



OPEN Pseudogenization of the *Slc23a4* gene is necessary for the survival of *Xdh*-deficient mice

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In most patients with type 1 xanthinuria caused by mutations in the xanthine dehydrogenase gene (*XDH*), no clinical complications, except for urinary stones, are observed. In contrast, all *Xdh*($-/-$) mice die due to renal failure before reaching adulthood at 8 weeks of age. Hypoxanthine or xanthine levels become excessive and thus toxic in *Xdh*($-/-$) mice because enhancing the activity of hypoxanthine phosphoribosyl transferase (HPRT), which is an enzyme that uses hypoxanthine as a substrate, slightly increases the life span of these mice. In this study, we targeted the mouse intestinal sodium-dependent nucleobase transporter (SNBT) gene (*Slc23a4*), which is a pseudogene in humans. *Hprt*(high)*Xdh*($-/-$)*Slc23a4*($-/-$) mice had a longer life span and reached adulthood. The urinary xanthine excretion of these mice was 20-fold greater than that of patients with type 1 xanthinuria. The urinary hypoxanthine/xanthine ratio of *Hprt*(high)*Xdh*($-/-$)*Slc23a4*($-/-$) mice was lower than that of patients with type 1 xanthinuria. *Hprt*(high)*Xdh*($-/-$)*Slc23a4*($-/-$) mice exhibited renal impairment, accompanied by high plasma creatinine levels and anemia. Moreover, female *Hprt*(high)*Xdh*($-/-$)*Slc23a4*($-/-$) mice produced offspring that did not survive. In conclusion, for the first time, we established that *Xdh*($-/-$) mice survive to adulthood.

Keywords Xanthine dehydrogenase, Sodium dependent nucleobase transporter, Hypoxanthine, Xanthine, Type 1 xanthinuria

The final step of purine metabolism in humans, namely, the breakdown of hypoxanthine to xanthine and xanthine to uric acid, is carried out intracellularly by xanthine dehydrogenase (XDH); this is a dehydrogenation reaction that uses a coenzyme NAD^+ . In type 1 xanthinuria, which is caused by a loss of XDH activity due to mutations in the xanthine dehydrogenase (*XDH*) gene, uric acid production in the body is lost, and the oxypurines xanthine and hypoxanthine are excreted as purine end products, resulting in the clinical symptom of urinary stones. However, similar to patients with renal hypouricemia, no clinical complications are observed in many patients with xanthinuria¹. On the other hand, it has been reported that all *Xdh*($-/-$) mice, which serve as a model of type 1 xanthinuria, die before reaching adulthood at 8 weeks of age^{2–4}. Therefore, it has not been possible to use adult *Xdh*($-/-$) mice to study the biological function of XDH by comparing mice with or without XDH expression.

Even among the most closely related mammals, namely, humans and mice, there are large species-specific differences in purine metabolism. In mammals other than apes, uric acid is further degraded, and allantoin is excreted as the final product of purine metabolism. However, apes, including humans, lack uricase (*Uox*), which converts uric acid to allantoin, so uric acid is excreted as the final product of purine metabolism⁵. *Uox*($-/-$) mice, which are considered a model of normal human physiology, die within 2 weeks after birth⁶, similar to *Xdh*($-/-$) mice, which die soon after birth. On the other hand, patients deficient in hypoxanthine phosphoribosyltransferase (HPRT), which converts hypoxanthine into inosine monophosphate (IMP) and reuses it as purine salvage pathway, develop Lesch-Nyhan disease and exhibit neurological symptoms, including recurrent self-injurious behavior⁷; however, *Hprt*($-/-$) mice are completely asymptomatic⁸. Thus, it has been impossible to use mice to study human purine metabolism because of the large differences between species.

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We hypothesized that the reason why *Xdh*($-/-$) mice die soon after birth is that the levels of hypoxanthine or xanthine, which should be degraded by XDH, become excessive and toxic. Since HPRT has low activity in laboratory mice, we generated *Hprt*(high) mice by introducing a high-activity *Hprt* allele derived from field mice⁹, and then, we generated *Hprt*(high)*Xdh*($-/-$) mice. Individual mice that survived until 24 weeks of age were obtained. This survival indicated that a reduction in intracellular hypoxanthine levels is necessary for increasing the lifespan of *Xdh*($-/-$) mice¹⁰.

There are species-specific differences between humans and mice not only in purine metabolism but also in purine kinetics. Because the Na^+ -dependent intestinal purine base transporter SNBT1 (*Slc23a4*), which is a pseudogene in humans, is expressed in mice¹¹, the intestinal absorption of hypoxanthine and xanthine may be greater in mice than in humans, and the abundance of these substances in the body may become excessive. In this study, we targeted *Slc23a4* with the aim of reducing hypoxanthine and xanthine absorption in the intestinal tract and extending the lifespan of *Hprt*(high)*Xdh*($-/-$) mice. The *Hprt*(high)*Xdh*($-/-$)*Slc23a4*($-/-$) mice that were generated in this study exhibited successfully extended lifespans compared to *Hprt*(low)*Xdh*($-/-$)*Slc23a4*($+/+$) mice.

Results

Survival of *Hprt*(high)*Xdh*($-/-$)*Slc23a4*($-/-$) mice

The median survival time of the 108 *Hprt*(high)*Xdh*($-/-$)*Slc23a4*($-/-$) mice was 191.8 days. However, the median survival times of the 6 *Hprt*(high)*Xdh*($-/-$)*Slc23a4*($+/+$) and 7 *Hprt*(high)*Xdh*($-/-$)*Slc23a4*($+/+$) mice were 64.05 and 83.3 days, respectively (Fig. 1A). There was no significant difference in median survival between *Hprt*(high)*Xdh*($-/-$)*Slc23a4*($+/+$) mice and *Hprt*(high)*Xdh*($-/-$)*Slc23a4*($+/+$) mice. On the other hand, the median survival of *Hprt*(high)*Xdh*($-/-$)*Slc23a4*($-/-$) mice was significantly longer than that of both *Hprt*(high)*Xdh*($-/-$)*Slc23a4*($+/+$) mice and *Hprt*(high)*Xdh*($-/-$)*Slc23a4*($+/+$) mice.

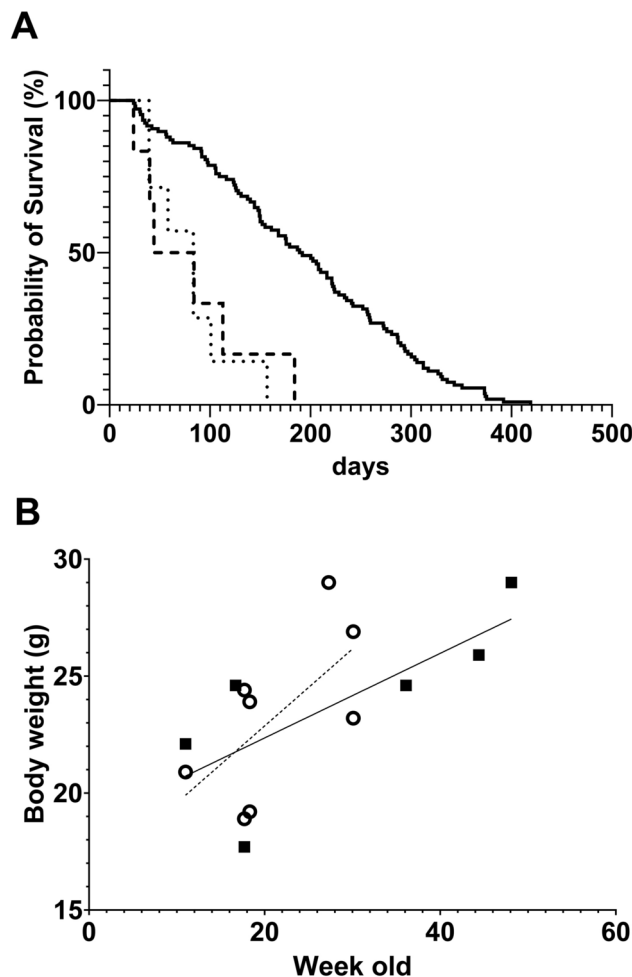


Fig. 1. (A) Kaplan–Meier curves depicting the survival of *Hprt*(high)*Xdh*($-/-$)*Slc23a4* mice. *Hprt*(high)*Xdh*($-/-$)*Slc23a4*($-/-$) mice (n = 108, solid line); *Hprt*(high)*Xdh*($-/-$)*Slc23a4*($+/+$) mice (n = 6, dashed line); *Hprt*(high)*Xdh*($-/-$)*Slc23a4*($+/+$) mice (n = 7, dotted line). (B) Body weights of *Hprt*(high)*Xdh*($-/-$)*Slc23a4*($-/-$) male mice (n = 6, black squares) compared with *Hprt*(high)*Xdh*($-/-$)*Slc23a4*($-/-$) female mice (n = 8, white circles).

Body weight increased with age in the six male and nine female *Hprt*(high)*Xdh*($-/-$)/*Slc23a4*($-/-$) mice that were used for blood and urine collection (Fig. 1B). The mating of male and female *Hprt*(high)*Xdh*($-/-$)/*Slc23a4*($-/-$) mice produced offspring several times. However, all the offspring died within a few days after birth. In contrast, the mating of male *Hprt*(high)*Xdh*($-/-$)/*Slc23a4*($-/-$) mice with female *Hprt*(high)*Xdh*($+/-$)/*Slc23a4*($-/-$) mice produced offspring that grew without problems. These results suggested that female *Xdh*($-/-$) mice could not raise offspring, which may have been caused by insufficient lactation.

Urinary oxypurine excretion in *Hprt*(high)*Xdh*($-/-$)/*Slc23a4*($-/-$) mice and the effect of a low-purine diet

The urinary hypoxanthine/creatinine ratio of *Hprt*(high)*Xdh*($-/-$)/*Slc23a4*($-/-$) mice that were fed a normal diet was 0.4335 ± 0.0864 (mol/mol Cr), whereas the ratio was significantly reduced to 0.0615 ± 0.0346 (mol/mol Cr) in the mice that were fed a low-purine purified diet (Fig. 2A). The urinary hypoxanthine/creatinine ratio of *Hprt*(high)*Xdh*($+/-$)/*Slc23a4*($-/-$) mice that were fed a normal diet was 0.0488 ± 0.0088 (mol/mol Cr), whereas it was 0.0290 ± 0.0041 (mol/mol Cr) in the mice that were fed a low-purine purified diet, indicating that there were no significant differences between the effects of the two diets. Compared with *Hprt*(high)*Xdh*($-/-$)/*Slc23a4*($-/-$) mice, the ratio was significantly reduced by the normal diet, but no significant difference was observed in mice that were fed the low-purine purified diet (Fig. 2A).

The urinary xanthine/creatinine ratio of *Hprt*(high)*Xdh*($-/-$)/*Slc23a4*($-/-$) mice that were fed a normal diet was 3.761 ± 0.299 (mol/mol Cr), whereas the ratio was significantly reduced by 40.4% to 1.520 ± 0.260 (mol/mol Cr) in mice that were fed the low-purine purified diet (Fig. 2B). The urinary xanthine/creatinine ratio of *Hprt*(high)*Xdh*($+/-$)/*Slc23a4*($-/-$) mice that were fed a normal diet was 0.015 ± 0.008 , whereas the ratio was 0.017 ± 0.007 in *Hprt*(high)*Xdh*($+/-$)/*Slc23a4*($-/-$) mice that were fed a low-purine purified diet, indicating that there were no significant differences between the effects of the two diets. But the urinary xanthine/creatinine ratio of *Hprt*(high)*Xdh*($+/-$)/*Slc23a4*($-/-$) mice was significantly lower than that in *Hprt*(high)*Xdh*($-/-$)/*Slc23a4*($-/-$) mice when the mice were fed both normal and low-purine diets (Fig. 2B). In addition, the urinary hypoxanthine/xanthine ratio of *Hprt*(high)*Xdh*($-/-$)/*Slc23a4*($-/-$) mice that were fed a normal diet was 0.115 (mol/mol Cr), whereas it was 0.040 (mol/mol Cr) in *Hprt*(high)*Xdh*($-/-$)/*Slc23a4*($-/-$) mice fed a low-purine purified diet (Fig. 2A, B).

The ratio of total urinary purine metabolites, including allantoin and uric acid, to creatinine in *Hprt*(high)*Xdh*($-/-$)/*Slc23a4*($-/-$) mice was 4.248 ± 0.3686 (mol/mol Cr) when the mice were fed a normal diet and 1.626 ± 0.2574 (mol/mol Cr) when the mice were fed a low-purine diet, which was a significant decrease of 61.7% (Fig. 2C). The ratio of *Hprt*(high)*Xdh*($+/-$)/*Slc23a4*($-/-$) mice was 6.478 ± 0.5065 (mol/mol Cr) when the mice were fed a normal diet and 3.977 ± 0.2700 (mol/mol Cr) when the mice were fed a low-purine diet, which was a significant decrease of 61.4%. Compared with that in *Hprt*(high)*Xdh*($+/-$)/*Slc23a4*($-/-$) mice, the total urinary purine/creatinine ratio in *Hprt*(high)*Xdh*($-/-$)/*Slc23a4*($-/-$) mice was significantly lower when the mice were fed both normal and low-purine diets (Fig. 2C).

Plasma oxypurine concentrations in *Hprt*(high)*Xdh*($-/-$)/*Slc23a4*($-/-$) mice

The plasma hypoxanthine concentration in *Hprt*(high)*Xdh*($-/-$)/*Slc23a4*($-/-$) mice was 0.4764 ± 0.0569 (mg/dL) when the mice were fed a normal diet and 0.3286 ± 0.0549 (mg/dL) when the mice were fed a low-purine purified diet, which was not significantly different (Fig. 3A). On the other hand, the plasma xanthine concentration was 1.855 ± 0.1595 (mg/dL) in the normal diet group and was significantly reduced to 0.9942 ± 0.07633 (mg/dL) in the low-purine purified diet group (Fig. 3B). Neither the plasma hypoxanthine concentration nor the plasma xanthine concentration in *Hprt*(high)*Xdh*($+/-$)/*Slc23a4*($-/-$) mice was detectable (data not shown).

The hypoxanthine clearance (CL_{HX})/creatinine clearance (CL_{Cr}) ratio of *Hprt*(high)*Xdh*($-/-$)/*Slc23a4*($-/-$) mice that were fed a normal diet was 0.4595 ± 0.0798 . Since it was less than 1.0, it was concluded that hypoxanthine was reabsorbed in the kidney (Fig. 3C). When the mice were fed a low-purine purified diet, this ratio significantly decreased to 0.0609 ± 0.0259 , and 94% of the hypoxanthine in the glomerular filtrate was reabsorbed in the kidney (Fig. 3C). On the other hand, the xanthine clearance (CL_{XA})/ CL_{Cr} ratio of *Hprt*(high)*Xdh*($-/-$)/*Slc23a4*($-/-$) mice that were fed a normal diet was 1.163 ± 0.090 , suggesting that xanthine may be slightly secreted by the kidney (Fig. 3D). When the mice were fed the low-purine purified diet, this ratio was significantly reduced to 0.7912 ± 0.0599 , and approximately 20% of the xanthine in the glomerular filtrate was reabsorbed in the kidney because of the decrease in purine intake (Fig. 3D).

Renal dysfunction in *Hprt*(high)*Xdh*($-/-$)/*Slc23a4*($-/-$) mice

The plasma creatinine concentration in *Hprt*(high)*Xdh*($-/-$)/*Slc23a4*($-/-$) mice was 0.4395 ± 0.0440 (mg/dL) when the mice were fed a normal diet and 0.4619 ± 0.0707 (mg/dL) when the mice were fed a low-purine diet, which were not significantly different (Fig. 4A). The plasma creatinine concentrations of *Hprt*(high)*Xdh*($+/-$)/*Slc23a4*($-/-$) mice were 0.06875 ± 0.01848 (mg/dL) when the mice were fed a normal diet and 0.05775 ± 0.008577 (mg/dL) when the mice were fed a low-purine diet, which were not significantly different (Fig. 4A). Therefore, the plasma creatinine concentrations of *Hprt*(high)*Xdh*($-/-$)/*Slc23a4*($-/-$) mice were significantly increased by 6.39-fold when the mice were fed a normal diet and by 8.00-fold when the mice were fed a low-purine purified diet compared with those of *Hprt*(high)*Xdh*($+/-$)/*Slc23a4*($-/-$) mice. Therefore, renal dysfunction was observed in *Hprt*(high)*Xdh*($-/-$)/*Slc23a4*($-/-$) mice.

The hematocrit value of *Hprt*(high)*Xdh*($-/-$)/*Slc23a4*($-/-$) mice that were fed a normal diet was $35.00 \pm 1.16\%$, whereas that of *Hprt*(high)*Xdh*($-/-$)/*Slc23a4*($-/-$) mice that were fed a low-purine purified diet was $34.30 \pm 1.52\%$, and no significant difference between the groups was observed (Fig. 4B). Similarly, the hematocrit values of *Hprt*(high)*Xdh*($+/-$)/*Slc23a4*($-/-$) mice that were fed normal diet were $59.50 \pm 0.96\%$ and $56.00 \pm 1.87\%$, respectively, with no significant difference between the groups (Fig. 4B).

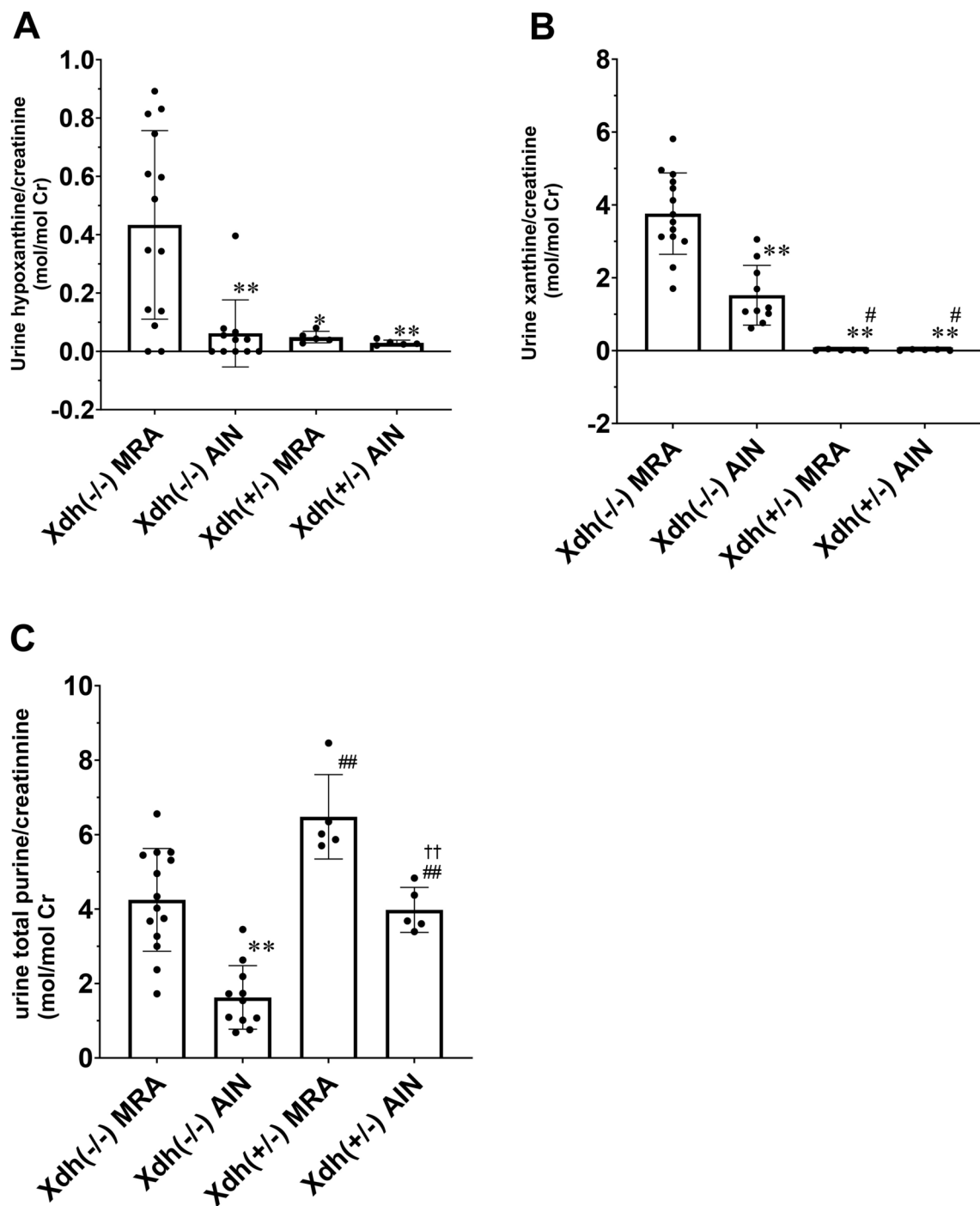


Fig. 2. Profiles of purine metabolism in *Hprt*(high)*Xdh*(-/-)*Slc23a4*(-/-) and *Hprt*(high)*Xdh*(+/-)*Slc23a4*(-/-) mice that were fed a normal diet or a low-purine purified diet. (A) urinary hypoxanthine/creatinine ratio, (B) urinary xanthine/creatinine ratio, (C) total urinary purine/creatinine ratio. The data represent the means \pm SDs. * $p < 0.05$, ** $p < 0.01$ vs. the *Xdh*(-/-) MRA group; # $p < 0.05$, ## $p < 0.01$ vs. the *Xdh*(-/-) AIN group; †† $p < 0.01$ vs. the *Xdh*(+/-) MRA group. P values were calculated via one-way ANOVA. Normal diet; MRA. Low-purine purified diet; AIN.

Therefore, the hematocrit values of *Hprt*(high)*Xdh*(-/-)*Slc23a4*(-/-) mice were significantly lower (0.588-fold when the mice were fed a normal diet and 0.613-fold when the mice were fed a low-purine purified diet) than those of *Hprt*(high)*Xdh*(+/-)*Slc23a4*(-/-) mice. Thus, the high creatinine level and anemia of *Hprt*(high)*Xdh*(-/-)*Slc23a4*(-/-) mice were not improved by the short-term attenuation of the renal oxypurine levels due to consumption of a low-purine diet.

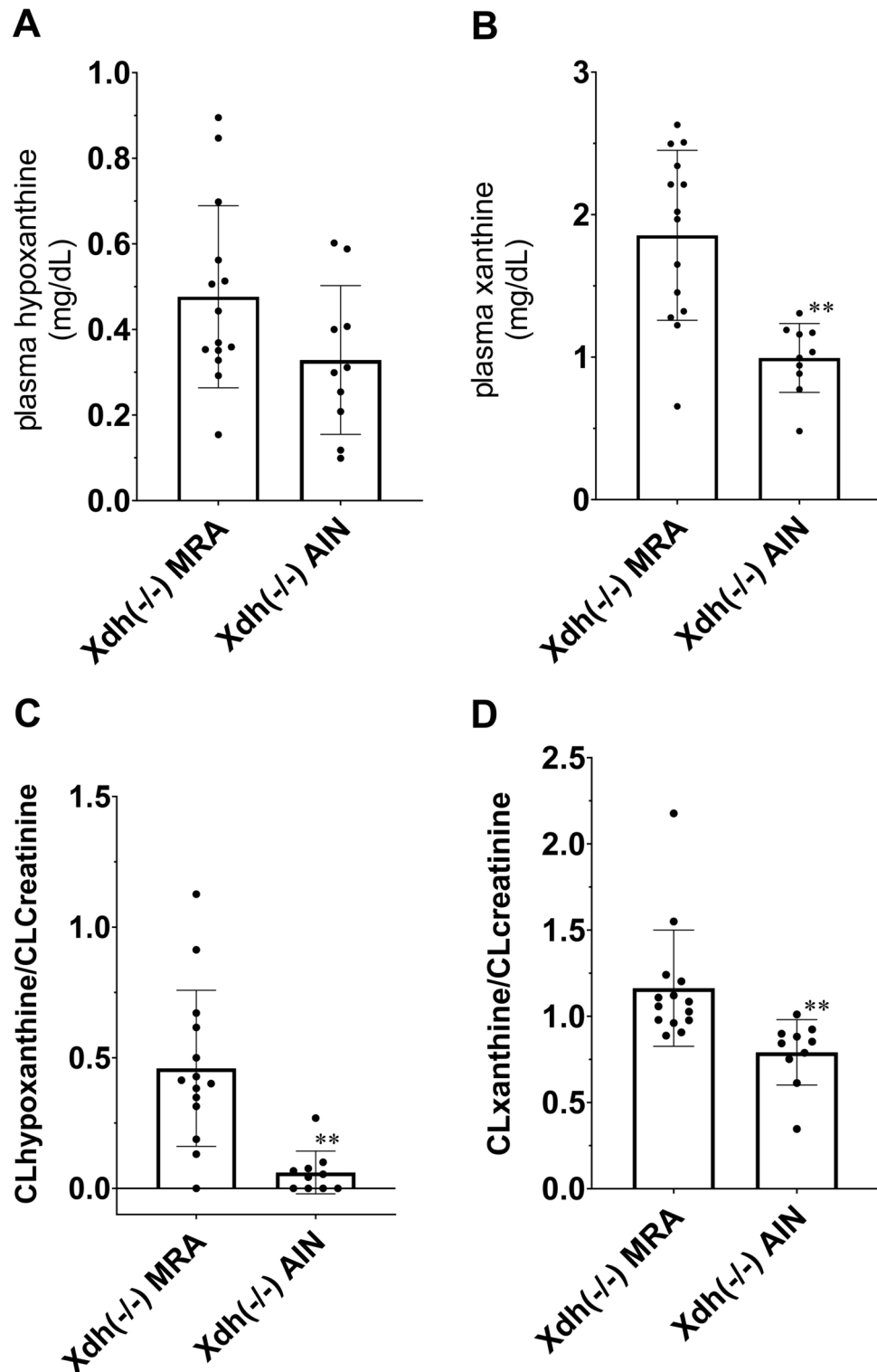


Fig. 3. Profiles of purine metabolism in *Hprt*(high)*Xdh*(-/-)*Slc23a4*(-/-) mice that were fed a normal diet or a low-purine purified diet. (A) plasma hypoxanthine concentration, (B) plasma xanthine concentration, (C) hypoxanthine clearance (CL_{HX})/creatinine clearance (CL_{Cr}) ratio, (D) xanthine clearance (CL_{XA})/ CL_{Cr} ratio. The data represent the means \pm SDs. ** $p < 0.01$ vs. the *Xdh*(-/-) MRA group. P values were calculated via Student's t test. Normal diet; MRA. Low-purine purified diet; AIN.

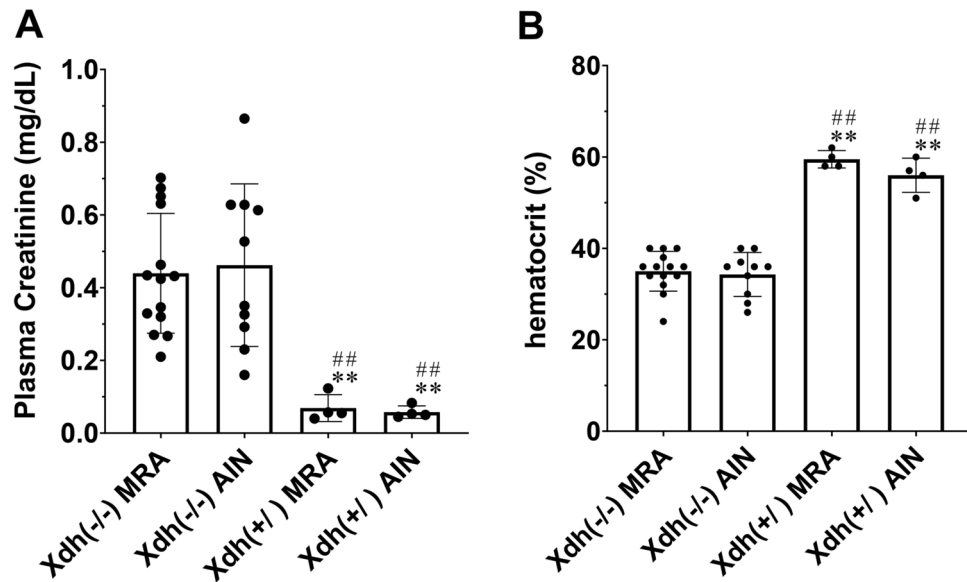


Fig. 4. Profiles of blood from *Hprt*(high)*Xdh*(-/-)*Slc23a4*(-/-) and *Hprt*(high)*Xdh*(+/-)*Slc23a4*(-/-) mice that were fed a normal diet or a low-purine purified diet. (A) plasma creatinine concentration. (B) hematocrit values. The data represent the means \pm SDs. * $p < 0.05$, ** $p < 0.01$ vs. the *Xdh*(-/-) MRA group; ## $p < 0.01$ vs. the *Xdh*(-/-) AIN group. P values were calculated via one-way ANOVA. Normal diet; MRA. Low-purine purified diet; AIN.

Discussion

Xanthinuria and *Xdh*(-/-) mice

All type 1 xanthinuria model mice (*Xdh*(-/-) mice) die by 8 weeks of age (56 days after birth)^{2–4}. It has also been reported that type 2 xanthinuria model mice die by 8 weeks of age (average life span of 28.4 days)¹². We reported that *Hprt*(high)*Xdh*(-/-) mice have an extended life span of up to 24 weeks of age, and we suggested that increased intracellular hypoxanthine levels may contribute to the death of *Xdh*(-/-) mice¹⁰. In this study, the median survival of *Hprt*(high)*Xdh*(-/-)*Slc23a4*(+/+) mice was 83.3 days, which is longer than that of *Hprt*(low)*Xdh*(-/-)*Slc23a4*(+/+) mice, which are reported to die within 56 days of birth. Therefore, *Xdh*(-/-) mice, which express high levels of *Hprt* similar to humans and field mice, are considered have longer lifespans than mice with low HPRT activity, such as laboratory mice. The transporter gene *Slc23a4*, which absorbs hypoxanthine and xanthine from the intestinal tract, is pseudogenized in humans. Therefore, we targeted *Slc23a4* to account for species-specific differences between mice and humans. Although the median survival of *Hprt*(high)*Xdh*(-/-)*Slc23a4*(+/-) mice was not longer than that of *Hprt*(high)*Xdh*(-/-)*Slc23a4*(+/+) mice, the median survival of *Hprt*(high)*Xdh*(-/-)*Slc23a4*(-/-) mice was significantly longer (191.8 days), making it possible, for the first time, to use adult *Xdh*(-/-) mice for breeding and research purposes.

A previous report on *Hprt*(low)*Xdh*(-/-)*Slc23a4*(+/+) mice, whose kidney have exhibited interstitial fibrosis through aberrant lipid and purine accumulation in renal tubules, revealed that the plasma hypoxanthine concentration was approximately 20 μ g/mL (2 mg/dL) and that the plasma xanthine concentration was approximately 30 μ g/mL (3 mg/dL) at 4 weeks of age¹³. In *Hprt*(high)*Xdh*(-/-)*Slc23a4*(-/-) mice, the plasma hypoxanthine concentration was approximately 0.5 mg/dL, which is a quarter of that in *Hprt*(low)*Xdh*(-/-)*Slc23a4*(+/+) mice. The plasma xanthine concentration was approximately 1.9 mg/dL, which is two-thirds that of *Hprt*(low)*Xdh*(-/-)*Slc23a4*(+/+) mice. In addition, both the urinary xanthine/creatinine ratio and hypoxanthine/creatinine ratio of 4-week-old *Hprt*(low)*Xdh*(-/-)*Slc23a4*(+/+) mice have been reported to be approximately 40¹³, whereas the urinary xanthine/creatinine ratio of *Hprt*(high)*Xdh*(-/-)*Slc23a4*(-/-) mice is approximately 3.8, which is 1/10 that of *Hprt*(low)*Xdh*(-/-)*Slc23a4*(+/+) mice. Moreover, the urinary hypoxanthine/creatinine ratio is approximately 0.4, which is 1/100 that of *Hprt*(low)*Xdh*(-/-)*Slc23a4*(+/+) mice. Although the difference in age may have an effect, targeting *Slc23a4* may decrease the absorption of hypoxanthine and xanthine in the intestine, contributing to the increased lifespan.

The activity of serum xanthine oxidase (XO), the oxidase form of *XDH* gene product, is high in mice but almost nonexistent in humans¹⁴, and the expression of *Xdh* in the mouse liver is 100-fold greater than that of human *XDH*¹⁵; thus, the *XDH* activity of *Xdh*(+/-) mice is much greater than that of wild-type humans. The total purine excretion in the urine of *Hprt*(high)*Xdh*(+/+)*Slc23a4*(+/+) mice that was previously reported¹⁶ is almost equal to that of *Hprt*(high)*Xdh*(+/-)*Slc23a4*(-/-) mice, as shown in Fig. 2C. If we assume that the common ancestor of mice and humans had high *XDH* activity similar to that of modern mice and that *XDH* activity decreased during the evolution of humans, the *Slc23a4* mutation is considered neutral because no difference in traits due to *Slc23a4* mutation appears when *XDH* activity is high. Furthermore, the effect of the *Slc23a4* mutation on total urinary purine excretion was apparent only in homozygous *Xdh* mutant mice after *XDH* activity was reduced. These findings suggest that when *XDH* activity decreased during human evolution,

		The urinary xanthine/ creatinine ratio (mmol/mol Cr)	The urinary hypoxanthine/ creatinine ratio (mmol/mol Cr)	The urinary hypoxanthine/ xanthine ratio	Plasma xanthine (μmol/L)	Plasma hypoxanthine (μmol/L)	Ref
Ichida 1997	#1	110	89	0.809	23.6	5.9	21
	#2	85.7	57.8	0.674	15.8	7.3	
	#3*	132	1.5	0.011	85	0.8	
	#4	108.2	38.9	0.360	38.5	1.6	
Gok, 2003	III-3	64.2	28.4	0.442	21.0	10.6	28
	III-1	227.2	28.5	0.125	14.2	14.2	
Arikyants, 2007	S.B	259	54	0.208	18	13	20
	S.B. sis	370	77	0.208	16	19	
Jurecka, 2010		192	44	0.229			29
Stiburkova, 2012	P1	190	64	0.337			30
	P2	170	89	0.524			
Eggermann, 2013		193	65.0	0.337			31
Mraz, 2015	Case 1	164.5	74.4	0.452	14	31	32
	Case 2	349.3	138.0	0.395	9	0	
	Case 3	328.2	61.8	0.188	14	15	
Iguchi, 2016		207.4	58.2	0.281	25.8	7.0	33
Xu, 2020		270	186	0.689			34
Peretz, 2021	F2V2	106	40	0.377			35
Goncalves, 2024	*	94.18	4.418	0.047			17
Maes, 2024		135	16	0.119			36
Mean ± SEM		187.8 ± 20.1	60.8 ± 9.7	0.375 ± 0.046	24.6 ± 5.9	10.4 ± 2.6	
<i>Hprt</i> (high) <i>Xdh</i> (-/-) <i>Slc23a4</i> (-/-) mice fed a normal diet		3761 ± 299	433.5 ± 86.4	0.115 ± 0.289	121.9 ± 10.4	35.0 ± 4.2	
<i>Hprt</i> (high) <i>Xdh</i> (-/-) <i>Slc23a4</i> (-/-) mice fed a purified diet		1520 ± 260	61.5 ± 34.6	0.040 ± 0.116	65.4 ± 5.0	24.1 ± 4.0	

Table 1. Oxypurine kinetics of patients with type 1 xanthuria. *Patients with type 1 xanthinuria with renal impairment.

only the homozygous *Slc23a4* mutant had a renoprotective effect, leading to selection and the development of a pseudogene.

Although female *Xdh*(-/-) mice gave birth to pups, the pups died a few days after birth, suggesting that female *Xdh*(-/-) mice may have lost the ability to secrete milk. Although lactation is reportedly affected in *Xdh*(+/-) mice², the *Xdh*(-/-) mouse strain that was used in this study could be maintained with *Xdh*(+/-) mice³, so it is believed that lactation is not affected in the *Xdh*(+/-) mice we used. By generating *Xdh*(-/-) mice of childbearing age, it was revealed for the first time that homozygous *Xdh* mutations affect lactation. Although agalactia has recently been reported in a patient with xanthinuria¹⁷, a study of the milk of a female patient with xanthinuria reported that no XO activity was detected in this patient's milk¹⁸. Therefore, it is unlikely that all female patients with xanthinuria exhibit agalactia. Moreover, mammary gland-specific *Xdh*-knockout mice exhibit delayed lactation but no problems¹⁹; thus, multiple factors other than *Xdh* deficiency may be related to agalactia in female *Xdh*(-/-) mice.

Oxypurine kinetics

As shown in Table 1, which listed oxypurine kinetics of patients with type 1 xanthinuria whose *XDH* gene mutation was confirmed by DNA sequencing, the urinary xanthine/creatinine ratio of patients with type 1 xanthinuria was 0.188 ± 0.021 (mol/mol Cr), and the urinary xanthine/creatinine ratio of *Hprt*(High)*Xdh*(-/-)*Slc23a4*(-/-) mice that were fed a normal diet and those that were fed a low-purine diet was 20.0-fold and 8.09-fold greater than that of patients with type 1 xanthinuria, respectively. Compared with patients with type 1 xanthinuria, *Hprt*(High)*Xdh*(-/-)*Slc23a4*(-/-) mice excrete excessive levels of xanthine in their urine. The urinary hypoxanthine/creatinine ratio of *Hprt*(High)*Xdh*(-/-)*Slc23a4*(-/-) mice was 0.061 ± 0.010 (mol/mol Cr) when the mice were fed a normal diet, which was 7.11-fold greater than that of patients with type 1 xanthinuria, but when the mice were fed a low-purine diet, it was reduced to almost the same level as that of patients with type 1 xanthinuria. Because the low-purine diet reduced urinary xanthine and hypoxanthine excretion, the purine metabolites that are excreted in excess in the urine as xanthine in *Xdh*(-/-) mice and as allantoin in *Xdh*(+/-) mice are thought to be the purines that are absorbed in the digestive tract. Compared with that in mice, intestinal purine base absorption that is mediated by *SNBT1/Slc23a4* is lost in humans, and the activity of *XDH*, which converts absorbed purines to uric acid in intestinal epithelial cells, might be attenuated in humans. Thus, the reduced purine absorption in the digestive tract may decrease urinary oxypurine excretion.

As shown in Table 1, the plasma xanthine concentration of patients with type 1 xanthinuria was 0.371 ± 0.090 (mg/dl), which was 5.00 and 2.68 times lower than that of *Hprt(High)Xdh(-/-)Slc23a4(-/-)* mice that were fed a normal diet and those that were a low-purine diet, respectively. The plasma hypoxanthine concentration of patients with type 1 xanthinuria was 0.141 ± 0.035 (mg/dl), which was 3.38 and 2.33 times lower than that of *Hprt(High)Xdh(-/-)Slc23a4(-/-)* mice that were fed a normal diet and those that were fed a low-purine diet, respectively. It is thought that the mechanism for hypoxanthine reabsorption in the kidneys was active and that the hypoxanthine plasma concentration was maintained even when purine intake was reduced. The renal tubular reabsorption rate of xanthine in patients with type 1 xanthinuria has been reported to be 3% to 35%, and that of hypoxanthine is 93% to 79%²⁰. The renal tubular reabsorption rates of xanthine and hypoxanthine in *Hprt(High)Xdh(-/-)Slc23a4(-/-)* mice were similar to those in patients with type 1 xanthinuria, suggesting that the hypoxanthine reabsorption mechanism in these mice may be similar to that in humans.

The urinary hypoxanthine/xanthine ratio of patients with type 1 xanthinuria, except two patients with renal impairment, was 0.378 ± 0.049 , which was higher than that of *Hprt(High)Xdh(-/-)Slc23a4(-/-)* mice, suggesting that remaining species-specific differences may contribute to the difference in the urinary hypoxanthine/xanthine ratios between humans and mice. The decrease in the urinary hypoxanthine/xanthine ratio indicates the oxidation or dehydrogenation of hypoxanthine to xanthine, but whether there are species-specific differences in this reaction pathway remains to be investigated. Moreover, the urinary hypoxanthine/xanthine ratios of patients with type 1 xanthinuria with renal impairment are low at 0.047 and 0.011^{17,21}, and these ratios are similar to those of *Hprt(High)Xdh(-/-)Slc23a4(-/-)* mice. Therefore, we cannot exclude the possibility that the low urinary hypoxanthine/xanthine ratio in *Hprt(High)Xdh(-/-)Slc23a4(-/-)* mice might occur due to renal dysfunction rather than to remaining species-specific differences.

Renal dysfunction associated with increased urinary excretion of oxypurine

All *Hprt(High)Xdh(-/-)Slc23a4(-/-)* mice exhibited chronic kidney damage accompanied by anemia. Although some patients with type 1 xanthinuria develop urinary stones and urinary stone-associated acute kidney injury, many patients are completely asymptomatic. Compared with patients with type 1 xanthinuria, *Hprt(High)Xdh(-/-)Slc23a4(-/-)* mice presented 7.11-fold increased urinary hypoxanthine excretion and 20.0-fold increased urinary xanthine excretion. Furthermore, the urinary hypoxanthine/xanthine ratio was decreased in the same way as that in patients with type 1 xanthinuria with renal dysfunction, suggesting that increased urinary xanthine excretion may be related to the cause of renal dysfunction.

Hypoxanthine and uric acid are reabsorbed at the renal tubules at more than 90% of the glomerular filtrate, but xanthine is excreted in the urine without significant reabsorption. Similar to patients with renal hypouricemia, in whom uric acid reaches the collecting duct without being reabsorbed by the renal tubules, it is assumed that urinary uric acid excretion increases due to purine breakdown during exercise in patients with renal hypouricemia, and it is known that these patients develop acute kidney injury after exercise^{22,23}. Therefore, it is possible that increased urinary excretion of xanthine, which is a purine metabolite that is not reabsorbed by the renal tubules, might be related to decreased renal function, similar to that in renal hypouricemia patients.

Allopurinol reportedly cannot suppress the progression of renal injury in patients with chronic kidney disease²⁴. *Xdh(-/-)* mice, which exhibit increased urinary xanthine excretion, exhibit renal injury, so it is possible that inhibition of XDH activity by allopurinol increases xanthine production in patients with chronic kidney disease and that the renal injury caused by increased urinary excretion of xanthine might offset the renoprotective effect by suppressing uric acid production.

Adenine nephropathy is a known mouse model of kidney damage that is caused by purine metabolites²⁵. When the mice are fed a 0.20% adenine diet (200 mg adenine/100 g diet), the amount of adenine, which is normally salvaged to adenine monophosphate (AMP) by adenine phosphoribosyltransferase (APRT), becomes excessive. Excess adenine is converted to 2,8-dihydroxyadenine by XDH and excreted in the urine, which causes kidney damage. However, normal human purine intake is approximately 10–20 mg/100 g diet, and 2,8-dihydroxyadenine is not excreted in urine except in patients with APRT deficiency^{26,27}. In contrast, xanthine, which is deaminated at the 6-position of 2,8-dihydroxyadenine, is excreted in urine even in healthy individuals. It is possible that the xanthine load per single nephron increases with decreasing nephron number, causing progressive deterioration of renal function. Therefore, *Hprt(High)Xdh(-/-)Slc23a4(-/-)* mice may be a better model of human chronic kidney disease.

In this study, targeting *Slc23a4* attenuated renal damage in *Xdh(-/-)* mice and increased their life span. Since *Slc23a4* is expressed in the intestine and its product is a transporter that functions in the absorption of purine bases, it is thought that purines that are absorbed in the intestine may play a role in causing renal damage. Uric acid did not contribute to the increased life span of *Hprt(High)Xdh(-/-)Slc23a4(-/-)* mice, suggesting a mechanism different from the initial hypothesis that the loss of uric acid resulting from XDH deficiency is the cause of renal damage in *Xdh(-/-)* mice. In addition, in this study, the mice were fed a low-purine diet for one week after being fed a normal diet, but no improvement was observed in the plasma creatinine concentration or hematocrit values. These results suggest that this model mouse has irreversible renal damage rather than a functional decrease in the GFR. In the future, it will be necessary to investigate whether *Hprt(High)Xdh(-/-)Slc23a4(-/-)* mice do not develop renal damage if they are fed a low-purine diet from birth.

Conclusion

We succeeded in extending the life span of *Xdh(-/-)* mice and obtaining adult mice by the induction of high HPRT activity and pseudogenization of *Slc23a4*, as observed in humans. However, there are still differences between *Xdh(-/-)* mice and patients with type 1 xanthinuria in terms of excessive urinary excretion of oxypurines, renal dysfunction with anemia in all cases, and failure of offspring to live, presumably due to

lactation failure in female *Xdh*($-/-$) mice. It is necessary to establish a mouse model of type 1 xanthinuria that is more similar to patients with type 1 xanthinuria by accounting for the species-specific differences between *Xdh*($-/-$) mice and humans not only in purine metabolism but also in purine kinetics.

Materials and methods

Establishment of *Hprt*(high)*Xdh*($-/-$)*Slc23a4*($-/-$) mice

Knockout-first allele: *Slc23a4*-targeted mice carrying the promoter-driven selection cassette were by importing cryopreserved sperm (049682-UCD-SPERM, C57BL/6N-*Slc23a4*^{tm1a(KOMP)Wtsi/Mmucd}) from MMRRC UC Davis; and recovering litters from the cryopreserved sperm at the RIKEN BioResource Center. Mating the recovered litters with *cre* mice²³ resulted in offspring with the *lac z* sequence that lost exon 4 of *Slc23a4* and the *neo* cassette. Mating the recovered litters first with *flp* mice²⁴ resulted in offspring that lost the *lac z* sequence and *neo* cassette. Then, mating the offspring from *flp* mice with *cre* mice resulted in *Slc23a4*($+/-$) mice without the *lac z* sequence. By crossing these *Slc23a4*($+/-$) mice with *Hprt*(high)*Xdh*($+/-$) mice on the B6 background, *Hprt*(high)*Xdh*($-/-$)*Slc23a4*($-/-$) mice and *Hprt*(high)*Xdh*($+/-$)*Slc23a4*($-/-$) mice were generated. In addition, *Hprt*(high)*Xdh*($-/-$)*Slc23a4*($+/+$) and *Hprt*(high)*Xdh*($-/-$)*Slc23a4*($+/-$) mice were obtained during the establishment process.

All animal experiments and the experimental protocols were approved as Teikyo Animal Ethics #21-007 by the animal ethics committee of Teikyo University. All methods were performed in accordance with the relevant guidelines and regulations. Anesthesia in this study was 3.5% isoflurane only at the ear punching for genotyping. Euthanasia was conducted with 100% CO₂ and deep freezing. All methods are reported in accordance with ARRIVE guidelines for the reporting of animal experiments.

Genotyping

The *Hprt* genotype was determined as previously reported⁹. The genotypes of *Xdh* and *Slc23a4* were determined via PCR via the use of 10 µL of the TaKaRa PCR Carryover Prevention Kit (Takara Bio Inc. Shiga, Japan) and DreamTaq™ Hot Start Green DNA Polymerase (Thermo Fisher Sci. USA) together with 0.5 µL of DNA extracted from an ear punch via Phire Tissue Direct PCR Master Mix (Thermo Fisher Sci. USA).

The PCR conditions for the *Xdh* gene were as follows: (25 °C for 10 min) - (95 °C for 2 min) - (95 °C for 10 s - 56 °C for 10 s - 72 °C for 40 s) × 40 cycles - (72 °C for 1 min) - 10 °C. The sense primer was 5'-gcaccgtgatgatctccaagta-3', and the wild-type antisense primer was 5'-ttctga ggcggaaagaaccag-3' to produce a 205-bp product of the wild-type amplicon. The sense primer was used with the targeted antisense primer 5'-aaaattctgggtgagaggaca-3' to produce a 445-bp product of the targeted amplicon.

The PCR conditions for the *Slc23a4* gene with the *lac z* sequence were as follows: (25 °C for 10 min) - (95 °C for 2 min) - (95 °C for 10 s - 55 °C for 10 s - 72 °C for 45 s) × 40 cycles - (72 °C for 1 min.) - 10 °C. The sense primer was 5'-agctagacacaccacaacgaaa-3' and the wild-type antisense primer was 5'-agtttgagacgggtgtgt-3' to produce a 175-bp product of the wild-type amplicon. The sense primer was used with the targeted antisense primer 5'-ataaagtgaccctccaacagc-3' to produce a 316-bp product of the targeted amplicon. The PCR conditions for the *Slc23a4* gene without the *lac z* sequence were the same as those described above, and the sense primer was used with another targeted antisense primer 5'-ggcttcactctctggagtt-3' to produce a 437-bp targeted amplicon.

Measurement of blood and urinary oxypurine levels

Mice of various species were bred using MR-A1 feed (Nihon Nosan) as a normal diet and AIN93M (Oriental Yeast Co., Ltd., Japan) as a low-purine purified diet for one week. The mice were placed in a plastic container and weighed, and 30 µL of spotted urine was collected. The mice were placed in a rodent restrainer, and the tail vein was punctured with a 27G blood collection needle to collect 20 µL of blood via a heparinized microcapillary (Drummond). After being sealed, the blood was centrifuged at 6000×g for 3 min to measure the hematocrit values, and plasma samples were obtained. The urine and plasma samples were stored at -20 °C until measurement.

The quantitative analysis of the measured substances was carried out via Chromaster (Hitachi) high-performance liquid chromatography (HPLC) with a Unison US-C18 250×2.0 mm column (Imtakt US026). The mobile phase was 10 mM ammonium formate 0.200 mL/min at 25 °C, and the absorbance was measured at 234 nm. For the urine samples, 2 µL of spotted urine was diluted with 100 µL of the mobile phase. For the plasma samples, 2 µL of plasma was diluted with 40 µL of 80% acetonitrile solution, deproteinized with a centrifugal filter, evaporated to dryness with a centrifugal concentrator, and redissolved in 10 µL of the mobile phase.

The supplemental figure shows typical chromatograms of standards (Suppl. Fig. A), urine samples of *Hprt*(high)*Xdh*($-/-$)*Slc23a4*($-/-$) mice (Suppl. Fig. B) and *Hprt*(high)*Xdh*($+/-$)*Slc23a4*($-/-$) mice (Suppl. Fig. C), and plasma samples of *Hprt*(high)*Xdh*($-/-$)*Slc23a4*($-/-$) mice (Suppl. Fig. D) and *Hprt*(high)*Xdh*($+/-$)*Slc23a4*($-/-$) mice (Suppl. Fig. E). The standards that were used were 10 mg/L allantoin, 1 mg/L sodium urate, creatinine, hypoxanthine, and sodium xanthine.

Statistical analysis

Statistical comparisons between groups and treatments were performed via one-way analysis of variance (ANOVA). Student's *t* tests were used to compare two groups. A *p* value of <0.05 was considered to indicate statistical significance. The data are presented as the means ± SDs.

Data availability

All data generated or analysed during this study are included in this published article and its supplementary information files.

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Author contributions

K. T. contributed draft of the work; T. W., N. Y., T. O., S. S. and K. I. contributed substantively revised it; and M.H. contributed all of this study.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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