# Development of a Novel Gene Therapy for Fabry Disease: Engineered Alpha-Galactosidase A Transgene for Improved Stability

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## Abstract

Fabry disease is an X-linked lysosomal storage disorder caused by mutations in alpha-galactosidase A (GLA). Multiple approved treatment options exist for Fabry patients, including infused recombinant human alpha-Gal A (rhα-Gal A), termed enzyme replacement therapy (ERT), and an orally administered molecular chaperone for patients with amenable GLA variants. Limitations of ERT include low physical stability, a short circulating half-life at neutral pH of the blood, and variable uptake into different disease-relevant tissues, which may limit the efficacy of ERT as well as gene therapies relying on cross-correction. Secretion-uptake of enzymes is desirable in some target organs that are less transduced by AAV, such as kidney or blood vessel walls. We hypothesize that a stabilized human alpha-galactosidase A (hGLA) produced in vivo through gene therapy, will provide a larger window for the enzyme to stay active while in circulation prior to being taken up into the target tissues. To establish proof-of-concept, we engineered stabilized versions of hGLA and delivered it through AAV-based gene therapy with the goal to develop a one-time effective gene therapy for Fabry disease. To enhance stability, we focused the protein engineering on stabilizing the hGLA homodimer. Several engineered hGLA constructs showed improved enzyme stability and half-life under plasma-like neutral pH conditions as well as in the lysosome after cellular uptake in vitro. Top candidates were delivered to *Gla* knockout mice via low dose AAV mediated gene delivery using a pantropic capsid and ubiquitous promoter. We demonstrated that one engineered hGLA construct had equal or slightly lower enzyme activity levels than wildtype hGLA with enhanced globotriaosylsphingosine (lyso-$Gb_3$) substrate reduction in plasma as well as globotriaosylceramide (GL-3) storage reduction in disease-relevant tissues at doses that were subtherapeutic with wildtype hGLA. Collectively, our data shows that our stabilized hGLA is more potent and we provide the first of its kind proof-of-concept for an enhanced AAV-based gene therapy at low doses of vector suggesting a safe and translational approach for Fabry disease.

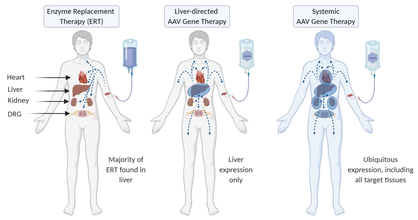
## Background

### Challenges of Delivering Lysosomal Enzymes to Target Tissues

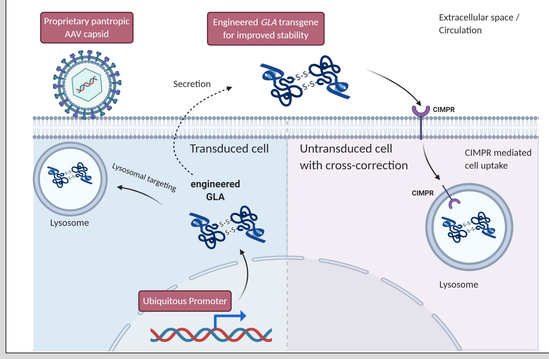
For Enzyme Replacement Therapy and AAV Gene Therapy the therapeutic enzymes need to pass through circulation to get to target tissues

**Problem:**
Lysosomal enzymes are optimized to function at pH <5, unstable at neutral pH of the blood pH 7.4

**Solution:**
- Improve tissue targeting/uptake: enzyme fusion with targeting motif
- Improve stability at neutral pH
  **=> Amicus Protein Engineering**




**Alpha-Galactosidase A** (GLA, defective in **Fabry disease**) is inactive after <1h in plasma

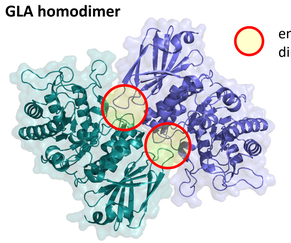



**Amicus Fabry Gene Therapy IND Candidate Approach**
- *GLA* transgene engineered for improved stability
- Proprietary pantropic AAV capsid
- Ubiquitous promoter

## Protein Engineering

### Rational design to enhance alpha-galactosidase stability

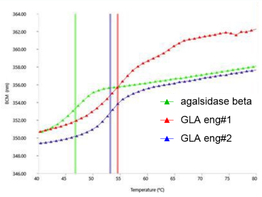
**GLA homodimer**




Variant GLA was rationally designed to introduce artificial disulfide (S-S) bridges for enhanced stability. Pairs of residues in contact on the homodimer interface were targeted for mutagenesis to cysteine; probable pairs were identified by torsional angles and inter-residue distance. The rotational axis of symmetry about the interface gives rise to two disulfide bridges per mutated pair.

## In vitro Characterization of Stabilized alpha-Galactosidase A Enzymes

### Thermostability of stabilized GLA proteins

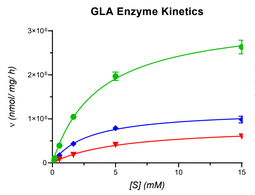



| Construct | Tm (°C) | Δ Tm (°C) |
|---|---|---|
| **agalsidase beta** | 47.0 | |
| **hGLA eng#1** | 54.5 | 7.5 |
| **hGLA eng#2** | 53.0 | 6.0 |

- Heat-induced melting profiles of different alpha-galactosidase A were recorded by thermal shift. Thermostability was assessed at neutral pH 7.4.
- Unfolding of GLA was monitored across a temperature range by the increase in intrinsic fluorescence as proteins undergo conformational changes.
- **Both Amicus engineered GLA proteins showed increased thermostability and a ~6-7°C increase in Tm at neutral pH.**


## In vitro Characterization of Stabilized alpha-Galactosidase A Enzymes

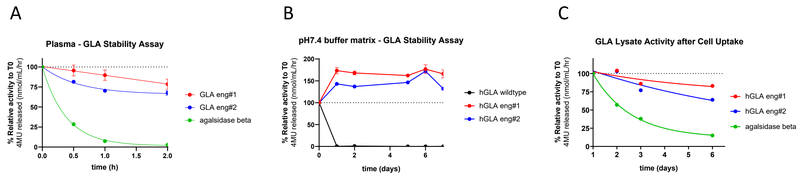
### Enzyme kinetics of stabilized GLA proteins




| Enzyme | $K_m$ (mM) | $k_{cat}$ ($s^{-1}$) | Specific Activity (nmol $mg^{-1}$ $h^{-1}$) |
|---|---|---|---|
| **agalsidase beta** | 3.65 | 83.6 | 2.26E+06 |
| **hGLA eng#1** | 5.42 | 20.9 | 2.78E+05 |
| **hGLA eng#2** | 2.87 | 30.3 | 6.25E+05 |

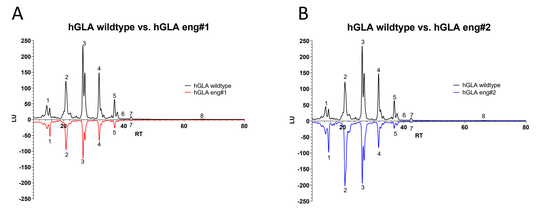
- Kinetic analysis was carried out using an artificial 4-MU substrate.
- **Characterization of both Amicus engineered recombinant alpha-galactosidase A constructs demonstrated they were enzymatically active with comparable $K_m$, but it revealed slightly reduced specific activity and $k_{cat}$ compared to the standard of care agalsidase beta.**

### Engineered GLA has enhanced stability extracellular at neutral pH and in the lysosome




- GLA stability at neutral pH and 37°C degrees was assessed short-term in plasma (**A**) and long-term in a pH 7.4 buffer matrix (**B**)
- In plasma, engineered Amicus hGLA constructs were stable over the course of 2 hours. However, agalsidase beta lost more than 50% of its activity within 30 min of incubation in plasma.
- In a pH 7.4 buffer matrix, engineered Amicus constructs are stable and showed no loss of activity over the course of 7-days, while wildtype GLA was inactive within the first day.
- Tracking intracellular GLA activity after cell uptake in Fabry patient fibroblasts (**C**) showed that stabilized GLA constructs have a 3-5 fold longer intracellular half-life in the lysosome.
- **Amicus stabilized GLA constructs showed increased stability at neutral pH and are expected to be more stable in vivo in circulation. Increased intracellular stability of engineered GLA constructs after uptake into the lysosomes is an added benefit.**

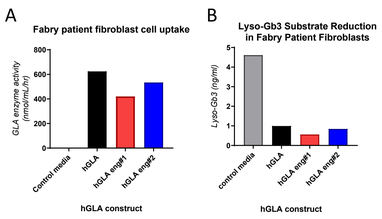
### N-glycan profiles are similar between wild-type hGLA and engineered constructs




| # | Glycan Type |
|---|---|
| 1 | High Mannose or De-Sialylated Complex |
| 2 | Mono-Sialylated |
| 3 | Di-Sialylated |
| 4 | Tri-Sialylated |
| 5 | Mono-Phosphorylated |
| 6 | Tetra-Sialylated |
| 7 | Hybrid-Phosphorylated |
| 8 | Bis-Phosphorylated |

- PNGase F released glycans were labelled with anthranilic acid (2-AA) and analyzed by HPLC-FLD.
- Comparison of hGLA eng#1 (**A**) and hGLA eng #2 (**B**) with wildtype hGLA revealed similar glycan profiles as well as low levels of mono-phosphorylated and bis-phosphorylated glycans types.
- **Protein engineering had no effect on the N-glycan profile.**

### Engineered alpha-galactosidase A is taken up by cells and reduces lyso-$Gb_3$ substrate




- Cell uptake experiment using Fabry patient fibroblasts with conditioned media containing recombinant hGLA constructs.
- After the uptake cell lysates were tested for GLA activity (**A**) and lyso-$Gb_3$ substrate reduction (**B**).
- **The GLA enzyme activity assay demonstrated that GLA wildtype and the engineered GLA constructs can be taken up into target cells. Lyso-$Gb_3$ substrate reduction in Fabry target cells confirmed that recombinant hGLA wildtype and the engineered hGLA constructs were able to utilize and break down endogenous substrates comparable to wildtype.**

## Study Design for Fabry Gene Therapy Preclinical POC Study

**Animal Model:**
- Fabry model *Gla* KO
- Wildtype *Gla +/+ (*Fabry model littermates)
- Gender: male (N=4) and female (N=4)

**Age:**
3-4 months at AAV dosing

**AAV vector:**
- Capsid: pantropic
- Promoter: ubiquitous

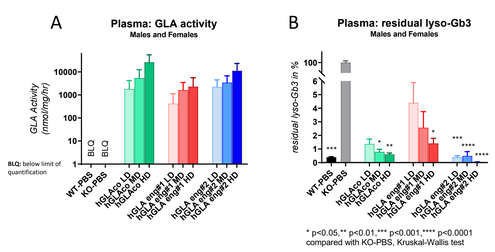
**AAV transgene:**
- Human GLA wildtype, codon optimized (hGLAco)
- 2 constructs with stabilized human hGLA, codon optimized (hGLA eng#1, hGLA eng#2)

**Dose / Route:**
- Dose ranging study (*) with low (LD), mid (MD) and high dose (HD)
- Tail Vein IV

**Duration**
Necropsy 4 weeks (day 28) after AAV administration

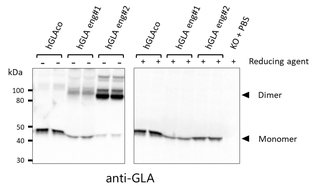
(*) LD and MD were chosen to best differentiate candidates, full dose range was analyzed with the lead candidate only

### hGLA eng#2 shows significant reduction in plasma lyso-$Gb_3$




- GLA enzyme activity and Lyso-$Gb_3$ substrate reduction were measured in AAV treated *Gla* KO animals and compared with untreated KO and WT mice (N = 8).
- High GLA enzyme levels of stabilized and wildtype hGLA were measured in plasma at day 28 in a dose dependent manner.
- **Stabilized construct hGLA eng#2 showed slightly lower GLA activity in plasma, but better in vivo efficacy reduction of Lyso-$Gb_3$ in D28 plasma compared to the wildtype hGLAco vector.**

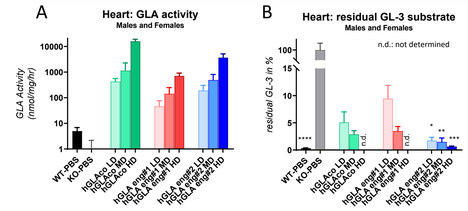
### Western Blot analysis of in vivo secreted GLA in plasma




- In vivo secreted alpha-Gal A from AAV treated animals was detected in plasma by Western Blot.
- As expected under non-reducing conditions the stabilized alpha-Gal A constructs with engineered disulfide bonds were mostly present as dimer.
- Addition of reducing agent disrupted the engineered disulfide bonds and the dimer indicated by the monomer band.
- **Introduction of artificial disulfide bridges into alpha-Gal A enzyme results in the formation of a stable secreted homodimer.**

## Fabry AAV Gene Therapy Preclinical POC Study

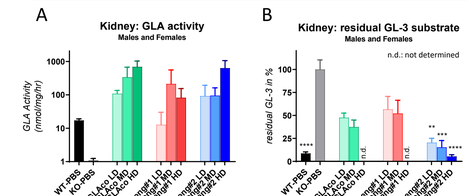
### hGLA eng#2 reduces GL-3 substrate in heart




* p<0.05,** p<0.01,*** p<0.001,**** p<0.0001 compared with KO-PBS, Kruskal-Wallis test

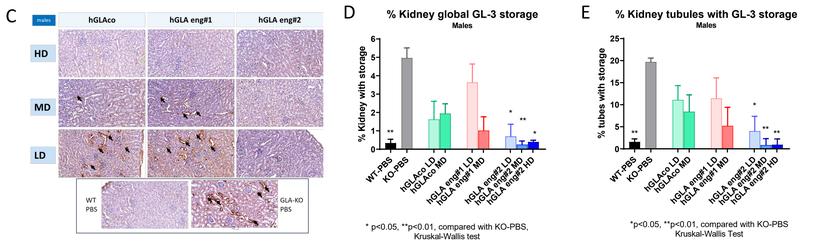
- Animals treated with high dose of our AAV vectors showed highly elevated alpha-Gal A activity in the heart compared to endogenous levels in untreated wildtype animals: hGLAco (~3.100-fold over wt), hGLA eng#1 (~140-fold over wt) and hGLA eng#2 (~730-fold over wt)
- **Amicus hGLA eng#2 stabilized construct showed lower alpha-Gal A enzyme activity in heart tissue (A), but significantly better in vivo efficacy indicated by greater reduction/prevention of GL-3 storage material compared to wildtype alpha-Gal A (hGLAco) (B).**

### hGLA eng#2 significantly reduces GL-3 substrate in kidney



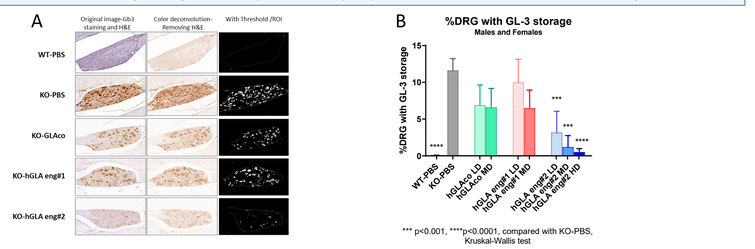

** p<0.01,*** p<0.001,**** p<0.0001 compared with KO-PBS, Kruskal-Wallis test

- Animals treated with high dose of our AAV vectors showed highly elevated alpha-Gal A activity in the kidney compared to endogenous levels in untreated wildtype animals: hGLAco (~40-fold over wt), hGLA eng#1 (~5-fold over wt) and hGLA eng#2 (~37-fold over wt)
- **Amicus hGLA eng#2 stabilized construct showed slightly lower or equal alpha-Gal A enzyme activity in kidney tissue (A), but significantly better in vivo efficacy indicated by greater reduction/prevention of GL-3 storage material compared to wildtype alpha-Gal A (hGLAco) (B).**




- GL-3 levels in kidney were examined by IHC on paraffin sections using an anti-GL-3 antibody. GL-3 signals are shown as brown spots in renal distal tubular epithelial cells (arrows) (**C**). Quantification of GL-3 IHC signal on kidney tissue sections is further evidence for in vivo efficacy (**D**, **E**).
- **Amicus hGLA eng#2 stabilized transgene was the only vector capable of fully clearing GL-3 storage material in male kidney samples at the lowest administered dose and showed significant reduction/prevention of GL-3 accumulation in the kidney globally (D) and more specifically in the tubules (E). It is remarkable that significantly lower GL-3 storage material was detected in all dosing groups down to the lowest administered dose.**

### hGLAeng#2 significantly reduced proportion of DRGs with GL-3 storage




- Dorsal root ganglia (DRG) sensory neurons are a critical disease relevant target tissue in Fabry disease. GL-3 substrate accumulation in DRG neurons is linked to neuropathic pain. Quantification of GL-3 IHC signal on tissue sections with DRG neurons (low dose treated males shown in **A**) is further evidence for in vivo efficacy (**B**).
- **Amicus hGLA eng#2 stabilized transgene was the only vector which achieved significant reduction/prevention of GL-3 accumulation in the DRG neurons (B). It is remarkable that significantly lower GL-3 storage material was detected in all dosing groups down to the lowest administered dose.**

## Conclusions

- In vitro characterization of two stabilized alpha-Gal A constructs with engineered disulfide bonds demonstrated stable homodimer formation, enhanced temperature, plasma, neutral pH stability compared to wildtype alpha-Gal A.
- **hGLA eng#2** in vivo efficacy is dose dependent in all analyzed tissues. Lowest tested dose in *Gla* ko mice showed partial substrate reduction while highest tested dose resulted in near complete substrate reduction.
- Our AAV vector with stabilized GLA transgene **hGLA eng#2** demonstrated significantly greater lyso-$Gb_3$ / GL-3 substrate reduction across all Fabry disease relevant tissues (DRG, kidney, heart), with reductions at low dose being equal to or greater than the reductions observed at higher doses with wildtype transgene.
- We hypothesize that despite equal or slightly lower enzyme activity levels of our stabilized alpha-Gal A construct **hGLA eng#2** in plasma and tissues the longer half-life pre- and post uptake did result in significantly superior lyso-$Gb_3$ / GL-3 substrate reduction/prevention compared to wildtype alpha-Gal A.
- We provided the first evidence for DRG storage reduction in a Fabry mouse model treated with AAV gene therapy.
- **hGLA eng#2** was selected as candidate (**AT-GTX-701**) for IND-enabling studies.
- This is a first of its kind proof-of-concept for an enhanced AAV-based gene therapy at low doses of vector suggesting a safe and translational approach for Fabry disease.

**Disclaimer:**
This work describes an investigational therapy not approved for treatment of any disease.

## Acknowledgement

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- GTP Program of Comparative Medicine
- Amicus Therapeutics: Kate Chang, Su Xu, Lukas M. Martin, Jessie Feng, Nastry Brignol, Leida Rassouli-Taylor, Joseph Conti, Anthony Perry, Matthew Pepper, Matthew Madrid, Renee Krampetz, Yi Lun, Andrea Gutierrez
- Graphics were created with BioRender.com

## Conflicts of interest

- All current employees of Amicus Therapeutics Inc. hold equity in the company.
- J.M. Wilson is an advisor to, holds equity in, and has a sponsored research agreement with Scout Bio and Passage Bio; he also has a sponsored research agreement with Ultragenyx, Biogen, Janssen, Precision Biosciences, Moderna Therapeutics, and Amicus Therapeutics who are licensees of Penn technology. JMW is an inventor on patents that have been licensed to various biopharmaceutical companies.
- Juliette Hordeaux is an inventor on provisional patent applications and patents related to AAV gene therapy.