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Research paper

Association of immune response with efficacy and safety outcomes in adults with phenylketonuria administered pegvaliase in phase 3 clinical trials

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ABSTRACT

Background: This study assessed the immunogenicity of pegvaliase (recombinant *Anabaena variabilis* phenylalanine [Phe] ammonia lyase [PAL] conjugated with polyethylene glycol [PEG]) treatment in adults with phenylketonuria (PKU) and its impact on safety and efficacy.

Methods: Immunogenicity was assessed during induction, upward titration, and maintenance dosing regimens in adults with PKU ($n = 261$). Total antidrug antibodies (ADA), neutralizing antibodies, immunoglobulin (Ig) M and IgG antibodies against PAL and PEG, IgG and IgM circulating immune complex (CIC) levels, complement components 3 and 4 (C3/C4), plasma Phe, and safety were assessed at baseline and throughout the study. Pegvaliase-specific IgE levels were measured in patients after hypersensitivity adverse events (HAE).

Findings: All patients developed ADA against PAL, peaking by 6 months and then stabilizing. Most developed transient antibody responses against PEG, peaking by 3 months, then returning to baseline by 9 months. Binding of ADA to pegvaliase led to CIC formation and complement activation, which were highest during early treatment. Blood Phe decreased over time as CIC levels and complement activation declined and pegvaliase dosage increased. HAEs were most frequent during early treatment and declined over time. No patient with acute systemic hypersensitivity events tested positive for pegvaliase-specific IgE near the time of the event. Laboratory evidence was consistent with immune complex-mediated type III hypersensitivity. No evidence of pegvaliase-associated IC-mediated end organ damage was noted.

Interpretation: Despite a universal ADA response post-pegvaliase administration, adult patients with PKU achieved substantial and sustained blood Phe reductions with a manageable safety profile.

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1. Introduction

Phenylketonuria (PKU; OMIM 261600), also known as phenylalanine hydroxylase (PAH) deficiency, is a rare, autosomal recessive disease resulting in high concentrations of blood phenylalanine (Phe). Elevated blood Phe in adults is associated with executive dysfunction and significant behavioral and psychiatric problems, which lead to a

negative impact on patient quality of life [1–4]. A recent survey of patients with PKU reported that only 23% of adult patients [5] were able to maintain blood Phe within the therapeutic range of 120 to 360 $\mu\text{mol/L}$ [6].

Treatment for PKU includes severe restriction of dietary Phe, supplemented with Phe-free or low-Phe amino acid-modified medical foods, alone or in combination with sapropterin dihydrochloride (KUVAN[®], BioMarin Pharmaceutical Inc., Novato, CA). However, for most adults with PKU, these treatments are insufficient to manage their chronic disease due to long-term adherence issues or inadequate Phe-lowering effects [1,5,7–9].

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Research in context

Evidence before this study

Phenylketonuria (PKU), an autosomal recessive disease caused by phenylalanine hydroxylase deficiency, results in high blood phenylalanine (Phe) levels leading to neuropsychiatric and neurocognitive symptoms. Pegvaliase is a PEGylated, bacterially produced, phenylalanine ammonia lyase (PAL) enzyme substitution therapy that lowers blood Phe by converting Phe to trans-cinnamic acid and ammonia. Protein therapeutics have been shown to induce antidrug antibodies (ADA), which can impact clinical safety and efficacy. Development of anti-polyethylene glycol (PEG) antibodies following treatment with PEGylated therapeutics has been reported, but the nature of these antibodies and their clinical consequences are not well established.

Added value of this study

This is the first time that the immunogenicity of a chronically administered, bacterially derived, PEGylated enzyme therapeutic and its impact on safety and efficacy have been characterized. During early treatment (≤ 6 months after initiation), patients developed an immune response composed of anti-PEG antibodies and anti-PAL immunoglobulin (Ig) M. During late treatment (> 6 months after initiation), anti-PEG antibodies reduced to baseline levels and the immune response was mainly anti-PAL IgG and IgM. Circulating immune complexes (CICs) and complement activation were highest in early treatment and decreased as the anti-PEG antibodies decreased, suggesting that the PEGylation was effective in shielding binding of PAL-specific antibodies to the drug and forming CICs. Hypersensitivity adverse events were most frequent in early treatment and decreased as CICs and complement activation lessened, but required slow titration of the drug to efficacious levels. Blood Phe concentrations decreased over time as the immune response matured and pegvaliase dosage increased.

Implications of all the available evidence

Patients treated with pegvaliase developed ADA responses that matured to a clinical non responsive state over time, allowing substantial and sustained blood Phe reduction to be achieved with a manageable safety profile for most patients.

Pegvaliase (Palynziq™, BioMarin Pharmaceutical Inc., Novato, CA) is a bacterially derived (recombinant *Anabaena variabilis* [rAv]) phenylalanine ammonia lyase (PAL) conjugated with polyethylene glycol (PEG) and produced in *Escherichia coli*. PEGylation of the PAL enzyme reduces immune recognition and improves pharmacodynamic stability. Pegvaliase, an enzyme substitution therapy, metabolizes Phe to trans-cinnamic acid and ammonia. Based on results from phase 3 studies showing substantial and sustained reductions in mean blood Phe and long-term improvements in symptoms of inattention and mood, pegvaliase is approved in the United States to treat adult patients with PKU and is the first bacterially derived therapeutic protein approved for the treatment of a chronic disease [4,7,10].

Biologics, including enzyme replacement therapies (ERTs), are used for a range of disease treatments. Many ERTs induce antidrug antibodies (ADAs) that result in adverse events (AEs) and reduced efficacy, and/or impact pharmacokinetics [11,12]. AEs may be due to the formation of drug/ADA circulating immune complex (CIC) intermediates with downstream effects [13]. Neutralizing antibody (NAb) formation can inhibit

enzymatic activity and efficacy [14,15]. Given that pegvaliase contains bacterially derived PAL, development of ADAs after treatment with pegvaliase is expected. Further, an increasing number of reports document development of anti-PEG antibodies following treatment with PEGylated therapeutics [16–18]. Anti-PEG antibodies have been associated with reduced efficacy and hypersensitivity with some therapeutics, but it is unclear why only some PEGylated therapeutics induce a clinically relevant anti-PEG antibody response [16,18]. Therefore, the objective of this study was to comprehensively characterize the immunogenicity profile after pegvaliase treatment, including anti-PEG and anti-PAL antibody responses and CIC and complement levels, and assess the impact of that response on safety and efficacy.

2. Materials and methods

The methodology has been previously described for the phase 3 PRISM-1 and PRISM-2 studies (ClinicalTrials.gov Identifiers: NCT01819727, NCT01889862) [4]. Patients in the phase 3 trials self-administered subcutaneous pegvaliase using an induction, titration, and maintenance dosing schedule. Patients were randomized in PRISM-1 to titrate to a maintenance dose of 20 mg/day or 40 mg/day of pegvaliase. In PRISM-2, dosing of 5 to 60 mg/day of pegvaliase was allowed [4]. Details on the structural and biochemical characteristics of rAvPAL (accession number: 3CZO) have previously been reported [19]. Baseline blood Phe and immunogenicity data are provided (Supplemental Table 1). Informed consent was obtained from each participant and the PRISM studies were conducted in accordance with the Declaration of Helsinki. Prior to initiating the study, the investigator at each study site obtained written confirmation that the institutional review board (IRB) was properly constituted and compliant with International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use and Good Clinical Practice requirements and applicable laws and local regulations. A complete list of the IRBs is provided in the Supplemental Text. An independent data monitoring committee governed the conduct of the phase 3 trials.

2.1. Assessments

Blood Phe (measured in plasma), immunogenicity (measured in serum), and safety assessments were conducted at baseline and at least monthly during the induction, titration, and maintenance periods until 1 year on treatment, at which point assessments were conducted bimonthly. Established CLIA/CAP methods were used to measure Phe, and ADA assays were validated to assess serum positivity and titers for anti-pegvaliase total antibodies (TAB), PAL immunoglobulin (Ig) M, PAL IgG, PEG IgM, PEG IgG, and antibodies that inhibit pegvaliase enzyme activity (NAb). IgG and IgM CICs were measured at baseline and at approximately 12, 36, 60, 84, and 108 weeks after first dose. For acute systemic hypersensitivity events (see “Safety measurements”), a clinic visit was required for measurement of anti-pegvaliase IgE and other clinical laboratory parameters (high-sensitivity C-reactive protein, complement components 3 and 4 [C3/C4] antigens, and trypsin).

2.2. Safety measurements

Safety was monitored using clinical laboratory tests (chemistry, hematology, and urinalysis), vital signs, physical examinations, electrocardiograms, and AEs (coded by MedDRA preferred terms [20]). AEs suggestive of immune complex (IC) formation were assessed; these included arthralgia/arthritis, generalized skin reaction lasting ≥ 14 days, urticaria lasting ≥ 14 days, lymphadenopathy lasting ≥ 14 days, serum sickness-like reaction, serious infections, and ischemic heart disease. Search strategies for laboratory data identified abnormalities that could be reflective of IC disease. Hypersensitivity adverse events (HAEs) were identified using broad modified hypersensitivity Standardized MedDRA Queries (SMQ), which included additional preferred

term AEs of arthralgia, arthritis, eye inflammation, eye irritation, eye pain, joint stiffness, joint swelling, pyrexia, blurred vision and polyarthritides, and the broad algorithmic anaphylactic reaction SMQ. The anaphylactic reaction SMQ, hypersensitivity SMQ, and MedDRA preferred terms were used to identify all AEs that could be component manifestations of acute systemic hypersensitivity events and/or reported as anaphylactic reactions. An allergist/immunologist independent of the clinical site and sponsor reviewed all potential episodes of acute systemic hypersensitivity events to identify the events consistent with clinical criteria of anaphylaxis defined by the National Institute of Allergy and Infectious Diseases/Food Allergy and Anaphylaxis Network [21] and Brown's severe criteria (ie, hypoxia, hypotension, or neurologic compromise) [22].

2.3. Assays

Semi-quantitative direct-format electrochemiluminescent titer assays were used to measure PAL-specific IgM, PAL-specific IgG, PEG-specific IgM, PEG-specific IgG, and anti-pegvaliasse TAB titers in serum. Pegvaliasse NAb titers were measured using a semi-quantitative hybrid ligand-binding liquid chromatography/tandem mass spectrometry method. Pegvaliasse-specific IgE was measured using an ImmunoCAP assay that was modified to include a step whereby IgG and IgM were depleted from serum before IgE assay. Complement-activating CICs containing IgG and complement C3d were measured in serum using the MicroVue CIC-Raji Cell Replacement (also referred to as CIC-C3d EIA) assay (Quidel Corporation, San Diego, CA). The MicroVue assay was modified to measure CIC containing IgM and C3d. Decreases in C3 and C4 levels were monitored in the serum as biomarkers of complement activation. Phe concentrations were measured in deproteinized plasma using ion exchange chromatography coupled with ninhydrin derivatization. Details of each assay method are provided (see Supplemental Text).

2.4. Statistical analyses

The analysis population included all patients who first initiated pegvaliasse in PRISM-1 and included all data collected in PRISM-1 and PRISM-2 as of September 23, 2016. Baseline values were defined as the last available measurement prior to the first administration of pegvaliasse in PRISM-1 (ie, pegvaliasse-naïve baseline). Blood Phe data were summarized at monthly intervals relative to baseline. The safety analyses included data on the incidence, exposure-adjusted event rate, and severity based on Common Terminology Criteria for Adverse Events grade (grade 1, mild; grade 2, moderate; grade 3, severe but not immediately life-threatening; grade 4, life-threatening; grade 5, death). An exposure-adjusted event rate analysis was performed to control for differences in duration of study participation.

Analysis of the association of CIC levels with blood Phe reduction was performed by dividing patients into quartiles based on mean IgG-CIC or IgM-CIC change from baseline over the entire treatment duration. To evaluate the relationship between antibody titers and HAEs, patients were divided into quartiles based on their mean antibody titer during early (≤ 6 months) or late (> 6 months) treatment phases plotted against the number of HAEs. To evaluate associations between CIC concentrations and exposure-adjusted AE rates, patients were divided into quartiles based on their mean CIC change from baseline level calculated from all available results. Mean percent change in blood Phe was calculated for each quartile at each evaluated time point. Patients with complement reduction $< 25\%$ and $\geq 25\%$ at Year 1 from baseline were compared to evaluate the association of complement reduction with AEs or labs of interest.

3. Results

Two hundred and sixty-one patients enrolled in PRISM-1, as described previously [4]. Mean $\pm$ standard deviation (SD) blood

concentration of Phe at baseline was $1232.7 \pm 386.36 \mu\text{mol/L}$, indicating poor control.

3.1. Development of a biphasic immune response against pegvaliasse

All patients developed TAB responses against pegvaliasse (Supplemental Table 2), comprising subpopulations of treatment-induced ADA to PAL and treatment-induced or treatment-boosted ADA to PEG. The antibody response occurred in a biphasic manner; the response in early treatment (≤ 6 months after treatment initiation) was primarily composed of anti-PEG IgM, anti-PEG IgG, and anti-PAL IgM, whereas the response in late treatment (> 6 months) was composed of anti-PAL IgM and anti-PAL IgG (Fig. 1a and b).

Anti-PEG antibody titers peaked at 1 to 3 months after treatment initiation and then returned to baseline in most patients by 6 to 9 months. The incidence of anti-PEG IgG and anti-PEG IgM at baseline was 52.3% and 45.0% (Supplemental Table 2), respectively, resulting from previous exposures to PEG, which is within the range reported in the literature [23–25]. The presence of anti-PEG antibodies at baseline was not associated with an increased number of hypersensitivity events (Supplemental Table 3).

Anti-PAL IgM and IgG titers peaked at 3 and 6 months, respectively, and remained stable through long-term treatment. The majority of patients developed a low-titer neutralizing ADA (NAb) response, which remained stable through long-term treatment (mean NAb titer at 6 months: 415 [$n = 192$]; 1 year: 608 [$n = 144$]; 2 years: 661 [$n = 91$]; Supplemental Table 2).

Mean CIC levels were highest during early treatment and then decreased, corresponding with the change in anti-PEG responses over time. Peak IgG-containing CIC levels were associated with declining C3 and C4 levels, indicating complement activation (Fig. 1c). Overall, there was a high degree of consistency in the pattern of IgG CIC and IgM CIC development over time, suggesting that CICs comprised both IgM and IgG and can be detected with either assay method.

Antibody titers and IgG CIC levels either decreased (ie, anti-PEG antibodies) or stabilized (ie, anti-PAL antibodies) over time and did not increase after dose increases (Fig. 1c, Supplemental Fig. 1).

3.2. Immunogenicity and efficacy

The immune response to pegvaliasse was associated with blood Phe reduction. During early treatment, anti-PEG IgG, anti-PEG IgM, anti-PAL IgM, and CIC levels were highest, C3/C4 levels were lowest, and plasma Phe concentrations were high (blood Phe [mean $\pm$ SD] = $922.5 \pm 525.1 \mu\text{mol/L}$, $n = 237$ at 3 months). In late treatment, anti-PEG IgG, anti-PEG IgM, CIC, and C3/C4 levels returned toward baseline values, pegvaliasse dose was adjusted to individual blood Phe response, and blood Phe concentrations decreased (blood Phe [mean $\pm$ SD] = $1232.7 \pm 386.4 \mu\text{mol/L}$ at baseline; $564.5 \pm 531.2 \mu\text{mol/L}$ at 12 months; $311.4 \pm 426.6 \mu\text{mol/L}$ at 24 months).

As shown in Fig. 2, the magnitude of the NAb immune response was inversely associated with the degree of blood Phe reduction. In early treatment, patients in the lowest antibody titer quartile (Q1) experienced the largest reductions in blood Phe. Following stabilization of antibody titers and dose adjustment, mean blood Phe concentrations continued to decrease, including in patients within higher antibody titer quartiles (Q2, Q3, and Q4). A similar trend was observed across all antibody types (non-neutralizing, neutralizing), analytes (anti-PAL, anti-PEG, TAB), and isotypes (IgM, IgG). A related pattern was also observed between the magnitude of the CIC levels and blood Phe reduction. Quartile analysis of IgG CIC levels (Fig. 3) indicated that patients with less change from baseline in IgG CIC had more rapid mean blood Phe reduction, although substantial blood Phe reduction was observed in all IgG CIC quartiles.

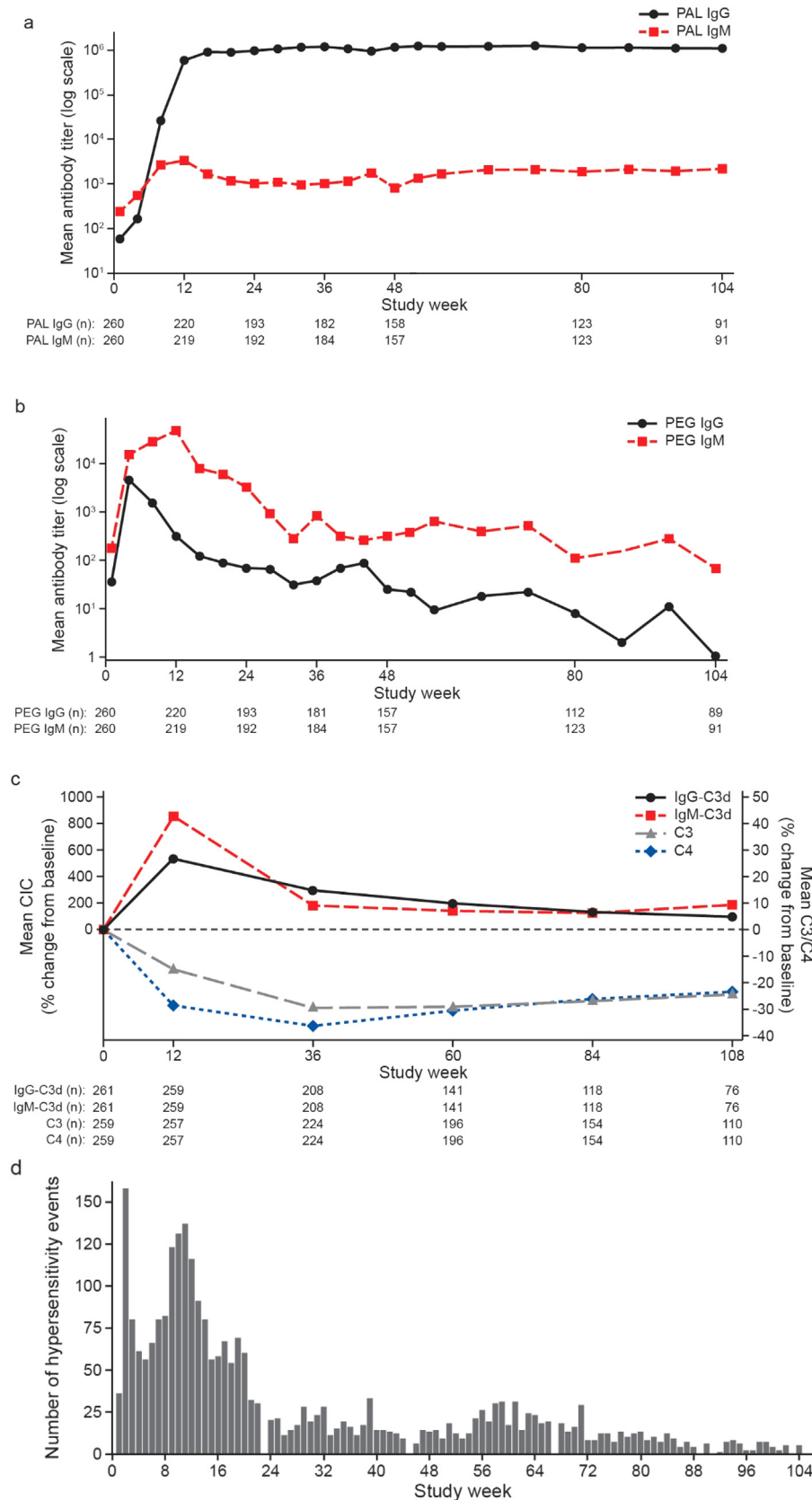


Fig. 1. Anti-pegvaliase ADA, CIC, and C3/C4 response (a) PAL IgG and PAL IgM titers, (b) PEG IgG and PEG IgM titers, (c) percent change in CIC and C3/C4 levels from baseline, (d) hypersensitivity adverse events. ADA, antidrug antibody; C3/C4 complement components 3 and 4; CIC, circulating immune complex; IgG, immunoglobulin G; IgM, immunoglobulin M; PAL, phenylalanine ammonia lyase; PEG, polyethylene glycol.

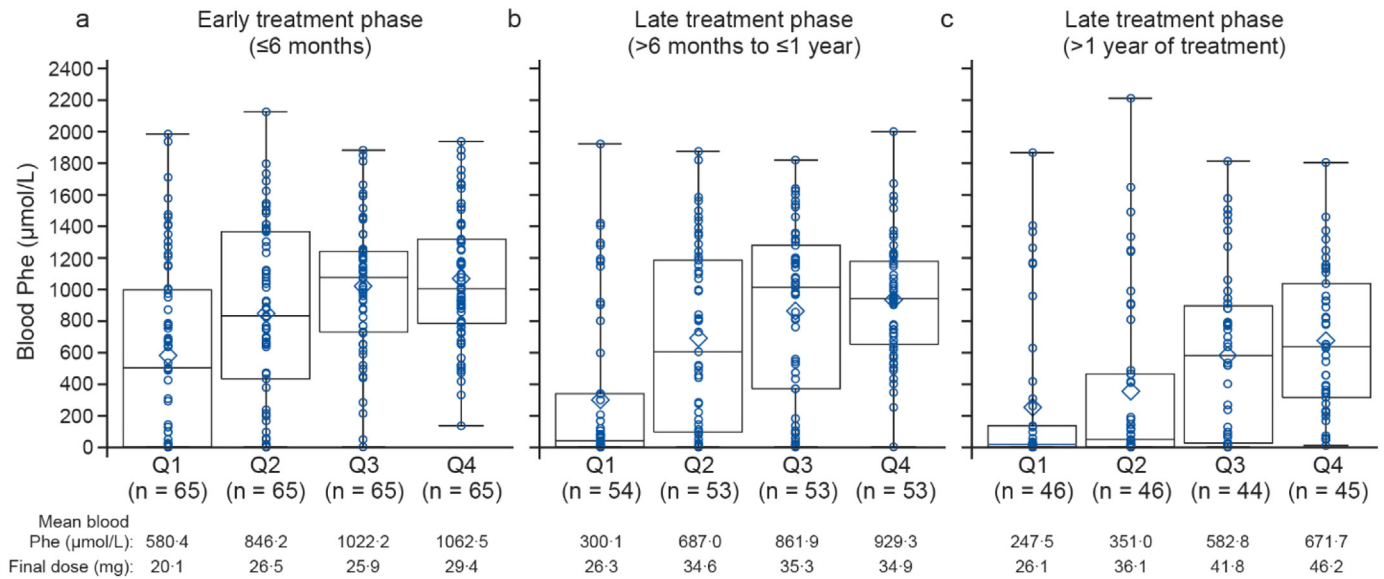


Fig. 2. NAb quartiles and blood Phe. Subject data were divided into quartiles based on mean post-baseline antibody titer (a) ≤6 months after start of treatment, (b) >6 months to ≤1 year after start of treatment, and (c) >1 year after start of treatment. Observed blood Phe was defined as the last available post-baseline blood Phe within each time interval. NAb, neutralizing antibody; Phe, phenylalanine; Q, quartile.

3.3. Immunogenicity and safety

Almost all patients (93.5%) experienced HAEs. HAEs occurred most frequently in early treatment (15.6 events/patient-year) when anti-PEG IgM, anti-PEG IgG, and anti-PAL IgM responses were peaking, CIC levels were high, and C3/C4 levels were low (Fig. 1d). The frequency of HAEs declined over time (HAE rate after 6 months = 4.0 events/patient-year), as the anti-PAL antibody response stabilized and the anti-PEG response, CIC, and complement activation decreased over time.

A total of 17 events in 12 patients (4.6%) were adjudicated by an independent allergist as acute systemic hypersensitivity events, with

15 of these events occurring in the first year of treatment. No patient required intubation or vasopressor treatment to treat acute hypersensitivity, and all the events resolved without sequelae. Pegvalias-specific IgE was not detected in serum from patients at or near the time of the event. Six patients discontinued from the study after these events while 6 continued dosing; of those, 2 had additional events that did not occur directly after restarting pegvalias [26].

Antibody titers were not associated with HAEs during any treatment phase, as demonstrated in Supplemental Fig. 2 using TAB titers as an example. There was significant overlap of HAEs in patients from all TAB quartiles; thus, antibody titers were not predictive of development of HAEs.

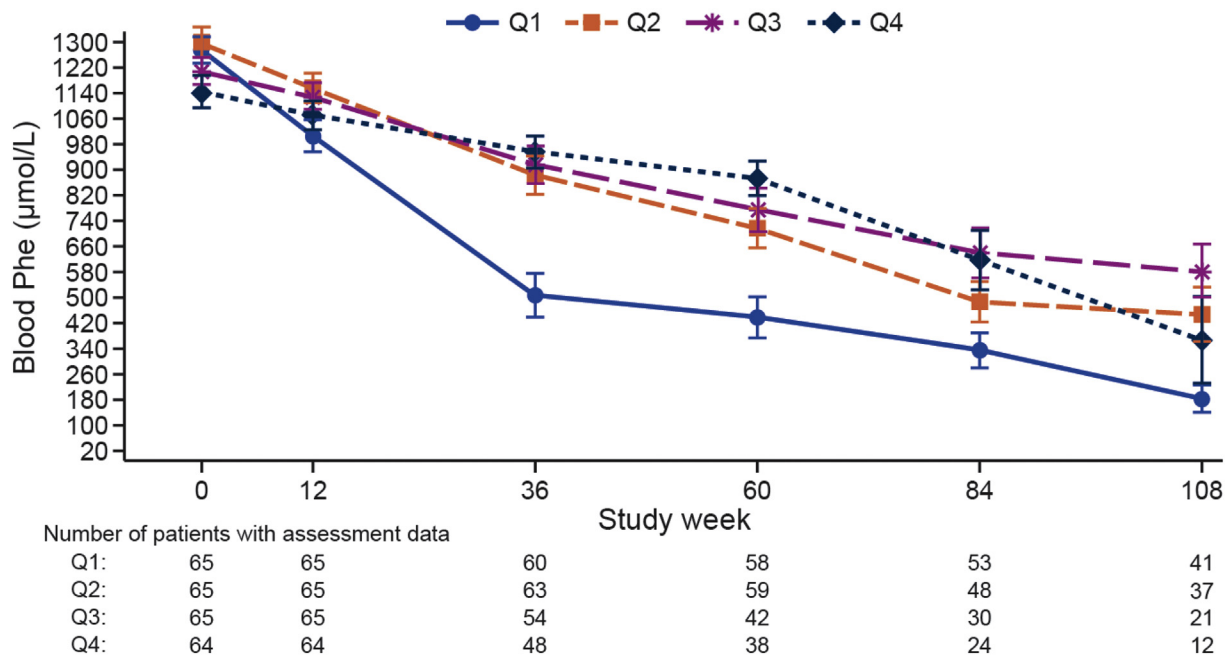


Fig. 3. IgG CIC quartiles and blood Phe. Subject data were divided into quartiles based on mean CIC change from baseline level calculated from all available results, which was plotted with mean blood Phe concentration for each quartile at each evaluated time point. The averages of all values within each interval are plotted. Due to differences in the duration of time on study for individual patients and discontinuation rates, there are a limited number of patients with data at time points after week 84. CIC, circulating immune complex; IgG, immunoglobulin G; Phe, phenylalanine; Q, quartile.

Table 1Adverse events by IgG circulating immune complex change from baseline quartiles (intent-to-treat population, $N = 261$).

	Quartile 1 ($n = 65$)	Quartile 2 ($n = 65$)	Quartile 3 ($n = 65$)	Quartile 4 ($n = 64$)	Total ($n = 259$)
IgG CIC change from baseline quartile range (min-max)	(-22.76-10.85)	(10.98-21.11)	(21.54-35.20)	(35.46-242.25)	
Total treatment exposure, person-years ^a	125.5	125.3	84.6	65.8	401.2
Event rate, per person-year (total number of events)					
Adverse events	22.58 (2833)	28.74 (3603)	34.37 (2909)	45.10 (2967)	30.69 (12312)
Serious adverse events	0.05 (6)	0.18 (22)	0.11 (9)	0.35 (23)	0.15 (60)
Hypersensitivity adverse events	5.83 (731)	7.67 (961)	7.46 (631)	9.41 (619)	7.33 (2942)
Acute systemic hypersensitivity events of anaphylaxis	0	0.02 (2)	0.04 (3)	0.18 (12)	0.04 (17)
Arthralgia/arthritis	2.45 (308)	2.39 (300)	2.71 (229)	4.09 (269)	2.76 (1106)
Generalized skin reaction ≥ 14 days duration	0.52 (65)	0.42 (53)	0.39 (33)	0.30 (20)	0.43 (171)
Urticaria ≥ 14 days duration	0.02 (3)	0.02 (3)	0.02 (2)	0.03 (2)	0.02 (10)
Lymphadenopathy ≥ 14 days duration	0.09 (11)	0.12 (15)	0.11 (9)	0.29 (19)	0.13 (54)
Serum sickness-like reaction	0.02 (2)	0.02 (2)	0.02 (2)	0.02 (1)	0.02 (7)
Infection and infestations ≥ 30 days duration	0.16 (20)	0.23 (29)	0.25 (21)	0.23 (15)	0.21 (85)
Infection serious adverse events	0.01 (1)	0.03 (4)	0	0.02 (1)	0.01 (1)
Ischemic heart disease	0	0	0	0	0

Event rate was calculated as total number of events divided by person-years of exposure.

^a Total treatment exposure was the aggregated duration of treatment across all patients (for each patient, time from the first dose to the last dose administered across all studies in which the patient was enrolled). Intervals of missing doses that were > 28 consecutive days were excluded from the calculation of treatment duration. CIC, circulating immune complex; IgG, immunoglobulin G.

As shown in Table 1, AEs, serious AEs (SAEs), HAEs, acute systemic hypersensitivity events, arthralgia/arthritis, and lymphadenopathy lasting ≥ 14 days were highest in patients with the largest IgG CIC increase from baseline. Results for IgM CIC were similar to IgG CIC (Supplemental Table 4). The trend observed with SAEs was driven by acute systemic hypersensitivity events.

Overall, exposure-adjusted AE rates were similar for patients with $<25\%$ or $> 25\%$ C3 reduction from baseline at 1 year with the exception of acute systemic hypersensitivity events, which occurred at a higher rate in patients with $\geq 25\%$ C3 reduction (Table 2). Results were similar for C3 and C4.

No specific antibody titer value, CIC level, or complement level was predictive of AEs, including acute systemic hypersensitivity events. Wide individual variability was observed in antibody titers, CIC levels, and complement levels between patients with and without acute systemic hypersensitivity events (Fig. 4).

No clinical observations or laboratory abnormalities suggestive of pegvaliase-associated IC-mediated end-organ damage, including renal failure, central nervous system manifestations (such as cerebrovascular accidents or transient ischemic attacks), myocardial ischemic events, or hemolytic anemia events were observed in any pegvaliase-treated patients.

4. Discussion

Historically, reports characterizing immunogenicity against biologics have been limited to a description of ADA incidence. However,

many of the immune-mediated clinical effects attributed to ADAs require the formation of a drug/ADA IC intermediate, which is seldom measured and can have a variety of downstream effects. In this study, we report on the immunogenicity of pegvaliase using an extensive monitoring strategy, including measurement of antibody titers of multiple isotypes and specificities, assessment of enzymatic neutralization, measurement of IgM and IgG IC, and analyses of biomarkers of complement activation. These findings lead to a clear understanding of the immunogenicity of pegvaliase and its impact on efficacy and safety of the drug, enabling the design of a dosing paradigm aimed to achieve maximum efficacy while minimizing AEs.

Patients treated daily with a subcutaneous injection of pegvaliase developed antibodies against PAL and PEG. The sustained anti-PAL response is consistent with a typical CD4-dependent antibody response induced against a foreign protein [27]. The transient nature of the anti-PEG response may result from a low-affinity, CD4-independent antibody response typically observed against non-protein repetitive antigens [13]. CD4-independent antibody responses to PEG have been reported for both nonclinical and clinical studies. In nearly all animal studies, anti-PEG antibody responses were largely IgM and were transient [28,29].

Two of 13 approved PEGylated biotherapeutics, pegaspargase and pegloticase, induce anti-PEG antibodies associated with loss of efficacy and increased risks for hypersensitivity reactions [16]. With both, the anti-PEG antibody responses were sustained and transitioned from a response predominantly IgM to an IgG isotype. This is in contrast to the transient and predominantly IgM and low-titer IgG responses to PEG

Table 2Adverse events by C3 reduction from baseline at year 1 (intent-to-treat population, $N = 233$).

Percent reduction in C3 from baseline at year 1	$<25\%$ ($n = 67$)	$\geq 25\%$ ($n = 98$)	$\geq 25\%$ and $< 50\%$ ($n = 59$)	$\geq 50\%$ ($n = 39$)
Total treatment exposure, person-years ^a	120.2	188.4	115.7	72.7
Event rate, per person-year (total number of events)				
Any adverse event	31.3 (3760)	29.5 (5562)	26.0 (3005)	35.2 (2557)
Any serious adverse event	0.1 (12)	0.1 (26)	0.1 (16)	0.1 (10)
Any hypersensitivity adverse event	7.1 (857)	7.2 (1363)	7.2 (834)	7.3 (529)
Acute systemic hypersensitivity event of anaphylaxis	0.01 (1)	0.06 (12)	0.05 (6)	0.08 (6)
Arthralgia/arthritis	2.9 (351)	2.6 (490)	2.3 (262)	3.1 (228)
Generalized skin reaction ≥ 14 days duration	0.6 (68)	0.4 (67)	0.4 (48)	0.3 (19)
Persistent urticaria ≥ 14 days duration	0.02 (2)	0.03 (5)	0.03 (3)	0.03 (2)

Event rate was calculated as total number of events divided by person-years of exposure.

^a Total treatment exposure was the aggregated duration of treatment across all patients (for each patient, time from the first dose to the last dose administered across all studies in which the patient was enrolled). Intervals of missing doses that were > 28 consecutive days were excluded from the calculation of treatment duration. C3, complement component 3.

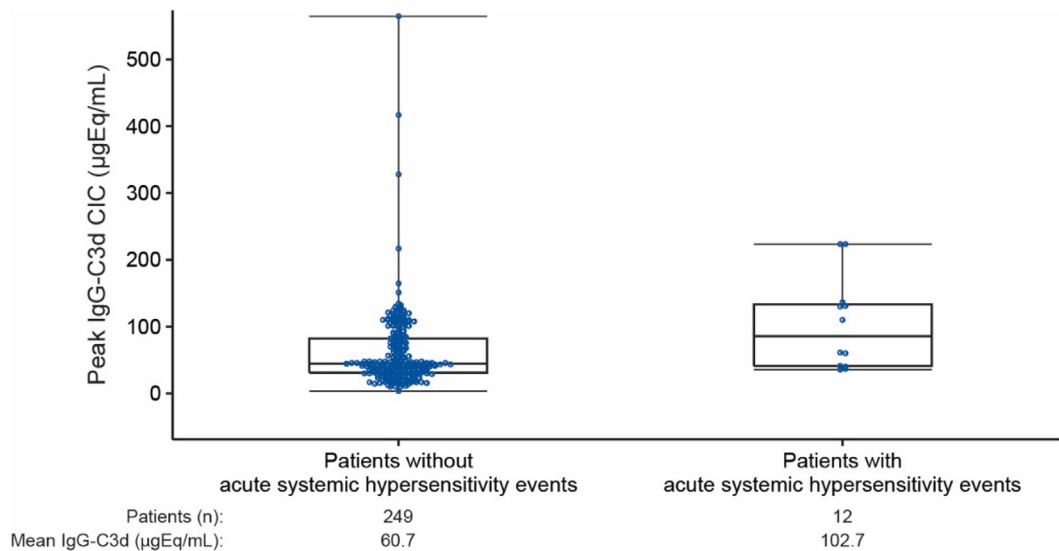


Fig. 4. Peak IgG-C3d CIC concentrations in patients with and without acute systemic hypersensitivity events. There was considerable overlap in IgG CIC levels for patients with and without acute systemic hypersensitivity events, and patients with the highest IgG CIC levels did not experience acute systemic hypersensitivity events. C3d, complement component 3d; CIC, circulating immune complex; IgG, immunoglobulin G.

observed with pegvaliase, perhaps attributable to the induction, titration, and daily maintenance dosing regimen leading to desensitization over time. Anti-PEG antibodies in healthy individuals have been reported due to exposure to PEG-containing compounds in cosmetics, pharmaceuticals, and processed food products, but the presence of these antibodies has not been associated with any pathology. A subset of patients in the PRISM trials enrolled with pre-existing anti-PEG IgG and/or anti-PEG IgM, consistent with the baseline positivity rates reported in the literature [24,25,27]. Presence of anti-PEG antibodies at baseline did not impact pegvaliase safety.

The data presented herein suggest the change in immune response over time influences the dosing necessary to achieve efficacy. High levels of IgM observed during early treatment (≤ 6 months after treatment initiation) are an efficient activator of the classical complement pathway. In contrast, the immune response in late treatment (> 6 months after treatment initiation) is composed predominately of anti-PAL IgG antibodies, which are less likely to bind to the drug product and fix complement due to the masking of PAL epitopes by extensive PEGylation on the surface of the drug. Fig. 5 illustrates our model that CIC levels are highest in early treatment despite sustained anti-PAL ADA levels.

The time course of ADA maturation, IC formation, and complement activation drove the need for an induction/titration dosing schedule necessary to achieve efficacious doses in later treatment while minimizing the occurrence of HAEs during early treatment. The suggested dosing schedule includes a low starting dose of 2.5 mg/week for 4 weeks, followed by a gradual titration over ≥ 5 weeks up to 20 mg/day and a

delayed escalation from 20 to 40 mg/day. The maintenance dose of 20 or 40 mg/day is individualized based on patient tolerability and blood Phe concentrations. For further details on the dosing schedule, please refer to the Palynziq prescribing information [10]. The overlap in the range of antibody titers between the different dose groups makes it difficult to associate a specific antibody titer with a specific dose required to achieve Phe reduction. For this reason, monitoring of blood Phe is recommended for managing patients to ensure the lowest dose is used to achieve reduction in blood Phe.

Data from this study demonstrated that there was an inverse association between the magnitude of the ADA response and the degree of blood Phe reduction. Nevertheless, patients with the highest ADA titers could still achieve a meaningful Phe response once the immune response matured and their dose increased with long-term treatment. The mechanisms by which ADA may impact pegvaliase efficacy include immune-mediated plasma clearance of the drug via the reticuloendothelial system and reduction of enzyme activity following NAb binding. In this study, patients with a wide range of NAb responses were able to attain sustained reductions in blood Phe, suggesting that the neutralizing response to pegvaliase was a minor component of the overall immune response and only partially inhibited *in vivo* enzymatic activity (Fig. 2). Overall, no specific threshold of antibody titer, isotype, or specificity, including NAb, was predictive of the degree of reduction or dose needed to achieve substantial reduction in blood Phe.

In this study, no specific CIC level was predictive of the occurrence of an acute systemic hypersensitivity event despite an association between the magnitude of CIC change from baseline and frequency of

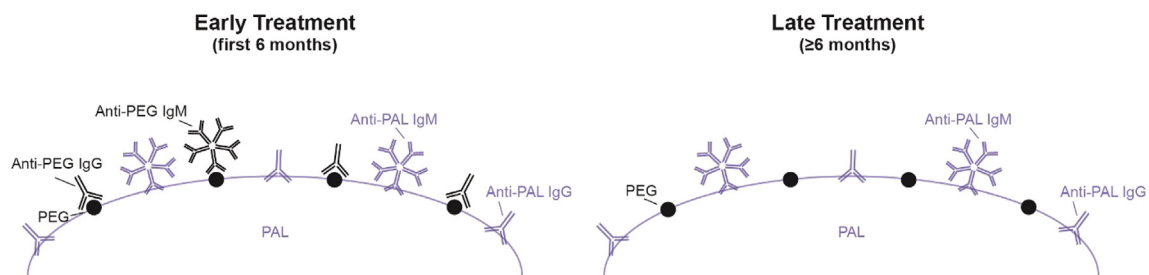


Fig. 5. Illustration of the changing CIC landscape in early vs. late treatment. Early in treatment (0–6 months), CICs contain anti-PEG IgG, anti-PEG IgM, anti-PAL IgG and anti-PAL IgM. Later in treatment (> 6 months), there are fewer CICs formed by anti-PAL IgG and anti-PAL IgM only. CIC, circulating immune complex; IgG, immunoglobulin G; IgM, immunoglobulin M; PAL, phenylalanine ammonia lyase; PEG, polyethylene glycol.

acute systemic hypersensitivity events. Reasons for this may be multifactorial, including the methods used to detect CICs, which detected all CICs rather than those that are antigen-specific, contribution of other mechanisms such as IgG-mediated hypersensitivity reactions via binding to Fc gamma receptors, and presence of other unidentified co-factors that might increase the risk of hypersensitivity in some patients. Although pegvaliase has potential for IC-mediated toxicity, a review of the safety data thus far has shown no evidence of pegvaliase-associated end-organ damage due to IC deposition with 401 patient-years of exposure, including 20 patients with >3 years of exposure.

In summary, these results support the efficacy of pegvaliase for the treatment of adults with PKU, with a manageable safety profile for most participants. Pegvaliase has been shown to have improved safety and efficacy when introduced slowly with incremental dose increases to allow the immune system to stabilize. The risk of AEs is highest in early treatment during induction/titration dosing, when the immune response is immature and patients are receiving low, sub-therapeutic doses. In late treatment, the immune response matures and stabilizes, and the occurrence of HAEs decreases due to a reduction in IC formation and complement activation, despite dose increases that allow the majority of patients to achieve substantial and durable blood Phe efficacy.

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Declaration of interests

SG, KL, RB, JO, KL, ZG, ML, OR, SJZ, HHW, and BS are employees and shareholders of BioMarin Pharmaceutical Inc.

COH reports personal fees and other from BioMarin Pharmaceutical Inc. during the conduct of the study; personal fees from Synlogic, Rubius Therapeutics, Inc., and Pfizer Inc.; and grants from StrideBio, Inc., outside the submitted work.

GS and JPA report personal fees from BioMarin Pharmaceutical Inc. during the conduct of the study.

VK reports grants from BioMarin Pharmaceutical Inc. during the conduct of the study.

Author contributions

SG, KL, COH, GS, RB, JPA, VK, JO, KL, ZG, ML, OR, SJZ, HHW, and BS contributed to critical data interpretation, intellectual content, review, and approval of this manuscript. COH was a primary investigator of the PRISM clinical trials. VK and JPA analyzed the data. VK performed laboratory analyses. All authors read and approved the final version of this manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ebiom.2018.10.038>.

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Supplemental Data

Supplemental Table 1. Baseline blood phenylalanine and immunogenicity data for patients enrolled in the PRISM-1 study.

Baseline analytes		PRISM-1 study population (n = 261)
Blood phenylalanine		
	n	261
	Mean (SD), $\mu\text{mol/L}$	1232.7 (386.36)
	Median, $\mu\text{mol/L}$	1221
	Min-max, $\mu\text{mol/L}$	285–2330
Total antibodies		
	n	260
	Mean (SD)	206 (949)
	Median	0
	Min-max	0–10,700
Neutralizing antibodies		
	n	258
	Mean (SD)	0 (1)
	Median	0
	Min-max	0–18
PEG IgG		
	n	260
	Mean (SD)	36 (119)
	Median	10
	Min-max	0–810
PEG IgM		
	n	260
	Mean (SD)	177 (1020)
	Median	0
	Min-max	0–15,300
PAL IgG		
	n	260
	Mean (SD)	59 (762)
	Median	0
	Min-max	0–12,150
PAL IgM		
	n	260
	Mean (SD)	243 (582)
	Median	130
	Min-max	0–8201
IgM-C3d CIC		
	n	259
	Mean (SD), $\mu\text{g Eq/mL}$	2.1 (2.4)
	Median, $\mu\text{g Eq/mL}$	1.13
	Min-max, $\mu\text{g Eq/mL}$	0.5–16.5
IgG-C3d CIC		
	n	259
	Mean (SD), $\mu\text{g Eq/mL}$	10.6 (5.3)
	Median, $\mu\text{g Eq/mL}$	9.4
	Min-max, $\mu\text{g Eq/mL}$	1.8–37.1
C3		
	n	258
	Mean (SD), g/L	1.19 (0.234)
	Median, g/L	1.2
	Min-max, g/L	0.7–2.2
C4		
	n	258
	Mean (SD), g/L	0.25 (0.097)
	Median, g/L	0.2
	Min-max, g/L	0.1–1.3

CIC, circulating immune complex; IgG, immunoglobulin G; IgM, immunoglobulin M; PAL, phenylalanine ammonia lyase; PEG, polyethylene glycol.

Supplemental Table 2. Incidence and mean titers of antibodies in the PRISM phase 3 studies over time (n = 261).

	Baseline	Week 4	Week 8	Week 12	Week 16	Week 20	Week 24	Week 36	Week 52	Week 104
Incidence (%)										
TAbs	30.8	91.1	96.3	98.6	99.0	99.0	100	98.9	100	98.9
NAb	0.4	0.8	14.0	53.7	67.5	73.9	79.2	79.7	81.3	72.5
PAL IgG	6.9	34.9	98.0	99.5	100	100	100	100	100	100
PAL IgM	50.0	74.4	98.4	98.6	98.5	95.0	92.2	87.5	84.0	83.5
PEG IgG	52.3	92.6	96.3	65.0	59.8	47.0	41.5	28.6	14.8	3.4
PEG IgM	45.0	86.4	96.3	91.3	87.2	82.6	76.6	67.9	59.7	57.1
Mean titer										
TAbs	206	38,949	29,279	14,536	17,530	12,884	13,895	16,174	14,858	16,286
NAb	0	0	9	248	332	415	415	505	608	661
PAL IgG	59	166	26,104	584,350	896,429	884,979	961,296	1,177,665	1,219,788	1,086,602
PAL IgM	243	557	2687	3372	1675	1183	1026	1024	1355	2190
PEG IgG	36	4600	1556	316	122	89	69	38	22	0
PEG IgM	177	15,466	28,735	48,689	7958	6019	3259	883	385	67

Patients treated with pegvaliase developed sustained ADA against the PAL protein, and transient antibodies against PEG. The development of the antibody response occurred in a biphasic manner, characterized by a difference in composition of the antibodies during early versus late treatment. The early antibody response was composed of PEG IgM, PEG IgG, and PAL IgM antibodies, whereas the late antibody response was composed of PAL IgG. Anti-PEG antibody titers peaked 1–3 months after treatment initiation and then returned to baseline in most patients by 6–9 months. PAL IgM and PAL IgG titers peaked at 3 and 6 months, respectively, and remained stable through long-term treatment. The majority of patients developed a low-titer NAb response over time. ADA, antidrug antibodies; IgG, immunoglobulin G; IgM, immunoglobulin M; NAb, neutralizing antibody; PAL, phenylalanine ammonia lyase; PEG, polyethylene glycol; TAb, total antibody.

Supplemental Table 3. Baseline anti-PEG positivity and frequency of HAEs (n = 261).

Anti-PEG IgG	Baseline positive N = 136	Baseline negative N = 123
Mean number of HAEs	5.9	7.6
Number of patients with HAEs	119	109

Anti-PEG IgM	Baseline positive N = 116	Baseline negative N = 143
Mean number of HAEs	6.7	6.7
Number of patients with HAEs	105	123

HAEs, hypersensitivity adverse events; IgG, immunoglobulin G; IgM, immunoglobulin M; PEG, polyethylene glycol.

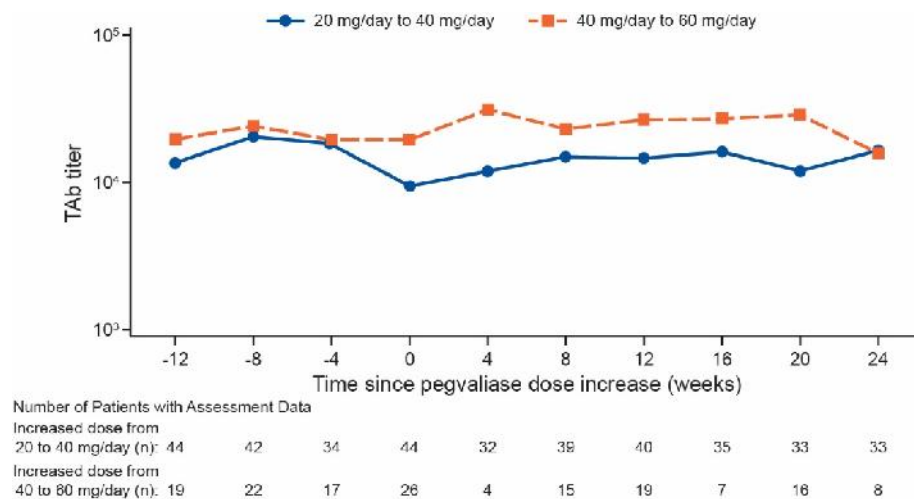
Supplemental Table 4. Adverse events by IgM circulating immune complex change from baseline quartiles (intent-to-treat population, N=261).

	Quartile 1 (n = 65)	Quartile 2 (n = 65)	Quartile 3 (n = 65)	Quartile 4 (n = 64)	Total (n = 259)
IgM CIC change from baseline quartile range (min-max)	-4.02, 0.79	0.79, 1.98	2.01, 5.53	5.59, 50.96	
Total treatment exposure, person-years ^a	118.0	120.1	102.6	60.5	401.2
Event rate, per person-year (total number of events)					
Adverse events	24.33 (2872)	25.79 (3098)	33.54 (3443)	47.95 (2899)	30.69 (12312)
Serious adverse events	0.11 (13)	0.12 (15)	0.13 (13)	0.31 (19)	0.15 (60)
Hypersensitivity adverse events	7.24 (854)	6.63 (796)	6.62 (679)	10.14 (613)	7.33 (2942)
Acute systemic hypersensitivity events of anaphylaxis	0.03 (3)	0.06 (7)	(2) (0.02)	0.08 (5)	0.04 (17)
Arthralgia/arthritis	2.14 (252)	3.11 (373)	(260) (2.53)	3.66 (221)	2.76 (1106)
Generalized skin reaction ≥14 days' duration	0.37 (44)	0.39 (47)	(51) (0.50)	0.48 (29)	0.43 (171)
Urticaria ≥14 days' duration	0.03 (3)	0.03 (4)	(1) (0.01)	0.03 (2)	0.02 (10)
Lymphadenopathy ≥14 days' duration	0.14 (16)	0.11 (13)	(15) (0.15)	0.17 (10)	0.13 (54)
Serum sickness-like reaction	0.01 (1)	0.01 (1)	(1) (0.01)	0.07 (4)	0.02 (7)
Infection and infestations ≥30 days' duration	0.24 (28)	0.22 (27)	(18) (0.18)	0.20 (12)	0.21 (85)
Infection serious adverse events	0.02 (2)	0.01 (1)	(2) (0.02)	0.02 (1)	0.01 (6)
Ischemic heart disease	0	0	0	0	0

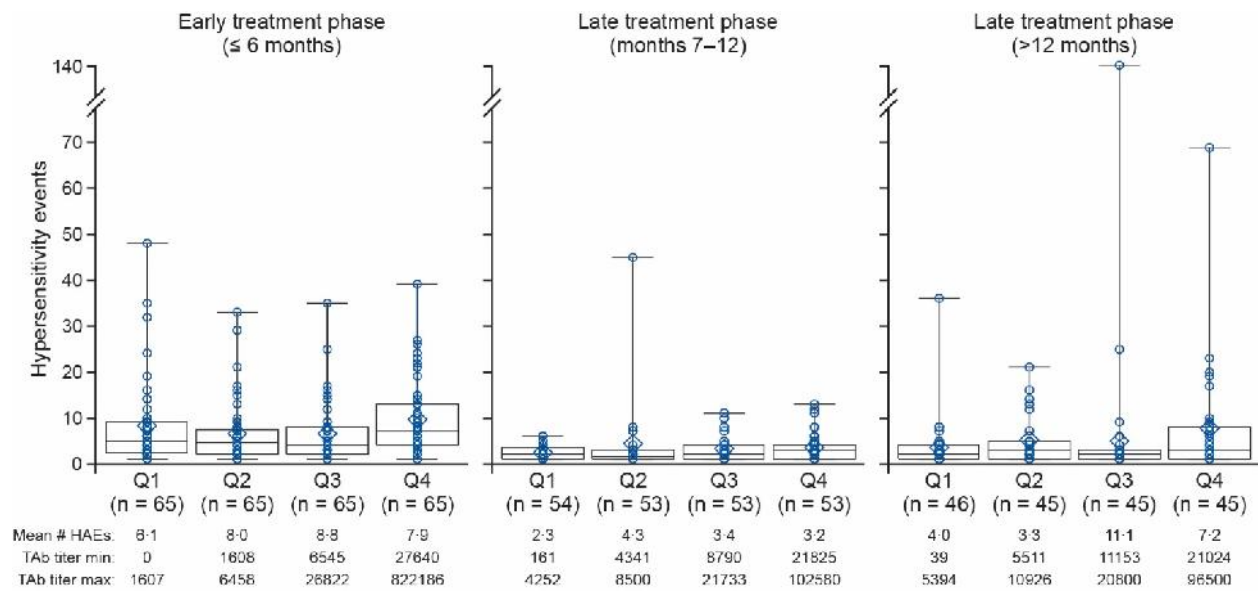
Event rate was calculated as total number of events divided by person-years of exposure.

^aTotal treatment exposure was the aggregated duration of treatment across all patients (for each patient, time from the first dose to the last dose administered across all studies in which the patient was enrolled). Intervals of missing doses that were >28 consecutive days were excluded from the calculation of treatment duration.

CIC, circulating immune complex; IgM, immunoglobulin M.



Supplemental Figure 1. Plot of mean dose increases on total anti-pegvaliasse antibody (TAb) titer in patients increasing dose. Increasing pegvaliasse dose was not associated with an increase in total anti-pegvaliasse antibody (TAb). Patients who were originally randomized to pegvaliasse 20 mg/day and had at least 4 consecutive weeks with 80% compliance on 40 mg/day dose were included in the 20 mg to 40 mg analysis. Patients who had at least 4 consecutive weeks with 80% compliance on 60 mg/day were included in the 40 mg to 60 mg analysis. TAb, total antibodies.



Supplemental Figure 2. Frequency of HAEs and TAb titer by treatment phase. Mean antibody titers for TAb were not associated with frequency of HAEs during any treatment phase. Additionally, there was considerable overlap within patients from all quartiles. Each dot represents one patient. Mean titer was calculated from all available antibody titers over the entire time on treatment for each patient. Antibody titers ≤ 1 are negative. HAE, hypersensitivity adverse event; TAb, total antibody.

Supplemental Text

Overview of analytical methods

Bioanalytical methods were developed, optimized and validated using the following documents as guidance: Bioanalytical Method Validation, FDA (CDER) Guidance for Industry, May 2001, Guideline on Bioanalytical Method Validation, EMA, February 2012; Guideline on Immunogenicity Assessment of Biotechnology-Derived Therapeutic Proteins, EMA April 2008; International Conference on Harmonization (ICH) Harmonized Tripartite Guideline: Validation of Analytical Procedures: Text and Methodology Q2 (R1), November 2005. Method validation studies were conducted to establish precision, sensitivity, specificity, selectivity, interference, and analyte stability. Pharmacokinetic assays were additionally validated for dilution linearity and accuracy.

Quantitative measurement of Phe in human plasma

Human plasma was processed and immediately frozen at clinical sites before shipping for analysis at a central laboratory. A quantitative ion exchange chromatography method to measure amino acids phenylalanine and tyrosine in human plasma was validated to support pegvaliase clinical studies. Amino acids in deproteinized plasma were injected into a cation exchange column (high-performance lithium column) and separated using a gradient of pH, ionic strength, and temperature. The amino acids eluting from the column were mixed with ninhydrin and passed through a high-temperature reaction coil to form colored adducts. The light absorbance of these adducts were measured with a photometer unit at 570 nm (amino acids) and integrated as a series of peaks with a specific retention time for each amino acid. The area of each peak was proportional to the concentration of the amino acid. The concentration of each amino acid, in $\mu\text{mol/L}$, was calculated using an internal standard (L-norleucine) to adjust for differences in extraction and/or injection.

Measurement of anti-rAvPAL IgG in human serum

A semi-quantitative direct format electrochemiluminescence assay (ECLA) method was validated to measure anti-PAL IgG antibodies in human serum samples. The method employed biotin-conjugated PAL coated on streptavidin plates to capture anti-PAL IgG from serum (1:50 minimum required sample dilution; MRD), and a ruthenium-labeled polyclonal goat antihuman IgG Fc antibody for detection. Assay plates were read on a mesoscale discovery plate reader, where the assay signal detected was proportional to the level, affinity, and avidity of the anti-PAL antibodies present in the sample. Samples that produced signal at or above the established screening cut point were considered reactive and were confirmed as PAL-specific by incubating with 204 $\mu\text{g/mL}$ soluble PAL. If antibody binding was specific, the signal was lower in the presence of unlabeled PAL relative to the signal generated in the absence of PAL. A semi-quantitative titer value was determined for confirmed positive samples by serially diluting the sample until it was no longer reactive. The interpolated dilution factor at which the signal of the dilution curve crosses the titer cut point was reported as the sample titer.

Measurement of anti-PEG IgG in human serum

A semi-quantitative, direct-format ECLA method was validated to measure anti-PEG IgG in human serum samples. The method employed biotin-conjugated methoxy PEG coated on streptavidin plates to capture anti-PEG IgG from serum (1:10 MRD), and a ruthenium-labeled polyclonal goat anti-human IgG Fc antibody for detection. Assay plates were read on an MSD plate reader, where the assay signal detected was proportional to the level, affinity, and avidity of the anti-PEG antibodies present in the sample. Samples that produced signal at or above the established screening cut point were considered reactive and were confirmed with 2.0 mg/mL excess PEG to compete for binding to the PEG-coated plate. A semi-quantitative titer value was determined for confirmed positive samples by serially diluting the sample until it was no longer reactive. The interpolated dilution factor at which the signal of the dilution curve crosses the titer cut point was reported as the sample titer.

Measurement of anti-rAvPAL IgM in human serum

A semi-quantitative, direct-format ECLA method was validated to detect anti-PAL IgM in human serum samples. Streptavidin-coated MSD plates were coated with biotin-conjugated PAL, washed, and blocked, and samples were incubated after dilution in buffer (1:10 MRD), to capture anti-PAL antibodies. Captured anti-PAL IgM was detected with a ruthenium-labeled polyclonal rabbit anti-human IgM Fc fragment-specific antibody. After washing, plates were read on an MSD Sector Imager reader. Due to a range of pre-existing anti-PAL IgM levels in normal human sera, a population of verified negative samples could not be identified, and therefore a conventional cut point delineating positive and negative could not be established. Instead a low-titer cut point was established below the lowest observed screening assay signal, in order to determine semi-quantitative titer results from all samples. Samples were serially diluted in assay diluent until the samples were no longer reactive above the established cut point. The interpolated dilution factor at which the signal of the dilution curve crossed the titer cut point was reported as the sample titer.

Measurement of anti-PEG IgM in human serum

A semi-quantitative, direct-format ECLA method was validated to detect anti-PEG IgM in human serum samples. Ninety-six well streptavidin-coated MSD plates were coated with biotin-conjugated methoxy PEG, washed, and blocked. Samples were diluted in buffer (1:20 MRD) and incubated on the plate to capture anti-PEG antibodies. Captured anti-PEG IgM was detected with a ruthenium-labeled polyclonal rabbit anti-human IgM Fc fragment-specific antibody. After washing, plates were read on an MSD Sector Imager reader. Samples were serially diluted in buffer until the samples were no longer reactive above the established cut point. The interpolated dilution factor at which the signal of the dilution curve crossed the titer cut point was reported as the sample titer.

Measurement of anti-pegvaliase IgE in human serum

Quantitative radioallergosorbent test (RAST) methods were developed and validated to measure anti-pegvaliase IgE and anti-PAL IgE in human serum samples. Biotin-conjugated pegvaliase or biotin-conjugated PAL were incubated with streptavidin-coated 6.3 mm paper discs. Antigen-coated discs were blocked, washed, and incubated with neat serum samples in individual tubes to capture anti-pegvaliase or anti-PAL antibodies. After washing of unbound serum, discs were incubated with an I^{125} -conjugated anti-human IgE detection reagent to detect captured anti-pegvaliase IgE and anti-PAL IgE. The discs were washed and raw assay gamma radioactivity counts were measured on a Wallac 1470 Wizard gamma counter. The gamma radioactivity adsorbed to each disc was proportional to the antigen-specific IgE concentration in each sample. The level of anti-pegvaliase or anti-PAL IgE in each sample was determined by interpolation of raw gamma counts against a human anti-rye grass IgE standard curve calibrated to the World Health Organization (WHO) international IgE- standard, captured on perennial rye grass-coated discs. The specificity of samples that screened positive was confirmed by pre-incubating the sample in the presence of soluble antigen to compete for binding to the antigen immobilized on the disc.

A quantitative method to measure anti-pegvaliase IgE in human serum was also developed and validated on the ImmunoCAP 1000 platform to confirm phase 3 study results. Pegvaliase-coupled ImmunoCAP cellulosic sponges were incubated with neat serum samples in the ImmunoCAP 1000 instrument to capture antigen-specific antibodies. After washing away nonspecific IgE, β -galactosidase-labeled monoclonal mouse anti-human IgE detection antibodies were added. Unbound detection reagent was washed away and the bound complex was incubated with the ImmunoCAP Development Solution containing 4-methylumbelliferyl- β -D-galactoside, a β -galactosidase fluorogenic substrate. After stopping the reaction, the fluorescence of the eluate was measured. The measured fluorescence is directly proportional to the concentration of drug-specific IgE in each patient sample. The concentration of antigen-specific IgE in each sample was determined by interpolation of raw assay signals against a human allergen-specific IgE standard curve calibrated with the WHO international IgE standard. The specificity of samples that screened positive was confirmed by signal inhibition in the presence of soluble antigen. Assay performance was monitored using quality control samples consisting of anti-pegvaliase IgE surrogate positive control (polyclonal rabbit anti-PAL IgG conjugated with human myeloma IgE) in normal human serum.

Measurement of anti-pegvaliase total antibodies in human serum

A semi-quantitative direct format ECLA method was validated to measure total anti-pegvaliase IgG, IgM, and IgA antibodies in human serum. Ninety-six-well MSD plates were coated with a rabbit anti-PEG monoclonal IgG capture antibody, washed, blocked, washed, coated with pegvaliase, and finally washed before addition of serum samples diluted in buffer (1:50 MRD), to capture anti-pegvaliase antibodies. Plates were incubated with a ruthenium-conjugated rabbit polyclonal as a pan-isotype detection reagent that reacts with human IgG, IgM, and IgA. MSD Read Buffer T (surfactant-free) was added to all wells, and the plates were read on the MSD Sector Imager instrument. A semi-quantitative titer value was determined for each sample by serially diluting the sample until it was no longer reactive. The interpolated dilution factor at which the signal of the dilution curve crosses the titer cut point was reported as the sample titer.

Measurement of anti-pegvaliase activity-neutralizing antibodies in human serum

A semi-quantitative hybrid ligand-binding: liquid chromatography/tandem mass spectrometry (LC/MS/MS) method was validated to measure anti-pegvaliase activity-neutralizing antibodies in human serum. The assay measured anti-pegvaliase antibodies in human serum that inhibit pegvaliase-mediated enzymatic conversion of the substrate L-phenylalanine (Phe) to the products *trans*-cinnamic acid (t-CA) and ammonia.

Ninety-six-well MSD plates containing immobilized rabbit monoclonal anti-PEG IgG were coated with pegvaliase. Serum samples were diluted in buffer (1:2 MRD) and acidified with glycine to dissociate antibody complexes. Acidified samples were then neutralized and added to the assay plate to capture anti-pegvaliase antibodies on the pegvaliase-coated plate. Unbound antibodies, pegvaliase, Phe, and t-CA were washed away and a Phe substrate solution was added and incubated overnight to allow enzymatic conversion of Phe to t-CA by pegvaliase. To quantify the amount of t-CA generated overnight, reaction samples were diluted in 96-deep-well plates using a solution of methanol with 0.01% acetic acid. Internal standard (*trans*-cinnamic-d7 acid) was added to the extraction

solution for all samples except the blank matrix sample. Extracted samples were analyzed through LC/MS/MS and levels of t-CA were quantified using analyte to internal standard peak area/height ratios. Concentrations of the calibration curve standards, quality control samples, and study samples were determined by the method of $1/x^2$ weighted least squares linear regression. The concentration of t-CA produced after overnight incubation was inversely proportional to the concentration of NAb in each sample. Enzyme-neutralizing activity was detected by a reduction of t-CA concentration beyond the cut point established in method validation based on a panel of normal drug-naïve serum samples. The specificity of neutralizing activity in samples that screened positive was confirmed by adding 1 mg/mL of free pegvaliasse to samples before the ligand-binding step. The resulting increase in t-CA corresponded to reduced neutralization. Samples that screened and confirmed positive were serially diluted in buffer and assayed to determine the relative level of NAb in each sample, reported as a semi-quantitative titer value.

Measurement of IgG circulating immune complexes in human serum

The Quidel MicroVue CIC-Raji Cell Replacement (also referred to as CIC-C3D EIA) assay kit was validated and used to measure circulating immune complexes (CIC) in serum samples. The assay relies on fragments of the third complement component, C3, that become covalently bound to complement-activating immune complexes. To capture immune complexes containing C3 activation fragments, standards and serum specimens in diluent (1:50 MRD) were added to microtiter plates coated with monoclonal antibodies that specifically bind the iC3b, C3d, g, and C3d fragments. After a wash cycle removed unbound serum proteins, horseradish peroxidase (HRP)-conjugated mouse anti-human IgG was added to each test well. The chromogenic substrate 2, 2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt was added to react with HRP, and oxalic acid was used to stop color development. The absorbance value (405 nm) was measured spectrophotometrically and was proportional to the amount of C3d containing complexes bound to the plate. A standard curve was generated by plotting the A405 values obtained with each standard versus its indicated concentration. The concentration of C3d immune complexes present in the test specimen was determined by interpolating values from a standard curve prepared from the surrogate positive control (heat-aggregated human gamma globulin; HAGG) added to pooled normal human serum. Samples with signals above the established quantitative range of the calibrator curve at 1:50 dilution factor were further diluted to generate results within the assay quantitative range. Results were expressed as micrograms of HAGG equivalents per mL ($\mu\text{g Eq/mL}$).

Measurement of IgM circulating immune complexes in human serum

The Quidel MicroVue C3d-CIC assay kit was modified to measure IgM-containing CIC, and was validated to support testing of human serum samples. The HRP-conjugated mouse anti-human IgG detection reagent supplied with the assay kit was replaced with a HRP-conjugated rabbit polyclonal anti-human IgM reagent to enable specific measurement of IgM-containing CIC. The kit standard calibrators and quality control (QC) samples, consisting of heat aggregated human gamma globulin, were replaced with analogous calibrators and QCs consisting of heat-aggregated human myeloma IgM. Other aspects of the assay method were unchanged from the C3d-CIC assay kit. Samples were initially tested at a dilution factor of 1:100. Samples with assay signals initially above the quantitative range of the calibrator curve were further diluted to generate results within the quantitative range. Results were expressed as micrograms of serum-treated HAGG equivalents per mL ($\mu\text{g Eq/mL}$).

Institutional review boards by site (chairperson/director)

Vanderbilt University Institutional Review Board (Steven L. Goudy, MD); Institutional Review Boards of UC San Diego Human Research Protections Program (Michael Caligiuri, PhD); Children's Hospital and Research Center Oakland, Institutional Review Board (Horst Fischer, PhD); Oregon Health & Science University Research Integrity Office (Kathryn Schuff, MD, MCR); Ann and Robert H Lurie Children's Institutional Review Board (Catherine A. Powers, RD, LDN, CCRC); Washington University in St. Louis Institutional Review Board (Amanda Unanue, BA); Boston Children's Hospital Office of Clinical Investigation (Steven Colan, MD); University of Utah Institutional Review Board (Gerald Treiman, MD); Western Institutional Review Board (R. Bert Wilkins, JD); IU Human Subjects Office (Shelley D. Bizila, MS, CIP); Drexel University Human Research Protection (Daniel Conway, MD); Wayne State University IRB Administration Office (Niels Beck, PhD); Chesapeake IRB (Anita Tarzian, PhD, RN); Children's Hospital and Health System Institutional Review Board (J. Paul Scott, MD); University of Texas Health Science Center at Houston Committee for the Protection of Human Subjects (L. Maximilian Buja, MD); University of Louisville Institutional Review Board Human Subjects Protection Program Office (Laura D. Clark, MD); University of Kentucky Office of Research Integrity (Jeffrey Carrico, PharmD); University of Nebraska Medical Center Institutional Review Board (Kevin J. Epperson, CIP); University of Oklahoma Institutional Review Board for the Protection of Human Subjects (Karen Beckman, MD); Cooper Health System Institutional Review Board (David Warshal, MD); and University of Miami Medical IRB (Thomas Sick, PhD).