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S-Methyl cysteine sulfoxide: its effects on cardiometabolic outcomes in high-fat fed C57BL/6 mice and relevance to human health

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S-Methyl cysteine sulfoxide (SMCSO) is an organosulfur compound with demonstrated hypocholesterolemic, anti-diabetic, and antioxidant benefits in rodents. However, the doses used have limited translatability for humans. We explored whether SMCSO could blunt development of diet-induced metabolic syndrome features in mice using doses that are potentially more translatable and achievable in humans. Male mice (C57BL/6; n = 54) were randomly assigned into one of five groups: [1] normal-diet; [2] high-fat-diet; or high-fat-diet with [3] 60 mg kg^{-1}, [4] 170 mg kg^{-1}, or [5] 350 mg per kg per body weight (BW) of SMCSO, respectively. Doses were administered five days per week for 12 weeks *via* gavage. Repeated measure analysis of variance (ANOVA) was used to determine changes over time. One-way ANOVA with Dunnett's *post hoc* analysis determined differences between groups for all parametric data, with Kruskal–Wallis with Dunn's test used for non-parametric data. The high-fat fed group (group 2) was the comparator for all analyses with statistical significance set at $p < 0.05$. High-fat fed mice experienced weight gain, impaired glucose tolerance, elevated total cholesterol, high-density lipoprotein-cholesterol (HDL-C), and measured low-density lipoprotein-cholesterol (mLDL-C), compared to normal-diet fed mice (all $p < 0.05$). Adding 170 mg kg^{-1} and 350 mg per kg BW per day of SMCSO reduced diet-induced elevations in HDL-C (–11 to –12%), whilst 350 mg per kg per BW day lowered triglycerides (–16%), compared to high-fat fed mice (all $p \leq 0.05$). Other cardiometabolic parameters were not significantly altered. SMCSO did not improve glucose tolerance, mLDL-C, or blunt weight gain in high-fat fed mice. The higher SMCSO doses (170 and 350 mg per kg BW per day) lowered diet-induced elevations in HDL-C, although the mechanism is unclear. Whether this finding has relevance to human health remains unknown.

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1. Introduction

Abdominal obesity, hypercholesterolemia, poor glucose control, and insulin resistance are all hallmarks of poor cardiometabolic health, and can lead to hypertension, atherogenic and vascular changes, type-2-diabetes, cognitive impairment, fatty liver, and overall poor health outcomes.[1–4] Cardiometabolic parameters such as elevated cholesterol, triglycerides, and insulin resistance, are central features of metabolic dysfunction, and often measured as indicators of cardiovascular damage.[2] The clustering of cardiometabolic risk factors, commonly referred to as metabolic syndrome, affects an estimated 25–35% of adults, with prevalence increasing globally.[1–3] Metabolic syndrome is associated with a 1.5-fold increased risk for all-cause mortality, as well as a two-fold increased risk for cardiovascular disease and a five-fold increased risk for type-2-diabetes.[5,6] Therefore, preventive efforts to reduce the development of metabolic syndrome is a high health priority globally.[2,4]

A higher intake of vegetables is associated with a lower risk of metabolic syndrome and its cardiometabolic features.[7–9] However, certain vegetables including dark green-leafy, cruciferous and alliums may offer greater cardiometabolic risk

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reduction, compared to other vegetable sub-types.[8,10,11] Cruciferous (also comprising many of the dark-green leafy vegetables) include broccoli, cauliflower, cabbage, kale, and Brussels sprouts; and alliums include onions, garlic, and leek.[12] One in fifteen Australian adults (~6.5%) met the recommended intake of vegetables in 2022, with only ~half a serving (1/4 cup) being from either green and/or cruciferous vegetables.[13,14] High intake of these vegetables is inversely related to type-2-diabetes prevalence, central obesity, hypertension, and mitigates many of the drivers associated with the development of cardiometabolic risk.[8,10,12,15,16] Many of the benefits of these vegetables are attributed to their abundance of sulfur compounds including glucosinolates (*e.g.*, glucoraphanin) and *S*-alk(en)yl cysteine sulfoxides, such as *S*-methyl cysteine sulfoxide (SMCSO; also referred to as methiin).[17] Emerging evidence suggests that these compounds have protective benefits for cholesterol and glucose regulation, act as anti-inflammatory and antioxidant agents, and impart anticarcinogenic, antimicrobial and neuroprotective effects.[12,18]

SMCSO is an organosulfur compound found almost exclusively in cruciferous and *allium* vegetables.[19] Recently, we reported that SMCSO contributes ~0.6–1.9% of the dry weight of cruciferous vegetables, a higher concentration than glucosinolates at ~0.3–1.2%.[20] Despite this higher concentration, research investigating the role of SMCSO in cardiometabolic health has frequently been overlooked.[21,22] Previous studies have shown that doses of 180–364 mg per kg body weight (BW) per day of SMCSO given to rats over 14–60 days lowered total cholesterol by ~18–33%[23–27] and LDL-C by ~26%.[28] Doses of 200 mg per kg BW per day lowered triglycerides by 26% in hypercholesterolemic rats after 45 days[26] and by 65% in diabetic rats after 30 days.[29] These doses (180–364 mg per kg BW per day) were also shown to lower hepatic cholesterol and lipid content (~10–18%, and 11–33%, respectively) after 14–45 days.[23,24,27] The mechanisms behind these cholesterol-lowering benefits have been identified to be through altered lipogenic enzymes (malic enzyme, lipoprotein lipase), increased fecal bile acids and cholesterol 7α-hydroxylase (CYP7A1) activity, as well as elevated levels of hydroxymethylglutaryl coenzyme A (HMG CoA) reductase, which collectively contribute to reduced cholesterol absorption.[25–27] Administering SMCSO at 200 mg per kg BW per day for 30–60 days has also been shown to lower blood glucose levels (~19–25%) in diabetic rats.[27–32] In these previous studies, SMCSO was shown to act as an insulin secretagogue, regulate hepatic glycogen stores *via* glucose-6-phosphatase and hexokinase activity[26,27,29,32] and blunt negative histological changes associated with diabetes within pancreatic tissue.[29] Some of the anti-diabetic mechanisms of SMCSO were reportedly due to its antioxidant and anti-inflammatory effects; blunting of malondialdehyde (−12%), hydroperoxides (−34%) and conjugated dienes (−12%),[31] and raising of hepatic superoxide dismutase to mitigate oxidative damage.[29,31] The most common dose of SMCSO across these studies was 200 mg per kg BW per day.[23–32] When accounting for differences in body surface area between rats and mice,[33] this range of doses is equivalent to mice receiving 360–728 mg per kg BW per day, with the most common dose equivalent to 400 mg per kg BW per day. Importantly, these SMCSO doses given in earlier rat studies would be equivalent to humans consuming ~54 serves of cruciferous and alliums daily,[33] based on a 75 gram serving size.[34] This level of intake is clearly not feasible with limited translatability to human intake. Taking these aforementioned doses into consideration, whilst also attempting a more translatable dose for humans, an upper dose of 350 mg per kg BW per day was selected. We have previously explored the effects of 43–256 mg per kg BW per day SMCSO.[35] This is equivalent to humans consuming between three and 18 serves of broccoli per day, based on a 75 g serving size.[34] We added SMCSO (43–256 mg per kg BW per day) into the high-fat feed of young five-week-old mice over 12-weeks and reported that these levels had no significant blunting effect on the development of metabolic syndrome.[35] However, for this previous study the dose of SMCSO consumed by each mouse was highly dependent on daily feed intake, it presumed an equal consumption of the provided feed by mice sharing a cage, and precision in calculating the amount of leftover feed. As such, the total amount of feed (and therefore SMCSO) provided, minus the leftover feed contained within the hopper plus that remaining on the floor, was divided by the number of mice per cage (n = 2). Uneaten feed was quantified by sifting the floor contents from each cage using a large-hole silicone collapsible colander (Woolworths), and resultant calculation equally divided by the number of mice per cage. It was not possible to determine if one mouse ate more or less SMCSO-containing feed than the other mouse in the cage. Subsequently, this current study explored SMCSO administered *via* oral gavage to ensure exact doses of SMCSO given to each mouse were administered according to the individual BW of each mouse, measured earlier that same day. In light of our previous non-significant results, we additionally used this current study as an opportunity to explore a higher upper SMCSO dose (≤350 mg per kg BW per day) and to increase the starting age of mice from 5 weeks[35] to 8 weeks as C57BL/6 mice aged ≥8 weeks are known to be more receptive to developing diet-induced features associated with metabolic syndrome.[36] Ultimately, the key differences in methodology between this study and our previous study[35] were how SMCSO was administered and dosage used. Oral gavage at a maximum dose of ≤350 mg per kg BW per day was used in the current study. In our previous study SMCSO was incorporated into feed pellets and therefore dosage was subject to how much each mouse consumed, providing an estimated ≤256 mg per kg BW per day.

This study aimed to explore whether SMCSO, given *via* oral gavage at doses of ≤350 mg per kg BW per day (equivalent to ≤24 serves per day in humans) blunts the development of high-fat diet induced metabolic syndrome features in C57BL/6 male mice (*i.e.*, weight gain, glucose and lipid profiles). We also sought to explore the effect of SMCSO at these doses on inflammation and hepatic lipid content. We hypothesized that SMCSO within these vegetables may act as a partial mediator of their cardiometabolic benefits, with the intention that the

doses used may subsequently inform dietary recommendations in humans.

2. Experimental

This study was approved by Animal Ethics Committees at Harry Perkins Institute of Medical Research (AE282-BALMER) and Edith Cowan University (24319 SHAFAEI DARESTANI). All components comply with the National Health and Medical Research Council Guidelines on the Care and Use of Laboratory Animals in Australia, the Australian code of practice for the care and use of animals for scientific purposes, the Conduct of Ethical Research and Teaching Involving Animals Policy and the Animal Welfare Act. This manuscript has adhered to the ARRIVE 2.0 guidelines for reporting animal research.[37]

2.1 Subjects and study design

Male mice (C57BL/6; n = 54), aged 7–8 weeks, were purchased through the Animal Resources Centre (Perth, Western Australia), selected from the same groups of parents, randomized, and numbered (*via* ear-notch), before being delivered to animal rooms at the Harry Perkins Institute of Medical Research (Nedlands, Western Australia). Incoming weights were recorded. Mice were maintained on a standard mouse feed during a one-week acclimatization period housed under a 12-hour light–dark cycle at 23 ± 2° Celsius (43 to 62% humidity) with two to three mice per cage. At the end of the first week, weight, urine, and blood were collected, before mice were placed into one of five pre-randomized intervention groups: [1] normal diet (ND, n = 10); [2] high-fat diet (HFD, n = 11, as the control); [3] HFD + 60 mg kg^{-1} of SMCSO, (n = 11); [4] HFD + 170 mg kg^{-1} of SMCSO, (n = 11) or [5] HFD + 350 mg kg^{-1} of SMCSO, (n = 11), per BW, *via* oral gavage (five days per week). The normal diet feed (group 1) consisted of 20% protein and 4.8% fat (providing 11% of digestible energy from lipids and 14 kJ g^{-1} of energy intake). The high-fat feed (groups 2 to 5) contained 23% protein and 24% fat (providing 43% of digestible energy from lipids and 19 kJ g^{-1} of energy intake). Both feeds were commercially pre-prepared (Specialty Feeds, Glen Forrest, Western Australia). All mice had unlimited access to their allocated feed, except during stated fasting periods, with *ad libitum* access to water and environmental enrichment (*e.g.*, paper cones, wooden sticks, cotton). Body weight and feed intake were recorded weekly. Mice were maintained on their respective diet for 12 weeks, with urine and blood samples collected at baseline, 6- and 12-week time points. At 12-weeks, mice were anaesthetized before collecting final blood samples and then euthanized before collecting tissue samples. Upon entry, the two investigators (CRH, LB) were responsible for all dispensing of feed, SMCSO, animal husbandry and procedures throughout the study, therefore, blinding was not feasible thereafter. Care was taken to analyze results in a blinded capacity, whenever possible.

2.2 SMCSO dose and administration

The SMCSO was purchased from Toronto Research Chemicals, Toronto, Canada (*via* PM Separations, Queensland, Australia: CAS No 6853-87-8). Prior to commencing, we confirmed the stability of SMCSO when mixed in saline and stored at 4–6° C for ≤seven days, *via* liquid chromatography triple quadrupole mass spectrometry. SMCSO solutions were prepared in saline, twice a week and stored at 4–6 °C. The entire volume was never stored beyond five days and was administered *via* oral gavage daily (Monday to Friday ~mid-afternoon) for the 12-week duration. Each dose was given within the animal housing room ensuring immediate return of each mouse to its cage after administration. Daily weights of all mice were recorded to determine the correct volume of solutions required for oral gavage. Mice in groups 1 and 2 received saline (*i.e.*, placebo) whilst mice in groups 3, 4 and 5 received SMCSO at either 60 mg kg^{-1}, 170 mg kg^{-1}, or 350 mg per kg of BW per day, as per their randomly allocated intervention.

2.3 Sample collections

2.3.1. Urines. At baseline, 6- and 12-week time points, urine was collected by having each mouse void into a single-use tray. Urines were transferred to microcentrifuge tubes and immediately frozen in liquid nitrogen and stored at −80 °C until analysis for the quantification of SMCSO levels by liquid chromatography triple quadrupole mass spectrometry, as previously described.[35]

2.3.2 Insulin and glucose tolerance. At weeks 11 and 12 respectively, both glucose tolerance and insulin tolerance were assessed after five hours of fasting. Neither anesthesia nor tail warming were used during testing as these procedures are both known to trigger hyperglycemia and/or physiological changes that could impact results.[38] Blood glucose was measured from the tail vein before (t = 0 minutes) and after (t = 15, t = 30, t = 45, t = 60, t = 90 and t = 120) administration of either 1 g glucose per kg for intraperitoneal glucose tolerance test (IPGTT) or 0.5 units insulin per kg per BW for intraperitoneal insulin tolerance test (IPITT). Blood glucose levels were assessed using Freestyle Glucose Strips and a Glucometer (Freestyle Optimum Neo, Australia). Calculations were performed by measuring the area under the curve (AUC) using the trapezoid rule, as previously described.[39]

2.3.3 Final blood and tissues. After 12 weeks, mice were fasted for five hours and anaesthetized with isoflurane prior to blood collection (retro-orbitally and *via* cardiac puncture). Serum was separated *via* centrifugation (3000 rpm, 4 °C, 10 minutes) and all samples were immediately frozen in liquid nitrogen before storing at −80 °C until analysis. Serum total cholesterol, measured low-density lipoprotein cholesterol (mLDL-C), high-density lipoprotein cholesterol (HDL-C), triglycerides, high-sensitivity C-reactive protein (hs-CRP), and glucose were analyzed using Alinity reagent kits (PathWest Laboratories, Fiona Stanley Hospital, Perth).

Following cervical dislocation, hepatic tissue was collected, washed in physiological saline, immediately frozen in liquid nitrogen, and stored at −80° C until analysis. Hepatic tissue

was used to measure total hepatic lipid content, analyzed using the Folch method.[40] For this, equal quantities of thawed hepatic tissue were weighed and recorded, evenly sliced, and placed into a solvent mix (2 : 1; chloroform : methanol) before being homogenized at eight watts for ~four minutes using a Sonicator (Branson Sonifier 150). The homogenate was then siphoned and filtered before being placed into a miVac Pressure concentrator (Genevac Ltd, Germany) at 45 °C until completely dry. The final weight of each sample was subtracted from its original to quantify the total hepatic lipid for each sample. For the histological analysis, hepatic, white adipose, pancreatic, and kidney tissues were immediately fixed in 4% formaldehyde overnight, incubated in 70% ethanol and embedded in paraffin. Tissues were then sliced into 4μm sections and stained with hematoxylin and eosin (Cell Central, University of Western Australia) before being photographed for histological features using a Nikon Ni-E microscope equipped with a Nikon DS-Qi2 camera (Tokyo, Japan).

2.4 Power and statistical analysis

Previous studies have shown that C57BL/6 mice fed a HFD develop obesity, poor glucose homeostasis and hypercholesterolemia from ≥eight weeks.[36] Using anticipated changes to fasting glucose, ten mice/group was calculated to provide >80% power at $\alpha = 0.05$ to detect a difference of 400 AUC units, with a standard deviation of 320. Prior to commencing analysis, we checked for outliers and normality (omnibus K2 D'Agostino & Pearson test). If outliers were identified and normal distribution was achieved through removal, parametric tests were used. If removing outliers failed to achieve normality, the outlier was left in, and non-parametric analyses were performed. Repeated measure analysis of variance (ANOVA) was used to determine changes over time. One-way ANOVA with Dunnett's *post hoc* analysis was used to determine if, and where, differences were between all groups. Mean ± standard error of the mean (SEM), or mean difference and confidence interval (CI) were presented, as appropriate. Kruskal–Wallis (with Dunn's test for multiple comparisons) was used for non-normally distributed data and was presented as median (interquartile range [IQR]). The high-fat group (*i.e.*, group 2; HFD) was used as the comparator for all analyses. Student *t*-tests were used to confirm metabolic syndrome features (*i.e.*, triglycerides, fasting glucose, insulin tolerance) that were not sufficiently powered to report statistical significance using ANOVA. To account for the lower weight gain observed in the SMCSO-fed groups, we additionally adjusted for total energy intake. Analyses were performed using Stata (version 16, StataCorp, USA) and GraphPad Prism, version 10 (LLC., USA). Statistical significance was set at a 2-sided type-1 error rate of $p < 0.05$.

3. Results

There were no significant differences in baseline weights across our five intervention groups prior to randomization ($p = 0.2897$). The high-fat fed mice experienced weight gain, impaired glucose tolerance, elevated total cholesterol, HDL-C, and mLDL-C, compared to normal-fed mice (all $p < 0.05$). Triglycerides, fasting glucose, and insulin tolerance in the high-fat fed mice were not significantly different from the mice fed the normal diet (all $p > 0.05$). However, using Student *t*-tests, high-fat fed mice had increased fasting glucose in comparison to normal diet fed mice (all $p < 0.05$), while triglycerides and insulin tolerance remained non-significant (SI Fig. S1).

3.1 Adiposity and feed intake

As expected, mice consuming a high-fat diet gained significantly more weight than mice consuming the normal diet over the 12 weeks [mean difference in grams (95% CI)]: 7.7 (4.6 to 11); $p < 0.05$ (Fig. 1a). Administering SMCSO (60 mg kg^{-1}, 170 mg kg^{-1}, or 350 mg per kg BW per day) in conjunction with the high-fat diet did not significantly blunt the weight gain when compared to high fat diet alone at the 12-week time point (Fig. 1b and SI Table S1); [mean difference in grams (95% CI)]: 2.1 (−0.91 to 5.1), 1.5 (−1.5 to 4.5) and −1.8 (−1.1 to 4.9), respectively (all $p > 0.05$). Although mice consumed slightly more feed in the two groups with lower SMCSO doses (60 mg kg^{-1} and 170 mg kg^{-1}), weight gain did not significantly differ, even after adjusting for total energy intake (SI Table S1). A significant difference in total energy intake was reported between the normal and high-fat fed groups at the 12-week time point (Fig. 1c and SI Table S1).

3.2 Glucose and insulin profiles

Mice fed a high-fat diet had significantly worsened glucose tolerance than those fed the normal diet, and this was not blunted by SMCSO (Fig. 2a and SI Table S1). There was no difference in fasting serum glucose across our five intervention groups after a 5-hour fasting period ($p > 0.05$) (Fig. 2b and SI Table S1). Although mice fed the high-fat diet appeared to have worsened insulin resistance, it did not reach statistical significance. Therefore, the observed trend of SMCSO improving insulin tolerance cannot be fully determined (see Fig. 2c and SI Table S1).

3.3 Lipid profiles

Both total cholesterol and mLDL-C were elevated in the high-fat diet group with neither being significantly reduced by administering SMCSO at 60 mg kg^{-1}, 170 mg kg^{-1}, or 350 mg per kg BW per day for 12 weeks (Fig. 3a, b and SI Table S1). Levels of HDL-C were elevated in the high-fat group [median (IQR): 2.2 (2.1–2.2)] compared to the normal diet [1.8 (1.7–1.9)] and this was lowered by the addition of 170 mg kg^{-1} [1.9 (1.8–2.1)] and 350 mg kg^{-1} [1.9 (1.8–2.1)] of SMCSO per BW per day ($p \leq 0.05$), but not by the lower-dose (60 mg kg^{-1}) of SMCSO per BW per day (Fig. 3c). Serum triglycerides were not significantly elevated in mice given the high-fat diet ($p > 0.05$). However, mice given SMCSO at 350 mg per kg BW per day in addition to the high-fat diet reported significantly lower triglyceride levels than those receiving the high-fat diet alone ($p <$

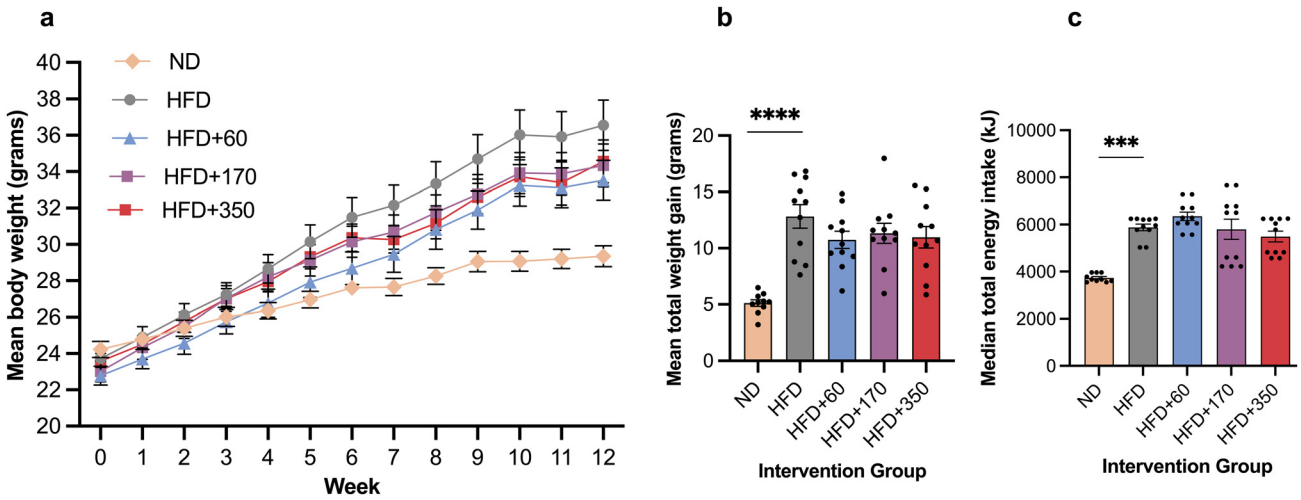

Fig. 1 (a) Weekly mean body weight measurements over 12 weeks; (b) mean total weight gain (g) differentiated by intervention group with error bars indicating standard error of the mean; and (c) median total energy intake (kJ) over 12 weeks differentiated by intervention group with error bars indicating group median and interquartile range (IQR). The data of the ND, HFD + 60, HFD + 170, and HFD + 350 groups were each statistically compared to the HFD group; ANOVA was performed for (a) and (b), whilst Kruskal–Wallis test was performed for (c). All intervention groups contained n = 11 except for ND, which contained n = 10, statistical significance as ***p < 0.001, ****p < 0.0001. BW, body weight; HFD, high-fat diet; HFD + 60, high-fat diet + 60 mg per kg BW per day of SMCSO; HFD + 170, high-fat diet + 170 mg per kg BW per day of SMCSO; HFD + 350, high-fat diet + 350 mg per kg BW per day of SMCSO; ND, normal diet; SMCSO, *S*-methyl cysteine sulfoxide.

0.05) (Fig. 3d). The median (IQR) triglyceride values per intervention group were as follows; ND: 0.59 (0.53–0.67); HFD: 0.64 (0.55–0.69); HFD + 60 mg kg^{-1}: 0.63 (0.56–0.69); HFD + 170 mg kg^{-1}: 0.61 (0.54–0.65); and HFD + 350 mg kg^{-1}: 0.54 (0.48–0.61).

3.4 Inflammatory, hepatic, and histological changes

Inflammation, as measured *via* hs-CRP, was not significantly elevated in mice fed the high-fat diet, nor was this blunted *via* the addition of SMCSO (both p > 0.05) (Fig. 4a and SI Table S1). Histology images (Fig. 5) suggest that larger adipocytes were present in the high-fat diet group (up to 150μm in diameter), compared to the SMCSO-treated (~50–100 μm) and the normal-diet fed groups (<50 μm). However, no statistically significant differences in hepatic lipid levels were observed across the five intervention groups when measured using the Folch method (Fig. 4b and SI Table S1). Greater variability in hepatic lipids was observed in mice receiving the higher dose of SMCSO (350 mg per kg BW per day). Further analysis was not conducted due to the non-significant changes reported in quantitative measurements. Final liver weights [mean grams (SEM)] after euthanasia were not significantly different between mice fed the normal diet [1.2 g (0.05)], HFD [1.1 g (0.05)], or HFD with SMCSO at 60, 170, or 350 mg per kg BW per day [1.1 g (0.04), 1.1 g (0.06), and 1.1 g (0.05), respectively], all p > 0.05. There were minimal histological alterations evident in pancreatic islets (ranging ~100–300 μm in size) whilst the architecture of renal corpuscles, glomeruli and renal tubules within the kidney also appeared unchanged in the mice supplemented with SMCSO (Fig. 5).

3.5 Urinary SMCSO levels

Urinary SMCSO levels increased in each of the SMCSO-administered groups (60 mg kg^{-1}, 170 mg kg^{-1}, and 350 mg per kg BW per day) at 6 weeks (100%, 757%, 1372%, respectively) (Fig. 6) before further increasing in the latter two SMCSO groups (13% and 25%, respectively) and reducing (−35%) in the lower-SMCSO group by week-12 (SI Table S2). The final urinary SMCSO levels measured at 12 weeks in the groups receiving 60 mg kg^{-1}, 170 mg kg^{-1}, and 350 mg per kg BW per day doses of SMCSO were [mean (SEM) μg ml^{-1}]: 0.64 (0.11), 2.7 (1.10), and 6.6 (1.40), respectively.

4. Discussion

Humans consume SMCSO regularly due to its abundance in cruciferous and *allium* vegetables, yet little is understood about this compound, the effectual doses, or its potential role in the health benefits of these vegetables. Administering a high-fat diet to C57BL/6 male mice induced several cardiometabolic risk factors, including weight gain, impaired glucose tolerance and cholesterol levels (*i.e.*, total cholesterol, mLDL-C, and HDL-C), compared to mice fed a normal diet (all p < 0.05). Adding SMCSO (60 mg kg^{-1}, 170 mg kg^{-1}, and 350 mg per kg BW per day) for five days per week over 12 weeks resulted in a statistically significant blunting of HDL-C and triglyceride levels but did not significantly impact other cardiometabolic parameters measured.

Whilst SMCSO has been previously shown to have significant hypolipidemic and hypoglycemic effects in rats (between 18 to 33%, and 19 to 25%, respectively),[23–30] this is only the

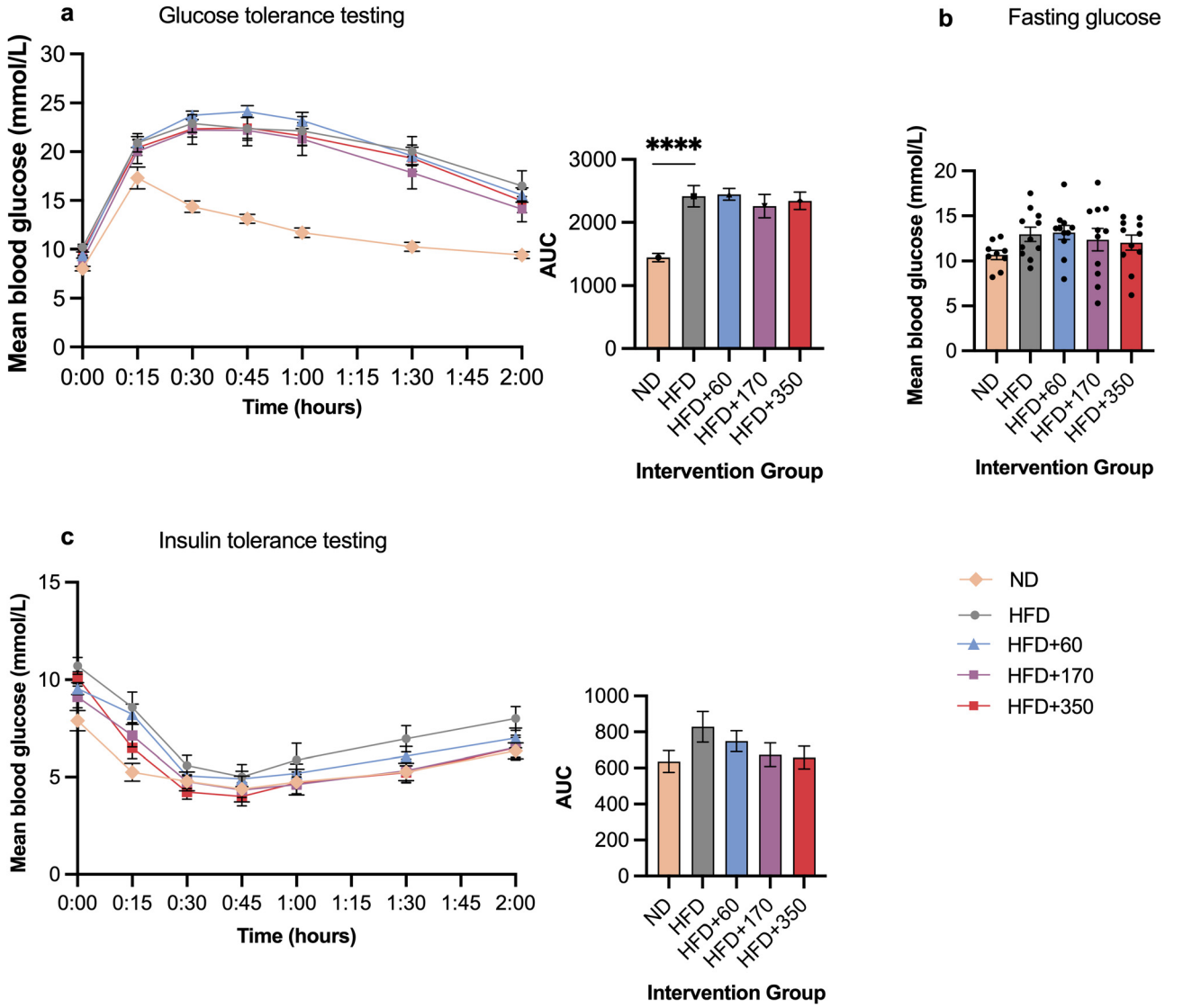

Fig. 2 Displayed as group mean and standard error of the mean for (a) blood glucose over 2 hours and corresponding area under the curve (AUC) following an intraperitoneal bolus of glucose, (b) circulating fasting serum glucose, and (c) blood glucose over 2 hours and corresponding AUC following an intraperitoneal bolus of insulin; all after 12 weeks of intervention. The data from the ND, HFD + 60, HFD + 170, and HFD + 350 groups were each statistically compared to the HFD group using ANOVA. All intervention groups contained $n = 11$ except for ND, which contained $n = 10$ for (a), $n = 9$ for (b) [as one mouse in our ND group did not provide adequate blood volume for analysis] and $n = 10$ for (c) [as the fasting sugar level of one mouse in our HFD + 350 group reached <2.0 mmol L^{-1} so was administered glucose and discontinued from this procedure as per facility protocol]. Statistical significance as ****$p < 0.0001$. BW, body weight; HFD, high-fat diet; HFD + 60, high-fat diet + 60 mg per kg BW per day of SMCSO; HFD + 170, high-fat diet + 170 mg per kg BW per day of SMCSO; HFD + 350, high-fat diet + 350 mg per kg BW per day of SMCSO; ND, normal diet; SMCSO, *S*-methyl cysteine sulfoxide.

second time that these outcomes have been explored in mice.[35] However, the effect of any given substance is impacted by pharmacokinetics and physiology of the animal species. To enhance translatability across differing animal species, we used an online calculator which accounts for differences in body surface area across animal species (*i.e.*, in this case, rodents) when converting, and translating, doses.[33] Larger animals, such as rats for example, have lower metabolic rates than mice, and as such the physiological response is slower with smaller doses per BW required.[33] Importantly, after accounting for these differences in body surface area between animals,[33] the predominant dose used previously in rats was 200 mg per kg BW per day, which is the equivalent of 400 mg per kg BW per day in mice, therefore, greater than the highest dose used in our current study (350 mg per kg BW per day).

Our team has previously supplemented SMCSO into high-fat mice feed (at doses of 43–256 mg per kg BW per day) and found no significant lowering of the high-fat induced cardiometabolic features after 12 weeks.[35] Two of the three mice groups supplemented with SMCSO in our previous study

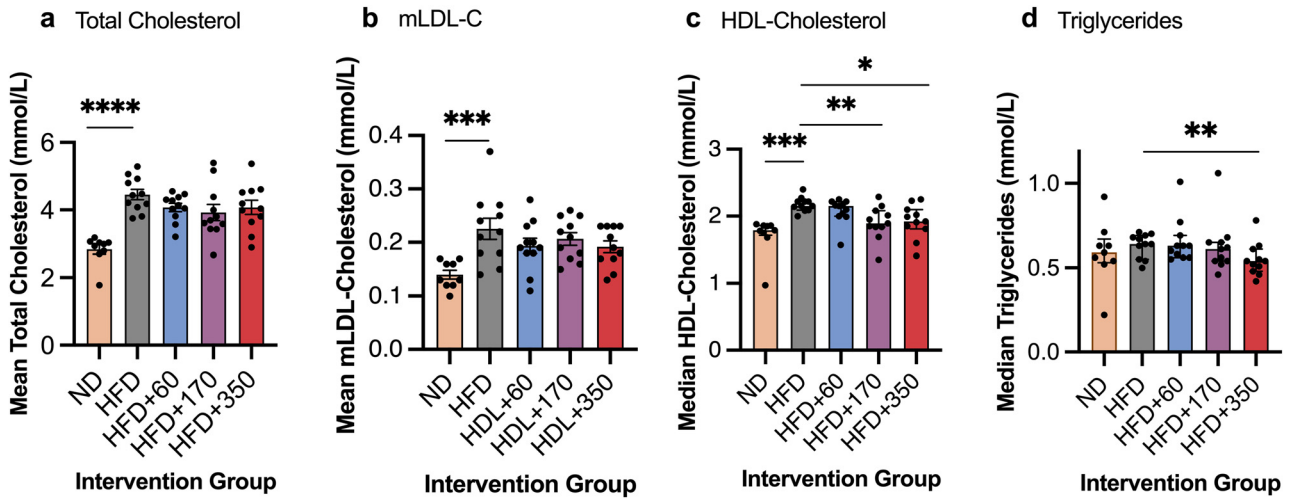

Fig. 3 The lipid profiles for fasting serum (a) total cholesterol, (b) measured LDL-C, (c) HDL-C, and (D) triglycerides in mice across our five intervention groups, after 12 weeks. The data from the ND, HFD + 60, HFD + 170, and HFD + 350 groups were each statistically compared to the HFD group. Data are presented as mean ± standard error of the mean *via* ANOVA (for a and b) and as median and interquartile range (for c and d) *via* Kruskal–Wallis test. All intervention groups contained $n = 11$ except for ND which contained $n = 9$ [as one mouse in our ND group did not provide adequate blood volume for analysis]. Statistical significance as $*p = 0.05$, $**p < 0.05$, $***p < 0.001$, $****p < 0.0001$. BW, body weight; HDL-C, high-density lipoprotein cholesterol; HFD, high-fat diet; HFD + 60, high-fat diet + 60 mg per kg BW per day of SMCSO; HFD + 170, high-fat diet + 170 mg per kg BW per day of SMCSO; HFD + 350, high-fat diet + 350 mg per kg BW per day of SMCSO; mLDL-C, measured low-density lipoprotein cholesterol; ND, normal diet; SMCSO *S*-methyl cysteine sulfoxide.

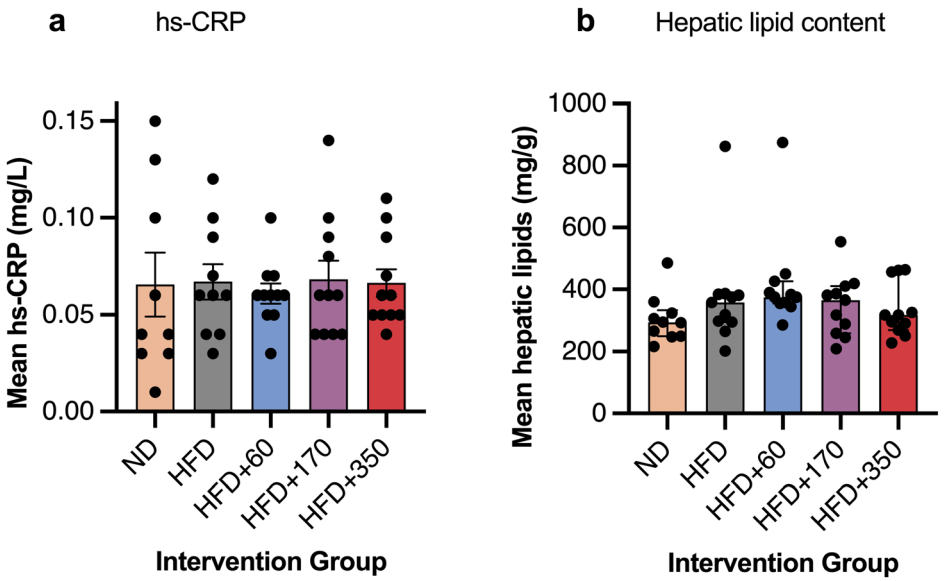

Fig. 4 (a) The difference in hs-C-reactive protein (hs-CRP) and (b) hepatic lipid content across our five intervention feed groups; ND, HFD, HFD + 60, HFD + 170, or HFD + 350, for 12 weeks, respectively. The data from the ND, HFD + 60, HFD + 170, and HFD + 350 were each statistically compared to the HFD group. Data are presented as mean ± standard error of the mean using ANOVA (for a) and as median and interquartile range (for b), using Kruskal–Wallis test. The intervention groups in (a) contained $n = 11$ except for ND which contains $n = 9$ [as one mouse in our ND group did not provide adequate blood volume for analysis], and HFD which contained $n = 10$; the intervention groups in (b) contained $n = 11$ except for ND which contained $n = 10$. BW, body weight; HFD + 60, high-fat diet + 60 mg per kg BW per day of SMCSO; HFD + 170, high-fat diet + 170 mg per kg BW per day of SMCSO; HFD + 350, high-fat diet + 350 mg per kg BW per day of SMCSO; ND, normal diet; SMCSO, *S*-methyl cysteine sulfoxide.

reported greater feed consumption, without significant weight gain.[35] However, the weight gain reported in our previous study was unable to be adjusted for, as the SMCSO was incorporated directly into the mice feed.[35] Nonetheless, this current study found a similar pattern with feed intake and weight gain to that observed previously. The lower two of the three SMCSO-administered groups (60 mg kg^{-1} and 170 mg kg^{-1}) consumed non-significantly more feed alongside non-significant changes

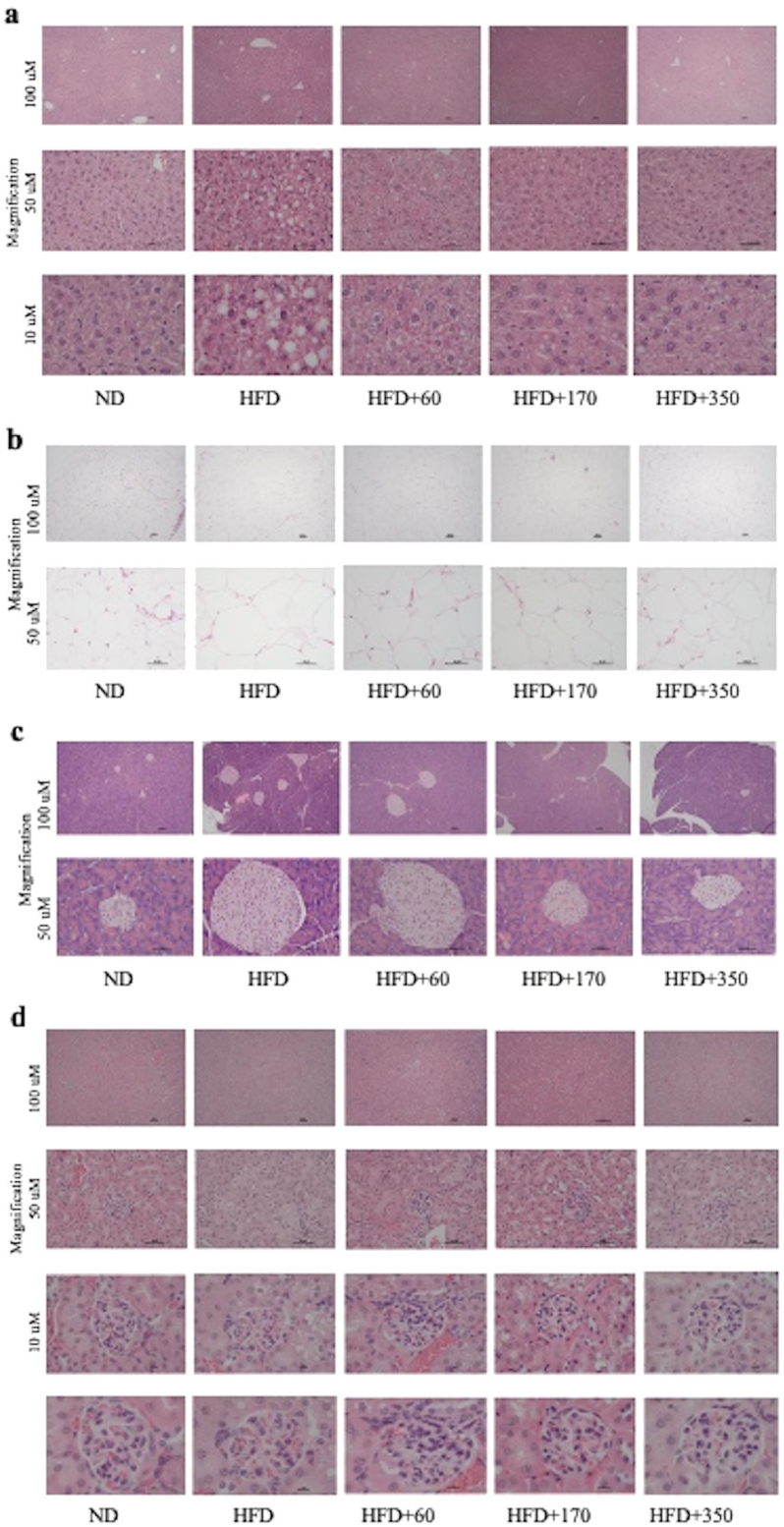

Fig. 5 Histological images of (a) hepatic, (b) white adipose, (c) pancreatic, and (d) kidney tissues after receiving either ND, HFD, HFD + 60, HFD + 170, or HFD + 350, for 12-weeks. Samples stained with hematoxylin and eosin. BW, body weight; HFD, high fat diet; HFD + 60, high-fat diet + 60 mg per kg BW per day of SMCSO; HFD + 170, high-fat diet + 170 mg per kg BW per day of SMCSO; HFD + 350, high-fat diet + 350 mg per kg BW per day of SMCSO; ND, normal diet; SMCSO, *S*-methyl cysteine sulfoxide.

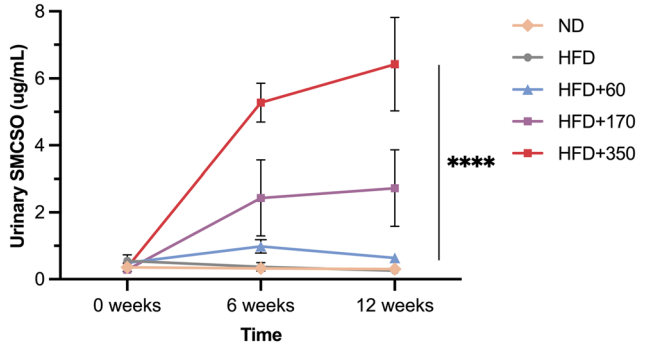

Fig. 6 The mean and standard error of the mean for urinary SMCSO levels (μg mL^{-1}) measured across intervention feed groups at baseline, 6-weeks, and 12-week time points. The data from the ND, HFD + 60, HFD + 170, and HFD + 350 groups were each statistically compared to the data from the HFD group, at 12- weeks. All intervention feed groups contained n = 11 except for ND which contains n = 10. Statistical significance at the 12-week time point as ****p < 0.001. BW, body weight; HFD + 60, high-fat diet + 60 mg per kg BW per day of SMCSO; HFD + 170, high-fat diet + 170 mg per kg BW per day of SMCSO; HFD + 350, high-fat diet + 350 mg per kg BW per day of SMCSO; ND, normal diet; SMCSO, *S*-methyl cysteine sulfoxide.

in weight gain, even after adjusting for total energy intake. No significant changes in appetite or weight have been reported from previous studies in rats.[25,30,31] We observed that urinary SMCSO levels increased dramatically at 6-weeks in the SMCSO-fed groups. This increase was lessened at 12-weeks, despite continued administration. A similar pattern was observed in our previous study[35] suggesting that SMCSO may accumulate within tissue per s, which has been reported by others.[41] A human dietary intervention study that provided a SMCSO/glucoraphanin-rich broccoli soup to male participants undergoing prostate surgery identified that SMCSO accumulated in *peri*-prostatic tissue, even in a subset of those assigned into the 'no soup' arm.[41] With urinary excretion of SMCSO intake known to take ≤2 weeks in humans, this suggests the potential for metabolic activity *in vivo*.[41,42] Recent evidence has identified a key metabolite of SMCSO (*S*-methyl methanethiosulfonate) also disrupts multiple cellular and energy metabolism pathways in human prostate cancer cells.[43] Whether SMCSO reaches a level of tissue saturation and/or accumulation with the potential for cellular or metabolic changes that may alter lipid metabolism in mice requires further exploration.

In this study, high-fat fed C57BL/6 mice increased total cholesterol by 50% and mLDL-C by 64%. Yet, administering SMCSO (even at our highest dose of 350 mg per kg BW per day) resulted in non-significant reductions of just 9% for total cholesterol and 17% for mLDL-C. Mice in the high-fat group had a non-significant increase in triglycerides (+8%). Further administering our highest dose SMCSO (350 mg per kg BW per day) in the high-fat fed group resulted in a statistically significant 16% reduction in triglycerides over those fed high-fat alone. The triglyceride lowering effect of SMCSO has been reported previously in rats (~26 to 65%).[26,29] Earlier rat studies have reported between 6–15% reductions in HDL-C after administering SMCSO.[26,27,29] These earlier studies identified that SMCSO lowered overall cholesterol absorption in rats whilst concomitantly aiding cholesterol clearance (*i.e.*, increasing HMG CoA reductase, CYP7A1 activity, bile acid and sterol excretion, and blunting hepatic lipogenic enzymes such as lipoprotein lipase, glucose-6-phosphate dehydrogenase and malic enzyme).[25,26] We identified comparable (−11 to −12%) reductions in HDL-C after administering 170 mg kg^{-1} and 350 mg per kg BW per day SMCSO doses, although the underlying mechanisms responsible for this effect are unknown. These findings raise some important considerations in terms of lipid metabolism between mice and humans, and the subsequent challenge in translation for humans.

The changes to lipid profiles of mice given high-fat feed are consistent with previous studies, with C57BL/6 mice particularly suspectable to developing dyslipidemia.[36] Despite known differences in circulating lipid profiles, rodents (*i.e.*, rats, mice) continue to be widely used pre-clinical models to assist in finding novel nutraceuticals to ameliorate metabolic dysfunction.[44] In this study, the mice receiving the HFD increased their HDL-C levels, which were significantly lowered in those mice administered our two upper doses of SMCSO (170 and 350 mg per kg BW per day). It is known that HDL-C is comprised of cholesterol, triglycerides, phospholipids, and approximately 90 different proteins (*i.e.*, apolipoproteins [Apo]), and is largely considered beneficial due to its major role in reverse cholesterol transport.[45,46] Less-widely known is that many of these HDL-associated proteins play functional roles in inflammation, immunity, proteolysis and in the transport of vitamins, with similarities observed between mice and humans.[46] The key apolipoproteins involved in HDL metabolism in humans include Apo A-1, Apo A-II, Apo C, and Apo E.[45,46] Apo A-1 is the major structural apolipoprotein comprising ~70% of total protein content in HDL and is synthesized in the liver and intestine, whilst Apo A-II comprises ~20% and is synthesized in the liver.[45] Apo C is produced in the liver and aids in the regulation of triglycerides, either through stimulatory (Apo C-II) or inhibitory (Apo C-III effects on lipoprotein lipase activity.[45,47] The movement, and incorporation of cholesterol and phospholipids from enterocytes and hepatocytes to the Apo A-1 protein is a key role of adenosine triphosphate (ATP) binding cassette subfamily A member 1 (ABCA1), necessary for HDL formation and sequestering of lipids.[45,47] Subsequently, HDL and its key proteins, play major roles in transporting excess cholesterol from peripheral tissues back to the liver, as part of the reverse cholesterol transport process. A similar pattern is also evident in C56BL/6 mice, with the HDL-C structure dominated by Apo A-I and Apo A-II lipoproteins.[46] Evidence suggests that despite the similarities, functional differences remain likely and thus caution in interpretation across species is always advised.[46] For example, administering an atherogenic diet to mice has been shown to increase HDL-C and stimulate reverse cholesterol transport,[48] but may subsequently promote the development of a more pro-inflammatory subtype of HDL-C.[48–51] Further research exploring this

inflammatory nature of HDL-C in mice is warranted, particularly when investigating potential targets for human translatability. It is possible that as dietary fat increased in the HFD group, hepatic production of HDL-C levels rose to transport excess cholesterol back to liver for excretion or for uptake into fecal sterols and bile acids to minimize build up in peripheral tissues, as previously reported.[25–27] The two upper doses of SMCSO in our study blunted this HDL-raising effect, thus the protective HDL-C requirements shifted. Similarly, this may have triggered the reduction in triglyceride levels observed between our highest SMCSO-group and the ND control group. Evidence also suggests that HDL-regulating genes involved in lipid metabolism [e.g., liver-X-receptor (LXR), peroxisome proliferator-activated receptors (PPARs), and ABCA1)] can each be activated by a HFD, subsequently raising HDL-C production to help mitigate inflammation and subsequent cardiovascular damage.[52] Gwon *et al.*[53] reported that another sulfur-based compound, phenethyl isothiocyanate which is also found in cruciferous vegetables, has been shown to blunt HFD-induced atherosclerosis and obesity in C57BL/6 mice, by upregulating the expression of these aforementioned genes; PPARγ LXR-α, and ABCA1. It is possible that SMCSO attenuated the HFD-induced increase in HDL-C through a similar mechanism, however without further testing, this remains hypothetical. An HFD can also trigger adiposity, particularly in the hepatic and adipose cells, which in turn may elevate lipoprotein metabolism (*i.e.*, HDL-C). However, aside from significant weight gain observed in our HFD group compared to our ND group, our findings from hepatic lipids do not support this mechanism for SMCSO at the tested doses. Additionally, diets rich in fats have been shown to promote synthesis of Apo A-1, which can subsequently raise HDL-C levels in mice.[54] Therefore, we acknowledge a species-specific difference in HDL-C response. An increase in HDL-C is typically considered beneficial in humans; however, SMCSO administered to mice in this study resulted in a favorable reduction in HDL-C levels. These findings may not directly translate to humans. Despite the identified similarities between humans and rodents,[46] the lipoproteome is less well characterized in mice, and known variations between humans and rodents remain,[46,55] making direct translations of this study to humans difficult. We additionally acknowledge that cholesterol ester transfer protein (CETP) is central to the metabolism of cholesterol in humans; a protein responsible for transferring cholesterol esters from HDL-C to the apolipoprotein B-containing lipoproteins, such as the low- and very-low density lipoproteins.[46,55] With humans carrying most of their cholesterol within LDL-C, and mice carrying theirs in HDL-C, this makes mice less likely to develop high LDL-C-induced atherosclerosis, as in humans.[46,56] Importantly, this may additionally explain why our high-fat fed mice increased their HDL-C by 20%, and why an 11–12% reduction in HDL-C (after administering SMCSO) is viewed as a favorable change in lipid profile.

Our high-fat fed group developed impaired glucose tolerance, although no other features of poor glucose control were reported. Despite SMCSO reducing overall fasting glucose (19–25%) in rats previously,[27–32] only one of these earlier studies reported an improvement in glucose tolerance testing.[28] As we did not induce an increase in fasting glucose, we were unable to observe the previously reported hypoglycemic effects of SMCSO. The liver is the primary site of glucose disposal in mice, whilst in humans it is the skeletal muscle.[44] As in humans, glucose and insulin control in mice can be influenced in response to stress which may explain this finding, along with the natural variations in basal metabolic rates and gut microbiota.[44]

Using an online calculator[33] and a database reporting dietary SMCSO levels in foods,[19] we estimated SMCSO doses administered in this study were equivalent to a human consuming ~four, 12 and 24 serves (300 g, 900 g and 1800 g) of mixed cruciferous (*e.g.*, broccoli, Brussels sprouts, cabbage) vegetables daily. For example, the average SMCSO content found in mixed cruciferous vegetables is 0.96 mg g^{-1},[19] or 72 mg per 75 g serving size[34] equating to four serves (*i.e.*, 300 mg SMCSO required/72 mg in a serve), 12 serves (*i.e.*, 900 mg/72 mg), and 24 (*i.e.*, 1800 mg/72 mg) serves per day. Brussels sprouts are reported to contain the highest concentration of SMCSO (*i.e.*, 3.18 mg g^{-1} or 239 mg per 75 g serving size),[19] therefore, these doses would alternatively be equivalent to a human consuming ~one, four, and seven serves of Brussels sprouts daily. We acknowledge these levels are typically not achievable through human habitual intake and nutraceutical supplementation would still be needed. Many food constituents (*i.e.*, bioactives) work synergistically.[57,58] It is possible that lower doses of SMCSO within whole foods work synergistically with other plant-derived bioactives and collectively contribute to the overall benefits of cruciferous and *allium* vegetables.

We acknowledge the strengths and weaknesses of our study. Firstly, our dose of SMCSO was given directly *via* oral gavage, therefore we were assured of the exact doses being accurately administered based on daily weight. Secondly, whilst our study was powered on changes to fasting glucose, we did not find any statistically significant changes, when using ANOVA. We did, however, find a statistically significant change in the AUC for glucose tolerance. Furthermore, statistical significance in fasting glucose was observed between our normal diet and high-fat control groups after performing a simple Student *t*-test. This indicates that whilst we initiated hyperglycemia through a high-fat diet, we lacked the power to see the full extent of differences across multiple groups. Therefore, we were likely underpowered to observe changes in lipids. Furthermore, the mechanism by which SMCSO impacts cholesterol (particularly HDL-C and triglycerides) in C57BL/6 mice remains unclear and needs further investigation. In light of our urinary excretion results, a deeper exploration is needed into possible accumulation of SMCSO into tissues, requiring further study. A further limitation is that we did not measure visceral fat weights and blood liver enzymes at the time of euthanasia, which may have identified other SMCSO-related effects. We also acknowledge that by including only males in this study, we have limited our ability to observe any

potential gender differences in response to SMCSO intake. Lastly, using animal models to infer generalizability in human physiology poses challenges as it is not always evident if results are directly translatable to humans.[59] Although the mouse lipoproteome is less well understood than the human lipoproteome, there are many shared similarities in the roles of lipid-associated LDL and HDL proteins between humans and mice.[46] These similarities support the use of this animal model when exploring effects on lipoprotein and cardiometabolic-related outcomes that may be relevant to humans.[44,46] Nonetheless, we acknowledge the differences in lipoprotein metabolism (particularly CETP and potential variation in HDL functionality) between human and mice as being substantial limitations. That said, animal models continue to offer a unique opportunity to investigate novel bioactive compounds on pathophysiological changes and provide insight into possible mechanisms that may aid human health and/or disease prevention.

Recent evidence has identified SMCSO as a key biomarker of cruciferous intake.[41,60,61] It has also emerged as a metabolite of interest in healthy dietary patterns, such as the Adherence to the Dietary Approaches to Stop Hypertension (DASH) diet, with higher SMCSO intake being inversely related to lower blood pressure.[62] Therefore, a better understanding of SMCSO is needed to comprehend the effects of this commonly consumed organosulfur compound.[17,21,41]

5. Conclusion

S-methyl cysteine sulfoxide administered to mice *via* oral gavage did not result in significant blunting of HFD-induced features of metabolic syndrome, except for HDL-C levels. Whether SMCSO has any effect on cholesterol metabolism in humans remains to be seen and more research is needed. Nonetheless, it is unlikely that the doses reported in this study could be achieved in humans through habitual dietary intake and nutraceutical supplementation would be required.

Author contributions

Caroline R. Hill: conceptualization, formal analysis, investigation, methodology, writing – original draft, writing – review & editing. Lois Balmer: conceptualization, formal analysis, investigation, methodology, supervision, writing – review & editing. Hayley Abbiss: investigation, writing – review & editing. Jonathan M. Hodgson: supervision, writing – review & editing. Joshua R. Lewis: supervision, writing – review & editing. Armaghan Shafaei: conceptualization, investigation, funding acquisition, methodology, supervision, writing – review & editing. Lauren C. Blekkenhorst: conceptualization, funding acquisition, methodology, supervision, writing – review & editing.

Conflicts of interest

The authors have no conflicts to declare.

Abbreviations

ANOVA	Analysis of variance
Apo	Apolipoproteins
ABCA1	Adenosine triphosphate binding cassette subfamily A member 1
ATP	Adenosine triphosphate
AUC	Area under the curve
BW	Body weight
CETP	Cholesterol ester transfer protein
CI	Confidence interval
HDL-C	High-density lipoprotein cholesterol
HFD	High-fat diet (group 2)
HFD+ 60	High-fat diet plus SMCSO at 60 mg per kg body weight per day (group 3)
HFD+ 170	High-fat diet plus SMCSO at 170 mg per kg body weight per day (group 4)
HFD+ 350	High-fat diet plus SMCSO at 350 mg per kg body weight per day (group 5)
hs-CRP	High-sensitivity C-reactive protein
IPGTT	Intraperitoneal glucose tolerance test
IPITT	Intraperitoneal insulin tolerance test
IQR	Interquartile range
LXR	Liver-X-receptor
mLDL-C	Measured low-density lipoprotein cholesterol
ND	Normal diet (group 1)
PPARs	Peroxisome proliferator-activated receptors
SEM	Standard error of the mean
SMCSO	*S*-Methyl cysteine sulfoxide

Ethics approval

This study was approved by Animal Ethics Committees at Harry Perkins Institute of Medical Research (AE282-BALMER) and Edith Cowan University (24319 SHAFAEI DARESTANI). All components comply with the National Health and Medical Research Council Guidelines on the Care and Use of Laboratory Animals in Australia, the Australian code of practice for the care and use of animals for scientific purposes, the Conduct of Ethical Research and Teaching Involving Animals Policy and the Animal Welfare Act.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Supplementary information (SI) is available. See DOI: https://doi.org/10.1039/d5fo04816h.

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