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# The Impact of Dietary Methionine Restriction on Biomarkers of Metabolic Health

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## Abstract

Calorie restriction without malnutrition, commonly referred to as dietary restriction (DR), results in a well-documented extension of life span. DR also produces significant, long-lasting improvements in biomarkers of metabolic health that begin to accrue soon after its introduction. The improvements are attributable in part to the effects of DR on energy balance, which limit fat accumulation through reduction in energy intake. Accumulation of excess body fat occurs when energy intake chronically exceeds the energy costs for growth and maintenance of existing tissue. The resulting obesity promotes the development of insulin resistance, disordered lipid metabolism, and increased expression of inflammatory markers in peripheral tissues. The link between the life-extending effects of DR and adiposity is the subject of an ongoing debate, but it is clear that decreased fat accumulation improves insulin sensitivity and produces beneficial effects on overall metabolic health. Over the last 20 years, dietary methionine restriction (MR) has emerged as a promising DR mimetic because it produces a comparable extension in life span, but surprisingly, does not require food restriction. Dietary MR also reduces adiposity but does so through a paradoxical increase in both energy intake and expenditure. The increase in energy expenditure fully compensates for increased energy intake and effectively limits fat deposition. Perhaps more importantly, the diet increases metabolic flexibility and overall insulin sensitivity and improves lipid metabolism while decreasing systemic inflammation. In this chapter, we describe recent advances in our understanding of the mechanisms and effects of dietary MR and discuss the remaining obstacles to implementing MR as a treatment for metabolic disease.

## 1. RATIONALE FOR STUDY OF DIETARY METHIONINE RESTRICTION

The essential amino acids (EAAs) (e.g., methionine, lysine, leucine, isoleucine, tryptophan, valine, threonine, phenylalanine, and histidine) cannot be synthesized endogenously, so 10–20 mg/kg body weight of each must be obtained in the diet each day from consumed protein. Moreover, dietary protein sources must contain the full array of EAAs because proteins deficient in one or more EAAs quickly produce an aversive feeding response that results in a significant decrease in consumption of the diet. Semisynthetic diets absent a single EAA have been used to explore the sensing and signaling mechanisms that mediate the behavioral and physiological responses to EAA deprivation.[1–6] An important implication of this work is that dietary amino acids are functioning much like receptor ligands in the sense that specific concentration ranges engage signaling systems linked to molecular responses that have biochemical and physiological consequences. An important distinction is that the

absence of an EAA (e.g., EAA deprivation) is the signal that initiates the full signaling response, suggesting that limitation of an EAA must attain some threshold of restriction for triggering the response. This assumption has been supported by substantial empirical evidence, but the somewhat surprising finding is that dietary restriction (DR) of EAAs within narrowly defined ranges has proven highly beneficial to metabolic status and overall health. In particular, the beneficial responses that result from restricting normal intakes of dietary methionine within a defined range are the subject of this chapter.

## 2. ORIGINS OF DIETARY METHIONINE RESTRICTION AS AN EXPERIMENTAL APPROACH TO INCREASE LONGEVITY

The initial reports of the health benefits of dietary methionine restriction (MR) were from the Orentreich group.[7,8] They found that removing cysteine and reducing dietary methionine from control levels of 0.86% (i.e., 8.6 g/kg diet) to restricted levels of 0.17% (1.7 g/kg diet) increased longevity by 30–40%. A significant difference between this and other models of DR is that no food restriction was involved and the rats were provided their diets ad libitum. In an important follow-up to their initial work, the authors showed that dietary MR increased longevity in a variety of rat strains with differing pathological profiles.[9] These findings showed that dietary MR decreased mortality from all causes of death and support the view that this dietary approach affects the overall rate of aging. In two of their studies,[7,9] Orentreich *et al*. pair-fed a second control group the control diet to the level of intake of the MR group. They found that life span was not extended in the pair-fed group, showing that restriction of methionine, and not overall DR, was responsible for the increase in longevity in the MR group.

Dietary MR also increases longevity in mice[10,11] and flies[12] so it seems likely that MR, like DR, will prove to be efficacious across multiple species. Grandison *et al*.[12] used *Drosophila* as a model organism to identify the nutrients being limited during DR that mediated the increase in longevity and decrease in fecundity. In nature, *Drosophila* eats yeast so implementation of DR is accomplished by diluting the yeasts and allowing unlimited consumption of the diluted diet. Using this approach, enhanced longevity and decreased fecundity appear to be mediated primarily by specific nutrients from the yeasts and independent of caloric intake.[13] Using flies as their model organism, Grandison *et al*.[12] investigated which nutrients were responsible for increased longevity and decreased fecundity by systematically adding back nutrients to the restricted diet. They found that adding back carbohydrate or fat had no effect, but adding back all 10 EAAs reduced longevity and increased fecundity to the same extent as full feeding. Interestingly, adding back methionine alone fully restored fecundity but did not decrease longevity. Thus, the limitation of methionine by DR was solely responsible for reduced fecundity, while limitation of methionine and additional EAAs was required for increased longevity.[12] Further study of the comparative mechanisms engaged by DR in flies and MR in rodents will be required to understand how limiting methionine functions to affect longevity and health span in the two models.

Additional work pointing to a role for methionine metabolism in longevity comes from approaches in model organisms, where manipulation of genes involved in the reduction of

oxidized methionine residues affected life span.[14,15] Oxidation of free methionine or methionine in proteins occurs naturally and produces a mixture of the two diastereomers, methionine-*R*-sulfoxide and methionine-*S*-sulfoxide. Methionine sulfoxide accumulates in tissue proteins over time and progressively compromises their function. The oxidation of methionine is normally reversed by methionine sulfoxide reductases A and B (MsrA and MsrB), which reduce the two diastereoisomers back to methionine. In yeast, overexpression of either MsrA or MsrB increased replicative life span of yeast, while deletion of either isoform reduced yeast life span.[14] To test the hypothesis that these manipulations were affecting longevity by increasing the supply of methionine, Koc *et al*.[14] manipulated media concentrations of methionine and found that increasing methionine decreased life span while limiting methionine increased the replicative life span of yeast. In complementary studies with *Drosophila*, Ruan *et al*.[15] showed that transgenic overexpression of MsrA markedly extends fly life span. In addition, targeted disruption of MsrA in mice significantly reduced their life span, increased their susceptibility to oxidative stress, and accentuated accumulation of oxidized proteins in their tissues.[16] Collectively, these studies make the case that genetic manipulation of the genes involved in repair of oxidized methionine affects longevity in multiple species. Although the underlying mechanisms are far from clear, limiting dietary methionine mimics the effects achieved by genetic enhancement of methionine repair, while increased dietary methionine accentuates the accumulation of oxidized methionine. As presented in subsequent sections, restriction of dietary methionine produces a number of other short- and long-term metabolic responses that may be equally important as mediators of its effects on longevity. Evaluation of their relative significance is the subject of growing interest in the field.

## 3. ANTI-INFLAMMATORY RESPONSES TO DIETARY METHIONINE RESTRICTION AND LONGEVITY

The original studies of dietary MR in rats showed that in addition to increasing life span, the diet produced a lifelong reduction in body weight and accumulation of adipose tissue.[7,8] Calorie restriction to 40% of ad libitum intake (DR) also produces a robust extension of mean and maximal life span across species,[17–19] along with the expected reduction in fat accumulation. In addition to comparable effects on longevity and adiposity, DR and MR share other beneficial outcomes including increased insulin sensitivity and a comprehensive improvement in biomarkers of metabolic health.[8,10,20–24] Previous studies have established that the reduction in adiposity produced by DR is associated with reduced expression of proinflammatory markers in peripheral tissues.[25] Thus, an emerging hypothesis is that DR delays death in part by reducing comorbidities associated with chronic inflammatory states such as obesity, diabetes, and cardiovascular disease.[26] The impact of dietary MR on inflammation has been largely unexplored, and a significant unanswered question is whether decreased adiposity, irrespective of mechanism, produces comparable reductions in systemic inflammation. Using transcriptional profiling of peripheral tissues after long-term DR and MR, we recently examined the systems biology of 59 networks annotated to the inflammatory process.[27] Despite comparable reductions in adiposity with both diets, the anti-inflammatory responses to MR were far more extensive than DR and targeted different inflammatory processes in both liver and white adipose tissue (WAT). In particular, the

primary pathways affected by MR in inguinal white adipose tissue (IWAT) involved phagocyte and macrophage migration, and the majority of genes within these pathways were downregulated by MR.[27] The primary pathways affected by MR in liver involved accumulation, activation, and morphology of leukocytes and macrophages, and like IWAT, the majority of affected genes were downregulated.[27] Another important observation is that the transcriptional changes appear temporally unrelated to improvements in metabolic biomarkers and occur well after the diet reduces fat deposition, suggesting that the anti-inflammatory effects of MR are not responsible for improved insulin sensitivity.[21,27] Collectively, these findings suggest the interesting possibility that the delays in age-associated inflammation by MR are secondary to the metabolic effects of the diet, rather than a direct result of reduced adiposity.

## 4. EFFECTS OF DIETARY MR ON ENERGY INTAKE AND ADIPOSITY

The initial reports that dietary MR increased longevity in rats reported that the diet decreased accumulation of body weight by ~40% over 2 years.[7,8] Paradoxically, the weight-adjusted food consumption of the MR group was ~90% higher than controls during the first 3 months and 62% higher after 2 years.[7] Dietary MR produces a similar increase in energy intake in mice, whether expressed per mouse (~20% increase) or on a weight-adjusted basis (~60% increase).[28,29] To evaluate the impact of dietary MR on energy balance and test for metabolic effects of the diet, the Orentreich group used a pair-feeding approach by feeding a third group of rats the control diet to the amounts consumed by the MR group.[7,22] The pair-fed group gained 75 g more than the MR group over the first 3 months[7] and 115 g more than the MR group after 63 weeks.[22] These findings provide prima facie evidence that dietary MR increases the energy costs of maintaining body weight and are highly suggestive that the mechanism involves increased energy expenditure (EE).

The effects of dietary MR on body composition were initially assessed by comparing fat pad weights to control rats after different times on the diet. For example, dietary MR for 18 months reduced dissectible visceral fat mass from 72 g in the control group to 20 g in the MR group. Expressed as a percent of body weight, the MR diet reduced visceral fat pad weights from 13.7% to 7.4% based on this method of assessing body composition.[22] More accurate and comprehensive assessments of body composition using both DEXA- and NMR-based methods have shown that in control rats, adiposity increases from ~16% to near 30% over a 2-year period after weaning.[21] In rats consuming the MR diet, the increase in adiposity is limited to a 4% increase from 16% to 20% over the same period.[21] This 4% increase in the MR group is achieved in the first 3 months after introduction of MR, so from 3 to 20 months, adiposity is essentially clamped at 20% in the MR group. In contrast, adiposity increases from 16% to 22% during the first 3 months on the control diet and from 22% to 26% from 3 to 9 months.[21] When dietary MR is initiated after physical maturity (e.g., 6 months of age), their initial adiposity (23%) remains unchanged over the following 6 months, while adiposity in the control group increases from 23% to 27% over the same period.[21] Body weight is also unchanged in the 6 months following introduction of dietary MR, while body weight in the control group increases from 385 to 480 g.[21] These studies show that introduction of MR in either a juvenile or adult context effectively limits fat deposition and restricts expansion of adipose tissue mass.

Postweaning dietary MR in mice produces similar but strain-specific changes in body weight and adiposity. In FVB mice, dietary MR effectively limited increases in body weight or adiposity during a 10-week study, whereas the adiposity of control mice increased from ~14% to ~25% over the same period.[28] Increases in adiposity were similarly limited by MR over a 10-week study in C57BL/6J mice and the increase in body weight was limited to 3 g (e.g., from ~18 to ~21 g).[29] In mice on the control diet, body weight increased from 18 to 28 g over the same period, while adiposity increased from 12% to 21%.[29] As well documented in rats, mice also respond to dietary MR by increasing both absolute and weight-adjusted food consumption,[28,29] supporting the view that the diet limits fat deposition despite producing an increase in energy intake. Collectively, these findings make a compelling case that dietary MR, regardless of when it is initiated, impacts nutrient partitioning between fat and protein deposition through effects on both energy intake and EE.

## 5. EFFECTS OF DIETARY MR ON ENERGY EXPENDITURE

Group differences in adiposity not attributable to differences in energy intake infer group differences in EE, and expanded access to small-animal indirect calorimetry has made it the method of choice for measuring EE in rodents. The computational challenges inherent in application of this method are substantial in cases where the genotype or diet results in significant group differences in body size and/or composition. One of the immutable laws of calorimetry, beginning with the eighteenth-century work of Lavoisier and Laplace and extending through the subsequent work of Rubner, Brody, and Kleiber,[30,31] is that EE is proportional to some function of body size. Thus, EE must be scaled accordingly to determine whether group differences remain after correcting for size. This is precisely the problem encountered after long-term MR when group differences in body weight and composition are vastly different. It is well accepted that such differences are not inconsequential since fat and lean tissue make unique mass-specific contributions to overall EE. Scaling to body weight assumes all tissues have the same rate of metabolism, while scaling to lean mass assumes that adipose tissue is metabolically inert. The respective assumptions are demonstrably incorrect, but the error introduced by scaling to body weight is far more significant and increases in proportion to the difference in mass of the groups being compared.[32–36] It is clear that scaling to body weight after long-term MR is particularly inappropriate.[22] Although not ideal, scaling EE to lean body mass introduces far less bias into group comparisons. Application of this approach in rats after 3 months of MR showed that nighttime EE (kJ/h/kg lean body mass) was 70% higher in the MR group than controls while daytime EE was only 25% higher.[21] After 9 months on the diet, both night- and daytime EE were ~90% higher in the MR group, whereas the difference decreased to 25% after 20 months on the diet.[21] When MR was introduced after physical maturity, the diet increased EE by 21% relative to controls and produced a matching 20% higher energy intake.[21] The comparable increases in energy intake and EE are consistent with the stable body weight and composition that was observed in the MR group over the 6-month period of study.[21]

Measurements of EE in mice after various intervals of dietary MR present the same challenges of ratio-based scaling of EE with groups differing in size and composition. For example, at the end of a 10-week study, FVB mice on the control diet are 19% heavier and

have 80% more fat in their carcasses than mice in the MR group.[28] However, despite these differences, lean body mass is comparable between mice on the two diets. EE scaled to lean mass was 31% higher in the MR group than controls by the end of the study.[28] Viewed collectively, the observations provide compelling evidence that dietary MR limits growth and fat deposition by decreasing metabolic efficiency through a mechanism that increases EE per unit lean mass.

An alternative to ratio-based normalization of EE is provided by analysis of covariance (ANCOVA), which uses least squares analysis to assess the impact of variation in fixed (e.g., genotype) and continuous (body composition, intake, and activity) variables in relation to variation in EE between animals. ANCOVA was initially recommended for analysis of rodent indirect calorimetry data in 2006,[33] and a series of recent papers have used the approach to assess the relative contributions of fat-free mass, fat mass, activity, and energy intake to total EE.[34,35,37] Application of ANCOVA to analyze the effects of dietary MR on EE, incorporating body composition, activity, and energy intake into the model, showed that 10 weeks of dietary MR increased EE in C57BL/6J mice by ~35%.[29] Although it may be somewhat surprising that ANCOVA and ratio-based scaling of EE provided similar assessments of the effects of MR on EE, two additional points should be considered. First, application of ANCOVA to indirect calorimetry data in both humans and rodents has shown that lean body mass accounts for ~70–75% of the variation in total daily EE.[38,39] Second, variation in voluntary activity accounts for ~20% of the variation in EE in humans[39,40] and presumably rodents. Therefore, if dietary MR produced changes in voluntary activity, accounting for this source of variation within ANCOVA would diminish the variation in EE attributable to MR. However, analyses of the responses of mice[28,29] and rats[21] to MR provide no evidence that the diet alters voluntary activity. This indicates that dietary MR is affecting energy balance through changes in activity-independent components of EE.

Lastly, several recent publications have addressed additional design considerations when applying indirect calorimetry to test for treatment differences in EE.[34,37,41] One recommendation is to test for group differences in EE in animals before the emergence of treatment-induced changes in size and/or body composition. In recent studies in the authors' laboratory, we have taken this approach and evaluated the acute effects of dietary MR on EE after first obtaining baseline measures of EE in mice on the control diet before introduction of dietary MR. We have found that on day 6 after initiation of MR, nighttime EE (kJ/h/mouse) is 20% higher than controls and the increase in EE occurs in conjunction with an increase in energy intake. Then, by day 10, both night- and daytime EEs are ~30% higher in mice on the MR diet compared to controls (authors' unpublished data). The observed increases in EE occurred well before diet-induced changes in body weight or composition, further supporting the conclusion that the MR-induced increases in EE are not an analytic artifact of ratio-based scaling of indirect calorimetry data. Lastly, continuous monitoring of voluntary activity before and after initiation of MR shows that activity did not differ between the dietary groups at any time during the study. Collectively, analysis of EE using indirect calorimetry after both short- and long-term dietary MR provides convincing support for the conclusion that the diet produces a rapidly developing and long-lasting increase in EE that uncouples peripheral fuel oxidation and increases the energy costs of maintenance

and growth. A significant ongoing challenge is to determine the molecular mechanism(s) of the uncoupling and identify the tissue sites where it is occurring.

## 6. EFFECTS OF DIETARY MR ON FUEL SELECTION AND METABOLIC FLEXIBILITY

The respiratory exchange ratio (RER) provides a real-time index of substrate utilization during the phases of the metabolic cycle. RER is calculated from the molar ratios of $O_2$ consumed and $CO_2$ produced during the oxidation of glucose (1.00), lipid (0.70), and protein (0.80).[42–44] RERs typically approach 1 during the switch to glucose utilization in the fed state and towards 0.7 during the switch to fat utilization during fasting. Metabolic flexibility measures how effectively substrate switching occurs during the transitions between fasted and fed states. The concept arose from the recognition by Kelley and Mandarino[45] that the daily shifts from fat to carbohydrate utilization were impaired by insulin resistance (IR). The impairment is recognized as a diminution in the normal increase in RER that occurs upon refeeding and has been attributed to compromised insulin-dependent glucose uptake in peripheral tissues.[46,47] The increase in glucose utilization associated with improved insulin sensitivity is reflected by a concomitant increase of RER in the fed state. Studies in rats show that dietary MR expands the dynamic range of RER excursions during the transitions between fed and fasted states,[21] supporting the conclusion that metabolic flexibility is significantly enhanced by dietary MR.

An increase in metabolic flexibility is indicative of an increase in overall insulin sensitivity and is often accompanied by a reduction in both fasting and postprandial insulin levels. Malloy et al.[22] reported that 80 weeks of dietary MR reduced basal insulin levels in rats by sevenfold compared to controls. This difference occurs because insulin does not increase with age in the MR group as it does in controls, where insulin levels increase from ~1 ng/ml after weaning to over 7 ng/ml after 80 weeks.[22] The authors also evaluated the ability of rats to clear a glucose challenge after 23 and 72 weeks on the respective diets and found modest improvements in glucose clearance in the MR group only after 72 weeks.[22] However, fasting insulin was lower in the MR group at both 23 and 72 weeks, and the increase in insulin required to clear the glucose challenge was far less than the increase in the control group at both time points.[22]

In mice, the reduction in basal insulin produced by dietary MR can be detected within 7 days of introduction of the diet, and after 8 weeks, the reduction is ~fourfold.[29] As observed in rats,[21] the dynamic range of day to night excursions in RER is also enhanced in mice after 8 weeks of dietary MR.[28] Collectively, these observations make a compelling case that overall insulin sensitivity is enhanced by dietary MR, but provide no insight into how the diet has changed insulin sensitivity in individual tissues. In recent studies conducted in collaboration with the Mouse Metabolic Phenotyping Center at Vanderbilt University, hyperinsulinemic–euglycemic clamps conducted after 8 weeks of dietary MR provided evidence that the diet significantly enhanced insulin-dependent glucose uptake in all peripheral tissues. The glucose infusion rate required to maintain euglycemia during the clamp was threefold higher in the MR group compared to controls (authors' unpublished results). This increase in glucose utilization is consistent with the three- to fourfold decrease in basal insulin observed

after 8 weeks of dietary MR. More importantly, the clamp data show that MR enhanced both the ability of insulin to suppress hepatic glucose production and insulin-dependent uptake of 2-deoxyglucose in muscle and adipose tissue (authors' unpublished data). An important ongoing challenge is to determine the specific mechanism(s) engaged by MR that produce this uniform improvement in insulin responsiveness among all tissues.

One attractive hypothesis is that endocrine changes produced by the diet work through a common mechanism in all tissues to enhance insulin sensitivity. For example, both the adipocyte hormone (adiponectin) and the liver hormone (fibroblast growth factor 21, FGF-21) improve insulin sensitivity and both are significantly increased by dietary MR.[21,22,24,29,48] The actions of both hormones are complex and include functions additional to regulation of insulin sensitivity. The role of each hormone in mediating the physiological responses to dietary MR is currently unknown but is being evaluated using loss-of-function approaches with adiponectin- and FGF-21-null mice. Clearly, the ability of dietary MR to limit expansion of adipose tissue also plays a contributing role and it seems likely that the endocrine changes produced by MR are not entirely secondary to reduced adiposity. In contrast, emerging evidence supports a role for FGF-21 in remodeling of adipose tissue, increases in adiponectin, regulation of EE, and enhancement of insulin action.[49–53] Therefore, the hypothesis that dietary MR produces several of its metabolic effects through modulation of circulating FGF-21 is intriguing and will no doubt be evaluated in the near future.

Dietary MR has been studied in preclinical settings that involve introduction of the diet soon after weaning and after physical maturity, but the translational potential of dietary MR will most certainly involve evaluations of its efficacy in an adult context and likely with obese subjects that manifest markers of metabolic disease. The evidence from preclinical experiments in rats involving introduction of dietary MR after physical maturity shows that the diet is fully effective in reducing basal insulin.[21] The efficacy of dietary MR has also been evaluated in obesity-prone Osborne–Mendel rats and C57BL/6J mice, both of which are sensitive to the obesogenic effects of high-fat diets. In both reports, methionine was limited in 60 kcal% high-fat diets and provided for ~4 months after weaning.[21,48] Dietary MR reduced basal insulin by ~threefold in each case, and in mice, the authors reported that glucose tolerance and the ability of insulin to lower blood glucose were both enhanced by the diet.[48] Thus, in both models of obesity, dietary MR is able to ameliorate the development of obesity and the associated development of IR.

The important remaining question is whether dietary MR can ameliorate and/or reverse existing obesity and IR. This is the most relevant translational context for dietary MR. Male C57BL/6J mice are one of the best studied preclinical models of diet-induced metabolic disease, developing IR and dysregulated lipid metabolism when fed a diet high in saturated fat.[54] The progression and severity of the IR depends on the amount of dietary fat and how long the diet is consumed. For example, very high-fat diet formulations (e.g., 55–60 kcal% fat) produce rapid deterioration in whole-body insulin sensitivity that becomes progressively worse and involves both hepatic and peripheral IR.[55–58] In a recent collaborative study with the Mouse Metabolic Phenotyping Center at Vanderbilt University, we tested the efficacy of short-term (e.g., 8 weeks) dietary MR to reverse IR established in C57BL/6J mice after

chronic consumption (e.g., 16 weeks) of a very high-fat diet (e.g., 58 kcal%). Hyperinsulinemic–euglycemic clamps conducted after remediating the mice for 8 subsequent weeks of MR revealed that the MR diet improved overall insulin sensitivity by 50% and reduced body weight by ~18% (authors' unpublished data). The improvement in overall insulin sensitivity was primarily the result of increased suppression of hepatic glucose production by insulin. Additional experiments with this model will be required to assess the ability of MR to enhance insulin sensitivity in other tissues. It will also be interesting to determine if longer-term consumption of dietary MR will fully reverse diet-induced obesity and IR. The collective view is that dietary MR is highly effective at enhancing insulin sensitivity in multiple biological contexts but more importantly is able to reverse preexisting IR associated with diet-induced obesity.

## 7. EFFECTS OF DIETARY MR ON HEPATIC LIPID METABOLISM: TRANSCRIPTIONAL MECHANISMS

Dysregulation of lipid metabolism and ectopic lipid accumulation in peripheral tissues are hallmarks of metabolic syndrome, while reductions in circulating and tissue lipid levels are biomarkers of improved metabolic health. The effects of dietary MR on lipid metabolism were initially described by Malloy *et al*.[22] who reported that MR attenuated the age-associated increase in circulating cholesterol and triglycerides in rats. The authors examined the responses in rats that had consumed the MR diet for different times after weaning. They found that serum triglycerides did not increase between week 16 and week 81 in rats on the MR diet, while in rats on the control diet, levels increased by ~70% over the same period. Serum cholesterol also increased by 65% in the controls between 16 and 105 weeks, while the increase was limited to 16% in the MR group.[22] In studies of shorter duration, MR produced a threefold reduction in serum triglycerides that was evident 1 month after introduction of the diet.[59] These findings indicate that dietary MR reduces serum lipids soon after its introduction, but they also show that it prevents the subsequent age-dependent increase that normally occurs in conjunction with increasing adiposity.[22,59]

Changes in serum lipids are often indicative of changes in hepatic lipogenesis and/or release, coupled with changes in the capacity of adipose tissue to release and/or store triglyceride. In addition to reducing serum lipids, dietary MR reduces hepatic lipids by two- to threefold in both mice[29] and rats,[60] suggesting the liver as an important target of MR and potentially responsible for the reduction in serum lipids. Excess fat accumulation in the liver typically results from some combination of increased delivery, increased synthesis, and decreased oxidation or decreased export.[61] Therefore, the dysfunctional steps that lead to increased liver fat provide a useful framework for evaluating the mechanisms through which dietary MR produces the opposite effect and reduces both plasma and hepatic triglycerides.

Studies to date have primarily addressed the transcriptional effects of MR on genes involved in lipogenesis or fatty acid oxidation[60,62] and have been coupled with *ex vivo* and *in vivo* assays to evaluate the biological significance of observed changes of genes within particular pathways. For example, the rate-limiting enzymes for *de novo* lipogenesis (acetyl-CoA carboxylase 1, ACC-1; fatty acid synthase, FASN) and triglyceride synthesis (stearoyl-CoA desaturase 1, SCD-1) were identified as transcriptional targets of MR in the liver.[62] Thus,

the observed decreases in FASN and ACC-1 mRNAs were predicted to reduce the capacity for hepatic *de novo* lipogenesis, while the reduction in SCD-1 mRNA should reduce formation of the preferred monounsaturated fatty acids (C16:1 and C18:1) used in the first committed step of TG synthesis. Therefore, the observed reductions in hepatic triglyceride by dietary MR are consistent with the predicted reductions in hepatic lipogenic capacity, but they do not establish a cause and effect relationship or that *in vivo* rates of hepatic lipogenesis are decreased by MR. However, recent studies in our laboratory assessed the physiological significance of the transcriptional changes in hepatic lipogenic genes[60] by measuring $^{2}H$-enrichment in palmitate 12 h after injecting control and MR mice with $^{2}H_{2}O$ (authors' unpublished data). As expected, the calculated rates of *de novo* lipogenesis in livers from MR mice were three- to fourfold lower than control mice. The effects of dietary MR on hepatic fatty acid oxidation have also been measured in rats where, after 9 months on the diet, the capacity to oxidize $^{14}C$-palmitate was increased ~40% compared to controls.[60] Lastly, the effects of MR on hepatic export of triglyceride have not been measured, but based on the significant reduction in serum lipids, it seems likely that the combination of increased oxidative capacity and reduced lipogenic function are the most dominant effects of the diet on hepatic lipid metabolism.

More comprehensive analyses of the transcriptional responses to dietary MR have used high-content microarray-based approaches to identify biological processes affected. For example, Perrone *et al*.[24] used gene set enrichment analysis of their microarray data to identify biological process that were differentially affected in peripheral tissues by 3 months of MR. A discussion of the extensive transcriptional effects of MR is beyond the scope of this chapter, but if attention is restricted to hepatic lipid metabolism, it is somewhat surprising that their gene set enrichment algorithm failed to identify lipid or fatty acid synthesis as pathways that were downregulated by MR. In contrast, both hepatic fatty acid oxidation and cholesterol/isoprenoid metabolism were identified as downregulated pathways.[24] These conclusions are somewhat at odds with their previous data showing significant downregulation of hepatic lipogenic genes, upregulation of oxidative genes, and increased hepatic citrate synthase activity.[59,62] To assess the functional significance of observed changes in gene expression, Perrone *et al*.[24] coupled unbiased metabolomics analysis of tissues and serum with their transcriptional analysis to assess the impact of dietary MR on the metabolome. This comprehensive analysis should provide a valuable ongoing resource for probing how transcriptional changes of genes in specific tissues affect the levels of associated metabolites in the serum.

Another recent high-content evaluation of the transcriptional responses to chronic MR identified lipid metabolism as among the top biological processes affected by the diet in liver and adipose tissue.[60] Recent developments in bioinformatics software not only include better annotation of the systems biology of transcriptional changes but also provide new algorithms that seek to identify transcriptional mechanisms from the observed changes in gene expression. This analytic strategy seeks to identify transcription factors (TFs) and nuclear receptors mediating dietary responses based on expected causal effects of TFs/nuclear receptors on known target genes relative to observed changes of those genes within the dataset. By examining direction of change in target gene expression, the algorithm

predicts whether specific TFs/nuclear receptors are activated or inhibited. In the liver, the coordinated downregulation of over 30 genes involved in lipid metabolism led to the prediction that SREBF1, SFEBF2, and MLXIPL were inhibited by MR.[60] Moreover, the fact that the majority of repressed genes receive transcriptional input from two or more of these TFs further corroborates the interconnectedness of the regulatory network. The diet-induced decrease in expression of hepatic SREBP-1c is consistent with the repressive effect of MR on lipogenic gene expression. SREBP-1c and ChRE-BP are also subject to regulation by cholesterol and glucose, and both are decreased by dietary MR.[23] However, it remains unclear whether decreases in cholesterol and glucose are the cause or product of transcriptional responses to MR. In either case, hepatic SREBP-1c appears to be a key target of the mechanism through which MR reduces hepatic *de novo* lipogenesis, triglyceride synthesis, and lipid content.[60] An important remaining challenge is to identify additional TFs/nuclear receptors recruited by dietary MR and explore the signaling mechanisms involved in their recruitment as mediators of the transcriptional responses to the diet.

## 8. TRANSCRIPTIONAL EFFECTS OF DIETARY MR ON ADIPOSE TISSUE

Dietary MR produces significant changes in adipose tissue mass, cell morphology, mitochondrial content, and endocrine function.[21,23,28,29,60,62] An evaluation of the transcriptional responses of peripheral tissues to MR (e.g., liver, IWAT, skeletal muscle, and brown adipose tissue (BAT)) shows that >75% of all differentially expressed genes were found in the liver and IWAT.[60] Lipid metabolism was the top molecular and cellular process affected by MR in both tissues, but interestingly, the diet produced opposite effects on genes associated with lipid synthesis in the two tissues.[60] For example, the downregulation of lipogenic genes in the liver is mirrored by a reciprocal upregulation of key lipogenic genes in WAT depots.[60] QRT-PCR and Western blots of the rate-limiting enzymes for *de novo* lipogenesis (FASN and ACC-1) and triglyceride synthesis (SCD-1) show that MR increased their mRNA and protein expression in all WAT depots.[60] These findings indicate that dietary MR has transformed WAT into a potentially important site of lipid synthesis to compensate for the loss of lipogenic capacity in the liver.[60] This conclusion is supported by two lines of evidence. First, RERs consistently exceed 1 at night in the MR group, indicative of high rates of glucose utilization coupled to interconversion to fat by *de novo* lipogenesis.[63,64] Given that lipogenic capacity of the liver is severely compromised by MR, it seems likely that WAT is the site where glucose inter-conversion to lipid is occurring. The second line of evidence comes from measuring $^{2}H$-enrichment in palmitate in mice acutely injected with $2H_2O$. As predicted, the calculated rates of *de novo* lipogenesis in retroperitoneal WAT, IWAT, epididymal WAT, and BAT from MR mice were three- to fourfold higher than control mice (authors' unpublished observations), establishing that both WAT and BAT are important sites of *de novo* lipogenesis after MR. Considered together, these findings indicate that dietary MR has produced a fundamental change in the respective functions of liver and adipose tissue, particularly in the fed state where the roles of adipose tissue and the liver in *de novo* lipogenesis have been reversed.

The transcriptional responses to MR in WAT also included genes involved in lipid oxidation, tricarboxylic acid (TCA) cycle, respiratory chain function, and adaptive thermogenesis.[21,24,28,60] To evaluate the impact of these changes, the *ex vivo* capacity of

freshly isolated tissues to oxidize fatty acids was measured using $^{14}$C-palmitate, coupled with measures of citrate synthase activity as a surrogate of TCA cycle flux. MR produced a fivefold increase in both palmitate oxidation and citrate synthase activity in IWAT, along with a doubling of mitochondrial numbers.[60] The transcriptional remodeling of WAT is similar to the "browning" of WAT that occurs in WAT during cold exposure and involves many of the same changes seen with MR, including reduction in cell size, formation of multilocular adipocytes, and increased uncoupling protein 1 (UCP1) expression.[21,28] It is well established that cold exposure, acting via norepinephrine, elicits a simultaneous increase in glucose uptake, lipogenesis, and β-oxidation in BAT.[65,66] Moreover, after chronic cold exposure, the increased number of brown adipocytes in WAT enhances glucose uptake and lipogenic function within these depots. Recent work has emphasized the importance of BAT to triglyceride clearance while documenting the regulatory role of sympathetic nervous system (SNS) input in the process.[67] Thus, the simultaneous increase in lipogenic and oxidative gene expression in WAT may be reflective of extensive remodeling of WAT depots produced by dietary MR through effects on SNS activity. This conclusion is supported by our previous work showing that dietary MR increased UCP1 mRNA 3- to 10-fold among WAT depots,[21,28] and recent observations that MR doubled mitochondrial density among WAT depots and increased oxidative capacity of IWAT by fivefold.[60] Many of these responses characteristic of the "browning" response in WAT are also produced or enhanced by FGF-21.[53] Given the significant upregulation of hepatic FGF-21 expression and release by MR,[24] it will be interesting to assess the relative roles of the SNS and FGF-21 as mediators of the remodeling of WAT and increase in EE produced by the diet.

Dietary MR increases EE by increasing uncoupled respiration in peripheral tissues, but a full accounting of sites involved and tissue-specific mechanisms is incomplete. The evidence is compelling that induction of UCP1 in BAT and WAT by MR increases uncoupled respiration in both tissues. The increase accounts for ~50% of the overall diet-induced increase in EE,[21,28] leaving ~50% of the MR-induced increase in total EE being mediated through UCP1-independent mechanisms. Recent studies with cold-adapted UCP1-null mice show that high rates of uncoupled respiration do not require the presence of UCP1.[68] While activation of thermogenic respiration during cold exposure increases β-oxidation of fatty acids, it also produces a coordinated increase in glucose utilization coupled with a paradoxical increase in *de novo* lipogenesis.[66,69,70] An interesting feature of the interconversion of glucose to lipid prior to oxidation is that it produces more heat and captures less of the potential energy normally obtained from direct oxidation of glucose.[66,70] Thus, by increasing heat loss and decreasing net ATP generation from glucose during interconversion to lipid, *de novo* lipogenesis represents a metabolically inefficient substrate cycle capable of making a significant UCP1-independent contribution to nonshivering thermogenesis, particularly during periods of high glucose utilization.[70] The interconversion of glucose to lipid prior to oxidation produces an RER of 1.0 when rates of *de novo* lipogenesis and lipid oxidation are equal.[43,44] Thus, the impact of this inefficient conversion of glucose to lipid is minimal when rates of fatty acid oxidation are low (e.g., fed state at ambient temperature). However, rates of fatty acid oxidation increase more than 12-fold in UCP1-null mice during cold exposure,[68] and nighttime RQs reached or exceeded 1.0, guaranteeing that rates of glucose conversion to lipid were proportionately increased to

match or exceed the increase in lipid oxidation. Stated another way, the increased flux of glucose through this metabolically inefficient pathway is capable of significantly increasing heat production and EE through a mechanism that does not require but can be enhanced by UCP1. In our studies with MR, the diet produces a consistent enhancement of *de novo* lipogenesis that is temporally matched with nighttime increases in EE. We hypothesize that enhanced substrate cycling of glucose through this mechanism at night is an important component of the uncoupled respiration and metabolic inefficiency produced by dietary MR. However, EE in animals on the MR diet is also higher than controls during the day when fat becomes the primary metabolic fuel and substrate cycling through a glucogenic/lipogenic mechanism is precluded. And since rodents primarily sleep during the day, group differences in resting EE suggest that dietary MR is also impacting overall EE by reducing the metabolic efficiency of fat oxidation. It is unclear whether this uncoupling of fat oxidation is restricted to specific tissues or whether MR is inducing metabolic inefficiency in multiple tissues. These questions are being explored using loss-of-function approaches with UCP1-null mice and with mice lacking β-adrenergic receptors. The respective mouse lines will allow us to determine the proportions of the increased EE that are dependent on UCP1 and the extent to which increased SNS activation is required for transcriptional remodeling of adipose tissue. Given the role of the SNS in regulating endocrine function of adipose tissue, it also seems likely that MR may be using the SNS as a motor arm to produce components of its energy balance phenotype through reduction of leptin expression and release.[21,22,28] For example, the reductions in plasma leptin produced by MR are disproportionate to the reductions in adipose tissue mass produced by the diet,[21,22,28] producing a strong orexigenic signal that is perhaps responsible in part for the hyperphagic response to dietary MR. An attractive model to explore this hypothesis is the *ob/ob* mouse, which lacks the ability to express functional leptin because of a mutation within the gene.[71] However, the *ob* mutation results in secondary changes in β-adrenergic signaling in adipose tissue that compromise the ability of both BAT and WAT to fully respond to SNS input.[72,73] We hypothesize that the MR-dependent increase in SNS input to adipose tissue is an important component of the mechanism through which the diet functions to affect energy balance. Therefore, it will be interesting to determine experimentally the extent to which the metabolic derangements of the *ob/ob* mouse compromise its ability to respond to dietary MR.

When considered in a physiological context, the transcriptional and morphological remodeling of WAT by dietary MR produces fundamental changes in the way WAT functions in both fed and fasted states. In the fed state, WAT becomes an important site of glucose uptake and utilization for interconversion to lipid, while in the fasted state, the increase in oxidative capacity expands its involvement in fatty acid oxidation. The net effect is to limit overall expansion of adipose tissue mass and ectopic lipid accumulation, but the changes also modify the respective roles of adipose tissue and the liver, particularly in terms of lipogenesis. When viewed collectively, the tissue-specific transcriptional responses to dietary MR make a compelling case that the diet has effectively remodeled the integration of lipid metabolism between the liver and adipose tissue in a manner that is beneficial to the overall metabolic profile of the animal.

## 9. SENSING OF DIETARY MR

An important unanswered question is how restriction of dietary methionine is detected and how sensing of the restriction is translated into highly integrated transcriptional responses in the liver and WAT. Restricting availability of EAAs effectively limits charging of tRNA with its cognate amino acid and activates the highly conserved and ubiquitously expressed protein kinase, GCN2 (general control nonderepressible 2), which limits ribosomal translation of most mRNAs.[74–76] Transcriptional effects of EAA deprivation on lipogenic genes were initially identified in human HepG2 cells, where media lacking single EAAs decreases transcriptional initiation and expression of FASN.[77] These studies suggest the interesting possibility that MR functions through GCN2 to decrease expression of lipogenic genes in the liver. However, MR increased lipogenic gene expression in WAT and muscle, arguing against a role for GCN2 and suggesting involvement of additional sensing systems in these tissues. Alternatively, WAT and muscle may be responding to endocrine signals originated in different tissues or to neural signals resulting from central sensing of reducing circulating methionine. These questions are being explored in GCN2-null mice at present and findings to date suggest that many of the responses to dietary MR are intact in the absence of GCN2. Although preliminary, these findings provide compelling evidence that sensing and signaling systems in addition to GCN2 are involved in detecting and mediating the physiological, biochemical, and transcriptional responses to dietary MR.

## 10. PERSPECTIVES AND FUTURE DIRECTIONS

In our work on dietary MR to date, we have identified a range of dietary methionine concentrations that produces profound improvements in biomarkers of metabolic health. The important next steps are to refine our understanding of the degree of MR linked to each component and extend this work to dietary restriction of other EAAs. A significant body of work has been devoted to the study of dietary leucine deprivation. We recently published a detailed accounting of the similarities and differences between the responses produced by the two diets.[29] One critical difference is that dietary MR produces hyperphagia while dietary leucine deprivation causes significant food aversion and a rapidly developing negative energy balance that cannot be sustained beyond a few weeks. It should be noted that restriction of dietary methionine to levels much lower than the 0.17% methionine provided in the MR diet formulation produces food aversion and the same detrimental effects as leucine deprivation. Therefore, a comparison of the physiological responses to dietary MR and leucine deprivation suggests that the EAAs are in a sense functioning as ligands with responses determined by the degree of the restriction. It follows from this that an important future objective will be to examine systematically the physiological responses to incremental restriction of methionine, leucine, and other EAAs. In addition to identifying a range of restrictions that are most beneficial, this approach could also provide important mechanistic insights regarding EAA-specific sensing mechanisms. For example, in preliminary studies to date, we have found that dietary leucine restriction reproduces some but not all of the physiological responses produced by dietary MR. These models promise to provide a fruitful approach to dissect and identify the unique mechanisms engaged by dietary restriction of methionine to improve metabolic health.

Another experimental strategy being taken to better understand the components of the complex responses to dietary MR is to study their spatial and temporal organization. For example, it is difficult to distinguish the direct effects of MR in a particular tissue from responses that are modulated by detection of the restriction in another anatomical site that may then provide secondary signaling or endocrine input to the initial site. Thus, in addition to its spatial organization, the individual component of the response to MR is also temporally organized, developing in a reproducible progression after introduction of the diet. In a recent review,[29] we proposed four potential sites of sensing dietary EAA restriction: (1) direct sensing of luminal or absorbed EAAs in the gut, (2) sensing of EAAs in the portal circulation and/or liver, (3) direct sensing of reduced EAAs by tissues, and (4) sensing of EAAs in various regions of the brain. There are enormous gaps in our understanding of how these and as yet unknown sensing components function together to mediate the integrated physiological responses to changes in dietary EAA content. It will be particularly important to identify the central amino acid-sensing systems and map how they are organized to provide integrated regulation of the components of energy balance and communication to peripheral tissues. A better understanding of how dietary MR enhances tissue-specific and overall insulin sensitivity is also a central focus. The overall metabolic phenotype produced by EAA deprivation versus restriction is the product of a series of responses that are anatomically and temporally organized and, in many cases, interdependent. Therefore, a significant ongoing challenge within the field will be to develop experimental approaches that distinguish between the direct, tissue-specific responses to MR and the responses perceived in one anatomical site and modulated in another.

Lastly, the translational potential of the concepts developed in preclinical studies of dietary MR was recently evaluated in a human cohort meeting the criteria for metabolic syndrome.[78] Dietary MR was accomplished using the semisynthetic medical food, Hominex-2® (Abbott Nutrition, Columbus, OH) in a short-term study (16 weeks) to evaluate the metabolic consequences of limiting dietary methionine from 35 mg/kg BW/d to 2 mg/kg BW/d. The experimental diet (Hominex-2®) is a commercial food designed to provide nutritional support for patients with pyridoxine-unresponsive homocystinuria or hypermethioninemia. It is comprised in part of elemental amino acids, and their associated low palatability resulted in high withdrawal rates and raised questions about compliance and achieving the desired degree of MR. Another poststudy concern stems from the fact that although Hominex-2® is methionine-deficient, it contains methionine-sparing cystine.[79] This could be significant because the rodent MR diet lacks cystine and a recent study reported that adding it back to the rodent MR formulation reversed many of the beneficial metabolic effects of MR.[59] Thus, the cystine in Hominex-2® may have limited the full efficacy of the MR achieved with this approach. Notwithstanding these experimental limitations, we found that dietary MR increased fat oxidation and reduced hepatic lipid content in subjects with metabolic disease.[78] Development of methods to produce highly palatable, methionine-depleted proteins represents a better approach because it will solve both the cystine and palatability problems. This represents an area of intense interest in our laboratory and is likely to provide the best strategy for testing the translational potential of dietary MR in the clinic.

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## ABBREVIATIONS

| | |
|---|---|
| **ACC-1** | acetyl-CoA carboxylase 1 |
| **ANCOVA** | analysis of covariance |
| **BAT** | brown adipose tissue |
| **DR** | dietary restriction |
| **EAAs** | essential amino acids |
| **EE** | energy expenditure |
| **FASN** | fatty acid synthase |
| **FGF-21** | fibroblast growth factor 21 |
| **GCN2** | general control nonderepressible 2 |
| **IR** | insulin resistance |
| **IWAT** | inguinal white adipose tissue |
| **MR** | methionine restriction |
| **MsrA and MsrB** | methionine sulfoxide reductase A and B |
| **RER** | respiratory exchange ratio |
| **SCD-1** | stearoyl-CoA desaturase 1 |
| **SNS** | sympathetic nervous system |
| **TCA** | tricarboxylic acid |
| **TF** | transcription factor |
| **UCP1** | uncoupling protein 1 |
| **WAT** | white adipose tissue |

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