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ORIGINAL ARTICLE

Methionine restriction delays aging-related urogenital diseases in male Fischer 344 rats

Despina Komninou · Virginia L. Malloy · Jay A. Zimmerman · Raghu Sinha · John P. Richie Jr

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Abstract Dietary methionine restriction (MR) has been found to enhance longevity across many species. We hypothesized that MR might enhance longevity in part by delaying or inhibiting age-related disease processes. To this end, male Fischer 344 rats were fed control (CF, 0.86% methionine) or MR (0.17% methionine) diets throughout their life until sacrifice at approximately 30 months of age, and histopathology was performed to identify the incidence and progression of two important aging-related pathologies, namely, chronic progressive nephropathy (CPN) and testicular tumorigenesis. Although kidney pathology was observed in 87% CF rats and CPN in 62% of CF animals, no evidence of kidney disease was observed in MR rats. Consistent with the absence of renal pathology, urinary albumin levels were lower in the MR group compared to controls throughout the study, with over a six-fold difference between the groups at 30 months of age. Biomarkers associated with renal disease, namely, clusterin, cystatin C, and β-2 microglobulin, were reduced following 18 months of MR. A reduction in testicular tumor incidence from 88% in CF to 22% in MR rats was also observed. These results suggest that MR may lead to metabolic and cellular changes providing protection against age-related diseases.

Keywords Aging · Nephropathy · Metabolic changes · Methionine restriction · Testicular cancer

Introduction

Dietary methionine restriction (MR) results in substantial increases in survival in both vertebrate and invertebrate organisms (Orentreich et al. 1993; Richie et al. 1994; Zimmerman et al. 2003; Miller et al. 2005; Johnson and Johnson 2014; Lee et al. 2014). Most of the evidence that MR promotes longevity comes from studies in rodent models. In both rats and mice, the effects of MR include reductions in adiposity, blood lipids, glucose, insulin, and IGF1 levels (Miller et al. 2005; Malloy et al. 2006; Dong et al. 2018). MR mice fed high-fat diets are resistant to adiposity and remain insulin sensitive (Ables et al. 2012). MR also increases

D. Komninou
Technological Educational Institute of Thessaloniki, Thessaloniki, Greece

V. L. Malloy · J. A. Zimmerman
Orentreich Foundation for the Advancement of Science, Inc., Cold Spring-on-Hudson, NY 10516, USA

R. Sinha (✉)
Department of Biochemistry and Molecular Biology, Penn State Cancer Institute, Penn State University College of Medicine, 500 University Dr., Hershey, PA 17033, USA
e-mail: rus15@psu.edu

J. P. Richie Jr, (✉)
Department of Public Health Sciences, Penn State Cancer Institute, Penn State University College of Medicine, 500 University Dr., Hershey, PA 17033, USA
e-mail: jrichie@pennstatehealth.psu.edu

adiponectin levels, energy expenditure, and mitochondrial biogenesis and aerobic capacity (Malloy et al. 2006; Hasek et al. 2010; Perrone et al. 2010). MR has been shown to inhibit colon, prostate, and mammary carcinogenesis (Komninou et al. 2006; Sinha et al., 2014; Hens et al. 2016) and delay the onset of numerous aging impairments, e.g., reduction in immune function and lens turbidity (Miller et al. 2005). In mice, MR was also effective at enhancing lifespan, even when initiated in middle age, suggesting that the anti-aging effect was not simply driven by a delay in growth and development (Sun et al. 2009). A salient feature of MR is decreased body weight and reduced food intake; however, when adjusted for body weight, MR animals are mildly hyperphagic relative to their control-fed (CF) counterparts. These effects of MR are not secondary to calorie restriction (CR), and this is supported by pair feeding (PF) studies in which CF were fed to the level consumed by MR animals. Growth of PF animals was slightly attenuated compared to CF; however, serum biomarkers of adiposity, visceral fat weight, and survival were comparable to CF (Orentreich et al. 1993; Zimmerman et al. 2003; Malloy et al. 2006).

The effects of MR on liver, fat, cardiac function, bone development, and epithelial tight junctions have all been reported (Ables et al. 2015; Lees et al. 2015; Mullin et al. 2016; Ouattara et al. 2016). For instance, increased plasma levels of fibroblast growth factor 21 (FGF21) and its increased expression in hepatic tissue appear to improve glucose metabolism in aged mice and in those fed high-fat diets (Ables et al. 2012; Lees et al. 2014). Interestingly, dietary MR also results in elevated plasma homocysteine levels (Elshorbagy et al. 2010). Data from both animal studies and clinical trials suggest an association between high homocysteine levels and increased risk for cardiovascular disease (Dayal et al. 2012; Ma et al. 2013). However, these results may not be definitive, as some reports show no association between hyperhomocystenemia and cardiovascular disease (Cioni et al. 2016; Lupton et al. 2016), and no deleterious effects on cardiac function were demonstrated in MR animals (Ables et al. 2015).

Effects of MR on kidney function have been recently described (Cooke et al. 2018). In young and aging mice, MR reduced urinary albumin and albumin-to-creatinine ratio and upregulated genes that are involved in ion transport. Utilizing unilateral nephrectomized and 5/6 nephrectomized (5/6Nx) mice, models of renal stress and injury, respectively, MR reduced urinary and plasma markers associated with renal disease. Moreover, in the 5/6Nx model, kidney lesions were reduced and molecular markers associated with fibrosis and inflammation were downregulated in response to MR (Cooke et al. 2018).

Male Fischer 344 rats develop several age-related pathologies (Coleman et al. 1977); two of the most prevalent are chronic progressive nephropathy (CPN) and testicular cancer (Coleman et al. 1977; Goodman et al. 1979; Hard et al. 2009). The delay of onset or prevention of chronic diseases such as nephropathy or cancer is a hallmark of delayed aging in CR animals (Yu et al. 1982; Maeda et al. 1985; Weindruch and Walford 1988). That MR was renoprotective in induced models of kidney disease led us to examine if progression of CPN and testicular tumors in male Fischer 344 rats could be attenuated in response to this dietary intervention.

Methods

Animals and experimental diets

Six-week-old male Fischer 344 (F344) rats were obtained from Taconic Farms (Germantown, NY). All studies were reviewed and approved by Orentreich Foundation for the Advancement of Science, Inc., IACUC committee and conducted following the National Research Council guidelines for laboratory animal use (Approval Number: 0789RbRMHB). Animals were housed in a conventional animal facility in groups of three in solid bottom cages lined with wood chips (3 animals per case), given free access to food and acidified water, and maintained on a 12-h light/dark cycle, 50% humidity, and 22 °C temperature throughout the study. A non-purified diet was given to all animals during the 1-week acclimation period, and then, they were randomly assigned to one of two groups receiving purified diets (Ziegler Brothers, Gardners, PA) containing either 0.86% methionine (CF, n = 8) or 0.17% methionine (MR, n = 9). Both of these chemically defined diets are based on the AIN-76 formulation with the protein replaced by an amino acid mixture where Met is the only source of sulfur amino acid (Orentreich et al. 1993; Richie et al. 1994; Malloy et al. 2006). The glutamic acid content of the MR diet is increased on an equal gram basis to compensate for the lower Met content and the choline content of both diets is 0.2%. This level of

MR was selected based on results of numerous previous studies demonstrating its effectiveness at increasing life span and beneficially impacting adiposity, oxidative stress, and metabolism (Orentreich et al. 1993; Richie et al. 1994; Malloy et al. 2006; Perrone et al. 2010; Hasek et al. 2010; Maddineni et al. 2013). Twenty-four hour food consumption was assessed monthly during months 1–5 and 19–24 after the initiation of MR by weighing the food ration for each cage at the beginning and end of feeding. Body weights of the animals were recorded throughout the study. Animals were sacrificed when found to be moribund (Toth 2000). CF and MR rats from two separate cohorts were used to obtain samples for blood and urine chemistries.

Pathology

Animals were autopsied after CO_2 euthanasia and examined for gross pathological lesions. Tissues were removed and immediately fixed in 10% neutral buffered formalin. Fixed tissues were embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin-eosin. A trained pathologist in a blinded fashion performed the histopathological examination. The grading system for CPN was based upon four categories as previously described (Yu et al. 1982): (1) no significant lesions to mild, (2) moderate, (3) marked, and (4) end-stage lesions.

Proliferating cellular nuclear antigen (PCNA) incorporation was analyzed on sections of paraffin-embedded testes by immunohistochemistry as previously described (Koshiji et al. 1998). Briefly, tissue paraffin sections were deparaffinized, rehydrated, and then incubated with 3% hydrogen peroxide for 10 min at 37 °C. Tissue sections were incubated with the anti-PCNA monoclonal antibody (DAKO, Santa Barbara, CA) optimally diluted 1:20 in phosphate-buffered saline (PBS) for 30 min at 37 °C. A biotinylated goat anti-mouse immunoglobulin was used as secondary antibody at a dilution 1:200 in PBS, in which sections were incubated for 30 min at 37 °C. This was followed by incubation by streptavidin at a dilution of 1:400 in PBS for 30 min at 37 °C. The slides were incubated with 0.05% diaminobenzidine solution for 10 min at room temperature. Each step was followed by three washes in PBS. The sections were lightly counterstained with Lillie-Mayer's alum hematoxylin. PBS replaced the PCNA monoclonal antibody as a negative control for immunohistochemical staining experiments. PCNA positivity was expressed as the ratio of PCNA-positive nuclei to total nuclei × 100 (labeling index) in each seminiferous tubule. A minimum of 10 seminiferous tubules per section was analyzed.

Clinical chemistry analyses

Urine and blood were collected from a different cohort every 3–6 months beginning at 14 weeks on diet. Rats were placed in plastic metabolic cages (Nalgene Company, Rochester, NY) and urine was collected for 24 h. The samples were frozen at − 70 °C until analysis. For blood collection, animals were fasted overnight and anesthetized with Metofane™ (Pitman Moore, Mundelin, Illinois) between 9:30 and 10:30 am to minimize possible diurnal variations. Blood was drawn from the retro-orbital sinus plexus into a test tube using a heparinized micropipette. After centrifugation at 2200 rpm for 15 min at 4 °C, the supernatant was stored at − 40 °C until analysis. Urinary albumin levels were measured spectrophotometrically using the bromocresol green method (Biotriol, distributed by Equal Diagnostics Exton, PA). The inter-assay coefficient of variation (CV) was 12.3%. Creatinine was determined spectrophotometrically using a commercial kit (Sigma Chemical Company, St. Louis, MO with a CV of 1.9%). Plasma cholesterol and triglycerides were analyzed using a Kodak Vitros clinical chemistry analyzer (CVs < 5%). Plasma clusterin, cystatin-C, and β-2 microglobulin levels were measured by Multiplex analysis using the Luminex xMAP platform (Rules Based Medicine, Austin, TX).

Statistical analysis

Data comparisons were performed using the Student's *t* test. Data are expressed as mean ± SEM. Fisher's exact test was employed to determine statistical differences in disease incidence rates. Albumin levels measured over time were compared between MR and CF group using repeated measures analysis of variance (ANOVA). Individual group trends were assessed using linear regression analysis.

Results

As observed previously (Orentreich et al. 1993; Richie et al. 1994; Malloy et al. 2006), the growth of MR rats

was substantially reduced compared to CF group with mean body weights of MR rats being ~ 40% lower than CF throughout the adult life span. This difference was reduced to 27% at the time of sacrifice, due to body weight losses in the moribund CF rats (Table 1). The mean age at sacrifice of MR rats was 3 months greater than that of CF rats ($P < 0.0001$). No differences in food intake (g/rat/day) were observed either during the first 5 months of MR, or between 19 and 24 months prior to the loss in body weight observed in MR rats compared to CF group (Fig. 1). While these food intake measurements were not performed in metabolic cages with singly housed animals, there was no evidence of major spillage of food or inter-individual differences in intake among the rats in each cage.

Diet-specific changes in organ weights were examined (Fig. 2). Decreases in kidney and testes weights of 12 and 17%, respectively, were observed in MR compared to CF rats at sacrifice. These differences in organ weights were less than that observed for overall body weight; however, organ to body weight ratios were 28% greater for kidney and 25% greater for testes in MR compared to CF rats ($P < 0.00001$).

Histopathologic examination of kidney tissues from CF rats revealed lesions in seven out of eight animals with CPN being the most common lesion (Fig. 3). Based upon previously described criteria (Yu et al. 1982), CPN lesions included one mild, one moderate, one marked, and two end-stage cases occurring as early as 26 months of age. Other lesions observed included hypernephrosis in one rat and pyelonephritis in another. These findings of CPN morphology and incidence are consistent with previous reports where the severity of this disease rapidly increased from about 5–15% at 6 months of age to 65–75% at 24 months of age in F344 rats (Gray et al. 1982). MR completely eliminated the development of CPN or any other renal pathology (Fig. 3). No evidence

Table 1 Age and body weights at the time of sacrifice

		Age (months)		Body weight (g)	
Diet group	*N*	Mean ± SEM	Range	Mean ± SEM	Range
CF[a]	8	29 ± 1.0	26–33	286 ± 16	215–337
MR	9	32 ± 0.9[b]	29–36	205 ± 14[b]	159–252

[a] Control-fed

[b] Significantly different from CF, $P < 0.0001$

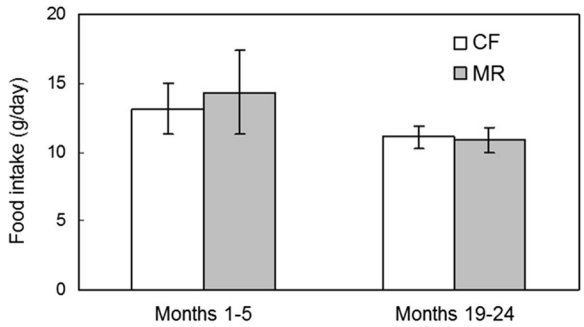

Fig. 1 Food intake in F344 rats on Met-restricted (MR) and control (CF) diets. Rats (8–9 per group) were randomized into CF and MR groups at 7 weeks of age. Twenty-four-hour food consumption was assessed monthly during months 1–5 and 19–24 after the initiation of MR by weighing the food ration for each cage at the beginning and end of feeding. Bars represent the mean daily food consumption during the 5-month period. Error bars represent SEM values

of kidney disease was observed even in animals as old as 36 months.

In order to assess renal function, 24-h urinary albumin levels were analyzed at different ages throughout the life span (Fig. 4). In CF animals, a progressive three-fold increase in urinary albumin was observed after 1 year continuing to a 19-fold increase at 2 years of age ($p < 0.05$). In contrast, lower urine albumin levels were observed in MR rats at all times with an over six-fold difference between the two groups at 2 years of age ($p < 0.0001$). Even at 28 months of age, when all CF rats were dead in this cohort, urine albumin levels in MR rats were similar to those of CF animals at 15 months of age (5.4 ± 1.6 mg/24 h). The effect of MR on urinary albumin levels was also observed when albumin was expressed per mg of urinary creatinine and was accompanied by similar reductions in urinary levels of total protein of 42% and 78% in 15- and 25-month-old rats, respectively (Table 2). The dramatic increase in albumin excretion observed in CF animals over time was not associated with a decrease in serum albumin or an increase in serum creatinine (data not shown). Decreased levels of blood lipids, cholesterol and triglycerides, were observed in MR compared to CF rats (Table 2).

The effect of MR on plasma markers for renal injury was also examined in a separate cohort of animals feeding on MR for 18 months. Chronic MR resulted in significant reduction of β-2 microglobulin, clusterin, and cystatin-C

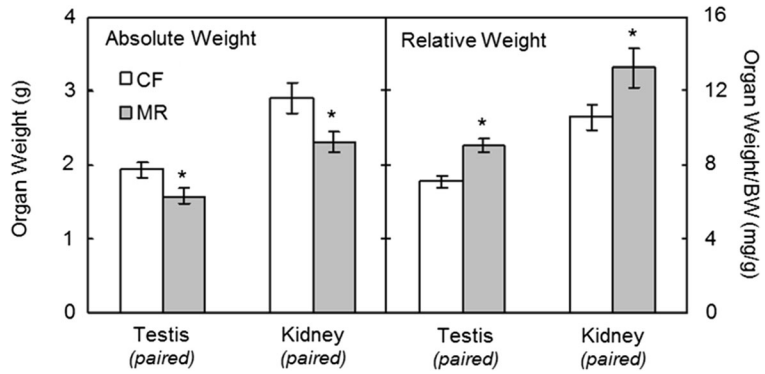

Fig. 2 Organ weight changes in F344 rats on Met-restricted (MR) and control (CF) diets. Rats (8–9 per group) were randomized into CF and MR groups at 7 weeks of age and body and organ weights were recorded at the time of sacrifice. Both absolute weights (left panel) and weights relative to total body weight (right panel) are presented. Bars represent the mean weights and error bars represent SD values. Significant differences ($P < 0.00001$) between CF and MR groups are designated by asterisks

by 48%, 34%, and 30%, respectively, compared to CF animals (Table 3). MR also inhibited the development of testicular tumors. The incidence of testicular lesions in the CF group was 87.5% with six out of eight CF rats having large Leydig (interstitial) cell tumors and one having early-stage Leydig cell foci (Fig. 5a). In the MR group, only two out of nine rats had tumors (22%), despite being slightly older at sacrifice. Two other MR rats showed signs of Leydig cell hyperplasia. Thus, MR resulted in a 75% reduction in testicular tumor formation as well as a delay in the progression of the disease from the hyperplastic stage. This reduction was associated with a decrease in cell proliferation as the PCNA labeling index was 16% lower in testicular tissue from MR compared to CF rats ($P < 0.003$) (Fig. 5b).

Discussion

The present findings indicate that MR is a potent inhibitor of two of the most common aging-related diseases in the F344 rat, namely CPN and testicular cancer. These data, together with previous findings that MR substantially enhances longevity in the rat (Orentreich et al. 1993; Richie et al. 1994), support the hypothesis that MR causes a delay in the biological aging process. MR is also associated with a reduction in fat accumulation, preservation of insulin activity, and a decreased production of reactive oxygen species and oxidative damage in heart and liver mitochondria in the rat (Malloy et al. 2006; Sanz et al. 2006). In the mouse, MR is associated with reduced levels of insulin, glucose, and thyroid

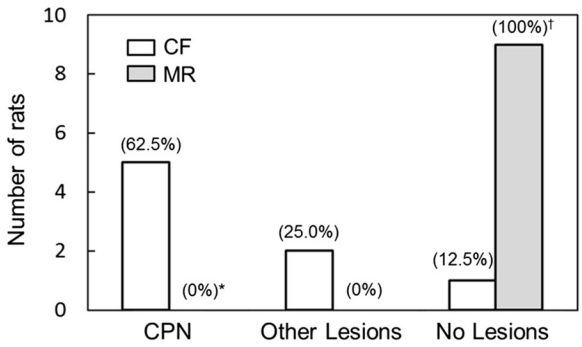

Fig. 3 MR eliminates chronic progressive nephropathy (CPN). Rats were randomized into CF ($n = 8$) and MR ($n = 9$) groups at 7 weeks of age. Upon sacrifice when rats became moribund (26–33 months for CF and 29–36 months for MR), kidneys were removed and processed for histopathologic analysis. *Significantly different from control group, $P < 0.04$. †Significantly different from control group, $P < 0.02$

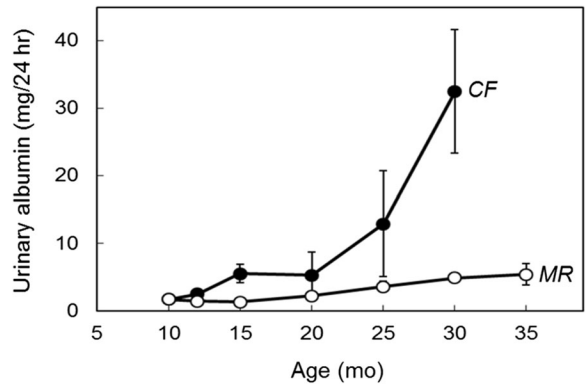

Fig. 4 Prevention of the age-related increase in albumin excretion by MR. Rats (6 per group) were randomized into CF and MR groups at 7 weeks of age and 24-h urine samples were collected every 5 months for albumin measurement. Values are mean ± SEM. $P < 0.05$ for CF vs. MR at 30 months

Table 2 Clinical chemistry values in control-fed (CF) and MR rats

Diet group	Age (months)	Plasma cholesterol (mg/dL)	Plasma triglycerides (mg/dL)	Urinary protein (mg/day)	Urinary albumin (mg/day)	Urinary creatinine (mg/day)	Urinary albumin/urinary creatinine
CF	15	136 ± 11.4	142 ± 21.1	26.9 ± 2.1	5.56 ± 1.33	3.92 ± 0.99	1.42 ± 0.39
MR	15	74.2 ± 9.30[a]	47.7 ± 8.70[a]	15.7 ± 1.1[a]	1.34 ± 0.51[b]	3.41 ± 1.22	0.37 ± 0.13[b]
CF	25	168 ± 10.20	179 ± 14.90	94.9 ± 10.3	12.9 ± 8.00	3.98 ± 1.71	3.24 ± 0.81
MR	25	84.1 ± 10.60[a]	61.5 ± 10.10[a]	21.0 ± 1.70[a]	3.64 ± 0.75	3.79 ± 0.87	0.96 ± 0.24[b]

Data are presented as mean ± SEM, $n = 6$.

[a] Significantly different from CF group, $P < 0.05$

[b] Significantly different from CF group, $P < 0.01$

hormone, slower development of lens turbidity and age-related changes in T-cell subsets and enhanced resistance to oxidative liver cell injury induced by administration of toxic doses of acetaminophen (Miller et al. 2005). Other evidence linking Met status with longevity includes the finding of a strong inverse correlation (r^2 = 0.93) between the protein Met content in heart tissue and maximum lifespan between mammalian species (Ruiz et al. 2005). Consistent with this relationship between reduced Met and longevity, recent findings in the extremely long-lived naked mole rat indicate that circulating Met levels are substantially lower than those observed in mice (Lewis et al. 2018), a likely result of higher levels of hepatic cystathionine β-synthase (CBS) activation observed in this species (Olecka et al. 2018).

The development of CPN in F344 rats is highly aging dependent with a progressive increase occurring with age in nearly all animals. Indeed, the subsequent glomerulosclerosis and renal failure resulting from CPN can account for most of the spontaneous deaths in these rats (Maeda et al. 1985; Masoro et al. 1989). Deposition of immune complexes in the glomerular basement membrane along with extensive cellular proliferation (Friend et al. 1978) indicates increased autoimmunity and inappropriate response of aging kidneys to different stresses. These animals also exhibit other age-related conditions, which appear to be

Table 3 Renal disease-associated plasma markers in control-fed (CF) and MR rats

Diet group	β-2 microglobulin (μg/mL)	Clusterin (μg/mL)	Cystatin-C (ng/mL)
CF	19 ± 2.2	64 ± 5.0	886 ± 85.5
MR	10 ± 1.2[c]	42 ± 2.5[b]	616 ± 47.5[a]

Data are presented as mean ± SEM, $n = 5$

[a] Significantly different from CF group, $P < 0.02$

[b] Significantly different from CF group, $P < 0.004$

[c] Significantly different from CF group, $P < 0.006$

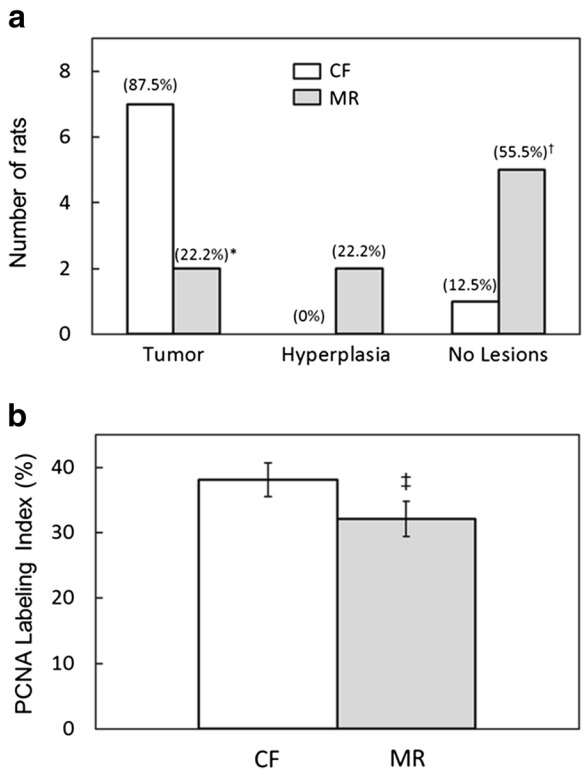

Fig. 5 MR reduces tumor development and cell proliferation in the aging rat. Rats were randomized into CF ($n = 8$) and MR ($n = 9$) groups at 7 weeks of age. Upon sacrifice when rats became moribund (26–33 months for CF and 29–36 months for MR), testes were removed and processed for analysis of histopathology (**a**) and cell proliferation by PCNA immunohistochemistry (**b**). *Significantly different from control group, $P < 0.04$. †Significantly different from control group, $P < 0.02$. ‡Significantly different from control group, $P < 0.003$

secondary to the nephropathy, such as cardiomyopathy, osteodystrophy, parathyroid hyperplasia, and calcium deposits in heart, skeletal muscle, and kidneys (Maeda et al. 1985). A similar age-associated increase in glomerulosclerosis occurs in humans (Anderson and Brenner 1986) and markedly progresses to renal failure when complicated by diabetes, obesity, hypertension, or atheromatosis (Matsumoto et al. 2000; Stern et al. 2001). The incidence of these conditions, often associated with insulin resistance and obesity, is escalating, reaching alarming levels in the USA and the rest of the Western world (Meigs 2002), and is likely to continue to grow as populations age and become more obese.

To our knowledge, this is the first report linking dietary reductions in Met intake with reductions in CPN in rats. Previously in the rat, 40% MR was associated with reductions in oxygen radical generation and oxidative DNA damage in the kidney (Caro et al. 2009). More recent findings indicate that MR is associated with improved glucose homeostasis and attenuation of kidney injury in mice (Grant et al. 2016; Cooke et al. 2018).

In rats, higher levels of total protein intake are associated with the development of nephropathy, while diets low in protein were protective against this disease (Bertani et al. 1989). A reduction in Met intake could be responsible, in part, for the protective effects of low protein intake on the development of CPN. In the case of CR, reduced incidence and severity of CPN occurred with or without protein restriction suggesting that this effect is independent of protein intake (Masoro et al. 1989). However, in studies where the intake of protein was increased, the protective effects of CR were abrogated (Everitt et al. 1982). The effects observed with MR, i.e., total inhibition of CPN, appear to be greater than those observed in CR, where mild nephropathy occurred in 25% of rats as early as 18 months and mild to moderate nephropathy in 40–60% of animals by the age of 24 months (Van Liew et al. 1992).

Since Met is often reduced in vegetarian diets, a restriction in Met intake could be responsible for reduced development of CPN in rats fed vegetarian diets. When casein in the diet of F344 rats was replaced by soy protein, a significant reduction in CPN development was observed (Iwasaki et al. 1988). Likewise, a lowered incidence of nephropathy-related structural and functional changes of the aging kidney was observed by feeding a diet of 2% fish and 15% vegetable protein to Wistar rats (Dodane et al. 1991). Indeed, these animals shared other characteristics with the MR animals including low body weights and an apparently long lifespan.

MR also prevented the age-dependent albuminuria observed in CF rats as early as 1 year of age and exponentially progressing thereafter (Fig. 4). This is similar to recent findings of MR in mice (Cooke et al. 2018). Increased albuminuria is commonly known to occur during aging (Weaver et al. 1975; Alt et al. 1980). In MR rats, albumin excretion was maintained at lower levels throughout the study in accordance with the observed elimination of nephropathy in these animals. Indeed, the protective effects of MR on albuminuria appeared to precede the observable protective effects on CPN development, consistent with the notion that albumin excretion is a sensitive and early biomarker of renal pathology in the aging kidney (Van Liew et al. 1992).

To further support the renoprotective actions of MR, we measured the levels of established markers of renal injury, β-2 microglobulin, clusterin, and cystatin-C in animals that were fed MR for 18 months. Similar to recent data reported in mice (Cooke et al. 2018), clusterin levels in MR rats were significantly reduced relative to CF. In addition, both β-2 microglobulin and cystatin C levels were decreased in MR rats. Lower cystatin C levels have been reported in MR mice that underwent 5/6 nephrectomy, a model of chronic kidney disease (Lim et al. 2014), however, in mice that underwent unilateral nephrectomy, levels were similar to CF. (Cooke et al. 2018). We did not conduct histological examinations on these animals; however, previous studies show that male F344 rats of comparable age have mild to moderate renal disease (Maeda et al. 1985).

Several hypotheses have been proposed for the pathogenesis of CPN, including glomerular hemodynamic alterations (Anderson and Brenner 1986) associated with diets high in caloric or protein intake, intraglomerular thrombosis (Klahr 1989), and hyperlipidemia (Diamond 1989). Oxidative stress associated with the accumulation of advanced glycosylation end-products along with alterations in macrophage function are also thought to play a role in age-related CPN (Nakatsuji et al. 1998). The elimination of CPN in MR may stem, in part, from the lifelong reduction in serum lipids, insulin, and IGF-1 levels (Malloy et al. 2006). Also, increased blood levels of glutathione, a major regulator of oxidative stress, induced by MR (Richie et al. 1994; Maddineni et al. 2013) may be playing a role

as glutathione depletion is observed during aging (Lang et al. 1992) and has been associated with the pathogenesis of diabetes (Murakami et al. 1989) and cancer (Ames and Gold 1991). In addition, increased levels of IGF-1 have been implicated in glomerulosclerosis, especially when it is complicated with diabetes (Lupia et al. 1999). Transgenic mice resistant to GH have reduced levels of IGF-1 and are resistant to diabetes-induced nephropathy, while those chronically expressing GH show high levels of mesangial cell proliferation and develop progressive glomerulosclerosis (Yang et al. 1993; Coschigano et al. 2000).

MR also had a remarkable preventive effect on the development of testicular tumors (Leydig cell type). According to the National Toxicological Program historical control database (Haseman et al. 1990), the interstitial cell tumor of testis is the most common spontaneous tumor in male F344 rats with an incidence of 88% (Goodman et al. 1979). This is similar to the tumor incidence observed in the CF rats in the present study. Lifelong feeding of the MR diet reduced this incidence to 22%. This was associated with a significant decrease in levels of cell proliferation. The mechanisms responsible for this dramatic inhibition of tumor formation are unknown but may involve a reduction in serum levels of a number of growth factors, such as IGF-1 and insulin which promote cell survival by protecting cells from a variety of pro-apoptotic stimuli (Prisco et al. 1999). Indeed, IGF-1 has been linked with a high risk of epithelial cancers including those of breast, lung, and colon (Pollak 2000). In support of this IGF-1 hypothesis, a significant decrease in colonic cell proliferation and formation of premalignant lesions has been observed in MR rats treated with a colon specific carcinogen (Komninou et al. 2006).

Met itself may also be playing a critical role in cancer development. Proliferation of many human and rodent cancer cells depends on Met, in contrast to normal cells, which are Met-independent (Mecham et al. 1983; Guo et al. 1993a). A possible explanation for Met dependence is the increased rate of transmethylation in cancer compared to normal cells (Judde et al. 1989). Indeed, a late S/G2 cell cycle arrest in Met-dependent tumor cell lines growing under conditions of limiting Met source was reported (Guo et al. 1993a). Furthermore, diets in which Met was either the only amino acid excluded from the protein composition or replaced by homocysteine resulted in regression of animal tumors and inhibition of metastasis in animal models (Breillout et al. 1987, 1990). In contrast, dietary restriction of other essential amino acids in tumor-bearing animals resulted either in no antitumor effect or in life-threatening toxicity (Sugimura et al. 1959). Methioninase, an enzyme that specifically degrades Met, was effective at inhibiting the growth of a variety of tumor types in animals (Tan et al. 1996; Kokkinakis et al. 1997; Yoshioka et al. 1998). In a clinical trial, when Met-free parenteral nutrition was combined with chemotherapy, response rates in gastric cancer were improved in comparison to those from only chemotherapy (Goseki et al. 1995). A phase I clinical study showed that enteral dietary methionine restriction is safe and tolerable in adults with metastatic tumor for as long as 39 weeks with some preliminary evidence of antitumor activity (Epner 2001).

Body weight may be an important yet unappreciated factor in relation to aging and cancer risk, and a reduction in body weight gain appears to be a common denominator in achieving disease inhibition. When Fischer rats were fed a diet supplemented with dehydroepiandrosterone (DHEA) to reduce body weight gain without energy restriction (Schwartz et al. 1981), Leydig cell hyperplasia and tumor development were completely inhibited (Rao et al. 1992). While an inhibition of tumor development by Met deprivation was observed in sarcoma-bearing nude mice with no apparent difference in body weight (Guo et al. 1993b), weight loss in control animals about the time of death masked the significant body weight decrease over time in the Met-deprived group. In the present study, MR resulted in a substantial reduction in body weight gain associated with an inhibition of Leydig tumor development suggesting that body weight could be a critical factor leading to many of the metabolic consequences of MR in rats.

Altogether, these results, together with numerous previous reports of the beneficial impact of MR diets on health status, metabolism, and longevity, provide further support that MR, like CR, is effective in delaying the aging process in rodent models. This includes findings of enhanced longevity in numerous animal models as well as decreased incidence and delay in age of onset for many aging-related diseases and disorders including cancer, cataracts, inflammation, and insulin insensitivity. These results are also consistent with prevailing concepts in the gerontological literature stressing the close relationship between metabolism and the aging process (e.g., epidemiological associations between

metabolic syndrome, frailty, and mortality in US adults) (Kane et al. 2017). Altogether, these results provide strong evidence for the importance of maintaining optimal sulfur amino acid nutrition in the aging process.

Further studies on the similarities and differences between these two models will help elucidate critical pathways involved in the development of aging-related diseases.

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Dedication We dedicate this paper to the memory of Norman Orentreich, MD. Dr. Orentreich was the first to describe methionine restriction as a means to extend lifespan and promote healthy aging. His commitment to the study of aging continues to inspire us as we move forward in our research.

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