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Pregnancy-Associated Plasma Protein-A Deficiency Improves Survival of Mice on a High Fat Diet

Running title: PAPP-A deficiency improves survival of mice on HFD

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ABBREVIATIONS

PAPP-A, pregnancy-associated plasma protein-A

HFD, high fat diet

KO, knock-out

WT, wild-type

NSP, no specific pathologies

ABSTRACT

Obesity is on the rise in Westernized countries, and visceral obesity in particular is associated with enhanced risk of developing metabolic disease and accelerated aging. Various dietary restriction regimens have been shown to extend healthy lifespan in a variety of species. However, identification of alternative approaches that could be more acceptable to humans is actively being pursued. We have shown previously that mice deficient in pregnancy-associated plasma protein-A (PAPP-A) have an extended healthy lifespan on a regular chow diet. In this study, we determined the lifespan of PAPP-A knock-out (KO) and wild-type (WT) littermates fed a high fat diet (HFD) starting at 12 months of age. PAPP-A KO and WT mice had equivalent weight gain as measured over 25 weeks on HFD. However, PAPP-A KO mice on HFD had a significant increase in lifespan (P = 0.018). Body composition and tissue pathology were assessed in a separate cohort of mice after 30 weeks on HFD. Percent body fat was equivalent in the two groups. However, there was a decrease in visceral fat depot weights and an increase in serum adiponectin levels in PAPP-A KO compared to WT mice. Major pathological differences were seen in kidney, heart and testes, with PAPP-A KO mice having little, if any, evidence of inflammation, mineralization, or degeneration in these tissues compared to WT mice. Thus, PAPP-A is a novel drug target with the potential to promote healthy longevity without a need for dietary restriction.

Key Words: pregnancy-associated plasma protein-A, lifespan, gene knock-out, mouse model, high fat diet

1. INTRODUCTION

To live an extended healthy life is a highly desirable human goal. The best studied anti-aging interventions, caloric/dietary restriction, have been shown to extend lifespan (and healthspan) across a variety of species (Masoro, 2005). However, people in the Westernized world like to eat sweets and fatty foods and long-term compliance with such a strict regimen would be understandably poor (Dirks & Leeuwenburgh, 2006). This has led to a search for treatments that would produce pro-longevity effects without extreme modification of diet (Ingram *et al.*, 2004).

Pregnancy-associated plasma protein-A (PAPP-A) is a novel zinc metalloproteinase that can promote local insulin-like growth factor (IGF) signaling through the proteolysis of inhibitory IGF binding proteins (reviewed in Conover, 2012). Reduced IGF signaling has been shown to be associated with longevity in a variety of species, and may be part of the effectiveness of caloric restriction (Kenyon, 2001; Sell, 2003). We found that PAPP-A knock-out (KO) mice with reduced local IGF bioavailability have an ~30% increase in median and maximum lifespan, and decreased incidence and severity of age-related degenerative diseases and delayed occurrence of cancer compared to wild-type (WT) littermates when maintained on a regular chow diet (Conover *et al.*, 2010). Since adiposity, especially visceral adiposity, is associated with accelerated aging (Muzumdar *et al.* 2008), we determined whether PAPP-A deficiency would increase lifespan in mice fed a high fat diet.

2. MATERIAL and METHODS

2.1. Mice. Male PAPP-A KO and WT mice from matings of heterozygous mice on a mixed C57BL/6 and 129/SvE background were used in these studies. Genotyping was performed as previously described (Conover *et al.* 2010). Mice were fed a high fat diet (HFD, 60% of calories from fat; Dyets, Inc., Bethlehem, PA; Suppl. Table 1) starting at 12 months of age. Male mice were chosen for this study because, unlike female mice, there was no developmental impact on the different fat depots (Conover *et al.*, 2013).

Mice were housed (4 to 5 per cage, mix of WT and KO mice) in static autoclaved HEPA-ventilated microisolator cages measuring 446 square centimeters (27 x 16.5 x 15.5 cm), which are opened only within Class II biosafety cabinets or animal change stations. These cages were located in a standard pathogen-free mouse facility. The facility has a 12-h light-dark cycle. Mouse colonies in this facility are monitored through contact sentinels (one cage per rodent rack) which are evaluated quarterly for (and are free of) the following agents: Sendai virus, pneumonia virus of mice (PVM), mouse hepatitis virus (MHV), minute virus of mice (MVM), Theiler's murine encephalomyelitis virus (TMEV strain GDVII), reovirus, rotavirus (EDIM), mouse parvovirus (MPV strain 1 and 2), murine norovirus (MNV), *Mycoplasma pulmonis*, *Aspiculuris tetraptera* and *Syphacia* spp. In addition, the following are annually screened for (and free of) these agents: lymphocytic choriomeningitis virus (LCMV), ectromelia virus (mousepox), K virus, polyoma virus, mouse adenovirus (MAV type 1 and 2), hantavirus, Prospect Hill virus (PHV), mouse cytomegalovirus (MCMV), lactate dehydrogenase dlevating virus (LDEV) *Encephalitozoon cuniculi*, cilia-associated respiratory bacillus (CARB), mouse thymic virus (MTV, MTLV), *Clostridium piliforme*, *Myobia* spp and *Myocoptes* spp. Autoclaved Enrich-o'Cobs (The Andersons Incorporated,

Maumee, OH, USA) were used as bedding. Cages and bedding were changed weekly. Mice had ad libitum access to municipal city water that was sterilized via ultraviolet light and irradiated pelleted diet. Room temperature was maintained between 21°C and 23°C. All procedures were approved by the Institutional Animal Care and Use Committee of Mayo Clinic.

Mice were examined daily, including weekends and holidays. For the longevity group, mice were considered to be at end of life and euthanized by carbon dioxide inhalation according to American Veterinary Medical Association Guidelines if they were moribund and demonstrated one or more clinical signs suggesting imminent death: nonresponsive to being touched, labored breathing, failure to eat or drink. Fourteen WT and 12 PAPP-A KO mice were euthanized. Two WT mice and one PAPP-A KO mouse were found dead-in-cage. In a separate experiment, 12-month-old mice were put on HFD for 30 weeks. Body composition was assessed, and mice then sacrificed and tissues harvested for pathological analyses.

2.2. Body composition. Body composition (total body lean and fat mass) was assessed in unanesthetized mice by quantitative magnetic resonance (EchoMRI-100, Houston, TX).

2.3. Pathology. Liver, lung, kidney, heart, testes, spleen, skeletal muscle, and adipose tissue (inguinal, mesenteric, epididymal depots) were fixed in 10% formalin and sent to the Small Animal Histology Core at Mayo Clinic, Scottsdale, AZ to be embedded, sectioned and stained with hematoxylin-eosin. These sections were then examined microscopically and assessed by an American College of Veterinary Pathologists board-certified veterinary

pathologist (RJM), who was blinded to mouse genotype. The histopathology definitions were based on standard descriptions and terminology. Lesions were given one of four subjective grades of minimal, mild, moderate and marked where appropriate.

2.4. Adiponectin. Serum adiponectin was measured using a double antibody radioimmunoassay kit (Linco Research Inc., St. Louis, MO).

2.5. Statistical analyses. Kaplan-Meier survival curves were compared using log-rank test in JMP Pro 9.0.1. Student's *t* test was used to compare WT and PAPP-A KO mice. Fisher's exact test was used to compare proportions of mice between groups. $P < 0.05$ was considered statistically significant.

3. RESULTS

3.1. Longevity. Male PAPP-A KO and WT mice were fed a high fat diet (HFD) starting at 12 months of age. Mice were weighed weekly up to 25 weeks on HFD, and the two groups showed equivalent weight gain (Fig. 1). At clinical signs of imminent mortality mice were considered at the endpoint of life. Survival distribution is presented in Figure 2. We found that PAPP-A KO mice had a significant extension of lifespan on the HFD ($P = 0.018$). Mean lifespan was 41 weeks of HFD (93 weeks of age) for WT mice, and 52 weeks of HFD (104 weeks of age) for PAPP-A KO mice. In a previous longevity study of male mice on chow diet, mean lifespan values were 97 weeks of age for WT mice and 121 weeks of age for PAPP-A KO mice (Conover *et al.*, 2010).

3.2. Pathology. As in the longevity study, a separate cohort of male PAPP-A KO and WT mice were fed HFD starting at 12 months of age. After 30 weeks on HFD, mice were weighed, body composition was assessed, and tissues were weighed and then fixed for pathological analyses. The percent fat, as determined by EchoMRI, was not different between WT (44 $\pm$ 3.4%) and PAPP-A KO (42 $\pm$ 3.6%) mice. Table 1 presents tissue weights of mice 30 weeks on HFD, normalized for body weight. There was a significant decrease in the mesenteric fat depot in PAPP-A KO mice compared to WT mice. Weights of the other fat depots and kidney, spleen, heart, lung and quadriceps muscle were not significantly different between WT and PAPP-A KO mice. Liver and testes weights were increased in PAPP-A KO mice. Absolute body and tissue weights (in grams) are presented in Supplemental Table 2.

Table 2 presents a summary of tissues from WT and PAPP-A KO mice that were diagnosed by the veterinary pathologist as having various age-related spontaneous pathologies. Ninety to 100% of the fat depots and the quadriceps muscles from both WT and PAPP-A KO mice had no specific pathologies (NSP) identified. Liver, spleen and lung had equivalent pathologies in WT and PAPP-A KO mice. For liver, the pathological assessment indicated hepatocellular vacuolization, hepatocellular carcinoma/adenoma, and hemangioma. Spleens showed inflammation and lymphoid hyperplasia. Lung had prominent peri-vascular/peri-bronchiolar lymphoid tissue, neoplasms, mineralization, and alveolar cell hyperplasia. On the other hand, kidney, heart and testes showed marked differences between WT and PAPP-A KO mice, and more detail on the pathology in these tissues is presented in Table 3. Twelve percent of kidneys from WT mice had NSP, whereas 50% of kidneys from PAPP-A KO mice had NSP. Seventy-five percent of WT kidneys, but only 20% of PAPP-A KO kidneys, showed obvious inflammatory infiltration, which was generally graded as more severe in WT kidneys. Likewise, 50% of WT kidneys had mineralization, whereas this was present in only 20% of PAPP-A KO kidneys. Two WT kidneys (25%) had indices of chronic nephropathy not seen in PAPP-A KO kidneys. Furthermore, 88% of WT kidneys and only 10% of PAPP-A KO kidneys had multiple abnormalities. All of the PAPP-A KO hearts were NSP, but only 50% of WT hearts were NSP. Heart abnormalities in the WT mice included inflammation and mineralization. Testes were also included in the histopathology, since we had seen marked differences in this tissue in WT and PAPP-A KO mice on chow diet (Conover *et al*., 2010). On HFD, the incidence and severity of testes degeneration and mineralization seen in WT mice was attenuated in PAPP-A KO mice. Although differences between PAPP-A KO and WT are indicated in the pathological analyses of kidney, heart and testes, the use of Fisher's exact test to compare proportions

did not yield statistically significant results, likely due to the small sample size. Therefore, conclusions about the data must be considered tentative.

4. DISCUSSION

The major new finding from this study is that, in the absence of PAPP-A, mice live significantly longer than WT mice, even when fed a high fat diet.

Body composition and tissue pathology were assessed after 12-month-old mice were 30 weeks on high fat diet, at a time when approximately 80% of WT would be expected to be alive, based on the survival curve, and at an age when differences in pathology were seen between WT and PAPP-A KO mice on chow diet (Conover *et al.*, 2010). On HFD, percent body fat was equivalent in WT and PAPP-A KO mice, but there was a significant decrease in mesenteric fat in PAPP-A KO mice. The relative decrease in weight of mesenteric fat, the visceral fat depot in mice, was also seen in young PAPP-A KO mice 20 weeks on a diet with 42% of calories from fat (Conover *et al*., 2013). In that study, adipocyte size was reduced and there was enhanced insulin-stimulated signaling in mesenteric fat compared to subcutaneous fat. Although not directly assessed in our HFD study, a recent study by Hill *et al*. (2015) demonstrated that female PAPP-A KO mice fed a high fat, high sucrose diet for 10 weeks starting at 12-14 months of age (similar to our protocol) were significantly more glucose tolerant and insulin sensitive than WT littermates. They speculated that this resistance to metabolic dysfunction with age could contribute to the enhanced longevity of PAPP-A KO mice.

Interestingly, the pathology of the other tissues assessed in WT and PAPP-A KO mice on HFD for 30 weeks (19.5 months of age) was very similar to what we found in 18-month-old mice on chow diet (Conover *et al.*, 2010). In particular, the evaluation of kidney, heart and testes indicated that PAPP-A KO mice had reduced incidence and severity of age-related,

but not necessarily diet-related, inflammation and degeneration. Serum adiponectin levels were significantly increased ($P < 0.05$) in PAPP-A KO compared to WT mice [6910 ± 251 ng/ml (N=8) versus 5770 ± 199 ng/ml (N=10)] 30 weeks on HFD. In another study, PAPP-A KO mice had significantly higher levels of circulating adiponectin compared to WT mice when on a low fat/low sucrose diet, and levels did not fall as they did in WT mice when on a 12-week high fat/high sucrose diet (Hill *et al.*, 2015). Adipose tissue-derived adiponectin plays a protective role in the development of cardiovascular disease and inflammation (Ouchi *et al*., 2006), and, therefore, may contribute to the healthy longevity of PAPP-A KO mice. Adiponectin levels are also increased with caloric restriction (Zhu *et al.*, 2004). Marked reduction in testicular degeneration in PAPP-A KO mice was of particular interest, since another approach to slow aging, i.e., rapamycin treatment, was found to accelerate testicular degeneration in mice (Wilkinson *et al*., 2012). When normalized to body weight, testes weight in PAPP-A KO mice was ~ 30% increased compared to WT mice. This relative increase in weight may reflect the reduction in testicular degeneration.

Liver had equivalent pathologies in WT and PAPP-A KO mice on HFD as was seen on chow diet (Conover *et al*. 2010). However, one difference was the cytovascular vacuolization in liver from both WT and PAPP-A KO mice, which was not seen in liver from mice on chow diet. This vacuolization is consistent with ectopic fat deposition in liver. It was not surprising that PAPP-A KO mice were provided little protective effect on liver pathology, such as that seen in kidney, heart and testes, since liver from WT mice expresses very little PAPP-A (Conover *et al*., 2011). It is unclear why the livers of PAPP-A KO mice on HFD were heavier than those of WT mice. Further studies will be necessary to determine if there are quantitative differences in the ectopic fat:stromal components that could contribute to this difference.

PAPP-A deletion in mice appears to provide many, if not all, of the beneficial effects of caloric restriction: longevity, resistance to age-related degenerative changes, reduced inflammation, and metabolic health. Although PAPP-A KO mice are small in size, like caloric restricted mice and several long-lived mouse strains, we have shown that rescuing the dwarf phenotype in PAPP-A KO mice does not impact their extended lifespan (Conover *et al*., 2010). Thus, inhibition of PAPP-A expression or activity could be a caloric restriction mimetic. Resveratrol has also been considered as a calorie restriction mimetic, and has been shown to increase the health and lifespan of mice on a high fat diet (Baur *et al*., 2006). However, its bioavailability in humans is controversial (Lam *et al.*, 2013). Interestingly, resveratrol is a potent inhibitor of PAPP-A expression in human coronary artery smooth muscle cells (Conover *et al.*, 2006) and visceral fat preadipocytes (Davidge-Pitts *et al*., 2014), and we have proposed that resveratrol-induced down-regulation of PAPP-A expression, secretion and local cell association could contribute to its beneficial effects. Thus, PAPP-A is a novel, accessible drug target with the potential to promote longevity without dietary restriction.

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The authors have no conflicts of interest.

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AUTHOR CONTRIBUTIONS

Cheryl A. Conover: Designed the experiments, analyzed the data, prepared the figures, drafted the manuscript, and approved the final version of the manuscript.

Laurie K. Bale: Performed the experiments, and reviewed and edited the manuscript.

Ronald J. Marler: Performed the pathological analyses in a blinded fashion, and reviewed and edited the manuscript.

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Table 1. Tissue weights of mice 30 weeks on HFD

Weight (% of body weight)

Fat Depots	**WT**	**PAPP-A KO**
Inguinal	4.8 ± 0.43	4.8 ± 0.60
Epididymal	7.6 ± 0.40	6.4 ± 0.65
Peri-renal	1.4 ± 0.14	1.2 ± 0.12
Mesenteric	1.8 ± 0.16	1.2 ± 0.18*
Pericardial	0.24 ± 0.04	0.18 ± 0.04
Other Tissues		
Kidney	0.9 ± 0.08	1.0 ± 0.05
Liver [a]	2.7 ± 0.11	3.6 ± 0.32*
Spleen	0.3 ± 0.04	0.2 ± 0.04
Heart	0.3 ± 0.01	0.3 ± 0.02
Lung [a]	0.6 ± 0.05	0.5 ± 0.05
Quadriceps	0.4 ± 0.03	0.4 ± 0.03
Testes	0.3 ± 0.04	0.4 ± 0.03#

Male WT (N=9) and PAPP-A KO (N=10) mice were fed a HFD for 30 weeks starting at 12 months of age. Results (tissue weight as a % of body weight) are mean ± SEM.

[a] Liver (2) and lung (1) with obvious tumors were excluded from weight calculations.

*$P < 0.05$

#$P < 0.06$

Table 2. Overview of Tissue Pathology

Age-related Spontaneous Pathology (% of Mice)

	WT	**PAPP-A KO**
Inguinal	0	0
Epididymal	0	10
Peri-renal	0	0
Mesenteric	0	10
Kidney	88	50 *
Liver	100	100
Spleen	38	40
Heart	50	0 *
Lung	50	40
Quadriceps	12	10
Testes	75	20 *

* Details in Table 3

Percent of WT (N=8) and PAPP-A KO (N=10) mice with the indicated tissues assessed as having age-related spontaneous pathology.

Table 3. Tissue Pathology

	WT		PAPP-A KO	
Kidney				
NSP	1	(12%)	5	(50%)
Inflammation	6	(75%)	2	(20%)
Nephropathy	2	(25%)	-	(0%)
Tubule degeneration / regeneration	2	(25%)	2	(20%)
Mineralization	4	(50%)	2	(20%)
Multiple abnormalities	7	(88%)	1	(10%)
Heart				
NSP	4	(50%)	10	(100%)
Inflammation	3	(38%)	-	(0%)
Mineralization	1	(12%)	-	(0%)
Testes				
NSP	2	(25%)	8	(80%)
Degeneration	3	(38%)	1	(10%)
Mineralization	4	(50%)	1	(10%)

Values presented are absolute numbers of mice (with percentage in parentheses) with the indicated pathological finding.

NSP – No specific pathology.

FIGURE LEGENDS

Figure 1. Wild-type (open squares) and PAPP-A KO (solid diamonds) mice on high fat diet: Weight gain.

Each point represents the mean of 16 WT and 13 PAPP-A KO mice. Starting weights were 40.9 $\pm$ 1.72 g and 27.1 $\pm$ 1.5 g for WT and PAPP-A KO mice, respectively. Error bars were omitted for clarity.

Figure 2. Wild-type (dotted line) and PAPP-A KO (solid line) mice on high fat diet: Survival curve.

Survival curves generated from the data on 16 WT and 13 PAPP-A KO mice were compared using Log-Rank test in JMP Pro 9.0.1.

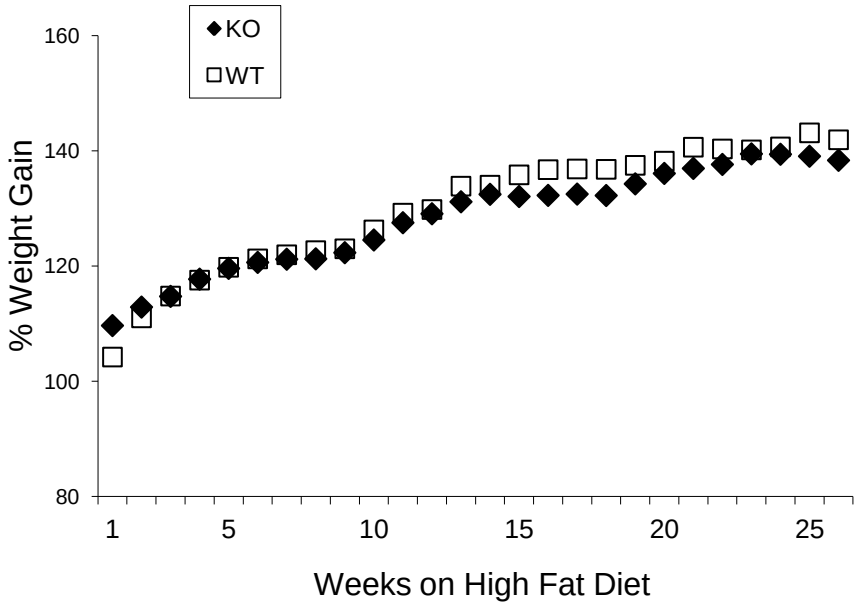

Figure 1

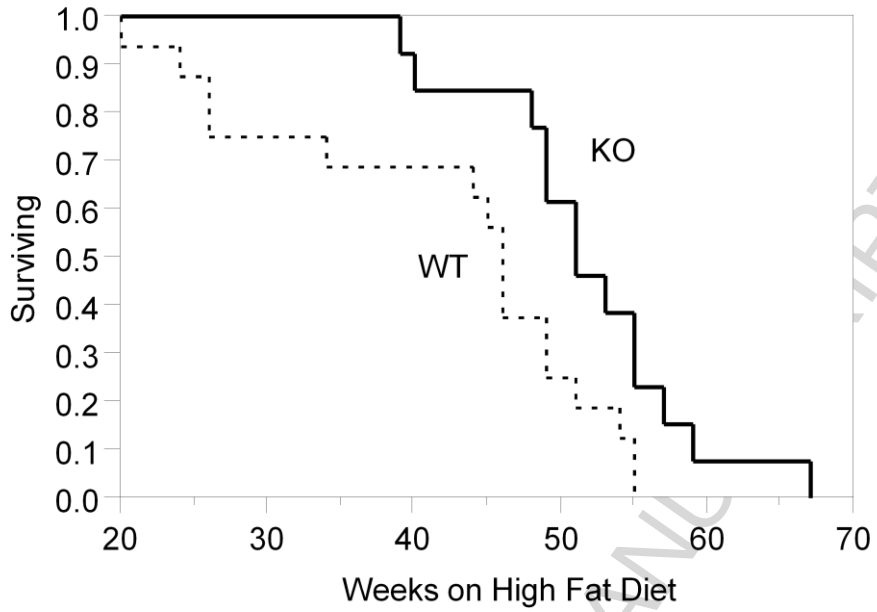

Figure 2