1 Microbiome Inhibition of IRAK-4 by Trimethylamine Mediates

2 Metabolic and Immune Benefits in High-Fat-Diet-induced Insulin

3 Resistance

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28 Referenced abstract

29 **The interaction between high-fat diet (HFD) feeding and the gut microbiome has a strong**
 30 **impact on the onset of insulin resistance (IR)¹⁻³. In particular, bacterial lipopolysaccharides**
 31 **(LPS) and dietary fats trigger low-grade inflammation⁴ through activation of *Toll*-like**
 32 **receptor 4 (TLR4), a process called metabolic endotoxemia⁵. However, little is known**
 33 **about how the microbiome can mitigate this process. Here, we investigate longitudinal**
 34 **physiological and metabotypical responses of C57BL/6 mice to HFD feeding. A series of *in***
 35 ***vivo* experiments with choline supplementation, then blocking trimethylamine (TMA)**
 36 **production and administering TMA, demonstrate that this microbiome-associated**
 37 **metabolite decouples inflammation and IR from obesity in HFD. Through *in vitro* kinome**
 38 **screens and *in silico* molecular dynamics studies, we reveal TMA specifically inhibits**
 39 **Interleukin-1 Receptor-associated Kinase 4 (IRAK-4), a central kinase integrating signals**
 40 **from various TLRs and cytokine receptors. Consistent with this, genetic ablation and**
 41 **chemical inhibition of IRAK-4 result in similar metabolic and immune improvements in**
 42 **HFD. In summary, TMA appears as a key microbial effector inhibiting IRAK-4 and**
 43 **mediating metabolic and immune effects with benefits upon HFD. Thereby we highlight**
 44 **the critical contribution of the microbial signalling metabolome in homeostatic regulation**
 45 **of host disease and the emerging role of the kinome⁶ in microbial–mammalian chemical**
 46 **crosstalk.**

48 Main Text

49 IR is key to a cluster of conditions such as impaired glucose tolerance (IGT), obesity,
 50 hyperlipidemia, hypertension and non-alcoholic fatty liver disease (NAFLD), increasing the
 51 risk of developing type 2 diabetes and cardiovascular diseases⁷. One of the hallmarks of
 52 these disorders is the early development of a systemic low-grade inflammation⁴.
 53 Increasing evidence supports that complex communication occurs between the gut
 54 microbiota and the innate immune system⁸, with consequences on metabolic homeostasis⁹.
 55 Whilst some of the functional signalling molecules mediating microbial–mammalian
 56 chemical crosstalk have been characterized, a limited number of metabolite classes and
 57 their targets have been identified (G protein-coupled¹⁰ and nuclear¹¹ receptors). It is,
 58 however, hypothesized that microbial metabolites potentially affect other pharmacological
 59 target classes such as kinases⁶. In previous studies, we and others have identified a family of

microbiome-associated metabolites, methylamines, associated with IR, NAFLD¹² and atherosclerosis¹³, but their mechanisms of action on mammalian hosts remain unclear. TMA is one of the most abundant microbial metabolites produced by the gut microbiota. We first reported that TMA may be associated with IR¹². TMA results from microbial metabolism of dietary choline, carnitine and TMAO¹⁴⁻¹⁸ before being absorbed and *N*-oxidized into TMAO by hepatic flavin-containing mono-oxygenase 3 (FMO3)¹⁹. After initial reports associating TMAO with adverse cardiovascular outcomes^{13,16}, it has since emerged that Fmo3 inactivation was beneficial for several metabolic outcomes²⁰⁻²³, strongly suggesting that TMA and TMAO have distinct biological roles. Here, we present a new paradigm to decipher the microbiome–host kinome chemical crosstalk in IR: i) identification of gut-derived microbial metabolites associated with HFD-induced IGT and obesity, ii) systematic pharmacological target screening of discriminant microbial metabolites, iii) molecular modelling of the interaction, and iv) mechanistic validation of the pathophysiological relevance of pharmacological interactions with *in vivo* models, thereby unravelling novel mechanisms by which gut microbial TMA acts as an IRAK-4 inhibitor and directly improves the host’s immunometabolism.

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77 **Longitudinal study leading to the choline supplementation hypothesis.**

We initially carried out a longitudinal pathophysiological monitoring in a cohort of C57BL/6 mice (n=472) rapidly developing obesity and IGT when fed a 65% kCal HFD (**Suppl. Fig. 1a-c**). Liver transcriptomics identified 1,641 significant differentially expressed genes between chow-fed (CHD-) and HFD-fed mice (**Suppl. Fig. 1d,e** and **Suppl. Tab. 1**). Gene ontology and signalling pathway impact analyses (**Suppl. Fig. 2, Suppl. Tab. 2-4**) demonstrated upregulation of protein processing in the endoplasmic reticulum, whereas carbohydrate metabolism, circadian rhythm and AMPK signalling were significantly inhibited, consistent with existing literature^{24,25}. Surprisingly, inflammation-associated pathways were not differentially affected: expression of genes coding for acute phase serum amyloid A proteins *Saa1* and *Saa2* in response to acute inflammation and associated with type 2 diabetes was reduced whereas expression of pro-inflammatory cytokines such as *Il6* and *Il1β* was not significant (**Suppl. Fig. 1d** and **Suppl. Tab. 4**). This is contrasting with established knowledge as low-grade inflammation is one of the key features associated with IR^{4,5}. Metabolic profiling of the urinary metabolome of this mouse cohort (CHD and HFD, 4 time-points) by

¹H-NMR spectroscopy followed by multivariate modelling showed diet is the main factor influencing the metabolic profiles, followed by age (**Suppl. Fig. 1f**), the model's goodness-of-prediction parameters being highly significant upon permutation testing (**Suppl. Fig. 1g**). The metabolic signature of HFD-feeding compared to CHD was mainly associated with TMA excretion (**Suppl. Fig. 1h**), consistent with our previous reports using this diet^{12,26}. We used variance components analysis to decompose the contribution of diet and age to the excretion of these three metabolites, showing that the HFD contribution overwhelms the contribution of age (**Suppl. Fig. 1i**). Since the HFD contains >15 g of choline per kg of diet, this led us to hypothesize that choline supplementation may explain some of the metabolic and immune variation in response to the HFD.

Choline supplementation corrects HFD-induced inflammation and IR.

To investigate the effect of choline supplementation on glucose tolerance and IR, we initiated a series of *in vivo*, *in vitro* and *in silico* studies. We first fed C57BL/6 mice with a 65% kCal HFD containing 2 g/kg or 17 g/kg of choline (i.e., LC-HFD and HC-HFD, respectively; **Fig. 1**). Body weight (BW) was significantly increased in mice fed a LC-HFD from weaning until 5 months of age compared to mice fed a chow diet and remarkably compared to mice fed HC-HFD (**Fig. 1a**), which was not related to food intake (**Suppl. Fig. 3a**). To evaluate metabolic homeostasis, we performed glucose tolerance tests (GTT), which displayed a similar pattern with normalisation of cumulative glycemia on HC-HFD compared to LC-HFD (**Fig. 1b-c**). We then profiled circulating pro-inflammatory cytokines such as IL6, IL1 β , TNF α (**Fig. 1d-f**) and phosphorylation of NF- κ B regulating their transcription (**Suppl. Fig. 3b,c**), which was also suggestive of an improvement of HFD-induced inflammation by choline supplementation.

Targeted analysis of choline and methylamine pathways.

To document the effect of choline supplementation on choline-related metabolic pathways, we further refined our isotopic UPLC-MS/MS quantification^{27,28} to evaluate plasma concentrations of choline- and carnitine-derived metabolites leading to TMA and TMAO (**Suppl. Fig. 3d,e**). An O-PLS-DA model significantly segregates the three treatment groups at 5 months and highlights a choline supplementation effect on the first predictive component (**Fig. 1g,h**). Quantifications all reflect an increase in TMA and TMAO in the HC-HFD in line

with choline supplementation (**Fig. 1i,j**). In particular, the circulating TMA levels were similar in the CHD and the LC-HFD (0.38 μ M in CHD vs. 0.3 μ M in LC-HFD) which was about 20 times less than in the HC-HFD (5.9 μ M). These results depict an increased microbial conversion of choline into TMA in our HC-HFD, an observation already made in a previous study¹². These results suggest that TMA could mediate the metabolic and immune benefits of choline supplementation.

Baseline metabolic phenotyping after 2-month HFD feeding.

To further assess the impact of choline supplementation on metabolic homeostasis and low-grade inflammation in HFD contexts, we performed a second experiment, feeding C57BL/6 mice a 60% kcal HFD with a time-frame comparable to the ones used in subsequent figures. Weekly BWs and the GTT showed a similar trend after 8 weeks of HFD-feeding (**Fig. 2a-c**). Choline supplementation not only improved glucose tolerance (**Fig. 2b,c**) but also the Matsuda index (**Fig. 2d**), a surrogate marker for IR²⁹. The normalisation of insulin sensitivity was confirmed through insulin tolerance tests (ITT) and hepatic Akt phosphorylation assays (**Fig. 2e-h**). We further characterized that HFD feeding increased hepatic NF- κ B phosphorylation compared to CHD, which was normalised by choline supplementation (**Fig. 2i,j**), a pattern that was also observed for expression of acute-phase proteins *Saa1*, *Saa2* and *Saa3* (**Fig. 2k-m**).

Chemical blockage of bacterial TMA biosynthesis and loss of metabolic benefits.

To test whether the beneficial effects of choline supplementation are mediated by its microbial product TMA, we sought to block bacterial TMA production in HC-HFD-fed mice both non-specifically and specifically using, respectively, a wide-spectrum antibiotics cocktail and 3,3-dimethyl-1-butanol (DMB), which inhibits microbial TMA-lyase³⁰. We first confirmed the functional blockage of TMA production in mice fed a HC-HFD, resulting in a drastic drop in circulating and excreted TMA and TMAO following 1% DMB administration (**Suppl. Fig. 4a-d**). In accordance with our hypothesis, both antibiotics and DMB treatments abolished the effects of choline supplementation-induced improvements in HFD, in particular for glucose tolerance, insulin sensitivity (as suggested by the Matsuda index, **Fig. 3a-c**, **Suppl. Fig. 4e,f**) and hepatic insulin sensitivity as shown by the absence of beneficial effect of choline supplementation on insulin-induced Akt phosphorylation (**Fig. 3d-g**). The

metabolic improvements were not associated with change in BW gain (**Suppl. Fig. 4e**), thereby strongly suggesting that the effects on glucose metabolism induced by inhibiting bacterial TMA production in HC-HFD were decoupled from obesity.

TMA treatment mimics choline supplementation.

To further confirm whether TMA mediates the beneficial effects of choline supplementation, we chronically treated LC-HFD-fed C57BL/6 mice with TMA for 6 weeks using subcutaneous osmotic minipumps and assessed their immunometabolism. We confirmed that chronic TMA treatment at 0.01 mM in a LC-HFD did not affect BW gain (**Suppl. Fig. 4g**) but effectively normalised glucose homeostasis and insulin sensitivity (**Fig. 3h-l, Suppl. Fig. 4d**). TMA treatment also improved the pro-inflammatory response to HFD-feeding and hepatic expression of acute phase proteins *Saa1*, *Saa2* and *Saa3* (**Fig. 3m-p**). These results suggest that chronic TMA treatment decouples BW gain and adiposity from low-grade inflammation and glucose homeostasis, thereby mimicking the immune and metabolic benefits observed in choline supplementation.

TMA is a specific IRAK-4 inhibitor.

To identify a direct mechanism linking TMA to metabolic and immune benefits in HFD-fed mice, we sought to identify its host pharmacological targets. The kinome, made of 518 kinases encoded the human genome⁶, represents a repertoire of critical signal transduction switches for metabolic homeostasis and inflammation. To discover physical interactions, we screened TMA against a panel of 456 clinically-relevant human kinases using a high-throughput assay^{31,32} and identified five preliminary hits for TMA (**Fig. 4a** and **Supplementary Tab. 5**). We then generated multiple-dose binding curves between TMA and each hit and confirmed that only TMA specifically binds IRAK-4 (dissociation constant $K_d = 14$ nM, **Fig. 4b**) whereas TMA did not bind the other four preliminary hits (flat dissociation curves with no K_d fit in **Supplementary Fig. 5a-d**, meaning no physical binding was observed). Since K_d only addresses a physical interaction in its simplest form (*i.e.* binding), we functionally tested TMA as an IRAK-4 inhibitor, by quantifying IRAK-4 phosphorylation activity in presence of ATP and increasing doses of TMA to derive a half-maximal relative inhibitory constant ($IC_{50} = 3.4$ μ M, **Fig. 4c**). We also confirmed that choline, TMAO and DMB do not inhibit IRAK-4 (**Suppl. Fig. 5e-g**) and that TMA does not inhibit IRAK-1 either (**Suppl.**

Fig. 5h), as *Irak1*^{-/-} mice fed a HFD were recently shown to have metabolic improvements similar to TMA treatment³³. Molecular dynamics simulations of IRAK-4 were performed in the presence of 1% TMA. *In silico* analysis of thousands of binding and unbinding events suggests that in solution TMA binds to several small hydrophobic cavities in IRAK-4 and to the ATP binding site (**Fig. 4d** and **Suppl. Fig. 5i-j**).

Since IRAK-4 is a central kinase in the TLR pathway sensing bacterial invasion and promoting a pyretic pro-inflammatory response in infectious contexts^{34,35}, we sought to test its inhibition in a HFD context. The main free fatty acid (FFA) in our HFD is palmitate, a saturated FFA which triggers TLR4. We therefore developed a palmitate-activated Kupffer cell model of HFD-induced low-grade inflammation based on murine KUP5 cell line³⁶ (**Suppl. Fig. 4k**). Palmitate treatment in KUP5 cells significantly increased both IL-6 and TNFα secretions, which were improved by co-treatment with 0.1 mM TMA (**Fig. 4e,f**). Our results reveal that TMA is a specific IRAK-4 inhibitor blunting the palmitate-induced low-grade inflammation *in vitro*, requiring further assessment in *Irak4*^{-/-} mice.

***Irak4*^{-/-} mice are protected against HFD-induced immune and metabolic dysregulations.**

IRAK-4 is a key kinase required for defence against pyogenic infections in acute contexts^{34,35}. To test whether this kinase plays a role in HFD-induced chronic low-grade inflammation and glucose homeostasis, we fed 5-week-old *Irak4*^{-/-} mice³⁷ and wild-type (WT) littermates in C57BL/6 background a LC-HFD, to avoid potential confounding effects from TMA for 8 weeks before assessing circulating cytokines, expression of hepatic inflammatory genes and acute-phase markers and metabolic homeostasis (**Fig. 5a-h**). *Irak4*^{-/-} mice presented improved glycemic control compared to WT littermates (**Fig. 5a,b**). Likewise, the inflammatory response to LC-HFD observed in WT was obliterated in *Irak4*^{-/-} littermates (**Fig. 5c-g**). There was a similar trend for *Saa3* (**Fig. 5h**), whilst there was no effect on BW gains (**Supplementary Fig. 6a**). Hence, genetic ablation of IRAK-4 abolishes the HFD-induced pro-inflammatory response and IGT thereby decoupling obesity from impaired glucose tolerance and low-grade inflammation with a similar phenotype to *Irak1* deficiency³³.

Pharmacological inhibition of *Irak4* normalises metabolism.

Since the *Irak4*^{-/-} mice lack the whole protein, we compared the knock-out phenotype with the phenotype of PF06650833, a recently discovered chemical inhibitor of the human IRAK4

protein³⁸ currently being assessed in a clinical trial (NCT02996500). Treatment with PF06650833 improved BW in LC-HFD mice (**Suppl. Fig. 6b**). This inhibitor also yielded significant improvements in plasma glycemia at the latter time-points of the GTT and ITT and in cumulative glycemia (**Fig. 5i-l**), which was mirrored by an increase in Akt phosphorylation (**Fig. 5m-n**). These results collectively show that specific chemical inhibition of IRAK-4 leads to significant improvements in glycemic control, insulin sensitivity and insulin signalling. Further, they suggest that IRAK-4 could constitute a clinically relevant target in IR and related disorders.

DISCUSSION

The discovery that TMA is a kinase inhibitor controlling IRAK-4, a central kinase involved in innate immunity, is a major finding which provides an attractive mechanism for the metabolic and immune improvements observed with choline-supplementation in HFD contexts (**Fig. 6**). IRAK-4 is the first regulatory checkpoint after MyD88, the adapter protein connecting IRAK-4 to at least six TLRs sensing bacterial compounds³⁹. IRAK-4 deficiency is associated with bacterial infections in humans³⁵ and in mice⁴⁰. Consistent with the involvement of the TLR signalling pathway at the crossroads between gut microbiota and dietary lipids^{41,42}, IRAK-4 deletion and its inhibition by TMA and PF06650833³⁸ rescued HFD-induced low-grade inflammation and IR^{4,43,44}, highlighting new unexpected roles for this microbial metabolite and its target kinase in immunometabolism. The relative IC₅₀ (3.4 µM) we determined is two times smaller than the plasma isotopic quantifications obtained by UPLC-MS/MS in the HFD group supplemented with choline. These quantifications are comparable to circulating TMA levels previously reported in normal human plasma, ranging between 0.42 and 48 µM^{45,46}, which makes this IC₅₀ particularly relevant with regard to pathophysiological mechanisms: dosing mice with 0.01 mM TMA (3x IC₅₀) was sufficient to improve inflammatory and metabolic responses. TMA can therefore be considered a “microbial signalling metabolite”⁴⁷ sending a negative feedback signal to a pathway triggered by influx of saturated free fatty acids in HFD contexts, this mechanism participating in maintaining a low immunological footprint and metabolic improvements in a symbiotic relationship. TMA’s role as an IRAK-4 inhibitor could for instance explain some of the beneficial effects reported for choline

supplementation in NAFLD patients⁴⁸. Altogether, our results on IRAK-4 inhibition and ablation provide further insight in the phenotypic convergence between *Myd88*, *Irak4* and *Irak1* knock-out mouse models^{33,49}, suggesting that the gut microbiota, through TMA, proceeds with a targeted hijacking of the TLR signal transduction machinery to the host's benefit resulting in metabolic and immune improvements.

In summary, through the combination of *in silico*, *ex vivo*, *in vitro* and *in vivo* approaches, we reveal a unique mechanism in which TMA acts as a gut microbial signalling metabolite inhibiting IRAK-4, a central molecular target in the TLR pathway, thereby allowing the gut microbiota to control HFD-induced pro-inflammatory response and IR. The kinome represents a key repertoire of regulatory host targets for microbial signals and the uncharted microbiome–kinome crosstalk requires further investigation. By highlighting the physiological and therapeutic roles of TMA and IRAK-4 on HFD-induced low-grade inflammation and IR, we anticipate that its immunomodulatory properties extend beyond IR to a wider range of human pathologies involving TLR signalling and modulation of innate immunity.

Author Contributions

JC and SG performed cell-based assays, JC performed multivariate statistics and interpreted the results. JC, FB, JFF, AE, HP, LZ, DS, ARR, PDC, SC and CG contributed *in vivo* experiments, RHB, AM, ALN, LMG, SG, CLB and SP performed various experiments. JEF and RCG designed and performed *in silico* experiments. LH analysed microarray data. JS, DG, JKN, PPL, PDC, NJG and MED conceived the project, mentored and supervised its participants and interpreted its results. MED performed metabolic profiling experiments, analysed data and interpreted the results. JC, NJG, DG, PPL, PDC, and MED wrote the manuscript. All authors edited the manuscript.

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Online Content.

Methods, along with any additional Supplementary display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Online Methods

Protocols

All experimental procedures involving mice were carried out in accordance with U.K. Home Office, Canadian Council on Animal Care, the ethics committee of the French Research Ministry (authorization number 00486.01), Belgian Law of May 29, 2013 regarding the protection of laboratory animals (agreement number LA1230314) and local guidelines on animal welfare and license conditions and the University of Oxford, University of Ottawa, Université Pierre et Marie Curie and Université Catholique de Louvain guidelines on animal welfare.

Cell-based assays.

C-myc-immortalised KUP5 cells, originally harvested from a C57BL/6 mouse, were purchased from RIKEN BioResource Center, which certified the cells were not contaminated by *Mycoplasma*. They were cultured in splitting medium at 37°C and 5% CO₂ atmosphere under sterile conditions. Cells were split every 48-72 h using splitting medium (DMEM GlutaMAX-I supplemented with 10% FBS, 1% penicillin and streptomycin, 0.1% insulin and 0.044% thioglycerol), maintained at a density between 5 x 10⁵ – 1 x 10⁶ cells/mL and used between passages 2 and 20. For palmitate (PA) treatment, 125,000 cells/cm² were seeded in treatment medium (DMEM GlutaMAX-I supplemented with 5% FBS, 1% penicillin and streptomycin, 0.1% insulin and 0.044% thioglycerol) on a 48-well plate with a working volume of 250 µL and incubated at 37°C and 5% CO₂ atmosphere overnight (16-18 h). 0.5 M PA stock solution was prepared in 100% ethanol and 0.5 mM PA treatment solution was prepared in treatment medium containing a final concentration of 0.5% low-endotoxin-fatty acid free BSA. TMA (0.1 mM) treatment was performed in a simultaneous fashion with PA. All solutions containing PA were prepared in borosilicate glass tubes. Upon overnight incubation of seeded cells, the medium was replaced with the same volume of treatment solutions and incubated at 37°C and 5% CO₂ atmosphere for 24 h before measurement of cytokines in the medium.

Mouse Models.

Longitudinal HFD-feeding in mice. All experiments were approved by the ethical committee of the University of Oxford. Male mice from C57BL/6J inbred strain were bred in our animal

facility by using a stock originating from The Jackson Laboratory. At 5 weeks of age, groups of n=8-10 mice were transferred to a 40 %w/w high fat diet (65% kcal) (Special Diets Services), containing 32 % lard and 8 % corn oil, whereas control groups remained on a 5 % Low Fat Diet (B & K Universal) for up to 6 months. Detailed diet formulations were published previously¹² and summarised in **Supplementary Table 6**.

Mice were housed under a 12 h–12 h light–dark cycle. For physiological profiling, several mouse groups fed CHD or HFD were tested to assess consistency of results and discard any impact of potential batch effects. Intra peritoneal glucose tolerance tests (ipGTT) were performed in mice after 2-, 3-, 5-and 7-month-old mice after an overnight fast, as previously published⁵⁰ (see also metabolic phenotyping below). Four days after the GTT, 24 h urinary samples (9 a.m. to 9 a.m.) were collected from mice maintained in individual metabolic cages. Urinary samples collected in a solution of 1 % (wt/vol) sodium azide were centrifuged to remove solid particles and kept at -80°C until assayed. After an overnight fast, mice were killed by exsanguination. Plasma was separated by centrifugation and stored at -80 °C until ¹H-NMR analysis.

Choline supplementation on HFD. At 5 weeks of age, mice were fed either a chow diet containing 2g of choline/kg of diet (Research Diets, D12450J), a low-choline HFD containing 2g of choline/kg of diet (Research diets, D12492), a high-choline HFD containing 17 g of choline/kg of diet (Research diets, D16100401), a HC-HFD containing 1% of DMB or a HC-HFD combined with a cocktail of antibiotics (0.5 g/L vancomycin hydrochloride, 1 g/L neomycin trisulfate, 1 g/L metronidazole, 1 g/L ampicillin sodium) in drinking bottles (n=6-10 per group) for 8 weeks (see diet formulations in **Supplementary Table 6**). Mice then were killed by decapitation and organ were dissected and weighed.

Irak4^{-/-} mice. *Irak4^{-/-}* mice on C57BL/6J background as already described³⁷ were bred with C57BL/6J female mice (The Jackson Laboratory), and the F1 offspring were subsequently bred to produce *Irak4^{-/-}* mice and WT littermate used for this study. After eight weeks of HFD-feeding, mice were killed by decapitation and organ were dissected and weighed.

Chronic TMA and PF06650833 treatment in LC-HFD-fed mice. Five-week-old C57BL/6J mice (Charles River) were housed a week before experiment in a controlled environment. Mice were housed under a 12 h–12 h light–dark cycle. At day 0, the 10-week-old mice were anaesthetised with isoflurane (ForeneH, Abbott). Mini-osmotic pumps were implanted subcutaneously (Model 2006, Alzet) (flow rate: 0.15 mL/h, total filling volume: 200 mL, delivery duration: 42 days) as previously described⁵¹. The osmotic mini-pump contains either vehicle or TMA (0.1 mM in circulation) or PF06650833 (50 nM in circulation). After six weeks of metabolite treatment, mice were killed by decapitation and organ were dissected and weighed.

Physiological phenotyping

After four weeks of treatment an intra-peritoneal glucose tolerance test (2g/kg) was performed in conscious mice following an overnight fast. Blood was collected from the tail vein before glucose injection and 30, 60, 90 and 120 minutes afterwards. Blood glucose levels were determined using an Accu-Check® Performa (Roche Diagnostics, Meylan, France). Additional blood samples were collected at baseline and 30 minutes after glucose injection in Microvette® CB 300 Lithium Heparin (Sarstedt, Marnay, France). Plasma was separated by centrifugation and stored at -80°C until insulin radioimmunoassay. Circulating insulin levels were determined using Insulin ELISA kits (Mercodia, Uppsala, Sweden). The Matsuda insulin sensitivity index was calculated as previously published²⁹. After five weeks, we performed an insulin tolerance test. 5 hour-fasted mice were injected intraperitoneally with insulin (0.75 mU/g, Actrapid, Novo Nordisk). Blood glucose levels were measured immediately before and 15, 30, 45, 60, 90 and 120 min after insulin injection with a standard glucose meter (Accu Check, Roche, Basel, Switzerland) on the tip of the tail vein.

Gene expression.

Groups of 6 mice showing consistent pathophysiological profile in response to CHD or HFD treatment were selected for microarray analysis performed as previously described⁵² and data were deposited in array express with accession number: E-MEXP-1755. The Bioconductor⁵³ package Limma⁵⁴ was used to generate the list of differentially expressed

genes. Gene ontology was implemented using Enrichr⁵⁵ and signalling pathway impact analysis using SPIA⁵⁶. For qPCR analysis, total RNA was prepared from tissues using TriPure reagent (Roche). Quantification and integrity analysis of total RNA were performed by analysing 1 µL of each sample in an Agilent 2100 Bioanalyzer (Agilent RNA 6000 Nano Kit, Agilent). cDNA was prepared by reverse transcription of 1 mg total RNA using a Reverse Transcription System kit (Promega). Real-time PCR was performed with the StepOnePlus real-time PCR system and software (Applied Biosystems) using Mesa Fast qPCR (Eurogentec) for detection according to the manufacturer's instructions. RPL19 RNA was chosen as the housekeeping gene. All samples were performed in duplicate in a single 96-well reaction plate and data were analysed according to the 2⁻DDCT method. The identity and purity of the amplified product were assessed by melting curve analysis at the end of amplification. The primer sequences for the targeted mouse genes are: SAA1 forward CAT-TTG-TTC-ACG-AGG-CTT-TCC, SAA1 reverse GTT-TTT-CCA-GTT-AGC-TTC-CTT-CAT-GT, SAA2 forward GGG-GTC-TGG-GCT-TCC-CAT-CT, SAA2 reverse CCA-TTC-TGA-AAC-CCT-TGT-GG, SAA3 forward CGC-AGC-ACG-AGC-AGG-AT, SAA3 reverse CCA-GGA-TCA-AGA-TGC-AAA-GAA-TG. Q-RT-PCR assays were performed in a single batch with the personnel blind to treatment groups.

Circulating cytokine quantification.

Circulating cytokines were quantified using MSD V-PLEX Plus Proinflammatory Panel 1 kit. Plasma samples were diluted 2 times in diluent 41 provided and experiment was processed as request by manufacturer and read on a SECTOR imager 2400. Cytokine assays were performed in a single batch with the personnel blind to treatment groups.

Western-blotting.

Western blot analyses were performed as described previously²⁸. To analyze the insulin signaling pathway, mice were allocated to either a saline-injected subgroup or an insulin-injected subgroup so that both subgroups were matched in terms of BW and fat mass. They then received 1 mU insulin/g BW (Actrapid; Novo Nordisk A/S, Denmark) or an equal volume of saline solution. Three minutes after injection, mice were killed and liver was harvested. 30mg of liver were homogenized in 680 µL of RIPA buffer containing a cocktail of protease and phosphatase inhibitors. The homogenate was then centrifuged at 12,000G for 20 min at

4 °C. Equal amounts of proteins were separated by SDS–PAGE and transferred to nitrocellulose membranes. Membranes were incubated overnight at 4 °C with antibodies diluted in Tris-buffered saline Tween-20 containing 1% bovine serum albumin: p-Akt Ser473 (1:1,000; #4060, Cell Signaling), Total Akt (1:1000 ,#9272S, Cell Signalling), p-NF-κB (1:3000, #ab86299, AbCam), Total NF- κB p65 (1:3000, #8242, Cell Signalling).

¹H-NMR spectroscopy and multivariate statistics.

Mouse urine and plasma samples were prepared and measured on a spectrometer (Bruker) operating at 600.22 MHz ¹H frequency; full resolution ¹H NMR spectra were then processed and analysed using orthogonal partial least square discriminant analysis as described previously¹². Variance component analysis was performed as described previously⁵⁷. ¹H NMR profiling was performed in a single batch with the personnel blind to treatment groups. The O-PLS-DA model was validated using 10,000 random permutations of the original class membership variable to explain (i.e. diets, treatments or genotypes), as described previously⁵⁷.

Plasma methylamine quantification by UPLC-MS/MS. Methylamines were quantified as previously described^{27,28}. Plasma samples (20 µL) were spiked with 10 µL Internal Standard (IS) solution (¹³C₃/¹⁵N-TMA, d₉-TMAO, d₄-choline, d₃-carnitine and d₉-betaine in water; 1 mg/L) and 45 µL of ethyl 2-bromoacetate solution (15 g/L ethyl 2-bromoacetate, 1% NH₄OH in acetonitrile) were added to derivatize methylamines (TMA and ¹³C₃/¹⁵N-TMA) to their ethoxy- analogues, completed after 30 min at room temperature. 935 µL of protein/lipid precipitation solution (94% acetonitrile/5% water/1% formic acid) was added; samples were centrifuged for 20 min (4°C, 20,000g) and were transferred to UPLC-autosampler vials. Sample injections (10 µL loop) were performed on a Waters Acquity UPLC-Xevo TQ-S UPLC-MS/MS system equipped with an Acquity BEH HILIC (2.1 × 100 mm, 1.7 µm) chromatographic column. An isocratic elution was applied with 10 mM ammonium formate in 95:5 (v/v) acetonitrile:water for 14min at 500 µL/min and 50 °C. Positive electrospray (ESI+) was used as ionization source and mass spectrometer parameters were set as follows: capillary, cone and source offset voltages at 500, 93 and 50 V, respectively, desolvation temperature at 600°C, desolvation/cone/nebulizer gases were high-purity nitrogen at 1000 L/hr, 150 L/hr and 7 bar respectively. Collision gas was high-purity argon. Mass

spectrometer was operated in multiple reactions monitoring (MRM) mode. The monitored transitions were the following: for derivatized-TMA, +146 → +118/59 *m/z* (23/27 V); for derivatised-¹³C₃/¹⁵N-TMA, +150 → +63 (27V); for TMAO, +76 → +59/58 *m/z* (12/13 V); for d₉-TMAO, +85 → +68 *m/z* (18 V); for choline, +104 → +60/45 *m/z* (14/16 V); for d₄-choline, +108 → +60 *m/z* (15 V); for γ-butyrobetaine, +146 → +60/87 *m/z* (12/12 V); for carnitine, +162 → +103/60 *m/z* (16/14 V); for d₃-carnitine, +165 → +103 *m/z* (16 V); for betaine, +118 → +59/58 *m/z* (16/16 V); for d₉-betaine, +127 → +68 *m/z* (16 V).

Kinome screen, *K_d* and IC₅₀ determination.

TMA was assessed using KdELECT screening service (DiscoverX) as described previously^{31,32}. This technique is based on a competition-binding assay that quantitatively measures the ability of a compound to compete with an immobilized, active site-directed ligand. The assay consists of DNA-tagged kinase, immobilized ligand, and the potent inhibitor. The ability of TMA to compete with the immobilized ligand was measured by quantitative PCR of the DNA tag. The binding constant (*K_d*) was then calculated from duplicate 11-point dose-response curve. Kinase interaction tree plots were generated using TREEspot™ Software Tool and reprinted with permission from KINOMEScan®, a division of DiscoverX Corporation, © Discoverx corporation 2015. The TMA IC₅₀ on IRAK-4 was determined using Kinexus kinase-inhibitor activity profiling service (Kinexus, Vancouver, Canada). The technique is based on the direct quantification of radiolabelled phosphate from ATP onto a protein substrate of a target protein kinase.

Molecular dynamics simulations.

Molecular dynamics simulations were performed within the Amber package⁵⁸ based on an X-ray structure of human IRAK-4⁵⁹. After removal of the bound ligand and sulfate ions, the system was prepared using protonate3d⁶⁰. Protein atoms were described using the Amber fore field 99SB-ILDN⁶¹, ligand TMA was described within the Generalized Amber Force Field⁶² after fitting partial charges using RESP at the Hartree-Fock/6-31G* level (see Supplementary 2e for resulting parameters). A periodic truncated octahedral shell of TIP3P water molecules was added⁶³ 10Å to our systems. 1% of approximately 10,000 water molecules with a minimum distance of 4Å to the protein were randomly replaced by TMA to study binding. After applying an elaborate equilibration protocol⁶⁴, we sampled each system

without restraints for 100 ns using the GPU implementation of pmemd⁵⁸. Analyses of simulation data were performed using ptraj and cpptraj from AmberTools⁶⁵. After performing standard stability checks for energy and overall structure, hydrogen bonding between protein and ligands was counted using cpptraj's default criteria. All protein–ligand contacts within a distance cutoff of 5Å were considered for contact analysis. Grid-based analyses of TMA positions were performed on a grid capturing the entire simulation box with a spacing of 0.5Å. The resulting grids were overlaid with an ATP-bound structure of IRAK-4 (PDB: 2OID)⁶⁶.

Reagents.

Glutamine (Glutamax, 35050061, Life Technologies), Fetal bovine serum (Life Technologies), crystal violet (C6158) and Trimethylamine (W324108) were from Sigma-Aldrich. Mouse IL-6 Quantikine ELISA kits (M6000B, R&D system), RNeasy Micro Kit (Qiagen). SuperScript II Reverse Transcriptase, IL6 Taqman probe Hs00174131_m1 and FAST master mix (Invitrogen). High Fat Diet (Special Diets Services) and Low Fat Diet (B & K Universal) were specifically formulated in¹². Control Diet (D12450K; Research diet), LC-HFD (60% kcal fat and 20% kcal carbohydrates, D12492, Research diet), HC-HFD (60% kcal fat and 20% kcal carbohydrates with 17g of choline/kg, D16100401i, Research diet). Mouse Insulin ELISA (10-1249-01, Mercodia). Isoflurane (10014451, Forene, Abbott). TriPure reagent (1667165, Roche). Reverse Transcription System kit (A3500, Promega). Mesa Fast qPCR (CS-CKIT-PROD, Eurogentec). MSD V-PLEX Plus Proinflammatory Panel 1 kit (K15048G Meso Scale Diagnostics).

Statistics.

Potential outliers were identified by a Grubbs test. For statistical comparisons between study groups, normality was tested using D'agostino-Pearson omnibus normality test, then one-way ANOVA was used, followed by Tukey's *post hoc* testing when data were normally distributed, otherwise groups were compared using the two-tailed Mann-Whitney test ($P < 0.05$ considered to be statistically significant). Data are displayed as mean $\pm$ s.e.m in all figures. All cell culture experiments included at least three biological replicates (as indicated in figure legends). All animal cohorts included at least five animals in each study group (as indicated in figure legends) and animals were randomized to treatment groups.

Figure Legends.

Figure 1. Choline supplementation improves glucose homeostasis and inflammation after 5 months of HFD.

5-week-old C57BL/6J mice were fed a CHD diet, a low-choline HFD or a low-choline HFD supplemented with choline (n=10) and were phenotyped after 5 months. **(a)** Body weight (BW) measured after 5 months of diet. **(b)** Plasma glucose concentration during an ipGTT. **(c)** Area under the curve of the plasma glucose concentration during an ipGTT. **(d-f)** Quantification of circulating cytokines: IL6 **(d)**, TNF α **(e)** and IL1 β **(f)**. **(g)** Scores plot from a cross-validated O-PLS-DA model segregating the three groups of mice according to diet based on isotopic quantification of methylamines by UPLC-MS/MS. **(h)** Empirical assessment of the significance of O-PLS goodness-of-fit parameters by generating a null distribution with 10,000 random permutations. **(i-j)** Quantification of circulating TMA **(i)** and TMAO **(j)** by UPLC-MS/MS. Data are means $\pm$ s.e.m. One-way ANOVA followed by Tukey's *post hoc* tests (superscript letters for factor levels $P < 0.05$) on log-transformed data except **(g-h)**.

Figure 2. Choline supplementation corrects HFD adverse effects on glucose homeostasis, insulin sensitivity and inflammation after 8 weeks of diet.

5-week-old C57BL/6J mice were fed a CHD diet, a low-choline HFD or a low-choline HFD supplemented with choline (n = 10) and were phenotyped after 8 weeks. **(a)** Body weight measured periodically during 8 weeks of diet. **(b)** Plasma glucose concentration during an ipGTT. **(c)** Area under the curve of the plasma glucose concentration during an ipGTT. **(d)** Matsuda insulin sensitivity index calculated from the ipGTT. **(e)** Plasma glucose concentration during an ipITT. **(f)** Area under the curve of the plasma glucose concentration during an ipITT. **(g-j)** Western-Blot analysis of liver Akt phosphorylation **(g-h)** and NF-kB phosphorylation **(i-j)**. **(k-m)** expression of hepatic acute phase inflammation proteins markers *Saa1* **(k)**, *Saa2* **(l)** and *Saa3* **(m)**. Data are means $\pm$ s.e.m. **(c, d, f, h-m)** One-way ANOVA followed by Tukey's *post hoc* tests (superscript letters for factor levels $P < 0.05$) on log-transformed data. **(a, b, e)** Non-parametric two-sided Mann-Whitney test was used for each timepoint, significance comparison signs are as follow: *=CHD vs LC-HFD, \$=LC-HFD vs HC-HFD and #=CHD vs HC-HFD.

Figure 3. Blocking TMA production from choline cancels metabolic benefits from choline supplementation and chronic TMA chronic treatment mimics it.

(a-g) 5-weeks-old C57BL/6J mice were fed a low-choline HFD or a low-choline HFD supplemented with choline (n = 10). Two strategies were used in parallel to block TMA production from choline by the gut microbiota using either 1% DMB in the diet or an antibiotic treatment. **(a)** Plasma glucose concentration during an ipGTT. **(b)** Area under the curve of the plasma glucose concentration during an ipGTT. **(c)** Matsuda insulin sensitivity index calculated from the ipGTT **(d)** Plasma glucose concentration during an ipITT. **(e)** Area under the curve of the plasma glucose concentration during an ipITT. **(f-g)** Western-blot analysis of liver Akt phosphorylation. **(h-p)** Mice were weaned at 3 weeks and fed a low-choline HFD before being fitted with osmotic minipumps delivering a chronic circulating dose of TMA (0.1 mM) for 6 weeks (n = 9 saline HFD, n = 8 TMA HFD). **(h)** Plasma glucose concentration during an ipGTT. **(i)** Area under the curve of the plasma glucose concentration during an ipGTT. **(j)** Matsuda insulin sensitivity index calculated from the ipGTT. **(k)** Plasma glucose concentration during an ipITT. **(l)** Area under the curve of the plasma glucose concentration during an ipITT. **(m)** Quantification of circulating IL6. **(n-p)** Expression of hepatic acute-phase inflammation protein markers *Saa1* **(n)**, *Saa2* **(o)** and *Saa3* **(p)**. Data are means ± s.e.m. Results were assessed by one-way analysis of variance followed by Tukey's *post hoc* tests (superscript letters for factor levels $P < 0.05$) on log-transformed data.

Figure 4. TMA inhibits IRAK-4 and rescues TLR4-mediated pro-inflammatory response.

(a) Kinome screen for a single dose of TMA (0.1 mM) against 456 kinases. **(b)** Confirmation of a physical interaction between TMA and IRAK-4 using TMA at concentrations ranging from 0.1 nM to 100 μ M, resulting in a dissociation constant (K_d) of 14 nM. **(c)** Functional characterization of the inhibition of IRAK-4 by TMA. IRAK-4 phosphorylation activity was determined in presence of various concentration of TMA and resulted in an IC_{50} of 3.4 μ M. **(d)** Preferred binding sites of TMA from molecular dynamic simulations of IRAK-4 in presence of 1% TMA. Blue grid points indicate regions with highest TMA occupancy, thereby identifying several small cavities and the ATP pocket as potential binding sites. **(e-f)** Assessment of the effect of 100 μ M of TMA treatment on KUP5 Kupffer cells activated with 0.5 mM of palmitate. **(e-f)** IL6 and TNF α secretion in cell medium (n=10 biological replicates). The kinases covered by the kinome scan assay are visualised using a

phylogenetic layout. Preliminary positive hits with potential binding >35 % vs control (DMSO) are represented by a red dot whilst non-binding kinases are represented by a green dot. Kinase group names: TK, Tyrosine Kinases; TKL, Tyrosine Kinase-like; STE, STE kinase group; CK1, Cell Kinase 1; AGC, Protein kinase A, G and C families; CAMK, Calcium and Calmodulin-regulated kinases; CMGC, CMGC kinase group. Data are means $\pm$ s.e.m. One-way analysis of variance followed by Tukey's *post hoc* tests (superscript letters for factor levels $P < 0.05$) on log-transformed data.

Figure 5. IRAK-4 deficiency corrects IGT and pro-inflammatory response after 8 weeks of low choline HFD feeding in C57BL/6 mice and a chemical inhibitor of IRAK-4 mimics the effects on glucose homeostasis. (a-g) 5-week-old *Irak4*^{-/-} mice and WT littermates were fed a low-choline HFD (10 WT HFD and 8 *Irak4*^{-/-} HFD) and were phenotyped after 8 weeks. (a) Plasma glucose concentration during an ipGTT. (b) Area under the curve of the plasma glucose concentration during an ipGTT. (c-e) Circulating cytokines (c) IL6, (d) TNF α , (e) IL1 β . (f-h) Liver mRNA expressions for *Saa1* (f), *Saa2* (g) and *Saa3* (h) genes. (i-n) Mice were weaned at 3 weeks and fed a low-choline HFD before being fitted with osmotic minipumps delivering a chronic circulating dose of PF06650833 (50 nM) for 6 weeks. (i) Plasma glucose concentration during an ipGTT. (j) Area under the curve of the plasma glucose concentration during an ipGTT. (k) Plasma glucose concentration during an ipITT. (l) Area under the curve of the plasma glucose concentration during an ipITT. (m-n) Western-blot analysis of liver Akt phosphorylation. Data are means $\pm$ s.e.m. Student's T-test ($P < 0.05$) on log-transformed data.

Fig. 6. Overview of effects of IRAK-4 inhibition by TMA on metabolic response to HFD. TMA, which is synthesized from dietary choline (1) by the gut microbiota (2) specifically inhibits IRAK-4 activity (3). This inhibition blunts the TLR signalling pathway (induced by LPS and saturated free fatty acids) leading to an improvement of metabolic response to HFD (e.g. glucose homeostasis).

Supplementary Figure Legends

Supplementary Figure 1. C57BL/6 mice fed HFD display low acute-phase gene expression

in liver paralleled by high TMA excretion. Male mice were weaned at 3 weeks and fed a CHD diet, before being randomly assigned to CHD group (n=270) or HFD (n=202) groups and monitored at 2 (72 CHD and 64 HFD), 3 (84 CHD and 57 HFD), 5 (68 CHD and 47 HFD) and 7 months (46 CHD and 34 HFD). (a-c) Effect of HFD-feeding on physiological parameters. (a) Body weight (BW), (b) glucose tolerance test (GTT) at 5 months and (c) cumulative glycemia during GTT (AUC). (d-e) Effect of HFD-feeding on liver transcriptome at 5 months. (d) Volcano plot of differentially expressed genes, highlighting that *Saa1* and *Saa2* are down-regulated in HFD. Red dots, significantly (FDR < 0.1) differentially expressed genes. (e) Heatmap of significantly (FDR < 0.1) differentially expressed genes. (f-i) Effect of HFD-feeding and age on urinary metabolic phenotypes analyzed by ¹H-NMR spectroscopy. (f) ¹H-NMR-based metabotyping scores plot from a cross-validated O-PLS-DA model segregating the eight groups of mice according to both diet and age. (g) Empirical assessment of the significance of O-PLS goodness-of-fit parameters by generating a null distribution with 10,000 random permutations. (h) Metabolic signature of diet on O-PLS model coefficient plot; line plot corresponds to covariance and the color-scale represents proportion of variance explained. (i) Assessment of the contribution of factors diet and age on choline, TMA and TMAO by O-PLS variance components analysis. Data are means ± s.e.m. Two-sided Mann-Whitney test (*P < 0.05 vs control). For the O-PLS-DA permutation test, the horizontal axis corresponds to the correlation between the original class membership variable (single dots on the right) and the randomly permuted class membership vectors (no longer correlated with the original class membership) (swarm of permuted models on the left). The y axis corresponds to goodness-of-fit parameter R² (in green) derived for each O-PLS-DA model and the goodness-of-prediction parameter Q² (in blue) derived by 7-fold cross-validation of each O-PLS-DA model. The R² and Q² parameters obtained from the original model in the top right corner are outside the confidence interval of the 10,000 randomly permuted models and therefore confirming the significance of the fitness and prediction capacity of the original O-PLS-DA model.

Supplementary Fig. 2. Pathway analysis for hepatic significant genes. (a) Gene ontology analysis for down- and up-regulated HFD-responsive genes, (b) Signaling pathway impact analysis for HFD-responsive genes. Pathways respectively on the right-hand side of the red and blue curves are significant after Bonferroni correction of the global p-values (pG) obtained by combining the pPERT and pNDE using the normal inversion method, and significant after a FDR correction of pG. Significant KEGG pathways: 04141, Protein processing in the endoplasmic reticulum; 04710, Circadian rhythm; 04152, AMPK signaling pathway.

Supplementary Fig. 3. Choline supplementation corrects HFD adverse effects and associated methylamine measurements. (a) Food intake measured on the three tested diets (CHD, LC-HFD and HC-HFD). (b-c) Western blot analysis of liver NF-kB phosphorylation state between LC-HFD and HC-HFD. (d) Plasma sample chromatogram for the simultaneous detection of TMA, TMAO, choline, γ -butyrobetaine, carnitine, betaine and their isotopically labelled analogues. (e) Principal Component Analysis Biplot of plasma methylamine quantifications by UPLC/MS-MS from mice fed CHD, LC-HFD and HC-HFD. Data are means $\pm$ s.e.m. Two-sided Mann-Whitney test (* $P < 0.05$ vs control).

Supplementary Fig. 4. Modulation of TMA production and effects. (a-d) Confirmation of blockage of production of TMA (a, c) and TMAO (b, d) by 1% DMB measured in plasma (a-b) and urine (c-d). (e) Body weight monitoring during the whole experiment of mice fed a LC-HFD and a HC-HFD supplemented or not with DMB or treated with antibiotics. (f) Plasma insulin concentration during GTT at 0, 30 and 120 min post glucose injection. (g) Body weight monitoring during the whole experiment of mice fed a LC-HFD and chronically treated with TMA. (h) Plasma insulin concentration during GTT at 0, 30 and 120 min post glucose injection. Data are means $\pm$ s.e.m. Two-sided Mann-Whitney test (* $P < 0.05$ vs LC-HFD control).

Supplementary Fig. 5. TMA does not bind with kinases ANKK1, HIPK2, JAK2, and NDR1 identified as candidates in the kinome screen, but makes significant contact with IRAK-4. (a-d) Physical interaction test between TMA and the four hits identified using a kinome screen, using TMA concentrations ranging from 0.1 nM to 100 μ M for (a) ANKK1, (b) HIPK2,

(c) JAK2 and (d) NDR1. (e-g) IRAK-4 activity is not inhibited by choline (e), TMAO (f) or DMB (g), while TMA does not have any inhibitory effect on IRAK-1 (h). (i-j) Molecular dynamic parameters and outputs (i) force-field parameters and (j) number of protein-ligand H bonds and contacts with 1% of TMA. (k) Optimisation of palmitate dose on IL6 secretion by KUP5 cells. Data are means $\pm$ s.e.m. One-way ANOVA followed by Tukey's *post hoc* tests (superscript letters for factor levels $P < 0.05$) on log-transformed data.

Supplementary Fig. 6. IRAK-4 knock-out does not affect body weight while chronic treatment with PF06650833 reduces it in mice fed a low-choline HFD. (a-b) Body weight monitoring during the whole experiment of *Irak4*^{-/-} mice (a) or PF06650833-treated mice (b) fed a LC-HFD.

Supplementary Tab. 1: Hepatic genes differentially expressed by HFD-feeding. Hepatic transcriptomes were analysed using Limma⁵⁴ and genes are considered significant if Fdr < 0.1.

Supplementary Tab. 2: Down-regulated HFD-responsive hepatic pathways. Gene ontology analysis was performed using Enrichr⁵⁵ and pathways are considered significant if Fdr < 0.1.

Supplementary Tab. 3: Up-regulated HFD-responsive hepatic genes. Gene ontology analysis was performed using Enrichr⁵⁵ and pathways are considered significant if Fdr < 0.1.

Supplementary Tab. 4: Hepatic signalling pathways impacted by HFD. Signalling pathway impact analysis was performed using SPIA⁵⁶ and pathways are considered significant if pGFWER < 0.1.

Supplementary Tab. 5. TMA kinome screen. Screening results for single-dose TMA binding to 456 kinases. "Percent control" corresponds to the percentage of DNA amplified by qPCR compared to the control condition. Values below 35 % (in green) are considered as positive hits.

825 **Supplementary Tab. 6. Diet formulations.** Sepcific fat, protein, carbohydrate and choline
 826 content in regimens formulated by Research Diet are summarized based on %w/w and
 827 %kcal.

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SIGNIFICANTLY DOWNREGULATED GENES

Biological Process

Cellular Component

Molecular Function

SIGNIFICANTLY UPREGULATED GENES

Biological Process

Cellular Component

Molecular Function

b

SPIA two-way evidence plot

S6

1

Choline-rich
food

Bacterial
cell wall
components

Flagellin
LPS
Saturated FAs

Liver cells

3

2

4

Improved metabolic response
to HFD