

RESEARCH ARTICLE

Chronic inorganic nitrate supplementation does not improve metabolic health and worsens disease progression in mice with diet-induced obesity

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Abstract

Inorganic nitrate (NO_3^-) has been proposed to be of therapeutic use as a dietary supplement in obesity and related conditions including the metabolic syndrome (MetS), type II diabetes, and metabolic dysfunction-associated steatotic liver disease (MASLD). Administration of NO_3^- to endothelial nitric oxide synthase-deficient mice reversed aspects of MetS; however, the impact of NO_3^- supplementation in diet-induced obesity is not well understood. Here we investigated the whole body metabolic phenotype and cardiac and hepatic metabolism in mice fed a high-fat, high-sucrose (HFHS) diet for up to 12 mo of age, supplemented with 1 mM NaNO_3 (or NaCl) in their drinking water. HFHS feeding was associated with a progressive obesogenic and diabetogenic phenotype, which was not ameliorated by NO_3^- . Furthermore, HFHS-fed mice supplemented with NO_3^- showed elevated levels of cardiac fibrosis and accelerated progression of MASLD including development of hepatocellular carcinoma in comparison with NaCl-supplemented mice. NO_3^- did not enhance mitochondrial β -oxidation capacity in any tissue assayed and did not suppress hepatic lipid accumulation, suggesting it does not prevent lipotoxicity. We conclude that NO_3^- is ineffective in preventing the metabolic consequences of an obesogenic diet and may instead be detrimental to metabolic health against the background of HFHS feeding. This is the first report of an unfavorable effect of long-term nitrate supplementation in the context of the metabolic challenges of overfeeding, warranting urgent further investigation into the mechanism of this interaction.

NEW & NOTEWORTHY Inorganic nitrate has been suggested to be of therapeutic benefit in obesity-related conditions, as it increases nitric oxide bioavailability, enhances mitochondrial β -oxidation, and reverses metabolic syndrome in $e\text{NOS}^{-/-}$ mice. However, we here show that over 12 months nitrate was ineffective in preventing metabolic consequences in high fat, high sucrose-fed mice and worsened aspects of metabolic health, impairing cholesterol handling, increasing cardiac fibrosis, and exacerbating steatotic liver disease progression, with acceleration to hepatocellular carcinoma.

inorganic nitrate; metabolic dysfunction-associated steatotic liver disease; metabolism; mitochondria; obesity

INTRODUCTION

The obesity epidemic remains a global health concern, and its prevalence continues to increase worldwide (1). Obesity is associated with high morbidity and mortality (2), largely due to the elevated risk of comorbidities such as type II diabetes mellitus (T2DM), cardiovascular disease (CVD), and metabolic dysfunction-associated steatotic liver disease (MASLD), a condition previously known as nonalcoholic fatty liver disease (NAFLD) (3–7).

Obesity and diabetes are associated with hypertension (8), which is in turn associated with endothelial dysfunction and reduced nitric oxide (NO) bioavailability. Mice

lacking endothelial nitric oxide synthase ($e\text{NOS}^{-/-}$ mice) are hypertensive (9) but also develop symptoms of the metabolic syndrome (MetS) including glucose intolerance and dyslipidemia, alongside hyperleptinemia (10, 11) and defective mitochondrial β -oxidation and biogenesis (12, 13). In humans, $e\text{NOS}$ polymorphisms have been associated with T2DM and MetS (14, 15), whereas patients with T2DM generate less NO from L-arginine than healthy control subjects (16). NO production and metabolism, particularly decreased NO bioavailability, are therefore considered central to the etiology of MetS/T2DM.

Canonical formation of NO occurs via oxidation of a guanidino nitrogen of L-arginine by one of three isoforms of



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NOS. However, it is now recognized that NO can also be produced via reduction of endogenously produced or dietary inorganic nitrate (NO_3^-) (17). In the circulation, NO generated by the vascular endothelium is oxidized to nitrite (NO_2^-) and nitrate. The latter is taken up from the blood and secreted into saliva, where it is reduced to nitrite by commensal bacterial flora of the mouth (18). Upon swallowing of saliva, NO_2^- is rapidly protonated in the stomach, forming nitrous acid (HNO_2), which spontaneously decomposes to form NO (19, 20). Remaining nitrate or nitrite can be absorbed in the intestine, where nitrite can be reduced to NO by enzymes including xanthine oxidoreductase, deoxyhemoglobin, and myoglobin (21–23). Circulating NO_3^- is eventually excreted by the kidneys, although ~25% is actively taken up by the salivary glands, allowing it to be concentrated in saliva and recirculated (17, 20). Thus, inorganic nitrate, once considered an end product of NO metabolism (24) and, along with nitrite, a potentially toxic residue of food preservation (25), is now recognized as a potentially important source of NO under hypoxic and acidic conditions (20, 26) and a route to modulate NO bioavailability via dietary manipulation (20). Both nitrite and nitrate were used medicinally long before any of these mechanisms had been discovered (27).

Dietary supplementation with inorganic nitrate for 8–10 wk reverses features of MetS in $e\text{NOS}^{-/-}$ mice, improving glucose handling, hypertension, and dyslipidemia (28). It is not established whether reduced NO bioavailability is a universal feature of MetS and/or T2DM; however, nitrite improved glycemia in *db/db* mice (1-mo treatment) (29) and *ob^{lep}* mice (1-wk treatment) (30), whereas nitrate improved insulin sensitivity in high fructose-fed rats over 10 wk (31), high-fat diet low-dose streptozotocin T2DM rats over 2 mo (32), and high fat, high fructose-fed mice over 1 mo (33). At a tissue level, dietary nitrate increased mitochondrial β -oxidation in the heart and skeletal muscle of rats and mice (34–36) and was associated with increased voluntary wheel running in mice (37). Dietary inorganic nitrate enhanced white adipose tissue browning in rats (38) and increased mitochondrial oxygen consumption (39) and glucose oxidation (40) in white adipocytes. In liver, NO (generated by *eNOS*) inhibits activation of proinflammatory Kupffer cells, a response characteristic of MASLD pathogenesis (41). Although long-term (17 mo) nitrate supplementation did not result in adverse health effects in healthy mice and was associated with improved insulin sensitivity (42), the long-term implications of nitrate supplementation in obese animals remain unknown. This could be of particular importance in light of the effects of overfeeding with lipids and carbohydrates on mitochondrial function and reactive oxygen species (ROS) production against the complex interaction with tissue and whole body redox regulation vis-à-vis aging-related alterations in metabolic regulation by liver and skeletal muscle.

We therefore sought to investigate whether dietary supplementation with a moderate dose of inorganic nitrate (similar to that achievable with a human diet rich in leafy vegetables) ameliorates the progression of metabolic comorbidities associated with diet-induced obesity in mice. We hypothesized that inorganic nitrate would delay the development of metabolic and mitochondrial dysregulation in high fat, high sucrose-fed mice via enhanced NO bioavailability and modulation of tissue mitochondrial function.

MATERIALS AND METHODS

Chemicals

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich (Merck Life Science UK, Gillingham, UK).

Ethical Approval

All studies were carried out in accordance with United Kingdom Home Office legislation under the Animals in Scientific Procedures (1986) Act and received prior approval from the University of Cambridge Animal Welfare and Ethical Review Board. All procedures were carried out by a personal license holder in accordance with these regulations.

Study Design

Male C57Bl/6J mice ($n = 95$; RRID: MGI:3028467) were purchased from a commercial breeder (Charles River Laboratories, Margate, UK) at 3 wk of age. Unless otherwise stated, all mice were group-housed in conventional cages under controlled environmental conditions (21°C, 54% humidity, 12-h photoperiod) and were allowed ad libitum access to food and water for the duration of the study.

In the first week of vivarium acclimatization, mice received a standard laboratory rodent chow diet [RM3(E); Special Diet Services, Essex, UK; 3.6 kcal·g⁻¹, 39.7% carbohydrate, 22.4% crude protein, 4.3% crude fat; referred to here as “chow”] and distilled water ad libitum. From 4 wk of age, mice were randomized into four experimental groups ($n = 23$ –24/group): two groups continued to receive chow, and the others received a high-saturated fat, high-sucrose (HFHS) diet (TD.88137; Envigo Teklad Diets, Madison, WI; 4.5 kcal·g⁻¹, 48.5% carbohydrate, 17.3% crude protein, 21.2% crude fat). Within each diet group, one experimental group received drinking water supplemented with 1 mM sodium nitrate (NaNO_3 ; TraceSELECT, no. 71752, Fluka; Honeywell Specialty Chemicals, Seelze, Germany) and the other received water supplemented with 1 mM sodium chloride (NaCl ; TraceSELECT, no. 38979, Fluka) to control for sodium intake. Mice were maintained on their respective diets/treatments until they reached 4, 8, or 12 mo of age ($n = 7$ or 8/group; Fig. 1A). Body mass, food, and water intake were measured weekly.

Five (± 2) days before the end of the treatment period, mice were fasted overnight before blood was sampled from the lateral tail vein for determination of fasting blood glucose (Accu-Chek Compact glucometer; Roche) and plasma separated (4,000 g, 10 min, 4°C, K₃EDTA anticoagulant) and snap frozen for clinical chemistry analyses.

At 4, 8, or 12 mo of age (± 2 days) mice were terminally anesthetized via an intraperitoneal injection of sodium pentobarbital (500 mg·kg⁻¹, Euthatal Solution; Merial Animal Health Ltd., Bracknell, UK). A terminal (fed state) blood sample was collected via cardiac puncture and the plasma separated and snap frozen for later analyses. The heart was rapidly excised, atria and extraneous tissue removed, and the heart weighed before the apex was removed and placed into ice-cold biopsy-preservation medium [BIOPS (in mM): 2.77 CaK₂EGTA, 7.23 K₂EGTA, 6.56 MgCl₂, 50 MES, 5.77 ATP, 15 phosphocreatine (PCr), 20 imidazole, 20 taurine, and 0.5

dithiothreitol, pH 7.1]. A transverse midsection 1–2 mm above the apex was carefully sectioned and placed into ice-cold paraformaldehyde (PFA) (4% in phosphate-buffered saline, no. J61899; Alfa-Asaer, Heysham, UK) for subsequent histological analysis, before the remaining left ventricle was separated and snap frozen. The liver was removed from the abdominal cavity and the left lateral lobe (LLL) quickly divided into three equal sections; one section was placed into ice-cold BIOPS, one section placed in neutral buffered formalin (no. 11699455; VWR International, Lutterworth, UK), and the remaining third snap frozen for molecular analyses. The right lateral lobe was fixed in isopentane precooled in dry ice.

After removal of the heart and liver, the carcass was weighed, nose-to-anus length measured, and body composition determined by dual-energy X-ray absorptiometry (DEXA) (Lunar PIXImus II; GE Medical Systems Ltd.). Body composition parameters were calculated with PIXImus software (Lunar Corporation, Madison, WI) after exclusion of the skull, per the manufacturer's instructions. After the DEXA scan, the right soleus and gastrocnemius muscles were dissected and placed into ice-cold BIOPS. The left tibia was also dissected and boiled for >5 h to remove all soft tissue, then precisely measured from the tibial plateau to the tip of the medial malleolus.

Metabolic Cages

For the 12-mo cohort, mice underwent a 48-h metabolic cage (Promethion; Sable Systems, Germany) protocol at 10 mo (± 6 days) of age. Mice were acclimatized to indirect calorimetry cages (Promethion; Sable Systems, Germany) for 24 h before data collection. Cages were housed in a thermostatic, light-cycling cabinet (CAB-16; Sable Systems) to maintain constant temperature, humidity, and photoperiod equivalent to home cages throughout the experimental period.

Each mouse had ad libitum access to food and water of its relevant dietary intervention throughout its time in the metabolic cages, which was continuously measured via MM-2 load cells (Promethion; Sable Systems) precalibrated to known masses. Indirect calorimetry was achieved through a pull-mode air flow generator (Promethion Core CGF; Sable Systems), calibrated to wet and dry air and 0 to 5,000 ppm carbon dioxide. Air was pulled through each metabolic cage at $2 \text{ L} \cdot \text{min}^{-1}$, with subsamples analyzed for oxygen, carbon dioxide, and water vapor, allowing measurement of oxygen consumption ($\dot{V}\text{O}_2$) and carbon dioxide production ($\dot{V}\text{CO}_2$) from each mouse.

Data were collected with a 1-Hz sampling rate and acquired and coordinated by the IM3 Interface Module (v20.0.6; Sable Systems), with real-time measurement of respiratory exchange ratio (RER) and calculation of energy expenditure (EE, via the Weir equation) (45). Data were processed with ExpeData (v1.9.27; Sable Systems) with Macro Interpreter (v2.32; Sable Systems) to process raw data into 5-min bins. Metabolic cage data were corrected to body mass, lean mass, or fat mass covariates, as appropriate (46), determined by time-domain nuclear magnetic resonance (TD-NMR) (LF50H Minispec; Bruker, Coventry, UK). TD-NMR data were acquired with minispec software

(v3.0, connected to OPUS v7.0; both Bruker). Data were analyzed with the CalR framework (RRID: SCR_015859) (47), adapted for a 2×2 factorial design, in R (48).

Blood Analyses

Insulin, free fatty acids (FFA), triacylglycerols (TAG), total cholesterol, and HDL cholesterol were measured in fasted blood samples by the Core Biochemical Assay Laboratory (Cambridge University Hospitals, NHS Foundation Trust, Cambridge, UK). LDL cholesterol was calculated with the Friedewald equation (49). Fasted glucose and insulin measurements were used to calculate the homeostatic model assessment (43) as a measurement of insulin resistance (HOMA-IR) adapted for murine models, as previously described (50).

Plasma nitrate and nitrite concentrations were determined in the fed state with a dedicated high-performance liquid chromatography analysis system (Eicom ENO-30, coupled to an AS-700 INSIGHT autosampler; Amuza Inc.) after methanol deproteinization as previously described (51). Plasma concentrations of the liver enzymes alanine transaminase (ALT) and aspartate transaminase (AST) were quantified via commercial ELISAs (no. ab28282 and no. ab263882; Abcam, Cambridge, UK). The antioxidant capacity of plasma was determined via the ferric reducing ability of plasma (FRAP) assay, as previously described (52). Lipid peroxidation was used as a marker of oxidative stress and quantified in plasma by measurement of thiobarbituric acid reactive substances (TBARS), as previously described (52).

Intestinal Morphology

For investigation of intestinal morphology, an additional cohort of male C57Bl/6J mice (aged 8 wk, $n = 7$) was purchased from the same commercial breeder and randomized to receive either standard laboratory chow [RM3(E)] or HFHS diet (TD.88137). After 14 wk, mice were terminally anesthetized and the gastrointestinal tract dissected from the pyloric sphincter to the rectum. After the cecum was removed, the intestinal tract was cut into four sections (colon, plus proximal, mid, and distal small intestine approximately equivalent to the duodenum, jejunum, and ileum) and prepared for histological processing, as previously described (53).

Hepatic Lipid Content

Hepatic lipid content was quantified with an optimized Folch extraction protocol for quantification of liver fat (54).

High-Resolution Respirometry

High-resolution respirometry was performed with an Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria) on saponin-permeabilized muscle fiber bundles from the cardiac apex, soleus and gastrocnemius muscles, and liver left lateral lobe (LLL) homogenate, prepared as previously described (55, 56). A substrate-uncoupler-inhibitor titration was carried out to assess mitochondrial capacity as described in Table 1.

Flux control ratios were calculated to determine the proportion of maximal oxidative phosphorylation (OXPHOS) that

could be supported by the F pathway via β -oxidation (FCR_F ; Eq. 1) and by the N pathway via complex I (FCR_N ; Eq. 2), as well as the relative capacity to support OXPHOS using octanoyl-carnitine and malate as substrates compared with

pyruvate and malate (FCR_{F+P} ; Eq. 3). Mitochondrial OXPHOS coupling efficiency (OCE; Eq. 4) was also calculated as a measure of the proportion of OXPHOS not limited by LEAK-state respiration.

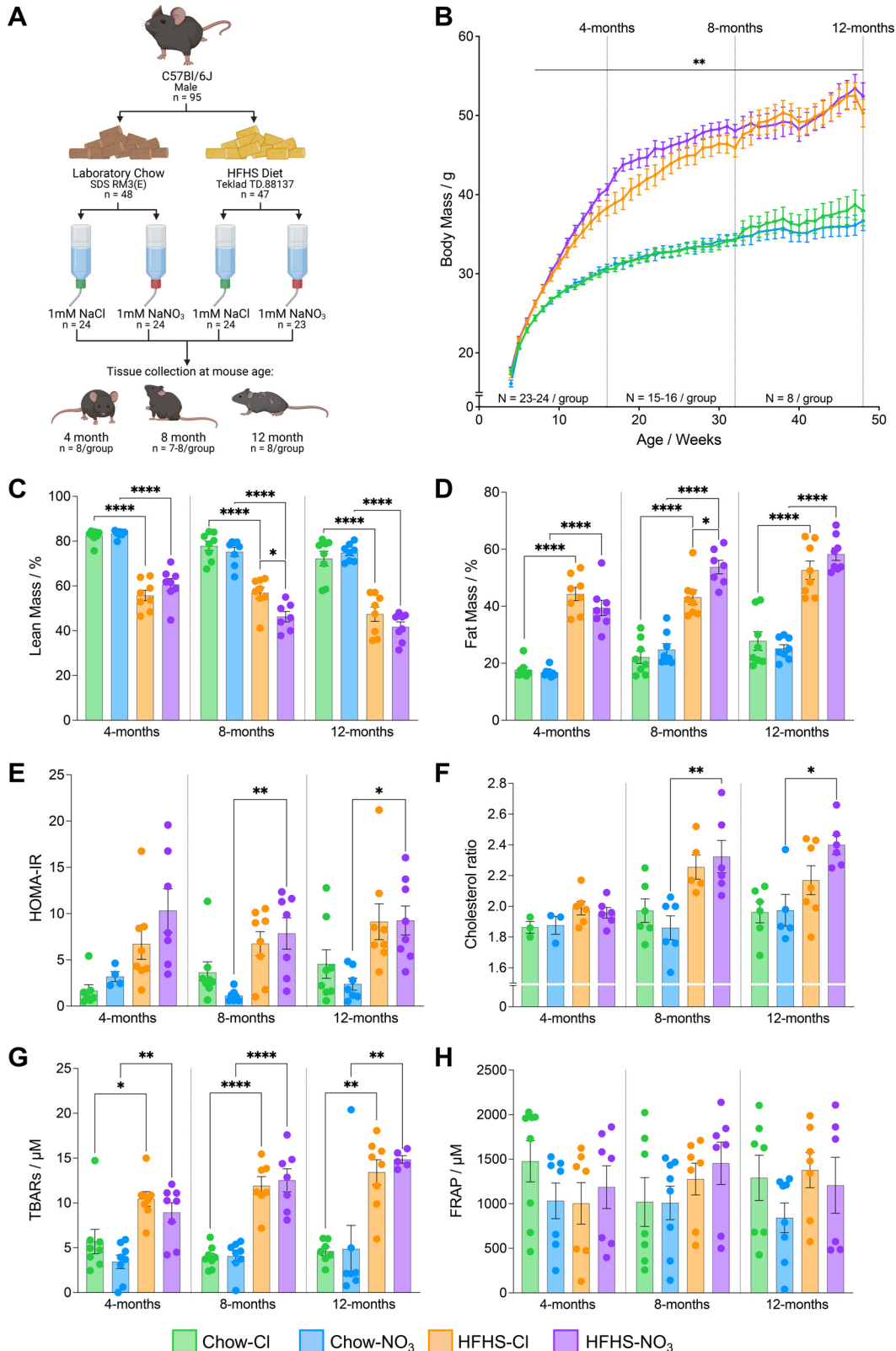


Table 1. Substrate-uncoupler-inhibitor titration used to investigate mitochondrial capacity in permeabilized heart fibers, skeletal muscle fibers, and liver homogenate

Substrate	Concentration, mM			Respiratory State
	Heart	Liver	Skeletal Muscle	
Malate	1	1	1	OctM _L
Octanoyl-carnitine	0.5	0.2	0.2	
ADP	10	10	10	OctM _P
Pyruvate	25	25	25	OctPM _P
Glutamate	10	10	10	GM _P
Cytochrome c	0.01	10	0.01	
Succinate	10	10	10	GMS _P
FCCP*		0.0005 titers	0.00025 titers	GMS _E
Rotenone	0.0005	0.0005	0.0005	S _E /S _P **

Two types of skeletal muscle (gastrocnemius and soleus) were assayed, using the same titration protocol. Cytochrome *c* was added as a quality control to check outer mitochondrial membrane integrity. *Carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) was titrated in the indicated quantities until the maximum rate was reached. No FCCP was added in the heart, as there was no reserve capacity measurable above maximal oxidative phosphorylation (OXPHOS) stimulated following saturation of the electron transport system (ETS) with glutamate and succinate. **In the heart assay, because of the absence of the uncoupler FCCP, addition of the complex I inhibitor rotenone led to measurement of OXPHOS supported by succinate via ETS complex II alone (state S_P), whereas in liver and muscle the uncoupling of the ETS from ATP synthase meant that the oxygen consumption rate measured reflected capacity of ETS complex II (state S_E).

$$FCR_F = \frac{\text{OctM}_P}{\text{GMS}_P} \quad (1) \quad \text{from chemical standards: glucose: } +179.000 \rightarrow +89.000, 2.6 \text{ min; glucose-6-phosphate: } +259.000 \rightarrow +97.077, 4.0 \text{ min;}$$

$$FCR_N = \frac{\text{GM}_P}{\text{GMS}_P} \quad (2) \quad \text{glucose-1-phosphate: } +259.000 \rightarrow +97.077, 3.9 \text{ min; UDP-glucose: } +565.078 \rightarrow +323.000, 3.88 \text{ min.}$$

$$FCR_{FA/P} = \frac{\text{OctM}_P}{\text{OctPM}_P} \quad (3) \quad \text{Analysis of acyl-carnitines in liver and heart was carried out using a reconstituted mix of half of each of the aqueous and organic phases, as previously described (56). Acyl-carnitine analysis was carried out on the same instrumentation as HILIC analysis, with data collection and processing in both cases carried out with Xcalibur software (v.2.2; Thermo Scientific; RRID:SCR_014593).}$$

$$\text{OCE} = \frac{\text{OctM}_P - \text{OctM}_L}{\text{OctM}_P} \quad (4) \quad \text{Open-profiling lipidomics was carried out as previously described (58) with lipid data collected using the Fourier transform mass spectrometer analyzer set in profile mode with a resolution of 60,000 (Ultimate 3000 UHPLC system coupled to an LTQ Orbitrap Elite Mass Spectrometer; both Thermo Fisher Scientific). A full scan was performed across an } m/z \text{ range of 110–2,000. Lipid metabolites were processed as previously described (58), with peaks identified by XCMS software (RRID: SCR_015538) (60) based on an approximate chromatographic peak detection [full width at half-maximum (FWHM)] of 5 s and a signal-to-noise threshold ratio of 5. Peaks were annotated by accurate mass, with an automated in-house R script and by comparison to the LipidMaps database (61).}$$

Liquid Chromatography-Mass Spectrometry-Based Metabolite Analyses

Aqueous and organic metabolites were extracted from ~25 mg of liver LLL or cardiac left ventricle with a modified Bligh and Dyer method (57), as previously described (58), dried under nitrogen, and stored at –80°C until analysis.

For analysis of cardiac glycogenesis intermediates, half the aqueous fraction was reconstituted for hydrophobic interaction liquid chromatography (HILIC), which was performed as previously described (59) with Vanquish UHPLC⁺ series coupled to a TSQ Quantiva Triple Quadrupole Mass Spectrometer (both Thermo Scientific). Identification of detected metabolites was performed by targeted analysis of parent/daughter ion mass-to-charge ratio (*m/z*) and retention times

from chemical standards: glucose: +179.000 → +89.000, 2.6 min; glucose-6-phosphate: +259.000 → +97.077, 4.0 min; glucose-1-phosphate: +259.000 → +97.077, 3.9 min; UDP-glucose: +565.078 → +323.000, 3.88 min.

Analysis of acyl-carnitines in liver and heart was carried out using a reconstituted mix of half of each of the aqueous and organic phases, as previously described (56). Acyl-carnitine analysis was carried out on the same instrumentation as HILIC analysis, with data collection and processing in both cases carried out with Xcalibur software (v.2.2; Thermo Scientific; RRID:SCR_014593).

Open-profiling lipidomics was carried out as previously described (58) with lipid data collected using the Fourier transform mass spectrometer analyzer set in profile mode with a resolution of 60,000 (Ultimate 3000 UHPLC system coupled to an LTQ Orbitrap Elite Mass Spectrometer; both Thermo Fisher Scientific). A full scan was performed across an *m/z* range of 110–2,000. Lipid metabolites were processed as previously described (58), with peaks identified by XCMS software (RRID: SCR_015538) (60) based on an approximate chromatographic peak detection [full width at half-maximum (FWHM)] of 5 s and a signal-to-noise threshold ratio of 5. Peaks were annotated by accurate mass, with an automated in-house R script and by comparison to the LipidMaps database (61).

All data were normalized to the intensity of appropriate internal standards and to sample protein concentration

Figure 1. Mice fed a high-fat, high-sucrose (HFHS) diet show progressive diet-induced obesity. **A:** experimental design. Male C57Bl/6J mice were randomly allocated to receive either standard laboratory chow [Special Diet Services RM3 (E)] or HFHS (Teklad TD.88137) from 4 wk of age. Simultaneously, mice were randomized to receive either sodium chloride (NaCl; 1 mM) or sodium nitrate (NaNO₃; 1 mM) via their drinking water. Mice continued to receive these dietary interventions ad libitum until they reached 4, 8, or 12 mo of age, when tissues were collected for subsequent metabolic analyses (*n* = 7 or 8/group). Created with BioRender.com. **B:** mouse body mass over the course of the study. Individual mouse body mass was measured once per week. ***P* < 0.01, HFHS compared with chow-fed groups supplemented with the same water treatment, for each week [2-way ANOVA with Benjamini–Hochberg false discovery rate (FDR) correction and Tukey's post hoc honest significant difference (HSD) test]. **C:** percentage (%) of the body carcass (after removal of the heart and liver) that represents lean mass determined via dual-energy X-ray absorptiometry. *N* = 7 or 8/group. **D:** percentage (%) of the body carcass (after removal of the heart and liver) that represents fat mass determined via dual-energy X-ray absorptiometry. *N* = 7 or 8/group. **E:** homeostatic model of insulin resistance (HOMA-IR) calculated according to Ref. 43 using measurements of fasted blood glucose and insulin. *N* = 4–8/group. **F:** cholesterol ratio (total cholesterol/HDL cholesterol), used clinically as a predictor of cardiovascular disease (44), calculated from clinical biochemistry measurements. *N* = 3–7 mice/group. **G:** concentration of thiobarbituric acid reactive substances (TBARS) in plasma, as an indication of levels of lipid peroxidation. *N* = 5–8 mice/group. **H:** ferric reducing ability of plasma (FRAP), as an indication of the antioxidant capacity of plasma. (*N* = 6–8 mice/group). For C–H, data represent means ± SE. **P* < 0.05, ***P* < 0.01, ****P* < 0.0001; 2-way ANOVA with Tukey's post hoc HSD test for multiple comparisons.

(Pierce BCA Assay; Thermo Scientific) to account for differences in sample size.

Reverse Transcription-Quantitative PCR

Total RNA was extracted from ~30 mg of liver LLL or ~25 mg of cardiac left ventricle with an RNeasy Fibrous Tissue Mini Kit (QIAGEN Ltd., Manchester, UK) according to the manufacturer's instructions. RNA (1 µg, quantified via NanoDrop 2000 spectrophotometer) was transcribed to cDNA with the QuantiTect Reverse Transcription kit (QIAGEN) and stored at -20°C before analysis. Real-time qPCR was carried out with a LightCycler 480 System (Roche) fitted with a 384-well block, using the QuantiNova SYBR Green PCR kit (QIAGEN) and QuantiTect primer assays (QIAGEN; Supplemental Table S1). Expression was determined in triplicate from each biological replicate. Fold change in gene expression was determined by the relative standard curve method, with expression of all genes normalized to the geometric mean of three housekeeping genes: *Rn18s*, *Srsf4*, and *Hmbs* in liver and *Actb*, *Ywhaz*, and *Rn18s* in heart.

Histology

Sections were fixed in PFA (cardiac midsections) or formalin (LLL and intestinal sections) and embedded into paraffin blocks by standard laboratory methods. For LLL sections, tissue was processed at the Histopathology Core (MRC Metabolic Diseases Unit, Cambridge). All tissue was sectioned at 7 µm with an RM2235 Microtome (Leica Biosystems).

Liver and intestinal morphology was determined via hematoxylin (no. ab220365, Abcam) and eosin (H&E) staining with standard laboratory protocols. Cardiac and hepatic fibrosis was determined by staining for collagen with picrosirius red (PSR) using 0.1% Direct Red in saturated picric acid (12 g·L⁻¹, no. A2520; AppliChem, Darmstadt, Germany) for 1 h. Cardiac glycogen was stained with periodic acid Schiff (PAS) using 0.5% periodic acid (5 min) and Schiff's reagent (10 min) with nuclei counterstained with hematoxylin. To correct for any PAS staining not due to glycogen deposition, one section from each sample was treated with 0.5% α -amylase (type VI-B from porcine pancreas, ~2.5 U/mL) for 20 min before staining. After staining, slides were dehydrated in ethanol, cleared in xylene, and mounted with DPX.

Fix-frozen right lateral lobe was embedded in optimum cutting temperature compound (no. KMA-0100-00A; CellPath) and sectioned at 10 µm in a cryostat (OTFAS-001; Bright Instruments, Luton, UK) at -20°C for histological assessment of hepatic steatosis. Sections were allowed to adhere to slides at room temperature for 10 min before sections were fixed in ice-cold formalin for 10 min. Neutral lipid was stained with oil red O [ORO; 0.3% (wt/vol) in 60% isopropanol] for 20 min. Nuclei were counterstained with hematoxylin. After staining, the slides were mounted in glycerin gelatin at 60°C and coverslips sealed with clear nail varnish once the mountant had set.

Slides were imaged with a NanoZoomer 2.0-RS with images collected with NDP.view2 (v2.9.25; Hamamatsu Photonics, Japan). At least three independent sections per biological replicate were examined and analyzed. To determine the level of

hepatic pathology, a MASLD activity score (MAS) was generated for each section using sections blinded for experimental condition (Supplemental Table S2) (62). Quantification of villus length was carried out manually in NDP.view2, with 10 intact villi measured per quadrant per intestinal section (63). Quantification of PSR and PAS in cardiac sections was determined after color deconvolution in ImageJ (RRID: SCR_003070) (64). PAS staining was corrected for that in control sections treated with α -amylase to account for any non-glycogen PAS staining.

Statistics

All data are presented as means $\pm$ standard error, unless otherwise stated, with differences accepted as statistically significant when $P \leq 0.05$. All univariate statistical tests were carried out in R (48) with graphs produced in Prism (v.10.0.3; GraphPad Software LLC., Boston, MA; RRID: SCR_002798). Multivariate statistical analysis was carried out with SIMCA-17 (Umetrics; Sartorius Stedim Data Analytics AB, Umeå, Sweden). Unless otherwise stated, statistical analysis was carried out in line with two a priori questions outlined before commencing the study: 1) at each time point, is there an effect of diet or nitrate supplementation? and 2) is there an effect of diet or nitrate supplementation on age-related and/or disease progression? For each question, two-way ANOVAs were carried out on the relevant experimental cohorts as appropriate, with correction for false discovery rates as required (Supplemental Fig. S1). When significant interactions were found, Tukey's post hoc honestly significant difference (HSD) test was used to consider relevant interactions (Supplemental Fig. S1).

RESULTS

Whole Body Metabolic Phenotype

Mice fed a HFHS diet developed obesity that persisted throughout the 44 wk of the study (Fig. 1B). After 3 wk on the respective diets HFHS-fed mice were 4.6% heavier than chow-fed mice ($P < 0.01$), and by 12 mo of age HFHS-fed mice were 37.6% heavier than their counterparts on the control chow diet ($P < 0.0001$; Fig. 1B). There was no effect of nitrate supplementation on body mass (Fig. 1B).

Food intake remained constant throughout the study, with chow-fed mice consuming 4.0 ± 0.03 g·mouse⁻¹·day⁻¹ and HFHS-fed mice eating 3.1 ± 0.05 g·mouse⁻¹·day⁻¹. However, as the HFHS diet was more calorie dense, there was no significant difference in energy intake between the experimental groups throughout the study (Table 2). HFHS-fed mice drank 27.9% less water per day ($P < 0.01$; Table 2) compared with chow-fed control mice, likely because of the higher sodium content of the latter diet (0.3% in HFHS vs. 10% in chow diet). Despite this, mice receiving drinking water supplemented with 1 mM NaNO₃ had a fivefold higher intake of nitrate compared with NaCl-supplemented control mice ($P < 0.0001$; Table 2). This resulted in a plasma nitrate concentration that was 1.9-fold higher than in NaCl-supplemented control mice at 4 and 8 mo of age ($P < 0.05$; Table 3). Circulating nitrite concentrations were significantly elevated only in 4-mo-old mice fed standard rodent chow (Table 3). At 12 mo

Table 2. Food, water, and NO_3^- intake across the experimental groups

	Food Intake, kcal·mouse ⁻¹ ·day ⁻¹	Water Intake, mL·mouse ⁻¹ ·day ⁻¹	NO_3^- Intake, mg·mouse ⁻¹ ·day ⁻¹
Chow/chloride	14.5 ± 0.1	5.8 ± 0.1	1.4 ± 0.0
Chow/nitrate	14.1 ± 0.1	5.3 ± 0.1	12.4 ± 0.2++++
HFHS/chloride	13.8 ± 0.2	4.1 ± 0.1**	1.9 ± 0.1
HFHS/nitrate	14.9 ± 0.2	3.9 ± 0.1**	8.2 ± 0.2*****††††

Values were calculated based on individual cage measurements ($n = 3$ or 4 mice/cage; 6 cages/group). Nitrate intake represents combined intake from supplemented water and food (using values provided from the diet suppliers). HFHS, high-fat, high-sucrose diet. Data show means ± SE. ** $P < 0.01$, **** $P < 0.0001$, compared with chow-fed mice receiving the same water treatment; †††† $P < 0.0001$, compared with chloride-supplemented mice receiving the same diet; 2-way ANOVA with Tukey's honestly significant difference (HSD) post hoc test for multiple comparisons.

there were no differences in plasma nitrate levels between any of the experimental groups (Table 3).

The greater body mass observed in HFHS-fed mice compared with chow-fed mice was reflected in a greater body fat percentage and, correspondingly, a lower lean body mass percentage ($P < 0.0001$; Fig. 1, C and D). Body fat was ~2- to 2.5-fold greater, whereas lean mass was approximately one-third less in mice fed a HFHS diet, compared with chow-fed control mice. In the 8-mo cohort, HFHS-fed mice also receiving NaNO_3 had 31.3% more body fat and 13.3% less lean mass

compared with chloride-supplemented counterparts on the HFHS diet ($P < 0.05$), although this exacerbation of obesity in nitrate-fed mice was not seen at 12 mo (Fig. 1, C and D).

Mice fed a HFHS diet had a fasted blood glucose concentration that was 36.7% higher than that of chow-fed mice across all ages assessed ($P < 0.01$), with no difference in fasted plasma insulin concentration (Table 3). Accordingly, HFHS-fed mice had approximately twofold higher HOMA-IR scores indicating evidence of insulin resistance, which reached statistical significance in mice supplemented with

Table 3. Clinical biochemistry measurements and other plasma parameters

	Mouse Age, mo	Chow-Cl	Chow- NO_3	HFHS-Cl	HFHS- NO_3
Glucose, mmol·L ⁻¹ ($n = 7$ or 8)	4	5.7 ± 0.3	6.2 ± 0.2	8.3 ± 0.4**	8.7 ± 0.7**
	8	6.8 ± 1.1	5.8 ± 0.4	7.9 ± 1.7	8.9 ± 1.5***
	12	6.5 ± 1.2	5.6 ± 0.5	8.5 ± 1.1***	7.8 ± 0.6***
Insulin, mg·L ⁻¹ ($n = 4$ –8)	4	0.19 ± 0.07	0.37 ± 0.07	0.53 ± 0.11	0.77 ± 0.14
	8	0.39 ± 0.07	0.14 ± 0.03	0.59 ± 0.12	0.59 ± 0.12
	12	0.48 ± 0.16	0.29 ± 0.08	0.76 ± 0.18	0.81 ± 0.13
FFA, mmol·L ⁻¹ ($n = 2$ –8)	4	1.8 ± 0.10	1.1 ± 0.10†	1.1 ± 0.10*	1.4 ± 0.08
	8	1.6 ± 0.07	1.4 ± 0.10	1.1 ± 0.10*	1.2 ± 0.06
	12	0.9 ± 0.06††§	1.4 ± 0.10†	0.8 ± 0.05	0.8 ± 0.10**††
TAG, mmol·L ⁻¹ ($n = 3$ –7)	4	0.80 ± 0.01	0.93 ± 0.01	0.84 ± 0.05	0.83 ± 0.08
	8	0.99 ± 0.04	1.10 ± 0.07	0.87 ± 0.04	0.88 ± 0.02**
	12	0.87 ± 0.11	0.83 ± 0.04	0.72 ± 0.04	0.74 ± 0.04
Cholesterol, mmol·L ⁻¹ ($n = 3$ –7)	4	2.4 ± 0.1	2.6 ± 0.0	5.7 ± 0.2**	5.1 ± 0.6*
	8	2.6 ± 0.2	2.4 ± 0.1	6.0 ± 0.8***	7.1 ± 0.5****
	12	2.7 ± 0.1	2.3 ± 0.1	6.5 ± 0.6***	8.6 ± 0.6*****†††
HDLc, mmol·L ⁻¹ ($n = 3$ –7)	4	1.3 ± 0.0	1.4 ± 0.1	2.9 ± 0.2**	2.6 ± 0.3*
	8	1.3 ± 0.1	1.3 ± 0.1	2.7 ± 0.4**	3.1 ± 0.2***
	12	1.4 ± 0.1	1.2 ± 0.1	3.0 ± 0.2****	3.6 ± 0.3****
LDLc, mmol·L ⁻¹ ($n = 3$ –6)	4	0.75 ± 0.08	0.77 ± 0.04	2.4 ± 0.1**	2.1 ± 0.3**
	8	0.82 ± 0.09	0.59 ± 0.08	2.9 ± 0.4***	3.5 ± 0.4****
	12	0.93 ± 0.07	0.75 ± 0.07	2.9 ± 0.4***	4.2 ± 0.5*****†††
ALT, mg·mL ⁻¹ ($n = 5$ –8)	4	0.22 ± 0.05	0.26 ± 0.06	0.39 ± 0.08	0.36 ± 0.11
	8	0.21 ± 0.03	0.15 ± 0.03	0.40 ± 0.07*	0.44 ± 0.07**
	12	0.24 ± 0.04	0.11 ± 0.02	0.47 ± 0.07**	0.54 ± 0.04****
AST, mg·mL ⁻¹ ($n = 7$ or 8)	4	0.92 ± 0.19	1.01 ± 0.12	1.65 ± 0.56	1.35 ± 0.36
	8	0.72 ± 0.10	0.59 ± 0.06	1.13 ± 0.17	1.02 ± 0.12
	12	0.76 ± 0.11	0.59 ± 0.11	1.35 ± 0.25	2.04 ± 0.32***
Nitrate, $\mu\text{mol} \cdot \text{L}^{-1}$ ($n = 7$ or 8)	4	32.9 ± 3.7	74.0 ± 7.4††††	27.6 ± 3.2	48.6 ± 4.6**†
	8	31.7 ± 3.2	51.7 ± 6.8	27.7 ± 3.4	57.4 ± 7.8††
	12	49.2 ± 6.1	65.0 ± 10.7	60.2 ± 13.1§	47.5 ± 3.6
Nitrite, $\mu\text{mol} \cdot \text{L}^{-1}$ ($n = 7$ or 8)	4	4.5 ± 0.43	7.9 ± 0.77*	4.6 ± 0.54	6.0 ± 1.74
	8	3.4 ± 0.53	5.4 ± 0.60	3.7 ± 0.81	4.0 ± 0.99
	12	5.1 ± 0.52	6.0 ± 1.13	4.9 ± 0.75	3.2 ± 0.42

All clinical biochemistry measurements were assayed from fasted plasma. Nitrate and nitrite concentrations and liver enzymes were measured from terminal blood samples (fed state). Data show means ± SE with numbers of mice assayed indicated. ALT, alanine transaminase; AST, aspartate transaminase; FFA, free fatty acids; HDLc, high-density lipoprotein cholesterol; HFHS, high-fat, high-sucrose diet; LDLc, low-density lipoprotein cholesterol; TAG, triacylglycerols. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, compared with chow-fed mice receiving the same water treatment; † $P < 0.05$, †† $P < 0.01$, †††† $P < 0.0001$, compared with chloride-supplemented mice receiving the same diet; ‡ $P < 0.01$, compared with 4-mo-old mice within the same treatment group; § $P < 0.05$ compared with 8-mo-old mice within the same treatment group; all statistics represent 2-way ANOVA with Tukey's honestly significant difference (HSD) post hoc test for multiple comparisons.

NO_3^- from 8 mo of age compared with their chow-fed counterparts ($P < 0.05$; Fig. 1E). Total, HDL, and LDL cholesterol were elevated in mice fed a HFHS diet across all ages compared with chow-fed control mice (2.6-fold, 2.1-fold, and 3.7-fold higher, respectively; $P < 0.05$; Table 3). Total and LDL cholesterol were also elevated in the 12-mo HFHS- NO_3^- cohort, compared both with the same group at 4 mo of age (69.1% total, 2.1-fold LDL; $P < 0.01$; Table 3) and with the 12-mo HFHS-Cl group (30.9% total, 1.5-fold LDL; $P < 0.05$; Table 3), suggesting an exacerbation of dyslipidemia with longer-term nitrate supplementation. Cholesterol ratio (total/HDL cholesterol) (44) was also elevated in HFHS- NO_3^- mice at 8 and 12 mo of age, compared with chow-fed control mice (23.1%; $P < 0.05$; Fig. 1F) and raised at both of these time points in comparison with 4-mo-old HFHS- NO_3^- -fed mice (20.2%, $P < 0.05$; Fig. 1F). There were, however, few clear differences in the plasma free fatty acids or triacylglycerol (TAG) concentrations as a result of age, diet, or nitrate treatment (Table 3).

We also detected elevations in plasma levels of the liver enzymes ALT and AST indicative of liver damage (65). In chloride-supplemented HFHS-fed mice, plasma ALT concentration was 92.4% higher at 8 mo ($P < 0.05$) and 96.4% higher at 12 mo ($P < 0.01$) in comparison with chow-fed control mice, whereas in those receiving nitrate plasma ALT concentration was 2.9-fold greater at 8 mo ($P < 0.01$) and 4.8-fold greater at 12 mo ($P < 0.0001$) compared with chow-matched control mice (Table 3). Plasma AST concentration was also elevated in HFHS-fed mice: in the 8-mo cohort plasma AST was 64.3% higher than in chow-fed mice ($P < 0.01$), whereas at 12 mo HFHS- NO_3^- -fed mice showed a plasma AST concentration 3.5-fold higher than chow- NO_3^- mice ($P < 0.001$; Table 3).

Quantification of TBARS in plasma showed that, at every time point, lipid peroxidation was higher in HFHS-fed mice compared with chow-fed control mice (2.6-fold; $P < 0.05$; Fig. 1G), with no effect of nitrate supplementation. This increase in lipid peroxidation was not associated with any differences in plasma antioxidant capacity, as shown by similar FRAP concentrations across all cohorts (Fig. 1H).

Taken together, these data do not support a protective effect of nitrate supplementation for metabolic health during diet-induced obesity.

Whole Body Energy Balance

Next, we sought to determine whether HFHS feeding or nitrate administration altered whole body energy balance, as has been previously reported (66, 67). We used indirect calorimetry and activity measurements to investigate energy utilization in the 12-mo cohort when mice were 10 mo of age (Fig. 2, A–E).

Throughout the 48-h metabolic cage protocol, chow-fed mice had a higher respiratory exchange ratio (RER) than HFHS-fed mice (0.84 ± 0.005 vs. 0.76 ± 0.002 ; $P < 0.001$), which was independent of nitrate supplementation (Fig. 2, A and B), indicating that HFHS-fed mice were more reliant on fat oxidation than their chow-fed counterparts (68). Chow-fed mice also showed a greater difference in RER between the peaks and troughs recorded during light and dark periods compared with HFHS-fed mice (Fig. 2, A–C), indicating greater metabolic flexibility to switch between the fed and

fasted states, and this reached statistical significance in NaCl-supplemented mice (47.0% lower in HFHS-Cl mice, $P < 0.05$; Fig. 2C).

HFHS-fed mice had a total energy expenditure (EE) that was 20.3% greater than mice maintained on the chow diet ($P < 0.0001$; Fig. 2D), with no effect of nitrate supplementation. When normalized to body mass, however, EE was 11.2% lower in HFHS-fed mice compared with chow-fed mice ($P < 0.05$; Fig. 2E). Again, there was no apparent effect of nitrate supplementation, although mass-corrected EE was 13.5% lower in the inactive light period in HFHS- NO_3^- mice compared with chow-fed control mice ($P < 0.05$; Fig. 2E). Regression analysis demonstrated that EE varied consistently with total body mass across all experimental groups (Supplemental Fig. S2A).

Given the relationship between EE and body mass, we investigated how the HFHS diet might exert an obesogenic effect despite similar caloric intake and EE compared with chow-fed control mice. First, we investigated mitochondrial respiratory capacity in the skeletal muscle, since this is the largest insulin-sensitive tissue in the body (69) and largely dominates the measurement of whole body RER (70). We found very little difference in mitochondrial respiration between cohorts in either soleus (oxidative type I muscle) or gastrocnemius [a more glycolytic type II muscle (71); Supplemental Fig. S2, B and C], with similar OXPHOS coupling efficiencies across treatment groups within given muscle types (Supplemental Fig. 2D).

However, contribution to maximal OXPHOS through the F pathway via β -oxidation (FCR_F) was 38.8% higher in gastrocnemius from 8-mo-old HFHS- NO_3^- mice compared with chow-fed control mice ($P < 0.05$; Fig. 2F), and the relative capacity for fatty acid oxidation ($\text{FCR}_{FA/P}$) was also higher in gastrocnemius from HFHS-fed mice compared with chow-fed control mice (26.0% higher in 8-mo-old HFHS- NO_3^- mice, 32.6% higher in 12-mo HFHS-Cl mice; $P < 0.05$; Fig. 2G). These differences were not apparent in soleus (Fig. 2, F and G).

These findings support a shift in mitochondrial substrate preference in the gastrocnemius of HFHS-fed mice but did not indicate a reduction in overall muscle oxidative capacity or altered mitochondrial efficiency.

Next, we investigated whether the HFHS diet resulted in changes in intestinal morphology similar to those previously reported to result from dietary fructose (44). Accordingly, a cohort of mice fed the HFHS diet for 8 wk showed longer villi across the small intestine and deeper colonic crypts than chow-fed control mice (62.8%, $P < 0.0001$; Fig. 2H, Supplemental Fig. S2E). This suggests that obesity in the HFHS-fed mice resulted from increased nutrient absorption rather than increased intake or reduced expenditure.

Cardiac Metabolism and Fibrosis

As ~65% of diabetes-related mortality arises from cardiovascular diseases (72), with alterations in myocardial metabolism considered central to the etiology (73), we sought to investigate whether inorganic nitrate supplementation altered cardiac metabolic function in HFHS-fed mice. There was no difference in either heart mass (Supplemental Fig. S3A) or heart mass normalized to tibia length (a proxy for body size) (74) as a result of age, diet, or nitrate supplementation (Fig. 3A).

Cardiac glycogen content was assessed in sections of cardiac midsection with PAS staining (Fig. 3, B and C). When corrected for staining in the presence of α -amylase, the hearts of 12-mo-old HFHS-fed mice showed 3.0-fold higher PAS staining than those of age-matched chow-fed control mice ($P < 0.0001$), indicative of increased glycogen content (Fig. 3C). The area staining positively for PAS was also 82.5% higher in the HFHS-Cl cohort than the HFHS-NO₃ cohort at 12 mo of age ($P < 0.01$; Fig. 3C), indicating that glycogen accumulation was prevented when mice were supplemented with nitrate.

Using HILIC-mass spectrometry, we quantified metabolites involved in glycogen synthesis in the left ventricle (Fig. 3, D–G). HFHS-fed mice had 59.6% higher cardiac glucose levels compared with chow-fed control mice across all cohorts (Fig. 3D), which may be due to the high sucrose content of the diet. Cardiac glucose-6-phosphate showed no difference across age, diet, or nitrate supplementation (Fig. 3E); however, glucose-1-phosphate levels were 60.3% lower in hearts from HFHS-NO₃ mice compared with HFHS-Cl mice at 4 mo of age ($P < 0.05$; Fig. 3F). Glucose-1-phosphate levels in HFHS-Cl fed mice, however, showed decreasing levels with age (Fig. 3F), being 33.2% lower in 8-mo-old mice compared to 4-mo-old mice and 73.5% lower in 12-mo-old mice fed HFHS-Cl ($P < 0.05$; Fig. 3F). There was no difference in cardiac levels of UDP-glucose as a result of diet or nitrate supplementation (Fig. 3G).

The expression of genes involved in glycogen synthesis was unaffected by diet or nitrate supplementation (Fig. 3, H–J); however, both glycogen synthase 1 (*Gys1*) and *Ppp1ca* (1 of the catalytic subunits of protein phosphatase 1) showed lower expression in the hearts of older HFHS-fed mice. In HFHS-fed mice receiving NaCl, *Gys1* expression was 50.2% lower ($P < 0.05$; Fig. 3H) and *Ppp1ca* was 51.8% lower ($P < 0.01$; Fig. 3I) at 12 mo than at 4 mo. However, in HFHS-NO₃-fed mice, lower expression was detectable in the 8-mo cohort, with *Gys1* expression 54.5% lower at 8 mo ($P < 0.001$) and 61.8% lower at 12 mo ($P < 0.0001$) compared with hearts from 4-mo-old mice (Fig. 3H) and *Ppp1ca* expression 42.8% lower at 8 mo ($P < 0.05$) and 56.5% lower at 12 mo ($P < 0.001$) compared with that at 4 mo (Fig. 3I). There were no differences in cardiac expression of *Gsk3a* as a result of age or disease progression (Fig. 3J).

Taken together, these findings suggest that nitrate supplementation minimizes glycogen accumulation as a result of long-term HFHS feeding. Glycogen accumulation, which can occur in the diabetic heart (75), has been linked to the cardiometabolic stress response (76).

We next investigated myocardial fat metabolism, given that fatty acids are the predominant source of ATP in the healthy heart (77) and alterations in substrate usage are canonical markers of both the diabetic and failing heart (73, 78). Total TAG content of the left ventricle [as determined by liquid chromatography-mass spectrometry (LC-MS) peak intensity] was 1.5-fold higher in HFHS-fed mice compared with chow-fed control mice, reaching statistical significance in 4- and 8-mo chloride-supplemented mice ($P < 0.05$; Fig. 4A). Similarly, total cardiac diacylglycerol (DAG) was 53.1% higher in HFHS-Cl mice at 4 mo and 79.8% higher at 8 mo compared with chow-fed control mice ($P < 0.0001$; Fig. 4B). HFHS-NO₃-fed mice showed no differences in cardiac DAG

levels compared with either HFHS-Cl or chow-NO₃-fed mice at any age.

Moreover, combined HFHS-NO₃ feeding prevented a reduction in the total acyl-carnitine pool that was observed in hearts from mice fed HFHS-Cl at 4 mo (–21.2%, $P < 0.05$) and 12 mo (–35.2%, $P < 0.01$) compared with chow-fed control mice (Supplemental Fig. S3B). However, HFHS feeding resulted in alterations to the composition of the cardiac acyl-carnitine pool, independent of nitrate supplementation (Fig. 4, C and D; Supplemental Fig. S3C), including a relative depletion in the contribution of short-chain (total carbons C2–C5) acyl-carnitines to the total cardiac carnitine pool and a higher contribution of long-chain acyl-carnitines (total carbons $\geq$ C13) to the total cardiac carnitine pool in 4- and 8-mo-old mice ($P < 0.05$; Fig. 4C). Medium-chain acyl-carnitines (total carbons C6–C12) were higher in HFHS-fed mice, regardless of age or nitrate supplementation ($P < 0.01$; Fig. 4, C and D).

To investigate whether these changes in metabolites were reflected in mitochondrial capacity, we investigated cardiac mitochondrial respiratory function in saponin-permeabilized fiber bundles. We found no differences in mass-specific respiration in any state assayed due to diet, nitrate, or age (Supplemental Fig. S3D). However, at 12 mo relative contribution of fatty acid oxidation (FCR_F) was 13.9% higher in the hearts of HFHS-fed mice than those of chow-fed mice regardless of nitrate supplementation ($P < 0.01$; Fig. 4E). The capacity for fatty acid oxidation relative to pyruvate oxidation ($FCR_{FA/P}$) was also higher in the hearts of 12-mo-old HFHS-fed mice: HFHS-Cl-fed mice had 11.8% higher $FCR_{FA/P}$ compared with chow-Cl mouse hearts ($P < 0.05$), whereas HFHS-NO₃ mice had a 20.9% higher cardiac $FCR_{FA/P}$ than chow-NO₃ mice ($P < 0.05$; Fig. 4F).

We also investigated cardiac fibrosis histologically in cardiac midsections, using picrosirius red (PSR) staining for collagen (Fig. 4G). Analysis of the area staining positive for collagen showed that, at all ages, HFHS-NO₃-fed mouse hearts had a 71.7% higher cardiac collagen content than chow-fed control mice ($P < 0.01$; Fig. 4H). At 8 mo, hearts from HFHS-NO₃ mice also showed a collagen content that was 45.4% higher than that of hearts from HFHS-Cl mice ($P < 0.05$). This trend of higher collagen content in HFHS-NO₃ mouse hearts compared with HFHS-Cl mice was also seen at 4 mo (40.9% higher; $P = 0.0624$) and 12 mo (23.8% higher; $P = 0.107$; Fig. 4H).

In summary, inorganic nitrate supplementation exerted a metabolic benefit in the HFHS-fed mouse heart, through minimizing cardiac lipid accumulation and delaying glycogen accumulation, but did not affect mitochondrial respiratory capacity and resulted in increased cardiac fibrosis from 4 mo.

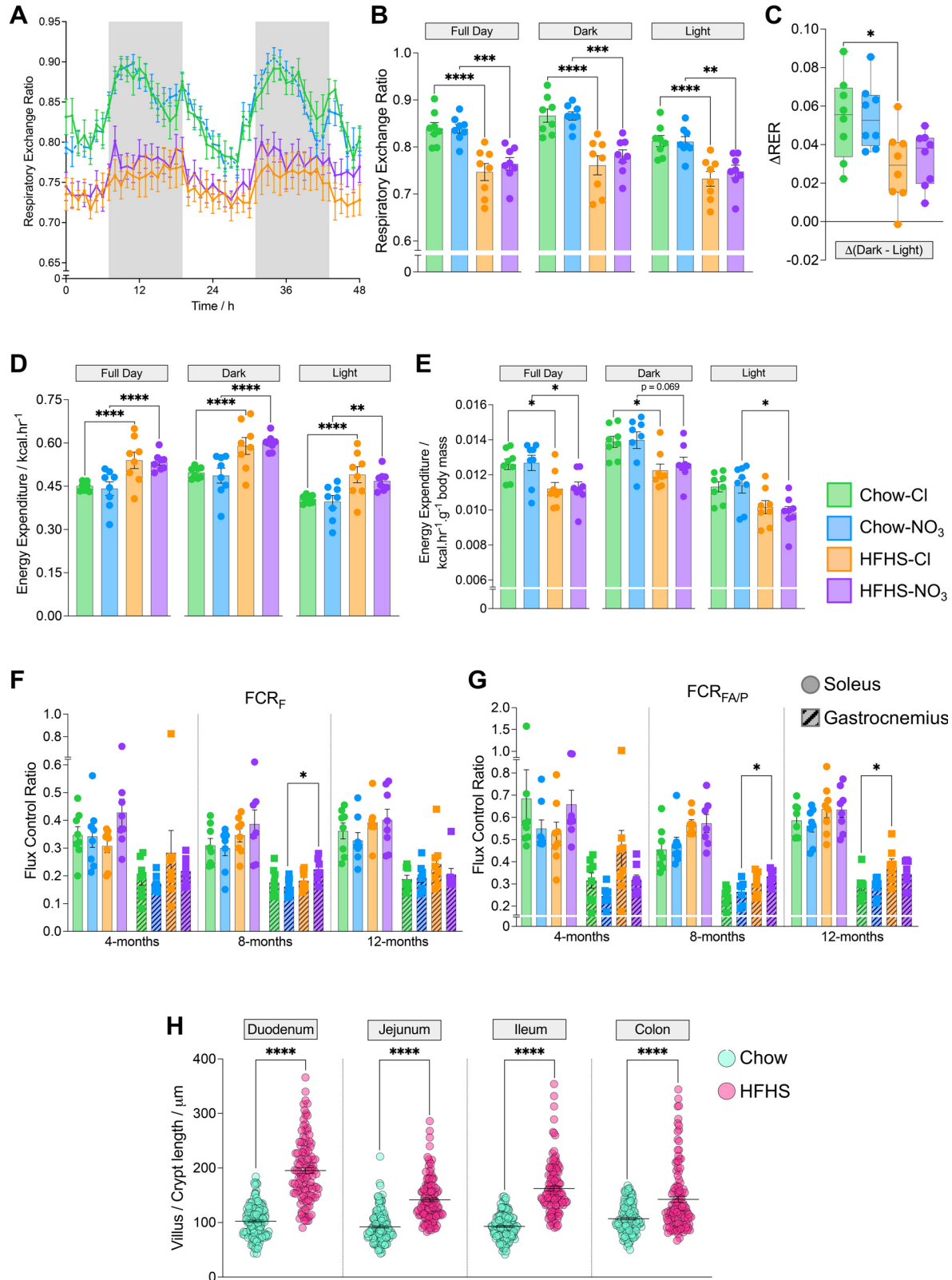
Inorganic Nitrate and MASLD Progression

A common, silent comorbidity of obesity is MASLD (79). We therefore investigated the effect of inorganic nitrate supplementation on liver metabolism and MASLD progression in HFHS-fed mice.

At 12 mo, mice fed a HFHS diet had livers that were 1.8-fold larger (relative to body mass) than chow-fed control mice ($P < 0.0001$; Fig. 5A). Interestingly, HFHS-NO₃-fed mice showed greater hepatomegaly than HFHS-Cl-fed mice (total liver mass being 1% heavier relative to body weight, $P < 0.05$; Fig. 5A). HFHS-NO₃ mice also had a greater prevalence of

tumors, with 50% of this group having visible tumors on the liver surface, compared to only one mouse in the HFHS-Cl group and none in chow-fed mice [χ^2 (df = 3, $N = 32$) = 10.2, $P < 0.05$; Fig. 5B].

As hepatocellular carcinoma (HCC) is a known consequence of MASLD, we investigated transcriptional markers of HCC (80, 81) in livers across all cohorts (Fig. 5, C–E). *Akr1b10*, a marker positively associated with HCC progression, was



elevated in 12-mo-old HFHS-fed mice compared with chow-fed control mice irrespective of nitrate supplementation (4.7-fold, $P < 0.01$; Fig. 5C). Moreover, HFHS-NO₃-fed mice showed specific elevation of the HCC marker *Spp1*, which was 3.2-fold higher than in chow-fed mice at 8 mo of age ($P < 0.01$) and by 12 mo showed expression that was 11.1-fold higher than in chow-fed control mice ($P < 0.0001$) and 2.4-fold higher than HFHS-Cl-fed mice ($p < 0.01$; Fig. 5D). Hepatic *Spp1* expression also showed a progressive increase in HFHS-NO₃-fed mice, with 8.6-fold higher ($P < 0.0001$) and 3.3-fold higher ($P < 0.001$) expression at 12 mo compared with 4 and 8 mo, respectively (Fig. 5D). Furthermore, *Hrg* (a marker negatively associated with HCC) was lower in livers of HFHS-NO₃-fed mice from 8 mo of age, reaching significance in the 12-mo cohort compared with chow-fed control mice (61.7%, $p < 0.05$; Fig. 5E). *Hrg* expression was also 64.7% lower in 8-mo-old mice ($P < 0.01$) and 77.2% lower in 12-mo-old mice ($P < 0.0001$) fed a HFHS-NO₃ diet, compared with the same cohort at 4 mo old (Fig. 5E).

We further undertook blind histological scoring of MASLD activity (MAS) in livers from mice of all cohorts (Fig. 5, F–I). Representative histological images are shown in Fig. 5, F–H. Mice fed a HFHS diet had livers with MAS that were, on average, 4.46 points higher than those maintained on chow ($P < 0.001$), independent of nitrate supplementation (Fig. 5I).

As the steatotic component of MAS was graded 2.1 points higher in HFHS-fed mice compared with chow-fed control mice at every time point ($P < 0.0001$; Supplemental Fig. S4A), hepatic lipid content was quantified to more accurately examine differences in hepatic lipid load across the experimental cohorts (Fig. 5J). Whereas HFHS-fed mice showed clear steatosis with 4.4-fold more fat per gram liver tissue compared with chow-fed control mice ($P < 0.01$; Fig. 5J), inorganic nitrate supplementation did not alter the steatotic component of MASLD.

In contrast, expression of the fibrotic markers *Serpine1* and *Pdgfb* indicated that fibrosis may be elevated at an earlier stage in HFHS-NO₃-fed mice compared with HFHS-Cl mice (Fig. 5, K and L). Whereas *Serpine1* expression was elevated 20.0-fold in livers from HFHS-Cl-fed mice at 12 mo compared with chow-fed control mice, there was no difference in expression of *Serpine1* in HFHS-Cl and chow-Cl livers from younger mice (Fig. 5K). In HFHS-fed mice supplemented with NO₃[−], however, *Serpine1* showed a 6.7-fold higher expression at 8 mo ($P < 0.05$) and 8.5-fold higher expression at 12 mo ($P = 0.0532$) compared with chow-fed control mice (Fig. 5K). Likewise, *Pdgfb* expression was 3.1-fold higher at 8 mo ($P < 0.01$) and 13.3-fold higher at 12 mo ($P < 0.05$) in the livers of HFHS-Cl mice compared with those

of chow-Cl mice, whereas in HFHS-NO₃-supplemented mice *Pdgfb* showed a 3.6-fold higher expression at 8 mo ($P < 0.0001$) and 13.2-fold higher expression at 12 mo ($P < 0.01$) compared with chow-fed control mice (Fig. 5L).

Taken together, these data suggest that nitrate supplementation accelerates MASLD progression in HFHS-fed mice, including greater incidence of hepatocellular carcinoma. Although nitrate did not alter steatosis, fibrosis was initiated earlier in HFHS-NO₃ livers than in those of HFHS-Cl mice.

Hepatic Lipid Metabolism and Mitochondrial Function

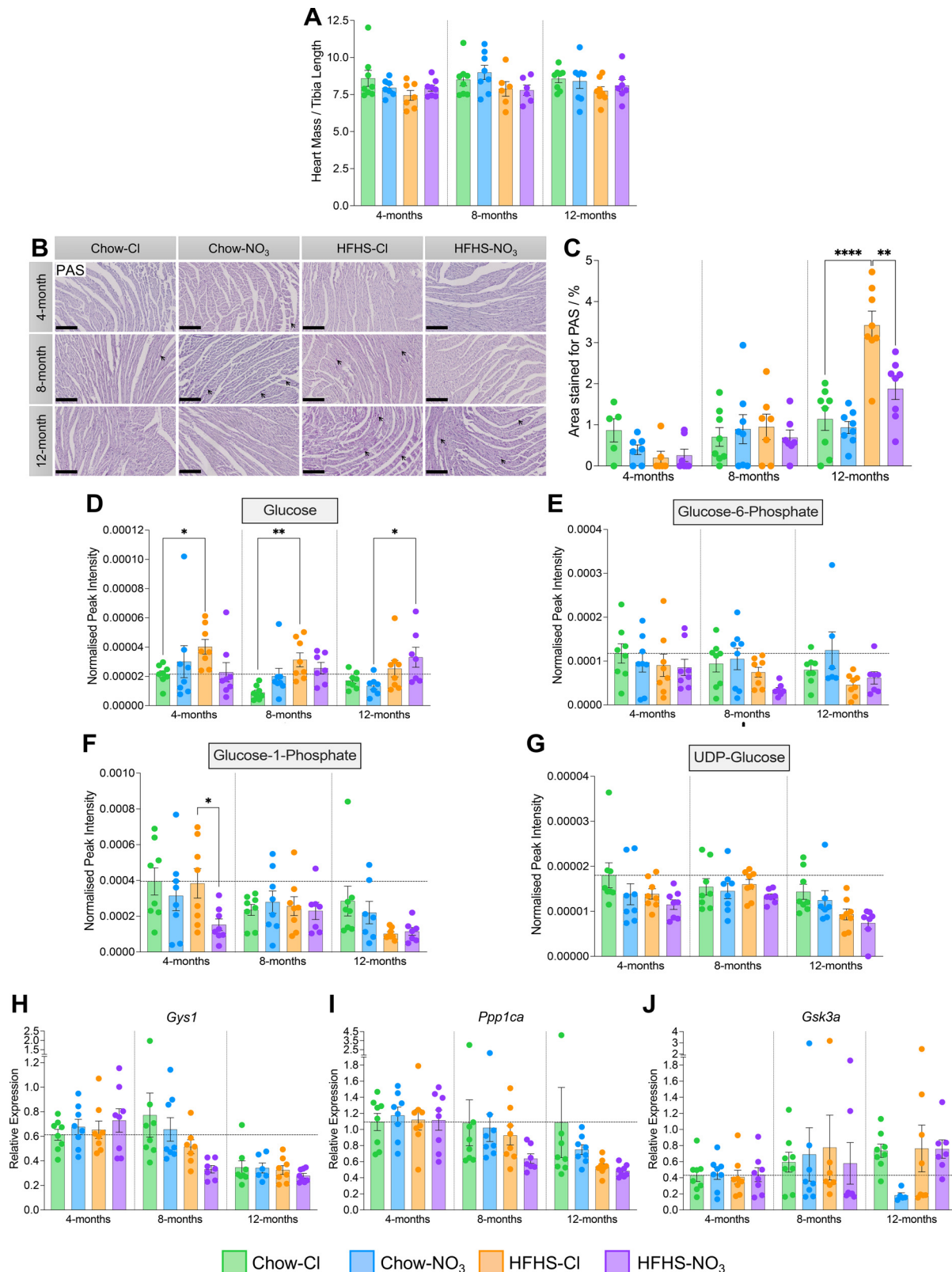
Finally, given the changes in steatosis in HFHS-fed mice, we sought to investigate whether the lipidome and, subsequently, fat metabolism were altered in mice fed a HFHS diet or inorganic nitrate. Open-profiling lipidomics identified >900 lipid species present in the livers of mice across all experimental conditions and time points. Principal component analysis (PCA) revealed that the hepatic lipidomes of HFHS-fed mice were distinct from those of chow-fed mice [$R^2X_{\text{(cum)}} = 88.3\%$, $Q^2_{\text{(cum)}} = 82.2\%$], irrespective of nitrate (Supplemental Fig. S4B). Plotting all lipids by species indicated clear differences due to diet in the phosphatidylserine (PS) and TAG complements of the lipidome (Supplemental Fig. S4C), with more subtle changes due to age and nitrate supplementation also appearing in these classes.

We therefore investigated the changes occurring in the PS and TAG complements in more depth. Partial least square-discriminant analysis (PLS-DA) of the PS [$R^2X_{\text{(cum)}} = 95.3\%$, $R^2Y_{\text{(cum)}} = 40.2\%$, $Q^2_{\text{(cum)}} = 24.1\%$, $P < 0.05$; Supplemental Fig. S4D] and TAG [$R^2X_{\text{(cum)}} = 84.6\%$, $R^2Y_{\text{(cum)}} = 28.5\%$, $Q^2_{\text{(cum)}} = 26\%$, $P < 0.0001$; Supplemental Fig. S4E] complements, respectively, in chloride-supplemented mice revealed that these lipid classes alone were sufficient to discriminate between livers of chow- and HFHS-fed mice. PS species were depleted across the board in livers of mice fed a HFHS diet, independent of nitrate supplementation (Fig. 6A), and although TAGs were generally increased in HFHS-fed mouse livers, there was greatest accumulation of species associated with de novo lipogenesis [total carbons 44 to 48 (82); Fig. 6B]. Interestingly, the top five species differentiating HFHS- from chow-fed livers [by variable importance parameter (VIP) score] for both PS and TAGs were highly correlated with the score given for steatosis in MAS (all $P < 0.0001$; Pearson's correlation). The most highly correlated PS (PS[34:2]; $R^2 = 0.7733$) and TAG (TG[46:1]; $R^2 = 0.8211$) were 99.9% lower ($P < 0.0001$; Supplemental Fig. S4F) and 42.3-fold higher ($P < 0.0001$; Supplemental Fig. S4F), respectively, in livers with histologically classified steatosis (MAS steatosis score

Figure 2. Whole body energy expenditure and skeletal muscle mitochondrial metabolism differences do not account for development of obesity in high-fat, high-sucrose (HFHS)-fed mice. A: changes in respiratory exchange ratio (RER), determined from oxygen consumption ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$). Shaded areas represent the dark period. B: average RER during the dark period, the light period, and across a full day, determined from $\dot{V}O_2$ and $\dot{V}CO_2$ in 10-mo-old mice during the 48-h metabolic cage protocol. C: difference in average RER between the active dark period and the quiet light period. D: energy expenditure across the dark period, light period, and full day. E: mass-normalized energy expenditure (normalized to total body mass) across the dark period, light period, and full day. For B–E, $N = 8$ mice/group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$, **** $P < 0.0001$, analyzed according to the CalR framework (47) adapted for a 2×2 design. F: contribution of the F pathway via β -oxidation to maximal oxidative phosphorylation (OXPHOS) (FCR_F) in saponin-permeabilized skeletal muscle fibers. G: capacity for OXPHOS supported by octanoyl-carnitine and malate relative to pyruvate and malate (FCR_{F/M}) in saponin-permeabilized skeletal muscle fibers. For F and G, $N = 7$ or 8 mice/group. * $P < 0.05$, 2-way ANOVA with Tukey's post hoc honestly significant difference (HSD) test for multiple comparisons. H: villus length (or colonic crypt depth) across regions of the intestine measured in histological sections (Supplemental Fig. S2D) from mice fed chow or HFHS diet for 8 wk. $N = 3$ or 4 mice/group. **** $P < 0.0001$, unpaired 2-tailed t test with Welch's correction. All data represent means $\pm$ SE.

2 or 3) compared with those without steatosis (MAS steatosis score 0 or 1), suggesting that these lipidomic differences are associated with MASLD not just increased dietary intake of lipid.

We next considered the expression of genes involved in hepatic lipid catabolism and anabolism. Lipogenesis genes were found to be generally upregulated in HFHS-fed mice (Fig. 6, C–E). Fatty acid synthase (*Fasn*) showed 7.4-fold



higher expression at 4 mo and 5.0-fold higher expression at 8 mo in HFHS-Cl mice compared with chow-fed control mice ($P < 0.01$; Fig. 6C), with no impact of inorganic nitrate supplementation. Stearoyl-CoA desaturase 1 (*Scd1*) was expressed 8.1-fold higher in livers from 4- and 8-mo-old HFHS-fed mice regardless of nitrate supplementation ($P < 0.05$; Fig. 6D). Similarly, acetyl-coA carboxylase (*Acc1*) was expressed 2.9-fold higher in 4- and 8-mo-old HFHS-fed mice ($P \leq 0.05$; Fig. 6E) but remained elevated in 12-mo-old HFHS-NO₃ mice, where expression was 3.6-fold higher than in chow-fed control mice ($P < 0.05$; Fig. 6E). Interestingly, the genes encoding the β -oxidation enzymes 3-hydroxyacyl-CoA dehydrogenase (*Hadh*) and long-chain acyl-coA dehydrogenase (*Acadl*) showed an age-dependent expression profile (Fig. 6, F and G). In 4-mo-old HFHS-Cl mice, *Hadh* expression was 4.7-fold higher ($P < 0.0001$; Fig. 6F) and *Acadl* expression was 2.2-fold higher ($P < 0.01$; Fig. 6G) compared with chow-fed control mice. However, there was no difference due to diet or nitrate supplementation in 8-mo-old mice, and by 12 mo-old *Hadh* expression was 60.3% lower in HFHS-NO₃ mice ($P < 0.05$; Fig. 6F) and *Acadl* expression was 45.4% lower in HFHS-Cl mice ($P < 0.05$, Fig. 6G) compared with chow-fed control mice. Chow-NO₃-fed mice also showed 44.0% lower *Acadl* expression at 12 mo compared with chow-Cl-fed mice ($P < 0.05$; Fig. 6G).

We next investigated the acyl-carnitine profile of the liver. The total acyl-carnitine pool was 40.7% lower in HFHS-fed mice compared with chow-fed mice (Supplemental Fig. S5A). However, as in the heart, there were alterations to the composition of the hepatic acyl-carnitine pool independent of nitrate supplementation (Fig. 6, H and I; Supplemental Fig. S5B). At all time points, relative levels of long-chain ($\geq C13$) acyl-carnitines were 3.4-fold higher in HFHS-fed mice ($P < 0.05$), and 8- and 12-mo-old HFHS-fed mice showed 22.8% lower contribution of free carnitine to the total carnitine pool (Fig. 6H). Surprisingly, there was no concurrent suppression in the relative level of short-chain (C2–C5) acyl-carnitines with the accumulation in long-chain acyl-carnitines, except in 12-mo-old HFHS-NO₃ mice, where the relative contribution of short-chain acyl-carnitines to the total carnitine pool was 18.2% lower than in chow-fed control mice ($P < 0.01$; Fig. 6H). There were no differences in relative levels of medium-chain acyl-carnitines (Fig. 6, H and I).

Finally, we assessed whether changes measured in the liver during MASLD progression were associated with alterations in mitochondrial respiratory function. Generally, mice fed a HFHS diet tended to have a lower mass-corrected respiratory capacity in all states at all ages, independent of nitrate supplementation. This was especially evident with β -oxidation-supported respiration (OctM_L and OctM_P) and after

stimulation of complex II with succinate (GMS_P, GMS_E and S_E; Supplemental Fig. S5C). In line with this, the capacity for fatty acid oxidation relative to pyruvate oxidation (FCR_{FA/P}) was 23.7% lower in the livers of 4-mo-old HFHS-fed mice ($P < 0.05$) and 30.3% lower in the livers of 8-mo-old HFHS-NO₃ mice ($P < 0.01$) compared with chow-fed control mice (Fig. 6J). However, the contribution of the N pathway to maximal OXPHOS (FCR_N) was 34.6% higher in 4-mo-old HFHS-fed mice regardless of nitrate supplementation ($P < 0.05$) and 42.1% higher in 4-mo-old HFHS-NO₃ mice ($P < 0.01$) compared with chow-fed control mice (Fig. 6K).

Taken together, these results highlight that hepatic lipid metabolism is impacted by HFHS feeding, with metabolic changes occurring early and persisting throughout life in mice fed a HFHS diet.

DISCUSSION

Inorganic nitrate has been suggested as a possible treatment for obesity-related metabolic disease owing to the relative ease of modifying intake through the diet (83) and favorable effects upon acute administration including increased bioavailability of NO (20), improvement of mitochondrial efficiency (66), and enhanced mitochondrial β -oxidation (35). Collectively, these have been proposed to be beneficial in treating metabolic diseases by ameliorating any effects of mitochondrial dysfunction and providing a mechanism for dissipating excess FFA. However, here we show that dietary supplementation with a moderate dose of inorganic nitrate supplementation (achievable in humans via a modest increase in leafy green vegetable consumption) (84) was not effective as a therapeutic intervention in mice with diet-induced obesity and was instead associated with adverse effects in HFHS-fed mice (Fig. 7). Most notably, dietary supplementation with inorganic nitrate resulted in elevated plasma LDL cholesterol, increased cardiac fibrosis, and accelerated MASLD severity in HFHS-fed mice, including a significant, detectable tumor burden in 12-mo-old HFHS-NO₃ fed mice.

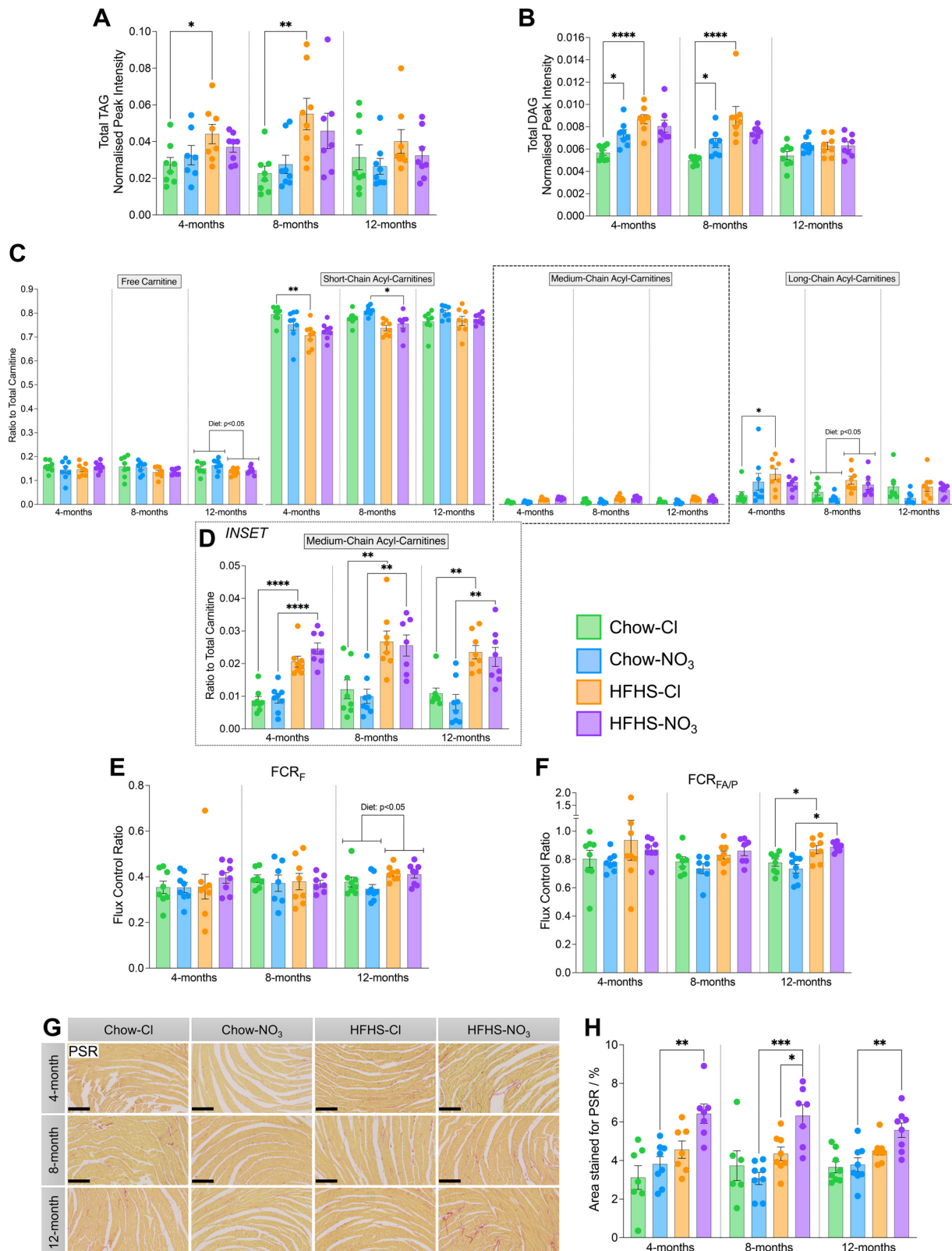
A major strength of this study is that we have investigated the metabolic phenotype at multiple time points over a sustained time frame. Many studies investigating metabolic alterations brought about by obesity or diabetes consider a single time point in comparison with a healthy control. However, type II diabetes is a progressive disease that exists on a spectrum from mild insulin resistance to overt glucose intolerance with β -cell impairment, and it is therefore important to consider when and where adverse metabolic remodeling occurs as the disease progresses. This temporal analysis has also allowed us to consider some of the longer-

Figure 3. Inorganic nitrate supplementation prevents glycogen accumulation in high-fat, high-sucrose (HFHS)-fed mouse heart. A: heart mass, normalized to tibial length, as a proxy for body size. B: representative cardiac midsections stained with periodic acid Schiff (PAS) stain at $\times 20$ magnification. Scale bars, 150 μ m. Glycogen deposits are stained magenta and marked with arrows (maximum 3 per slide shown). C: quantification of area staining positive for PAS. Percentages were corrected for the staining present in control sides pretreated with α -amylase, thereby excluding any staining resulting from other glycans and mucins. D: relative cardiac concentration of glucose, determined by hydrophobic interaction liquid chromatography (HILIC)-mass spectrometry (MS). E: relative cardiac concentration of glucose-6-phosphate, determined by HILIC-MS. F: relative cardiac concentration of glucose-1-phosphate, determined by HILIC-MS. G: relative cardiac concentration of UDP-glucose, determined by HILIC-MS. H: relative cardiac expression of *Gys1*, determined by RT-qPCR. I: relative cardiac expression of *Ppp1ca*, determined by RT-qPCR. J: relative cardiac expression of *Gsk3a*, determined by RT-qPCR. Data represent means $\pm$ SE; $N = 6$ –8 mice/group. Data in D–G are normalized to an appropriate internal standard and sample protein concentration. For H–J, horizontal line indicates relative expression in 4-mo chow/chloride mice. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$, 2-way ANOVA with Tukey's post hoc honestly significant difference (HSD) test for multiple comparisons.

term effects associated with nitrate supplementation, which are not apparent over a shorter time frame. Furthermore, we have utilized a range of gold-standard techniques allowing an in-depth dissection of the progressive obesity phenotype

alongside changes in mitochondrial function, tissue metabolism, and morphology.

Our findings add clarity to the time course of metabolic derailment occurring over the development of obesity and



related comorbidities. This is evident, for instance, in the liver, where our in-depth analysis of the MASLD phenotype, accompanied with whole lipidome analysis and mitochondrial functional measurements, has allowed a comprehensive assessment of the metabolic changes occurring from a healthy tissue all the way to severe disease. For instance, although PS depletion has previously been shown to occur in metabolic dysfunction-associated steatohepatitis (MASH) patients (85), our data highlight that PS depletion occurs from the very earliest time points of metabolic derailment, before progression to MASH. Furthermore, combined suppression of PSS1 and PSS2 (enzymes involved in the synthesis of PS) lowered hepatic PS in mice and concurrently increased hepatic TAG (86), in agreement with the changes reported here. However, we additionally show that these changes are accompanied by alterations in the balance between hepatic lipid catabolism and anabolism, alongside changes in mitochondrial respiratory function.

Our finding of detrimental consequences of inorganic nitrate in HFHS diet-fed mice (Fig. 7) can be considered alongside previously published benefits of nitrate supplementation in healthy rodents. For instance, up to 17 mo of inorganic nitrate supplementation at an equivalent dose was shown to have no adverse effects in male C57Bl/6 mice and improved fasting insulin in these mice (42); however, these mice were maintained on a standard laboratory chow (with depleted nitrate) (42). The adverse effects observed in the HFHS-NO₃ mice here are likely to result from an interaction between the obesogenic diet and the nitrate itself; indeed, we also observed no adverse effects in nitrate-supplemented mice fed a standard laboratory chow diet. Of note, however, nitrate supplementation did not result in any improvement in fasting insulin in chow-fed mice in our study, contrary to that previously observed (42).

Furthermore, inorganic nitrate has been shown to increase mitochondrial β -oxidation capacity in rat skeletal muscle (35, 87), a finding that gave rise to some of the hypothesized benefits of inorganic nitrate supplementation in obesity-related comorbidities. However, this effect was not observed in NO₃⁻-supplemented mice here, regardless of whether they received standard chow or HFHS diet or whether soleus or gastrocnemius muscle was analyzed. This apparent discrepancy is likely due to the lower baseline nitrate levels of rats in comparison to mice (88), resulting in reduced elevations in plasma nitrate levels achievable in mice, as used in this study, through dietary supplementation with NaNO₃. Although plasma levels of nitrate in rats are more similar to those in humans (88, 89), one of the significant advantages of this work was that by using mice rather than rats a long-term study could be carried out (90), allowing us to

observe the metabolic alterations over the entire course of obesity progression.

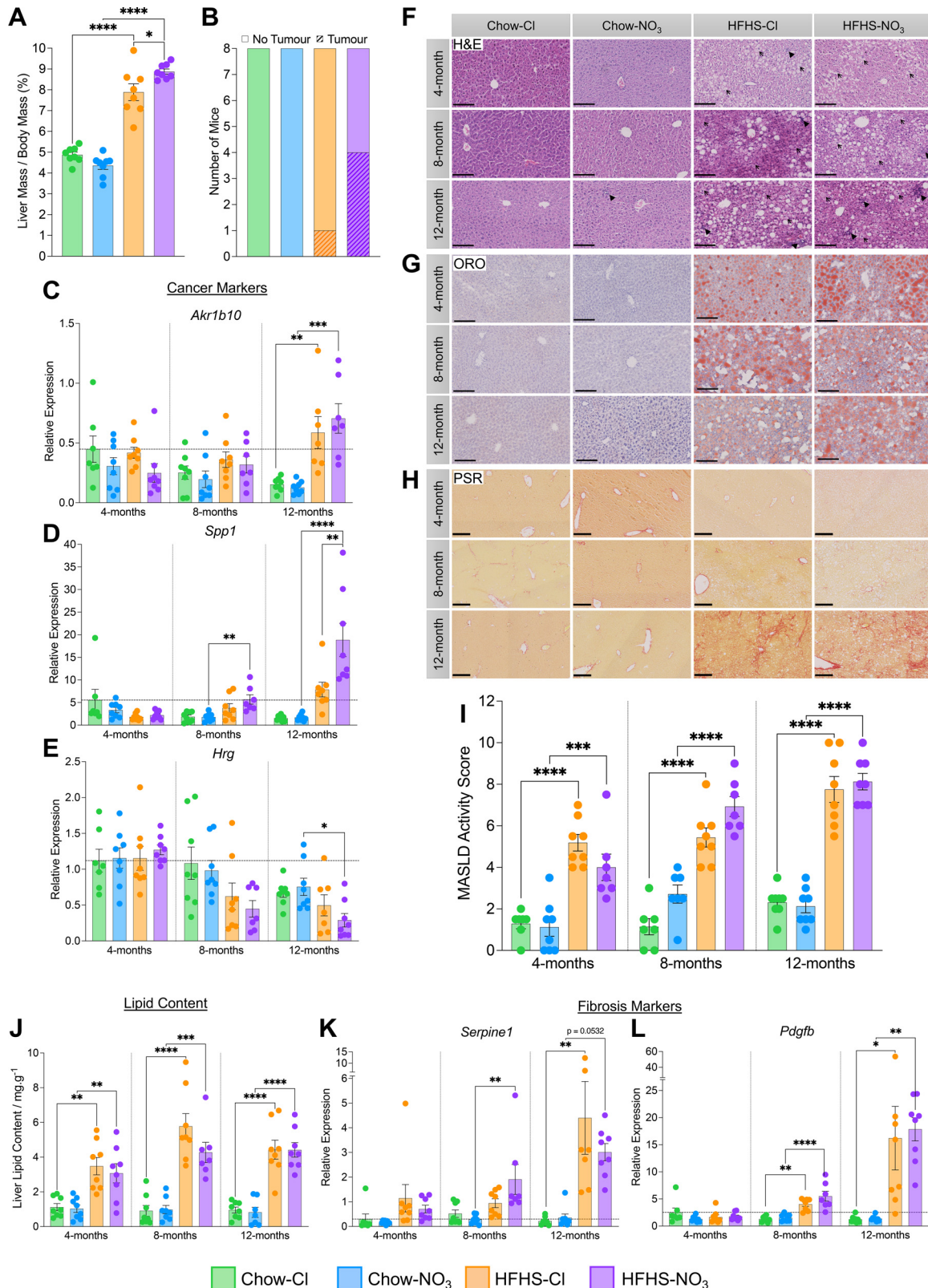
There may also be alternative explanations for the lack of an apparent beneficial effect of nitrate supplementation in this study. For instance, given that commensal oral microbes are vital for the reduction of NO₃⁻ to NO₂⁻ (18), alterations to the oral microbiome are known to affect NO production from inorganic nitrate (91). Consumption of inorganic nitrate can itself cause alterations to the oral microbiome (92). Furthermore, both aging (93, 94) and consumption of a sugar-rich diet (95) are known to alter the composition of the oral microbiota, and changes in the oral microbiome have also been identified in individuals with obesity, reflective of concomitant changes in the gastrointestinal tract (96). Specifically, sugar-rich diets can result in a facultative shift in the oral flora toward saccharolytic, acidogenic, and aciduric species (95), and aging is associated with decreased species diversity and increased numbers of opportunistic pathogens (93). Given that specific species of *Streptococcus*, *Staphylococcus*, *Nocardia*, and *Corynebacterium* are key for nitrate reduction (97, 98), an interesting avenue for future research would be to investigate whether those species are maintained during prolonged HFHS feeding and aging.

Additionally, our finding of increased cardiac fibrosis in HFHS-NO₃ mice contrasts with previous studies that have shown a nitrate-driven amelioration of fibrosis in the kidney and heart as a result of obesity or hypertension in rodent models (99–102). This apparent discrepancy may be attributable to the significantly longer timescale investigated here, compared with a maximum of 10 wk in previous studies. Alternatively, an endothelial-to-mesenchymal transition due to inflammation or a perturbation in redox homeostasis, along with the known reduced effectiveness of the Nrf2 system (and thus fibrosis resolution) with aging (103, 104), may account for the increased cardiac fibrosis seen here. This elevation in cardiac fibrosis was particularly surprising to find in HFHS-NO₃ mice, given the anti-inflammatory effects purported for nitro-fatty acids (nitro-FAs): endogenously produced electrophilic metabolites resulting from the nonenzymatic reaction between unsaturated fatty acids and NO/NO₂⁻ oxidation products (105, 106). Whereas the HFHS diet given to these mice was predominantly high in saturated fats, the unsaturated fat content was thrice that of the standard laboratory chow [6.6% vs. 2.2% (wt/wt), according to manufacturer analyses], which would be suspected to result in increased nitro-FAs concomitantly. However, as the anti-inflammatory effects of nitro-FAs occur, at least partially, through activation of the Nrf2 system (106, 107), perhaps the age-related decline in effectiveness also accounts for the surprising increase in fibrosis. If not, there may perhaps be an alteration of the balance of nitro-FA formation and the relationship with Nrf2-mediated processes that leads to an

Figure 4. Inorganic nitrate supplementation offers minor benefits to cardiac fat metabolism in high-fat, high-sucrose (HFHS)-fed mice but worsens cardiac fibrosis. **A:** total cardiac triacylglycerol (TAG) content, determined by liquid chromatography-mass spectrometry (LC-MS), measured as total peak area ratio normalized to an appropriate internal standard and sample protein concentration. **B:** total cardiac diacylglycerol (DAG) content, determined by LC-MS, measured as total peak area ratio normalized to an appropriate internal standard and sample protein concentration. **C:** relative levels of free (no associated acyl chain), short-chain (C2–C5), medium-chain (C6–C12), and long-chain ($\geq$ C13) acyl-carnitines to the total carnitine pool as measured by LC-MS from cardiac tissue. **D:** relative levels of medium-chain acyl-carnitines to the total carnitine pool (from **H**) shown as an expanded inset. **E:** contribution of the F pathway via β -oxidation to maximal oxidative phosphorylation (OXPHOS) (FCR_F) in saponin-permeabilized cardiac fibers. **F:** capacity for OXPHOS supported by octanoyl-carnitine and malate relative to pyruvate and malate (FCR_{F+M}) in saponin-permeabilized cardiac fibers. **G:** representative cardiac midsections stained with picosirius red (PSR) at $\times 10$ magnification. Scale bars, 250 μ m. Collagen fibers are stained red, identifying regions of fibrosis. **H:** quantification of area staining position for PSR, as an indicator of cardiac fibrosis. Data represent means $\pm$ SE; $N = 6$ –8 mice/group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, 2-way ANOVA with Tukey's post hoc honestly significant difference (HSD) test for multiple comparisons.

unfavorable shift in the wrong direction in HFHS-NO₃-fed mice. In future work, it would also be interesting to examine the impact of combined long-term HFHS feeding with NO₃⁻ supplementation on vascular function and blood pressure,

considering the elevated fibrosis elucidated here. Although not examined in the present study, the well-known hypotensive effects of NO (108, 109), which have been observed to be achievable via dietary inorganic nitrate (110–112), are likely to



interact with the hypertensive effects of a HFHS diet (8, 113, 114), warranting further investigation into nitrate-based approaches to treat hypertension over longer timescales against the background of obesogenic diets.

An unexpected finding in this study was the increased incidence of liver tumors in 12-mo HFHS-NO₃-fed mice, with gene expression analysis suggesting that concurrent HFHS-NO₃ feeding accelerated progression from MASLD to HCC. The pathogenesis of MASLD-associated HCC is incompletely understood, as, despite MASLD being the most common underlying risk factor for HCC (115), only a minority of MASLD patients go on to develop HCC (116). The current hypothesized factors underlying the increased HCC risk in MASLD include compensatory hyperproliferation to counter hepatocyte cell death (as a result of lipotoxicity or DNA damage from oxidative damage), activation of hepatic stellate cells resulting in increased fibrosis, and elevated inflammatory factors (116). Although inorganic nitrate was administered to mice at a low dose, it was expected to increase NO bioavailability, which, while not considered to be damaging to DNA alone, can produce reactive nitrogen species (RNS) upon reacting with reactive oxygen species (ROS) that can damage DNA through both base mutations and strand breaks (117). MASLD has been associated with increased ROS production as a result of fatty acid overload, which may reduce mitochondrial coupling and increase inner mitochondrial membrane electron leak (118). The combined increase of ROS (as a result of the HFHS diet) and NO (as a result of dietary nitrate) may therefore be a key event precipitating the increased incidence of apparent HCC with HFHS-NO₃ feeding. Although the reduction of nitrate to nitrite is likely slow and quantitatively moderate, tissue nitrite may exceed levels in the circulation (88); thus we cannot exclude the possibility that liver tumors may be induced as a result of enhanced nitrosamine formation. The high hepatic concentration of key reductive enzymes, such as xanthine oxidoreductase and (under hypoxic conditions) cytochrome P-450 enzymes (119–121), although not measured in our study, may also facilitate an elevated level of RNS in cases of HFHS-NO₃ feeding, thus contributing to the unexpected malignancies observed here. However, the exact products and posttranslational modifications formed and the subsequent reactions leading to oncogenesis remain unknown and warrant further investigation.

It is unlikely that nitrite/NO-adducts themselves are directly causing the increased malignancy; instead, our results are more likely to reflect the outcome of a complex systems response to changes triggered by the obesogenic diet and

chronic nitrate supplementation administered concomitantly. For instance, pathogenic extracellular matrix (ECM) remodeling has been shown to occur in both the brain (122) and skeletal muscle (123) after obesogenic diets and thus is likely to occur in all metabolically active tissues. Such ECM-driven alterations will lead to inflammation, which will alter the local redox landscape within which cells communicate, which, in turn, will affect DNA repair processes and may possibly increase the propensity for malignancy. The combined effect of nitrate supplementation may escalate these redox changes by, for instance, altering cellular pH (124) and thus elevate this malignancy risk further. Whether such a mechanism is indeed at play here would be an interesting avenue for future research.

Several studies have highlighted the potential benefits of nitrate supplementation in obesity-related metabolic diseases (28, 31–33, 125). Much of this work stems from the initial observation that dietary inorganic nitrate increased NO bioavailability, offsetting the fall in endogenous NO production, which may be a key pathological event in the development of MetS (28). However, these experiments were carried out on eNOS-deficient mice (28), raising the possibility that nitrate may only be beneficial against the background of reduced endogenous NO production. Inorganic nitrate supplementation may be less effective in cases of MetS or T2DM, where endogenous NOS enzymes are functional, as, although there can be impaired eNOS activity in MetS and T2DM (126, 127), there is a more subtle endogenous NO depletion than that seen in eNOS-deficient mice. Support for this arises from the observation that 12-wk nitrate supplementation did not ameliorate MetS development in high-fat diet-fed mice (128), much as nitrate supplementation did not improve adiposity or HOMA-IR in the HFHS-fed mice in this study.

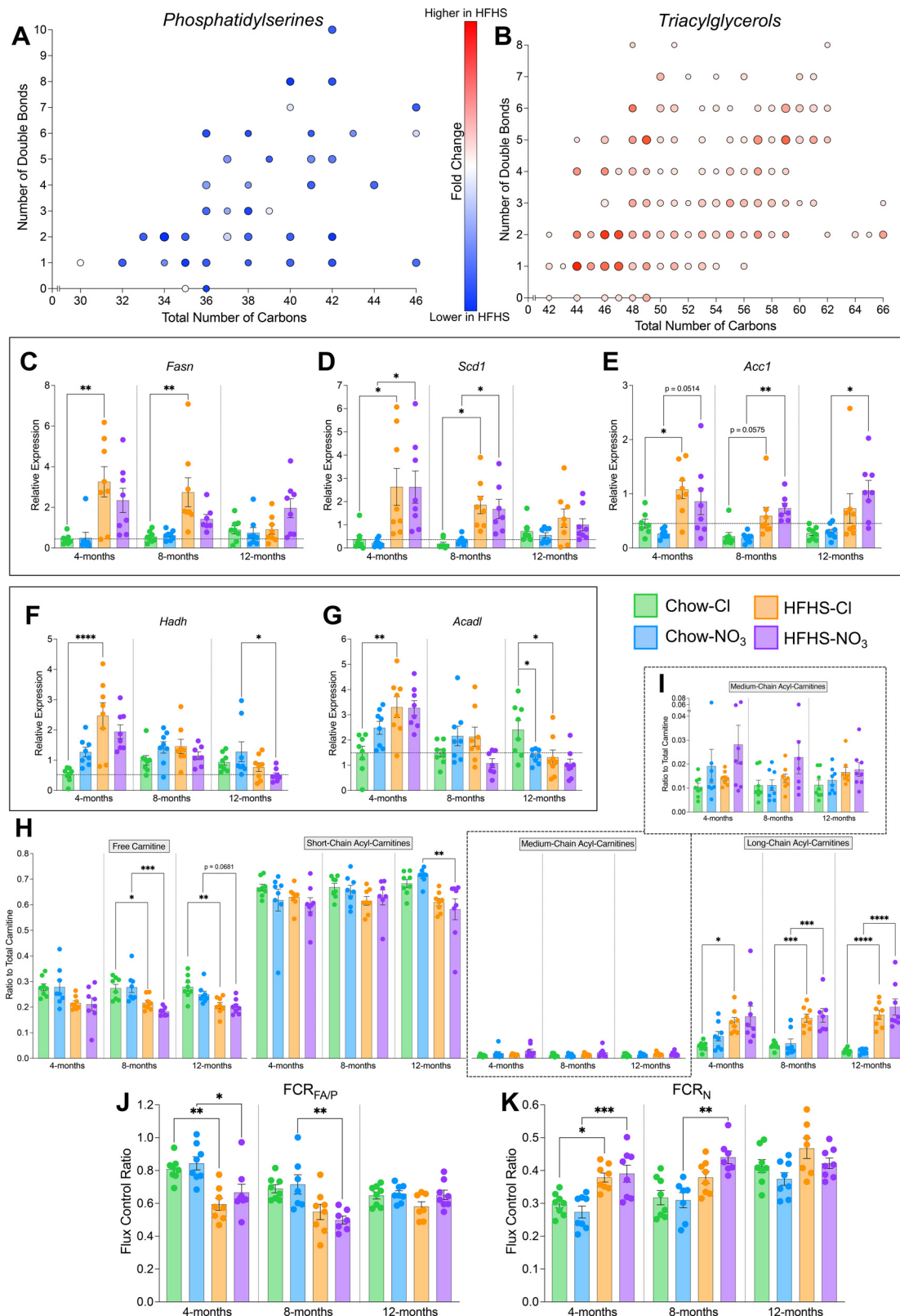
Despite these negative findings, nitrate may yet hold benefit as a therapeutic agent in certain individuals with obesity-related metabolic disease. eNOS polymorphisms have been associated with T2DM and MetS (14, 15), highlighting a group of patients in which nitrate supplementation may prove to be a beneficial treatment strategy. Furthermore, a higher intake of leafy green vegetables (the largest dietary source of inorganic nitrate) (129) is associated with a 14% reduced risk of T2DM development (130). Although this may be a result of general dietary habits (i.e., individuals who consume more leafy vegetables may consume less processed foods), it highlights the possibility of a preventative role of nitrate in T2DM development as opposed to using it as a therapeutic agent for the treatment of the condition.

Nevertheless, our work highlights that nitrate supplementation is largely ineffective in protecting against the significant

Figure 5. Inorganic nitrate supplementation accelerates the severity of metabolic dysfunction-associated steatotic liver disease (MASLD) in mice. **A:** liver mass, normalized to total body mass, in 12-mo-old mice. **B:** prevalence of tumors, visible with the naked eye, in livers from 12-mo-old mice. **C:** relative hepatic expression of *Akr1b10*, a marker known to positively associate with hepatocellular carcinoma (HCC) progression, determined by RT-qPCR. **D:** relative hepatic expression of *Spp1*, a marker known to positively associate with HCC progression, determined by RT-qPCR. **E:** relative hepatic expression of *Hrg*, a marker known to negatively associate with HCC progression, determined by RT-qPCR. **F:** representative liver sections stained with hematoxylin and eosin (H&E) at $\times 20$ magnification. Scale bars, 150 μ m. Arrows highlight examples of hepatocyte ballooning; arrowheads highlight examples of immune invasion foci. HFHS, high-fat, high-sucrose diet. **G:** representative liver sections stained with oil red O (ORO) at $\times 20$ magnification. Scale bars, 150 μ m. Neutral lipids and triglycerides are stained red. **H:** representative liver sections stained with picrosirius red (PSR) at $\times 10$ magnification. Scale bars, 250 μ m. Collagen fibers are stained red, identifying regions of fibrosis. **I:** MASLD activity score (MAS) determined from blinded histological scoring (Supplemental Table S2). **J:** hepatic lipid content determined by modified Folch extraction of lipids. **K:** relative hepatic expression of *Serpine1*, a marker of fibrosis, determined by RT-qPCR. **L:** relative hepatic expression of *Pdgfrb*, a marker of fibrosis, determined by RT-qPCR. Data represent means $\pm$ SE. $N = 7$ or 8 mice/group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, 2-way ANOVA with Tukey's post hoc honestly significant difference (HSD) test for multiple comparisons. For C–E, K, and L, horizontal line indicates relative expression in 4-mo chow/chloride mice.

consequences of long-term HFHS feeding. Furthermore, we have uncovered that negative consequences of nitrate can emerge against the background of an obesogenic diet. Thus, nitrate supplementation is unlikely to be an effective

approach to improve metabolic health in obesity, without wider alterations to the diet. Given the recent change in public perception of nitrate as being beneficial rather than detrimental, there is an urgent need to reproduce our findings in other



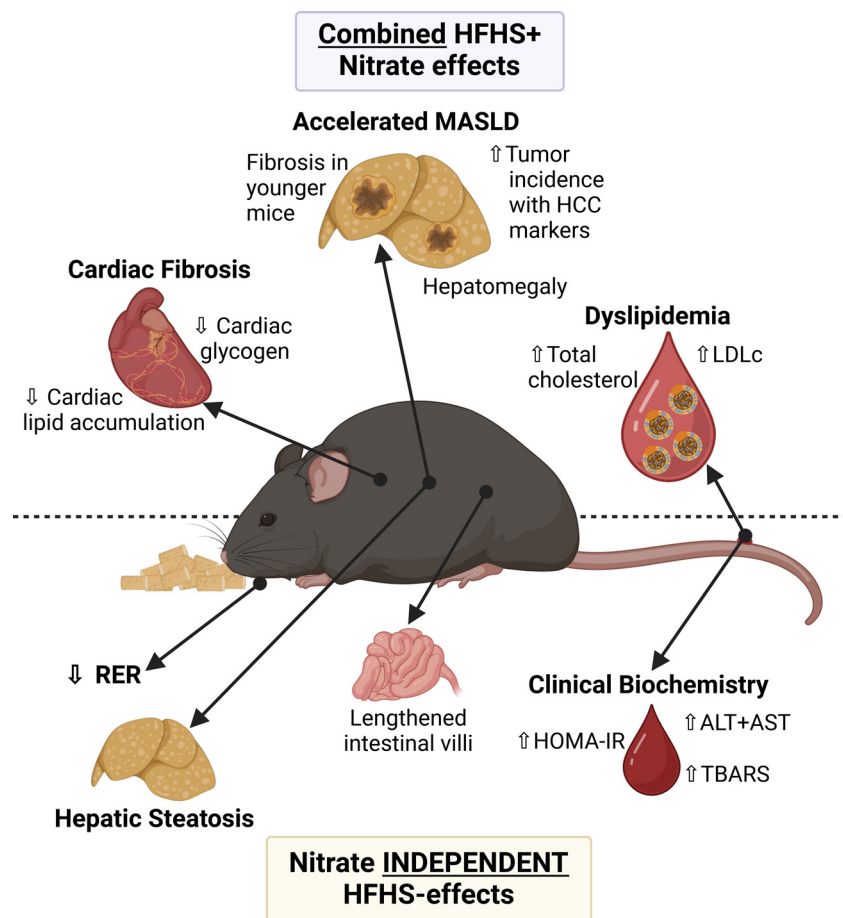


Figure 7. Summary of diet-induced obesity effects determined in this study as a result of high-fat, high-sucrose (HFHS) feeding (independent of nitrate supplementation) or as a result of inorganic nitrate supplementation in combination with HFHS diet. Upward arrow indicates higher in HFHS (compared with chow) or HFHS-NO₃ (compared with HFHS-Cl), respectively; downward arrow indicates lower in HFHS (compared with chow) or HFHS-NO₃ (compared with HFHS-Cl), respectively. ALT, alanine transaminase; AST, aspartate transaminase; HCC, hepatocellular carcinoma; HOMA-IR, homeostatic model assessment of insulin resistance; LDLc, low-density lipoprotein cholesterol; MASLD, metabolic dysfunction-associated steatotic liver disease; RER, respiratory exchange ratio; TBARS, thiobarbituric acid reactive substances. Created with BioRender.com.

laboratories and animal models and to investigate the mechanisms of enhanced cardiac fibrosis and hepatic malignancy. This is especially important since epidemiological evidence is emerging that the effects of enhanced nitrate intake on cardiovascular health may be source specific (131).

DATA AVAILABILITY

The datasets supporting the results presented in this article are freely available via the Cambridge University Repository (<https://doi.org/10.17863/CAM.112799>). Raw metabolomics data are available in the EMBL-EBI MetaboLights database (132) with the study identifier MTBLS9526.

SUPPLEMENTAL MATERIAL

Supplemental Figs. S1–S5 and Supplemental Tables S1 and S2: <https://doi.org/10.17863/CAM.112799>.

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Figure 6. High-fat, high-sucrose (HFHS) feeding is associated with remodeling of the hepatic lipidome and rewiring of lipid metabolism in an age-dependent manner. **A:** hepatic phosphatidylserine (PS) species found to be differentially affected by HFHS feeding in mice [determined by variable importance parameter (VIP) score from partial least square-discriminant analysis (PLS-DA; Supplemental Fig. S4D) > 1], plotted by total number of carbons and double bonds in the acyl chains. **B:** hepatic triacylglycerol (TAG) species found to be differentially affected by HFHS feeding in mice [VIP score from PLS-DA (Supplemental Fig. S4E) > 1], plotted by total number of carbons and double bonds in the acyl chains. For **A** and **B**, size of the point represents the VIP score for the PLS-DA model, and color represents relative fold change in livers from HFHS-fed mice compared to chow-fed mice. **C:** relative hepatic expression of *Fasn*, determined by RT-qPCR. Horizontal line indicates relative expression in 4-mo chow/chloride mice. **D:** relative hepatic expression of *Scd1*, determined by RT-qPCR. Horizontal line indicates relative expression in 4-mo chow/chloride mice. **E:** relative hepatic expression of *Acc1*, determined by RT-qPCR. Horizontal line indicates relative expression in 4-mo chow/chloride mice. **F:** relative hepatic expression of *Hadh*, determined by RT-qPCR. Horizontal line indicates relative expression in 4-mo chow/chloride mice. **G:** relative hepatic expression of *Acadl*, determined by RT-qPCR. Horizontal line indicates relative expression in 4-mo chow/chloride mice. **H:** relative levels of free (no associated acyl chain), short-chain (C2–C5), medium-chain (C6–C12), and long-chain (≥C13) acyl-carnitines to the total carnitine pool as measured by liquid chromatography-mass spectrometry (LC-MS) from liver tissue. **I:** relative levels of medium-chain acyl-carnitines to the total carnitine pool (from **H**) shown as an expanded inset. **J:** capacity for oxidative phosphorylation (OXPHOS) supported by octanoyl-carnitine and malate relative to pyruvate and malate (FCR_{FA/P}) in liver homogenate. **K:** contribution of the N pathway via complex I to maximal OXPHOS (FCR_N) in liver homogenate. For **C–K**, data represent means ± SE; N = 7 or 8 mice/group. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001, 2-way ANOVA with Tukey's post hoc honestly significant difference (HSD) test for multiple comparisons.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

A.P.S., M.V., M.F., J.L.G., and A.J.M. conceived and designed research; A.P.S., L.M.W.H., F.N.K., R.B., G.M., D.K.K., M.M., K.A.O., M.C.H., P.M.D., and B.D.T. performed experiments; A.P.S. analyzed data; A.P.S. interpreted results of experiments; A.P.S. prepared figures; A.P.S. drafted manuscript; A.P.S., M.F., J.L.G., and A.J.M. edited and revised manuscript; A.P.S., L.M.W.H., F.N.K., R.B., G.M., D.K.K., M.M., K.A.O., M.C.H., P.M.D., B.D.T., M.V., M.F., J.L.G., and A.J.M. approved final version of manuscript.

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