Supporting Information: An LC-MS/MS Based Method for the Quantification of Pyridox(am)ine 5′-Phosphate Oxidase Activity in Dried Blood Spots from Patients with Epilepsy

Matthew P. Wilson,[1] Emma J. Footitt,[2] Apostolos Papandreou,[1] Mari-Liis Uudelepp,[1] Ronit Pressler,[3] Danielle Stevenson,[2] Camila Gabriel,[2] Mel McSweeney,[2] Matthew Baggot,[4] Derek Burke,[4] Tommy Stödberg,[5] Kate Riney,[6] Manuel Schiff,[7] Simon J. H. Heales,[1,4,8] Kevin A. Mills,[1] Paul Gissen, [1,2] Peter T. Clayton,*[1] Philippa B. Mills*[1]

[1] Genetics and Genomic Medicine, UCL GOS Institute of Child Health, 30 Guilford St, London WC1N 1EH, UK.

[2] Metabolic Medicine Unit, Great Ormond Street Hospital for Children NHS Foundation Trust, London WC1N 3JH, UK.

[3] Neurology Department, Great Ormond Street Hospital for Children NHS Foundation Trust, London WC1N 3JH, UK.

[4] Chemical Pathology, Great Ormond Street Hospital for Children NHS Foundation Trust, London WC1N 3JH, UK.

[5] Neuropediatric Unit, Karolinska University Hospital, SE-171 76, Stockholm, Sweden.

[6] Neurosciences Unit, The Lady Cilento Children's Hospital, 501 Stanley Street, South Brisbane 4101, QLD, Australia.

[7] Reference Center for Inborn Errors of Metabolism, Robert Debré University Hospital, APHP, Paris 75019, France.

[8] Neurometabolic Unit, National Hospital for Neurology and Neurosurgery, Queen Square, London WC1N 3BG.

Corresponding Authors:

* E-mail: peter.clayton@ucl.ac.uk (Peter T. Clayton)

* E-mail: p.mills@ucl.ac.uk (Philippa B. Mills)

CONTENTS:

Table S-1: Profile for reversed-phase gradient elution of B_6 vitamers……Page S-2
Table S-2: MS Parameters for MRM-Based Quantification of the B_6 vitamers. ……Page S-3
Table S-3: Effect of pH on PNPO Activity ……Page S-4
Table S-4: Effect of exogenous PLP on PNPO activity……Page S-4
Table S-5: Summary of subjects with mutations identified in PNPO……Page S-5

Figure S-1: LC-MS/MS chromatrogram of the B_6 vitamers and pyridoxic acid……Page S-7
Figure S-2: Effect of buffer upon pyridoxal formation due to PLP hydrolysis……Page S-8
Figure S-3: Effect of incubation buffer on pyridoxal kinase activity……Page S-9
Figure S-4: Effect of substrate concentration on PNPO activity……Page S-10
Figure S-5: Effect of FMN concentration and substrate on PLP formation.……Page S-11
Figure S-6: PNPO activity as a function of DBS weight……Page S-12
Figure S-7: PNPO activity as a function of PLP formation from 0-120 min.……Page S-13

Figure S-8: PM/PA ratios in subjects receiving vitamin B_6 supplementation……....Page S-14

Table S-1: Profile for Reversed-Phase Gradient Elution of B_6 vitamers.

Time (min)	Percent mobile phase		Flow rate (ml/min)
	A	B	
0.00	97.5	2.5	0.40
0.40	97.5	2.5	0.40
3.75	50.0	50.0	0.40
4.25	0.1	99.9	0.40
5.00	97.5	2.5	0.40
6.50	97.5	2.5	0.40

Table S-2: MS Parameters for MRM-Based Quantification of the B_6 Vitamers.

Analyte	Retention time (minutes)	Precursor ion (m/z)	Product ion (m/z)	Cone voltage (V)	Collision energy (V)
Pyridoxamine 5'-phosphate	0.64	249.04	134.05	27	22
Pyridoxal 5'-phosphate	0.84	248.00	150.01	27	16
Pyridoxine 5'-phosphate	0.84	250.16	134.13	58	20
d_3-Pyridoxal 5'-phosphate	0.84	251.16	152.18	30	18
Pyridoxic acid	0.92	184.06	147.99	18	18
d_2-Pyridoxic acid	0.92	186.06	149.99	18	18
Pyridoxal	1.08	168.1	150.1	21	12
d_3-Pyridoxal	1.08	171.1	153.1	21	12
Pyridoxamine	1.17	169.12	134.04	22	20
d_3-Pyridoxamine	1.17	172.12	137.04	22	20
Pyridoxine	1.42	170.1	134.0	27	19
d_2-Pyridoxine	1.42	172.1	136.0	27	19

Table S-3: Effect of pH on PNPO Activity

pH	PNPO Activity (pmol DBS^{-1} h^{-1})
7.0	16.72 ±0.80
7.2	20.37 ±0.36
7.4	18.43 ±0.98
7.6	19.88 ±1.06
7.8	24.90 ±1.32
8.0	22.72 ±1.78

PNPO activity measured in a healthy adult control at pH 7 - 8. Incubation conditions: 20 mmol/L KPO_4 (pH adjusted appropriately); 1.5 µmol/L FMN; 400 nmol/L PNP; 37 °C; 300 rpm agitation; 1 x 3 mm DBS; 30 min incubation; n = 3. Error = SEM.

Table S-4: Effect of Exogenous PLP on PNPO Activity.

PLP added to reaction buffer (nmol/L)	% activity*
0	100.0
25	101.5
50	95.8
100	76.5
150	79.1
200	58.7

PNPO activity measured in a healthy adult control with varying amounts of PLP added to the reaction buffer. *100% = Activity with no exogenous PLP. Incubation conditions: 20 mmol/L $TrisPO_4$ pH 7.6; 1.5 µmol/L FMN; 400 nmol/L PNP; 37 °C; 300 rpm agitation; 1 x 3 mm DBS; 30 min incubation. (n=1).

Table S-5: Summary of Subjects with Mutations Identified in PNPO.

Subject	Age at sampling	Seizure Onset	Mutation/sequence variant	Presumed effect	PNPO activity (pmol DBS^{-1} h^{-1})	PM/PA ratio	References
1	2y	5d	c.[98A>T] (M) + c.[576C>A] (P)	p.D33V (M) + Y157* (P)	0.00	0.45	Novel
2	1d	None *	c.[364-1G>C] + [364-1G>C]	Splice errors + Splice errors	0.65	0.13	Novel
3	9y	5m	c.[347G>A] + c.[347G>A]	p.R116Q + p.R116Q	0.00	0.00	Mills et al. 2014
4	5y	None ***	c.[347G>A] + c.[347G>A]	p.R116Q + p.R116Q	0.13	0.00	Novel
5	25y	3h	c.[264-21_ 264-1delinsC] (M) + c.[98A>T] (P)	Splice errors (M) + p.D33V (P)	1.96	0.10	Mills et al. 2014
6	7y	5h	c.[641dupA] + ? **	p.Q214fs + ? **	0.00	0.16	Mills et al. 2014; Raimondi et al 2015
7	6y	30 min	c.[284G>A] (M) + c.[148G> A]; c.[364-1G>A] (P)	p.R95H (M) + p.E50K; Splice errors (P)	0.47	0.44	Mills et al. 2014
8	16y	n/a	c.[363+5G>A] + c.[363+5G>A]	Splice errors + Splice errors	1.83	0.01	Novel
9	17y	n/a	c.[363+5G>A] + c.[363+5G>A]	Splice errors + Splice errors	1.77	0.00	Novel
10	12y	10h	c.[347G>A];c.[674G>A] + c.[347G >A];c.[674G>A]	p.R116Q;p.R225H + p.R116Q;p.R225H	0.00	0.55	Mills et al. 2014

11	13y	<12h	c.[148G> A]; c.[364-1G>A] + c.[148G> A]; c.[364-1G>A]	p.E50K;Splice errors + p.E50K;Splice errors	3.11	0.39	Mills et al. 2005
12	5m	n/a	c.[673C>T] + c.[673C>T]	p.R225C + p.R225C	0.00	0.24	Novel
13	3y	n/a	c.[347G>A] + c.[347G>A]	p.R116Q + p.R116Q	0.00	0.00	Novel
14	5y	90 min	c.[637C>T] + c.[637C>T]	p.P213S + p.P213S	2.32	0.74	Mills et al. 2014; Hatch et al. 2015
15	3y	No neonatal seizures *	c.[637C>T] + c.[637C>T]	p.P213S + p.P213S	4.67	0.70	Mills et al. 2014; Hatch et al. 2015
16	11m	3w	c.[194G>T] + c.[194G>T]	p.W65L + p.W65L	0.00	0.11	Novel
17	6y	6h	c.[98A>T] + c.[98A>T]	p.D33V + p.D33V	0.04	0.95	Mills et al. 2014
18	4y	n/a	c.[263+2T>C] + c.[263+2T>C]	Splice errors + Splice Errors	3.58	0.13	Novel

Control ranges; Children receiving B_6 supplementation: 23.0 – 85.9 pmol DBS^{-1} h^{-1} (n=16); Children not receiving B_6 supplementation: 10.0 – 95.0 pmol DBS^{-1} h^{-1} (n=37); Healthy adults: 13.8 - 44.0 pmol DBS^{-1} h^{-1} (n=7).

*No seizures due to prophylactic treatment – in infancy/childhood presented with two seizures due to late PLP doses (first at 10m).

**No second mutation found.

*** Mother recipient of multivitamin containing pyridoxine during pregnancy.

n/a = not available; d = day; m = month; y = year

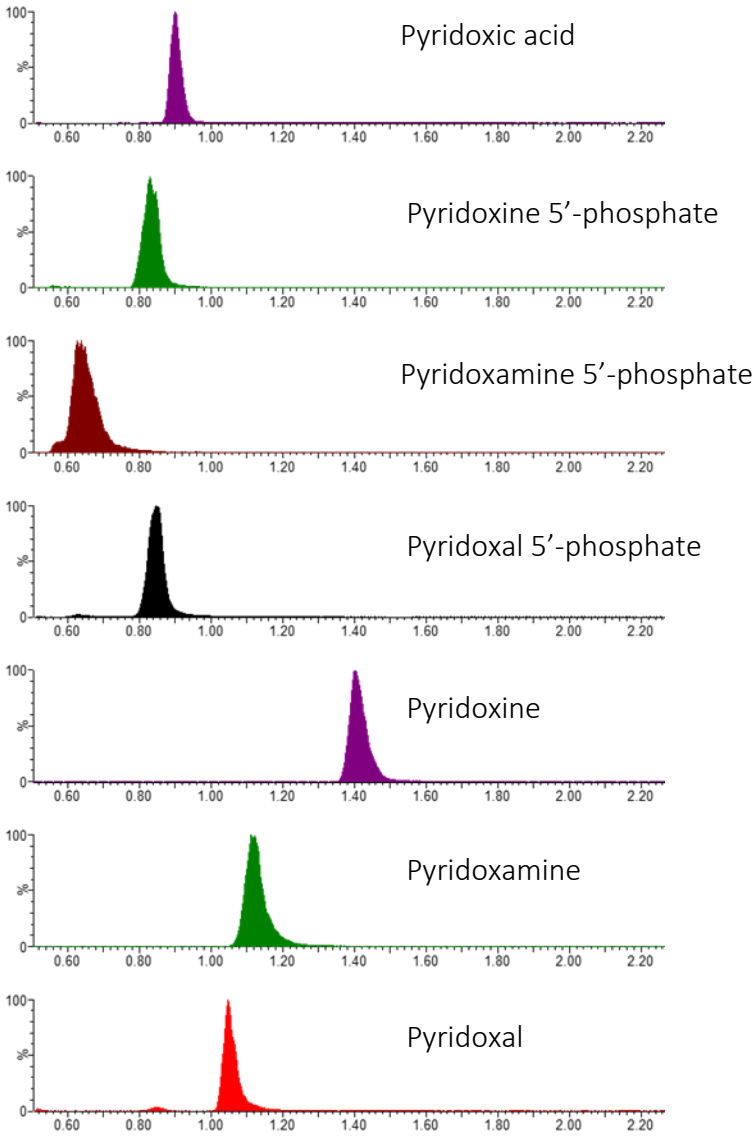

Figure S-1: LC-MS/MS chromatrogram showing the elution profile of the B_6 vitamers and pyridoxic acid.

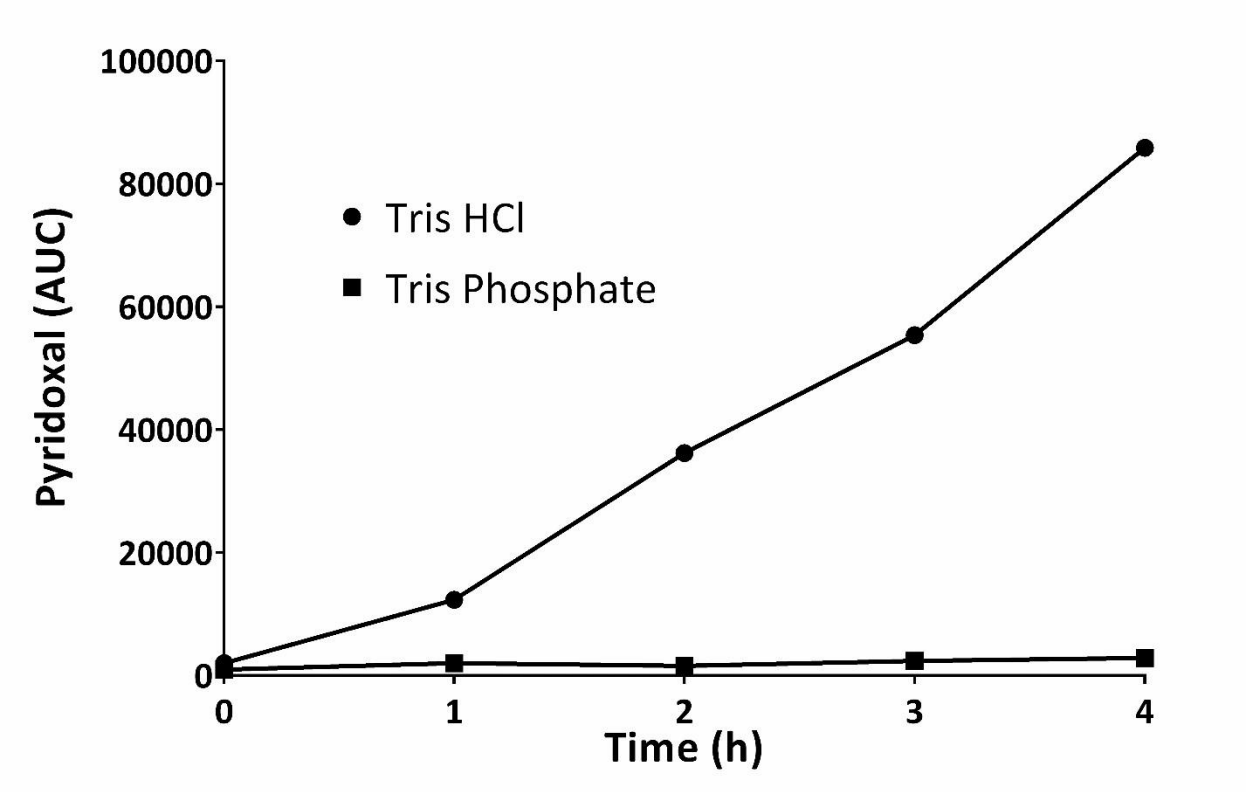

Figure S-2: Effect of buffer on pyridoxal formation. Incubation conditions: 20 mmol/L TrisPO_4/Cl pH 7.6; 1.5 µmol/L FMN; 400 nmol/L PNP; 37 °C; 300 rpm agitation; 1 x 3 mm DBS. DBS from a healthy adult control. AUC = area under curve. (n=1).

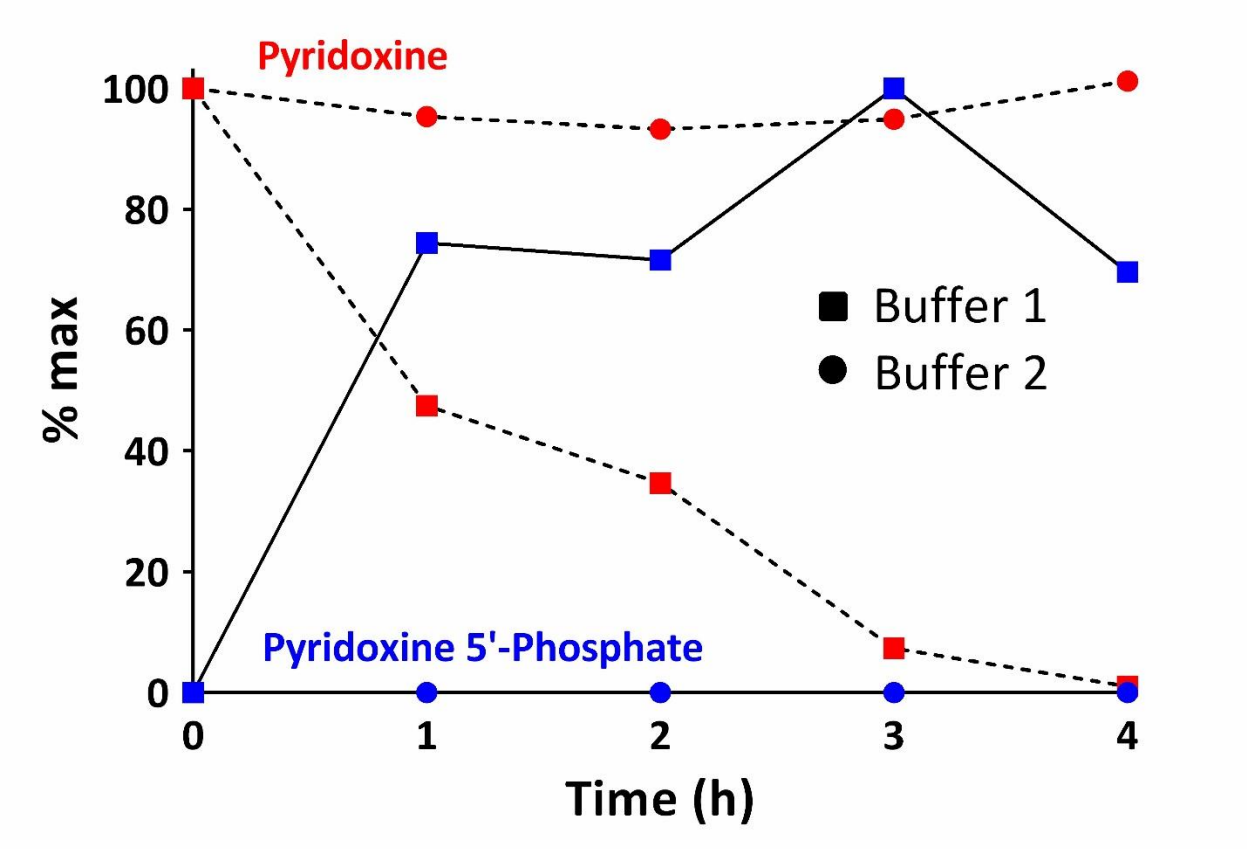

Figure S-3: Effect of incubation buffer on pyridoxal kinase activity. % maximum concentrations at specific time points (0-4 h) Filled line = PNP; dashed line = PN. **Buffer 1 includes cofactor reported to be necessary for optimal pyridoxal kinase activity:** 20 mmol/L KCl pH 7.6; 1.5 µmol/L FMN; 0.3 mmol/L ATP; 3 mmol/L $MgCl_2$; 400 nmol/L PN; 37 °C; 300 rpm agitation. **Buffer 2 lacks PK cofactors:** 20 mmol/L KCl pH 7.6; 1.5 µM FMN; 400 nmol/L PN; 37 °C; 300 rpm agitation. (n=1).

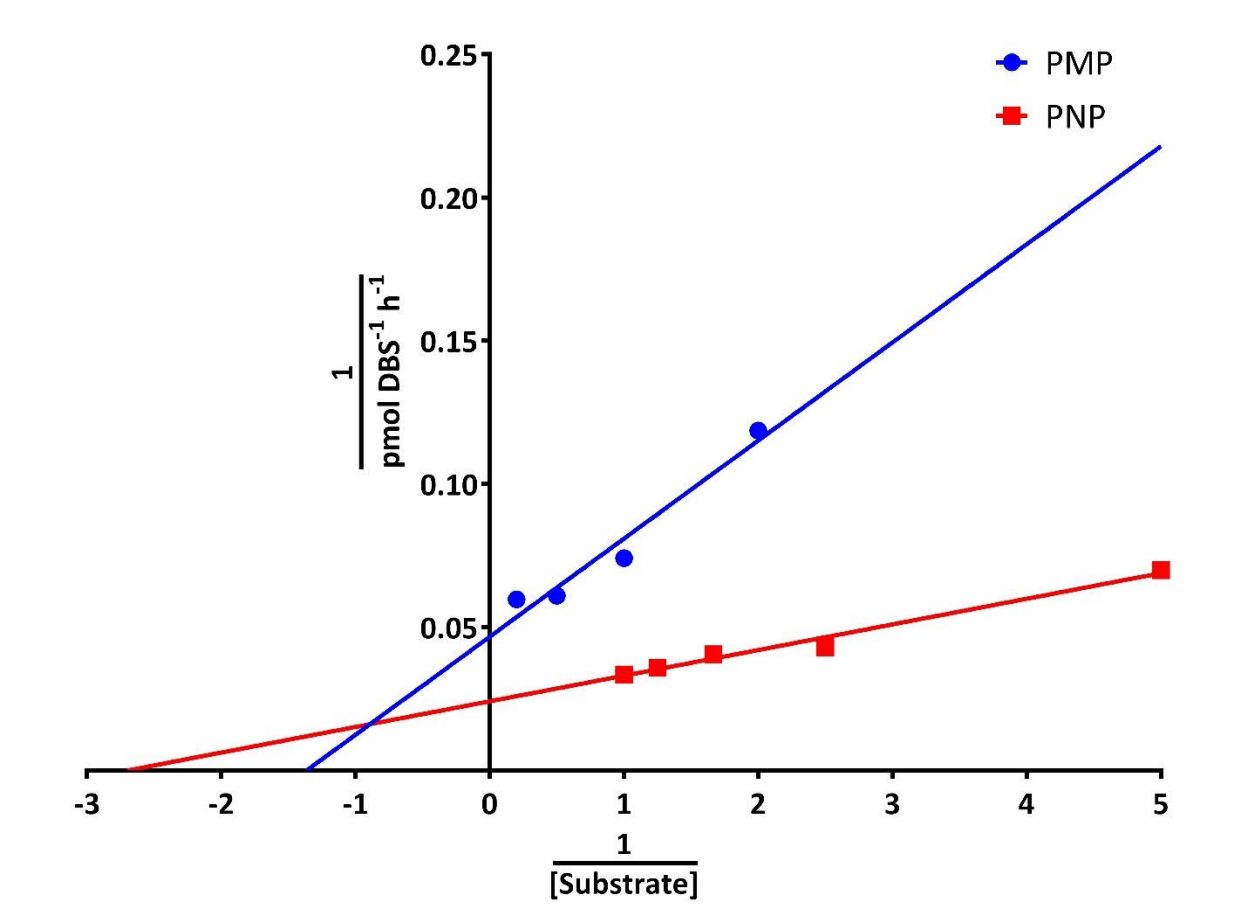

Figure S-4: Lineweaver-Burk plot showing the effect of substrate concentration and type on PLP formation. Incubation conditions: 20 mmol/L $TrisPO_4$ pH 7.6; 1.5 µmol/L FMN; 0 - 1 µmol/L PNP or 0 – 5 µmol/L PMP; 37 °C; 300 rpm agitation; 1 x 3 mm DBS from a healthy adult control. Calculated V_{max}: PNP 39.55 ±3.55 pmol DBS^{-1} h^{-1}; PMP 19.76 ±1.42 pmol DBS^{-1} h^{-1}. Calculated K_m: PNP 0.319 ±0.081 µmol/L; PMP 0.530 ±0.143 µmol/L. Michaelis-Menten kinetics calculated using GraphPad Prism 6.05.

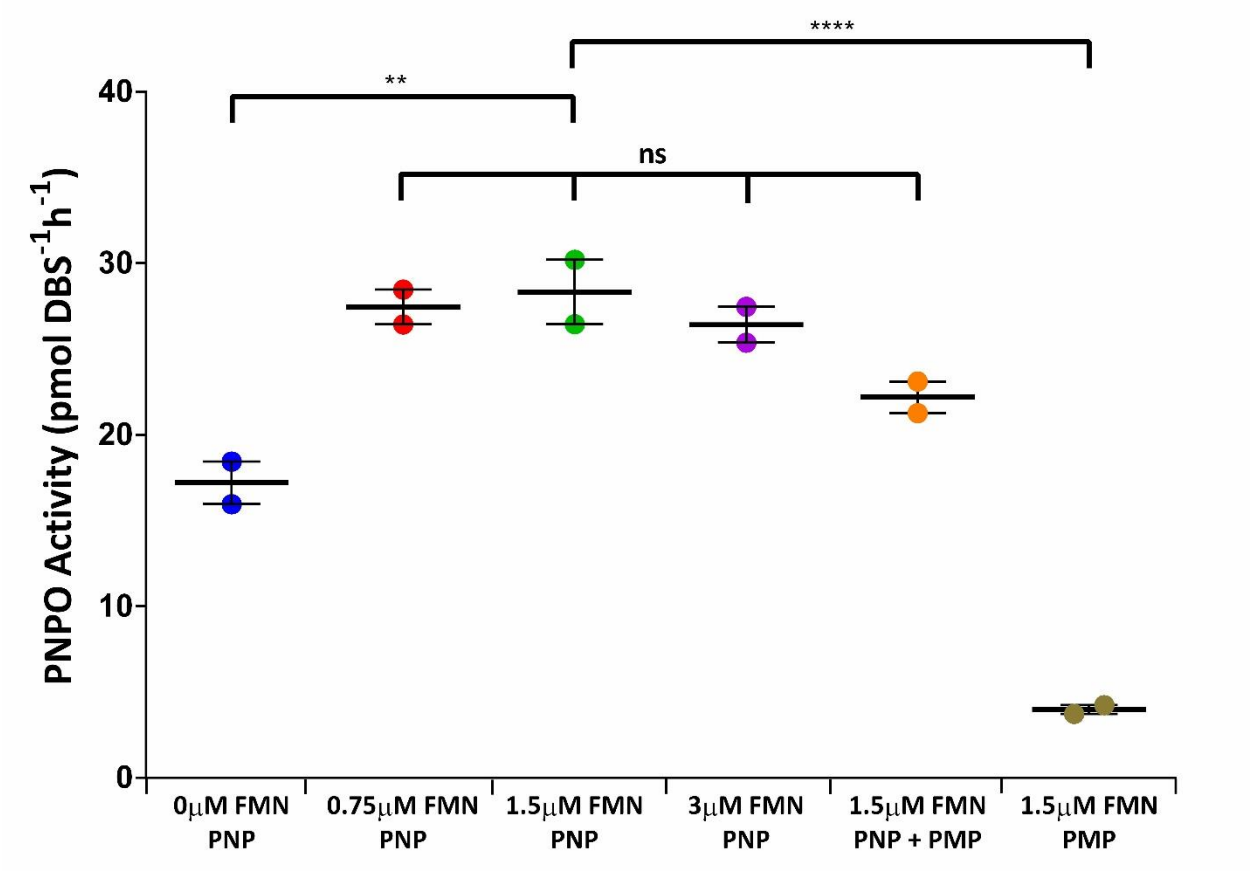

Figure S-5: Effect of FMN concentration and substrate on PLP formation. Incubation conditions as follows: 20 mmol/L TrisPO_4 pH 7.6; 400nmol/L PNP and/or PMP; 37 °C; 300 rpm agitation; 30 min incubation; 1 x 3 mm DBS. Error bars = SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test: ns = not significant; ** = P<0.01; **** = P<0.0001. (n=2).

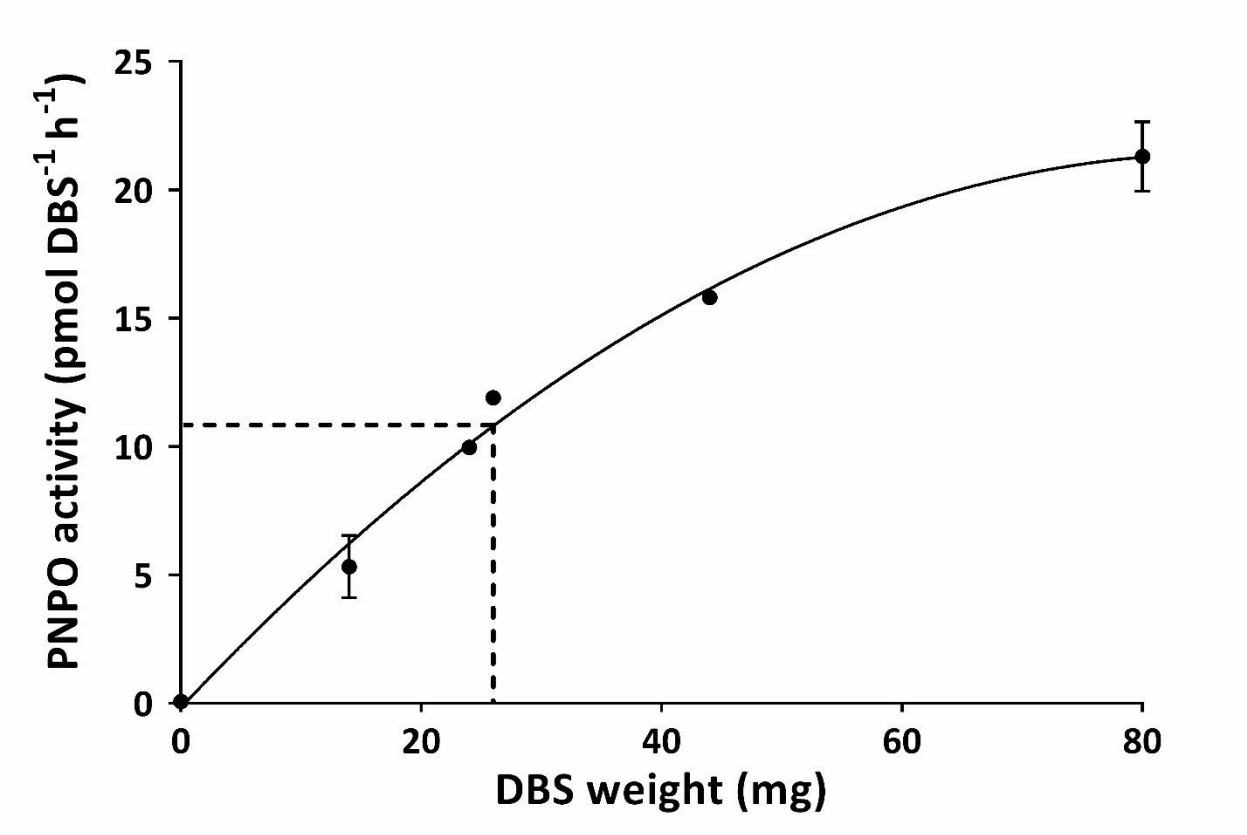

Figure S-6: The effect of DBS weight on PNPO activity. Data point at 0 mg corresponds to a 3mm punch containing no blood. Unbroken line is a second order polynomial best-fit of the data shown. Dashed line corresponds to the approximate weight of a 3mm punch filled with dried blood (26 mg). Incubation conditions: 20 mmol/L $TrisPO_4$ pH 7.6; 1.5 µmol/L FMN; 400 nmol/L PNP; 37 °C; 300 rpm agitation; 30 min incubation. Error bars = SEM (n=2).

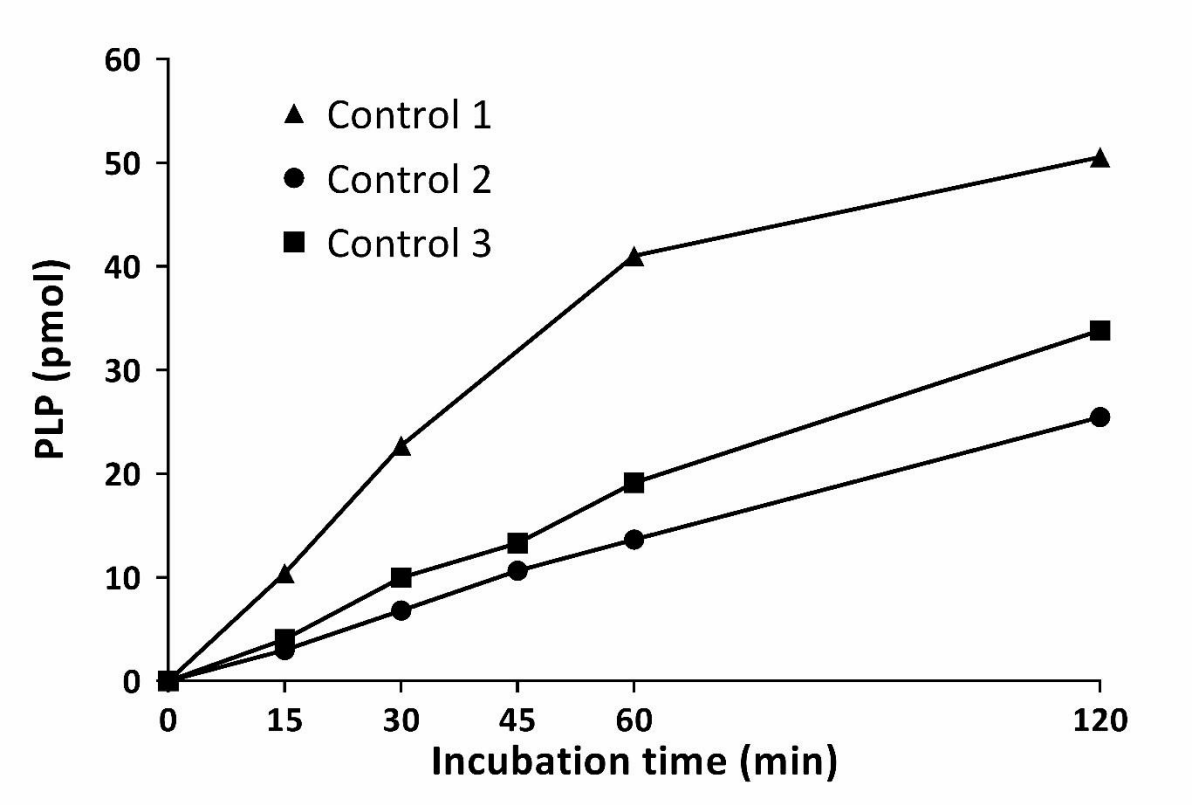

Figure S-7: PNPO activity as a function of PLP formation from 0-120 min. Control 1; Child hospital control (7 m); Control 2; Healthy male adult control (33 y); Control 3; Healthy female adult control (45 y). Incubation conditions: 20 mmol/L $TrisPO_4$ pH 7.6; 1.5 µmol/L FMN; 400 nmol/L PNP; 37 °C; 300 rpm agitation; 1 x 3 mm DBS. Each data point represents n=1.

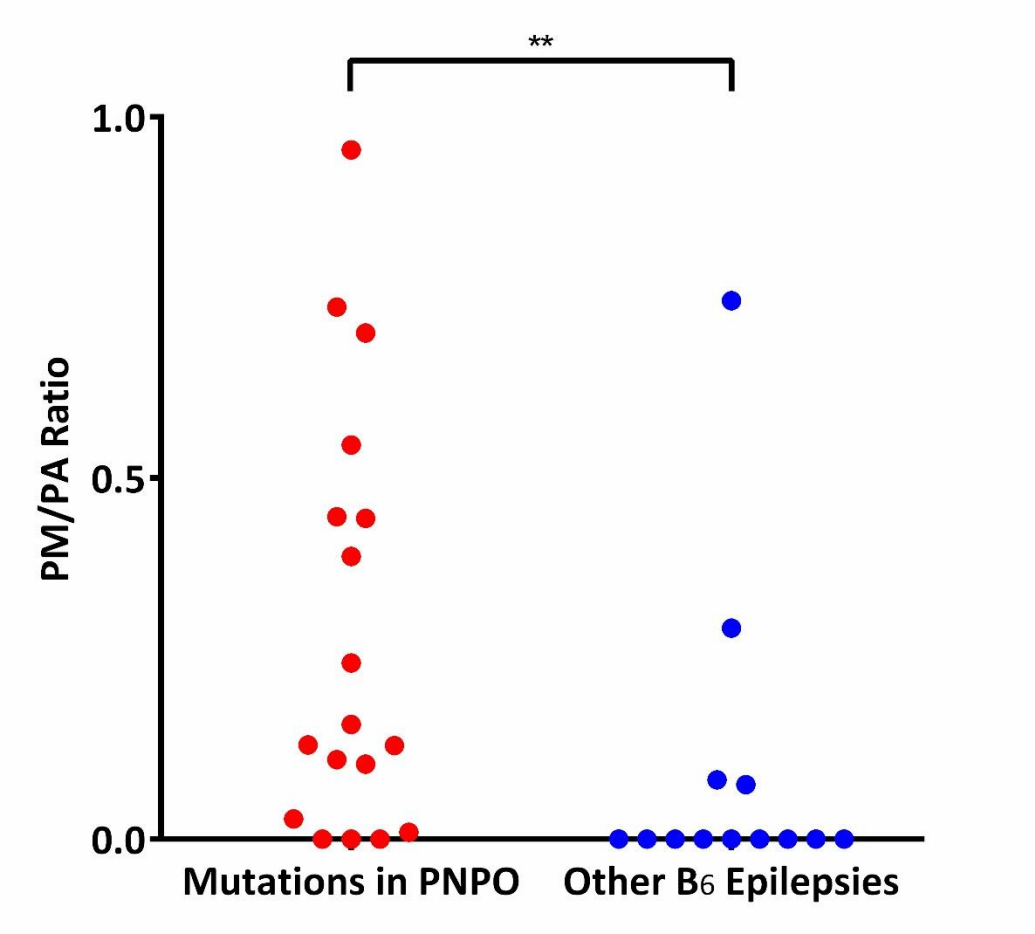

Figure S-8: PM/PA ratios in subjects with mutations in PNPO (red) and other subjects receiving vitamin B_6 supplementation for seizure treatment (blue). Statistical analysis was performed using a Mann-Whitney U test. ** = $P<0.01$.