Information regarding the potential for *ex vivo* PAL (phenylalanine ammonia lyase) enzymatic activity at ambient temperature in samples used for blood Phe testing

This letter is to inform you of an important sample handling issue when treating PKU patients with Palynziq® (pegvaliase-pqpz) injection.

There is the potential for *ex vivo* PAL enzyme activity in Palynziq-treated patient plasma samples kept at ambient temperature, rather than processed per the procedures outlined in the pegvaliase clinical trial laboratory manuals (described in recommendation #1, below). Not following these procedures has the potential to cause inaccurate plasma Phe measurements. If plasma specimens cannot be processed immediately, placing the tube on ice or sending a dried blood spot may help mitigate the effect of *ex vivo* PAL enzyme activity.

The bench experiments performed suggest that the rate of Phe degradation may be related to both blood Phe concentration, as well as the pegvaliase plasma concentration [see Fig 2 below].

Based on the results of a series of *ex vivo* analyses, we have the following recommendations for blood Phe testing in patients on Palynziq to minimize the effect of ex vivo PAL enzymatic activity:

1. When assessing blood Phe via plasma amino acid analysis, sample processing should be completed within 30 minutes of collection into a sodium heparin tube, which includes a 15-minute centrifuge time (at 1500 to 2000 x g), followed by immediate freezing of plasma at –20C° or colder. If plasma amino acids are not analyzed in-house, the frozen plasma should be shipped on dry ice. These practices will minimize the time the sample spends at ambient temperature, helping to ensure that the test results are as accurate as possible [see Figs. 1&2 below].

2. If a delay in processing the freshly drawn sodium heparin tube is anticipated, experimental evidence (conducted over 180 minutes) suggests that placing the tube on ice may mitigate Phe degradation [see Fig 3 below].

3. If you have been collecting dried blood spots (DBS) by spotting blood from a patient's pricked finger directly onto filter paper, no change is recommended at this time. While there may be some *ex vivo* PAL enzyme activity during the time it takes the spot to dry, existing data suggest that the effect is minimal [see Fig 4a-b below]. This would be the specimen collection technique of choice if plasma amino acids are not collected and processed as above.

4. DBS collected by spotting blood from a tube onto filter paper may be at risk for measurement error if application to the filter paper is not done immediately after the blood draw [see Fig 4a-b below].

If you have any questions about this topic, please contact medinfo@bmrn.com.

Global Medical Affairs
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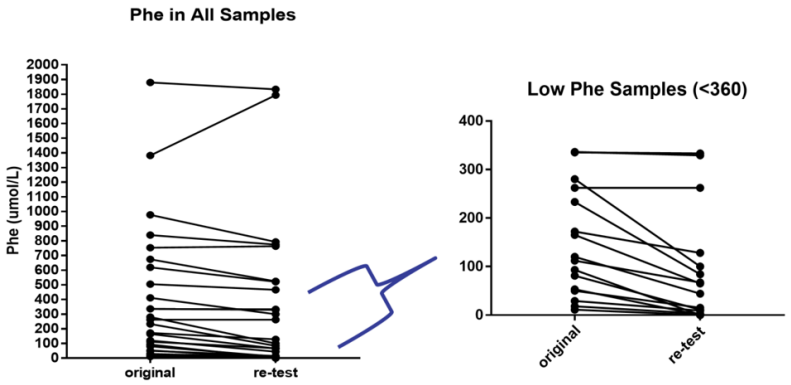
Supplemental Information

Experiment 1: Phe levels were lower in thawed and retested plasma samples

Fig. 1



Difference in Absolute Phe Results (All Samples)
Original vs. Retest

Mean Difference	-47
Median Difference	-47
Min Difference	412
Max Difference	-184

Difference in Absolute Phe Results (Low Phe Samples)
Original vs. Retest

Mean Difference	-56
Median Difference	-45
Min Difference	0
Max Difference	-180

Experimental design:

- Plasma samples from 165-301 patients were thawed and left at room temperature for an *uncontrolled* period of time before being re-tested

Interpretation:

- Majority of Phe results were lower when tested the second time, particularly in samples with original blood Phe < 360 µmol/L
- Tyr results in the same samples were not impacted
- Phe and Tyr from PKU patient samples not containing pegvaliase were not impacted

Experiment 2*: In vitro* plasma Phe reduction proportional to added pegvaliase concentration

Fig. 2a

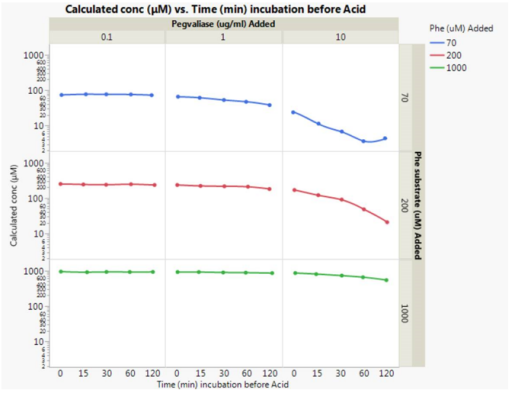


Fig. 2b

		0.1 ug/ml pegvaliase	1.0 ug/ml pegvaliase	10 ug/ml pegvaliase
Phe (uM)	Time (min)	Calculated Phe (µM)		
70	0	73.2	65.3	23.7
	15	76.5	60.9	11.4
	30	76.2	52.7	6.83
	60	75.6	46.9	3.64
	120	72.1	37.9	4.4
200	0	250	234	169
	15	242	220	121
	30	238	215	90.8
	60	246	209	48.2
	120	234	180	20.9
1000	0	934	908	851
	15	898	906	792
	30	914	882	722
	60	909	871	646
	120	914	847	536

Experimental design:

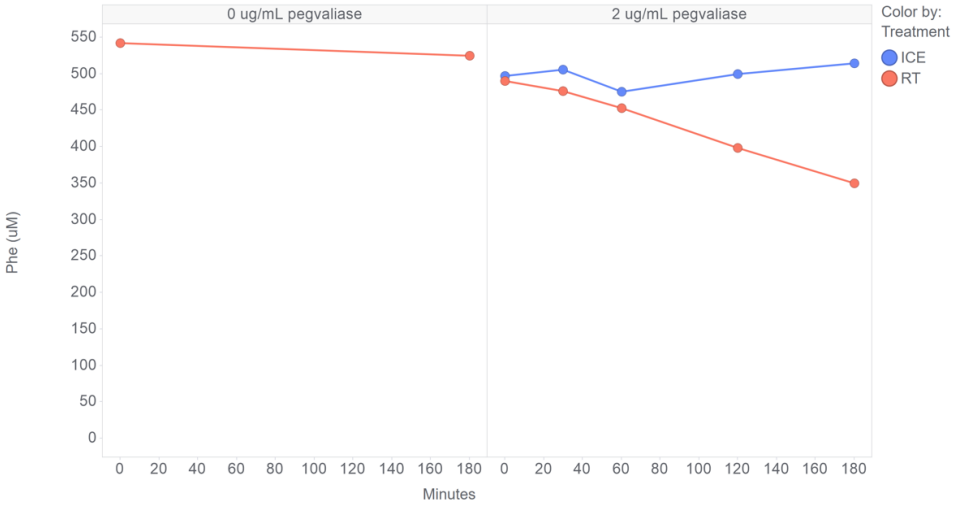
- Phe and pegvaliase added to pooled unaffected donor plasma at different concentrations
- Pegvaliase activity stopped by addition of acid at different time points

Interpretation:

- Minimal effect seen on plasma Phe samples over time with lower pegvaliase concentration (up to 1 µg/mL)
- More significant effect seen on plasma Phe reduction over time at pegvaliase concentration of 10 µg/mL (which approximates the mean pegvaliase plasma concentration seen in PRISM patients with low blood Phe levels)
- Caveat: Addition of pegvaliase to unaffected donor plasma may not accurately represent samples from pegvaliase treated patients containing antidrug antibodies, immune complexes, etc.

Experiment 3: Phe levels in plasma containing pegvaliase are stable on ice but not at room temperature (RT)

Fig. 3



Experimental design:

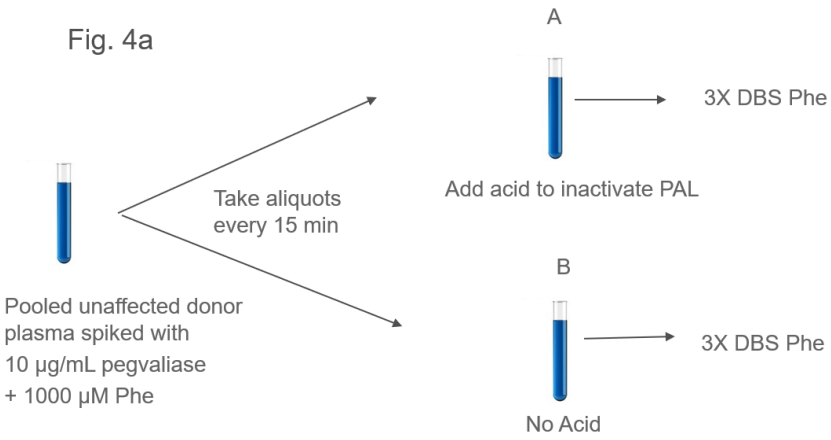
- Plasma samples containing 0 or 2 µg/mL pegvaliase and ~500 µM Phe were held at room temperature (red lines) or on ice (blue line) for 180 minutes

Interpretation:

- In the presence of pegvaliase, plasma Phe concentrations declined over time at room temperature, but remained stable when held on ice

Experiment 4: Investigation using plasma spotted on DBS cards

Fig. 4a

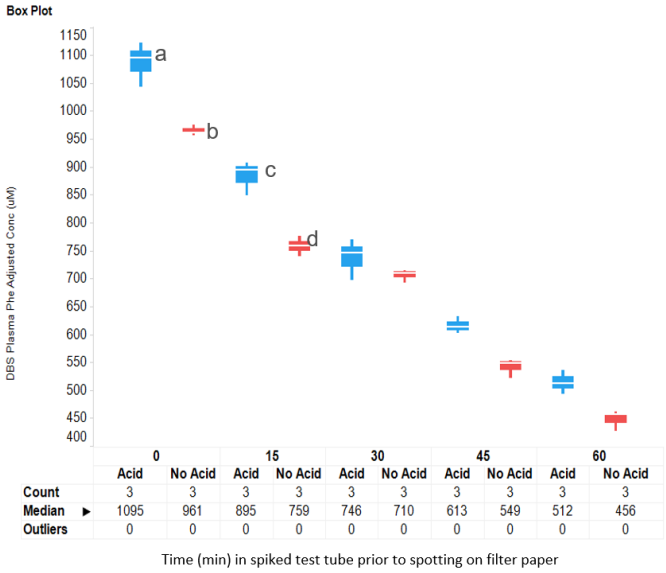


Experimental design:

- Pooled unaffected donor plasma was spiked with 10 µg/mL pegvaliase and 1000 µM Phe
- Aliquots were taken every 15 minutes into 2 tubes, one of which was acidified to inactivate pegvaliase, and then spotted onto filter paper cards
- The cards were allowed to dry over night prior to analyzing Phe levels

Experiment 4: Reduction of plasma Phe while drying on DBS

Fig. 4b



	0		15		30		45		60	
	Acid	No Acid	Acid	No Acid	Acid	No Acid	Acid	No Acid	Acid	No Acid
Count	3	3	3	3	3	3	3	3	3	3
Median ▸	1095	961	895	759	746	710	613	549	512	456
Outliers	0	0	0	0	0	0	0	0	0	0

Time (min) in spiked test tube prior to spotting on filter paper

Interpretation:

- The difference seen between acidified and non-acidified samples reflects the extent of Phe reduction while blood spot dries on the filter paper card:

[a] Acidified baseline sample spotted on filter paper at time 0. No change observed from expected baseline, demonstrating that immediate acidification eliminated all residual PAL metabolism of Phe in the sample during the drying time.

[b] Unacidified baseline sample spotted on filter paper at time 0. There is some Phe reduction compared to the acidified sample (a), occurring during the drying time, indicating residual PAL activity.

[c] Sample acidified after 15 minutes at ambient temperature before being spotted and drying on filter paper. More substantial Phe metabolism has occurred relative to (a)

[d] Sample remains unacidified for 15 minutes at ambient temperature before being spotted and drying on filter paper. More substantial Phe metabolism has occurred relative to (b)