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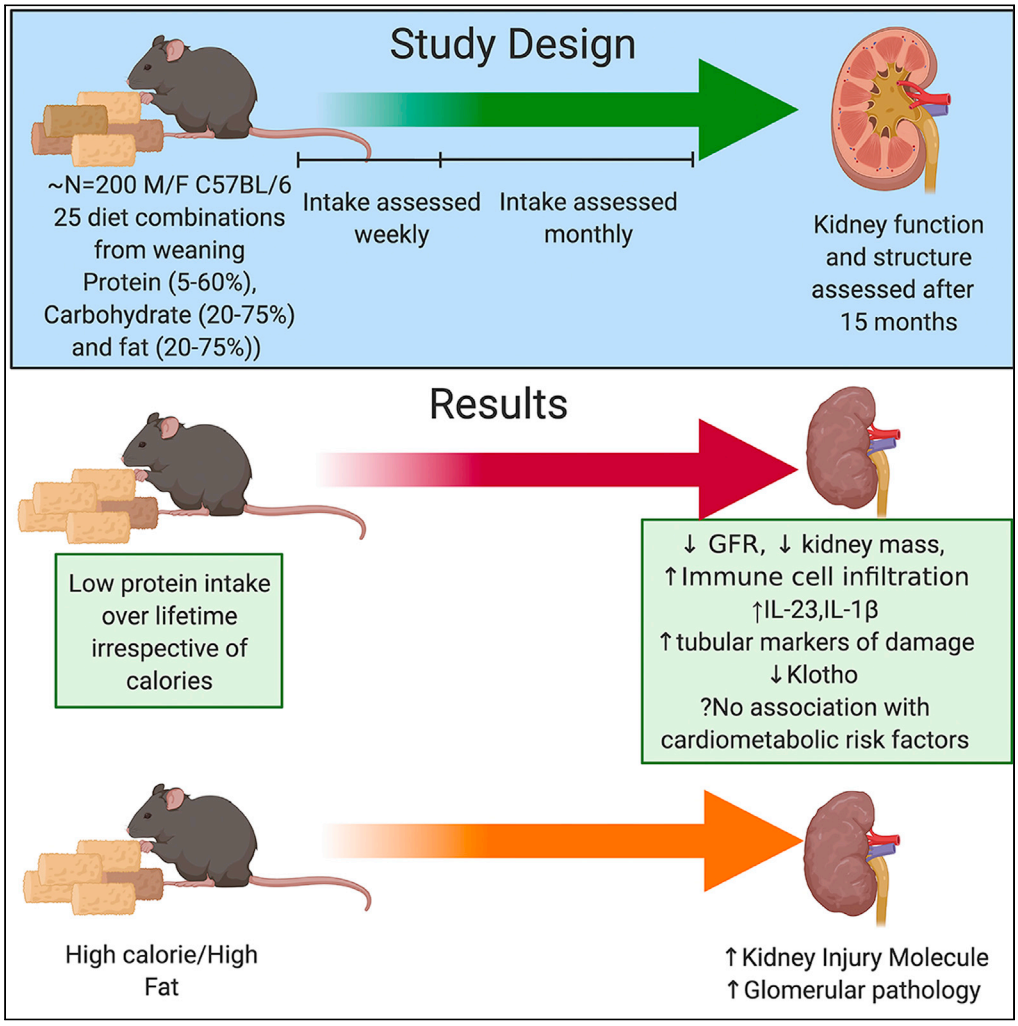

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Highlights

Chronic high macronutrient intake from any source increases kidney function (GFR)

Low protein intake led to greater kidney tubular structural injury and inflammation

Lower protein intake decreased kidney mass and glomerular filtration capacity

Kidney outcomes did not align with longevity or cardiometabolic outcomes

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Kidney disease risk factors do not explain impacts of low dietary protein on kidney function and structure

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SUMMARY

The kidneys balance many byproducts of the metabolism of dietary components. Previous studies examining dietary effects on kidney health are generally of short duration and manipulate a single macronutrient. Here, kidney function and structure were examined in C57BL/6J mice randomized to consume one of a spectrum of macronutrient combinations (protein [5%–60%], carbohydrate [20%–75%], and fat [20%–75%]) from weaning to late-middle age (15 months). Individual and interactive impacts of macronutrients on kidney health were modeled. Dietary protein had the greatest influence on kidney function, where chronic low protein intake decreased glomerular filtration rates and kidney mass, whereas it increased kidney immune infiltration and structural injury. Kidney outcomes did not align with cardiometabolic risk factors including glucose intolerance, overweight/obesity, dyslipidemia, and hypertension in mice with chronic low protein consumption. This study highlights that protein intake over a lifespan is an important determinant of kidney function independent of cardiometabolic changes.

INTRODUCTION

Affecting ~15% of the global population, chronic kidney disease (CKD) incidence is rising, reducing life expectancy and presenting a significant economic burden (Jha et al., 2013). CKD is a spectrum of disorders characterized by reduced kidney function and pathological structural changes, which commonly include leukocyte infiltration, fibrosis in the tubular compartment, and glomerulosclerosis (Levey and Coresh, 2012; Schlondorff, 2008), and age is among the most important initiators of CKD (Taal and Brenner, 2006). Many of these kidney pathologies also occur with aging (O'Sullivan et al., 2017; Zhou et al., 2008), and there is significant evidence that kidney function is a major predictor of all-cause mortality in the general population (Go et al., 2004; Waheed et al., 2013; Schmieder et al., 2011; Hallan et al., 2012). Hence, uncovering the determinants of kidney function with aging is of particular importance.

Nutritional imbalances drive many risk factors for CKD (Lozano et al., 2012; Hall et al., 2004) and adverse kidney aging (Odden et al., 2010; de Boer et al., 2009), as evidenced by large population-based studies in which factors that increase adiposity (Pinto-Sietsma et al., 2003; Foster et al., 2008; Chang et al., 2013; Kramer et al., 2005; Ejerblad et al., 2006; Gelber et al., 2005), cause glucose intolerance, exacerbate hypertension (Hall et al., 2014), or promote chronic inflammation (Ghigliotti et al., 2014; Hunley et al., 2010) or dyslipidemia (Hall et al., 2014; Joles et al., 2000) associate with leakage of albumin into the urine (albuminuria), declining renal function and causing end-stage kidney disease (Munkhaugen et al., 2009; Iseki et al., 2004; Vivante et al., 2012; Hsu et al., 2006; Asghari et al., 2018). Not surprisingly, those individuals with greater consumption of fruits and vegetables, but less non-dairy animal protein, show less microalbuminuria and reduced CKD risk (Lin et al., 2011; Nettleton et al., 2008; Asghari et al., 2018). However, most nutritional studies in kidney health, kidney aging, and CKD have focused on dietary consumption of individual macronutrients. For protein, there is a persisting hypothesis that habitual consumption of protein above the daily recommendation of 0.8 g/kg promotes CKD by increasing glomerular pressure and hyperfiltration (Metges and Barth, 2000; Brenner et al., 1982). In physiological studies high dietary protein has both acute

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Continued

and chronic effects on kidney function (Kontessis et al., 1990; Chan et al., 1988; Bergstrom et al., 1985; Jones et al., 1987), whereas population-based studies suggest that this only occurs where there is baseline kidney functional insufficiency (Knight et al., 2003). For other macronutrients, the findings are also contentious. In rodent studies, high-fat feeding does induce kidney injury, characterized by inflammation, glomerular swelling (hypertrophy), and abnormalities in kidney tubule function (Declèves et al., 2014; Harcourt et al., 2011). Whether these findings are recapitulated in humans is less clear (Yuzbashian et al., 2015; Diaz-Lopez et al., 2013). In the largest study to date, Lin et al. found no association between total dietary fat intake and albuminuria or loss of kidney function, although saturated fat intake did associate with albuminuria (Lin et al., 2010). Gross carbohydrate intake has garnered the least amount of attention, where carbohydrate levels exceeding 50% of total calories impair kidney function in healthy rodents (Nakayama et al., 2010) and exacerbate obesity/diabetes-induced nephropathy (Velasquez et al., 1989). However, it should be noted that the source of carbohydrates in those studies were sucrose and fructose, which also affect glucose tolerance. Recent clinical data do support a positive relationship between carbohydrate intake and CKD risk (Nam et al., 2019), but in cross-sectional studies, high complex carbohydrate and fiber are associated with favorable kidney outcomes for aging kidneys (Gopinath et al., 2011; Mirmiran et al., 2018). Although these studies provide some insight, single-nutrient manipulations fail to provide a complete story.

Using a multidimensional modeling platform called the nutritional geometry framework (NGF), our previous studies showed that individually and additively, macronutrients have powerful effects on appetite, reproduction, cardiometabolic health, and aging over a lifetime (Solon-Biet et al., 2014, 2015; Cogger et al., 2016; Wahl et al., 2017). For the first time here, we utilize the NGF to systematically explore the complex effects of dietary macronutrients and energy on kidney structure and function in the longer term in aging mice.

RESULTS

Lower protein consumption into late-middle age reduces kidney function in a manner dependent on kidney mass

Ten different combinations of macronutrients across three caloric tiers (30 diets) were tested in mice without a specific genetic predisposition to kidney disease from weaning to late-middle age (15 months; Figure 1A, Table 1). Five very-low-protein, low-energy diets were discontinued at an early time point as they did not sustain a predefined growth rate following weaning (Figure 1A and Table 1). Individual and interactive effects of macronutrients were explored in a three-dimensional nutrient space and interpreted by generalized additive models (GAMs). Each surface (blue-red spectrum) shows two macronutrient planes, with the third macronutrient fixed at the median.

At 15 months of age, kidney function using glomerular filtration rate (GFR) was estimated using plasma cystatin C, which has an inverse relationship to GFR (Simonsen et al., 1985). Plasma cystatin C ranged from 126 to 1,007 ng/mL (Figure S1A). Dietary protein ($p = 0.027$) and carbohydrate (CHO) intake ($p = 0.012$) had the greatest influence on kidney function (Figures 1B and 1D), where GFR was the lowest in mice with low protein in combination with high carbohydrate intake (red areas; Figure 1B). Since GFR calculations in clinical settings consider body size (Cockcroft and Gault, 1976), cystatin C was also adjusted for lean muscle mass, strengthening the relationship between protein intake and GFR ($P < 0.0001$; Figures 1C and 1E) and the interactive effects of dietary protein and carbohydrate consumption on GFR ($P = 0.029$, Figures 1C and 1E). These relationships generally did not differ between male and female mice (Figure S1B–1I).

The kidneys also play an important role in maintaining the body's nitrogen balance (Weiner et al., 2015). Blood urea nitrogen (BUN), representing the balance between hepatic/renal urea production and renal urea excretion, is elevated with aging and in CKD following a decline in GFR (Seki et al., 2019; Fehrman-Ekholm and Skeppholm, 2004), and also reflects dietary protein intake (Frank et al., 2009) and biological age (Corless et al., 1975; Fehrman-Ekholm and Skeppholm, 2004). BUN measured at 15 months ranged from 2.1 to 20.7 mmol/L (Figure S2A) and was negatively related to cystatin C (Figure S2B), with the highest BUN concentrations seen in mice with greater protein consumption and lowest CHO and fat intake (Figures S2C–S2F). Unlike GFR, however, GAMs found that intake of each macronutrients was an independent determinant of BUN such that greater intake of each increased BUN ($p < 0.001$; Figures S2C–S2F). Circulating concentrations of major ions Na^+, K^+, and Cl^- were also measured, but no associations between macronutrient intake and the concentrations of these ions were found (Figures S3A–S3D).

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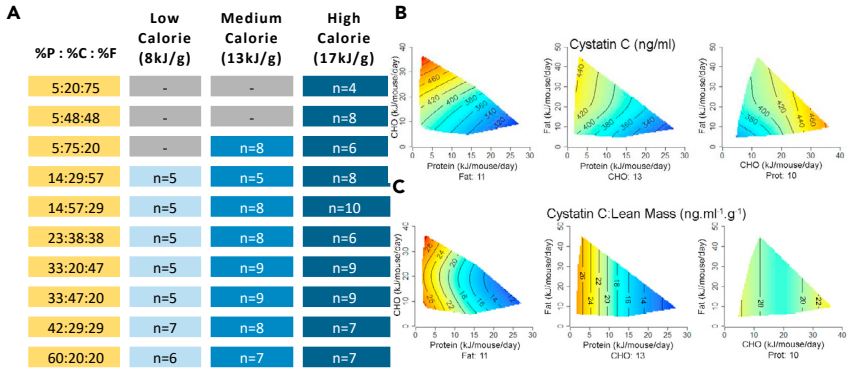
A

%P : %C : %F	Low Calorie (8kJ/g)	Medium Calorie (13kJ/g)	High Calorie (17kJ/g)
5:20:75	-	-	n=4
5:48:48	-	-	n=8
5:75:20	-	n=8	n=6
14:29:57	n=5	n=5	n=8
14:57:29	n=5	n=8	n=10
23:38:38	n=5	n=8	n=6
33:20:47	n=5	n=9	n=9
33:47:20	n=5	n=9	n=9
42:29:29	n=7	n=8	n=7
60:20:20	n=6	n=7	n=7

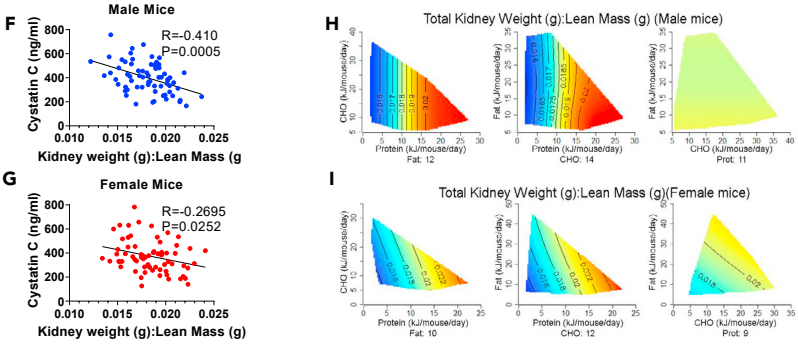


D

Coefficients of the GAMs for Cystatin C (ng/ml)	edf	Ref.df	F	p-value
Protein	0.772	8	0.423	0.0268 *
CHO	0.823	8	0.582	0.0124 *
Fat	0.462	8	0.107	0.1354
Protein and CHO	8.163×10^{-5}	3	0	0.5914
Protein and Fat	1.667×10^{-4}	3	0	0.4525
CHO and Fat	0.731	3	0.396	0.1684
Protein, CHO and Fat	2.655×10^{-4}	7	0	0.5537

E

Coefficients of the GAM for Cystatin C : lean mass (ng.ml^{-1}.g^{-1})	edf	Ref.df	F	p-value
Protein	0.965	8	3.426	<0.0001***
CHO	1.680×10^{-4}	8	0	0.475
Fat	4.208×10^{-6}	8	0	1
Protein and CHO	1.99	3	2.066	0.029 *
Protein and Fat	3.303×10^{-6}	3	0	0.944
CHO and Fat	4.882×10^{-6}	3	0	0.934
Protein, CHO and Fat	9.052×10^{-6}	7	0	0.998



J

Coefficients of GAM for total kidney weigh (g): lean mass (g) (males)	edf	Ref.df	F	p-value
Protein	2.706	8	7.84	<0.0001 ***
CHO	1.706×10^{-4}	8	0	0.91
Fat	5.413×10^{-5}	8	0	0.657
Protein and CHO	2.428×10^{-5}	3	0	0.692
Protein and Fat	0.615	3	0.325	0.197
CHO and Fat	8.404×10^{-5}	3	0	0.503
Protein, CHO and Fat	1.242×10^{-4}	7	0	0.787

K

Coefficients of GAM for total kidney weigh (g): lean mass (g) (females)	edf	Ref.df	F	p-value
Protein	1.311	8	16.227	<0.0001 ***
CHO	0.933	8	1.733	0.000157 ***
Fat	1.344	8	2.863	<0.0001 ***
Protein and CHO	8.794×10^{-5}	3	0	0.39882
Protein and Fat	8.803×10^{-5}	3	0	0.486262
CHO and Fat	1.107×10^{-4}	3	0	0.618179
Protein, CHO and Fat	5.151×10^{-5}	7	0	0.861342

Figure 1. Lifelong lower protein consumption into middle age reduces kidney function

(A) Diets used in this study and their respective n numbers; 10 dietary macronutrient combinations were selected, shown as the relative ratios of protein (%P), carbohydrate (%C), and fat (%F) content (yellow) and further refined into three tiers of energy content (kJ/g), low (light blue), medium (med, blue), and high (dark blue) to produce 30 distinct diets. Five of these diets (-, gray) were excluded at an early time point as they did not sustain a pre-defined growth rate. C57BL6/J mice were randomized to consume one of these diets from weaning (4 weeks of age) to 15 months of age in N = 174 mice (81 male:93 female, n = 4–10 mice per diet, shown in the blue boxes).

(B–E) Kidney function was assessed by plasma cystatin C, an inverse marker of glomerular filtration rate (GFR) in 145 of the GFN mice for which plasma was available. (B) Response surfaces showing the effect of macronutrient intake on unadjusted plasma cystatin C (N = 145). (C) Response surfaces showing the effect of macronutrient intake on lean mass-adjusted plasma cystatin C (N = 145).

(D) Coefficients for the GAMs for plasma cystatin C.

(E) Coefficients for the GAMs for plasma cystatin C adjusted for lean mass.

(F–K) Assessment of kidney weight (g) with relation to lean mass (g), sex, and kidney function in GFN mice. (F) Spearman's correlations between lean mass-adjusted plasma cystatin C and kidney weight for males (n = 68). (G) Pearson's correlations between lean mass-adjusted plasma cystatin C and kidney weight for females. (n = 69). (H) Response surfaces showing the effect of macronutrient intake on total kidney weight adjusted for lean mass in males (n = 77). (I) Response surfaces showing the effect of macronutrient intake on total kidney weight adjusted for lean mass in females (n = 85). (J) GAMs coefficients for total kidney weight adjusted for lean mass in males. Significant values shown in blue. (K) GAMs

Figure 1. *Continued*
coefficients for total kidney weight, lean mass adjusted in females. In all response surfaces, red represents the highest value, whereas dark blue represents the lowest. Colors are standardized across all slices. For all coefficients tables significant values are highlighted in blue. *p < 0.05, ***p < 0.001.

Renal mass commonly decreases with both kidney disease progression and aging (Weinstein and Anderson, 2010). Total kidney weight ranged from 0.170 to 0.538 g (Figure S4A), with males having greater renal mass (male, 0.391 [0.125] g versus female, 0.329 [0.094] g; p < 0.0001). When adjusted for lean mass, kidney mass did not differ between sexes (male, 0.018 ± 0.002 versus female, 0.018 ± 0.003 g/lean muscle mass; p = 0.5). Using univariate analyses, a negative relationship between cystatin C and kidney mass was demonstrable in both male (Figure 1F) and female (Figure 1G) mice, confirming that GFRs were lower as kidney mass:lean mass decreased. In male mice only protein intake significantly influenced kidney:lean mass (Figures 1H and 1J), although unadjusted kidney weight was influenced by all three macronutrients (Figure S4E and S4I). Conversely in female mice, kidney:lean mass was influenced independently by all three macronutrients (protein: p < 0.00001; carbohydrate, p = 0.00015; fat, p < 0.0001; Figures 1I and 1K).

Lower dietary protein intake into late-middle age results in kidney leukocyte infiltration and increases in pro-inflammatory cytokines

Leukocyte infiltration is an early pathological feature of CKD (Eddy, 2005) and occurs in kidney aging (O'Sullivan et al., 2017). The relationship between dietary intake of macronutrients and leukocyte infiltration into the kidney cortex was independently scored by a veterinary pathologist (Figure 2A, representative images of scoring) and examined using ordinal regression (Figure 2B). Here, less leukocyte infiltration (score of 1, red line; Figure 2B, left panel) was evident with greater dietary protein intake, whereas scores for higher infiltrate (score of 2, moderate infiltrate, green line; score of 3, high degree of infiltrate, blue line) showed a decrease as protein intake increased. Meanwhile, increasing fat intake was associated with increasing leukocyte infiltration (score of 3, blue line; Figure 2B, right panel). As dietary protein intake increased from <20 through to >40 kJ/day according to tertiles of intake, the chance of a lower leukocyte infiltration score also increased (Figure 2C, p = 0.0004). The opposite relationship was observed when tertiles of fat intake (low <8.58 kJ/mouse/day, medium [med] 8.59–13.8 kJ/mouse/day, high 13.8–44.9 kJ/mouse/day) were compared (Figure 2D; p = 0.012). T cell immunolabeling (CD3, CD4, FoxP3) was performed to characterize infiltrate in a small number of high-scoring (leukocyte score of 3) kidney samples (Figures 2E and S5A). CD3+ and CD3+, FoxP3+ Treg cells as well as CD3− non–T cells were present in infiltrates in kidneys.

Given the increases in leukocyte infiltration identified with chronic consumption of a low-protein diet, kidney cortical pro-inflammatory cytokines were assessed. These cytokines mediate inflammation and form key links between innate and adaptive immunity. Five of the thirteen cytokines measured were significantly associated with specific dietary macronutrient intake, particularly protein (Figures 2F–2J; Table S1). Kidney pro-inflammatory cytokines interleukin-23 (IL-23) and IL-1β increased as daily protein consumption decreased, independent of fat and carbohydrate intake (p = 0.003 and 0.011, respectively, Figures 2F–2H, Table S1). Kidney concentrations of interferon γ (IFNγ) and IL-12p70 also increased as dietary protein consumption declined in combination with greater dietary carbohydrate intake (Figures 2F, 2I, and 2J; Table S1). More dietary fat intake was associated with higher kidney IL-1β (*P* = 0.037, Figures 2F and 2H, Table S1). Kidney IL-1α and monocyte chemoattractant protein 1 (MCP-1) were affected by ratios of carbohydrate and fat consumption as interactive variables, but this did not reach significance for MCP-1 (p = 0.0583, Figures 2F, Figure S5C and 5D, Table S1). Macronutrient intake did not affect kidney concentrations of other cytokines analyzed (Figure 2F, Table S1).

Increase in the protein kidney injury molecule-1 (KIM-1) is considered a marker of kidney injury (Song et al., 2019; Bonventre, 2009). In the present study, kidney KIM-1 concentrations ranged from 6.72 to 216.9 pg/μg protein (Figure S6A) and were greater in male than in female mice (male, 106.7 (71.86) versus female, 77.14 (58.96) pg/μg of total protein; p = 0.001; Figure S6A). GAMs showed an independent association between carbohydrate intake and KIM-1 concentrations and intake of all three macronutrients (p = 0.00014; Figure 3A and 3B) with KIM-1 increasing modestly as protein increased and CHO intake decreased. KIM-1 concentrations were highest with greatest fat intake and lowest carbohydrate intake. However, neither protein nor fat intake was independent predictor of cortical KIM-1, suggesting that total energy intake may be an important determinant of kidney KIM-1 concentrations. Similar effects were seen when males and females were analyzed independently with increasing carbohydrate intake associated with decreasing KIM-1 and

Table 1. The range of dietary intakes of macronutrients achieved by mice on all diets consumed in the study

		Low			Med			High		
%Prot:CHO:Fat		Prot	CHO	Fat	Prot	CHO	Fat	Prot	CHO	Fat
5%: 75%: 20%	(kJ/kg D.wt)				628.69	9,414.00	2,510.40	838.25	12,552.00	3,347.20
	(kJ/mse/cage/d)				1.82 (1.68–2.44)	27.27 (25.08–36.49)	7.27 (6.69–9.73)	2.02 (1.53–2.44)	30.21 (22.91–36.49)	8.06 (6.11–9.73)
	g/kg of b.wt.day				4.91 (4.37–5.46)	73.50 (65.60–80.08)	8.68 (7.75–9.46)	5.04 (3.99–6.04)	75.45 (58.60–98.28)	8.91 (6.92–11.61)
5%: 20%: 75%	(kJ/kg D.wt)							838.25	3,347.20	12,552.00
	(kJ/mse/cage/d)							2.53 (2.13–3.00)	10.11 (8.5–11.98)	37.90 (31.89–44.93)
	g/kg of b.wt.day							6.58 (5.67–7.38)	26.3 (24.08–28.27)	43.69 (40.01–46.97)
5%: 48%: 48%	(kJ/kg D.wt)							838.25	8,033.28	8,033.28
	(kJ/mse/cage/d)							2.26 (2.05–2.45)	21.66 (19.61–23.49)	21.66 (19.61–23.49)
	g/kg of b.wt.day							5.74 (4.91–6.32)	55.038 (45.10–65.62)	24.38 (19.98–29.07)
14%: 29%: 57%	(kJ/kg D.wt)	1,173.55	2,426.72	4,769.76	1,760.33	3,640.08	7,154.64	2,347.10	4,853.44	9,539.52
	(kJ/mse/cage/d)	4.22 (3.39–4.72)	8.73 (7.02–9.77)	17.16 (13.70–19.20)	6.39 (5.94–6.81)	13.21 (12.30–14.08)	25.96 (24.17–27.67)	7.14 (5.79–8.53)	14.77 (11.97–17.64)	29.03 (24.84–34.68)
	g/kg of b.wt.day	11.88 (10.34–13.28)	24.85 (17.75–29.77)	21.63 (15.45–25.91)	15.11 (12.33–18.10)	31.95 (24.33–37.75)	27.82 (21.19–32.87)	12.14 (9.38–16.47)	26.2 (19.39–34.05)	22.81 (16.89–29.65)
14 : 57%: 29%	(kJ/kg D.wt)	1,173.55	4,769.76	2,426.72	1,760.33	7,154.64	3,640.08	2,347.10	9,539.52	4,853.44
	(kJ/mse/cage/d)	4.05 (3.75–4.43)	16.47 (15.22–18.00)	8.38 (7.74–9.16)	4.94 (4.25–5.69)	20.08 (17.27–23.11)	10.22 (8.79–11.76)	5.70 (4.68–6.68)	23.16 (19.04–27.16)	11.78 (9.69–13.81)
	g/kg of b.wt.day	11.89 (10.36–12.84)	48.41 (44.09–52.66)	10.91 (9.94–11.87)	10.43 (9.78–11.76)	42.79 (33.81–52.08)	9.64 (7.62–11.74)	11.44 (8.19–14.47)	47.17 (33.30–58.81)	10.63 (7.5–13.25)
23%:38%: 38%	(kJ/kg D.wt)	1,927.97	3,179.84	3,179.84	2,891.96	4,769.76	4,769.76	3,855.95	6,359.68	6,359.68
	(kJ/mse/cage/d)	7.6 (7.11–8.42)	12.54 (11.72–13.89)	12.54 (11.72–13.89)	8.52 (7.11–10.63)	14.05 (11.73–17.53)	14.05 (11.73–17.53)	10.30 (7.46–13.16)	16.98 (12.31–21.70)	16.98 (12.31–21.70)
	g/kg of b.wt.day	19.17 (18.36–20.13)	31.66 (28.54–35.60)	14.02 (12.64–15.77)	18.98 (15.52–22.83)	31.31 (25.59–37.65)	13.87 (11.34–16.68)	17.58 (14.28–20.52)	29.13 (23.55–33.85)	12.91 (10.43–14.99)
33%: 20%: 47%	(kJ/kg D.wt)	2,766.23	1,673.60	4,016.64	4,149.34	2,510.40	6,024.96	5,532.45	3,347.20	8,033.28
	(kJ/mse/cage/d)	10.01 (9.28–11.03)	6.06 (5.62–6.68)	14.53 (13.48–16.02)	12.33 (11.35–13.81)	7.46 (6.87–8.35)	17.91 (16.48–20.05)	13.04 (11.26–14.42)	7.89 (6.81–8.78)	18.94 (16.35–21.08)
	g/kg of b.wt.day	28.33 (27.45–29.56)	17.18 (16.61–17.91)	18.27 (17.66–19.04)	29.59 (26.56–36.05)	18.09 (16.36–21.80)	19.23 (17.08–23.18)	25.39 (21.64–33.58)	15.56 (13.09–20.31)	16.54 (13.92–21.60)

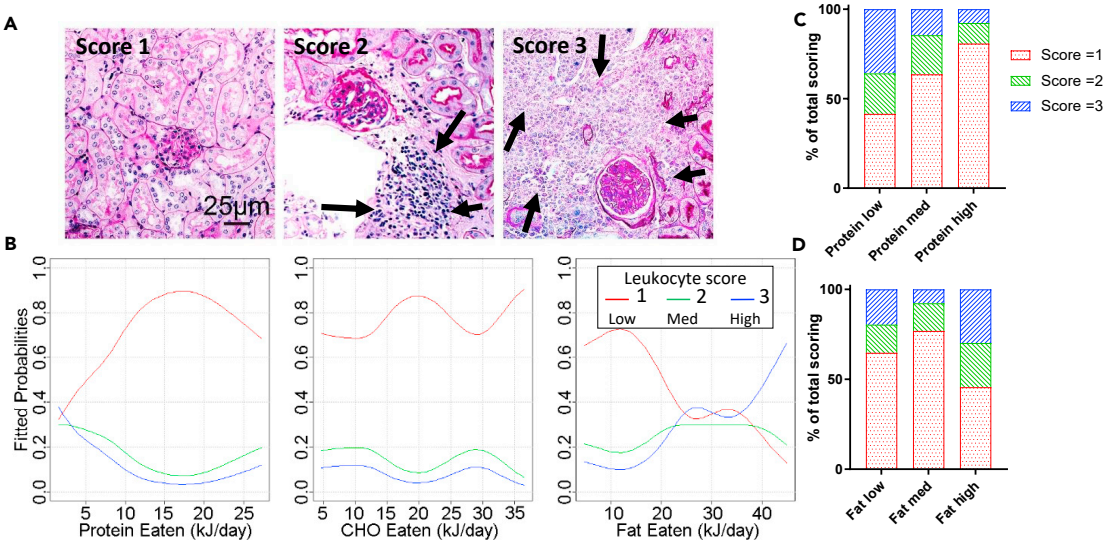
(*Continued on next page*)

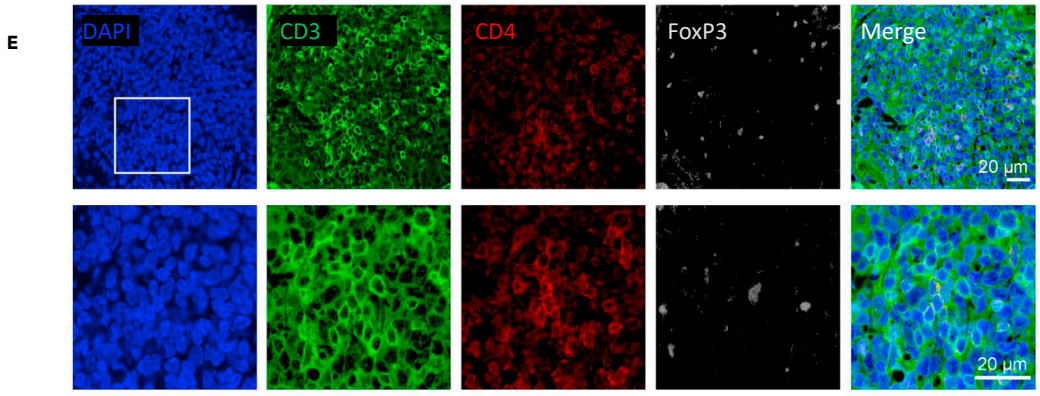
Table 1. *Continued*

		Low			Med			High		
%Prot:CHO:Fat		Prot	CHO	Fat	Prot	CHO	Fat	Prot	CHO	Fat
33%: 47%: 20%	(kJ/kg D.wt)	2,766.23	4,016.64	1,673.60	4,149.34	2,510.40	6,024.96	5,532.46	8,033.28	3,347.20
	(kJ/mse/cage/d)	**9.87** (8.93–11.25)	**14.34** (12.97–16.34)	**5.97** (5.40–6.81)	**12.33** (11.35–13.81)	**7.46** (6.87–8.35)	**17.91** (16.48–20.05)	**12.98** (10.64–16.37)	**18.84** (15.54–23.77)	**7.85** (6.44–9.90)
	g/kg of b.wt.day	**23.88** (22.22–27.73)	**34.98** (32.28–40.27)	**6.46** (5.96–7.43)	**29.59** (26.56–36.03)	**18.09** (16.07–21.08)	**19.23** (17.08–23.18)	**25.63** (21.04–28.88)	**37.38** (30.55–41.93)	**6.90** (5.64–7.74)
42%: 29%: 29%	(kJ/kg D.wt)	3,520.65	2,426.72	2,426.72	5,280.97	3,640.08	3,640.08	7,041.30	4,853.44	4,853.44
	(kJ/mse/cage/d)	**13.00** (10.51–15.24)	**8.96** (7.25-10.50)	**8.96** (7.25–10.50)	**14.00** (12.21–15.70)	**9.65** 8.42–10.82)	**9.65** (8.42–10.82)	**15.06** (12.75–17.48)	**10.38** (8.79–12.05)	**10.38** (8.79–12.05)
	g/kg of b.wt.day	**34.80** (29.57–42.82)	**24.33** (20.38-29.52)	**10.78** (9.03–13.08)	**32.04** (22.93–46.29)	**22.66** (15.81–31.91)	**10.04** (7.00–14.13)	**26.9** (21.06–37.77)	**19.54** (14.52–26.03)	**8.66** (6.43–11.53)
60%: 20%: 20%	(kJ/kg D.wt)	5,029.50	1,673.60	1,673.60	7,544.25	2,510.40	2,510.40	10,059.01	3,347.20	3,347.20
	(kJ/mse/cage/d)	**15.88** (14.09–17.98)	**5.29** (4.69–5.99)	**5.29** (4.69–5.99)	**19.66** (16.11–27.25)	**6.54** (5.36–9.07)	**6.54** (5.36–9.07)	**22.47** (18.24–25.82)	**7.48** (6.07–8.59)	**7.48** (6.07–8.59)
	g/kg of b.wt.day	**45.23** (40.10–54.37)	**15.27** (13.34–18.09)	**6.76** (5.91–8.02)	**45.05** (34.07–55.28)	**14.99** (11.34–18.39)	**6.64** (5.02–8.15)	**51.32** (41.35–64.77)	**17.73** (13.76–21.55)	**7.86** (6.09–9.55)

D, diet; Med, medium; mse, mouse.

Intakes are represented either by kJ/mouse/cage/day (kJ/mse/cage/d) or by g of diet per kg of bodyweight per day (g/kg of b.wt.day). Mean group intake is shown in bold with range of intakes shown in brackets. Total group intake is shown in bold.





F

Inflammatory Cytokine	Protein	Carbohydrate	Fat
IL-23	0.003	NS	NS
IL-1β	0.011	NS	0.037
IFNγ	0.0074		NS
IL-12p70	0.0521		NS
IL-1α		0.0282	
MCP-1		0.0583	
IL-17A	0.0629		
IFNβ	NS	NS	0.0694
IL-10	NS	NS	NS
IL-6	NS	NS	NS
IL-27	NS	NS	NS
GM-CSF	NS	NS	NS
TNFα	NS	NS	NS

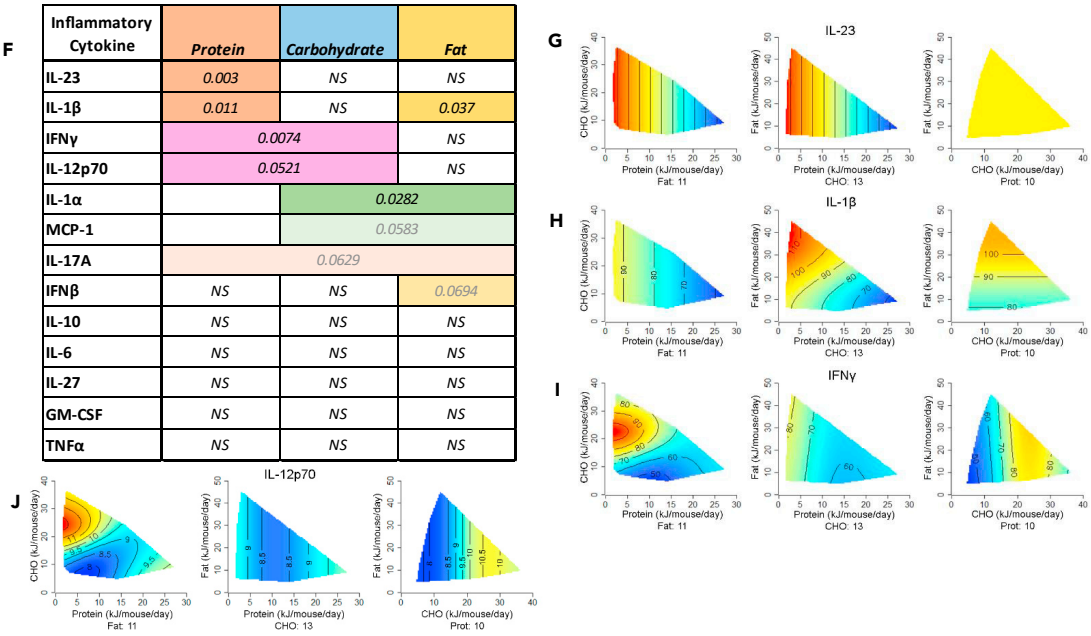


Figure 2. Lower dietary protein intake into middle age results in kidney leukocyte infiltration and inflammation

(A) Representative photomicrographs of leukocyte infiltration in periodic acid-Schiff (PAS)-stained kidney cortices according to score 1–3 (×200 magnification) (arrows indicate regions of leukocyte infiltrate).

(B) Ordinal regression was used to model the relationship between leukocyte infiltration and macronutrient intake, and plots show the probability of obtaining a score of 1 (red, low infiltration), 2 (green, moderate infiltration), or 3 (blue, high infiltration) as protein (left panel), CHO (middle panel), and fat (right panel) intake increased (n = 160).

(C) Kidney leukocyte infiltration scores presented by tertiles of dietary protein intake (low < 6.5 kJ/mouse/day, med 6.5–12 kJ/mouse/day, high 12.1–30 kJ/mouse/day).

(D) Kidney leukocyte infiltration scores presented by tertiles of dietary fat intake (low <8.58 kJ/mouse/day, med 8.59–13.8 kJ/mouse/day, high 13.8–44.9 kJ/mouse/day).

(E) Representative photomicrographs of regions of the renal cortex with leukocyte infiltrate (×800) stained for T cell markers CD3, CD4, FoxP3.

(F) Summarized p values for coefficients for the GAMs of kidney cytokine profiling using Legendplex 13-plex pro-inflammatory cytokine array. p values for individual effects (single column) or interactive effects (spanning 2 or more columns) of macronutrients on cortical cytokine profiles are shown (NS, non-significant; n numbers and full p values in Table S1).

(G–J) Response surfaces showing the effect of macronutrient intake on kidney cortex concentrations of select inflammatory cytokines (pg/μg of total protein). (G) IL-23, (H) IL-1β, (I) IFNγ, (J) IL-12p70.

protein intake an important interactive variable; however, fat intake was more important for male mice (Figures S6B–S6D).

Klotho, an important protein in mineral metabolism and aging (Kuro-o et al., 1997), is primarily produced by the kidney and decreases in CKD (Koh et al., 2001; Torres et al., 2007; Sugiura et al., 2012). Knockdown of klotho in mice results in accelerated aging and significantly shortened lifespan (Kuro-o et al., 1997), whereas overexpression increases the lifespan (Kurosu et al., 2005). Here, renal *Klotho* gene expression tended to reduce as dietary protein consumption decreased (Figure 3C) with mice consuming the most kilojoukes from protein, expressing more renal *Klotho*, although this did not reach significance (Figures 3C and 3D; $p = 0.06$). This relationship to dietary protein remained when stratified by sex but was not significant (Figures S6E–S6G). Immunofluorescence localized kidney klotho to aquaporin-1-positive tubules (Figure 3E), suggesting localization to the proximal convoluted tubule (Brandt et al., 2012).

Kidney markers of injury are exacerbated by chronic low dietary protein intake

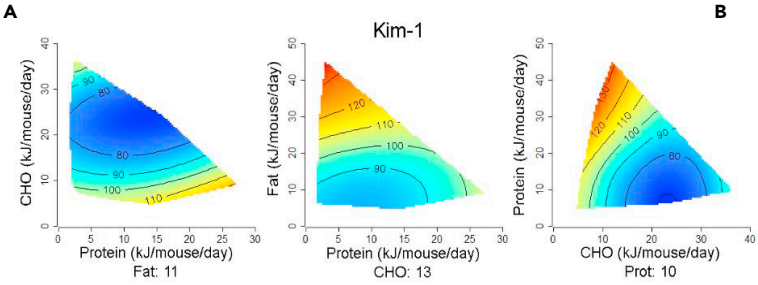
Injury to the vascularized filtration units of the kidney, the glomeruli, is common in CKD and with aging (Schlondorff, 2008). This includes thickening of the glomerular basement membrane and fibrosis within glomeruli (El Nahas and Bello, 2005; Webster et al., 2017). Kidney sections were scored by an independent veterinary pathologist blinded to the diets (representative images Figure 4A) and examined using ordinal regression (Figure 4B). Here, greater protein intake led to a decrease in the probability of glomerular injury (Figure 4B); however, this did not reach significance when examined by chi-square test (Figure 4C, $p = 0.27$).

Damage to the tubular compartment of the kidney is also characteristic of disease progression in CKD (Schlondorff, 2008). Again, greater protein intake was associated with decreased probability of tubular casts (Figure 4D, top left panel) and to a lesser extent tubular epithelial damage (Figure 4D, bottom left panel; Figure 4E, $p < 0.0001$ and $p = 0.0024$, respectively). Increasing fat intake also appeared to increase the probability of tubular epithelial damage (Figures 4D and 4E, bottom panels, $p = 0.0038$). However, macronutrient intake did not influence tubulointerstitial fibrosis as assessed using either Masson trichrome (Figures 4F–4H) or picrosirius red (Figures S7A and S7B).

Traditional cardiometabolic risk factors do not predict kidney injury seen with chronic low dietary protein intake

Growing evidence suggests that many cardiometabolic risk factors are accelerators rather than initiators of kidney injury (Forbes and Fotheringham, 2017). The correlogram (Figure 5A) displays the bivariate relationships among cardiometabolic risk factors and markers of renal health. Most cardiometabolic risk factors showed strong positive associations with body weight, fat mass, and lean mass (Figure 5A). Cystatin C and glomerular injury did not show any associations with CKD risk factors (Figure 5A), except a modest negative association for glomerular injury with one measure of glucose homeostasis, area under the curve (AUC) for an intraperitoneal glucose tolerance test (i.p.GTT). Cystatin C was positively related to tubular epithelial cell damage (Figure 5A), whereas glomerular injury, on the other hand, showed strong positive associations with other histological measures of kidney injury (tubular epithelial cell damage, tubular cast formation, and leukocyte infiltration) (Figure 5A). Measures of inflammation in the kidney such as

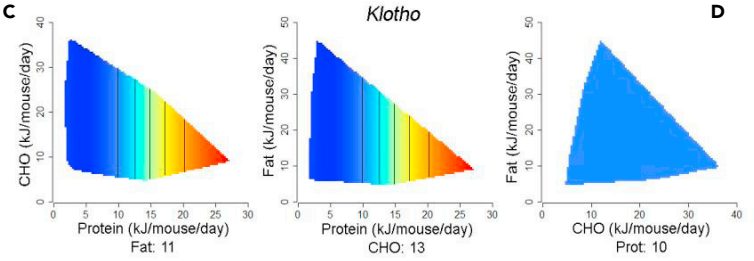
A



B

Coefficients of GAM Kidney Injury Molecule (pg/ug of protein) (All mice, N=152)				
	edf	Ref.df	F	p-value
Protein	1.908×10^{-4}	8	0	0.527
CHO	1.7560	8	1.79	0.00014 ***
Fat	2.144×10^{-4}	8	0	0.37654
Protein and CHO	1.018×10^{-4}	3	0	0.2878
Protein and Fat	8.099×10^{-4}	3	0	0.52711
CHO and Fat	2.886×10^{-5}	3	0	0.98321
Protein, CHO and Fat	1.2930	7	1.415	0.00139 **

C



D

Coefficients of GAM for *Klotho* (all mice) (N=149)				
	edf	Ref.df	F	p-value
Protein	1.25	8	0.441	0.0601 .
CHO	1.775×10^{-4}	8	0.00	0.9914
Fat	2.44×10^{-4}	8	0	0.5912
Protein and CHO	1.3×10^{-4}	3	0	0.6506
Protein and Fat	1.109×10^{-4}	3	0	0.6457
CHO and Fat	1.208×10^{-4}	3	0	0.8115
Protein, CHO and Fat	2.778×10^{-4}	7	0	1

E

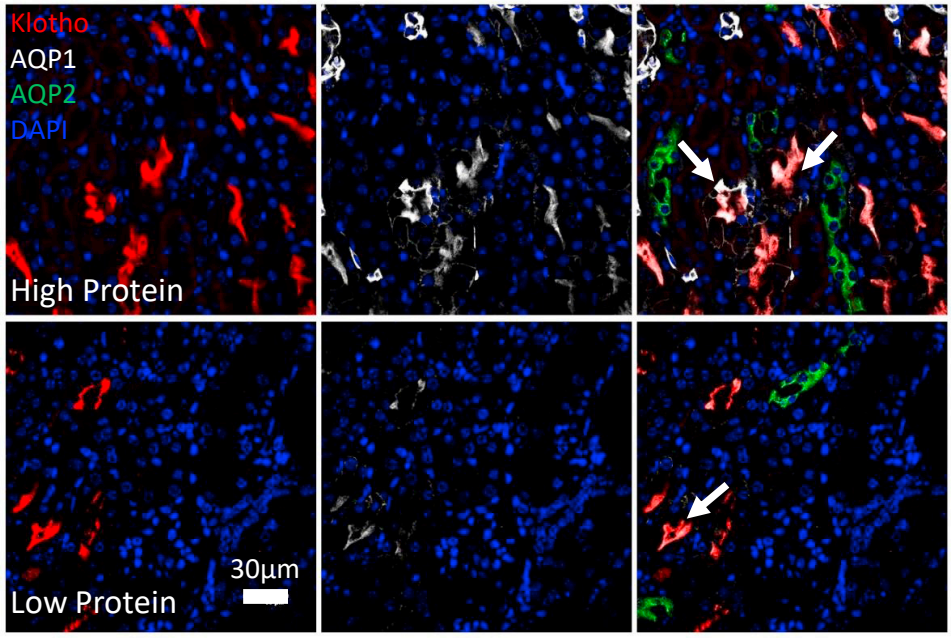


Figure 3. The relationship of pro-inflammatory protein kidney injury molecule-1 and anti-aging protein Klotho to macronutrient intake

(A) Response surfaces showing the effect of macronutrient intake on kidney cortex concentration of KIM-1 (pg/μg of total protein) in GFN mice (N = 152).

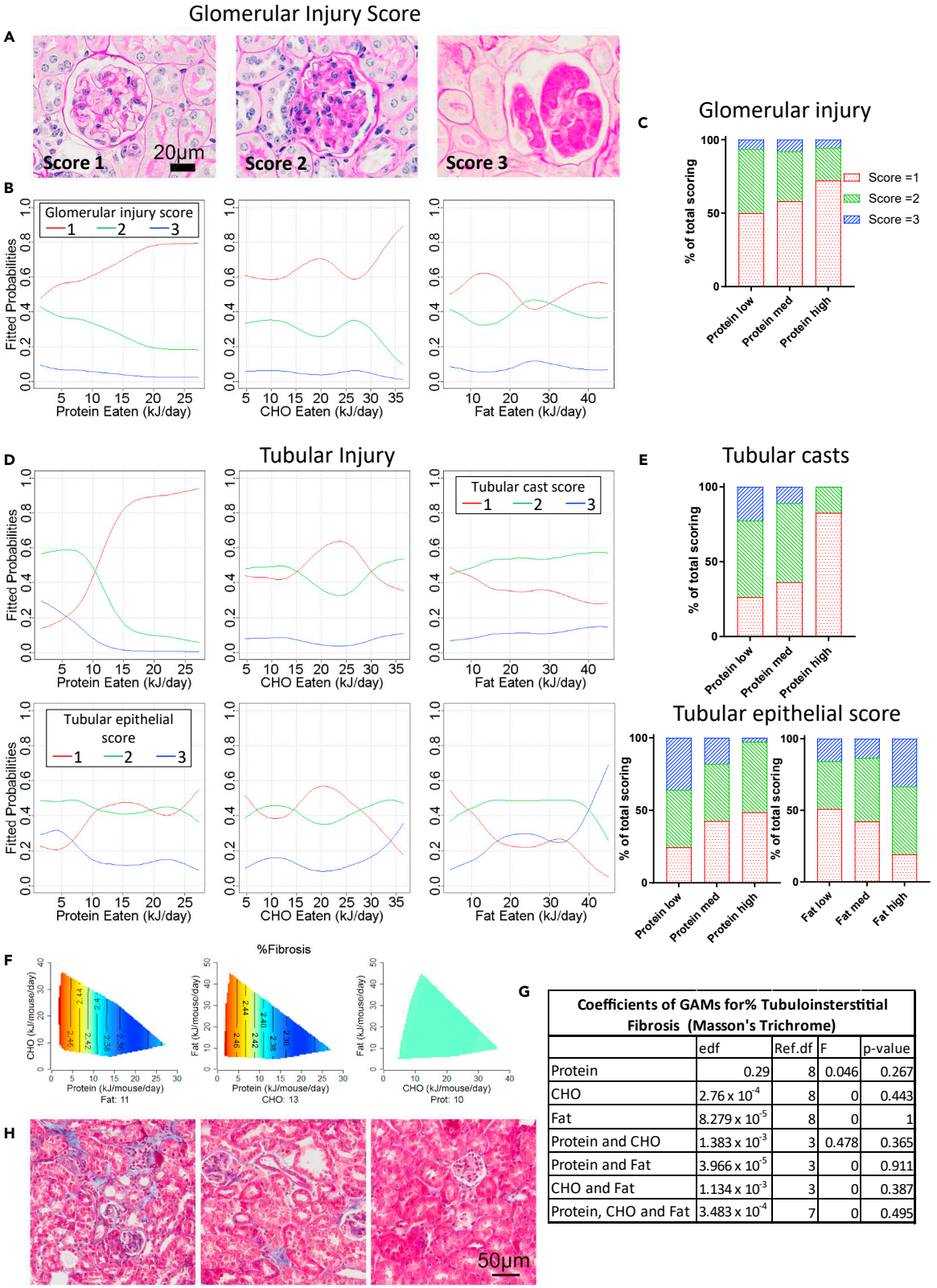
(B) GAMs coefficients for macronutrient-mediated effects on kidney injury molecule-1 (KIM-1) concentrations.

(C) Response surfaces for the effect of macronutrient intake on kidney cortex klotho expression (2^-(ΔCT), N = 149).

(D) Coefficients for GAMs for cortical expression of the gene klotho.

(E) Representative photomicrographs of the renal cortex (×400) depicting co-localization of klotho (red, left) with aquaporin 1 (white, center) and aquaporin 2 (green) in a high-klotho-expression kidney tissue (top panel) and a low-klotho-expression kidney tissue (lower panel). Nuclei are stained with DAPI (blue).

leukocyte infiltration and KIM-1 concentrations showed weak to modest negative associations with body weight, bone mineral density, and AUC during i.p.GTT (Figure 5A). KIM-1 additionally showed negative associations with other measures of adiposity and glucose homeostasis such as fasting plasma glucose and insulin (Figure 5A). When cardiometabolic risk factors in these mice were examined by GAMs, adiposity, blood pressure, glucose homeostasis (i.p.GTT AUC and insulin), and cholesterol did not show consistent responses to macronutrient intake (Figures 5B–5H, Table S2), although all but blood pressure did show significant relationships to macronutrient intakes (Table S2).



G

Coefficients of GAMs for% Tubuloinsterstitial Fibrosis (Masson's Trichrome)				
	edf	Ref.df	F	p-value
Protein	0.29	8	0.046	0.267
CHO	2.76×10^{-4}	8	0	0.443
Fat	8.279×10^{-5}	8	0	1
Protein and CHO	1.383×10^{-3}	3	0.478	0.365
Protein and Fat	3.966×10^{-5}	3	0	0.911
CHO and Fat	1.134×10^{-3}	3	0	0.387
Protein, CHO and Fat	3.483×10^{-4}	7	0	0.495

Figure 4. Markers of kidney injury are exacerbated by chronic low dietary protein intake

(A–C) Glomerular injury was scored on PAS-stained sections. (A) Representative photomicrographs of glomeruli scoring from PAS-stained kidney cortices (400×). (B) Ordinal regression plots showing the relationship between glomerular injury score and macronutrient intake. Higher score indicates greater degree of damage (red, score 1; green, score 2; blue, score 3; N = 160). (C) Scoring of glomerular injury by tertiles of dietary protein intake (upper panel, low <6.5 kJ/mouse/day, med 6.5–12 kJ/mouse/day, high 12.1–30 kJ/mouse/day) and by fat intake (lower panel, low <8.58 kJ/mouse/day, med 8.59–13.8 kJ/mouse/day, high 13.8–44.9 kJ/mouse/day).

(D) Ordinal regression plots showing the relationship between tubular injury score and each macronutrient. Tubular injury was defined by cast formation (upper panel) and epithelial damage (lower panel; sloughing, depolarization, loss of brush border, vacuolization). Higher score indicates greater degree of damage (red, score 1; green, score 2; blue, score 3; N = 160).

(E) Scoring of tubular injury by tertiles of dietary protein intake (tubular casts, upper panel) and protein and fat intake (tubular epithelial score, lower panel). Protein tertiles: low <6.5 kJ/mouse/day, med 6.5–12 kJ/mouse/day, high 12.1–30 kJ/mouse/day. Fat tertiles: low <8.58 kJ/mouse/day, med 8.59–13.8 kJ/mouse/day, high 13.8–44.9 kJ/mouse/day.

(F) Response surfaces showing the effect of macronutrient intake by 15 months of age on tubular interstitial fibrosis (N = 157).

(G) GAMs coefficients for kidney tubular interstitial fibrosis (N = 157).

(H) Representative photomicrographs of Masson trichrome-stained kidney cortices taken at each tertile of protein intake from mice consuming diets within the medium energy tier with an equivalent dietary fat content (×200 magnification).

DISCUSSION

Here, a multidimensional nutritional study design was used to examine the independent and interactive influence of dietary macronutrients and energy intake on markers of kidney structure and function in aging mice. Lower protein intake consistently had the greatest negative influence on renal parameters. Specifically, mice consuming less daily kilojoules from protein had the lowest GFRs and kidney mass:lean mass, highest plasma urea relative to lean mass, lowest klotho expression, and greatest kidney leukocyte infiltration and cytokine concentrations including IL-23, IL-1β, IFNγ, and Il-12p70. Furthermore, mice consuming less protein scored higher for markers of tubular atrophy and tubular epithelial cell damage, aligning with worse renal outcomes in the long term. Although there were some mice at the extremes of kidney pathology, much of this variation captured by surfaces and GAMs occurred within a subclinical, pre-pathological range. Importantly, high protein intake over a lifespan into late-middle age was not detrimental to overall kidney health, and instead greater protein intake, habitually over a life course, resulted in positive outcomes with regard to kidney size, GFR, inflammation, and immune cell infiltration in this mouse strain.

Protein intake increases GFR but does not worsen other renal outcomes

A GFR increase commensurate to protein intake has been reported many times (Kontessis et al., 1990; Chan et al., 1988; Bergstrom et al., 1985; Jones et al., 1987; Juraschek et al., 2013) and was not surprising given the kidney's role in the excretion of nitrogenous waste. As a nitrogenous byproduct of protein metabolism, urea has been reported to rise in the circulation proportionate to protein intake (Young et al., 2000) and can also influence GFR (Weiner et al., 2015; Oba et al., 2020). This has previously led to the postulate that this rise in GFR in the context of high protein metabolism, particularly in the context of the modern *ad libitum* diet, puts increased strain on the kidney increasing the risk for kidney disease (Bankir et al., 1996; Brenner et al., 1982, 1996). Indeed, unadjusted circulating urea concentrations (BUN) increased with increasing protein intake and likely contributed to changes in GFR observed in this study. However, when urea was adjusted for lean mass and body weight, both factors also influencing BUN, this relationship with protein was not observed.Furthermore, data in this present study do not support the hypotheses that greater dietary protein intake increases kidney damage in the absence of CKD. In the present study in which all genetic and environmental variables beyond diet were controlled, chronic high intake of dietary protein did elevate GFR, but was also protective against tubular damage and increases in inflammatory markers. Conversely, chronic low protein consumption resulted in lower GFRs and increases in markers of kidney damage. This is consistent with findings in humans where meta-analyses have revealed a mean increase in GFR with increasing protein intake in both men and women, but no loss of GFR in the long term (Schwingshackl and Hoffmann, 2014; Devries et al., 2018). Furthermore, the Modification of Diet in Renal Disease Study also showed a greater decline in GFR in the earlier stages of CKD with lower dietary protein intake (Levey et al., 2006). There is a paucity of long-term renal studies examining whether low protein intake in healthy humans affects the kidneys and their function. Certainly, malnutrition disorders and anorexia nervosa each have both short- and long-term renal and cardiovascular consequences (Klahr and Alleyne, 1973; Bouquegneau et al., 2012). It is important to highlight, however, that both in humans and rodent models of CKD, low to very low protein diets prevent progressive loss of GFR (Nath et al., 1986; Pedrini et al., 1996; Kasiske et al., 1998) and prolong progression to renal replacement therapy (Pedrini et al., 1996; Fouque et al., 1992, 2000). Considering this, the timing at which a diet is implemented may

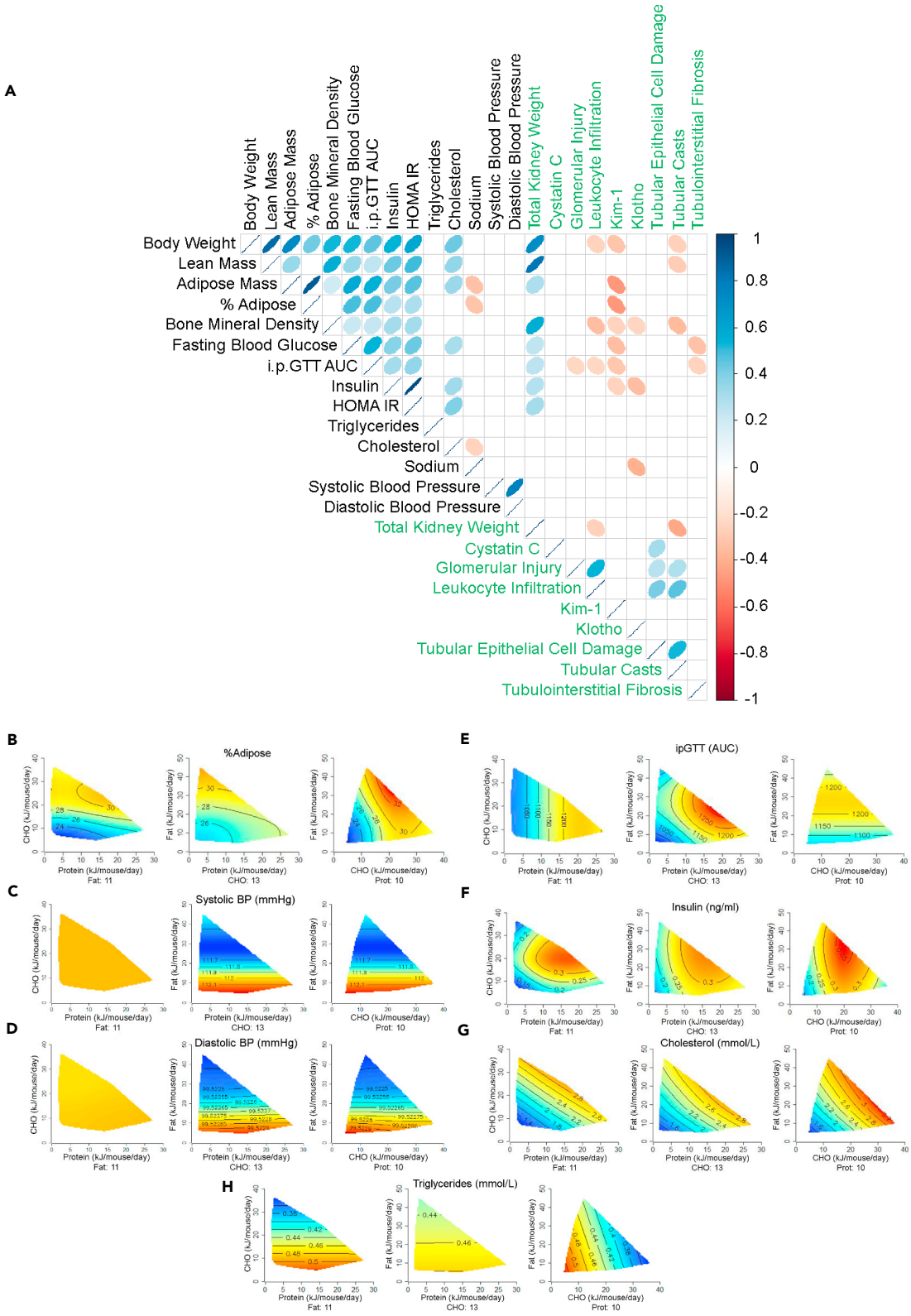

A
Body Weight
Lean Mass
Adipose Mass
% Adipose
Bone Mineral Density
Fasting Blood Glucose
i.p.GTT AUC
Insulin
HOMA IR
Triglycerides
Cholesterol
Sodium
Systolic Blood Pressure
Diastolic Blood Pressure
Total Kidney Weight
Cystatin C
Glomerular Injury
Leukocyte Infiltration
Kim-1
Klotho
Tubular Epithelial Cell Damage
Tubular Casts
Tubulointerstitial Fibrosis
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
B
%Adipose
E
ipGTT (AUC)
C
Systolic BP (mmHg)
F
Insulin (ng/ml)
D
Diastolic BP (mmHg)
G
Cholesterol (mmol/L)
H
Triglycerides (mmol/L)
CHO (kJ/mouse/day)
Fat (kJ/mouse/day)
Protein (kJ/mouse/day)
CHO (kJ/mouse/day)
Fat: 11
CHO: 13
Prot: 10

Figure 5. Traditional cardiometabolic risk factors do not associate with kidney injury seen with chronic low dietary protein intake

(A) Correlogram of Spearman's univariate correlations among traditional cardiometabolic risk factors (black) and kidney function (green) examined in GFN mice at 15 months of age. Color intensity and circle size are proportional to the correlation coefficients, with directionality of relationship indicated by the color (blue positive, red negative) and directionality of the ellipse. Only correlations where $p < 0.05$ (Bonferroni's correction applied) are indicated by an ellipse.

(B–H) Response surfaces showing the relationship between macronutrient consumption and cardiometabolic risk factors, (B) percentage adipose tissue, (C) systolic blood pressure, (D) diastolic blood pressure, (E) AUC glucose for 120 min i.p.GTT, (F) fasting plasma insulin, (G) plasma cholesterol, and (H) plasma triglycerides in GFN mice (n numbers and full p values supplied in Table S2).

be an important determinant in these findings. Here, mice began their allocated diets immediately following weaning, consuming this diet for their entire lifespan and during important growth phases, providing ample time for long-term physiological adaptation to the prevailing diet. In contrast, the majority of studies implement dietary changes only once disease has developed. Timing of dietary macronutrient intake with regard to growth and development may explain the disparity between our findings and previous work in models of CKD or individuals with renal disease. Further work in this area using direct measures of GFR and renal adaptability to dietary changes, as well as testing of renal functional reserve, are required. Examining the ability of the kidney to cope with a significant change in macronutrient intake following long-term adaption to a particular dietary macronutrient profile would be especially interesting.

The importance of protein intake may depend on life stage, a role for kidney size in renal outcomes with aging

Smaller kidney size and hence lower numbers of functional units (nephrons) proportional to body weight is also an important risk factor for kidney disease. This most commonly manifests in children with low birth weight (Barker et al., 2009; Ruggajo et al., 2016), occurs with obesity (Li et al., 2014; Blaslov et al., 2015) or premature birth (Crump et al., 2019), and significantly increases risk for kidney and cardiovascular disease in adulthood (Luyckx et al., 2013). This aligns with our data where lower protein intake and smaller kidney mass to body mass or lean mass ratios were seen in concert with lower GFR and increases in markers of kidney damage. Furthermore, malnutrition in humans, particularly in early life during growth and development, is an important driver of inferior kidney outcomes (Lv et al., 2020). Risk for kidney disease is also increased, where malnutrition in childhood is followed by adequate or over-nutrition in adulthood (Hoppe et al., 2007). Although we excluded several of the lowest protein diets that did not support adequate whole body growth and development early in the study, our data support that adequate dietary protein intake is important to facilitate adequate nephron growth and function. Notably, much of this benefit may be conferred by early life nutrition, warranting further investigation.

Cardiometabolic risk and longevity did not align with diet-induced renal outcomes with aging

Among the most surprising findings of the present study was that renal outcomes seen with protein consumption did not correlate with cardiometabolic risk factors such as systolic blood pressure and glucose tolerance measured at the same time point (Solon-Biet et al., 2014). Low-protein, high-carbohydrate diets are beneficial for cardiometabolic health and extend lifespan in mice at a variety of life stages (Solon-Biet et al., 2014; Li et al., 2018; Maida et al., 2016) and concomitantly improve numerous risk factors for CKD (Li et al., 2018; Maida et al., 2016). Hence, the lack of alignment between known risk factors for poor kidney health and kidney damage might have been related to the time point analyzed. Also, as alluded to earlier, early life nutrition including insufficient protein intake may confer greater risk for kidney disease, and this in combination with genetic and other environmental risk factors for cardiometabolic disease, not present in our study, may precipitate kidney disease in susceptible individuals. Indeed, the dietary patterns that promoted kidney damage and decreased the longevity protein klotho in our study were also those that promoted longevity in a previous study (Solon-Biet et al., 2014). This was unexpected given that kidney function, including modest changes such as microalbuminuria, independently predict future risk for cardiac events such as myocardial infarction (Mogensen, 1984; Go et al., 2004; Waheed et al., 2013; Matsushita et al., 2015) and all-cause mortality in humans (Go et al., 2004; Waheed et al., 2013; Schmieder et al., 2011; Hallan et al., 2012). Whether these same associations are as interdependent in mouse models is not known.

Inflammation and TIF: links to low protein intake

Inflammation is a key driver of glomerular and tubular damage in the kidney, with increased recruitment of immune cells to the tubulointerstitium seen before fibrosis onset (Levey and Coresh, 2012; Schlondorff,

2008). Consistent with functional data, kidney concentrations of the pro-inflammatory cytokines IL-23, IL-12p70, IFNγ, and IL-1β increased with lower protein consumption, in conjunction with probability for greater kidney leukocyte infiltration. Increases in renal IL-1β concentrations have been reported to occur before interstitial fibrosis (Vesey et al., 2002; Kitching et al., 2009; Lemos et al., 2018). Interestingly, renal IL-1β, IL-12p70, and IFNγ are increased in many CKDs of mostly immunological origins, including autoimmune and immune complex deposition-based diseases, with florid immune cell infiltration and fibrosis (Anders, 2016; Tucci et al., 2008; Law et al., 2017; Ortega and Fornoni, 2010). These identified factors mostly drive activation of T cell subsets including Th1 and Th17 cells. Of note is that IL-23, which was also increased in the kidney by chronic low protein intake, is postulated to be produced by dendritic cells (DCs) and a potent inducer of Th17 cells, a subset of CD4+ T cells that are critical mediators of cellular immunity, recruiting macrophages and neutrophils to affected tissues (Myer et al., 2010). Dysregulation of the Th17 response is implicated in inflammation and autoimmune diseases, including those of the kidney (Tesmer et al., 2008; Paust et al., 2009), and IL-23 production has been reported to increase up to 40-fold in DCs from aged mice (12 months or older) (Myer et al., 2010), whereas Th17 cells are over-represented in both aging mice (Huang et al., 2008) and humans (Myer et al., 2010). Taken together with our data, this suggests that dietary protein intake may be an important mediator of kidney leukocyte infiltration, thereby contributing to the known age-related decline in kidney function. However, no significant impact of macronutrient intake was seen on kidney fibrosis, suggesting that at this single snapshot of time during kidney aging immune events precede fibrosis. However, mouse models, particularly C57Bl6/J mice, are generally resistant to developing kidney fibrosis akin to that seen in human kidneys affected by the same diseases (Susztak et al., 2008).

Beyond protein intake: a role for fat intake

The present findings provide unique insights into kidney aging and pathogenesis. For example, tubular damage markers and inflammation in the kidney were strongly influenced by dietary protein consumption, but several markers such as epithelial damage and KIM-1 were also closely aligned with fat intake. Indeed, metabolic determinants of injury need not be the same and can occur quite independently of each other. Inflammatory cytokines such as IL-1β and IFNβ were also elevated as fat intake as a proportion of daily calories increased, as was the likelihood of leukocyte infiltrate in the kidney. This is consistent with the well-established relationship between dietary fat and inflammation. As for KIM-1, we suspect this could be driven by increasing total energy intake, which tends to be higher in diets with higher proportion of fat (Solon-Biet et al., 2014; Hu et al., 2018). Overall, changes in the kidney in response to chronic dietary fat intake did not appear to be as severe as previously reported from studies using equivalent dietary fat proportions, where significant renal pathology was observed in shorter timeframes (van der Heijden et al., 2015; Wakefield et al., 2011; Harcourt et al., 2011). However, we also did not see the onset of obesity except in mice that consumed diets both high in calories and fat, suggesting that these factors in concert may be more pathological to the kidney than dietary fat alone. Typically, fats and proteins sourced from animal products are postulated as more detrimental than vegetable-derived fats and proteins, affecting many risk factors for CKD such as glucose tolerance (Buettner et al., 2007) and inducing kidney damage (Yuzbashian et al., 2015; van der Heijden et al., 2015; Williams et al., 1987). In the present study, the fat source was soybean oil, which may explain this discrepancy. Furthermore, the inclusion of insoluble fiber cellulose in diets to manipulate the energy density of the diets may have ameliorated some of the macronutrient-driven effects on the kidney (Krishnamurthy et al., 2012; Xu et al., 2014).

Taken together, these data suggest that lifelong exposure to increased dietary protein intake was not detrimental to the kidney aging and resulted in favorable renal outcomes with regard to GFR, kidney size, tubular damage, and inflammation. Furthermore, the effects captured here with regard to long-term macronutrient consumption and renal outcomes appear to be independent of major risk factors for CKD such as circulating lipids, obesity, blood pressure, and glucose tolerance, with a spectrum of kidney changes occurring in these mice, much of which may fall within a subclinical or pre-pathological range. This study highlights the novelty and utility of nutritional geometry to further elucidate the relationships among macronutrient intake, kidney damage, and risk factors for renal functional decline. We suggest that future studies using the geometric framework should focus on mouse strains susceptible to kidney disease or be extrapolated directly to human renal studies.

Limitations of the study

This study has a number of methodological limitations. First, despite serum cystatin C being an excellent surrogate for GFR in both human and animal studies (Ferguson and Waikar, 2012), it would be desirable to

confirm our findings using direct GFR measurement and determine this over time from adolescence through to the study completion at 15 months. Changes in GFR over time, even within a normal range, have been shown to be a useful marker of CKD risk in humans (Palatini, 2012; Magee et al., 2009). Concomitant urine collections to examine urinary albumin:creatinine ratios would also have provided further information about whether the changes in kidney function over time were actually pathological. Second, the source of fat in these studies was soybean oil. Typically, fats sourced from animal products are more pathological to the kidney than vegetable-derived fats, affecting both risk factors for CKD such as glucose tolerance (Buettner et al., 2007) and kidney pathology (van der Heijden et al., 2015). Conversely, soybean oil is comparatively high in poly-ω-6 fatty acids (Gunstone, 2009) but dietary studies have shown increased inflammation, lower GFR, and greater risk of CKD in at least one human cohort (Diaz-Lopez et al., 2013). Third, the addition of indigestible fiber (cellulose) to the diets at the medium- and low-energy tiers may have counterbalanced adverse effects of protein and fat in the medium- and low-caloric tiered diets. Increased dietary fiber intake has consistently shown benefits for cardiovascular disease outcomes (King, 2005), and on kidney function thought to be an important mediator of these benefits (Krishnamurthy et al., 2012). Direct causation has yet to be established, but studies in humans have consistently found a positive association with high dietary fiber intake and better kidney function (Krishnamurthy et al., 2012; Xu et al., 2014). However, it must be noted that higher fiber intake is generally associated with higher diet quality in participants, including increased consumption of fruit and vegetables. Last, the C57BL/6 mouse is relatively resistant to renal disease especially in the context of diabetes. Hence, in future dietary studies, using FVB or DBA/2 mice, which have greater susceptibility to CKD, could provide additional insight into the impact of lifelong macronutrient intakes on kidney function.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

A.K.F. assisted with design of the study, conducted biochemical assays, analyzed the data and wrote the manuscript. S.M.S.-B. performed original animal study, performed biochemical analysis and reviewed/edited the manuscript. H.B.-O. performed analysis of renal pathology and reviewed/edited the manuscript. D.A.M. performed biochemical analysis and reviewed the manuscript. I.L. assisted with analysis. M.A.S. assisted with pathology analysis and reviewed the manuscript. R.O.W. assisted with pathology analysis and reviewed the manuscript. D.J.B. reviewed/edited manuscript. V.C.C. performed biochemical analysis and reviewed the manuscript. W.O.B. performed biochemical analysis and reviewed the manuscript. K.R. assisted with statistical analysis and reviewed the manuscript. N.T. performed biochemical analysis and reviewed the manuscript. R.G.M. performed biochemical analysis and reviewed the manuscript. D.R. designed the animal study and edited the manuscript. D.G.L.G. designed the animal study and edited the manuscript. S.J.S. designed the animal study reviewed/edited the manuscript. J.M.F. conceived the renal study design, edited the manuscript, and is the guarantor of this research.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

The authors worked to ensure sex balance in the selection of non-human subjects. One or more of the authors of this paper received support from a program designed to increase minority representation in science. While citing references scientifically relevant to this work, we also endeavored to promote gender balance.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-klotho rabbit polyclonal IgG	Bioss antibodies	Cat# bs-2925R; RRID:AB_11078665
Anti-aquaporin 2 goat polyclonal IgG	Santa Cruz Biotechnology	Cat# sc-9882
Alexa Fluor® 647 Anti-aquaporin 1 rabbit monoclonal IgG-	Abcam	Cat# ab225225 Clone ID EPR11588(B)
Alexa Fluor® 568 donkey anti-rabbit IgG	ThermoFisher Scientific	Cat# A10042; RRID:AB_2534017
Alexa Fluor® 488 donkey anti-goat IgG	ThermoFisher Scientific	Cat# A11055; RRID:AB_2534102
Anti-CD3 rabbit monoclonal IgG	Abcam	Cat# ab16669; RRID:AB_443425 Clone SP7 Lot# GR320743-5
Anti-CD4 goat polyclonal IgG	R&D Systems	Cat# AF554; RRID:AB_35543 Lot# EPE21807B
Anti-FoxP3 Biotinylated rat monoclonal IgG	eBioscience	Cat# 13-5773-82; RRID:AB_763540 Clone FJK-16s Lot# 4305913
Alexa Fluor® 488 chicken anti-rabbit polyclonal IgG	ThermoFisher Scientific	Cat #A-21441; RRID:AB_2535859 Lot#1003212
Alexa Fluor® 568 donkey anti goat polyclonal IgG	ThermoFisher Scientific	Cat# A-11057; RRID:AB_2534104 Lot# 1235787
Alexa Fluor® 647 Streptavidin	ThermoFisher Scientific	Cat# s32357
Chemicals		
Sodium citrate tribasic dihydrate	Sigma-Aldrich	Cat# S4641-500G
IGEPAL® CA-630	Sigma-Aldrich	Cat# I8896-50ML
Glycerol	Sigma-Aldrich	Cat# G7893-1L
Ethylenediaminetetraacetic acid	Sigma-Aldrich	Cat# 431788-100G
Trizma® base	Sigma-Aldrich	Cat# T1503-250G
Critical commercial assays		
Mouse cystatin C ELISA	BioVendor Research and Diagnostic Products	Cat# RD291009200R Lot# E15-122
Mouse ELISA for Kidney Injury Molecule 1	Cloud Clone Corp	SEA785Mu
RNEasy Mini Kit	Qiagen	Cat# 74106
LEGENDPlex™ Mouse Inflammation Panel (13-plex)	BioLegend	Cat# 740446 Lot# B242043
Experimental models: Organisms/strains		
C57Bl/6	Animal Resources Centre, WA, Australia	
Oligonucleotides		
Taqman Gene Expression assay for *αklotho*	ThermoFisher Scientific	Mm00502002_m1
Taqman Gene Expression assay for *Rn18s*	ThermoFisher Scientific	Mm03928990_g1

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
R version (3.4.0)	R Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.	
GraphPad Prism version 8.0.0 for Windows	GraphPad Software, San Diego, California USA, www.graphpad.com	

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Professor Josephine Forbes josephine.forbes@mater.uq.edu.au. A key resources table can be accessed here: https://star-methods.com/?rid=KRT606e73c6902d8.

Materials availability

This study did not generate new unique reagents.

Data and code availability

The datasets supporting the current study have not been deposited in a public repository but are available from the corresponding author on request. This study did not generate any code.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Nutritional geometry framework

Mice without specific genetic predisposition to chronic kidney disease were studied to separate the impact of the diet alone on kidney structure and function. Full details of animal experiments have been described previously (Solon-Biet et al., 2014, 2015; Holmes et al., 2017). In brief, three-week-old, male and female C57BL/6J mice (N = 174; M: F = 81:93, Animal Resources Centre, WA, Australia) were randomised to consume one of twenty-five diets *ad libitum*, (Specialty Feeds, Perth, Australia). The manufactured diets were systematically varied in their composition of protein, carbohydrate and fat, and calories manipulated by the addition of indigestible cellulose yielding energy density regimens fixed at 8, 13, and 17 kJ/g (Figure 1A). Mice remained on the diets from week 3 to 15 months of life and had their food consumption measured weekly for the first 6 months of the study and monthly for the remaining 9 months. Cardio-metabolic factors were assessed at 15 months of age, this has been previously published (Solon-Biet et al., 2014). Mice were euthanized at 15 months of age, blood was collected and the kidneys were snap frozen for protein and gene expression analysis. One pole of the kidney was removed and bisected, with half being embedded in OCT mountant and frozen and half fixed in 10% NBF and paraffin embedded. Where possible every tissue or sample available was analysed however for some analyses a slightly reduced subset was used due to constraints on tissue or serum availability. All protocols were approved by the Sydney Local Health District Animal Welfare Committee (Protocol No. 2009/003) and mice were group housed in pathogen free conditions (24°C–26°C and 44%–46% humidity under a 12 hr light:12 hr dark photoperiod, with lights on at 0600) at the Molecular Physiology Unit of the ANZAC Research Institute.

METHOD DETAILS

Histology

Glomerular Injury was evaluated by a trained veterinary pathologist, using 2 μm kidney sections stained with Periodic Acid-Schiff (PAS). The degree of interstitial fibrosis in the kidney cortical sections was quantified in 4 μm kidney sections stained with Masson's Trichrome or picrosirius red. Masson's Trichrome staining was performed by the Translational Research Institute Histology Core Facility (Woolloongabba, QLD Australia). Picrosirius red was performed by staining dewaxed, hydrated sections for 1 hour in Picrosirius red solution (0.1% direct red 80 dye (Sigma Aldrich, St Louis, Missouri, USA, Cat#365548) dissolved in picric acid solution, (1.3%, hydrated; Sigma Aldrich, Cat# P6744-1GA), washing twice in acidified water before dehydrating and mounting. Stained slides were imaged using the slide scanner (Virtual Slide System VS120,

Olympus, Tokyo, Japan) at a magnification of 400X. Photomicrographs of eight to twenty fields of view encompassing the stained kidney cortex without overlap were taken and analysed in image J using colour thresholding. In brief, colour thresholding was used to select collagens (stained blue Masson's Trichrome or Red- picrosirrius red) and the selected area was determined as a percentage of the total field of view area as described previously (Zhuang et al., 2017). The median selected area for all FOVs was calculated and plotted. Histopathology was assessed using both PAS and Masson's Trichrome stained sections by a veterinary pathologist blinded to treatment groups. Changes to tubular epithelium and glomeruli, presence of tubular casts and interstitial leukocyte infiltration was assessed individually and assigned a score based on pathology severity: 1, within normal limits to minimal change, 2; mild to moderate changes; 3, marked to severe changes.

Immunofluorescence

Paraffin embedded kidney sections (4 μm) were dewaxed, hydrated and heat induced epitope retrieval performed by boiling sections for 20 minutes in either sodium citrate buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0- Klotho, and tubular markers Aquaporin 1 and 2 staining) or ctric acid buffer (10mM Citric Acid, 0.05% Tween 20, pH 6.0– T cell staining). Sections were washed (Tris-buffered saline; TBS) and blocked (10% donkey serum, Merck, Darmstadt, Germany), and incubated overnight at 4°C with a cocktail containing anti-klotho, rabbit polyclonal IgG (10 μg/ml- bs-2925R; Bioss, Massachusetts, USA), and anti-aquaporin 2 (AQP2 (c-17), goat polyclonal IgG (1 μg/ml, sc-9882; Santa Cruz Biotechnology, Texas, USA) for klotho and tubular staining or a cocktail containing anti-CD3 rabbit monoclonal IgG (0.5 μg/ml #ab16669, clone SP7 -, Abcam, Cambridge, The United Kingdom), anti-CD4 goat polyclonal IgG (10 μg/ml, #AF554 R&D Systems, Minneapolis, USA) and anti-FoxP3 biotinylated rat monoclonal IgG (5 μg/ml, # 13-5773-82, clone FJK-16s – eBioscience, San Diego, USA) for T cell staining. For klotho and tubular staining, sections were washed (TBS- 0.05% Tween) and stained for 1 hour at RT with donkey anti-rabbit Alexa Fluor® 568 (5μg/ml- A10042, Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and donkey anti-goat Alexa Fluor® 488 (5 μg/ml- A11055, Invitrogen). Sections were then blocked in 10% donkey serum and incubated overnight at 4°C with anti-aquaporin 1 antibody (EPR11588(B), Alexa Fluor® 647; Rabbit monoclonal IgG (2.5 μg/ml ab225225; abcam, Cambridge, The United Kingdom) prior to staining of nuclei with DAPI (2 μg/ml, Invitrogen). For T cell staining, sections were washed (TBS- 0.05% Tween) and stained for 1 hour at RT with secondary antibody cocktail containing Alexa Fluor® 488 chicken anti-rabbit polyclonal IgG (5 μg/ml, #A-21441 ThermoFisher Scientific), Alexa Fluor® 568 donkey anti-goat polyclonal IgG (5 μg/ml, A-11057, ThermoFisher Scientific) and Alexa Fluor® 647 Streptavidin (5 μg/ml ThermoFisher Scientific, #s32357), prior to staining of the nuclei with DAPI (2 μg/ml, Invitrogen). Images were acquired using an Olympus FV3000 confocal microscope (Olympus, Tokyo, Japan).

Protein extraction

Protein was extracted from a cortex enriched piece of kidney tissue (20-40 mg). Tissue was homogenised in lysis buffer (10 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 10% Glycerol, 5 mM EDTA) including a protease inhibitor cocktail (Roche, Complete EDTA free, Sigma Aldrich, St Louis, Missouri, USA) using a Bullet Blender at 4°C (Next Advance, Troy, NY, USA; as per the manufacturer's instructions). The resulting homogenate was then spun at 12000 x *g* for 20 minutes to clear the lysate. Cleared lysate was aliquoted and stored at -80°C. The protein concentration of the cleared lysate was measured by BCA protein assay kit (Thermo Fischer Scientific).

Biochemical assays

Cystatin C. Glomerular filtration rate was estimated by measuring plasma cystatin C concentrations by ELISA (Mouse Cystatin C ELISA, BioVendor Research and Diagnostic Products, Karasek, Czech Republic) according to the manufacturer's instructions.

Urea. Plasma urea was measured at the Concord Hospital Pathology Department. Urea levels were adjusted to body weight and lean mass as per cystatin C.

Kidney injury Molecule-1. Kidney Injury Molecule-1 (KIM-1) concentration was measured in duplicate in protein extracted from kidney tissue by ELISA (Mouse ELISA for Kidney Injury Molecule 1, Cloud Clone Corp, Houston, Texas). One hundred μg of protein was loaded per well. Curve fitting and analysis was performed in GraphPad Prism 7.

Inflammatory cytokines. A LEGENDplex cytokine assay (Mouse Inflammatory cytokines panel 13plex; Bio legend, San Diego, CA, USA) was performed as per the manufacturer's instructions, in kidney cortex-enriched protein extract, loading 180 μg of protein per replicate. Quantification was performed on a CytoFLEX flow cytometer (Beckman and Coulter, Brea, CA, USA). Analysis was performed using LEGENDplex v.8.0 (Manufacturer provided).

RNA extraction and real-time qPCR

RNA was extracted using an adapted Phenol/Chloroform extraction in conjunction with RNeasy column (QIAGEN, Venlo, Netherlands). 20 mg of frozen kidney cortex tissue was homogenised in 500 μl of chilled QIAZOL (QIAGEN, Venlo, Netherlands), containing 0.5 mm zirconium oxide beads (Next Advance, Troy, USA) using a Bullet Blender (Next Advance, Troy, NY, USA). Chloroform was added (100 μl) to the homogenate, the solution was vortexed for 15 seconds and then incubated at room temperature for 15 minutes before being centrifuged at 12000 x *g* for 15 minutes at 4°C. RNA was extracted from 200 μl of the aqueous phase using the RNeasy column (QIAGEN, Venlo, Netherlands) according to the manufacturer's instructions. Total RNA was quantified using the NanoDrop1000 micro-volume spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

One microgram of RNA was DNase treated, (DNase 1, amplification grade, Invitrogen, California, USA) and then reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad, California, USA), as per the manufacturer's instructions. Gene-expression analysis was performed using the TaqMan fast advance platform (Thermo Fisher Scientific, Waltham, Massachusetts, USA), with gene expression probe and primer kits for murine αklotho (Mm00502002_m1) and *Rn18S* (Mm03928990_g1) was used as the housekeeping gene. Delta Ct was calculated using Applied Biosystem- Quant Studio Real Time PCR Software Vs 1.1 by subtracting mean Ct for the housekeeping gene from mean Ct of the gene of interest this was then expressed as $2^{-(\text{deltaCt})}$.

Cardiometabolic risk factors

Body weight, fat mass, lean mass, bone mineral density, glucose (ip.GTT), fasting plasma insulin, HOMA IR, triglycerides, cholesterol and Na+ were measured as described previously [41].

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistics

The effects of macronutrient intake on kidney outcomes was analysed using the Geometric Framework approach. Generalised additive models (GAMs) with thin plate splines were used to model the response variables (structural and functional markers of kidney disease) over mouse macronutrient intake spaces, as per Solon-Biet & McMahon et al., 2014 (Solon-Biet et al., 2014). This was performed in R (version 3.4.0) using the mgcv package for the R language (R Core team, version 1.8-17). To support the GAMs analysis, three heat map "response surfaces", cut as slices through the median intake for each macronutrient (shown on the x axis in parentheses) were used to visualise the relationship between the response variable and each paired combination of the macronutrients. Comparison of the distributions of response variables between males and females was performed by Student's T test or non-parametric equivalent in GraphPad Prism 7. Correlations were performed using the Rcorr package (version 2.3-2) in R (version 3.4.0) and a correlogram visualised using corrplot (version 0.84). Ordinal data such as histological scoring was modelled using ordinal regression (proportional odds) using the VGAM package (Version 1.1-1) in R (version 3.4.0) as previously described (Solon-Biet et al., 2016). Ordinal variables were examined by Chi squared test in Graph Pad Prism 8, following division of the mice according to tertiles of protein or fat intake and generation of a contingency table. Statistical significance was determined as $P \leq 0.05$ and is indicated in the figure legends.