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Depletion of interfering IgG and IgM is critical to determine the role of IgE in pegvaliase-associated hypersensitivity

Kevin Larimore[a,*], Thom Nguyen[a], Benjamin Badillo[a], Kelly Lau[a], Roberto Zori[b], Gillian Shepherd[c], Stephen J. Zoog[a], Haoling H. Weng[a], Soumi Gupta[a]

[a] BioMarin Pharmaceutical Inc., 105 Digital Drive, Novato, CA 94949, USA
[b] University of Florida, Gainesville, P.O. Box 100296, FL 32610, USA
[c] Weill Medical College of Cornell University, 47 East 91st Street, New York, NY 10128, USA

A B S T R A C T

Pegvaliase is an enzyme substitution therapy developed to lower blood phenylalanine (Phe) in adults with phenylketonuria (PKU). In phase 3 clinical studies, pegvaliase substantially reduced mean blood Phe in adult subjects with PKU. The most common type of adverse event observed in the pegvaliase clinical program was hypersensitivity adverse events (HAEs), which included occurrences of arthralgia, rash, and pruritis. The most clinically relevant HAEs were acute systemic hypersensitivity reactions consistent with anaphylaxis observed in 4.6% of phase 3 patients. HAEs were more commonly observed around the time of high circulating immune complex (CIC) levels and complement activation, and the majority of subjects that experienced acute systemic hypersensitivity events were able to continue treatment, which is atypical for a classical IgE-mediated anaphylactic event, but common for type III hypersensitivity reactions.

To investigate the alternative mechanism of type III hypersensitivity, serum samples collected shortly after hypersensitivity events (in phase 2 and 3 studies) were tested for anti-pegvaliase IgE using custom radioallergosorbent test and/or ImmunoCAP® (ThermoFisher Scientific, Waltham, MA) assay methods. All subjects with acute systemic hypersensitivity that were tested for anti-pegvaliase IgE at or near the time of event with one or both assays tested negative for IgE. As presented here, an investigation using selected study samples with high anti-drug antibody (ADA) titers demonstrated that presence of IgM and/or IgG ADA can interfere with measurement of a human anti-pegvaliase IgE surrogate positive control. A depletion method was therefore developed using protein A- and G-conjugated Sepharose to remove interfering IgG and IgM in serum samples to low levels (< 45 mg/dL) before IgE testing. A final 2× concentration step brought the IgE concentration in the depleted sample to approximately the same level of the starting serum. Phase 3 study samples with sufficient volume remaining that previously tested negative for anti-pegvaliase IgE were re-tested after depletion of IgG and IgM. All samples again tested negative, confirming the original test results. Taken together, the clinical presentation, temporal association of HAEs with CIC levels and complement activation, and lack of anti-pegvaliase IgE suggest pegvaliase-associated acute systemic hypersensitivity events were not IgE-mediated. Furthermore, we describe a universal method that is broadly applicable to enzyme therapies for detection of low concentrations of drug-specific IgE in the presence of high titer anti-drug antibodies of different isotypes.

1. Introduction

Phenylketonuria (PKU; OMIM 261600), also known as phenylalanine hydroxylase (PAH) deficiency, is a rare, autosomal recessive disease associated with high blood phenylalanine (Phe). Pegvaliase (Palynziq, BioMarin Pharmaceutical Inc., Novato, CA), recombinant *Anabaena variabilis* phenylalanine ammonia lyase (PAL) conjugated with polyethylene glycol (PEG), is an exogenous enzyme indicated to reduce blood phenylalanine (Phe) in adults with PKU (Bell et al., 2017; Blau and Longo, 2015). Pegvaliase converts Phe to trans-cinnamic acid (t-CA) and ammonia, substituting for the deficient PAH enzyme needed to lower blood Phe. PEGylation of the PAL enzyme is intended to reduce immune recognition of this bacterially derived protein and improve pharmacodynamic stability. In the phase 3 PRISM-1 and PRISM-2 studies of pegvaliase treatment in adults with PKU, pegvaliase was associated with substantial reductions in blood Phe levels. While hypersensitivity adverse events (HAEs) were observed in most (93.5%) subjects, acute systemic hypersensitivity reactions consistent with National Institute of Allergy and Infectious Diseases (NIAID) criteria for anaphylaxis, as adjudicated by an independent allergist, were relatively infrequent (4.6% of phase 3 subjects) (Thomas et al., 2018).

Most protein therapeutics, including enzyme replacement and enzyme substitution therapies (ERTs and ESTs) induce anti-drug antibodies (ADAs) that have been associated with HAEs (Mire-Sluis et al., 2004). Hypersensitivity reactions associated with ERT and EST treatment can be classic IgE-mediated (type I), or immune complex-mediated (type III). In rare cases, anti-drug IgE antibodies are associated with acute systemic hypersensitivity reactions, which can be life-threatening. Therefore, accurate and sensitive laboratory testing to detect anti-drug IgE in serum from ERT- and EST-treated patients

* Corresponding author.
E-mail address: KLarimore@bmrn.com (K. Larimore).

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during clinical development is critical to characterize the mechanism of immune-mediated hypersensitivity (Wachholz et al., 2005; Wu et al., 2013).

All phase 3 subjects treated with pegvaliase in the clinical trials developed anti-drug antibodies (ADA) against both the PAL enzyme and the PEG. The antibody response occurred in a biphasic manner; the early antibody response (< 6 months after treatment initiation) was comprised predominantly of anti-PEG antibodies (anti-PEG IgG peaked at Week 4 with a mean titer of 4600; anti-PEG IgM peaked at Week 12 with a mean titer of 48,689; Table S1) and anti-PAL IgM (peaked at Week 12 with a mean titer of 3372). In contrast, the immune response in late treatment (> 6 months after treatment initiation) was composed predominately of anti-PAL IgG (mean anti-PAL IgG titer at Week 24 = 961,296), with anti-PEG IgG titers near baseline levels (mean anti-PEG IgG titers at baseline = 36, at week 36 = 38). This change in composition of the immune response over time is referred to as the "maturation" of the immune response. The maturation of the pegvaliase ADA response was associated with reduced hypersensitivity and improved efficacy (Gupta et al., 2018). Specifically, HAEs were temporally associated with the peak of the early immune response when circulating immune complex (CIC) levels were at their highest and complement component 3/4 (C3/C4) levels were at their lowest. High levels of anti-PEG antibodies during early treatment could readily form CICs and activate the classical complement pathway. In contrast, the immune response in late treatment was composed predominately of anti-PAL IgG antibodies, which cannot bind to the drug as efficiently to form complement-activating immune complexes due to masking of PAL epitopes by extensive PEGylation on the surface of the drug.

While the association of hypersensitivity events with high CIC levels and complement activation suggested that the reactions were due to immune complex-mediated type III hypersensitivity, it is critical to directly investigate whether there is any evidence of an anti-pegvaliase IgE-mediated allergic response. While these assessments, based on assay methods intended for research use only, are not intended to facilitate point-of-care or clinical surveillance decisions, determination of the predominant biological mechanism driving treatment-associated hypersensitivity is expected to facilitate a better understanding of the nature of adverse events that occurred in pegvaliase clinical trials.

Detection and quantification of drug-specific IgE is challenging because a high sensitivity (due to generally low IgE levels) and high specificity assay is required, and anti-drug IgE positive samples can be rare (Dević et al., 2014; Jaoko et al., 2001; Wu et al., 2013). Furthermore, the presence of IgG and IgM ADA reportedly interfere with IgE detection (Dević et al., 2014; Kadooka et al., 2000; Lehrer et al., 2004; US Food and Drug Administration, 2016). The goals of this study were to investigate anti-pegvaliase IgE assay interference, to develop a depletion method to reduce IgG and IgM in serum samples while retaining IgE, and to elucidate the predominant mechanism of pegvaliase treatment-associated hypersensitivity.

2. Methods

2.1. Clinical trial design and patient samples collected

Pegvaliase was evaluated in four phase 2 clinical studies (ClinicalTrials.gov Identifiers: NCT00925054, NCT01212744, NCT00924703, NCT01560286) and two phase 3 clinical studies (ClinicalTrials.gov Identifiers: NCT01819727, NCT01889862). The methods and results for clinical studies have been reported (Adams et al., 2015; Harding et al., 2018; Longo et al., 2018; Thomas et al., 2018). In phase 2 clinical studies, testing for anti-pegvaliase IgE was conducted using the radioallergosorbent test (RAST) assay platform, with serum samples collected at baseline and at all study and hypersensitivity reaction visits. In phase 3 studies, testing was performed using RAST and/or ImmunoCAP IgE assays, with serum samples collected at hypersensitivity reaction visits; the phase 3 study is ongoing and results described here are as of September 23, 2016. Informed consent was obtained from each participant, and the studies were conducted in accordance with the Declaration of Helsinki.

2.2. Anti-PAL IgG electrochemiluminescent assay

An electrochemiluminescent assay (ECLA) method was developed and validated to support these studies. Multi-Array 96-well streptavidin gold plates (MSD; MesoScale Discovery, Rockville, MD) were blocked with 150 µL per well of diluent buffer (StabilGuard buffer [SurModics IVD, Eden Prairie, MN] containing 0.1% Tween 20) for approximately 2 h, washed 6 times with 300 µL per well of wash buffer (Dubecco's Phosphate Buffered Saline without Calcium and Magnesium [Corning, Corning, NY] with 0.05% Proclin 300 and 0.1% Tween 20) using an automated plate washer (Bio-Tek ELx405 Select or equivalent), and a solution of recombinant *Anabaena variabilis* phenylalanine ammonia lyase (PAL; BioMarin Pharmaceutical, Novato, CA) was added (50 µL per well) and incubated for 16–24 h at 2–8 °C. Plates were incubated for 1 h at room temperature on an orbital shaker at approximately 450 RPM before washing the plate 6 times with 300 µL wash buffer per well. Samples (including unknowns and quality controls) were diluted a minimum of 1:50 in diluent buffer and added to the assay plate (50 µL per well). Anti-PAL antibodies present in samples were captured to the PAL-coated plate during a 1-h incubation at room temperature on an orbital shaker at approximately 450 RPM. After washing the plate 6 times with wash buffer, a ruthenium-labeled polyclonal goat anti-human IgG Fc antibody was added to all wells (50 µL per well), and the plate was incubated for 1 h at room temperature on an orbital shaker at approximately 450 RPM while protected from ambient light. The plate was then washed 6 times with 300 µL wash buffer per well; tripropylamine-containing 2× read buffer (MSD) was added (150 µL per well), and plates were read on an MSD Sector Imager plate reader within 1 min of read buffer addition. In the presence of tris(2-pyridylmethyl) amine (TPA), ruthenium produces a chemiluminescent signal when a voltage is applied by the plate reader. The signal produced is proportional to the amount of anti-PAL IgG antibody present in each well. Samples that produced signal at or above the established screening cut point (statistically defined at the upper 95th percentile of the distribution of signals generated by a panel of drug-naïve human sera) were considered reactive and were confirmed as PAL-specific by assay signal inhibition in the presence of 200 µg/mL soluble PAL. A semi-quantitative titer value was determined for confirmed positive samples by serially diluting the sample until the signal was below the cut point. The interpolated dilution factor at which the signal curve crossed the titer cut point was reported as the sample titer. Assay performance was monitored using quality control samples consisting of an affinity purified polyclonal monkey anti-PAL IgG positive control in human serum, or pooled human sera containing anti-PAL IgG.

2.3. Human anti-pegvaliase IgE ImmunoCAP assay

A semi-quantitative method to measure anti-pegvaliase IgE in human serum was developed and validated on the ImmunoCAP 1000 platform (Phadia AB, Uppsala, Sweden). Pegvaliase was covalently bound to ImmunoCAP cellulosic sponges (Phadia AB) at a concentration of 320 µg/mL, *via* a poly-L-lysine linker. Pegvaliase-coupled ImmunoCAP assays were incubated with 40 µL neat serum samples for 30 min in the ImmunoCAP 1000 instrument to capture drug-specific antibodies. 50 µL of β-galactosidase-labeled monoclonal mouse anti-human IgE detection antibodies were then added and incubated for 24 min. The bound complex was incubated with 50 µL of Development Solution (Phadia AB) containing 4-methylumbelliferyl-β-D-galactoside, a β-galactosidase fluorogenic substrate, for 9 min. 600 µL of Stop Solution (Phadia AB) was added to stop colorimetric development. After stopping the reaction, the fluorescence of the eluate was measured by the ImmunoCAP instrument. The measured fluorescence is directly

proportional to the concentration of pegvaliase-specific IgE in each patient sample. The concentration of anti-pegvaliase IgE in each sample was determined by interpolation of raw assay signals against a human IgE standard curve calibrated with the World Health Organization (WHO) reference preparation for IgE (2nd IRP 75/502) where 1 U equals 2.42 ng of IgE. The specificity of samples that screened positive was confirmed by assay signal inhibition in the presence of 500 μg/mL soluble pegvaliase. The validated anti-pegvaliase IgE ImmunoCAP method had a lower limit of quantitation of 0.10 kU/L. Assay performance was monitored using quality control samples consisting of an anti-pegvaliase IgE surrogate positive control (SPC) in normal human serum. The SPC was generated by conjugation of a pegvaliase-specific rabbit polyclonal IgG antibody with human myeloma IgE. Conjugation was performed using N-Succinimidyl 3-(2-pyridyldithio) propionate (SPDP) technology followed by size exclusion chromatography to isolate IgG:IgE dimers (Superdex 200, AKTA explorer, GE Healthcare Bio-Sciences, Piscataway, NJ).

2.4. Human anti-pegvaliase IgE radioallergosorbent test

A semi-quantitative RAST method was validated to measure anti-pegvaliase IgE in human serum samples. Biotin-conjugated pegvaliase was incubated with streptavidin-coated 6.5 mm paper discs (10 μg/disc). Antigen-coated discs were blocked, washed, and incubated with 50 μL neat serum samples for a minimum of 3 h at 18–25 °C in individual tubes protected from light to capture anti-pegvaliase antibodies. After washing of unbound serum with working RAST wash solution (Viracor-IBT, Lee's Summit, MO), discs were incubated with 50 μL ^{125}I-conjugated anti-human IgE detection reagent (Hycor, Garden Grove, CA) for 14–20 h at 18–25 °C while protected from light to detect captured anti-pegvaliase 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 is proportional to the antigen-specific IgE concentration in each sample. The level of anti-pegvaliase IgE in each sample was determined by interpolation of raw gamma counts against a human anti-rye grass IgE standard curve calibrated with the WHO international IgE standard, captured on perennial rye grass-coated discs. The specificity of samples that screened positive was confirmed by pre-incubating 60 μL of the sample in the presence of 5 μL of 1000 μg/mL soluble pegvaliase to compete for binding to the antigen immobilized on the disc. The validated human anti-pegvaliase IgE RAST method had a lower limit of quantitation of 0.10 kU/L. Assay performance was monitored using Ragweed IgE positive controls and a negative control disc (Viracor-IBT).

2.5. Total human IgE ImmunoCAP assay

Total human IgE concentrations in sera were determined by ImmunoCAP assay on the automated ImmunoCAP 1000 instrument. Anti-human IgE covalently coupled to ImmunoCAP cellulosic sponges (Phadia AB) reacted with total IgE in the patient specimen. The standard ImmunoCAP detection system was used, as described above. The total human IgE ImmunoCAP assay had a lower limit of quantitation of 2.0 kU/L. Calibrators are traceable to the 2nd International Reference Preparation 75/502 of Human Serum Immunoglobulin E from WHO. Calibrator curves were stored by the instrument for 30 days maximum and accurate quantitation on each run was verified by assay of three control samples in each run.

2.6. Total human IgG and IgM SPA assays

Total human IgG and IgM concentrations were determined by turbidimetric methods (Scintillation Proximity Assay - SPA) using the semi-automated SPAPLUS® analyzer (The Binding Site, Birmingham, UK). Specific antiserum to human IgG and IgM reacted with total human IgG and IgM in serum specimens forming insoluble complexes. The amount of transmitted light measured was inversely proportional to the specific protein concentration in the test sample. Concentrations were automatically calculated by reference to a calibrator curve stored within the SPAPLUS analyzer. Two levels of quality controls per analyte were run each day of unknown sample testing to monitor assay performance. Calibrators and quality controls consisted of pooled human serum with known concentrations of IgG and IgM.

2.7. Depletion of IgG and IgM from human sera

Sepharose-conjugated Protein A and G (Genscript, Piscataway, NJ) were used to deplete IgG and IgM antibodies in human serum. Protein G-Sepharose (375 μL) and Protein A-Sepharose (480 μL) resins were mixed with serum (600 μL) and incubated overnight at 2–8 °C to allow IgG and IgM from serum samples to bind to the resins. Following overnight incubation, the supernatant was isolated from the resin mix by filtration, using a 0.30 μm filter disc. After removing the supernatant, the resin mixture was washed sequentially with 0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3 and 0.1 M citric acid, 0.1 M trisodium citrate, pH 3.0. Following the wash steps, the acidified wash filtrates were neutralized, combined with recovered supernatant, and concentrated using a CentriVAP DNA vacuum concentrator for 240 min, 1700 RPM, at 37 °C to a final volume of 300 μL.

3. Results

3.1. Characterization of human anti-pegvaliase IgE surrogate positive control

To assess and monitor performance of the human anti-pegvaliase IgE ImmunoCAP assay in the absence of a *bona fide* human anti-pegvaliase IgE antibody, a surrogate positive control (SPC) consisting of rabbit polyclonal anti-pegvaliase IgG (confers specificity for pegvaliase) conjugated to human myeloma IgE (confers reactivity with anti-human IgE detection reagents) was generated by Phadia AB (Uppsala, Sweden; see Methods). Sequential chemistry was used to conjugate the hetero-bifunctional linker N-Succinimidyl 3-(2-pyridyldithio) propionate (SPDP) first to IgG then to IgE resulting in formation of hetero-dimers but not homo-dimers, resulting in IgG-IgE but not IgG-IgG or IgE-IgE conjugates (Carlsson et al., 1978). After conjugation with SPDP, IgG-IgE dimers were purified from monomeric IgG or IgE and larger multimers by size exclusion chromatography. A chromatogram of the purification step (Fig. S1) shows well separated peaks corresponding to monomeric rabbit anti-PAL IgG, monomeric human IgE, and overlapping peaks representing IgG-IgE dimers and larger multimers. The shaded peak in Fig. S1, representing IgG-IgE dimers, was collected as the final purified reagent. Based on the separation of peaks in the chromatogram, we expect the final SPC reagent to be predominantly composed of IgG-IgE dimers, with potential low-level contamination with larger conjugates, with very little or no monomeric IgG or IgE. Although the reagent was likely < 100% pure, it was still detectable at the limit of quantitation of the ImmunoCAP platform (0.1 kU/L), suggesting excellent assay sensitivity.

Parallelism of the SPC diluted in human serum with the ImmunoCAP Specific IgE Calibrator curve was assessed with samples consisting of SPC in pooled normal human serum at 2 concentrations (11 and 55 kU/L), diluted in normal serum with dilution factors of 1 (undiluted), 2, 3, 5, 10, and 20. Anti-pegvaliase IgE ImmunoCAP assay results (duplicate measurements) from the three 6-fold dilution series demonstrated parallelism of the SPC in serum with the calibrator curve (inter-dilution %CV = 2.5 and 4.4; Table 1).

Specificity of the SPC for pegvaliase was demonstrated by assay signal inhibition in the presence of 100 μg/mL soluble pegvaliase (Table 2). To evaluate nonspecific binding, the SPC was added to normal human serum at a high concentration of 100 kU/L and tested against five different standard ImmunoCAP tests with unrelated

Table 1
Parallelism of surrogate positive control (SPC) with ImmunoCAP calibrators.

Dilution factor	11 kU/L sample			55 kU/L sample		
	kU/L	%CV	%Recovery	kU/L	%CV	%Recovery
1 (undiluted)	11	2.2	–	55	3.6	–
2	5.4	5.6	97	29	4.5	107
3	3.6	9.4	95	21	6.8	114
5	2.1	3.6	95	12	2.6	110
10	1.0	4.0	92	5.8	2.2	105
20	0.52	5.1	92	2.9	1.8	105
Inter-dilution %CV	3.2			4.4		

CV: coefficient of variation; kU/L: specific IgE concentration.
Recovery calculated relative to undiluted sample result.

Table 2
Surrogate positive control is specific for pegvaliase.

Dilution factor	100 µg/mL pegvaliase		0 µg/mL pegvaliase	
	kU/L	%CV	kU/L	%CV
1	10	0.45	66	4.4
3	3.1	3.1	26	2
9	0.98	3.4	8.8	2.5
27	0.31	3.6	2.9	5.8
81	< 0.10	–	0.81	3.5
243	< 0.10	–	0.24	3.9

CV: coefficient of variation between duplicate measurements; kU/L: specific IgE concentration.

Table 3
Minimal surrogate positive control binding to unrelated antigens.

ImmunoCAP	RU	%CV (RU)	kU/L
U1194 PAL (positive control)	20,957	1.2	> 100
e101, rCan f 1 dog	21	0.41	< 0.10
f233, nGal d 1 Ovomucoid, egg	17	10	< 0.10
f334, nBos d Bovine lactoferrin, milk	20	4.6	< 0.10
k218, rHev b 5 latex	18	5.5	< 0.10
t215, rBet v 1 PR-10, birch	77	1.6	0.11

CV: coefficient of variation; kU/L: specific IgE concentration; RU: response units (assay raw signal).

allergen proteins (dog, egg, milk, latex, and birch allergens) in 2 replicates in the ImmunoCAP Specific IgE assay (Table 3). Results were negative (< 0.10 kU/L) for all unrelated ImmunoCAP tests except for the birch allergen, with a result of 0.11 kU/L. The results suggest the rabbit IgG-human IgE conjugate is a reasonable surrogate positive control for the human anti-pegvaliase IgE ImmunoCAP assay.

3.2. Serum sample collection for IgE testing in phase 2 and 3 studies

In phase 2 studies, over 2000 serum samples collected routinely from 80 subjects tested negative in the anti-pegvaliase IgE RAST assay. Due to the lack of anti-drug IgE detected in specimens collected at every clinic visit, the IgE assessment schedule was amended to be conducted only at *ad hoc* visits after observed hypersensitivity events in the phase 2 extension study, PAL-003, and in the phase 3 clinical trials (Longo et al., 2018; Thomas et al., 2018). In phase 3 clinical studies, a total of 12 subjects experienced 17 acute systemic hypersensitivity events considered by expert adjudication to be consistent with NIAID anaphylaxis criteria (Thomas et al., 2018). Of these 17 events, 8 events in 8 subjects were identified at the time of the event; serum samples were drawn (on the day of the event or up to 9 days post event) and were tested for anti-pegvaliase IgE (Table S7). No anti-pegvaliase IgE was detected in any of these 8 serum samples using either the RAST assay ($n = 5$) or the ImmunoCAP assay ($n = 3$). An additional 9 acute systemic hypersensitivity events that occurred in 7 subjects were identified retrospectively. Consequently, serum samples were not collected contemporaneously with these hypersensitivity reactions. However, serum samples from these 7 subjects were available from visits before and after the date of the acute systemic hypersensitivity events and these samples were tested for anti-pegvaliase IgE using the ImmunoCAP assay after IgG and IgM depletion. No anti-pegvaliase IgE was detected in any of these 7 subjects from serum samples before or after the acute systemic hypersensitivity events.

3.3. Skin prick reaction testing

In phase 2 clinical studies, skin prick testing was explored as a means to identify subjects at risk for hypersensitivity reactions. Testing, including exposure to pegvaliase, saline (negative control), or histamine (positive control), was performed on 22 of 44 trial subjects, of which 31 subjects had experienced hypersensitivity, and 3 subjects had AEs consistent with the NIAID/FAAN criteria for anaphylaxis. All skin prick test results were negative. To confirm and extend these skin findings, sensitive *in vitro* pegvaliase-specific IgE assays were developed with orthogonal and more specific platforms.

3.4. Investigation of potential IgG interference with anti-pegvaliase IgE detection

After the first 3 months of treatment in the phase 3 study 165–301, anti-PAL IgG titers were sustained at levels that were on average 10,000-fold above baseline levels (Gupta et al., 2018), raising concern that high levels of anti-pegvalise IgG could interfere with detection of low concentration anti-pegvaliase IgE. To determine if anti-pegvaliase IgG could interfere with the detection of anti-pegvaliase IgE, positive control samples were prepared in normal human serum by adding low (0.6 kU/L) or high (12 kU/L) levels of anti-pegvaliase IgE SPC and increasing levels of polyclonal rabbit anti-pegvaliase IgG. When tested in the human anti-pegvaliase IgE ImmunoCAP assay, the results confirmed that the presence of high levels of rabbit anti-pegvaliase IgG interfered with measurement of human anti-pegvaliase IgE SPC (Fig. 1).

While there was little to no interference with the detection of SPC at low levels of rabbit anti-pegvaliase IgG (≤1 µg/mL), higher levels (≥10 µg/mL) caused a significant and proportionate decrease in the level of anti-pegvaliase IgE detected, leading to an underestimation of the concentration of SPC added. The interference was particularly

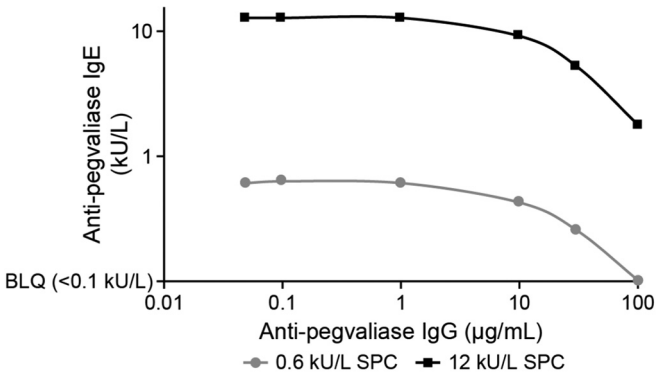

Fig. 1. High levels of anti-pegvaliase IgG interfered with anti-pegvaliase IgE positive control measurement. Rabbit polyclonal anti-pegvaliase IgG was added at 0 to 100 µg/mL (x axis) to normal human serum containing 0.6 kU/L black or 12 kU/L (grey) human anti-pegvaliase IgE SPC. The y axis shows anti-pegvaliase IgE concentrations in each sample measured once by the ImmunoCAP assay. The experiment was repeated a second time with equivalent results. Samples containing 0.6 kU/L SPC and 100 µg/mL anti-pegvaliase IgG yielded results below the assay limit of quantitation (BLQ; < 0.1 kU/L), plotted on the x axis for visualization.

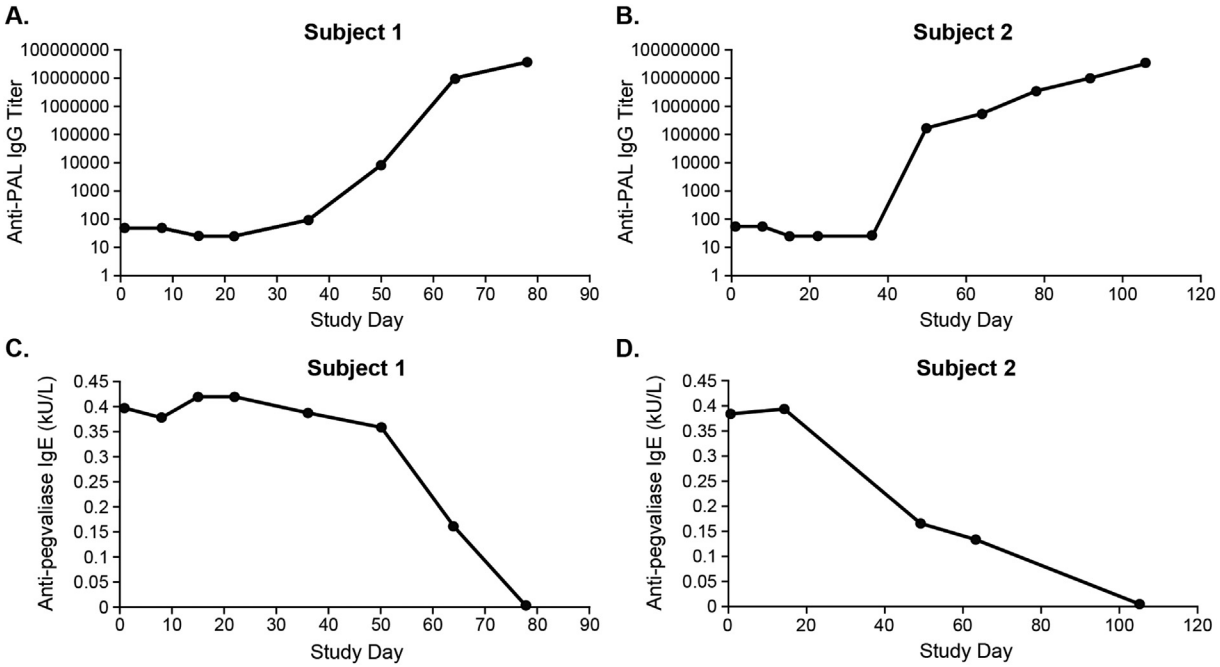

Fig. 2. High titer treatment-induced anti-PAL IgG can interfere with anti-pegvaliase IgE SPC detection in clinical samples. Anti-pegvaliase IgE surrogate positive control was added at approximately 0.4 kU/L to serial serum samples collected from two subjects receiving pegvaliase. Panels A and B show anti-PAL IgG titers over time in unspiked samples. Panels C and D show anti-pegvaliase IgE ImmunoCAP assay results from spiked serial samples. The x axis depicts the number of study days after first exposure to pegvaliase (administered subcutaneously on a daily basis). The lower limit of quantitation for the ImmunoCAP assay is 0.1 kU/L; samples that tested below the limit of quantitation are plotted at 0.1 kU/L for visualization.

Table 4
Titration of protein G and protein A-Sepharose resins for depletion of IgG and IgM from human serum.

SampleID	Sample volume (μL)	Protein G volume (μL)	Protein A volume (μL)	IgG (mg/dL)	IgM (mg/dL)	Total IgE (kU/L)	%IgG recovery	%IgM recovery	%Total IgE recovery
1	800	3200	0	BLQ	45.7	188.4	BLQ	54.7	62.4
2	800	2880	320	BLQ	33.4	177.5	BLQ	40.0	58.8
3	800	2560	640	BLQ	25.9	165.9	BLQ	31.0	54.9
4	800	2240	960	BLQ	27.5	172.6	BLQ	32.9	57.2
5	800	1920	1280	BLQ	24.8	164.7	BLQ	29.7	54.5
6	800	1600	1600	BLQ	25.4	171.2	BLQ	30.4	56.7
7	800	0	3200	33	26.1	174.9	2.7	31.3	57.9
Control	800	0	0	1213	83.5	302	100	100	100

BLQ: Results were below the limit of quantitation, < 17 mg/dL; percent recovery could not be calculated.
Percent recovery = 100*(depleted sample concentration/input serum concentration).

apparent with low concentrations of anti-pegvaliase IgE SPC (0.6 kU/L) and high concentrations of rabbit anti-pegvaliase IgG (100 μg/mL), leading to results below the IgE assay limit of quantitation (< 0.10 kU/L). These data suggested that clinical samples with low levels of anti-pegvaliase IgE and high levels of anti-pegvaliase IgG could result in a false-negative.

To further confirm whether pegvaliase treatment-induced ADA from actual human PKU subjects interferes with anti-pegvaliase IgE measurement, 2 subjects were identified that had low pretreatment anti-PAL IgG serum titers (<100) and high posttreatment anti-PAL IgG serum titers (> 30,000,000). Anti-pegvaliase IgE SPC was added at a low level (0.4 kU/L) to serum samples collected from these subjects over several months of treatment and samples were analyzed using the anti-pegvaliase IgE ImmunoCAP assay (Fig. 2).

Treatment-induced anti-PAL IgG titers > 100,000 interfered with measurement of anti-pegvaliase IgE. Increasing anti-PAL IgG serum titers were associated with under-recovery of the SPC added to samples at 0.4 kU/L. At the highest anti-PAL IgG levels in each subject (titer > 30,000,000), the SPC added at 0.4 kU/L generated false negative results below the assay limit of quantitation (< 0.1 kU/L).

3.5. Development of a method to deplete IgG and IgM from human sera

To reduce the risk of false-negative anti-pegvaliase IgE results due to ADA interference, a method was developed to deplete potentially interfering IgG and IgM ADA from serum samples prior to the IgE assessment, using a mixture of protein A and protein G Sepharose resins. Because protein A and protein G have substantially different affinities for IgG, IgM, and IgE

(Peng et al., 1994), the ratio of input serum: protein A-Sepharose: protein G-Sepharose was empirically optimized to maximize IgG and IgM depletion and IgE retention.

Pooled sera from healthy donors was tested for total IgG and IgM levels using Scintillation Proximity Assays (SPAs), and total IgE levels were measured using the total IgE ImmunoCAP assay. The pooled sera contained 1213 mg/dL IgG, 83.5 mg/dL IgM, and 302 kU/L IgE (Table 4) before depletion. To optimize the depletion method, 800 μL aliquots of pooled serum were treated with protein A Sepharose, protein G Sepharose, or mixtures of the Sepharose resins, and the depleted supernatant from each treatment was tested for total IgG, IgM, and IgE levels. The relative percent recovery of IgG, IgM, and IgE

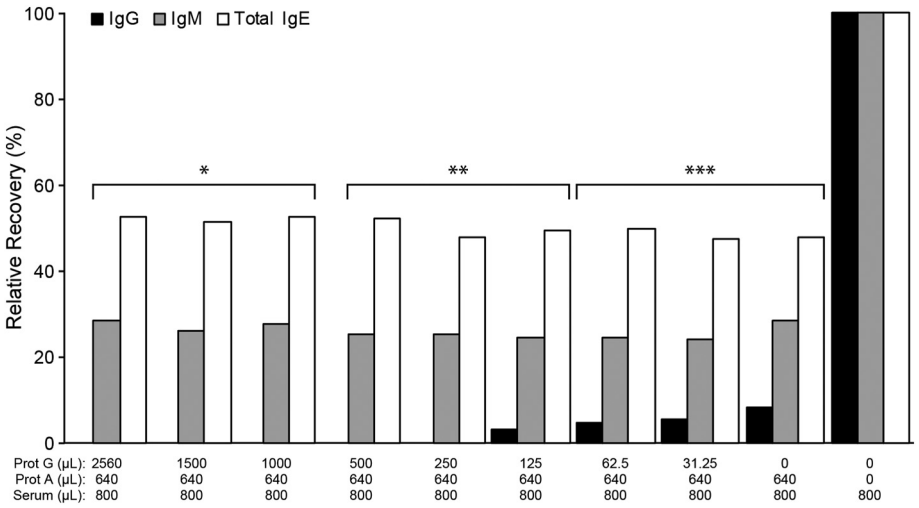

Fig. 3. Optimization of IgG and IgM depletion method. The x axis shows the volume of protein G Sepharose, protein A Sepharose, and serum in each sample, and the y axis shows the relative recovery of total IgG (black bars), IgM (grey bars), and IgE (open bars) concentrations in each depleted sample, compared with the concentration measured in the input serum. Relative recovery was calculated with the formula 100*(depleted sample concentration/input serum concentration). **Condition tested in experiment 1. **Condition tested in experiments 1 and 2; data represent the average of both experiments. ***Condition tested in experiment 2.

concentrations compared with the input serum sample was calculated with the formula shown in Table 4.

Protein G Sepharose alone at 3200 µL was sufficient to reduce IgG concentrations below the assay limit of quantitation (< 17 mg/dL), while protein A Sepharose alone was less efficient at IgG removal (33 mg/dL after depletion). In contrast, protein A alone removed more IgM than protein G alone (26.1 mg/dL after depletion *vs.* 45.7 mg/dL). Keeping the total resin volume constant and varying the ratio of protein G: protein A resin demonstrated similar IgG recovery under all conditions, but IgM depletion was enhanced by inclusion of 640 µL protein A. Further experiments demonstrated that the protein G volume could be reduced to 500 µL with similar IgG and IgM depletion (Fig. 3; Table S2). Under these conditions, (800 µL serum/ 500 µL protein G/ 640 µL protein A), the normal pooled serum sample demonstrated ≈25% relative recovery of total IgM, 50% relative recovery of total IgE, and total IgG was depleted from > 1200 mg/dL to below the assay limit of quantitation (< 17 mg/dL).

The depletion method efficiently removed IgG and IgM but also reduced the total IgE concentration in the sample by ≈50%, which could impact the sensitivity of the downstream anti-drug IgE assay. Therefore, a final 2-fold concentration step was implemented with the goal of restoring ≈100% relative IgE recovery of the concentrated supernatant compared with the IgE concentration in the input serum. Because the minimum volume required for the anti-pegvaliase IgE ImmunoCAP screening assay is 250 µL, the optimized serum: protein G: protein A ratio determined using 800 µL of serum was scaled down for a final sample volume of 300 µL after the 2-fold concentration step. Thus, the final method was characterized using 600 µL serum, 375 µL protein G-Sepharose, and 480 µL protein A-Sepharose, and concentration of the supernatant after depletion to 300 µL by vacuum centrifugation (2-fold concentration relative to input serum volume; Table 5). Under these concentrated conditions, the final sensitivity of anti-drug IgE detection should not be negatively impacted, despite substantial reduction of both total IgG and IgM to < 45 mg/dL.

3.6. Characterization of optimized depletion method

The precision and reproducibility of the optimized depletion method was characterized with 5 independent replicates of normal pooled serum on each of 4 runs over 2 days. On average the method depleted 95.3% of total IgG (inter-assay CV 1.5%; Table S3), and 47.8% of total IgM (inter-assay CV 9.4%; Table S4), with 99.1% relative recovery of total IgE from the input serum (inter-assay CV 6.9%; Table S5), after initial depletion of IgG and IgM and the final 2× concentration step. Reproducibility of the depletion method across individuals was assessed with 10 serum samples from healthy donors. For all individual donors, relative IgG recovery was < 9% and relative IgM recovery was < 57% (Table S6). Total IgE concentrations in individual input sera were variable (4 to 857 kU/L), which may have contributed to variable relative IgE recovery across individuals (31.1 to 107.7%). Overall, the mean IgE recovery of the ten individuals was 83.8% (range 31.3 to 107.7%) compared with the IgE concentration in the input serum, with 8/10 samples > 70% relative IgE recovery.

We further characterized the depletion method using samples consisting of pooled normal human serum alone, or after addition of human anti-house dust mite IgE or human anti-silver birch IgE positive control sera from ImmunoCAP allergen-specific IgE assays. We evaluated differences in the concentration of total protein, total IgG, IgM, and IgE, and allergen-specific IgE before and after depletion using protein A and G-sepharose resins, or using un-conjugated sepharose. After depletion and 2× concentration, the protein concentration in samples was increased, with percent increase ranging from 7.3% to 31.7% using protein A/G, and was increased from 15.3% to 21.1% with sepharose resin and concentration alone (Table S7). The increase in protein concentration was attributed to the final concentration step. Total IgE recovery ranged from 80.6% to 103.8% using protein A/G resin, and ranged from 105.1% to 117.3% using un-conjugated sepharose resin. Total IgG concentrations after protein A/G depletion were below the IgG assay limit of quantitation (< 17 mg/dL), demonstrating IgG recovery of < 3.1%. In contrast, total IgG recovery using un-conjugated sepharose resin ranged from 104.9% to 108.7%. Total IgM recoveries after protein A/G depletion ranged from 39.0% to 55.4%, or from 101.4% to 112.0% with un-conjugated sepharose. The data demonstrate depletion of total IgG and IgM from human serum by protein A/G-conjugated sepharose, but not un-conjugated sepharose. Importantly, similar levels of allergen-specific human IgE were detected

Table 5
Total IgG, IgM, and IgE % recovery after serum depletion and concentration.

Serum volume (µL)	Protein G volume (µL)	Protein A volume (µL)	IgG (mg/dL)	IgM (mg/dL)	Total IgE (kU/L)	%IgG recovery	%IgM recovery	%Total IgE recovery
600	375	480	44	43.5	308	3.5	53.2	107.3
Control (serum before depletion)			1262	81.8	287	100	100	100

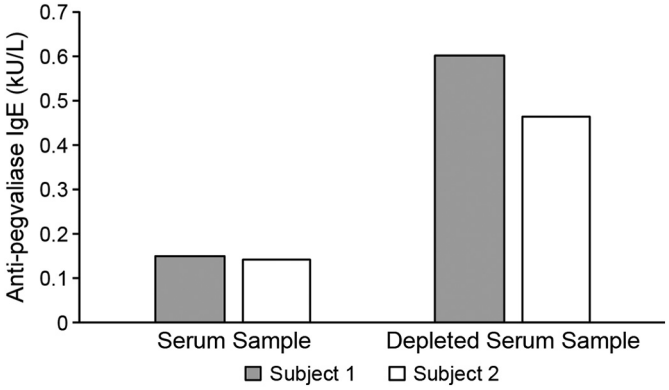

Fig. 4. Depletion method removes interfering ADA from clinical samples prior to IgE assessment. Anti-pegvaliase IgE surrogate positive control was added at approximately 0.4 kU/L to 2 individual serum samples (shaded subject 1, open subject 2) from the phase 3 study containing high ADA levels, with or without prior depletion of IgM and IgG. The spiked samples were each tested once in the anti-pegvaliase IgE ImmunoCAP assay. The y axis shows the concentration of anti-pegvalise IgE detected in each sample as interpolated from a heterologous calibrator curve.

before and after depletion with protein A/G (2.35 kU/L pre-depletion *vs.* 1.60 kU/L post-depletion for human anti-house dust mite IgE; 0.80 kU/L pre-depletion *vs.* 0.73 kU/L post-depletion for human anti-silver birch IgE). Although some IgE was lost in the process, the results suggest that the majority of allergen-specific IgE was retained in samples after depletion, and loss of IgE was not antigen-specific.

We next confirmed that the depletion method removed interfering anti-pegvaliase ADA from clinical samples, as intended. Serum samples containing high levels of anti-PAL IgG, collected from two subjects receiving pegvaliase, were each divided into 2 aliquots. IgG and IgM were depleted from one aliquot of each sample, while the second aliquot was untreated. Human anti-pegvaliase IgE SPC was added (≈0.4 kU/L) to each sample, and samples were tested by the ImmunoCAP assay. It was necessary to spike anti-pegvaliase IgE SPC after depletion because, unlike human anti-pegvaliase IgE, the surrogate control contains rabbit IgG framework and would be removed by the depletion resin. When added to serum without pre-depletion, the IgG and IgM ADAs interfered with SPC detection, and signal was near the assay limit of quantitation (0.14–0.15 kU/L; Fig. 4). In contrast, the measured concentration of SPC was higher in the IgM/IgG depleted samples (0.45 to 0.60 kU/L), demonstrating removal of interfering ADA by the depletion step.

3.7. Clinical sample results following IgM and IgG depletion confirm original anti-drug IgE negative results

To determine whether any phase 3 study anti-drug IgE test results were false-negative as a result of ADA interference, the samples were re-tested using the ImmunoCAP assay after depletion of IgM and IgG using the methodology described (Table S8). All 17 samples from the 12 subjects with acute hypersensitivity reactions tested negative for anti-pegvaliase IgE after depletion of IgG and IgM, confirming the original interpretation that samples were IgE-negative.

4. Discussion

Accurate and sensitive measurement of anti-drug IgE is critical to characterize the mechanism of treatment-associated hypersensitivity events (Leach et al., 2014; Riedl and Casillas, 2003). The detection of anti-drug IgE antibodies in serum is more precise than traditional skin tests and is not influenced by antihistamines or anti-inflammatory therapy (Portnoy, 2015). However, non-IgE ADAs (*e.g.*, IgG, IgM) can interfere with the detection of drug-specific IgE (Dević et al., 2014; Kadooka et al., 2000; Lehrer et al., 2004; US Food and Drug Administration, 2016). We confirmed this interference can occur with measurement of anti-pegvaliase IgE by demonstrating that rabbit anti-pegvaliase IgG, purified from animal sera after prime-boost immunization with drug, interfered with accurate measurement of a human anti-pegvaliase IgE SPC reagent in a concentration-dependent manner. We further demonstrated that pegvaliase treatment-induced ADA responses in clinical samples reach high enough levels to interfere with anti-drug IgE measurement in actual study samples. These results suggest a relatively low, but potentially clinically significant anti-pegvaliase IgE response (*e.g.*, 0.4 kU/L or less) in a subject with high anti-PAL IgG titers could be missed (*i.e.*, reported negative for anti-drug IgE) as a result of ADA interference. Interference from lower levels of IgG or with higher levels of IgE could result in underestimation of the true anti-drug IgE concentration.

While interference from anti-drug IgM was not specifically demonstrated in this study, it is logical that any non-IgE drug-specific antibody can potentially interfere with accurate measurement of anti-drug IgE concentration. We therefore developed a method to remove both IgM and IgG from serum samples, as these are the most abundant antibody isotypes. Both anti-pegvaliase IgG and IgM are induced by treatment and could potentially interfere with anti-pegvaliase IgE measurement. Differential binding of IgG, IgM, and IgE by protein A and protein G-Sepharose allowed selection of conditions that favored removal of IgG and IgM over IgE. Immunoglobulins were not depleted with un-conjugated sepharose alone. Although some IgE was lost after exposure to protein A and protein G-Sepharose, a 2-fold concentration step brought the IgE concentration of the final sample back to approximately the same concentration as the input serum, with final IgG and IgM concentrations < 45 mg/dL each. Confirming previous studies (Dević et al., 2014; Jaoko et al., 2001; Kadooka et al., 2000; Lehrer et al., 2004), a depletion step reduced total IgG and IgM concentrations (regardless of specificity) and improved the sensitivity of pegvaliase IgE detection. An advantage of this particular approach is that the method can be employed without modification upstream of any drug-specific IgE assay to reduce potential ADA interference.

The method demonstrated reproducible depletion of IgG and IgM across individual samples. While the majority of samples (8/10) showed relative IgE recovery > 70%, with mean relative IgE recovery of 83.8% overall, 2 of 8 individual samples unexpectedly had lower relative IgE recovery (31.3% and 62.9%). Therefore, while most samples retain the bulk of serum IgE, there is a risk that the depletion method could reduce total IgE concentration in some study samples, which could negatively affect downstream IgE assay sensitivity. Nevertheless, if IgG and IgM interference is significant, some reduction in total IgE concentration may be an acceptable trade-off for more sensitive detection of anti-drug IgE in the presence of non-IgE ADA. One potential strategy to mitigate the risk of IgE loss during the depletion step would be to confirm testing results with and without IgG and IgM depletion, similar to the approach used in the study presented here. Alternatively, the total IgE concentration in each sample could be monitored before and after depletion to identify samples where total IgE was significantly reduced, for repeat analysis. In this study, sample volumes were limited, making this approach infeasible. Lastly, risk of IgE over-depletion could also be prospectively mitigated by collecting multiple samples from a subject over time after hypersensitivity to provide additional volume for potential follow-up testing. Given the relatively short half-life of IgE in circulation, estimated to be 2–3 days (Lawrence et al., 2017), sample collection after hypersensitivity should be performed as soon as possible.

Removal of IgE during the depletion step is expected to be largely independent of antibody specificity. However, this hypothesis could not be tested for anti-pegvalise IgE specifically due to the lack of a *bona fide* human anti-pegvaliase IgE positive sample. The anti-pegvaliase SPC reagent contains rabbit IgG, which would be removed by protein A/G-sepharose. Thus, specific removal of anti-pegvaliase IgE by protein A and G could not be ruled out, but is not expected. When the depletion

method was applied to samples containing human anti-allergen IgE, the detected concentrations were modestly reduced after depletion. This effect was attributed to binding of IgE by protein A, which has been reported by others (Peng et al., 1994). The results suggest the modest reduction of IgE during the depletion step impacts IgE clones of any specificity. The total protein concentration was modestly increased in samples after the concentration step, which could have led to increased matrix interference and some reduction of assay signal, although this was not specifically tested.

Measurement of drug-specific IgE is challenging for multiple reasons: very low levels can be clinically significant, requiring sensitive assay methods; drug-specific IgE may be cell-associated *in vivo*, further reducing the concentration in blood; and free drug or other ADA isotypes (such as anti-drug IgG) in samples can potentially interfere with specific IgE measurement. With these limitations, and despite our improvements to reduce IgG interference, it is not possible to absolutely prove all samples are truly negative for anti-drug IgE based on negative test results alone. However, in the case of pegvaliase, it is important to acknowledge the preponderance of evidence. *In vivo* skin prick test results were negative, suggesting a lack of drug-specific IgE, blood samples collected around the time of hypersensitivity tested negative with sensitive RAST and ImmunoCAP methods, and samples again tested negative after removal of potentially interfering IgG and IgM. While these *in vitro* data and multiple aspects of the clinical presentation of hypersensitivity events suggest reactions were likely immune complex-mediated (Type III) rather than IgE-mediated (Type I), the presence of IgE below the assay limit of detection (0.1 kU/L), the presence of other interfering factors that preclude detection, or loss of anti-pegvaliase IgE during depletion cannot be completely ruled out. Further approaches that may be explored include *ex vivo* or *in vitro* cell-based histamine release or mast cell activation assays, enrichment of total IgE before testing (for example using anti-human IgE-conjugated sepharose resin), enrichment of IgE followed by depletion of IgG/IgM before testing, or attempts to develop ultra-sensitive methods with detection limits below 0.1 kU/L using cutting-edge assay platform technologies. Such techniques would need to be carefully developed and validated before clinical use.

In addition to sensitive and accurate anti-drug IgE test results, the clinical presentation of hypersensitivity events may inform interpretation of the mechanism of treatment-associated hypersensitivity. For example, classical IgE-mediated acute systemic hypersensitivity is caused by the continued presence of antigen-specific IgE; therefore, subsequent events of acute systemic hypersensitivity often occur directly after re-exposure to the allergen (Riedl and Casillas, 2003). Importantly, no subject who continued pegvaliase after experiencing acute systemic hypersensitivity events had an event immediately upon the next administration. In a study unrelated to pegvaliase, Aun et al. (2014) evaluated subjects with drug-induced IgE-mediated (type I) hypersensitivity anaphylactic and anaphylactoid events, and found 80% of patients were hospitalized, 50% required intensive care unit admission, and 45% were intubated, with rates several times lower for non-IgE-related reactions. In contrast, subjects treated with pegvaliase in clinical studies had HAEs associated with rash, arthralgia, and urticaria, which are clinical presentations consistent with immune complex-mediated (type III) hypersensitivity reactions (Leach et al., 2014; Riedl and Casillas, 2003). One subject required intensive care unit admission and no subjects were intubated. Consistent with a predominant type III etiology, hypersensitivity events were most frequent during the first six months of therapy when anti-PEG ADA, anti-PAL IgM and CIC levels were highest, and complement C3/C4 concentrations were lowest, compared with after the first year (Gupta et al., 2018).

Although CIC levels and complement activation were temporally associated with hypersensitivity events, neither CIC nor C3/C4 concentration was predictive of the risk of hypersensitivity in individuals, as virtually all subjects showed evidence of complement activation but only a minor subset experienced acute systemic hypersensitivity. Identification of analytes measurable in the blood that can predict the relative risk of acute systemic hypersensitivity in individual subjects would be extremely valuable to guard patient safety during clinical development of ERTs and ESTs. The risk to pegvaliase-treated patients was managed clinically by limiting patient exposure to the drug initially, with low dosages used during the induction period when CIC levels were highest and hypersensitivity reactions were most frequent. Dose concentration and frequency were gradually escalated in the titration period as the immune response matured, CIC levels waned, and hypersensitivity events became less frequent, until a daily maintenance dose associated with control of Phe levels was achieved.

Relatively low anti-drug IgE concentrations can potentially drive type I HAEs, necessitating sensitive assays for anti-drug IgE measurement in clinical studies, but interfering non-IgE ADA can be present at much higher concentrations than IgE in ERT- and EST-treated patients. In this situation, potential IgG and IgM interference makes interpretation of anti-drug IgE test results and understanding the mechanism of HAEs particularly challenging. No serum samples collected from pegvaliase-treated subjects that experienced acute systemic hypersensitivity events in phase 3 studies contained detectable anti-pegvaliase IgE, even when the depletion method was used to remove IgG and IgM before testing, confirming the initial results generated without IgM and IgG depletion. Taken together, the clinical presentation and ability to re-administer pegvaliase after hypersensitivity without adverse effects, temporal association with CIC levels and complement activation, and lack of anti-pegvaliase IgE suggest pegvaliase-associated hypersensitivity events were not IgE-mediated. The depletion method presented here offers a simple universal technique for dramatically reducing ADA interference from serum samples before anti-drug IgE measurement, to improve confidence in the interpretation of anti-drug IgE test results during clinical development.

Conflict of interest

KL, TN, BB, KL, SZ, HHW, and SG are employees and stockholders of BioMarin Pharmaceutical Inc., which manufactures and distributes pegvaliase. GS is a consultant and has received consulting fees from BioMarin. RZ is a consultant for BioMarin and has received grants and consulting fees from BioMarin.

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Author contributions

KL, HHW, and SG wrote the draft manuscript. TN and BB performed experiments and analysis. SZ contributed to method development and design of experiments. KLau and SG interpreted and described clinical immunogenicity data. GS is a clinical associate professor of Medicine at Weill Cornell Medical School and was the allergy-immunologist who reviewed the adverse drug reactions to determine consistency with anaphylaxis. HHW, GS, and RZ provided interpretation of the clinical presentation of hypersensitivity reactions. All authors provided critical review of the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2019.03.004.

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