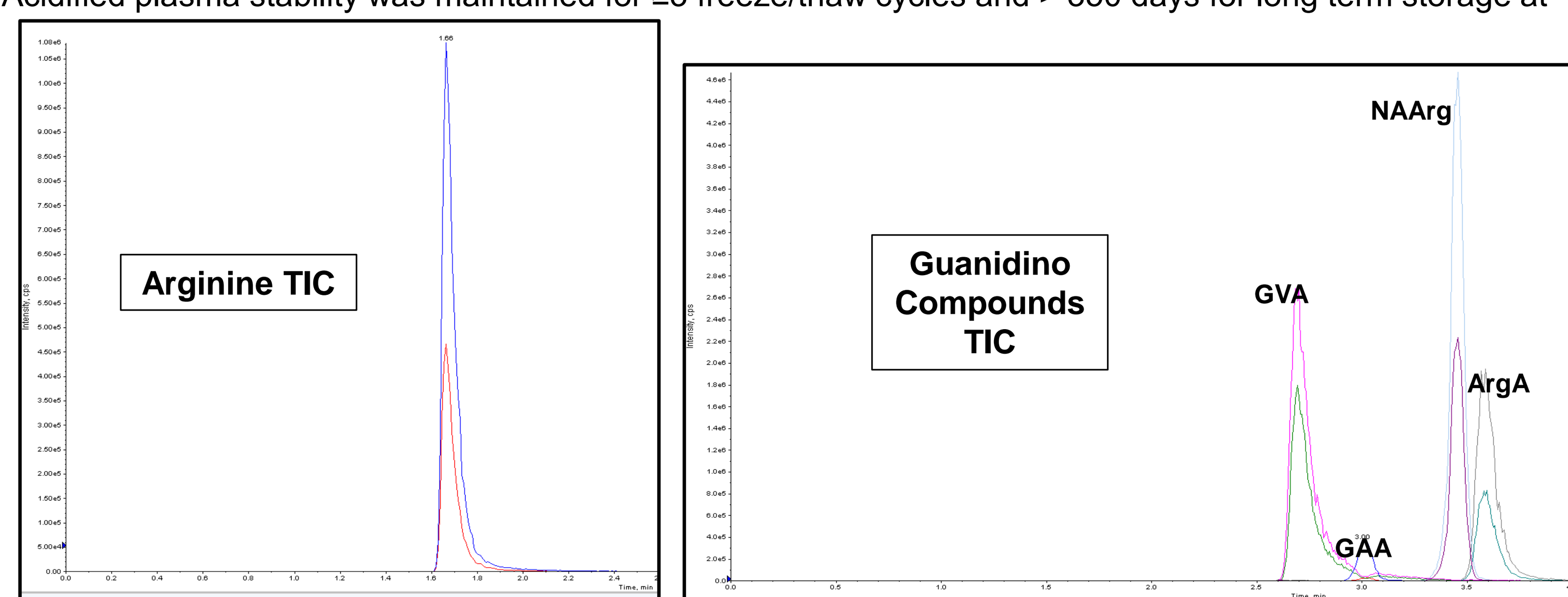

Figure 3: Above, to the left is a total ion current (TIC) of the upper limit of quantitation (ULOQ) calibration standard in PBS for arginine and its internal standard arginine-$^{13}C_6$. To the right is the TIC of the ULOQ calibration standard in PBS for the GCs and the corresponding internal standards (GVA-$^{13}C_6$, GAA-$^{13}C_2$, NAArg-$^{13}C_6$, and ArgA-$^{13}C_6$).

	Short-Term	Freeze-Thaw	Long-Term (-70°C)
Acidified Arginine	5.5 Hours	3 Cycles	1149 Days
Non-Acidified Arginine	Add glacial acetic acid prior to thaw	Add glacial acetic acid prior to thaw, however 1 cycle is supported	536 Days
Acidified GCs	16 Hours	4 Cycles	380 Days
Non-Acidified GCs	16 Hours	4 Cycles	380 Days

Table 2: Above are stability results (short-term on ice, freeze-thaw on ice, and long-term storage at approximately -70°C) for Arginine and the GCs in plasma stabilized with nor-NOHA and with and without glacial acetic acid.

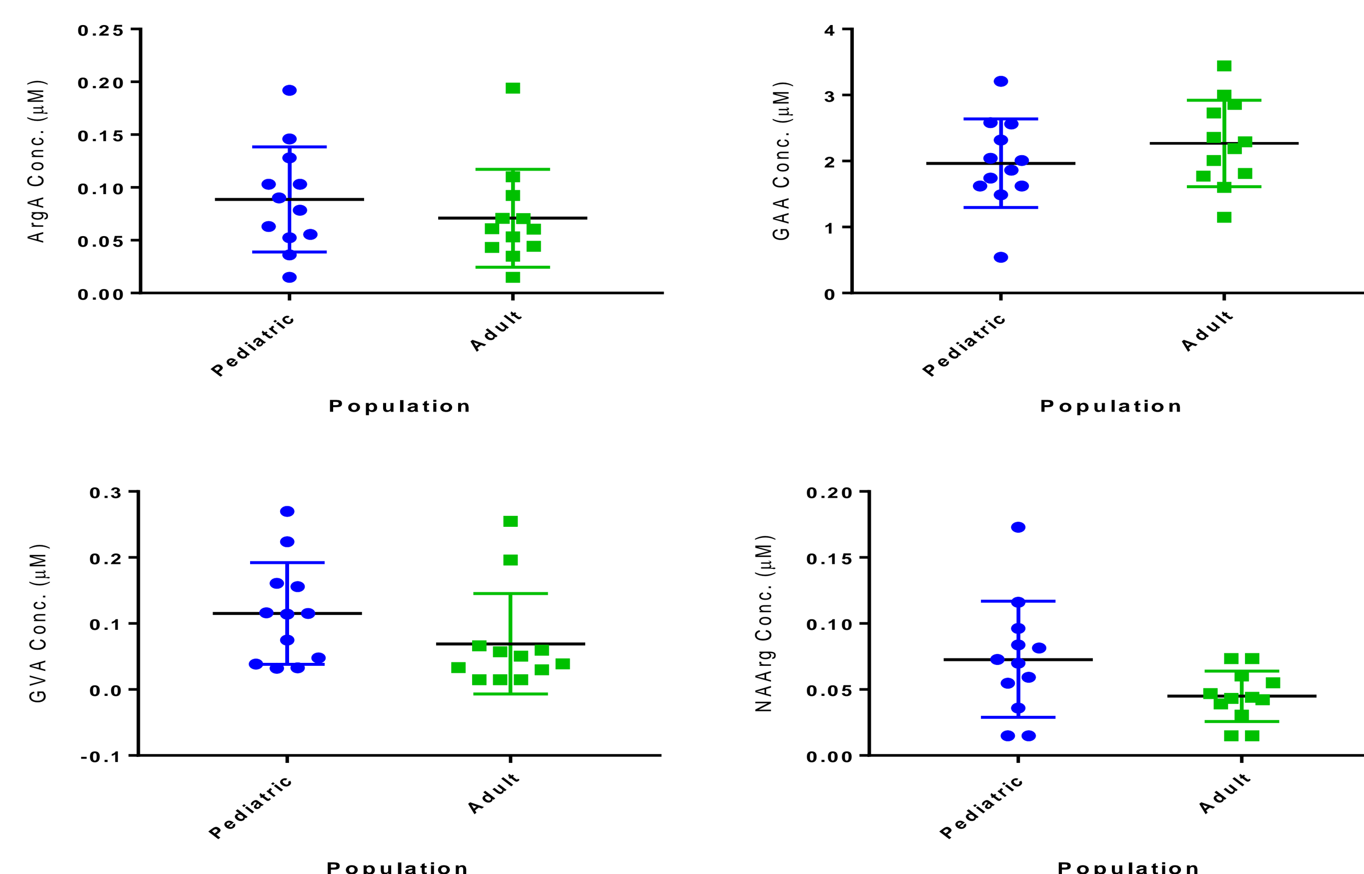

Figure 4: Baseline GCs in healthy pediatric and adult population (n=12) using the validated assay. Used to establish the Upper Limit of Normal (ULN, in µM) for each GC, as follows: ArgA: 0.2, GAA: 3.7, GVA: 0.3, NAArg: 0.17.

4 CLINICAL RESULTS (Continued)

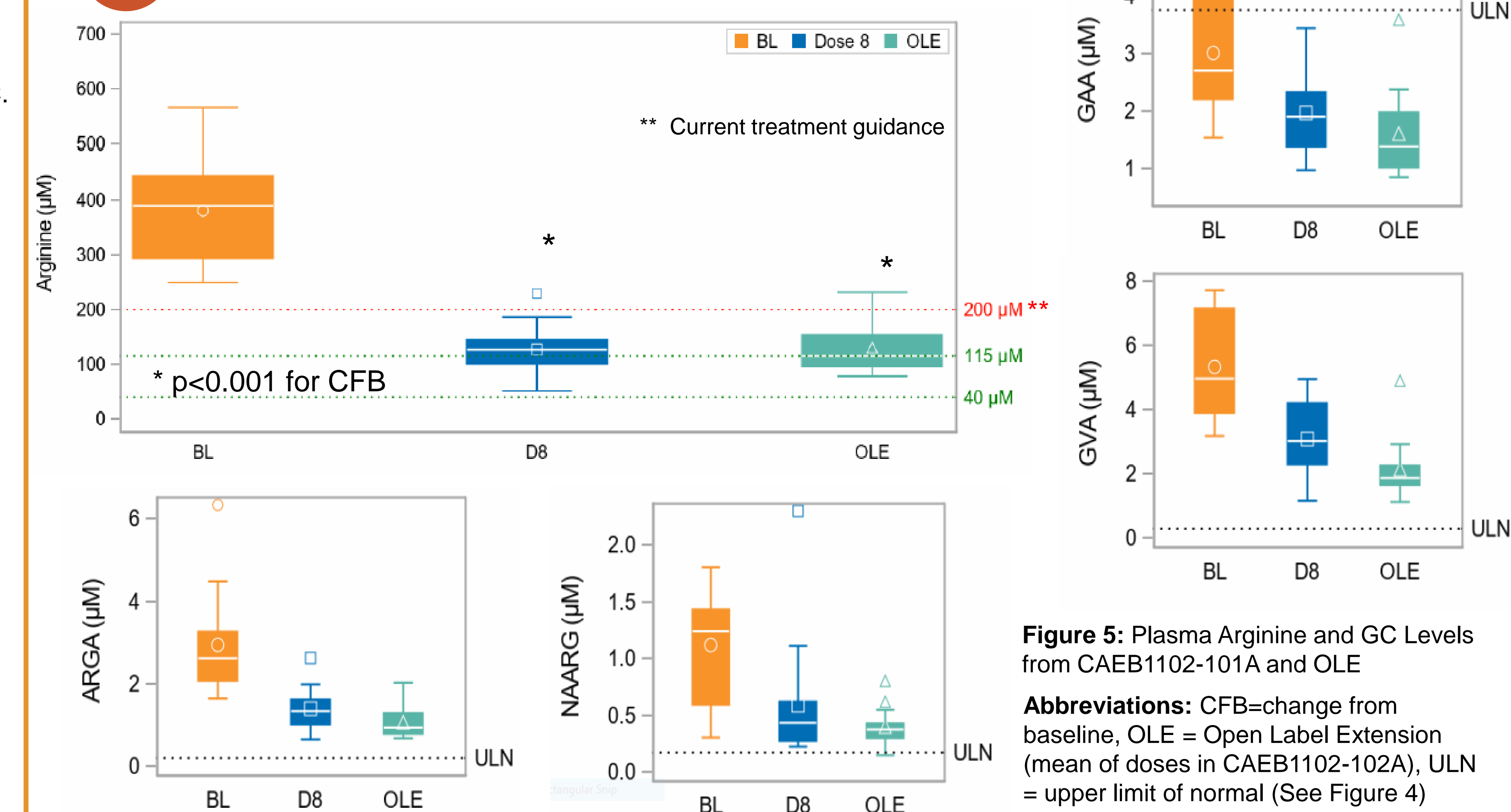

Figure 5: Plasma Arginine and GC Levels from CAEB1102-101A and OLE

Abbreviations: CFB=change from baseline, OLE = Open Label Extension (mean of doses in CAEB1102-102A), ULN = upper limit of normal (See Figure 4)

- Using this validated assay, baseline Arg, ArgA, GVA and NAArg were shown to be highly elevated in all ARG1-D patients while baseline GAA was above the ULN for 4 out of 14 ARG1-D patients.
- GAA was the most abundant GC in the plasma in normal subjects (Figure 4) and GVA was the most abundant in ARG1-D patients at baseline (Figure 5)
  - Baseline ArgA, GVA and NAArg were highly elevated in all ARG1-D patients
  - Baseline GAA was above the ULN for 4 out of 14 ARG1-D patients
- Plasma arginine reductions with pegzilarginase were accompanied by reductions in plasma GC levels
  - Median % plasma arginine reductions after 20 doses of pegzilarginase was 68.9%
  - Median % reductions in ArgA, GAA, GVA, and NAArg were 67.8%, 48.2%, 65.1%, and 70.1%, respectively

5 CONCLUSION

Use of nor-NOHA and glacial acetic acid as enzyme inhibitors are necessary to achieve an accurate quantitation of Arg and GCs in the presence of pegzilarginase. Using these validated assays it was shown that pegzilarginase (20 doses) treated patients experienced a median % plasma Arg reduction of 68.9% and median % reductions in ArgA, GAA, GVA, and NAArg of 67.8%, 48.2%, 65.1%, and 70.1%, respectively.

6 ACKNOWLEDGEMENTS

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7 REFERENCES

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