SUPPLEMENTAL MATERIAL

Study design

Patients were randomly assigned 2:1 to receive intravenous (IV) pegunigalsidase alfa or agalsidase beta, 1 mg/kg every-2-weeks for 24 months. Randomization was stratified by screening spot urine protein-to-creatinine ratio (UPCR) of <1 or ≥1 g/g. The primary objective was to demonstrate that pegunigalsidase alfa was noninferior to agalsidase beta with respect to median annualized change in estimated glomerular filtration rate (eGFR; eGFR slope), based on a prespecified margin. The secondary efficacy endpoint reported here was change in plasma lyso-Gb3 concentration. Remaining secondary endpoints are available on request, including cardiac magnetic resonance imaging left ventricular mass index, UPCR category, severity and progression of clinical signs of Fabry disease (FD) (by Mainz Severity Score Index), pain medication use, and quality of life. Safety endpoints included treatment-emergent adverse events (TEAEs), infusion-related reactions, premedication use, and antidrug antibody (ADA) status.

After the first 3 months of treatment, infusion duration could be gradually reduced from 3 to 1.5 hours, depending on the investigator's judgment of patient tolerability. Premedication was administered at the first infusion, if previously used for agalsidase beta treatment, and then tapered down over 3 months based on the investigator's judgement of patient tolerability. The initial 3 months of infusions (approximately 6–7 infusions) were administered at the study center; patients could then receive home infusions, with investigator and medical monitor approval, based on patient tolerability, clinical condition, and local practices and regulations. After study completion, patients were invited to participate in an open-label extension study to receive 1 mg/kg pegunigalsidase alfa every-2-weeks (PB-102-F60; BRILLIANCE; NCT03566017).

The study was conducted at 29 study centers in 12 countries from 22 August 2016 to 12 October 2021: 15 study centers in the United States, 4 in the United Kingdom, and 1 each in the Netherlands, Spain, France, Italy, Norway, Slovenia, Switzerland, Finland, Hungary, and the Czech Republic.

Inclusion and exclusion criteria

Inclusion criteria

- Symptomatic adult Fabry disease (FD) patients, age 18-60 years
- Males: Plasma and/or leucocyte alpha galactosidase activity (by activity assay) less than 30% mean normal levels and one or more of the characteristic features of FD:
    - neuropathic pain
    - cornea verticillata
    - clustered angiokeratoma
- Females:
    - Historical genetic test results consistent with Fabry pathogenic mutation and one or more of the described characteristic features of FD
        - neuropathic pain
        - cornea verticillata
        - clustered angiokeratoma

    - Or in the case of novel mutations a first-degree male family member with Fabry disease with the same mutation, and one or more of the characteristic features of Fabry disease
        - neuropathic pain
        - cornea verticillata
        - clustered angiokeratoma
- Screening eGFR by Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation 40–120 mL/min/1.73 m²[1]
- Linear negative slope of eGFR based on at least 3 serum creatinine values over approximately 1 year (range of 9 to 18 months, including the value obtained at the screening visit) of ≥ 2 mL/min/1.73 m²/year
- Treatment with a dose of 1 mg/kg agalsidase beta per infusion every 2 weeks for at least one year and at least 80% of 13 (10.4) mg/kg total dose over the last 6 months
- Female patients and male patients whose co-partners are of child-bearing potential agree to use a medically accepted method of contraception, not including the rhythm method

Inclusion of the negative historical slope was based on the previous open label FAACET Study that assessed the impact of control of proteinuria to a pre-specified goal of <0.5 g/g creatinine with angiotensin converting enzyme (ACE) inhibitors or angiotensin II receptor blockers (ARBs) in patients with classical FD who were being treated with agalsidase beta 1 mg/kg every 2 weeks [2]. This threshold for the historical eGFR slope is similar but not identical to the threshold for the “high renal-risk” group classification that was based on baseline eGFR and renal biopsy findings [3].

Exclusion criteria

- History of anaphylaxis or Type 1 hypersensitivity reaction to agalsidase beta
- Known non-pathogenic Fabry mutations
- History of renal dialysis or transplantation
- History of acute kidney injury in the 12 months before screening, including specific kidney diseases (e.g., acute interstitial nephritis, acute glomerular and vasculitic renal diseases); non-specific conditions (eg, ischemia, toxic injury); as well as extrarenal pathology (e.g., prerenal azotemia, and acute postrenal obstructive nephropathy)
- Angiotensin converting enzyme (ACE) inhibitor or angiotensin receptor blocker (ARB) therapy initiated or dose changed in the 4 weeks before screening
- Patient with a screening eGFR value between 91–120 mL/min/1.73 m², having ahistorical eGFR value higher than 120 mL/min/1.73 m² (during 9 to 18 months before screening)
- Urine protein to creatinine ratio (UPCR) > 0.5 g/g and not treated with an ACE inhibitor or ARB
- Cardiovascular event (myocardial infarction, unstable angina) in the 6 months before randomization
- Congestive heart failure New York Heart Association (NYHA) class IV

- Cerebrovascular event (stroke, transient ischemic attack) in the 6 months before randomization
- Known history of hypersensitivity to gadolinium contrast agent that is not managed by the use of pre-medication
- Female patients who are pregnant, planning to become pregnant during the study, or are breastfeeding
- Presence of any medical, emotional, behavioral or psychological condition that, in the judgment of the investigator and/or medical director, would interfere with the patient's compliance with the requirements of the study

Important changes to methods after trial commencement

Version 4 dated 29 September 2016: Sample size was reduced from 78 to 66.

Version 5 dated 14 July 2017:

- Study design: Added that no more than 50% of the patients enrolled will be female
- Inclusion/ exclusion: To allow the inclusion of patients who are progressors and were excluded by the previous versions: eGFR by CKD-EPI equation 40 to 120 mL/min/1.73 m^2 (instead of 40–90) while keeping the rate of deterioration (slope). This amendment did not change the main study population characteristics.
- Inclusion/ exclusion: Alpha galactosidase activity criterion: Different inclusion criteria were set to male and female due to the different nature of the genetic characteristics of the disease in males and females.

Important changes to outcomes after trial commencement

Changes to safety endpoints included the addition of the ability to taper off infusion premedication throughout the first 3 months of the study. Changes to the overall study design and plan included the demonstration of the noninferiority of pegunigalsidase alfa compared with agalsidase beta at 12 months and superiority at 24 months for regulatory submissions to the European Medicines Agency (EMA) and Food and Drug Administration (FDA), respectively. Changes to planned statistical analyses included analyses of infusion-related reactions, occurrence of Fabry clinical events, achievement of Fabry Kidney Disease Therapeutic Goals, and a change in the primary study analysis from superiority to non-inferiority. Changes in the planned analyses were due to knowledge and insights that were gained in the overall clinical program for pegunigalsidase alfa since the finalization of the protocol.

Determination of sample size

We initially estimated that a sample size of 66 patients randomly assigned 2:1 would yield at least 90% power to demonstrate the noninferiority of pegunigalsidase alfa compared with agalsidase beta in terms of annualized change (slope) in eGFR. This study sample size was planned to demonstrate noninferiority after 1 year in an interim analysis and superiority after 2 years in the final analysis. Anticipating a dropout rate as high as 15%, it was planned for approximately 78 patients (pegunigalsidase alfa, n=52; agalsidase beta, n=26) to be randomly assigned. With these assumptions, the power for showing superiority at 2 years would be approximately 80%. After the approval of agalsidase beta and discussions with the

FDA, the final analysis was changed to non-inferiority at 2 years. Power and sample size calculations were performed using PASS-13 Group Sequential Tests for Two Means procedure (Hintze, J. 2014. PASS 13. NCSS, LLC. Kaysville, UT, USA. www.ncss.com). The power was computed assuming a one-sided two-sample *t*-test with a one-sided alpha level of 0.025 and a noninferiority margin of −3.0 mL/min/1.73 m^2/year. The true difference in slopes was assumed to be 1.1 mL/min/1.73 m^2/year in favor of pegunigalsidase alfa, with the standard deviation of the slopes being 1.5 mL/min/1.73 m^2/year in each group.

Randomization

Investigators obtained signed and dated written informed consent from each patient before exposure to any protocol-specific procedure. Patients were randomly assigned 2:1 (pegunigalsidase alfa:agalsidase beta) using a fixed block randomization list (52 blocks of 3 per stratum) stratified at baseline by UPCR incorporated into a Target e*CRF system that generated patient randomization ID numbers once patient eligibility was confirmed by the Protalix medical director. Most persons involved in the study and patients were blinded to the treatment assignment. The pharmacist preparing the study drug was given unblinded access to the e*CRF system and recorded information regarding the prepared dose for each patient. Drug accountability staff were unblinded to treatment.

Ancillary analyses

The primary analysis was conducted on all patient subgroups. Other subgroup analyses were conducted for selected efficacy and safety endpoints. If a patient was missing a value at baseline for one or more of the subgroups, the classification was based on the value at screening.

Prespecified supportive and sensitivity analyses for robustness were conducted for intent-to-treat (ITT) and per protocol (PP) analysis sets on the primary endpoint including, both a random intercept random slope (RIRS) longitudinal mixed model and random intercept (RI) longitudinal model to compare eGFR slopes between arms, and a UPCR-adjusted quantile regression and an analysis for missing data. Noninferiority of pegunigalsidase alfa was confirmed by supportive analyses (i.e., RIRS and RI models) and sensitivity analyses of eGFR slope (i.e., quantile regression for the PP, UPCR-adjusted sensitivity and adjustment for sex) (data not shown).

Anti-drug antibody evaluation

The presence of anti-pegunigalsidase alfa or anti-agalsidase beta immunoglobulin G (IgG) antibodies were determined by a multitiered approach of three sequential tests: a screening test, immunodepletion test, and titer determination, using a validated direct enzyme-linked immunosorbent assays (ELISA). In these ELISA, anti-pegunigalsidase alfa or anti-agalsidase beta antibodies in the patient's serum sample bind pegunigalsidase alfa- or agalsidase beta-coated plates and are detected by alkaline-phosphatase (ALP)-conjugated antihuman-IgG antibodies. The minimal required dilution (MRD) for the assay was 60-fold. Samples were defined as "positive" when the mean Optical Density (OD) was greater than the plate-specific cutpoint using a Floating Cutpoint, defined during the assay validation, and after a confirmatory step.

ADA-positive samples were further characterized for their neutralizing activity by evaluating the ADA inhibitory effect on the enzymatic activity of a known drug concentration. Undiluted patient's serum was incubated with the drug and a synthetic substrate (4 Methylumbelliferyl β-D-galactopyranoside), and the formation of 4-Methylumbelliferone product was measured. The inhibition of enzyme activity by an ADA-positive sample was compared to a pooled-normal-serum sample.

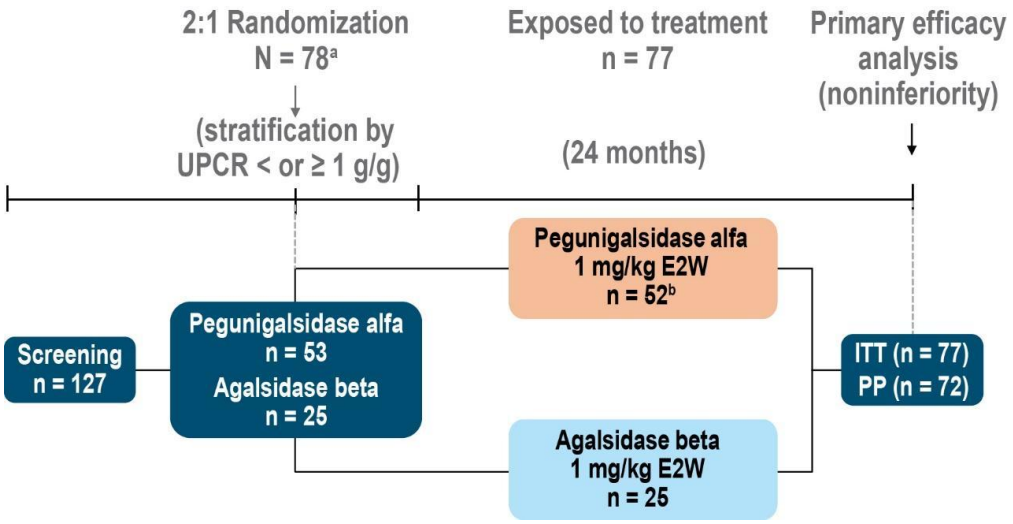
All assays were validated according to FDA and EMA immunogenicity guidelines. Patients were assessed for IgG ADAs at screening, baseline, 2 weeks after baseline, and monthly from month 1 to month 6, and then every 3 months up to the end of the study.

Those in the pegunigalsidase alfa arm who were positive for IgG were tested for epitope specificity (enzyme moiety, polyethylene glycol moiety, or plant glycan moiety). Patients with a suspected hypersensitivity reaction were also evaluated for antidrug IgE antibodies; one assessment was performed on the sample taken at the time of the event, and one on a retroactive assessment of the sample taken at baseline.

It should be noted that the ADA assay (especially for measuring anti-pegunigalsidase alfa antibodies) was designed to have a good drug tolerance to ensure that ADA will be detected even in the presence of a drug in the ADA serum sample. In the pharmacokinetics study, the lack of drug interference was demonstrated by showing a clear relationship between lower ADA titers and higher plasma pegunigalsidase alfa levels. In most patients, the drug levels at the time of ADA sampling were below the assay drug tolerance level. Yet, to be cautious, since pegunigalsidase alfa may be present in the blood at the time of ADA sampling, we cannot completely rule out the possibility that part of the drug remains bound to the ADA and therefore interferes with low titer ADA detection.

Covance Central Laboratory Services (Geneva, Switzerland), Covance (Asia) Pte. Ltd (Singapore), and Covance Central Laboratory Services, Inc. (Indianapolis, USA) were responsible for central laboratory sample shipments. Protalix LTD (Carmiel, Israel) was responsible for pharmacokinetics and antibody assessments. Waters-CHUS Expertise Centre in CIUSSS de l'Estrie-CHUS Hospital Fleurimont, (Sherbrooke, QC Canada) was responsible for plasma lyso-Gb3 assessments. The Academic Medical Center (AMC) Department of Clinical Genetics (Amsterdam, Netherlands) was responsible for the DNA analysis.

Online Supplemental Figure 1. Study design



[a]Reasons for screen failure included inclusion/exclusion criteria (n=39), consent withdrawn before randomization (n=3), and "other" (n=7).

[b]1 Randomly assigned patient withdrew consent prior to the first dose.

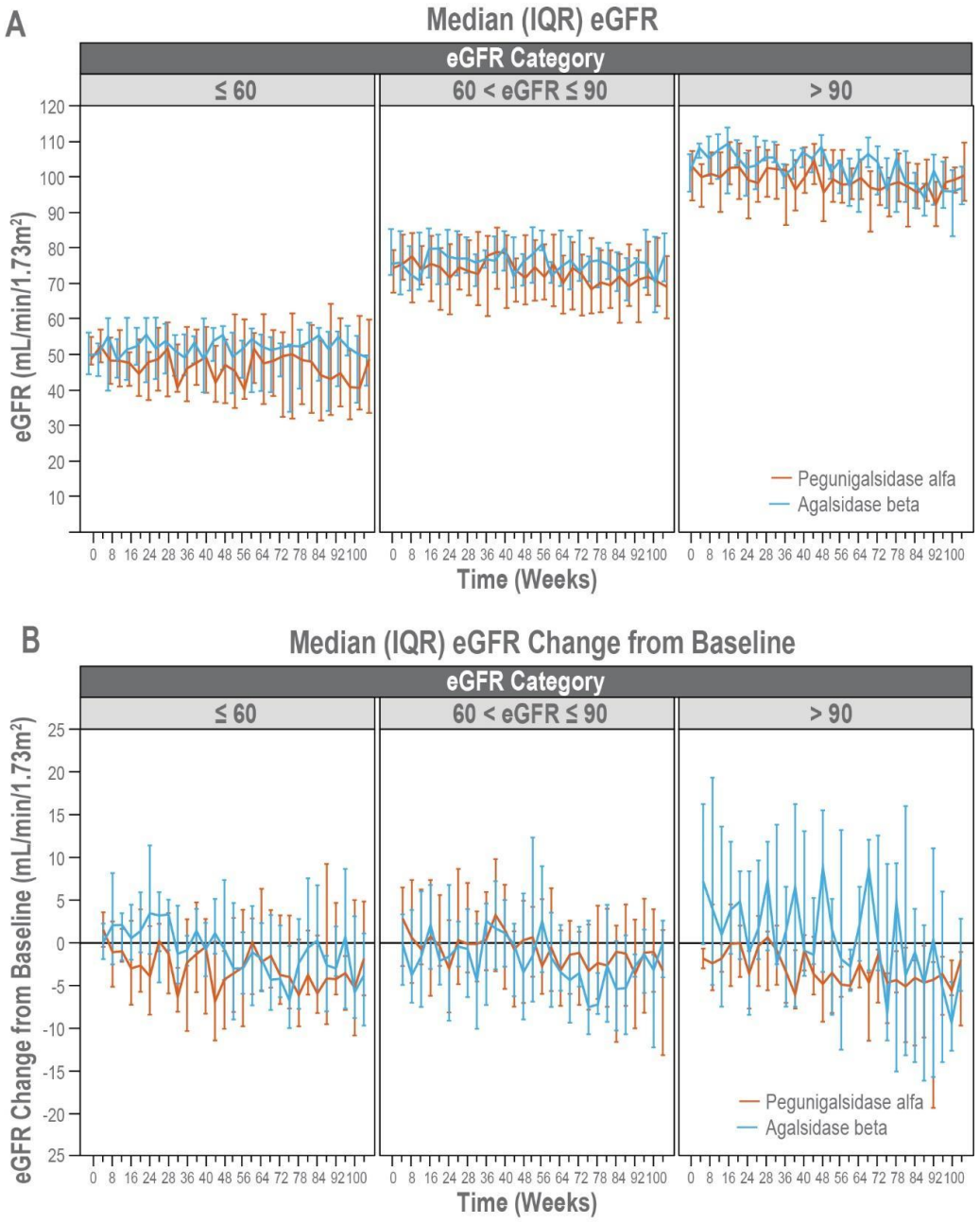
E2W, every-2-weeks; ITT, intent-to-treat; PP, per protocol; UPCR, urine protein creatinine ratio.

Online Supplemental Table 1. *GLA* variant analysis

Variant	Predicted protein change	Number of patients (%)
NM_000169.3:c.679C>T	p.(Arg227Ter)	6 (7.8)
NM_000169.3:c.680G>A	p.(Arg227Gln)	6 (7.8)
NM_000169.3:c.127G>A	p.(Gly43Ser)	3 (3.9)
NM_000169.3:c.966C>A	p.(Asp322Glu)	3 (3.9)
NM_000169.3:c.644A>G	p.(Asn215Ser)	2 (2.6)
NM_000169.3:c.668G>A	p.(Cys223Tyr)	2 (2.6)
NM_000169.3:c.791A>T	p.(Asp264Val)	2 (2.6)
NM_000169.3:c.836A>G	p.(Gln279Arg)	2 (2.6)
NM_000169.3:c.1042dup	p.(Ala348fs)	2 (2.6)
NM_000169.3:c.940A>T	p.(Lys314Ter)	2 (2.6)
NM_000169.3:c.547G>A	p.(Gly183Ser)	1 (1.3)
NM_000169.3:c.242G>A	p.(Trp81Ter)	1 (1.3)
NM_000169.3:c.2T>C	p.(Met1Thr)	1 (1.3)
NM_000169.3:c.41T>C	p.(Leu14Pro)	1 (1.3)
NM_000169.3:c.45del	p.(Leu16PhefsTer105)	1 (1.3)
NM_000169.3:c.132G>C	p.(Trp44Cys)	1 (1.3)
NM_000169.3:c.146G>C	p.(Arg49Pro)	1 (1.3)
NM_000169.3:c.157_160del	p.(Asn53LeufsTer67)	1 (1.3)
NM_000169.3:c.379A>T	p.(Lys127Ter)	1 (1.3)
NM_000169.3:c.396del	p.(Ile133PhefsTer32)	1 (1.3)
NM_000169.3:c.493G>C	p.(Asp165His)	1 (1.3)
NM_000169.3:c.514T>C	p.(Cys172Arg)	1 (1.3)
NM_000169.3:c.548G>T	p.(Gly183Val)	1 (1.3)
NM_000169.3:c.568del	p.(Ala190fs)	1 (1.3)
NM_000169.3:c.605G>A	p.(Cys202Tyr)	1 (1.3)
NM_000169.3:c.620A>G	p.(Tyr207Cys)	1 (1.3)
NM_000169.3:c.639+4A>T	Not available	1 (1.3)
NM_000169.3:c.674_732del	p.(His225LeufsTer5)	1 (1.3)
NM_000169.3:c.677G>A	p.(Trp226Ter)	1 (1.3)
NM_000169.3:c.707G>A	p.(Trp236Ter)	1 (1.3)
NM_000169.3:c.718_719del	p.(Lys240fs)	1 (1.3)
NM_000169.3:c.728_744del	p.(Leu243Ter)	1 (1.3)
NM_000169.3:c.778G>A	p.(Gly260Arg)	1 (1.3)
NM_000169.3:c.797A>T	p.(Asp266Val)	1 (1.3)
NM_000169.3:c.802-3_802-2del	Not available	1 (1.3)
NM_000169.3:c.830G>A	p.(Trp277Ter)	1 (1.3)
NM_000169.3:c.848A>G	p.(Gln283Arg)	1 (1.3)
NM_000169.3:c.861G>A	p.(Trp287Ter)	1 (1.3)
NM_000169.3:c.865A>T	p.(Ile289Phe)	1 (1.3)
NM_000169.3:c.890C>A	p.(Ser297Tyr)	1 (1.3)
NM_000169.3:c.902dup	p.(His302ThrfsTer13)	1 (1.3)
NM_000169.3:c.961C>T	p.(Gln321Ter)	1 (1.3)
NM_000169.3:c.983G>T	p.(Gly328Val)	1 (1.3)
NM_000169.3:c.1012G>T	p.(Glu338Ter)	1 (1.3)

NM_000169.3:c.1021G>A	p.(Glu341Lys)	1 (1.3)
NM_000169.3:c.1025G>A	p.(Arg342Gln)	1 (1.3)
NM_000169.3:c.1033_1034del	p.(Ser345fs)	1 (1.3)
NM_000169.3:c.1040dup	p.(Leu347fs)	1 (1.3)
NM_000169.3:c.1045T>A	p.(Trp349Arg)	1 (1.3)
NM_000169.3:c.1046G>A	p.(Trp349Ter)	1 (1.3)
NM_000169.3:c.1074_1075del	p.(Glu358AspfsTer16)	1 (1.3)
NM_000169.3:c.1088G>C	p.(Arg363Pro)	1 (1.3)
NM_000169.3:c.1193_1196del	p.(Glu398GlyfsTer5)	1 (1.3)
NM_000169.3:c.1212_1214del	p.(Arg404del)	1 (1.3)
NM_000169.3: c.(194+1_195-1)_(369+1_370-1)del	p.(Ser65Argfs*7)	1 (1.3)
NM_000169.2:c.(?_195)_(*1_?)del	Deletion	1 (1.3)
Not available	Not available	1 (1.3)

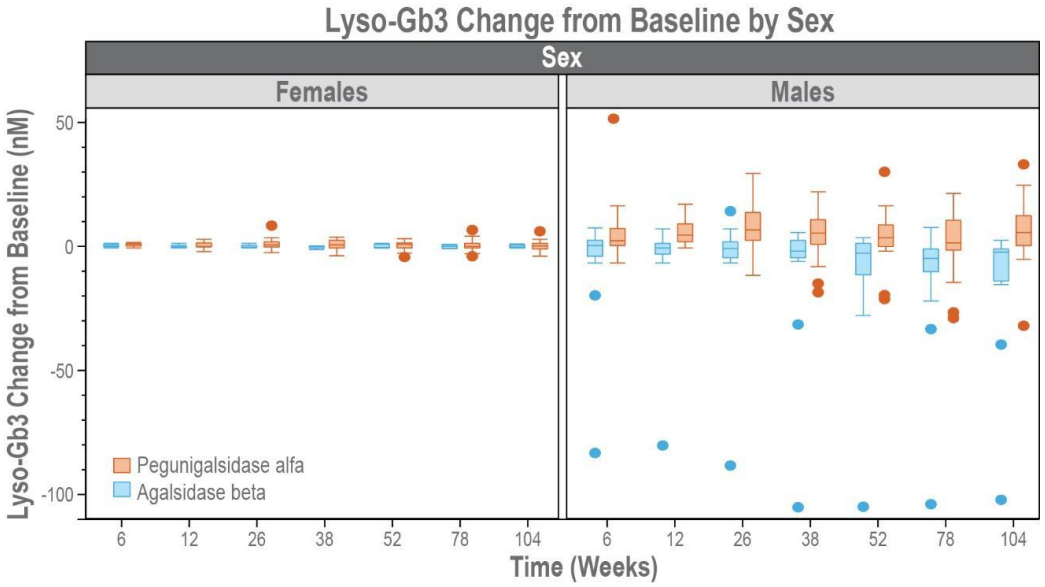
Online Supplemental Figure 2. Median (A) eGFR and (B) eGFR change from baseline over time by baseline eGFR category



Number of patients with baseline eGFR ≤60, 60<eGFR≤90, and >90 mL/min/1.73 m^2: pegunigalsidase alfa, n=13, n=28, and n=11, respectively; agalsidase beta, n=8, n=11, and n=6, respectively.

eGFR, estimated glomerular filtration rate; IQR, interquartile range.

Online Supplemental Figure 3. Plasma lyso-Gb3 change from baseline over time by sex



Boxes and whiskers represent the median and quartiles, with outliers as circles.

Lyso-Gb3, globotriaosylsphingosine.

Online Supplemental Table 2. TEAEs by system organ class and preferred term occurring in ≥10% of patients in any treatment arm

System Organ Class Preferred term	Pegunigalsidase alfa (n = 52) Patients, n (%)	 Events, n	Agalsidase beta (n = 25) Patients, n (%)	 Events, n
At least one TEAE	**47 (90.4)**	**561**	**24 (96.0)**	**406**
Blood and lymphatic system disorders	4 (7.7)	4	3 (12.0)	3
Cardiac disorders	16 (30.8)	25	10 (40.0)	17
Ear and labyrinth disorders	10 (19.2)	14	4 (16.0)	4
Eye disorders	4 (7.7)	6	4 (16.0)	6
Gastrointestinal disorders	24 (46.2)	54	17 (68.0)	47
Diarrhea	10 (19.2)	15	6 (24.0)	10
Nausea	9 (17.3)	10	3 (12.0)	3
Abdominal pain	6 (11.5)	6	0	0
Vomiting	6 (11.5)	8	3 (12.0)	8
Abdominal pain upper	2 (3.8)	2	4 (16.0)	7
Abdominal discomfort	1 (1.9)	1	3 (12.0)	3
General disorders and administration site conditions	22 (42.3)	48	14 (56.0)	38
Fatigue	9 (17.3)	10	4 (16.0)	6
Pyrexia	5 (9.6)	5	3 (12.0)	4
Oedema peripheral	4 (7.7)	9	3 (12.0)	3
Pain	2 (3.8)	3	3 (12.0)	5
Chest pain	1 (1.9)	1	3 (12.0)	3
Influenza like illness	1 (1.9)	1	3 (12.0)	4
Immune system disorders	6 (11.5)	8	2 (8.0)	6
Infections and infestations	38 (73.1)	105	16 (64.0)	74
Nasopharyngitis	11 (21.2)	21	4 (16.0)	6
Sinusitis	8 (15.4)	9	3 (12.0)	5
Upper respiratory tract infection	6 (11.5)	12	4 (16.0)	7
Urinary tract infection	6 (11.5)	6	3 (12.0)	4
Bronchitis	5 (9.6)	6	5 (20.0)	7
Viral infection	3 (5.8)	3	3 (12.0)	5
Pharyngitis	1 (1.9)	1	4 (16.0)	4
Injury, poisoning and procedural complications	15 (28.8)	22	12 (48.0)	30
Infusion-related reaction	2 (3.8)	2	1 (4.0)	5
Fall	1 (1.9)	1	3 (12.0)	4
Investigations	16 (30.8)	43	8 (32.0)	14
Blood creatinine increased	2 (3.8)	5	4 (16.0)	5
Metabolism and nutrition disorders	4 (7.7)	5	6 (24.0)	7
Musculoskeletal and connective tissue disorders	28 (53.8)	58	11 (44.0)	31
Back pain	8 (15.4)	12	5 (20.0)	6
Pain in extremity	8 (15.4)	15	4 (16.0)	5
Muscle spasms	5 (9.6)	6	3 (12.0)	3
Nervous system disorders	29 (55.8)	62	14 (56.0)	32
Headache	11 (21.2)	19	5 (20.0)	9
Dizziness	6 (11.5)	8	2 (8.0)	2
Paresthesia	2 (3.8)	2	4 (16.0)	8
Psychiatric disorders	6 (11.5)	8	6 (24.0)	6
Renal and urinary disorders	12 (23.1)	23	2 (8.0)	2
Proteinuria	6 (11.5)	7	0	0

Reproductive system and breast disorders	3 (5.8)	5	3 (12.0)	3
Respiratory, thoracic, and mediastinal disorders	19 (36.5)	34	13 (52.0)	29
Cough	6 (11.5)	7	5 (20.0)	7
Oropharyngeal pain	3 (5.8)	4	3 (12.0)	3
Rhinorrhea	1 (1.9)	1	3 (12.0)	3
Skin and subcutaneous tissue disorders	17 (32.7)	19	9 (36.0)	48
Pruritis	0	0	3 (12.0)	23
Vascular disorders	7 (13.5)	9	3 (12.0)	3

Note: Infusion-related reactions may be listed under more than one system organ class.
TEAE, treatment-emergent adverse event.

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