Multi-omic analysis in transgenic mice implicates omega-6/omega-3 fatty acid imbalance as a risk factor for chronic disease

Kanakaraju Kaliannan1, Xiang-Yong Li1, Bin Wang1, Qian Pan1, Chih-Yu Chen1, Lei Hao1, Shanfu Xie1 & Jing X. Kang1

An unbalanced increase in dietary omega-6 (n-6) polyunsaturated fatty acids (PUFA) and decrease in omega-3 (n-3) PUFA in the Western diet coincides with the global rise in chronic diseases. Whether n-6 and n-3 PUFA oppositely contribute to the development of chronic disease remains controversial. By using transgenic mice capable of synthesizing PUFA to eliminate confounding factors of diet, we show here that alteration of the tissue n-6/n-3 PUFA ratio leads to correlated changes in the gut microbiome and fecal and serum metabolites. Transgenic mice able to overproduce n-6 PUFA and achieve a high tissue n-6/n-3 PUFA ratio exhibit an increased risk for metabolic diseases and cancer, whereas mice able to convert n-6 to n-3 PUFA, and that have a lower n-6/n-3 ratio, show healthy phenotypes. Our study demonstrates that n-6 PUFA may be harmful in excess and suggests the importance of a low tissue n-6/n-3 ratio in reducing the risk for chronic diseases.

1Laboratory for Lipid Medicine and Technology, Department of Medicine, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02129, USA. Correspondence and requests for materials should be addressed to J.X.K. (email: kang.jing@mgh.harvard.edu)
Chronic illnesses, including obesity, type 2 diabetes, cardiovascular disease, cancer, and Alzheimer’s disease, are rising exponentially in the modern world. These diseases are multi-factorial in nature, but their prevalence coincides with the unbalanced increase in dietary omega-6 (n-6) polyunsaturated fatty acids (PUFA) and decrease in omega-3 (n-3) PUFA in today’s diets, suggesting that there may be differential effects of n-6 and n-3 PUFA on the development of chronic disease. Recent research has focused mainly on overall PUFA levels or on the level of n-3 PUFA alone, ignoring the important interplay between n-6 and n-3 PUFA levels. It is challenging to clarify the differential effects of n-6 and n-3 PUFA or varying n-6/n-3 PUFA ratios due to the confounding factors of diet. As a result, studies on PUFA show inconsistent results about the role of PUFA in health and disease. Therefore, it is critical to establish a model that allows us to accurately study the importance of the n-6/n-3 ratio and the potential dangers of excess n-6 PUFA.

Chronic low-grade inflammation, often caused by metabolic endotoxemia, is considered to be a critical contributor to the development of many modern chronic diseases. Metabolic endotoxemia can often result from gut microbiota dysbiosis and intestinal barrier dysfunction. Previous work has shown that diet is an important modulating factor for both metabolic endotoxemia and chronic low-grade inflammation. Thus, identifying dietary components that can optimize the gut microbiome is important for research into chronic disease prevention and treatment.

The transgenic FAT-1 mouse model was previously developed in our lab to understand the importance of the PUFA ratio in health and disease. This mouse model contains the FAT-1 gene from Caenorhabditis elegans, encoding an enzyme that can endogenously convert n-6 to n-3 PUFA. This conversion enables FAT-1 mice to markedly increase their tissue levels of n-3 PUFA and decrease the levels of n-6 PUFA correspondently, resulting in a significant reduction of tissue ratio of n-6/n-3 PUFA, close to 1:1, with no need for additional supplementation of PUFA in the diet. This unique feature allows for studies to be performed without the confounding factors of diet, and these mice have been widely used over the last decade to study the beneficial effects of increased tissue levels of n-3 PUFA and balanced (reduced or low) tissue ratio of n-6/n-3 PUFA (i.e. changing a ratio from a high to a relatively lower one) on various diseases. Recently, we have also developed another mouse model (FAT-2) to evaluate the effects of increased tissue levels of n-6 PUFA and a high tissue ratio of n-6/n-3 PUFA on the development of chronic diseases. FAT-2 mice were engineered to express the FAT-2 gene from C. elegans and are capable of converting monounsaturated fatty acids (MUFA) into n-6 PUFA. Therefore, FAT-2 mice have increased tissue levels of n-6 PUFA and a high tissue ratio of n-6/n-3 PUFA. With the availability of FAT-1 and FAT-2 mice and crossbreeding, we were able to create a compound transgenic mouse model, namely the FAT-1+/2 mouse, carrying both FAT-1 and FAT-2 genes that can endogenously synthesize both n-6 and n-3 PUFA. Therefore, FAT-1+/2 mice exhibit high tissue levels of both omega-6 and omega-3 fatty acids, with a balanced ratio of close to 1:1. Consequently, we have four genotypes of mice for use: wild type (capable of producing essential fatty acids), FAT-1 (producing n-3 fatty acids), FAT-2 (producing only n-6 fatty acids), and FAT-1+/2 (producing both n-6 and n-3 fatty acids). These mice exhibit four distinct PUFA phenotypes varying in the quantity of PUFA and n-6/n-3 ratio, even though they are all fed an identical diet with no need for dietary supplementation with corresponding PUFA. Thus, use of these transgenic mice allows us to evaluate the authentic effects of different quantities and ratios of PUFA without the confounding factors of diet.
groups (Fig. 1d), and whitening of brown fat (an increase in large adipocytes) was observed in the brown adipose tissue of the FAT-2 and wild type mice, compared to the FAT-1 and FAT-1+2 groups (Fig. 1e). Likewise, glucose tolerance testing showed that FAT-1 and FAT-1+2 mice exhibited better glucose tolerance than the wild type and FAT-2 mice (Fig. 1f, Supplementary Fig. 3a).

Oil red O staining revealed striking differences in lipid accumulation among the four groups; specifically, the FAT-2 mice exhibited more lipid accumulation (5+ grade based on the number and size of stained fat droplets) and fatty liver development compared to their wild-type littermates (3+ grade), while lipid accumulation was markedly reduced in the FAT-1 and FAT-1+2 mice (1+ grade) (Fig. 1g). Furthermore, estimation of hepatic fibrosis showed a dramatically higher collagen deposition...
Fig. 1  Tissue omega-6/omega-3 PUFA ratio influences the development of metabolic disorders and cancer. Wild type (WT) and FAT-2, FAT-1, and FAT-1 +2 transgenic mice fed an identical Western diet for 16 months were subjected to several types of analyses at different time points. a Body weight at the age of 8 months. b Visceral white adipose tissue weight (g) after sacrificing the mice. c Nuclear magnetic resonance technique-based body composition analysis at the age of 16 months. Histopathological analysis (hematoxylin & eosin staining) of subcutaneous white adipose tissue (d) and inter-scapular brown adipose tissue (e) after sacrificing the mice. f Oral glucose tolerance test results obtained at the age of 8 months. g Fatty liver analysis performed on Oil Red O-stained liver specimens after sacrificing the mice. h Masson’s trichrome staining (red, keratin and muscle fibers; blue, collagen; light red or pink, cytoplasms and dark brown to black, cell nuclei) performed on liver specimens to estimate the extent of fibrosis. i Anatomical shape and gross appearance of the livers with tumors from the FAT-2 mice. j Differences in the incidence rate of liver cancer between wild type, FAT-2, FAT-1, and FAT-1+2 mice. Markers of metabolic endotoxemia (lipopolysaccharides (LPS) (k), LPS-binding protein (LBP) (l), soluble CD14 (m) and intestinal permeability (serum levels of FITC-dextran macromolecules) (n)) and chronic low-grade inflammation (tumor necrosis factor-α (TNF-α) (o), interleukin-6 (IL-6) (p), IL-1β (q), and monocyte chemoattractant protein 1 (MCP-1) (r)) measured at the age of 12 months. For c, n = 6 per group. For f, k-n and p-r, wild type (n = 7), FAT-2, FAT-1, and FAT-1+2 (n = 8 per group). For o, wild type (n = 7), FAT-2 and FAT-1 (n = 8 per group), FAT-1+2 (n = 7 per group). For others, wild type (n = 9), FAT-2, FAT-1, and FAT-1+2 (n = 10 per group). Data shown as mean ± standard error of mean. Data with different superscript letters are significantly different (P < 0.05) according to ordinary one-way (a-c, k-m, o-r) or repeated measures two-way ANOVA (f) or Kruskal-Wallis test (n) followed by Tukey’s or Dunn’s multiple comparisons test. *FAT-2 vs. FAT-1 and FAT-2 vs. FAT-1+2; † wild type vs. FAT-2; ‡ wild type vs. FAT-1. Scale bar for images d and e: 1000 μm; g: 2000 μm; h: 3000 μm.

(cirrhosis) in the FAT-2 liver specimens compared to the FAT-1 and FAT-1+2 groups (Fig. 1h). Strikingly, when the animals were sacrificed at the age of 16 months, we discovered that most of the FAT-2 mice (86%) had developed liver tumors (nodules and irregularities), while only very few mice from each of the other groups had liver tumors (Fig. 1i, j). Collectively, these findings indicate an increased risk for metabolic disorders and cancer in the groups with a high n-6/n-3 PUFA ratio, especially the FAT-2 mice that have the highest tissue level of n-6 PUFA, compared to the FAT-1 and FAT-1+2 groups with a balanced (lower) n-6/n-3 PUFA ratio.

Chronic low-grade inflammation is an underlying factor of many chronic diseases and closely associated with gut dysbiosis and metabolic endotoxemia16. We found that markers of metabolic endotoxemia (serum LPS, LBP, and sCD14, and intestinal permeability) (Fig. 1k–n) and chronic low-grade inflammation (TNF-α, IL-6, IL-1β, and MCP-1) (Fig. 1o–r) were increased in the FAT-2 mice compared to the wild-type mice, while these markers were reduced in the FAT-1 and FAT-1+2 mice. Furthermore, immunohistochemical staining showed that the expression of Toll-like receptor-4 (TLR4), a key regulator of the inflammatory pathway, was upregulated in the ileum, liver, and epididymal adipose tissue of the FAT-2 mice (Supplementary Fig. 3b–d), while expression of ZO-1, a tight junction protein, was downregulated in ileal tissue of the FAT-2 mice, compared to the other groups (Supplementary Fig. 3e), consistent with the increased metabolic endotoxemia and inflammation observed in the FAT-2 mice.

n-6/n-3 PUFA ratio alters the composition of gut microbiota.

To examine the relationship between these divergent tissue fatty acid profiles and the microbial communities hosted by mice of differing genotypes, we performed high-throughput metagenomic sequencing and metabolomics of fecal samples. With principal coordinate analysis (Fig. 2a, Supplementary Fig. 4a) and hierarchical clustering (Supplementary Fig. 4b) methods, we discovered a distinct clustering of global microbiota composition among wild type, FAT-2, FAT-1, and FAT-1+2 genotypes, with the FAT-1 and FAT-1+2 genotypes forming a single cluster at one end, the wild-type cluster in the middle, and the FAT-2 cluster on the opposite end of the primary ordination axis (PERMANOVA results showed differences between groups except between FAT-1 and FAT-1+2 groups). The distribution of operational taxonomic units (OTUs) of seven bacterial groups with the largest magnitudes also showed distinct separation between wild type, FAT-2, and FAT-1 plus FAT-1+2 groups (Fig. 2b). Notably, OTUs belonging to the Enterobacteriaceae and Veromicrobiaceae families were most abundant in FAT-2 mice, followed by wild-type mice, while OTUs from the Bifidobacteriaceae, Desulfovibrionaceae, and Bacteroidiaceae families were enriched in FAT-1 and FAT-1+2 mice. Furthermore, hierarchical clustering based on the relative abundance of representative OTUs (P < 0.05) separated the mice as well as the bacterial groups into two primary clusters (Fig. 2c), with wild type and FAT-2 samples forming two distinct clusters in one clade and FAT-1 and FAT-1+2 samples combined in another clade. Biomarker analysis showed that Proteobacteria were increased in FAT-2 mice compared to the wild-type group, Firmicutes and Bacteroidetes were more abundant in FAT-1 mice, and Deltaproteobacteria and Actinobacteria were enriched in FAT-1+2 mice (Fig. 2d, Supplementary Fig. 4c). Furthermore, comparisons at the phylum level showed that FAT-2 mice had the highest abundance of Proteobacteria and the lowest abundance of Bacteroidetes, compared with the other genotypes (Fig. 2e). Both FAT-2 and wild type had lower abundances of Actinobacteria than FAT-1 and FAT-1+2 (Fig. 2e). Notably, we found that there was a striking difference in the Enterobacteriaceae and Bifidobacteriaceae between wild type and FAT-2 mice versus FAT-1 and FAT-1+2 mice (Fig. 2f, g). Relative quantification of bacterial groups by quantitative PCR (qPCR) was in accordance with these findings (Supplementary Fig. 4d, e). There were no differences between groups in the α-diversity measures (Supplementary Fig. 4f–j). Together, these results indicate that the n-6/n-3 PUFA ratio can affect gut microbiota composition. In particular, FAT-2 mice with an increased n-6/n-3 ratio exhibit greater abundance of Enterobacteriaceae and depleted Bifidobacteriaceae; in contrast, FAT-1 and FAT-1+2 mice with a balanced (lower) n-6/n-3 ratio exhibit enriched Bifidobacteriaceae and reduced Enterobacteriaceae. These findings support the notion that n-6 and n-3 PUFA have opposing effects on the gut microbiota.

Furthermore, similar to the patterns shown above in gut microbial profiles, the abundance of genes involved in functional KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways were distinctly clustered by genotype (PERMANOVA results showed differences between groups except between FAT-1 and FAT-1+2 groups) (Fig. 2h). Next, from the list of differentially expressed KEGG pathways (false discovery rate (FDR)-corrected P < 0.05) represented by the inferred genomic content, we selected those associated with metabolic syndrome (MS), inflammation, bacterial translocation, non-alcoholic fatty liver disease (NAFLD), and cancer (Supplementary Table 1) and then analyzed the abundance of these pathways among the different mouse genotypes. We found that these KEGG pathways were upregulated in the FAT-2 mice compared to wild type mice, and
the level of these pathways was markedly reduced in the FAT-1/+2 groups (Fig. 2i–k). These findings further support that the changes in gut microbiota due to the n-6/n-3 ratio alteration may be associated with disease development.

n-6/n-3 ratio alters fecal and serum metabolite profiles. Next, we examined the potential differences in fecal metabolite profiles between the four mouse genotypes using an untargeted metabolomics approach together with partial least squares-discriminant
Fig. 2 Alterations in the tissue-generated omega-6/omega-3 PUFA ratio impact the composition of fecal microbiota. Fecal microbiome analyses (V4 16S rRNA sequencing and predicted microbial functions) were performed on male wild type (WT), FAT-2, FAT-1, and FAT-1+2 transgenic mice (n = 5 per group) fed an identical Western diet for 12 months. a β-Diversity analysis performed on whole microbiota relative abundance using principal coordinate analysis (PCOA) with the Bray-Curtis dissimilarity index (BCD) followed by permutational multivariate analysis of variance (PERMANOVA) significance test. b Similarity percentage analysis with BCD was used to identify the specific genera with the greatest contribution to the differences observed between the groups, followed by principal component analysis (PCA) (variance-covariance type) showing the top eight operational taxonomic unit (OTU) scores included as vectors. The magnitude and direction correspond to the weights. c Hierarchical clustering with a heat map shows the relative abundance of representative OTUs (those with greatest difference between the four genotypes) group means (normalized to %) from each family selected for false discovery rate (FDR) corrected P < 0.05, obtained with differential abundance analysis. The OTUs are shown as phylum and family. d Cladogram generated from linear discriminant analysis (LDA) effect size showing the relationship between taxons (the levels represent, from the inner to outer rings, phylum, class, order, family, and genus). e Analysis at the phylum level using relative abundance (%). f, g Relative abundance (Y = sqrt(Y)) transformed of family_Enterobacteriaceae (E) and family_Bifidobacteriaceae (%). h Relative abundance of predicted microbial genes related to metabolism was identified using PICRUSt analysis followed by PCOA analysis with BCD and PERMANOVA significance test. i Heat map shows the relative abundance (%) of representative predicted microbial genes (those with greatest difference between four genotypes) group means from each family selected for FDR-corrected P < 0.05, obtained with differential abundance analysis. BT, bacterial translocation; DM, diabetes mellitus; NAFLD, non-alcoholic fatty liver disease. j, k Relative abundance (Y = sqrt(Y) transformed) of predicted bacterial genes involved in LPS biosynthesis and LPS biosynthesis proteins. Data shown as mean ± standard error of mean. n = 5/group. Data with different superscript letters are significantly different (P < 0.05) according to Mann-Whitney test (f, g, j, k).

Fig. 3 Alterations in the tissue-generated omega-6/omega-3 PUFA ratio impact the composition of fecal microbiota. Fecal microbiome analyses (V4 16S rRNA sequencing and predicted microbial functions) were performed on male wild type (WT), FAT-2, FAT-1, and FAT-1+2 transgenic mice (n = 5 per group) fed an identical Western diet for 12 months. a β-Diversity analysis performed on whole microbiota relative abundance using principal coordinate analysis (PCOA) with the Bray-Curtis dissimilarity index (BCD) followed by permutational multivariate analysis of variance (PERMANOVA) significance test. b Similarity percentage analysis with BCD was used to identify the specific genera with the greatest contribution to the differences observed between the groups, followed by principal component analysis (PCA) (variance-covariance type) showing the top eight operational taxonomic unit (OTU) scores included as vectors. The magnitude and direction correspond to the weights. c Hierarchical clustering with a heat map shows the relative abundance of representative OTUs (those with greatest difference between the four genotypes) group means (normalized to %) from each family selected for false discovery rate (FDR) corrected P < 0.05, obtained with differential abundance analysis. The OTUs are shown as phylum and family. d Cladogram generated from linear discriminant analysis (LDA) effect size showing the relationship between taxons (the levels represent, from the inner to outer rings, phylum, class, order, family, and genus). e Analysis at the phylum level using relative abundance (%). f, g Relative abundance (Y = sqrt(Y)) transformed of family_Enterobacteriaceae (E) and family_Bifidobacteriaceae (%). h Relative abundance of predicted microbial genes related to metabolism was identified using PICRUSt analysis followed by PCOA analysis with BCD and PERMANOVA significance test. i Heat map shows the relative abundance (%) of representative predicted microbial genes (those with greatest difference between four genotypes) group means from each family selected for FDR-corrected P < 0.05, obtained with differential abundance analysis. BT, bacterial translocation; DM, diabetes mellitus; NAFLD, non-alcoholic fatty liver disease. j, k Relative abundance (Y = sqrt(Y) transformed) of predicted bacterial genes involved in LPS biosynthesis and LPS biosynthesis proteins. Data shown as mean ± standard error of mean. n = 5/group. Data with different superscript letters are significantly different (P < 0.05) according to Mann-Whitney test (f, g, j, k).

ARTICLE COMMUNICATIONS BIOLOGY | https://doi.org/10.1038/s42003-019-0521-4

Fig. 4 Alterations in the tissue-generated omega-6/omega-3 PUFA ratio impact the composition of fecal microbiota. Fecal microbiome analyses (V4 16S rRNA sequencing and predicted microbial functions) were performed on male wild type (WT), FAT-2, FAT-1, and FAT-1+2 transgenic mice (n = 5 per group) fed an identical Western diet for 12 months. a β-Diversity analysis performed on whole microbiota relative abundance using principal coordinate analysis (PCOA) with the Bray-Curtis dissimilarity index (BCD) followed by permutational multivariate analysis of variance (PERMANOVA) significance test. b Similarity percentage analysis with BCD was used to identify the specific genera with the greatest contribution to the differences observed between the groups, followed by principal component analysis (PCA) (variance-covariance type) showing the top eight operational taxonomic unit (OTU) scores included as vectors. The magnitude and direction correspond to the weights. c Hierarchical clustering with a heat map shows the relative abundance of representative OTUs (those with greatest difference between the four genotypes) group means (normalized to %) from each family selected for false discovery rate (FDR) corrected P < 0.05, obtained with differential abundance analysis. The OTUs are shown as phylum and family. d Cladogram generated from linear discriminant analysis (LDA) effect size showing the relationship between taxons (the levels represent, from the inner to outer rings, phylum, class, order, family, and genus). e Analysis at the phylum level using relative abundance (%). f, g Relative abundance (Y = sqrt(Y)) transformed of family_Enterobacteriaceae (E) and family_Bifidobacteriaceae (%). h Relative abundance of predicted microbial genes related to metabolism was identified using PICRUSt analysis followed by PCOA analysis with BCD and PERMANOVA significance test. i Heat map shows the relative abundance (%) of representative predicted microbial genes (those with greatest difference between four genotypes) group means from each family selected for FDR-corrected P < 0.05, obtained with differential abundance analysis. BT, bacterial translocation; DM, diabetes mellitus; NAFLD, non-alcoholic fatty liver disease. j, k Relative abundance (Y = sqrt(Y) transformed) of predicted bacterial genes involved in LPS biosynthesis and LPS biosynthesis proteins. Data shown as mean ± standard error of mean. n = 5/group. Data with different superscript letters are significantly different (P < 0.05) according to Mann-Whitney test (f, g, j, k).

**n-6/n-3 ratio influences microbial–metabolite interactions.** A functional relationship between the microbiome and metabolome is suggested by the similarity between OTU types and metabolotypes. The three data sets (microbiome, fecal, and serum metabolome) showed a high degree of concordance that was statistically significant in Monte Carlo simulations with a P value of 0.02. Superimposed microbiome and metabolomics data were separated not only by n-6/n-3 PUFA status but also by OTU type and metabolotype. Next, an inter-omic network (Fig. 4c, d) was constructed using microbes and metabolites showed 8334 statistically significant correlations (Spearman’s non-parametric rank correlation coefficient; P < 0.05) between two microbes, two metabolites, or a microbe and metabolite. The top three OTUs (Enterobacteriaceae, Desulfovibrionaceae, and Bifidobacterium) and fecal (pyruvate, n-3 EPA, and n-6 DPA) and serum (n-3 EPA, n-6 DPA) and n-6 adrenate) metabolites were found to have a high inter-omic centrality as shown in the first two largest modules of the network (Fig. 4e, f). Betweenness centrality, which signifies the “bottleneck nodes (Supplementary Fig. 5a) that are crucial to the communication within the network,” further highlights that this network strategy yields relevant key microbes and metabolites altered by tissue n-6/n-3 PUFA ratio.

**n-6/n-3 PUFA ratio influences host–microbe interactions.** RV coefficient (0.73; P = 0.001) showed an overall measure of association between the tissue n-6/n-3 ratio-induced metabolic changes, microbes, and metabolites. Network-based analytical approaches extract complex host–microbe interactions. Correlation analysis resulted in a correlation network (Fig. 5a) consists of 1089 edges, 237 nodes, and 4 largest modules (Fig. 5b–e) (biologically important elementary units). It is
important to note that, according to the degree values, total n-6 PUFA (73), n-6/n-3 ratio (50), and intestinal permeability (IP) (52) in the first largest module, serum LPS (45) and LBP (43) and HCC (40) in the second module, serum IL-6 (37) and TNF-α (36) and body weight (34) in the third module, and total PUFA (14) and n-3 PUFA (27) in the fourth module were linked with microbes and metabolites identified as biologically important with microbiome-metabolite inter-omic analysis (Supplementary Fig. 5a). Next, parameters contributing to the multivariate PLS models were compared with the corresponding identified modules (Fig. 5b–e) in the correlation networks. A variability of 97%, 92%, 93%, 79%, 86%, 89%, and 82% for IP, LPS, LBP, HCC, IL-6,
TNF-α, and body weight, respectively, were explained by the combination of key microbes and metabolites (Fig. 5f, Supplementary Data 1) altered by the tissue n-6/n-3 PUFA ratio. Correlation network and principal component analysis (PCA) analyses on selected key parameters showed that the FAT-2 group was found to be closely associated with factors related to increased levels of n-6 PUFA metabolites, LPS production, gut permeability, inflammation, obesity, diabetes, fatty liver, and cancer, whereas FAT-1 and FAT-1+2 groups were associated with anti-inflammatory factors (Fig. 6a, b). Among the many correlations we found, the most important correlations are shown in Supplementary Fig. 5b–m. Notably, among the fecal PUFA tested, only n-6 PUFA were positively correlated with visceral adiposity (Supplementary Fig. 5m). In summary, elevated tissue omega-6 PUFA status with an increased tissue n-6/n-3 PUFA ratio alters gut microbiota and gut microbial functional pathways, fecal metabolites production, and eventually microbiota-derived serum metabolites levels. These alterations plus adverse changes in the levels of serum non-microbiota-derived metabolites impacted directly by the elevated tissue n-6/n-3 ratio lead to increased intestinal permeability, metabolic endotoxemia, and chronic low-grade inflammation, resulting in the occurrence of chronic disease and cancer (Fig. 6c).

Discussion

By using unique transgenic animal models and multi-omics technologies, our study has uncovered a potential pathway for the development of modern chronic diseases and cancer stemming from the dietary imbalance between n-6 and n-3 PUFA. Our discovery not only provides new insights into the etiology of chronic disease epidemics but also highlights the importance of balancing n-6 and n-3 PUFA in the diet to achieve a healthy tissue n-6/n-3 PUFA ratio as a key strategy for the management of chronic diseases. Our results emphasize that n-6 PUFA and n-3 PUFA are not equal, but actually exert differential or opposite effects on certain chronic health problems, indicating a need for reducing n-6 PUFA intake and increasing n-3 PUFA intake for improving health. This challenges many current dietary guidelines issued by governmental or health organizations, including the United States Dietary Guidelines (2015–2020), which recommends increasing intake of PUFA in general (mainly n-6 PUFA).

We present a unique model for nutritional intervention studies to reliably elucidate the relationship between n-6/n-3 ratio and chronic disease by integrated multi-omics measurements of a series of inter-related biomarkers. Biomarkers including lipidome (profiles of fatty acids and their metabolites), gut microbiota, and metabolic endotoxemia (elevated Enterobacteriaceae to Bifidobacterium ratio, serum LPS/LBP/gut permeability markers), inflammatory markers (e.g. TNF-a, IL-6, CRP), microbiota-derived metabolites (e.g. TMAO), host metabolomics, and pathological parameters can be accurately used for this model. Our unique genetic approach using transgenic mice allowed us to eliminate the confounding factors of diet (e.g. different types of diet30 and the choice of control diet26,31) affecting gut microbiota composition and metabolite production28. Dietary modification is conventionally used to investigate the effects of different fatty acid profiles on gut microbiota and chronic disease development. However, this method is problematic since the diets used between study groups may contain not only different fatty acids but also variations in impurities, flavor, calories, or other components, as confounding factors that complicate interpretation of results6,8,9.

Our model demonstrates the authentic effects of absolute amounts of n-6 and n-3 PUFA, total PUFA, and n-6/n-3 PUFA ratios in a single study. Given the fact that the Western diet consumption is associated with the imbalanced tissue n-6/n-3 ratio32,33, our preclinical results provide evidence that balancing the n-6/n-3 ratio would be more important for better health outcomes. Whereas increasing the total PUFA consumption by only increasing the intake of n-6 PUFA resulted in adverse health outcomes with FAT-2 mice (Fig. 6c), this was not the case for FAT-1+2 mice because of their lower n-6/n-3 ratio, although both groups showed similar total PUFA. Likewise, increasing the total PUFA intake might not provide additional benefits when a balanced n-6/n-3 ratio is achieved because FAT-1 and FAT-1+2 mice showed similar healthy outcomes even though the FAT-1 mice have lower total PUFA than FAT-1+2. Similarly, although the total PUFA was the same between wild type and FAT-1 mice, lowering the n-6/n-3 ratio by elevating the n-3 PUFA (FAT-1 mice) resulted in better health outcomes. Overall, our results provide evidence that a balance between n-6 and n-3 PUFA is critical for good health and suggest that tissue n-6/n-3 ratio may be an important health biomarker. In this context, excessive intake of n-6 PUFA might be associated with adverse health outcomes and a simultaneous increase in n-3 PUFA consumption to balance the n-6/n-3 ratio is needed for good health.

In addition to the uniqueness of the mouse model, the other strengths of our study are highlighted as follows. Firstly, integrated multi-omics analyses is a powerful tool because the true power of our study design comes from the ability to examine results across the different omics levels to provide an integrated systems picture26,34,35. Secondly, network-based analytical approaches have the potential to help disentangle complex higher-order microbe–microbe, microbe–metabolite and microbe–host interactions, thereby broadening the applicability of microbiome research to personalized medicine and public health36. Finally, understanding host–microbe interactions is critical during times of disease, and balanced host–microbe interactions are necessary for maintaining homeostasis.
Our results showed a marked increase in LPS-producing and/or pro-inflammatory bacteria (e.g. Proteobacteria) and reductions in LPS-suppressing and/or anti-inflammatory bacteria (e.g. Bifidobacterium) observed in FAT-2 mice result in elevation of endotoxemia and inflammation. In support of this key observation, we found elevated microbial functions related to LPS biosynthesis and related proteins in the FAT-2 mice. Chronically altered changes in the gut–liver axis and its role in the development of metabolic endotoxemia and associated chronic low-grade inflammation have been shown central to the development of chronic disease such as obesity, metabolic syndrome, and liver cancer. Increased LPS production due to alterations in LPS-related gut bacteria and/or gut barrier function lead to the development of metabolic endotoxemia and associated chronic low-grade inflammation. Recent studies have shown associations between markers of metabolic endotoxemia (LBP and sCD14) and systemic inflammation (TNF-α and IL-6) in subjects with obesity and metabolic syndrome. Likewise, increasing
evidence suggests that the presence of endotoxiaemia is of substantial clinical relevance to patients with HCC.45 In this context, decreasing the abundance of LPS-producing bacteria is a key mechanism for the reduction of metabolic endotoxiaemia, chronic low-grade inflammation, and the occurrence of chronic disease in the FAT-1 and FAT-1flmice with a balanced n-6/n-3 ratio. A recent study has shown that male FAT-1 mice fed high-fat diet for 6 weeks at young age showed a lean phenotype associated with higher energy expenditure than wild-type counterparts.46 In the present study, the sustained increase in fat mass and eventually body weight observed in FAT-2 mice could be partially due to their lower energy expenditure, which may be another mechanism underlying the body weight gain in FAT-2 mice. The altered energy homeostasis (lower energy expenditure, CO2 production and O2 consumption) in the FAT-2 mice with elevated tissue n-6/n-3 ratio could be associated with impaired browning process47 and altered intestinal endocannabinoid system.48 However, this assumption warrants further investigation.

The altered gut microbiota-derived metabolites (GM-DMs) and their translocation to the liver and systemic circulation have been shown to have a role in metabolic syndrome49 and hepatocellular carcinoma.50 In our study, several fecal (e.g. indolepropionate) and serum (e.g. ursodeoxycholate) GM-DMs, which have been shown in the pathogenesis of metabolic syndrome and hepatocellular carcinoma (Supplementary Tables 2 and 3), were altered in the FAT-2 mice and these alterations were prevented in the FAT-1 and FAT-1flmice. Notably, increased abundance of TMAO-producing bacteria (e.g. Erysipelotrichaceae and Enterobacteriaceae) was associated with elevated levels of fecal and serum TMAO (associated with insulin sensitivity, glucose metabolism, and atherosclerosis and modified by fat intake) were observed in FAT-2 mice. In addition to LPS and GM-DMs, alterations in the non-microbiota host-derived metabolites may also play a role in the disease phenotypes of FAT-2 mice. A recent study identified 40 serum metabolites that were elevated in hepatocellular carcinoma and cirrhosis subjects. Interestingly, the same 40 metabolites (e.g. UDCA and 12-HETE) were higher in the FAT-2 mice and lower in the FAT-1+2 mice. Taken together, unfavorable host–microbe interactions played a major role in the development of metabolic phenotypes and liver cancer due to imbalanced tissue n-6/n-3 ratios in the FAT-2 mice with the exact opposite situation occurring in the FAT-1 and FAT-1+2 mice. This conclusion is further supported by our high-throughput integrated multi-omic and host–microbe interaction analyses. Modules derived from these analyses clearly showed a microbe–microbe, microbe–metabolite, and metabolite–metabolite interactions, and a strong association between tissue n-6/n-3 ratio with LPS-related bacterial groups, several fecal and serum metabolites involved in chronic disease and markers of metabolic endotoxiaemia and chronic low-grade inflammation. This is important because a “module” in the network is an elementary unit of any biological network and biologically important when considered in isolation. Overall, our results indicate the necessity of having a balanced tissue n-6/n-3 ratio to create the balanced microbiome essential for the management of chronic disease.

The Western human diet has shifted dramatically in the last few decades from the diets that were consumed during most of human evolution. Key changes include increases in saturated fat, carbohydrates, and n-6 PUFA, and a decrease in n-3 PUFA.3,32 As a result, many people today have an n-6 to n-3 PUFA ratio that favors n-6 PUFA by as much as 20:1. Historically, this ratio would have been closer to 1:1, and the discrepancy may contribute to modern health problems, including chronic diseases such as cancer. Our discovery that elevating tissue n-3 PUFA status and lowering the n-6/n-3 PUFA ratio can improve the gut microbiome profile, create a balanced host–microbiome interaction landscape, and suppress metabolic endotoxiaemia, and chronic low-grade inflammation provides two major implications for dealing with modern health problems. First, it supports the hypothesis that an excess of n-6 PUFA and deficiency of n-3 PUFA in the Western diet can contribute to modern chronic diseases (Fig. 6c). Second, it provides a new strategy for the prevention and treatment of chronic diseases by reducing the tissue n-6/n-3 ratio through n-3 PUFA supplementation and reducing n-6 PUFA intake. Further, the methodologies and results of our preclinical study emphasize the potential importance of reducing n-6 PUFA intake and/or increasing n-3 PUFA intake, rather than just increasing the total PUFA intake mainly through foods rich in n-6 PUFA.

In order to bridge the gap to clinical implementation, our results should be validated with future large-scale multi-omic analyses of the human gastrointestinal microbiome through nutritional intervention studies. Our work suggests that nutritional policies should be established that emphasize the differential effects of n-6 and n-3 PUFA rather than simply replacing saturated fatty acids with PUFA for the prevention of chronic disease. Finally, we acknowledge that a limitation of our study is the use of only male mice; future research in female mice is necessary because our findings obtained with male mice may not be translational to females.

In conclusion, integrated multi-omic analyses of our unique transgenic animal models uncover the tissue omega-6/omega-3 fatty acid imbalance as a critical risk factor for chronic disease.
Decreased tissue n-6/n-3 ratio leads to beneficial changes in gut microbiota and gut microbial functional pathways. Subsequently, the positive alterations in fecal metabolite production and microbiota-derived and non-microbiota-derived serum metabolites suppress the development of metabolic endotoxemia and chronic low-grade inflammation and thereby reduce the risks for chronic diseases and cancer. Overall, this study demonstrates the importance of a low tissue omega-6/omega-3 PUFA ratio for maintaining good health and for the management of chronic diseases and cancer.
Network interactions uncover host-microbiome interactions driven by tissue n-6/n-3 PUFA status. a-e Host-microbiota interaction network built from Spearman’s non-parametric rank correlation coefficient (P < 0.05) between 11 host parameters (serum total PUFA and n-6 and n-3 PUFA, n-6/n-3 ratio, body weight, HCC incidence, iP, serum LPS, LBP, TNF-α and IL-6) (n = 6 per group) and 66 microbial (n = 5 per group) and 159 metabolite parameters (n = 6 per group) with FDR-corrected P values < 0.05. Two hundred and thirty-seven nodes (filled circles) represents host parameters (cytochrome P450, microbe (pink), fecal (green), and serum (olive) metabolites. Node size reflects betweenness centrality—a measure of how many shortest paths within the entire network passes through the node in question (crucial to the communication within the network). In total, 1089 lines (edges) represent statistically significant correlations (P < 0.05) and are colored gray for 779 positive and blue for 310 negative correlations. The full network (a) with edges showing all the correlations and the four (b-e) largest modules (biologically important elementary units of any biological network), which were separated from the full network according to the modularity scores. Partial least square (PLS)-regression loading score plot illustrating the association between host parameters (dependent variables — Y; blue triangles) and serum PUFA and microbial and metabolite parameters (explanatory variables — X; red dots). Exploratory variables of interest with variable importance in the projection (VIP) scores >1 were labeled with circles on the red dots. Samples from four different groups (wild type/FAT-2/FAT-1/FAT-1+2) were observed (green dots) and grouped using circles based on where they clustered on the plot. Leave-one-out cross-validation (LOO-CV) was applied. Ebac, Enterobacteriaceae; Bifido, Bifidobacteriaceae; HCC, hepatocellular carcinoma; iP, intestinal permeability; LPS, lipopolysaccharides; LBP, LPS-binding protein.

**Methods**

**Generation of transgenic mice.** As we described previously, we generated a novel transgenic mouse model that can endogenously synthesize all essential fatty acids. Our strategy was to first create a FAT-2 transgenic mouse, possessing the C. elegans FAT-2 gene encoding an enzyme that converts monounsaturated fatty acids (MUFA) into n-6 fatty acids (fat), and then cross the FAT-2 transgenic mice, with n-6 fatty acids, to a FAT-1 transgenic mouse, which we generated previously to possess the C. elegans FAT-1 gene, encoding an enzyme that converts n-6 to n-3 PUFA. Through this procedure, we generated a compound FAT-1+2 transgenic mouse (Omega mouse) — that is capable of producing both n-3 and n-6 PUFA from a diet containing only saturated fat.

As described in our previous study, genotyping was carried out by removing the tip of the tail to acquire a DNA sample for reverse transcription (RT)-PCR, which was performed with the following primers: FAT-2 forward, GGCGCCGAACCCGACATAC; and FAT-2 reverse, GGCGGCAGTGGACGGTTGTTGA. PCR products were run through gel electrophoresis on 2% agarose gel. Phenotyping by fatty acid composition analysis using gas chromatography (GC) was performed as previously described. Tissue samples were ground to powder under liquid nitrogen and total lipids were extracted using chloroform/methanol (2:1, v/v). Fatty acids were then methylated by heating them at 100°C for 1 h under 14% boron trifluoride (BF3)-methanol reagent (Sigma-Aldrich), and hexane (Sigma-Aldrich). Fatty acid methyl esters were analyzed by GC using a fully automated 6890N Network GC System (Agilent Technologies, Santa Clara, CA) equipped with a flame-ionization detector and an Omegawax 250 capillary column (30 m × 0.25 mm ID). Fatty acid standards (Nuchek Prep, Elysian, MN) were used to identify peaks of resolved fatty acids, and area percentages for all resolved peaks were analyzed using GC ChemStation Software (Agilent). The fatty acid C20:5 (20 mg per sample) was used as an internal standard to calculate the amount of each fatty acid measured. After identifying the genotype and phenotype, the FAT-2 mice were mated with wild-type C57BL6 mice to create the F1 generation. The F1 generation was then backcrossed with wild-type C57BL6 mice at least five times in order to certify that the gene is transmitted hereditarily and that the transgenic background is stable, so that FAT-2 lines could be maintained with a significant phenotype. Each generation was subjected to genotyping by RT-PCR and phenotyping by GC. The compound FAT-1+2 transgenic mice were created by crossingbreeding heterozygous FAT-2 transgenic mice with heterozygous FAT-1 transgenic mice, which were previously generated by our group. Genotyping by RT-PCR of the Omega mice was carried out with the following primers: FAT-1 forward, TGTTTCAGGCTCTCTTTCCTTTTCCT; FAT-1 reverse, GGCCGATCC TCAAACTTGGA; FAT-2 forward, GGCGGCAGACCCGACATAC; FAT-2 reverse, GGCGGCAGTGGACGGTTGTTGA. Phenotyping by fatty acid composition analysis using GC was performed as previously described.

Animals in this study were maintained in accordance with the guidelines prepared by the institutional animal care and use committee (IACUC) at MGH based on the Care And Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council [Department of Health, Education and Human Resources, Publication 86-22 (National Institutes of Health), revised 1985]. All animal protocols were reviewed and approved by the IACUC at MGH. Animals were sacrificed by the Animal Veterinary Medical Association (AVMA)-approved protocol of i.p. injection of pentobarbital (200 mg/kg).

**Animal experiments.** Mice were housed in a biosafety level 2 room in hard top cages with three or two mice per cage. Mice were maintained in a temperature-controlled room (22–24°C) with a strictly followed 12-h light/12-h dark diurnal cycle with food and water ad libitum. Four-week-old male wild type (wild type), FAT-2, FAT-1, and FAT-1+2 mice (n = 9–10 per group; 2 or 3 mice per cage), which were bred at the MGH animal facility, were fed an identical modified Western diet for 16 months, with no additional PUFA supplementation. All four groups of mice were subjected to several kinds of analysis at different time points. Animal body weight was measured at different time points and food intake was measured weekly. After 16 months of follow-up, mice were placed into individual cages in the Ozymax Comprehensive Laboratory Animal Monitoring System (CLAMS, Columbus Instruments) for an additional 24 h (at 23°C with a 12 h light/12 h dark cycle) for indirect calorimetry measurements for long-term phenotyping and assessment of energy expenditure, as previously described in Mearny et al. Water and food were available ad libitum. The Ozymax system is equipped with an indirect calorimeter for lab animal research allowing the measurement of oxygen consumption (VO2), respiratory exchange ratio, and activity levels of mice. VO2 is a measure of the volume of oxygen used to convert energy substrate into ATP. The respiratory exchange ratio is the ratio of carbon dioxide production (VCO2) divided by VO2, and can be used to estimate the fuel source for energy production based on the difference in the number of oxygen molecules required for the oxidation of glucose versus fatty acids. A respiratory exchange ratio of 0.7 indicates that fatty acids are the primary substrate for oxidative metabolism, while a respiratory exchange ratio of 1.0 indicates that carbohydrate is the primary energy substrate. Activity was calculated by summing the X axis movement counts associated with horizontal movement. Body composition (fat and lean mass and fluid weight) of each mouse was determined by dual-energy X-ray absorptiometry (DEXA) according to the manufacturer’s instructions (GE Lunar Piximus 2). All values (g) were normalized using each mouse body weight and body composition was expressed as percentages. Mice were fasted for 6 h during the light phase period and blood was collected from the facial vein unless otherwise specified. Mouse feces and blood were collected at different time points as mentioned above and sacrificed after sacrifice of the appropriate organs (intestine, adipose tissue, liver, spleen, bone, brain, testis, etc.) were flash frozen using liquid nitrogen and then stored at −80°C for further analysis.

**Extraction and purification of DNA from fecal samples.** At the age of 12 months, bacterial genomic DNA was extracted from fresh stool samples (~100–180 mg) from wild type, FAT-2, FAT-1, and FAT-1+2 mice using the QiAamp DNA Stool Mini Kit (Qiagen, Valencia, CA), following the manufacturer’s instructions. In order to increase its effectiveness, the lysis temperature was increased to 95°C. The eluted DNA was treated with RNase, concentration was determined by absorbance at 260 nm (A260), and purity was estimated by determining the A260/A280 ratio with a Nanodrop spectrophotometer (Biotech, Winooski, VT). DNA samples were diluted to 30 ng μl⁻¹, and this concentration was confirmed using both spectrophotometry (A260) and fluorometry (DNAQF-1KT; Sigma, USA).

**Genomic DNA library preparation.** Genomic DNA samples (n = 5 per group) were sent to the microbiome analysis company (Second Genome, Inc., CA, USA) to perform V4 16S rRNA gene sequencing. As described previously, to enrich the sample for bacterial V4 16S rRNA region, DNA was amplified using fusion primers designed against the surrounding conserved regions which are tagged with sequences to incorporate Illumina (San Diego, CA) adapters and indexing barcodes. Each sample was PCR amplified with two differently barcoded V4 fusion primers. Samples that met the post-PCR quantification minimum were advanced for pooling and sequencing. For each sample, amplified products were concentrated using a solid-phase reversible immobilization method for the purification of PCR products and quantified by qPCR.

**Microbiome profiling.** A pool containing 16S V4-enriched, amplified, barcoded samples was loaded into a MiSeq® reagent cartridge, and then onto the instrument.

ARTICLE COMMUNICATIONS BIOLOGY | https://doi.org/10.1038/s42003-019-0521-4

COMMUNICATIONS BIOLOGY | (2019) 2:276 | https://doi.org/10.1038/s42003-019-0521-4 | www.nature.com/commsbio
along with the flow cell. After cluster formation on the MiSeq instrument, the amplicons were sequenced for 250 cycles with custom primers designed for paired-end sequencing. Samples are processed in a Good Laboratory Practices (GLP) compliant service laboratory running Quality Management Systems for sample and data tracking. The laboratory implements detailed standard operating procedures (SOPs), equipment and process validation, training, audits, and document control measures. Quality control (QC) and assurance (QA) metrics were maintained for all samples.

**Microbiome data analysis.** The full data analysis pipeline for Second Genome’s Microbial Profiling Service incorporates several separate stages: pre-processing, summarization, normalization, alpha-diversity metrics (within sample diversity), beta-diversity metrics (sample-to-sample similarity), ordination/clustering, sample classification, and significance testing. The report was generated using second-genomER package: 0.2.4.

For OTU selection, paired-end reads were merged, quality filtered, and dereplicated with USEARCH\(^1\). Resulting unique sequences were then clustered at 97% similarity by UPARSE (de novo OTU clustering) and a representative consensus sequence per de novo OTU was determined. The clustering algorithm also performs chimera filtering to discard likely chimeric OTUs. Sequences that passed quality filtering were then mapped to a set of representative consensus sequences to generate an OTU abundance table. Representative OTU sequences were assigned taxonomic classification via mothur’s Bayesian classifier trained against the Greengenes reference database of 16S rRNA gene sequences clustered at 99%. After taxa were identified for inclusion in the analysis, the values used for

---

**Figure a:** Network of gut microbial functional pathways and fecal metabolites.

**Figure b:** PCA biplots of the correlation of fecal and serum metabolites with metabolic endotoxemia.

**Figure c:** Tissue PUFA profile showing the relationship between gut microbiota and serum metabolites.
each taxon-sample intersection were populated with the abundance of reads assigned to each OTU in an “OTU table.” A corresponding table of OTU GREENgenes classification was generated as well.

**Alpha-diversity (within sample diversity) metric:** “Observed” diversity is the simply the sum of unique OTUs found in each sample, also known as sample richness. Chao1 calculates the estimated sample richness (number of OTU(s) based on sequencing depth and taking into account rare taxa that may be present in a sample. Shannon diversity uses the richness of a sample along with the relative abundance of the present OTUs to calculate a diversity index.

**Beta-diversity (sample-to-sample dissimilarity) metrics:** All profiles were inter-compared in a pairwise fashion to determine the similarity score and store it in a distance dissimilarity matrix. Distance functions produce low dissimilarity scores when comparing similar samples. Abundance-weighted sample pairwise differences were calculated using the Bray–Curtis dissimilarity — the ratio of the summed absolute differences in counts to the sum of abundances in the two samples. The binary dissimilarity values were calculated with the Jaccard index. This metric compares the number of mismatches (OTUs present in one but absent in the other) in two samples relative to the number of OTUs present in at least one of the samples.

**Ordination, clustering, and classification methods:** Two-dimensional ordinations and hierarchical clustering maps of the samples in the form of dendrograms were created to graphically summarize the inter-sample relationships. Principal Coordinate Analysis is a method of two-dimensional ordination plotting that is used to help visualize complex relationships between samples. Principal Coordinate Analysis uses the sample-to-sample dissimilarity values to position the points relative to each other by maximizing the correlation between the dissimilarity values and the plot distances. To create dendrograms, the samples from the distance matrix are clustered hierarchically using the ward method.

**Whole microbiome significance testing:** Permutational Analysis of Variance (PERMANOVA) was used for finding significant differences among discrete categories or continuous variables. In this randomization permutation test, the samples are randomly reassigned to the various sample categories, and the between-category differences are compared to the true between-category differences. PERMANOVA utilizes the sample-to-sample distance matrix directly, not a derived ordination or clustering outcome.

**Taxonomic significance testing:** Univariate differential abundance of OTUs was tested using a negative binomial noise model for the overdispersion and Poisson process intrinsic to this data, as implemented in the DESeq2 package, and described for microbiome applications in ref. 68. It takes into account both biological and technical variability between experimental conditions. DESeq was run under default settings and q-values were calculated with the Benjamini–Hochberg procedure to correct P values, controlling for false discovery rates.

**Identification of metagenomic biomarkers.** The SIMPER (Similarity Percentage analysis) method was applied to whole microbiome relative abundance data to identify the top 10 taxa by abundance. Their contribution to groups (between and within groups) was analyzed as previously described using the PCA variance–covariance type ordination (FAST version 3.11 software) method. Differential abundance analysis (non-parametric ANOVA with Benjamini–Hochberg FDR-corrected P values<0.05) was performed on the relative abundance data at different levels of taxonomy to identify taxa with FDR-corrected P values<0.05 (XLSTAT software; Addinsoft, USA). Their relative abundance (normalized to percentage) was then shown by heat map with hierarchical clustering analysis. Also, pairwise comparisons were also performed between groups using GraphPad Prism version 8 (GraphPad Software, La Jolla, CA)31. The Kruskal–Wallis test was performed for each variable and then the Dunn’s multiple comparison post-hoc test was used to determine which groups differed from each other.

**Quantitative real-time PCR (qRT-PCR) assessment of fecal microbiota.** Targe fecal microbial profiling was performed as previously described. Briefly, qRT-PCR was performed with a PRISM 9000 Light Cycler (Applied Biosystems, USA) using the iTaq universal SYBR Green Supermix (Bio-Rad, USA) and group
specific primers (Supplementary Table S5) for total bacteria, family Enterobacteriaceae, genus Escherichia and genus Bifidobacterium. Samples (n = 9–10 per group) and controls were run in duplicate in total volume of 20 μL, containing 500 nM primers and 40 ng genomic DNA. Amplification and data acquisition was performed according to the protocol provided with SYBR Green (Bio-Rad, Hercules, CA). By subtracting the cycle threshold (Ct) values of total bacteria from the Ct values of each bacterial group, we estimated and compared the relative quantification of a specific bacterial group.

**Global metabolic profiling by Metabolon Inc.** Sample collection: At 14 months of age, fecal materials were collected from wild type, FAT-2, FAT-1, and FAT-1/fl mice (n = 6 per group) using sterile 2 ml tubes, weighed, flash frozen in liquid nitrogen, and stored at −80°C until shipping. Whole blood was drawn from the facial vein in 6 h fasted mice, left at room temperature for 25–30 min, clear serum-separated by centrifugation (6000 RPM for 6 min), flash frozen in liquid nitrogen, and stored at −80°C until processing. Consistency in sample handling was maintained by minimizing operational variation (collection technique, time of sampling, time to freezer, etc.). Both feces and serum samples were shipped to Metabolon Inc. (Durham, NC) where they were extracted and prepared for analysis using a previously described standard solvent extraction method.

Sample accessioning: Following receipt by Metabolon Inc., samples were inventoried and immediately stored at −80°C. Each sample received was accessioned into the Metabolon Laboratory Information Management System (LIMS system) and was assigned by the LIMS a unique identifier that was associated with the original source identifier only. This identifier was used to track all sample handling, tasks, results, etc. The samples (and all derived aliquots) were tracked by the LIMS system. All portions of any sample were automatically assigned an identifier when a new task was created; the relationship of these samples was also tracked. All samples were maintained at −80°C until processed.

Sample preparation: Samples were prepared using the automated MicroLab STAR® system from Hamilton Company (https://www.hamiltoncompany.com/).

QA/QC: Several types of controls were analyzed in concert with the experimental samples: a pooled metric matrix prepared by mixing a small volume of each experimental sample, or alternatively, a pool of well-characterized reference plasma, served as a technical replicate throughout the data set; extract water samples served as process blanks; and a cocktail of QC standards that were carefully chosen not to interfere with the measurement of endogenous compounds. The resulting extracts were divided into four fractions: two for analysis by reverse phase (RP)/ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) methods with positive ion mode electrospray ionization, one for analysis by RP/UPLC-MS/MS with negative ion mode electrospray ionization, one for analysis by HILIC/UPLC-MS/MS with negative ion mode electrospray ionization, and one sample that was reserved for reanalysis. Samples were reconstituted briefly on a TurboVap® (Zymark) to remove the organic solvent. The sample extracts were stored overnight under nitrogen before preparation for analysis.

UPLC-MS/MS: All methods used a Waters ACQUITY UPLC and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 FWHM. The Q-Exactive was set in data dependent manner such that a compact description of the data and, consequently, in their associated scores and loadings, allowing the visualization and understanding of different patterns and relations in the data. PCA is able to find low-dimensional embedding of multivariate data in a manner that optimally preserves the structure of the data. PCA transforms a data set into a smaller number of new latent variables called principal components (PCs), which are uncorrelated to each other and which account for decreasing proportions of the total variance of the original variables. Each new PC is a linear combination of the original variation such that a compact description of the variation within the data set is generated. Observations are assigned scores according to the variation measured by the PC with those having similar scores being clustered together.

PLS-DA is a classification technique that encompasses the properties of partial least-squares regression with the power of discriminant analysis. From a mathematical point of view, PLS-DA is a supervised extension of PCA used to distinguish two or more classes. In this approach, the axes are calculated to maximize class separation and can be used to examine separation that would otherwise be across three or more principal components. PLS-DA model quality.
was validated using Q^2 cum, R^2 Y cum and R^2 X cum values and CV-ANOVA was used to test the significance of the models (P < 0.05). Four groups and pairwise comparisons were clearly discriminated by the model quality parameters we used^{37}. In addition, the PLS-DA models were validated by a permutation test. R intercept and Q intercept values were checked to see whether the models were not over fitted^{37}. The "variable importance in project" (VIP) plots were generated to identify metabolites contributing significantly to the separation of the four genotypes. A cutoff value of 0.7–0.8 for the VIP is generally acceptable. In this study, the cutoff value was set at 1.0 (ref. 67).

Measurement of cytokine levels and other circulating factors. The coefficient of variation was 100 times the standard deviation of a group of values divided by the mean and is expressed as a percent. The coefficient of variation absorbance was less than 10%.

Measurement of cytokine levels and other circulating factors. Serum samples (n = 9–10 per group) were analyzed for levels of TNF-a (LLOD:1.4 pg ml^{-1}), IL-1β (LLOD: 9.4 pg ml^{-1}), IL-6 (LLOD: 0.2 pg ml^{-1}) and MCP-1 (LLOD: 3.7 pg ml^{-1}) by Bio-Plex immunoassays (assay range: 2-3000 pg ml^{-1}; intra-assay coefficient of variation: <10%; inter-assay coefficient of variation: <30%) formatted on magnetic beads (Bio-Rad Laboratories Inc, CA, USA), following the manufacturer’s instructions^{37}. Xponent software (Luminex, Austin, TX) was used for data acquisition and analysis. ELISA kits were used to analyze serum levels of sCD14 (LLOD: 0.06 ng ml^{-1}; inter- and intra-assay coefficients of variation were <12 and <8%, respectively) (MyBioSource, San Diego, CA) and LBP (LLOD: 0.4 ng ml^{-1}; coefficient of variation %<6) (NeoBioLab, Cambridge, MA), according to the manufacturer’s instructions. For all the assays mentioned previously, 5–6 standards including blank (negative control) were used.

Determination of fatty acid composition of mouse tissues and diets. Fatty acid profiles of mouse diets and tail tissues (n = 9–10 per group) were analyzed by GC as described previously^{38,39}. Briefly, tissue or food samples were ground to powder under liquid nitrogen and subjected to total lipid extraction and fatty acid methylation by 14% boron trifluoride (BF3)-methanol reagent (Sigma-Aldrich) at 100°C for 1 h. Fatty acid methyl esters were analyzed using a fully automated HP5890 GC system equipped with a flame-ionization detector (Agilent Technologies, Palo Alto, CA). The fatty acid peaks were identified by comparing their relative retention times with the commercial mixed standards (NuChek Prep, Elysian, MN), and area percentage for all resolved peaks was analyzed by using a software (ChemStation, Agilent Technol-

Liver histopathology and hepatic steatosis scoring. Liver histology was performed after 90 min from GTT and was scored using the Secondgenome R package: 0.2.4, DESeq2 package^{59}, PAST version 3.11 (ref.37), XLSTAT (2017.6) for MS Excel (Addinsoft SARL, Paris, France)^{37} and SIMCA 14.1 (Umetrics, Stockholm, Sweden)^{64} as explained in the Methods section. Gephi Graph Visualization and Manipulation software version 0.9.2 (ref. 37) was used to visualize the network. ImageJ software was utilized to draw scale bars on histopathological pictures. Univariate analyses (t-tests, ANOVA and correlation analysis) were performed using Prism 8.0 (GraphPad Software, Inc.) and PAST version 3.11 and SIMCA 14.1. Heat maps were generated using XLSTAT software version 2017.6 and Prism 8.0 (GraphPad Software, Inc.). Statistical differences among four groups in other data were evaluated by ordinary one-way analysis of variance (ANOVA) with Tukey’s or non-parametric Kruskal–Wallis test with Dunn’s multiple comparison post-tests (GraphPad Prism 8). Data were checked for heteroscedastic variance with the Bartlett’s and Brown-Forsythe tests or the F test. If unequal variance was detected, data were analyzed using non-parametric tests. If parametric and non-parametric analyses did not show statistical differences, actual data were used for analysis without transformation and parametric analysis results were presented. The significance was considered to be at P < 0.05.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Source data for figures, OTU tables, raw data, taxonomy, FASTA files, KEGG and KO pathways and abundances data, metadata for 165 rRNA gene sequence analysis as well as metabolomics data generated in this study have been made publicly available in Figshare^{68} (https://figshare.com/s/89fd8283ec58f967c1e97; https://doi.org/10.6084/m9. figshare.7607441). Figures that have associated raw data are Figs. 1-6, and Supplementary Figs. 1, 4 and 5.

Received: 2 January 2019 Accepted: 28 June 2019 Published online: 26 July 2019

References

1. Bauer, U. E., Briss, P. A., Goodman, R. A. & Bowmann, B. A. Prevention of chronic disease in the 21st century: elimination of the leading preventable causes of premature death and disability in the USA. Lancet 384, 45–52 (2014).

2. Leaf, A. & Weaver, P. C. A new era for science in nutrition. Am. J. Clin. Nutr. 105, 4088–1033 (1987).

3. Simopoulos, A. P. The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. Exp. Biol. Med.233, 674–688 (2008).

4. Kang, J. X. Differential effects of omega-6 and omega-3 fatty acids on telomere length. Am. J. Clin. Nutr. 92, 1276–1277 (2010).

5. Dyall, S. C. Interplay between n-3 and n-6 long-chain polyunsaturated fatty acids and the endocannabinoid system in pain protection and repair. Lipids 52, 885–900 (2017).

6. Kang, J. X. Fat-1 transgenic mice: a new model for omega-3 research. Prostaglandins Leukot. Essent. Fatty Acids 77, 263–267 (2007).

7. Pellizzon, M. A. & Ricci, M. R. The common use of improper control diets in diet-induced metabolic disease research confounds data interpretation: the fiber factor. Nutr. Metab. 15, 3 (2018).

8. Dyall, S. C. Methodological issues and inconsistencies in the field of omega-3 fatty acids research. Prostaglandins Leukot. Essent. Fatty Acids 85, 281–285 (2011).
9. Weilandt, K. H. et al. Omega-3 polyunsaturated fatty acids: the way forward in times of mixed evidence. *Biomed. Res. Int.* 2015, 143109 (2015).

10. Kang, J. X., Wang, J., Wu, L. & Kang, Z. Transgenic mice: fat-1 mice convert n-6 to n-3 fatty acids. *Nature 427*, 504 (2004).

11. Pai, V. J., Wang, B., Li, X., Wu, L. & Kang, J. X. Transgenic mice convert carbohydrates to essential fatty acids. *PLoS ONE 9*, e97367 (2014).

12. Ruiz-Nunez, B., Pruimboom, L., Djick-Brouwer, D. A. & Muskiet, F. A. Lipid metabolism and nutritional imbalances associated with Western diseases: causes and consequences of chronic systemic low-grade inflammation in an evolutionary context. *J. Nutr. Biochem.* 24, 1183–1201 (2013).

13. Cani, P. D., Osto, M., Geurts, L. & Everard, A. Involvement of gut microbiota in the development of low-grade inflammation and type 2 diabetes associated with obesity. *Gut Microbes* 3, 279–288 (2012).

14. Kaliamann, K. et al. Intestinal alkaline phosphatase prevents metabolic syndrome in mice. *Proc. Natl Acad. Sci. USA 110*, 7003–7008 (2013).

15. Cani, P. D. et al. Metabolic endobiont initiates obesity and insulin resistance. *Diabetes 56*, 1761–1772 (2007).

16. Kaliamann, K., Wang, B., Li, X., Kim, K. J. & Kang, J. X. A host-microbiome interaction mediates the opposing effects of omega-6 and omega-3 fatty acids on metabolic endobiosis. *Sci. Rep. 5*, 11276 (2015).

17. Kang, J. X. A transgenic mouse model for gene-nutrient interactions. *J. Nutr. Nutr. 1*, 172–177 (2008).

18. Kang, J. X. Balance of omega-6/omega-3 essential fatty acids is important for health. The evidence from gene transfer studies. *World Rev. Nutr. Diet. 95*, 93–107 (2008).

19. Bidu, C. et al. The transplantation of omega3 PUFA-altered gut microbiota of fat-1 mice to wild-type littermates prevents obesity and associated metabolic disorders. *Diabetes 67*, 1512–1523 (2018).

20. Lopez-Vicario, C. et al. Molecular interplay between Delta5/Delta6 desaturases and long-chain fatty acids in the pathogenesis of non-alcoholic steatohepatitis. *Diabetes Obes. Metab.* 22, 345–355 (2020).

21. Hudert, C. A. et al. Transgenic mice rich in endogenous omega-3 fatty acids are protected from colitis. *Proc. Natl Acad. Sci. USA 103*, 11126–11128 (2006).

22. Xia, S. et al. Melanoma growth is reduced in fat-1 transgenic mice: impact of omega-6/omega-3 essential fatty acids. *Proc. Natl Acad. Sci. USA 103*, 12499–12504 (2006).

23. He, C., Qiu, X., Chu, L., Wang, J. & Kang, J. X. Improved spatial learning performance of fat-1 mice is associated with enhanced neurogenesis and neurotogenesis by docosahexaenoic acid. *BMC Neurosci.* 18, 56 (2017).

24. Robertson, R. C. et al. Maternal omega-3 fatty acids regulate offspring obesity through persistent modulation of gut microbiota. *Microbiome 6*, 95 (2018).

25. Fitian, A. I. et al. Integrated metabolomic profiling of hepatocellular carcinoma in hepatitis C cirrhosis through GC/MS and UPLC/MS-MS. *Liver Int.* 34, 1428–1444 (2014).

26. Jacobs, J. P. et al. A disease-associated microbiome and metabolomic state in lifestyle and nutritional imbalances associated with Western diseases: causes and consequences of chronic systemic low-grade inflammation in an evolutionary context. *J. Nutr. Biochem.* 24, 1183–1201 (2013).

27. Schlotter, F. et al. Spatiotemporal multi-omics mapping generates a molecular evolutionary context. *Sci. Rep.* 3, 1949 (2013).

28. Layeghifard, M., Hwang, D. M. & Guttman, D. S. Disentangling interactions of serum indolepropionic acid, a gut microbiota metabolite, with type 2 diabetes and low-grade inflammation in high-risk individuals. *Nutr. Diabetes 8*, 35 (2018).

29. Gregory, J. C. et al. Transmission of atherosclerosis susceptibility with gut microbiota transplantation. *J. Biol. Chem.* 290, 5647–5660 (2015).

30. Hoyles, L. et al. Metabolic retroconversion of trimethylamine N-oxide and the gut microbiota. *Microbiome 6*, 73 (2018).

31. Heianza, Y. et al. Gut microbiota metabolites, amino acid metabolites and improvements in insulin sensitivity and glucose metabolism: the POUNDS Lost trial. *Gut 68*, 263–270 (2018).

32. Bennett, B. J. et al. Trimethylamine-N-oxide, a metabolite associated with atherosclerosis, exhibits complex genetic and dietary regulation. *Cell Metab.* 17, 49–60 (2013).

33. Barone, M. et al. Influence of ursoodeoxycholate-enriched diet on liver tumor growth in HBV transgenic mice. *Hepatology 37*, 880–886 (2003).

34. Uderhardt, S. & Kronke, G. 12/15-lipoxygenase during the regulation of inflammation, immunity, and self-tolerance. *J. Mol. Med. 90*, 1247–1256 (2012).

35. Kang, J. X. & Wang, J. A simplified method for analysis of polyunsaturated fatty acids. *BMC Biochem.* 6, 5 (2005).

36. Marvyn, P. M., Bradley, R. M., Mardian, E. B., Marks, K. A. & Duncan, R. E. Data on oxygen consumption rate, respiratory exchange ratio, and movement in *C57BL/6j* female mice on the third day of consuming a high-fat diet. *Data Brief 7*, 472–475 (2016).

37. Ahn, J., Son, S., Oliveira, S. C. & Barber, G. N. STING-dependent signaling underlies IL-10 controlled inflammatory colitis. *Cell Rep.* 21, 3873–3884 (2017).

38. Edgar, R. C. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods 10*, 996–998 (2013).

39. Love, M. L., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15, 550 (2014).

40. McMurdie, P. J. & Holmes, S. Waste not, want not: why rarefying microbiome data is inadmissible. *PLoS Comput. Biol.* 10, e1003531 (2014).

41. Chevalier, C. et al. Gut microbiota orchestrates energy homeostasis during cold. *Cell 163*, 1360–1374 (2015).

42. Segata, N. et al. Metagenomic biomarker discovery and explanation. *Genome Biol.* 12, R60 (2011).

43. Dharwal, A. et al. MicrobiomeAnalyzer: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. *Nucleic Acids Res. 45*, W180–W188 (2017).

44. Stewart, C. J. et al. Gut microbiota of type 1 diabetes patients with good glycaemic control and high physical fitness is similar to people without diabetes: an observational study. *Diabet. Med.* 34, 127–134 (2017).

45. Iwa, S. et al. Piphillin: improved prediction of metagenomic content by direct inference from human microbiomes. *PLoS ONE 11*, e0166104 (2016).

46. Sreekumar, A. et al. Metabolomic profiles delineate potential role for sarcosine in prostate cancer progression. *Nature 457*, 910–914 (2009).
Acknowledgements

This study was supported by the generous funding from Sansun Life Sciences and the Fortune Education Foundation. The authors are also grateful to Marina Kang for her editorial assistance.

Author contributions

J.X.K. and K.K. conceived and designed the study; K.K. collected serum, fecal, and tissue samples for metagenomics and metabolomics and immunohistochemical analyses, performed qPCR-based bacterial quantification, measured the markers of metabolic endotoxemia and chronic low-grade inflammation, analyzed all the data and performed univariate and multivariate statistical analyses including network construction and PLS models for inter-omic and host–microbiome interaction analyses; X.-Y.L. and B.W. prepared the transgenic mouse models (animal breeding, genotyping and phenotyping) and measured the markers of metabolic syndrome (body weight gain, body composition analysis, fatty liver, and glucose tolerance test) and liver cancer. Q.P. performed brown and white adipose tissue histopathological analyses, body composition analysis, and energy metabolism experiments; C.-Y.C., L.H. and S.X., contributed to fatty acid analysis and samples collection; K.K. and J.X.K. wrote the manuscript; All authors approved the final version of the manuscript.

Additional information

Supplementary information accompanies this paper at https://doi.org/10.1038/s42003-019-0521-4.

Competing interests: The authors declare no competing interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.