

RESEARCH PAPER



Adiposity and metabolic health in mice deficient in intestinal alkaline phosphatase

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ABSTRACT

Intestinal alkaline phosphatase 3 (AKP3) is an enzyme that was reported to play a role in lipid metabolism and to prevent high fat diet-induced metabolic syndrome in mice.

To investigate a potential functional role of AKP3 in diet-induced adiposity and metabolic health, we have kept male and female wild-type or AKP3 deficient mice on a high fat diet for 15 weeks to induce obesity and compared those with mice kept on standard fat diet.

Body weight as well as adipose tissue mass were statistically significantly higher upon high fat diet feeding for mice of both genders and genotypes. Female mice of either genotype kept on high fat diet gained less weight, resulting in smaller adipose tissue depots with smaller adipocytes. However, AKP3 deficiency had no significant effect on body weight gain or adipose tissue mass and did not affect adipocyte size or density. Gene expression analysis revealed no effect of the genotype on inflammatory parameters in adipose tissue, except for tumor necrosis factor alpha, which was higher in mesenteric adipose tissue of female obese mice. Plasma glucose and insulin levels were also not affected in obese AKP3 deficient mice.

Overall, our data do not support a functional role of AKP3 in adipose tissue development, or insulin sensitivity.

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Introduction

Over the last decades obesity and its consequences worldwide have become a major health problem. Obesity is frequently associated with metabolic abnormalities such as impaired glucose tolerance, hyperinsulinemia, dyslipidemia with elevated triglyceride level, decreased high-density lipoprotein cholesterol concentration and increased proportion of small dense lipoparticles. This cluster of metabolic disturbances is called the metabolic syndrome.¹ In addition, obesity is associated with an increased prevalence of cardiovascular disease, some forms of cancer, diabetes, liver and kidney disease.²

Intestinal alkaline phosphatase (AKP3) is an ectoenzyme that detoxifies a variety of bacterial toxins.³ It is associated with the brush border of the intestinal epithelium, with the highest level in the duodenum.^{4,5} Administration of the enzyme to mice prevented high fat diet (HFD)-induced endotoxemia as well as the metabolic syndrome.⁶ Mice with deficiency of AKP3 (KO) did not show apparent phenotype abnormalities.⁷ When fed a HFD, however, the KO mice showed faster weight gain and accelerated

transport of fat droplets through the intestinal epithelium.⁷ AKP3 deficiency in mice was also shown to be associated with visceral fat accumulation and hepatic steatosis.⁸ In addition, KO mice were reported to have glucose intolerance and insulin resistance.⁶ Interestingly, enhanced weight gain and liver steatosis were mainly restricted to female mice. However, none of these studies reported specific effects of AKP3 on adipose tissue (AT) biology such as adipogenesis or adipose tissue development and inflammation.

In the present study, we have further evaluated a potential functional role of AKP3 in AT development using an established model of diet-induced obesity in wild type (WT) and AKP3 deficient mice.

Materials and methods

Nutritionally induced obesity model

AKP3 deficient mice (AKP3 knockout or KO) and wild-type littermates (WT) (genetic background 75% 129/Sv and 25% C57Bl6/J) were obtained from heterozygous breeding couples, and were genotyped in the KU Leuven animal facility, as described.⁷

Male and female mice, from the age of 5 weeks on, were cohoused per sex in small groups (3 to maximum 5 mice) in microisolation cages on a 12h day/night cycle. All cages were enriched with nesting material, cotton cylinders and wooden autoclaved brick to reduce possible stress. Mice were fed for 15 weeks with a high fat diet (HFD, Ssniff, Soest Germany; 42% kcal as fat, caloric value 20.1 kJ/g) or a standard fat diet (SFD, Ssniff, Soest Germany; 13% kcal as fat, caloric value 10.9 kJ/g). Water was always available *ad libitum*. The HFD is a western type diet consisting of 43% kJ sucrose, 42% kJ saturated fat (butter fat) and 15kJ protein. The SFD consists of 30% kJ sucrose, 30% kJ saturated fat and 40% kJ protein.

Food intake was measured daily and body weight at weekly intervals. At the end of the experiments, after 6h fasting, mice were anesthetized by intraperitoneal injection of 60 mg/kg Nembutal (Abbott Laboratories, North Chicago, IL). Blood was collected via the retro-orbital sinus on trisodium citrate (final concentration 0.01 M) and plasma was stored at -80°C . Intra-abdominal gonadal (GN) or mesenteric (MES) and inguinal subcutaneous (SC) fat pads, colon and liver were removed and weighed; portions were snap-frozen in liquid nitrogen for RNA extraction and paraffin sections (10 μm) were prepared for histology.

All animal experiments were approved by the local ethical committee (KU Leuven P011-2014) and performed in accordance with the NIH Guide for the Care and use of Laboratory Animals (1996).

mRNA analysis

Taqman gene expression assays (Thermo Fisher Scientific, Merelbeke, Belgium) were used to analyze mRNA levels of the following genes in duodenum by quantitative RT-PCR: AKP3 (Mm00475848_g1), AKP6 (Mm01285814_g1), CD36 (Mm00432403_m1), tumor necrosis factor (TNF)- α (Mm00443258_m1), F4/80 (Mm00802529_m1) and adiponectin (Mm00456425_m1) according to a protocol described earlier.⁹ Data were obtained as cycle threshold (Ct) values and were normalized first to the housekeeping gene β -actin ($\Delta\text{Ct} = \text{Ct}_{\text{target}} - \text{Ct}_{\beta\text{-actin}}$) and subsequently to the control group, WT mice fed with SFD ($\Delta\Delta\text{Ct}$). For each group the data were represented as an average of $2^{-\Delta\Delta\text{Ct}} \pm \text{SEM}$. A non-template control, without cDNA in the reaction was used as a negative control.

Histological analysis and metabolic parameters

The size and density of adipocytes in AT sections, and steatosis in liver sections were determined by staining with haematoxylin/eosin under standard conditions. Analysis was performed using a Zeiss Axioplan 2 microscope with the AxioVision rel. 8.2 software (Carl Zeiss, Oberkochen, Germany). The area of

interest was marked and the individual cells were manually counted. The software provides the size of the marked area, the density and the total cell count. The average cell size was calculated as the ratio of the marked area divided by the total count in that area. The average density was calculated as the ratio of the cell count divided by the total area. Steatosis was scored as follows: 0–20% score 0; 20–40% score 1, 40–60% score 2, 60–80% score 3 and 80–100% score 4.

Blood glucose concentrations were measured using the Accu-chek performa meter and blood glucose test strips (Roche Diagnostics, Basel, Switzerland). Triglycerides, cholesterol, alkaline phosphatases, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were quantitated using routine clinical assays. Insulin and TNF- α levels in plasma were determined with a specific ELISA (Mercodia, Upsala, Sweden and Thermo Fisher Scientific, respectively). A blank was included in each ELISA test. Plasma endotoxin levels were measured using the Pierce™ LAL Chromogenic Endotoxin Quantitation Kit (Thermo Fisher Scientific); as positive control plasma from LPS triggered mice was used. Liver triglyceride levels were determined as described previously.⁹

Statistical analysis

Data are reported as means \pm SEM. Statistical significance between two groups is analysed by non-parametric Mann-Whitney U test. Progress curves are analysed by two-way ANOVA. Values of $p < 0.05$ are considered statistically significant. Analysis of the data was performed using Prism 7 (GraphPad Software Inc., San Diego, CA, USA)

Results

AKP3 deficiency in male and female littermate mice was confirmed by genotyping, as described.⁷ Furthermore, AKP3 mRNA was undetectable in the duodenum of male and female KO mice.

The higher caloric intake on HFD as compared to SFD resulted in higher weight gain, total body weight and increased subcutaneous and visceral fat depots for male as well as female mice of both genotypes, supporting diet-induced obesity. No differences between genotypes were observed in weight gain or body weight, compatible with comparable caloric intake on both diets for KO and WT mice of either gender (Table 1). Body weight gain of female WT as well as KO mice on both diets was significantly lower as compared to their male counterparts, resulting in lower total body weights (Table 1 and Figure 1). Colon weight was lower on HFD as compared to SFD, but comparable for males and females of both genotypes. SC, GN and MES fat

Table 1. Effect of AKP3 deficiency on fat mass and organ weight of male or female mice kept on SFD or HFD for 15 weeks.

	male				female			
	WT SFD n = 6	KO SFD n = 5	WT HFD n = 6	KO HFD n = 5	WT SFD n = 6	KO SFD n = 8	WT HFD n = 10	KO HFD n = 10
Bodyweight (g)	26.8 ± 0.95	28.8 ± 1.3	39.7 ± 1.7**	40.5 ± 1.02*	20.3 ± 0.5 ^{oo}	21.9 ± 0.3 ^{oo}	27.1 ± 1.7*** ^{oo}	28.3 ± 1.2*** ^{oo}
Bodyweight gain (g)	9.0 ± 0.8	9.6 ± 0.9	17.2 ± 1.4**	21.4 ± 1.5*	4.6 ± 0.3 ^{oo}	4.9 ± 0.6 ^{oo}	10.7 ± 1.4*** ^{oo}	11.5 ± 0.9*** ^{oo}
Food intake (g/mouse/ day)	4.0 ± 0.05	4.0 ± 0.09	3.5 ± 0.06***	3.3 ± 0.04***	3.6 ± 0.6 ^{oo}	3.8 ± 0.04 ^{oo}	3.4 ± 0.06**	3.5 ± 0.08***
Food intake (Kcal/day)	12.2 ± 0.2	12.2 ± 0.1	16.1 ± 0.3***	15 ± 0.2***££	10.9 ± 0.2 ^{oo}	11.6 ± 0.1£ ^{oo}	15.3 ± 0.2*** ^{oo}	16.2 ± 0.4*** ^{oo}
Liver (mg)	1049 ± 44	1173 ± 79	2440 ± 343**	2882 ± 216*	780 ± 21 ^{oo}	913 ± 29£ ^{oo}	1337 ± 113*** ^{oo}	1381 ± 89*** ^{oo}
Colon (mg)	183 ± 7.3	183 ± 8.3	113 ± 7.9**	105 ± 10.5*	173 ± 8.2	185 ± 7.5	109 ± 4.9***	108 ± 6.2
Colon length (cm)	9.4 ± 1.4	9.9 ± 0.2	8.1 ± 0.3*	7.8 ± 0.2*	8.9 ± 0.2	9.5 ± 0.2	8.2 ± 0.3	8.5 ± 0.2*
Colon mass/length	19.7 ± 1.4	18.5 ± 0.9	14.0 ± 1.1*	13.6 ± 1.7*	19.3 ± 0.7	19.6 ± 0.9	13.5 ± 0.7***	12.7 ± 0.7***
SC (% of bodyweight)	0.83 ± 0.02	1.2 ± 0.2	2.9 ± 0.27**	3.3 ± 0.09*	1.04 ± 0.05°	0.88 ± 0.05	1.9 ± 0.33	2.01 ± 0.2*** ^{oo}
GN (% of bodyweight)	1.5 ± 0.06	2.3 ± 0.4	5.4 ± 0.4**	6.4 ± 0.36*	1.1 ± 0.07 ^{oo}	1.3 ± 0.1°	2.5 ± 0.5 ^{oo}	2.7 ± 0.34*** ^{oo}
MES (% of bodyweight)	0.6 ± 0.08	0.9 ± 0.18	1.8 ± 0.19**	1.9 ± 0.14*	0.57 ± 0.08	0.48 ± 0.03	1.01 ± 0.19°	1.23 ± 0.17* ^{oo}

Data are means ± SEM of n experiments. WT, Wild-type; KO, AKP3 deficient mice; SC, subcutaneous; GN, gonadal; MES, mesenteric adipose tissue.

*, **, *** p < 0.05, 0.01 and 0.001 respectively versus SFD.

£, ££ p < 0.05 and 0.01 respectively versus WT on the same diet.

°, °°, °°° p < 0.05, 0.01 and 0.001 respectively versus males.

Males

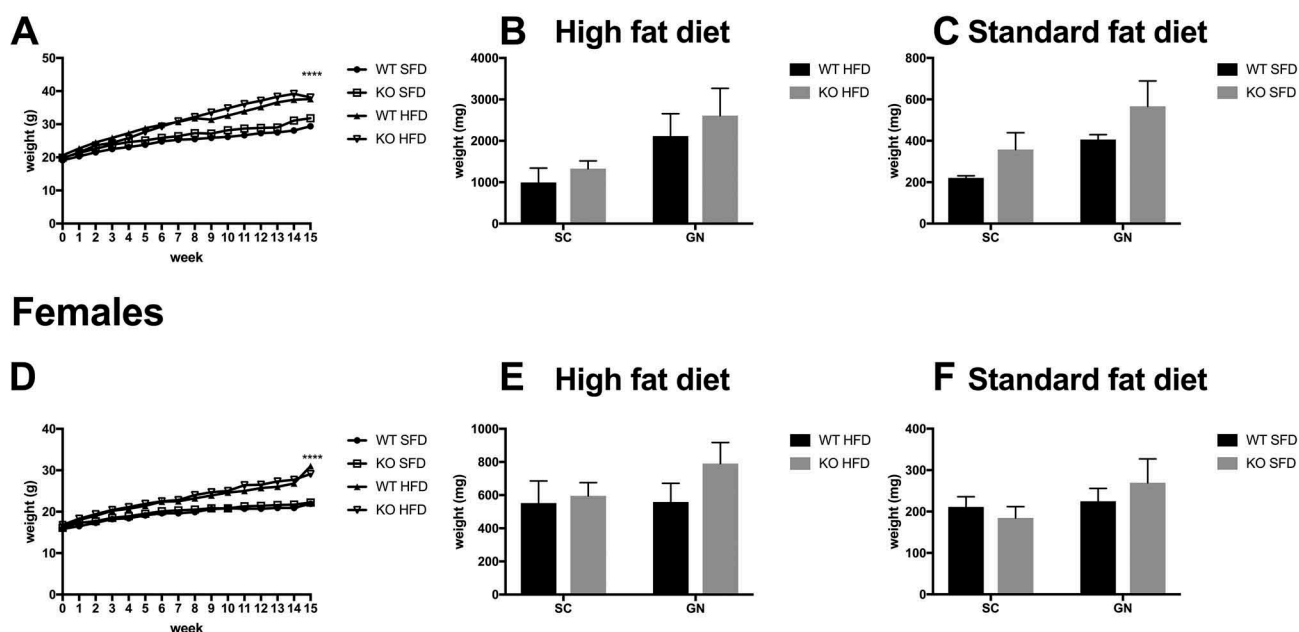
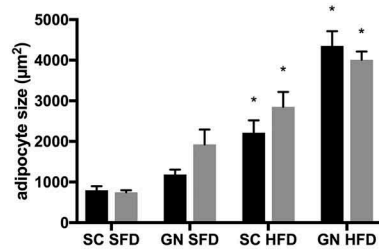
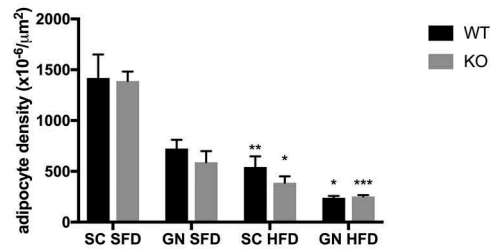


Figure 1. Effect of AKP3 deficiency on diet induced obesity. Evolution of body weight (A, D) and weight of SC and GN adipose tissue from male and female wild-type (WT) and AKP3 deficient (KO) mice kept on SFD (C, F) or HFD (B, E) for 15 weeks. Data are means ± SEM, ****, p < 0.001 as compared to SFD by two-way ANOVA.

depots had a larger fat mass weight on HFD as compared to SFD for both genotypes and genders, and were significantly smaller for females on HFD without, however, differences between genotypes (Figure 1). A similar pattern emerged when fat masses are expressed as percentage of body weight (Table 1). In addition, histological analysis of adipocyte size and density in SC and GN ATs revealed smaller adipocytes at higher density for female as compared to male mice on HFD, without effect of AKP3 deficiency (Figure 2; illustrated in Figure 3 A, B for female mice on HFD).

Gene expression analysis revealed lower expression of AKP3 in the duodenum of obese (HFD) versus lean (SFD) WT mice of both genders (3.7-fold for males, p = 0.017; 3.8-fold for females, p = 0.05), whereas no effect of genotype was observed on expression of AKP6 or CD36 (data not shown). Furthermore, no effect of genotype was seen on expression of F4/80, TNF- α or adiponectin in SC or GN ATs of either male or female mice. In MES AT, expression of TNF- α was significantly higher only in obese female KO as compared to WT mice (p = 0.017).

Males

A**B**

Females

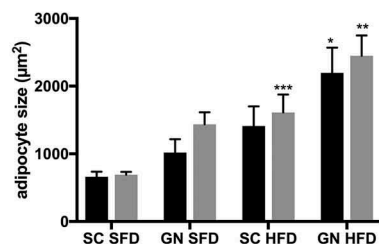
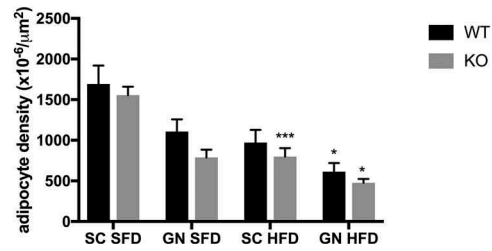
C**D**

Figure 2. Effect of AKP3 deficiency on adipocyte size and density in SC or GN adipose tissue from male (A,B) or female (C,D) wild-type (WT) and AKP3 deficient (KO) mice kept on SFD or HFD for 15 weeks. Data are means \pm SEM. *, **, ***, $p < 0.05$, 0.01 and 0.001 respectively, versus SFD.

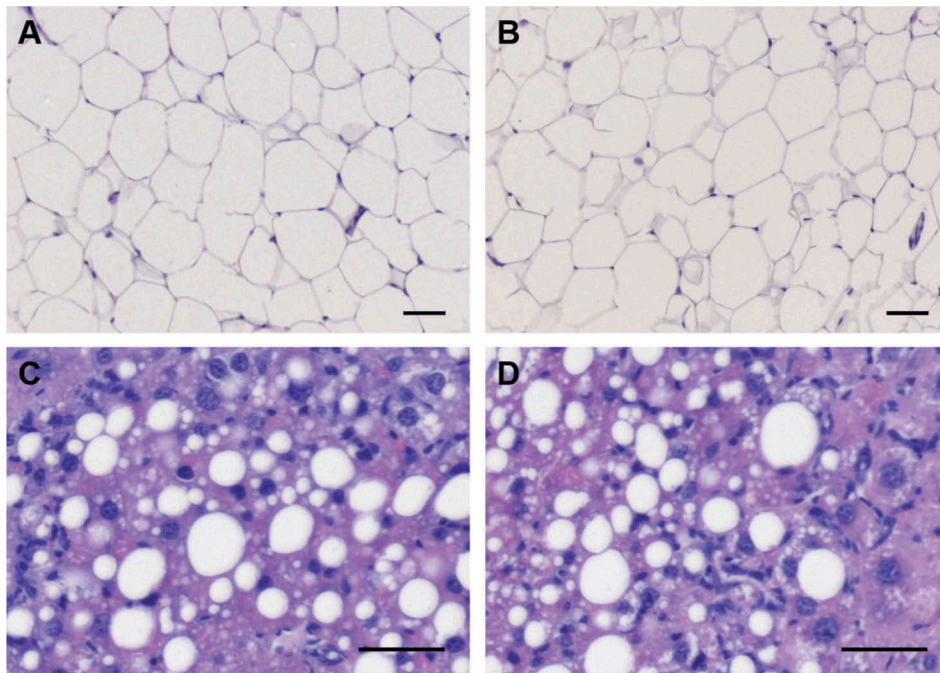


Figure 3. H&E staining of GN adipose tissue sections (A,B) or liver sections (C,D) of a female wild-type (A, C) or a AKP3 deficient mouse (B, D) kept on HFD for 15 weeks. Scale bars represent 50 μm . Magnification is 100x (A, B) or 200x (C, D).

Table 2. Effect of AKP3 deficiency on metabolic parameters in plasma and liver triglycerides of male or female mice kept on SFD or HFD for 15 weeks.

	male				female			
	WT SFD n = 6	KO SFD n = 5	WT HFD n = 6	KO HFD n = 5	WT SFD n = 6	KO SFD n = 8	WT HFD n = 10	KO HFD n = 10
Endotoxin (EU/ml)	14 ± 0.36	14 ± 0.24	14 ± 0.38	14 ± 0.67	6.3 ± 0.20 ^{oo}	7.5 ± 0.47 ^o	6.8 ± 0.23 ^{oo}	6.9 ± 0.22 ^{oo}
TNF-alpha (pg/ml)	0.8 ± 0.022	0.75 ± 0.00	3.1 ± 0.52*	3.4 ± 1.1	2.1 ± 0.66	2.1 ± 0.39	2.6 ± 0.71	2.1 ± 0.53
Glycemia (mg/dl)	127 ± 8	128 ± 12	196 ± 26	167 ± 9	112 ± 7	121 ± 6	157 ± 11*	163 ± 11*
Plasma insulin (ng/ml)	0.18 ± 0.03	0.52 ± 0.09 [£]	1.73 ± 0.23*	1.68 ± 0.59	0.20 ± 0.024	0.40 ± 0.048 [£]	0.71 ± 0.13 ^{oo}	0.59 ± 0.13 ^o
HOMA-IR	1.7 ± 0.5	4.3 ± 0.45 [£]	27.2 ± 4.1*	19.4 ± 6.4*	1.5 ± 0.15	3.5 ± 0.4 [£]	8.3 ± 1.8 ^{oo}	7.4 ± 1.9
Total cholesterol (mg/dl)	58 ± 4.5	59 ± 3.9	141 ± 24	149 ± 33	42 ± 0.01 ^{oo}	42 ± 4.3 ^o	89 ± 9.4 ^{oo}	71 ± 6.2 ^{**}
Plasma triglycerides (mg/dl)	44 ± 1.9	40 ± 3.2	41 ± 5.5	38 ± 1.9	37 ± 1.1 ^o	33 ± 0.75 [£]	32 ± 1.8	33 ± 1.9
Liver Triglycerides (mg/dl)	15 ± 2.6	11 ± 1.9	96 ± 15 ^{**}	114 ± 24*	12 ± 2.7	10 ± 0.85	43 ± 4.7 ^{oo}	43 ± 3.9 ^{oo}
Alk. Phosphatase (U/l)	57 ± 2.8	52 ± 3.2	90 ± 16.4	87 ± 6.8 ^{**}	82 ± 3.7 ^{oo}	87 ± 3.1 ^{oo}	82 ± 7.0	95 ± 11
AST (U/l)	47 ± 3.8	48 ± 5.3	131 ± 30 ^{**}	140 ± 13*	55 ± 8.4	61 ± 7.3	85 ± 18	138 ± 29*
ALT (U/l)	27 ± 5.5	28 ± 3.2	125 ± 33 ^{**}	125 ± 29*	23 ± 2.8	38 ± 4.9	41 ± 9.3 ^o	75 ± 17

Data are means ± SEM of n experiments. WT, Wild-type; KO, AKP3 deficient mice.

*, **, *** p < 0.05, 0.01 and 0.001 respectively versus SFD.

£ p < 0.05 versus WT on the same diet.

°, °° p < 0.05, and 0.01 respectively versus males.

Analysis of metabolic parameters did not reveal significant differences in glucose levels between male or female WT or KO mice on either diet (Table 2). Plasma insulin levels of male and female WT and KO mice were enhanced on HFD as compared to SFD feeding, and were higher for both male and female KO mice as compared to WT, on SFD but not on HFD (Table 2). Thus, on SFD HOMA-IR was significantly higher for male as well as female KO as compared to WT mice. Total cholesterol levels were enhanced by HFD feeding and were lower for females, but not different between WT and KO mice (Table 2).

Liver weight was markedly enhanced after HFD feeding and lower for females, but not different between genotypes (Table 1). For both genotypes, hepatic triglyceride levels were markedly higher after HFD as compared to SFD feeding without, however, differences between WT and KO mice (Table 2). Histologic examination of H&E stained liver sections did not reveal significant steatosis for mice kept on SFD in contrast to HFD. Semi-quantitative scoring of steatosis did not reveal significant effects of AKP3 deficiency for either gender on HFD (2.6 ± 0.47 vs 2.6 ± 0.42 for wild type and AKP3 deficient male mice respectively; 1.8 ± 0.38 vs 1.5 ± 0.27 for wild type and AKP3 deficient female mice respectively; illustrated in Figure 3 C, D for female mice on HFD). Furthermore, liver enzymes including total alkaline phosphatases, AST and ALT were not affected by AKP3 deficiency (Table 2).

Plasma TNF-α levels for the KO mice were not significantly enhanced as compared to WT mice (Table 2). Plasma endotoxin levels were statistically different between KO and WT female mice on SFD, but not on HFD and were lower for female as compared to male mice on both diets and for both genotypes (Table 2).

Discussion

Intestinal alkaline phosphatase (AKP3) in mice has been reported to play a role in AT accumulation, insulin resistance, the metabolic syndrome and non-alcoholic liver steatosis, predominantly in female mice.⁶⁻⁸ We have re-evaluated a potential functional role of AKP3 in these processes using a well-established diet-induced obesity model in male and female WT and AKP3 deficient (KO) mice. The KO and WT mice were littermates generated from heterozygous breeding couples and genotyped as described.⁷ The KO genotype was confirmed by the absence of AKP3 mRNA in the duodenum, but not supported functionally by higher plasma levels of endotoxin or TNF-α, as reported previously.⁶ In our study endotoxin levels were only slightly increased in female KO versus WT mice on SFD, but not in female KO mice on HFD, nor in male KO mice on either diet. In another study by Yang et al basal intestinal TNF-α expression was similar in adult AKP3 KO versus WT mice.¹⁰ The HFD model of this study (42% kcal as fat) has been used previously in many studies showing effects of gene deficiencies in mice on metabolism and AT development.¹¹⁻¹⁴

In contrast to previous studies using these KO mice, we did not observe a significant effect of AKP3 deficiency on body weight or AT mass, either in males or females. In addition, adipocyte size was not affected by the deficiency, which was not reported previously. Nakano et al⁸ observed higher body weight and visceral fat mass only in female KO mice, using a 30% fat chow for 10 weeks. In the study of Narisawa et al⁷ however, a 11% fat diet given up to 30 weeks resulted in enhanced body weight for male KO mice. Kaliannan et al⁶ also reported higher body weight for male KO as

compared to WT mice; they used a HFD with a lower carb content and higher protein and fat content, but with caloric value comparable to our HFD (20.9 versus 20.1 kJ/g). In this study, the lower body weight of WT as compared to KO mice appeared to be due to the fact that the increase in body weight of WT mice on HFD levelled off after about 5 weeks, whereas the KO mice progressively gained more weight up to 16 weeks.⁶ In our study, weight progress curves on HFD feeding steadily increased over time (Figure 1). On SFD, none of these studies, including ours, showed an effect of AKP3 deficiency on adiposity. It is possible that the effects of AKP3 on body weight are related to different compositions of the diet, but the reported differences between male and female KO mice remain unexplained. We indeed observed higher AT mass in male as compared to female mice, but no effect of AKP3 deficiency. Hong et al¹⁵ reported previously a higher susceptibility to obesity of male versus female mice. Another possible explanation might be the influence of sex driven hormonal synthesis on adiposity.¹⁶ In addition, it was earlier shown *in vitro* that tissue alkaline phosphatase knockdown increased the mRNA levels of adipokines including adiponectin but decreased leptin levels,¹⁷ indicating an effect of alkaline phosphatases on lipid metabolism and adipokine secretion. It therefore, might be possible that sex and AKP3 deficiency may have synergistic effects.

Furthermore, as shown in a previous report⁶ using a low fat diet (14% kcal from fat), we also observed mild insulin resistance in KO mice on SFD but not on HFD. Whereas on SFD the KO mice of both genders had higher plasma insulin levels as compared to WT mice, this difference was not observed after HFD feeding. Plasma glucose levels were moderately enhanced by the HFD in our study, but not different between genotypes. Also in the study of Nakano et al⁸ fasting glucose levels were comparable for males and females, and not affected by AKP3 deficiency.

Finally, we did not find evidence for an effect of AKP3 on non-alcoholic liver steatosis in male or female mice on HFD. In contrast to a previous study showing enhanced liver triglycerides only in female KO as compared to WT mice, we did not find differences in hepatic triglycerides between genotypes, either male or female. Plasma triglyceride levels were also not affected by genotype. Furthermore, histopathologic examination of liver sections did not reveal enhanced steatosis for the KO mice (Figure 3 C, D).

Several factors, besides diet composition, may contribute to the different phenotype observed in different studies.¹⁸ Thus, a minor difference in genetic background may alter the phenotype, as shown in several studies on diet-induced obesity and metabolism.^{19,20}

However, the mice we used are derived from the original KO strain,⁷ which was also used in the studies of Nakano et al⁸ and Kaliannan et al.⁶ An important factor may be the different housing environment, which may alter the gut microbiota and affect the metabolic state.^{21,22} Therefore, well controlled experiments and gut microbiome standardization may be mandatory in order to reduce variability and allow correct interpretation of experimental results.²³

In summary, the phenotype of AKP3 deficient mice under our experimental conditions differs fundamentally from that previously reported. It is reassuring that previous studies have also shown that oral supplementation of AKP3 to mice prevents and reverses the metabolic syndrome and improves the lipid profile on standard low fat chow.⁶ Furthermore, acute inhibition of AKP3 in the small intestine of mice attenuated the postprandial triglyceride increase in serum.⁸

Thus, conclusions on a functional *in vivo* role of AKP3 derived from studies with gene-deficient mice should be handled with care, and supported by additional *in vivo* experiments such as tissue-specific knockout, *in vivo* gene silencing or phenotype rescue.

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Disclosure of interest

The authors report no conflict of interest.

Disclosure statement

No potential conflict of interest was reported by the authors.

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