Supplemental Data:

Supplemental Method S1: Purification of citrulline-d4

The activity of OTC in plasma and serum was determined in the reverse reaction; thus, isotopically labeled citrulline-d4 was used as a substrate. Because the commercially available citrulline-d4 contains traces of ornithine-d4 (approximately 0.1%), which would interfere with the product of the enzyme reaction in the LC-MS/MS analysis, we purified the substance on a column filled with cation exchanger (Dowex 50WX8). The purified solution of the substrate citrulline-d4 contained less than 0.001% ornithine-d4.

The purification of deuterated citrulline-d4 from ornithine-d4 was performed in two steps. In the first step, citrulline-d4 was purified from ornithine-d4 contaminant, and in the second step, the purified citrulline-d4 fraction was transferred into potassium phosphate buffer.

The preparation of the chromatographic column: Ion exchanger (Dowex® 50WX8 hydrogen form, 200-400 mesh, Sigma-Aldrich, Saint Louis, MO, USA) was prepared according to the supplier's instructions (http://sigma-aldrich.custhelp.com/app/answers/detail/a_id/3082/p/19,2684/related/1). An aqueous suspension of activated ion exchanger was poured into a glass chromatography column (Econo-Column® Chromatography Columns, 1.0 × 5 cm, BIO-RAD, Hercules, CA, USA) to form a 3 cm high column. Prior to its use, the column was washed with 20 mL of lithium citrate buffer 1 (see the table showing the composition of lithium citrate buffers below).

First purification step: Citrulline-d4 was dissolved in dilution buffer at a pH of 2.2 to yield the final concentration of 100 mmol/L, and 2 mL of the solution was applied to the column. Subsequently, the column was washed with 8 mL of lithium citrate buffer 2. The elution was performed with 9 mL of lithium citrate buffer 3, and fractions containing approximately 1.5 mL were collected. All of the elution fractions were analyzed using an automatic amino acid analyzer (AAA 4000, Ingos, Czech Republic), and LC-MS/MS was performed using an

EZ:faast kit (see the Materials and Methods section) to determine citrulline-d4 and ornithine-d4 concentrations in the fractions.

Second purification step: The selected fractions without the ornithine-d4 contaminant were pooled together, diluted with water at a ratio of 1:5 and acidified with 5 mol/L HCl to a pH of 2.5. The solution (approximately 30 mL) was applied onto the column filled with Dowex® 50WX8. The column was washed with 30 mL of 50 mmol/L potassium phosphate buffer at a pH of 2.5. The elution was performed with 9 mL of 200 mmol/L potassium phosphate buffer at a pH of 8.0, and 1.5 mL fractions were collected. The citrulline-d4 and ornithine-d4 concentrations in all of the elution fractions were analyzed by LC-MS/MS using EZ:faast kit (see the Materials and Methods section), and selected fractions containing citrulline-d4 were pooled together and diluted with 200 mmol/L potassium phosphate buffer with a pH of 7.4 to yield a final concentration of 25 mmol/L citrulline-d4. The pH of the citrulline-d4 solution was adjusted with KOH to 7.4.

Composition of lithium citrate buffers

	Dilution buffer	**Buffer 1**	**Buffer 2**	**Buffer 3**
	pH 2.2	pH 2.5	pH 3.4	pH 4.2
	0.1 mol/L Li^+	0.18 mol/L Li^+	0.35 mol/L Li^+	0.33 mol/L Li^+
Citric acid	-	1.95 g	2.01 g	1.9 g
Lithium citrate	1.9 g	0.37 g	1.25 g	3.09 g
Lithium chloride	-	1.354 g	2.396 g	1.4 g
Water added to the final volume	200 mL	200 mL	200 mL	200 mL

Supplemental Method S2: Preparation of ornithine-d4 calibrators

The calibration standard solution of ornithine-d4 was prepared as follows. To a mixture of 50 µL of 200 mmol/L citrulline-d4, 5 µL of shrimp alkaline phosphatase (1U/µL; Thermo Fisher Scientific, Waltham, MA, USA), 20 µL of 300 mmol/L phosphate buffer (pH 8), and 5 µL of OTC from *Streptococcus faecalis* (100 U/mL; Sigma-Aldrich, Prague, Czech Republic) were added. After incubation for 6 hours at 37ºC, the mixture was heated for 5 minutes at 95ºC to stop the enzymatic reaction. The mixture was centrifuged for 2 minutes at 5000 g. The concentration of ornithine-d4 in the supernatant was determined using an automatic amino acid analyzer (AAA 4000, Ingos, Czech Republic), and finally, the supernatant (25 mmol/L ornithine-d4) was diluted with 0.01 N HCl to yield concentrations of 5, 1, 0.2 and 0.05 µmol/L.

The calibrators were processed as follows: 25 µL of the calibration standard was mixed with 100 µL of EZ:faast Reagent 1 supplemented with 2 µmol/L of internal standard ornithine-d6, and this solution was further processed according to the EZ:faast kit manual.

Supplemental Method S3: Method validation

pH optimum. We examined the ornithine-d4 production as a function of pH in the reverse reaction. Plasma sample (12.5 µL) was mixed with 5 uL of 1 mol/L phosphate buffers with various pH adjusted in the range 5-9, 0.5 µL of shrimp alkaline phosphatase (SAP, 1U/µL) and 4.5 µL of H_2O in a 0.2 mL PCR tube. The reaction was initiated with 2.5 µL of 100 mmol/L solution of citrulline-d4 in water. The sample was further processed according to the protocol described in the section OTC assay in plasma. The results are showed in Supplemental Figure 1.

Concentration of substrates. We examined the optimal substrate concentrations for the assay by determining the OTC activity using different citrulline-d4 concentrations (0, 0.62, 1.25, 2.5, 5, 7.5, 10, 12.5, 15 and 20 mmol/L) and phosphate (Pi) concentrations (0, 5, 25, 50, 75, 100, 125, 150, 175, 200 and 250 mmol/L) in the assay mixture. The effect of SAP in the assay was tested by assaying OTC activity with 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, 1, 1.2, 1.5, 2 and 2.5 units of SAP in the assay mixture. A control plasma sample was used in these experiments; the measurements were performed in duplicate for each tested concentration.

Assay linearity. To determine the linearity of the assay, we incubated the assay mixture containing control a plasma sample for different time intervals (1, 2, 4 and 6 hours). The assay mixture containing inactivated plasma blank sample was incubated for the same time intervals to evaluate the non-enzymatic production of ornithine-d4 from citrulline-d4. We also examined the relationship between the amount of plasma in the assay (0, 5, 7.5, 10, 12.5, 15 and 17.5 µL) and the amount of enzymatic product after 4 hours of incubation of the reaction mixture. All of the experiments were performed in duplicate, and the mean values of the produced ornithine-d4 were used for evaluation of the linearity.

Matrix effects. To explore the influence of different sample matrices on OTC activity, we performed the assay using samples from one healthy volunteer following blood collection into

EDTA- and heparin-containing tubes and into serum collection tubes. The OTC activity measurements for all of the sample matrices were performed in triplicate.

Enzyme stability in plasma sample. Sample stability was assessed by assaying control plasma separated within 20 minutes after venipuncture and after one, two, four and ten cycles of freezing and thawing. The control plasma aliquot was also repeatedly assayed during storage at -20ºC for a period of 24 weeks and after incubation of the plasma for 2, 4 and 6 hours at room temperature.

Assay recovery and imprecision. The assay recovery was evaluated by spiking the plasma blank sample with 100, 200 and 2000 nmol/L of ornithine-d4, which corresponded to 5, 10 and 100% ornithine-d4 concentrations produced by OTC in control plasma, respectively. The assay mixtures were prepared in triplicate, and the determined ornithine-d4 amounts were compared to the theoretical amounts (i.e., ornithine-d4 spiked into the assay mixture + ornithine-d4 in the assay mixture originating from residual contamination of purified citrulline-d4). We assessed the intra- and inter-assay imprecision by repetitive measurements of the OTC activity in the plasma from one healthy volunteer, one OTCD patient and one subject with an elevated ALT activity. For the intra-assay imprecision, the activity was determined using 5 replicates for each plasma sample. For the inter-assay imprecision, the activity in plasma samples was determined on five different days within a period of 6 weeks.

Supplemental Method S4: X-inactivation

The DNA methylation-based assay exploited polymorphic repeat regions located at the following three loci: *AR* (Xq12), *CNKSR2* (Xp22.12) and *RP2* (Xp11.3). The methods for the individual loci were described by Racchi 1998, Musalkova 2015 and Machado 2014, respectively. The analysis was performed according to published protocols, using digestion with the methylation-sensitive enzyme *HpaII*. In addition to *HpaII*, an auxiliary restrictase *RsaI* was used in examinations of the *AR* and *CNKSR2* loci. An internal check for the completeness of template digestion was performed by a DNA analysis of male subjects. Only female subjects carrying alleles differing by at least two polymorphic repeats were enrolled in the study.

Two highly polymorphic single-nucleotide polymorphisms (SNPs), c.156A>T (rs12097) in *LAMP2* (Xq24) and c.438C>T (rs1141608) in *IDS* (Xq28), were used in the transcription-based assay. If heterozygosity for the SNPs was detected in the gDNA, the cDNA transcript was PCR amplified using the primers described in the table below. The resulting PCR products were subjected to massive parallel sequencing for the accurate determination of transcriptional SNP allele frequencies, representative of the XCI ratios (Mossner 2013).

The massive parallel sequencing was performed under standard protocols using the NexteraXT kit and the MiSeq reagent kit (2x250), respectively. Paired-end sequence reads were generated using the MiSeq platform (Illumina, San Diego, CA, USA). Sequencing data were demultiplexed and trimmed for low quality and duplicates using MiSeq reporter, version 2.4. A secondary analysis of the cDNA data was performed using TopHat software, version 2.0.13 (Kim 2013). The human hg19 genome sequence was used as a reference.

The resulting XCI status was calculated as the mean of the results from all of the informative loci.

If only one locus was informative, the analysis was repeated two to four times. In the case that the results of XCI in one individual differed by more than 10%, the analysis was repeated, and the outlier was excluded.

Primer sequences for amplification of PCR products exploited in the transcription-based assay.

Template	**Gene -Fragment/ Exon**	**Primer sequence**
cDNA	*IDS* - Fr 2 U	TAATACGACTCACTATAGGAGCCGCGTTTCTTTCCTCA
	IDS - Fr 2 L	TGAAACAGCTATGACCATGCCAGGGGTTGTAGGCCACAG
gDNA	*IDS* - Ex 4 U	TAATACGACTCACTATAGCCTTCCACAGAGCCTAGCAC
	IDS - Ex 4 L	TGAAACAGCTATGACCATGCAGCTTCACAGAACATGCAG
cDNA	*LAMP2* - Fr1 U	GGTCGGTGGTCATCAGTGCT
	LAMP2 - Fr1 L	ATTCTGATGGCCAAAAGTTCAT
gDNA	*LAMP2* - Ex 2 U	TAATACGACTCACTATAG TTTAGAGCTGGTTGAACTTC
	LAMP2 - Ex 2 L	TGAAACAGCTATGACCATG TCAAAGGATAAAGTCAATTAAA

Upper (U) and lower (L) specific primers (excepting LAMP2 cDNA) contain T7 and RP universal sequences, respectively, at the 5′ end.

References

Kim D, Pertea G, Trapnell C et al. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol 2013: 14: R36.

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Mossner M, Nolte F, Hutter G et al. Skewed X-inactivation patterns in ageing healthy and myelodysplastic haematopoiesis determined by a pyrosequencing based transcriptional clonality assay. J Med Genet 2013: 50: 108-117.

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Supplemental Table S1: OTC patients.

Number of patient	Present age, years/ months/ weeks	Nucleotide change	Predicted amino acid change	Clinical classification[a]	Ascertainment	First clinical presentation			XCI, %[c]	Activity in individual samples		
						Age of onset , years/ months/ days	Presenting symptoms[b]	Highest ammonia at presentation, µmol/L		OTC, pkat/L	OTC/ALT, x 10^{-6}	OTC/AST, x10^{-6}
OTCD hemizygotes												
P1	deceased (2y 10m)	c.1065A>T	p.*355Cysext*15	neonatal	family screening	first week	encephalopathy, vomiting, hepatopathy	154		10	48	18
P2	24y 1m	c.829C>T	p.Arg277Trp	late	symptomatic	7y 7m	encephalopathy	300		8.2	22	27
										3.9	6.4	7.6
										6.0	10	14
P3	2y 6m	c.929_931delAAG	p.Glu310del	late	symptomatic	14m	encephalopathy	500		23	12	15
										6.0	0.6	3.0
										6.3	2.1	7.5
										5.3	14	10
										5.4	4.8	7.0
										18	16	20
										8.1	8.5	8.9
										0	0	0
P4	deceased (1w)	c.78-2113_ 216+73del2325	del Ex2	neonatal	symptomatic	3d	encephalopathy	2410		72	13	2.9

P5	23y 10m	c.829C>T	p.Arg277Trp	late	symptomatic	9y 1m	encephalopathy	197		0.7	3.9	1.2
										4.6	12	13
										4.2	19	13
P6	23y 10m	c.829C>T	p.Arg277Trp	late	family screening	9y 3m	vomiting	121		9.2	61	17
										6.1	51	19
										8.6	27	23
P7	28y 8m	c.829C>T	p.Arg277Trp	late	symptomatic	3y 9m	severe hepatopathy	elevated		4.0	11	9.2
										4.4	8.4	11
P8	6y 8m	c.929_931delAAG	p.Glu310del	late	symptomatic	8m	encephalopathy	396		2.8	40	11
										5.6	69	17
P9	23y 11m	c.829C>T	p.Arg277Trp	late	family screening	8y 7m	encephalopathy	298		1.9	4.2	6.1
P10	24y 9m	c.829C>T	p.Arg277Trp	late	family screening	12y	vomiting	elevated		4.2	10	13
										3.6	10	12
P11	38y 5m	c.829C>T	p.Arg277Trp	late	family screening	18y	vomiting	n.d		4.2	7.6	12
										0.4	0.9	1.0
Symptomatic OTCD heterozygotes												
P12	17y 9m	c.491C>A	p.Ser164*	late	family screening	3y 9m	vomiting	204	53	96	174	281
										83	208	244
										121	183	257
										46	202	202
										50	208	238
P13	16y 2m	c.628A>C	p.Lys210Gln	neonatal	symptomatic	3d	encephalopathy	1000	13	53	232	137
										74	323	165
										63	453	148
										31	148	84
P14	19y	g.57467_ 82002delinsCCT	del Ex9+10	late	symptomatic	3y 4m	encephalopathy	292	54	77	197	226
										78	201	178
										66	179	201

P15	22y 8m	c.386G>A	p.Arg129His	late	family screening	1y 8m	vomiting	65	9	33	252	59
										65	147	151
										31	148	86
P16	7y 6m	c.996G>T	p.Trp332Cys	late	symptomatic	6y 2 m	encephalopathy	249		86	278	137
										267	290	381
										82	146	122
										356	310	451
										157	103	173
										692	231	323
P17	22y 3m	c.274C>T	p.Arg92*	late	family screening	7y	encephalopathy	57	16	112	207	106
										571	794	664
P18	19y 9m	c.717+1G>T		late	symptomatic	6y	encefalopathy	360	35	85	316	276
P19	22y 3m	c.628A>G	p.Lys210Glu	late	symptomatic	4m	vomiting, hepatopathy	294	47	98	491	363
P20	17y 9m	c.1065A>T	p.*355Cysext*15	late	family screening	14y 9m	encephalopathy, vomiting	elevated	33	915	654	654
P21	9y 11m	c.1065A>T	p.*355Cysext*15	late	symptomatic	4y 6m	encefalopathy, hepatopathy	300	30	57	246	132
P22	6y 8m	c.1065A>T	p.*355Cysext*15	late	family screening	2y 3m	encephalopathy, vomiting	elevated	66	92	486	243
P23	5y 7m	c.663+1G>A		late	symptomatic	4y 5m	hepatopathy, vomoting, encephalopathy	303		294	132	498
										1015	368	479
										5709	229	484
										663	82	721
										141	38	256
Asymptomatic OTCD heterozygotes												
P24	32y 6m	c.78-2113_ 216+73del2325	del Ex2		family screening		asymptomatic		68	143	1101	211
P25	32y 9m	c.929_931delAAG	p.Glu310del		family screening		asymptomatic		92	118	909	408

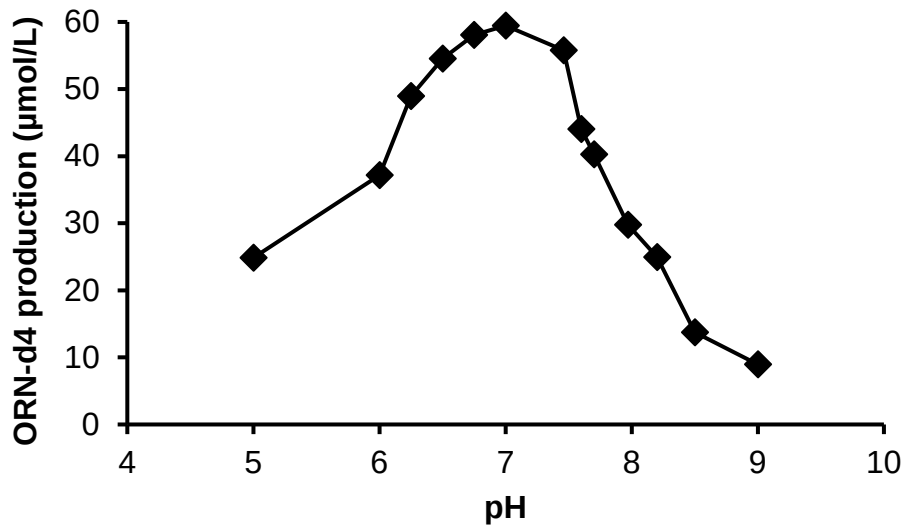
P26	47y 9m	c.491C>A	p.Ser164*		family screening		asymptomatic		29	181	1004	420
P27	28y 10m	c.929_931delAAG	p.Glu310del		family screening		asymptomatic		22	222	966	673
P28	62y	c.386G>A	p.Arg129His		family screening		asymptomatic		78	83	435	88
P29	40y 10m	c.304G>C	p.Ala102Pro		family screening		asymptomatic		89	91	253	115
P30	46y 7m	c.1065A>T	p.*355Cysext*15		family screening		asymptomatic		76	329	1267	515
P31	25y 5m	c.1065A>T	p.*355Cysext*15		family screening		asymptomatic		56	143	683	135
P32	51y 7m	c.829C>T	p.Arg277Trp		family screening		asymptomatic			219	950	437

[a] clinical classification; neonatal, onset before day 28; late, onset after day 28

[b] encephalopathy = altered consciousness and/or presence of seizures

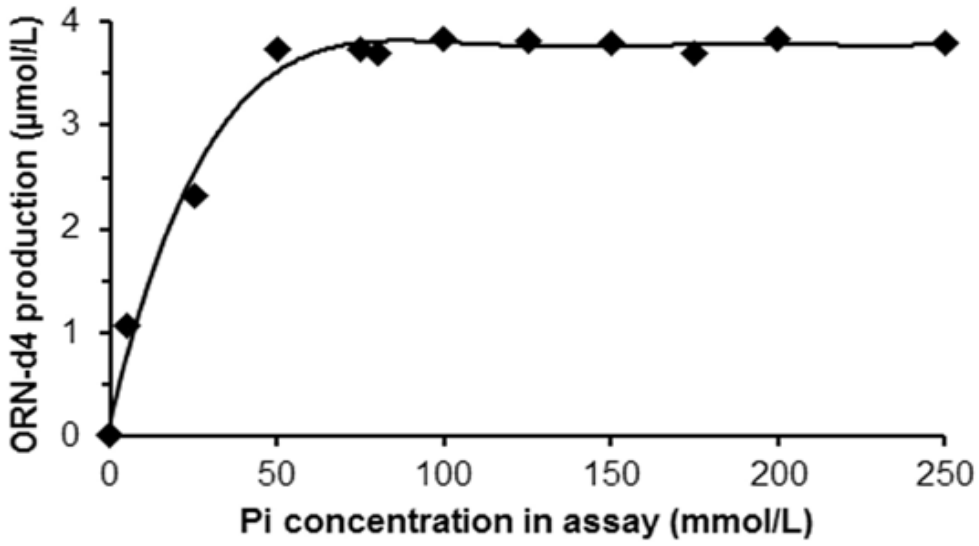
[c] the degree of the wild-type chromosome X expression

Supplemental Figure S1: pH optimum of assay



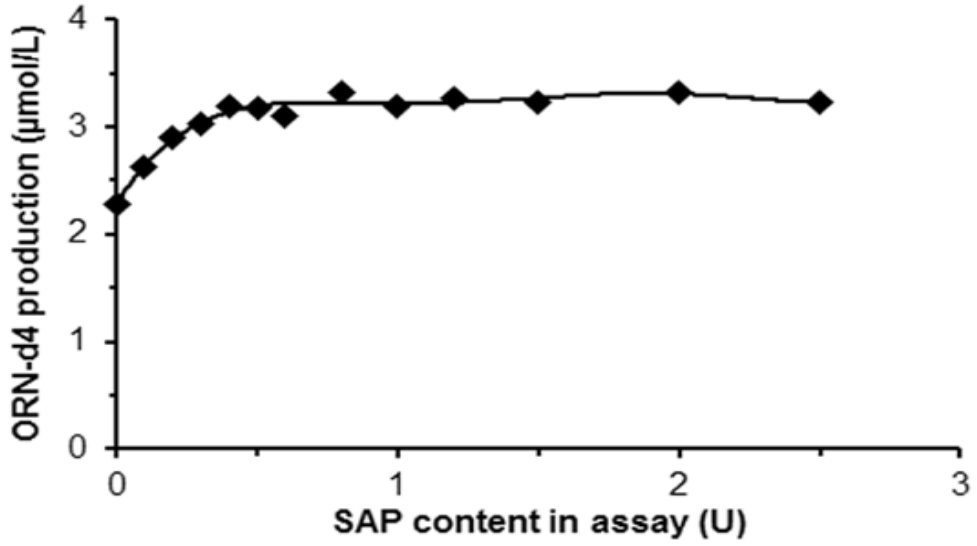
Supplemental Figure S1: pH optimum of assay. Effect of pH on the ornithine-d4 production in the OTC reverse reaction.

Supplemental Figure S2: Optimization of Pi concentration in assay.



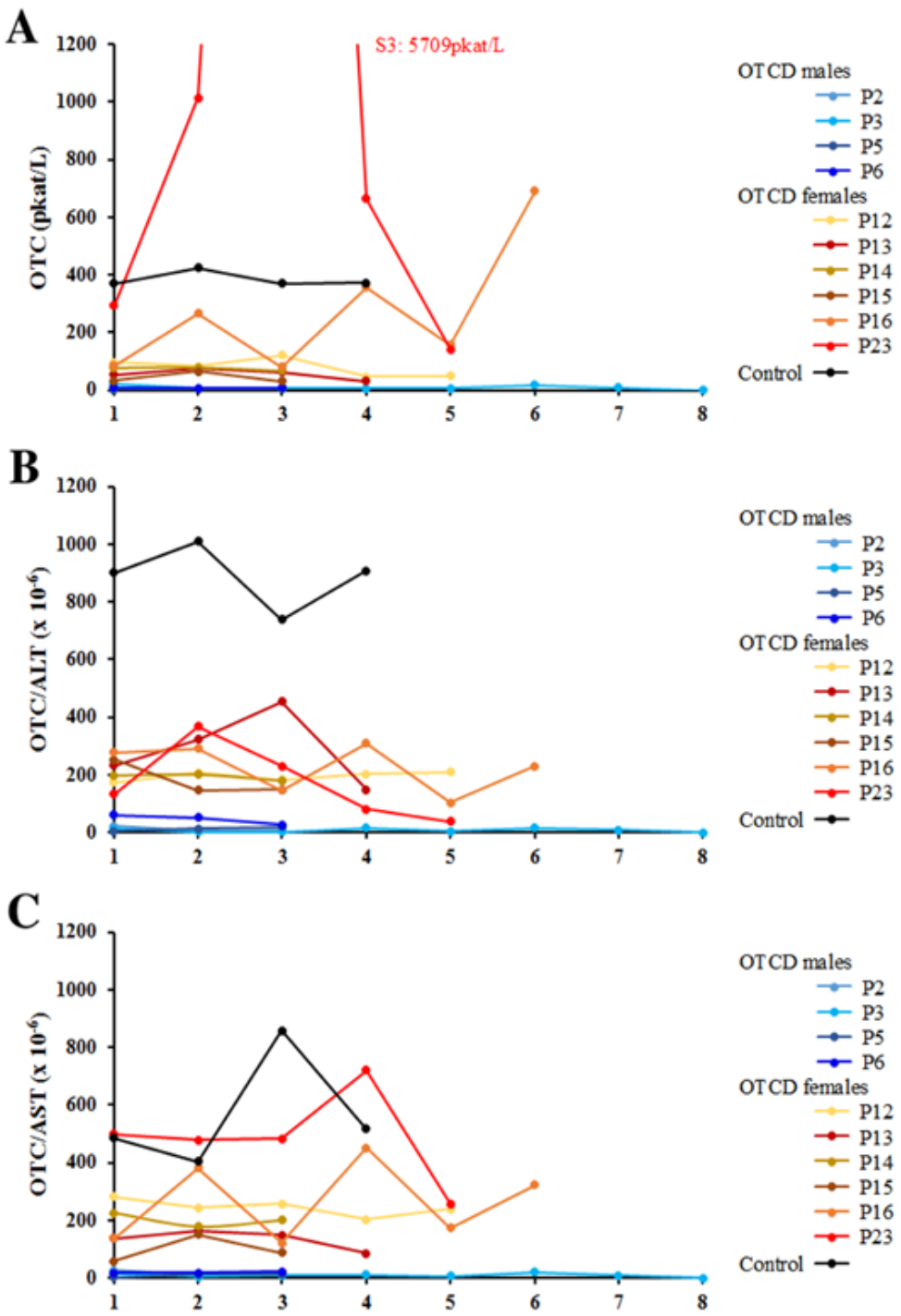
Supplemental Figure S2: Optimization of Pi concentration in assay. Signal of ornithine-d4 (ORN-d4) in the assay mixture in response to the phosphate (Pi) concentration in assay.

Supplemental Figure S3: Optimization of SAP content in assay.



Supplemental Figure S3: Optimization of SAP content in assay. Signal of ornithine-d4 in the assay mixture in response to content of alkaline phosphatase (SAP) in assay.

Supplemental Figure S4: Intraindividual variability



Supplemental Figure S4: Intraindividual variability of plasma or serum OTC activities. The graphs show serial OTC activities (**A**), OTC/ALT ratio (**B**) and OTC/AST ratio (**C**) in one control and in 10 OTCD deficient individuals in whom specimens were collected on 3 or more different days.