The gut microbiota as an environmental factor that regulates fat storage

Fredrik Bäckhed*†‡, Hao Ding‡§¶, Ting Wang‖, Lora V. Hooper†**, Gou Young Koh††, Andras Nagy§‡‡, Clay F. Semenkovich§§, and Jeffrey I. Gordon*†¶¶

*Center for Genome Sciences and Departments of †Molecular Biology and Pharmacology, ‖Genetics, and §§Medicine, Cell Biology, and Physiology, Washington University School of Medicine, St. Louis, MO 63110; §Samuel Luenfeld Research Institute, Mount Sinai Hospital, Toronto, ON, Canada M5G 1X5; ††Biomedical Center, Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon, 305-701, Republic of Korea; and ‡‡Department of Medical Genetics and Microbiology, University of Toronto, Toronto, ON, Canada M5S 1A8

Contributed by Jeffrey I. Gordon, September 24, 2004

New therapeutic targets for noncognitive reductions in energy intake, absorption, or storage are crucial given the worldwide epidemic of obesity. The gut microbial community (microbiota) is essential for processing dietary polysaccharides. We found that conventionalization of adult germ-free (GF) C57BL/6 mice with a normal microbiota harvested from the distal intestine (cecum) of conventionally raised animals produces a 60% increase in body fat content and insulin resistance within 14 days despite reduced food intake. Studies of GF and conventionalized mice revealed that the microbiota promotes absorption of monosaccharides from the gut lumen, with resulting induction of *de novo* hepatic lipogenesis. Fasting-induced adipocyte factor (Fiaf), a member of the angiopoietin-like family of proteins, is selectively suppressed in the intestinal epithelium of normal mice by conventionalization. Analysis of GF and conventionalized, normal and *Fiaf* knockout mice established that Fiaf is a circulating lipoprotein lipase inhibitor and that its suppression is essential for the microbiota-induced deposition of triglycerides in adipocytes. Studies of *Rag1*−/− animals indicate that these host responses do not require mature lymphocytes. Our findings suggest that the gut microbiota is an important environmental factor that affects energy harvest from the diet and energy storage in the host.

symbiosis | nutrient processing | energy storage | adiposity | fasting-induced adipose factor

There are now >500 million adult humans in the world who are overweight [body mass index (BMI) of 25.0–29.9 kg/m^2] and 250 million who are obese (BMI ≥ 30 kg/m^2) (1). This growing epidemic threatens both industrialized and developing countries and has been accompanied by worldwide increases in obesity-related disorders, including type II diabetes, hypertension, cardiovascular pathology, and nonalcoholic fatty liver disease. In the United States, 64% of adults are overweight or obese (2), prompting the Surgeon General to designate this condition as the most important public health challenge of our time (3). Most people are unable to make willful, lifelong dietary changes needed for weight management (4). Therefore, developing foods or identifying new therapeutic targets that produce noncognitive reductions in total energy intake, absorption, or storage has considerable importance for public health.

The human gut contains an immense number of microorganisms, collectively known as the microbiota. This community consists of at least 10^{13} citizens, is dominated by anaerobic bacteria, and includes ≈500–1,000 species whose collective genomes are estimated to contain 100 times more genes than our own human genome (5, 6). The microbiota can be viewed as a metabolic "organ" exquisitely tuned to our physiology that performs functions that we have not had to evolve on our own. These functions include the ability to process otherwise indigestible components of our diet, such as plant polysaccharides. Defining host signaling pathways regulated by the microbiota provides an opportunity to identify new therapeutic targets for promoting health. In the current study, we use normal and genetically engineered gnotobiotic mice to address the hypothesis that the microbiota acts through an integrated host signaling pathway to regulate energy storage in the host.

Materials and Methods

Animals. C57BL/6J (B6) WT and *Rag1*−/− mice were purchased from The Jackson Laboratory. B6 peroxisome proliferator-activator receptor-α (*Ppara*)−/− mice were kindly provided by F. J. Gonzales (National Institutes of Health, Bethesda) (7). Fasting-induced adipocyte factor (*Fiaf*) +/− heterozygotes on a mixed B6:129/Sv background were generated as described below, and *Fiaf*+/+, *Fiaf*+/−, and *Fiaf*−/− littermates, obtained from crosses of *Fiaf*+/− heterozygotes were compared. Animals were genotyped by using PCR protocols outlined in *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site.

Conventionally raised (CONV-R) WT and knockout mice were rederived as germ-free (GF) as described (8). GF animals were maintained in gnotobiotic isolators (8), under a strict 12-h light cycle (lights on at 0600 hours), and fed an autoclaved chow diet (B & K Universal, East Yorkshire, U.K.) ad libitum. All manipulations of mice were performed by using protocols approved by the Washington University Animal Studies Committee.

Colonization of GF Mice. The cecal contents of each 8-week-old CONV-R mouse were resuspended in 10 ml of sterile PBS, and 2-ml aliquots were spread on the fur of 7- to 10-week-old GF recipients. The resulting conventionalized (CONV-D) mice were housed in gnotobiotic isolators for 10–28 d under the same conditions and fed the same diet as their GF counterparts.

CONV-R animals were maintained in microisolator cages in a specified pathogen-free state in a barrier facility on the autoclaved B & K diet. They were transferred to gnotobiotic isolators 2 weeks before they were killed at 8–10 weeks of age to mimic the housing conditions of GF and CONV-D mice.

Eight- to 10-week-old GF mice were orally gavaged with 10^9 *Bacteroides thetaiotaomicron* strain VPI-5482. Colonization den-

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Abbreviations: GF, germ-free; Fiaf, fasting-induced adipocyte factor; B6, C57BL/6J; PPAR, peroxisome proliferator-activator receptor; CONV-R, conventionally raised; CONV-D, conventionalized; qRT-PCR, quantitative RT-PCR; LPL, lipoprotein lipase; Acc1, acetyl-CoA carboxylase; Fas, fatty acid synthase; SREBP-1, sterol response element binding protein 1; ChREBP, carbohydrate response element binding protein.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AY667702–AY668946).

‡F.B. and H.D. contributed equally to this work.

¶Present address: Department of Biochemistry and Medical Genetics, University of Manitoba, Winnipeg, MB, Canada R3E OW3.

**Present address: Center for Immunology, University of Texas Southwestern Medical Center, Dallas, TX 75390.

¶¶To whom correspondence should be addressed. E-mail: jgordon@molecool.wustl.edu.

sity in the distal intestine, cecum, and colon ranged from 10^8 to 10^{11} colony-forming units/ml luminal contents, as defined by culturing samples of luminal contents on BHI blood agar for 2–3 d at 37°C under anaerobic conditions.

Measurement of Total Body Fat Content and Metabolic Rate (Oxygen Consumption). Total body fat content was determined 5 min after mice were anesthesized with an i.p. injection of ketamine (10 mg/kg body weight) and xylazine (10 mg/kg). The protocol used for dual-energy x-ray absorptiometry (Lunar PIXImus Mouse, GE Medical Systems, Waukesha, WI) has been described (9).

Oxygen consumption was determined in conscious, individually caged mice, in a fed state, by using open-circuit indirect calorimetry (single-chamber small-animal Oxymax system, Columbus Instruments, Columbus, OH). Animals were allowed to adapt to the metabolic chamber for 20 min before VO_2 was measured every 30 s for 1 h.

SYBR-Green-Based Real-Time Quantitative RT-PCR (qRT-PCR). RNA was isolated as described in *Supporting Materials and Methods* and reverse-transcribed by using SuperScript II and dT_{15} primers (Invitrogen). qRT-PCR assays were performed as described (10), except that each 25-μl reaction contained cDNA corresponding to 1 ng of total RNA and 900 nM gene-specific primers (Table 1, which is published as supporting information on the PNAS web site). All assays were performed in triplicate with an ABI Prism 7700 Sequence Detector (Applied Biosystems). Data were normalized to L32 RNA ($\Delta\Delta C_T$ analysis).

Assays of Lipoprotein Lipase (LPL). LPL activity in epididymal fat pads was determined according to ref. 11. For details see *Supporting Materials and Methods*.

Statistics. Statistically significant differences were determined by using Student's *t* tests. Comparisons between more than two groups of mice were made by a one-way ANOVA followed by Tukey's post hoc multiple comparison test.

Results and Discussion

Introduction of a Gut Microbiota into Adult GF Mice Produces a Rapid Increase in Body Fat Content Despite Reduced Chow Consumption. Comparisons of 8- to 10-week-old male B6 mice raised in the absence of any microorganisms (GF) with mice that harbored a microbiota beginning at birth (CONV-R) revealed that CONV-R animals contain 42% more total body fat, as defined by dual energy x-ray absorptiometry (Fig. 1*A*). Epididymal fat pad weights were also significantly greater (47%; Fig. 1*B*). The higher levels of body fat observed in CONV-R animals is intriguing given that their daily consumption of a standard rodent chow diet (57% carbohydrates, 5% fat) was 29% less than their GF counterparts (Fig. 1*C*).

A 14-d colonization of 8- to 10-week-old male GF B6 recipients with an unfractionated microbiota harvested from the distal intestines (cecums) of adult CONV-R donors, a process known as conventionalization, produced a dramatic 57% increase in their total body fat content (Fig. 1*A*) and a 61% increase in epididymal fat weight (Fig. 1*B*). The increase in body fat was associated with a 7% decrease in lean body mass, resulting in no significant differences in total body weight between the two groups [23.5 ± 2.6 g (GF) versus 23.4 ± 2.6 g (CONV-D); $n = 21$; $P > 0.05$]. Fasting serum triglyceride values were similar ($P > 0.05$) in both GF and CONV-D mice (data not shown).

A similar increase in total body fat content was observed after a shorter, 10-d conventionalization (66%; $P > 0.05$ compared to 14 d). A more prolonged conventionalization (28 d) did not produce further increments in total body fat content or epididymal fat pad weight (data not shown). The increased fat storage

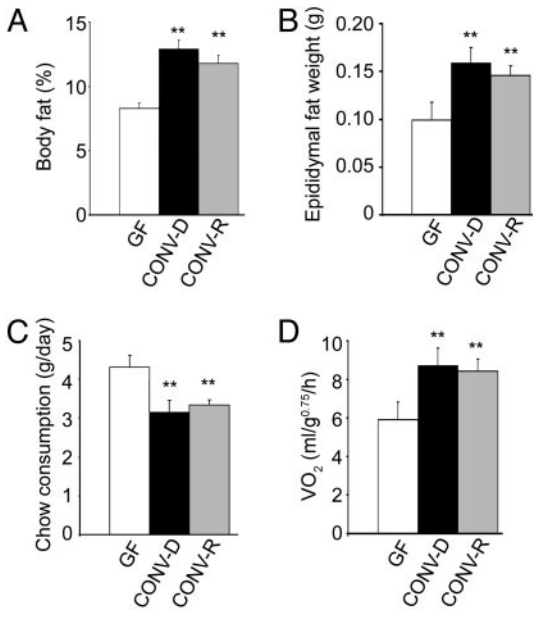

Fig. 1. Phenotyping WT gnotobiotic mice. Three groups of 8- to 10-week-old adult male B6 mice [those raised in a GF state, those allowed to acquire a microbiota from birth to adulthood (CONV-R), and those raised GF until adulthood and then colonized for 2 weeks with an unfractionated cecal microbiota harvested from CONV-R donors (CONV-D)] were analyzed for total body fat content by dual energy x-ray absorptiometry ($n = 21$–25 per group) (*A*), epididymal fat weight ($n = 10$–20 per group) (*B*), chow consumption (average daily value over the 3 d before termination of the experiment; $n = 10$ per group) (*C*), and oxygen consumption (defined by open circuit calorimetry just before the animals were killed; $n = 10$ per group) (*D*). Mean values ± SEM are plotted. **, $P < 0.01$ compared with GF.

produced by a 14-d conventionalization also occurred in the face of decreased chow consumption (27% lower than GF; Fig. 1*C*).

These effects were not unique to males: CONV-D B6 females exhibited increases in total body fat (85%) and reductions in lean body mass (9%) that were not significantly different from age-matched males ($P > 0.05$). In addition, the fat storage phenotype was not limited to the B6 inbred strain: a 14-d conventionalization of 8-week-old male mice belonging to the NMRI inbred strain produced a 90% increase in total body fat content ($P < 0.01$ compared with GF) and a 31% decrease in chow consumption ($P < 0.05$).

Sequence-based 16S rDNA enumeration studies of the cecal microbiota revealed great similarities in the fractional representation of the predominant species in CONV-R donors and CONV-D B6 recipients (Fig. 6 and Table 2, which are published as supporting information on the PNAS web site). As in many humans, Bacteroides and Clostridium were the most prevalent genera. *B. thetaiotaomicron* is a prominent member of the human distal gut microbiota with an extraordinary capacity for acquiring and degrading plant polysaccharides (12). For example, its proteome contains 172 glycosylhydrolases that are predicted to cleave most glycosidic linkages encountered in human diets. Studies in GF mice colonized with *B. thetaiotaomicron* have shown that its polysaccharide processing activity is associated with induction of host monosaccharide transporters (13). Therefore, we colonized 8-week-old male GF B6 mice for 2 weeks with the sequenced strain (VPI-5482) to determine whether a single saccharolytic bacterial species could, by itself, effect host fat storage. Colonization produced a statistically significant increase in total body fat content, although the magnitude of the increase was less than that obtained with an unfractionated cecal micro-

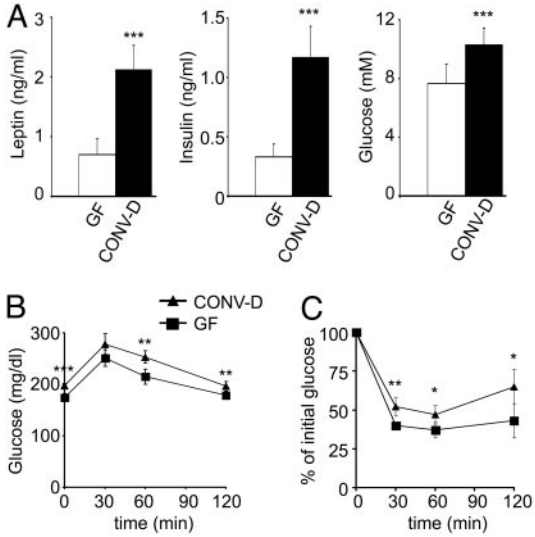

Fig. 2. A 14-d conventionalization of WT GF B6 mice increases circulating leptin levels and decreases sensitivity to insulin. (*A*) Sera were obtained after a 4-h fast and analyzed for leptin, insulin, and glucose ($n = 8$ animals per group). Numbers represent mean values ± SEM. Glucose tolerance (*B*) and insulin tolerance (*C*) tests were performed after a 4-h fast ($n = 8$ mice per group). Mean values ± SEM are plotted. ***, $P < 0.001$; **, $P < 0.01$; and *, $P < 0.05$ compared with GF.

biota (23% versus 57%, respectively; $n = 10$ mice per group; $P < 0.01$).

Metabolic Rate Is Higher in CONV-D Mice Than in Their GF Counterparts. Because the microbiota-mediated increase in body fat content was not caused by increased chow consumption, open-circuit indirect calorimetry was performed to determine whether it reflected decreased energy expenditure. This explanation was excluded when we found that the leaner GF mice had a metabolic rate (VO_2) that was 27% lower than age- and gender-matched (male) B6 mice conventionalized for 14 d ($P < 0.01$; Fig. 1*D*). CONV-D mice had VO_2 values that were not significantly different from age- and gender-matched CONV-R animals (Fig. 1*D*).

The increase in VO_2 observed with conventionalization could reflect increased metabolic rate in the host and/or the metabolic activity of their recently acquired microbial community. There are no available methods for measuring the metabolic activity of the microbiota *in vivo*. However, microanalytic biochemical assays of freeze-clamped gastrocnemius muscle and liver revealed significant increases in the steady-state levels of tricarboxylic acid cycle intermediates in CONV-D versus GF animals. Despite this evidence of increased cycle activity, there were no significant alterations in tissue high-energy phosphate stores ($n = 5$ animals per group; Table 3, which is published as supporting information on the PNAS web site). Increasing oxygen consumption without increasing high-energy phosphate stores implies the presence of futile cycles, a biochemical correlate of inefficient metabolism in the host (see below).

Leptin is an adipocyte-derived hormone whose expression correlates with adipocyte lipid content (14). Moreover, leptin is known to reduce food intake and increase energy expenditure in mice (15). Leptin levels increase upon colonization (Fig. 2*A*). The increase is proportional to the increase in body fat ($r^2 = 0.977$).

The increase in fat content was accompanied by statistically significant elevations in fasting glucose and insulin levels (Fig. 2*A*) and an insulin-resistant state, as defined by glucose and insulin tolerance tests (Fig. 2 *B* and *C*).

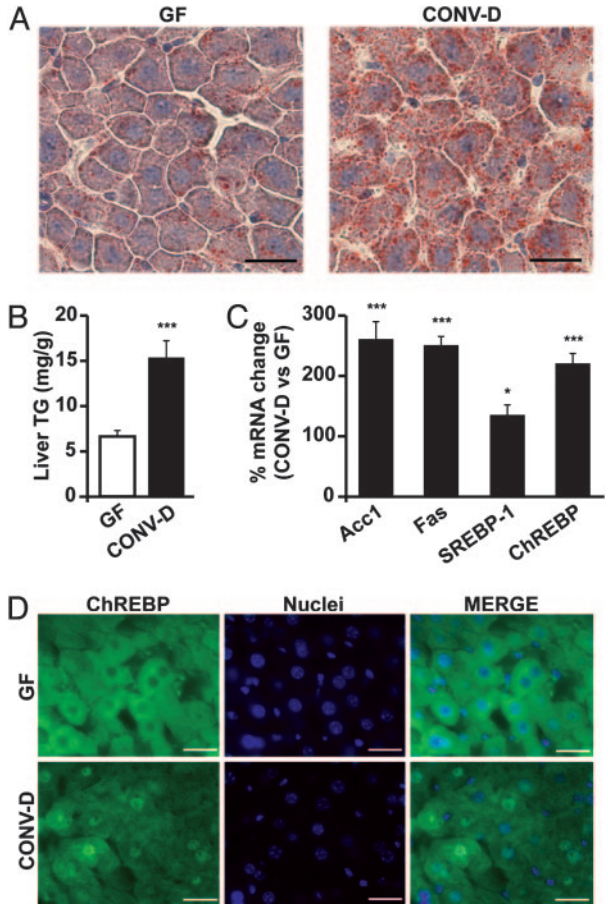

Fig. 3. Conventionalization induces hepatic lipogenesis and nuclear import of the basic helix–loop–helix transcription factor ChREBP. (*A*) Oil-red O stains of paraformaldehyde-fixed liver sections prepared from 8-week-old WT male GF and CONV-D B6 mice. (*B*) Liver triglyceride (TG) levels ($n = 5$ per group; mean values ± SEM; ***, $P < 0.001$ compared to GF). (*C*) qRT-PCR assays of liver RNAs from GF and CONV-D mice [$n = 15$ per group; mean values ± SEM are expressed relative to levels in GF animals (GF set at 100%); *, $P < 0.05$; ***, $P < 0.001$ compared with GF]. (*D*) Immunohistochemical study of paraformaldehyde-fixed sections of livers from GF or CONV-D mice. Sections were stained with rabbit polyclonal antibodies to mouse ChREBP (green). Nuclei are labeled dark blue with 4′,6-diamidino-2-phenylindole. (Bars: 25 μm.)

The Microbiota Directs the Host to Increase Hepatic Production of Triglycerides. Glucose and insulin are known to induce expression of lipogenic enzymes in the liver (16). A 14-d conventionalization of GF mice produced a 2.3-fold increase in liver triglyceride content (Fig. 3 *A* and *B*), but no appreciable changes in total liver free fatty acids or cholesterol ($P > 0.05$; data not shown). qRT-PCR assays confirmed that conventionalization was accompanied by statistically significant elevations in liver mRNAs encoding two key enzymes in the *de novo* fatty acid biosynthetic pathway, acetyl-CoA carboxylase (Acc1), and fatty acid synthase (Fas) (Fig. 3*C*).

Sterol response element binding protein 1 (SREBP-1) and carbohydrate response element binding protein (ChREBP), two basic helix–loop–helix/leucine zipper transcription factors, mediate hepatocyte lipogenic responses to insulin and glucose and appear to act synergistically (17). Both *Acc1* and *Fas* are known targets of ChREBP and SREBP-1 (16). qRT-PCR assays of liver RNAs revealed that conventionalization increases liver

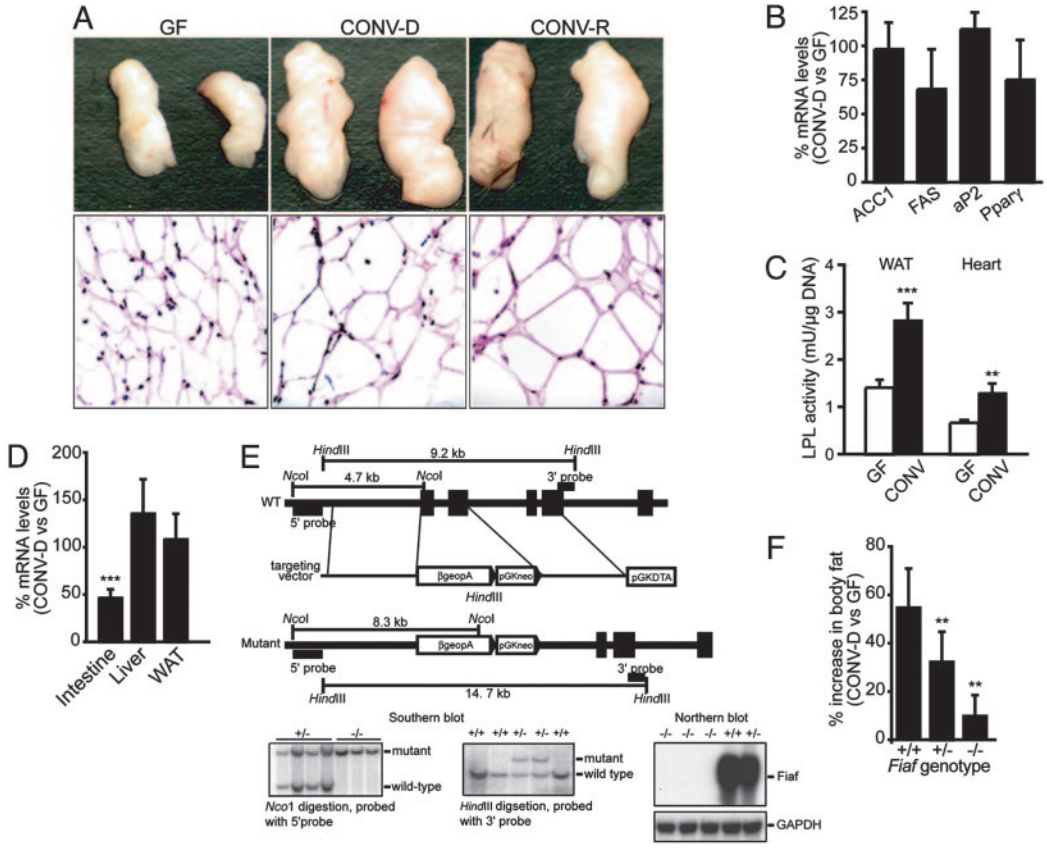

Fig. 4. Conventionalization promotes adipocyte hypertrophy by suppressing *Fiaf* expression in the intestine. (*A Upper*) Epididymal fat pads from 8-week-old WT male GF, CONV-D, and CONV-R B6 mice. (*A Lower*) The corresponding hematoxylin- and eosin-stained sections are shown. (*B*) qRT-PCR assays of epididymal fat pad RNAs harvested from WT mice reveal that conventionalization does not produce significant changes in expression of mediators or biomarkers of lipogenesis and adipogenesis (mean values ± SEM are plotted; $P > 0.05$; $n = 15$ per group). (*C*) LPL activity is increased upon conventionalization in both epididymal fat pads and heart of WT mice (**, $P < 0.01$ and ***, $P < 0.001$ compared to GF; $n = 5$ per group). (*D*) qRT-PCR assays of *Fiaf* expression in WT animals [***, $P < 0.001$ compared to GF (set at 100%); $n = 13$]. (*E*) Generation of *Fiaf* knockout mice. Structures are shown for the WT *Fiaf* locus, the targeting vector, and the mutated locus with exons 1–3 replaced by a βgeopA cassette. The desired disruption was verified by Southern blot analysis. Northern blots of adipocyte RNA establish the absence of detectable Fiaf mRNA in *Fiaf*−/− animals. (*F*) The absence of Fiaf markedly attenuates the increase in total body fat content after a 14-d conventionalization (**, $P < 0.01$ compared with WT; $n = 7–8$ per group).

ChREBP mRNA, and to a lesser extent, SREBP-1 mRNA levels (Fig. 3*C*).

ChREBP is translocated from the cytoplasm to the nucleus after it is dephosphorylated by the serine/threonine phosphatase PP2A (18, 19). PP2A, in turn, is activated by xylulose-5-phosphate (Xu5P) (20), an intermediate in the hexose monophosphate shunt. Mice colonized with a microbiota had elevated levels of liver Xu5P compared with their GF counterparts (1.6 ± 0.4 versus 2.6 ± 0.3 μmol/g wet weight of liver; $P < 0.01$; see Scheme 1, which is published as supporting information on the PNAS web site for assay) and more nuclear-localized ChREBP (Fig. 3*D*).

We obtained direct biochemical evidence that the presence of the microbiota promotes increased monosaccharide uptake from the gut. GF mice and their CONV-D counterparts ($n = 4$ per group) were given a single gavage of 100 μl of a mixture of 5 mM glucose and 0.2 mM 2-deoxyglucose and killed 15 min later, and 2-deoxyglucose 6-phosphate levels were measured in their distal intestines. Levels were 2-fold higher in CONV-D mice (1.15 ± 0.013 versus 0.55 ± 0.04 pmol/μg protein; $P <$ 0.001). Once taken up into the intestine, transfer of monosaccharides to the portal circulation is facilitated through an additional effect of the microbiota. We have shown previously that conventionalization results in a doubling of the density of capillaries that underlie the small intestinal villus epithelium to levels equivalent to that of age-matched CONV-R animals (21).

Together, these findings are consistent with an increase in processing of dietary polysaccharides by microbial glycosylhydrolases in CONV-D mice, increased delivery of monosaccharides to their livers, and increased transactivation of lipogenic enzymes by ChREBP and perhaps SREBP-1. The liver has at least two ways of responding to this augmented delivery of calories: increasing inefficient metabolism (futile cycles) and exporting these calories in the form of fat for deposition in peripheral tissues.

The Microbiota Promotes Storage of Triglycerides in Adipocytes Through Suppression of Intestinal Expression of a Circulating LPL Inhibitor. The DNA content of epididymal fat pads recovered from GF and CONV-D mice were not significantly different. This observation, together with histochemical studies, allowed us to conclude that the microbiota-induced increase in epididymal fat pad weight reflected adipocyte hypertrophy (Fig. 4*A*). Moreover, qRT-PCR analyses of fat pad RNA revealed that neither biomarkers of adipogenesis (aP2 and PPAR-γ) or lipogenesis (Acc1 and Fas) were significantly changed after conventionalization (Fig. 4*B*).

LPL is a key regulator of fatty acid release from triglyceride-rich lipoproteins in muscle, heart, and fat (22). Increased adi-

pocyte LPL activity leads to increased cellular uptake of fatty acids and adipocyte triglyceride accumulation. In white fat, LPL is regulated posttranscriptionally by nutritional status: fasting reduces and refeeding increases enzyme activity (23). Intriguingly, we found that a 14-d conventionalization increased LPL activity 122% in epididymal fat pads (Fig. 4*C*). Moreover, the effect was not confined to fat: enzymatic assays of heart revealed a 99% increase with conventionalization (Fig. 4*C*). Increased insulin levels produce reductions in muscle LPL activity (24). Therefore, our findings indicated that the microbiota induces the observed general increase in LPL through another mechanism.

Fiaf, also known as angiopoietin-like protein 4, is produced by brown and white fat, liver, and intestine (13, 25, 26). This secreted protein is an inhibitor of LPL *in vitro* [IC_{50} = 200 nM (27)]. qRT-PCR analysis of intestinal *Fiaf* expression during the postnatal period disclosed that the gene is induced in GF mice during the suckling-weaning transition. Induction does not occur in CONV-R animals, resulting in significantly lower levels of Fiaf mRNA in adult CONV-R versus GF intestine (Fig. 7, which is published as supporting information on the PNAS web site). During the suckling-weaning transition, the diet switches from lipid/lactose-rich mother's milk to low-fat/polysaccharide-rich chow, with coincident expansion of the microbiota and a shift from facultative to obligate anaerobes (e.g., Bacteroides). These observations suggested that *Fiaf* could provide a signal that links conventionalization with a change in host energy partitioning.

qRT-PCR assays disclosed that conventionalization of adult GF mice suppressed *Fiaf* expression in their small intestines (ileum), but not in their livers or white fat (Fig. 4*D*). Follow-up qRT-PCR studies of laser capture microdissected intestinal crypt and villus epithelium, and the villus mesenchyme, established that microbial suppression of *Fiaf* occurs in differentiated villus epithelial cells (data not shown).

Together these findings suggest that the microbiota acts to stimulate hepatic triglyceride production through effects mediated by transcription factors such as ChREBP and to promote LPL-directed incorporation of these triglycerides into adipocytes through transcriptional suppression of an intestinal epithelial gene encoding a circulating LPL inhibitor. We tested this hypothesis by generating mice with a null *Fiaf* allele (Fig. 4*E*) and rederiving them as GF.

Eight-week-old male GF *Fiaf*$-/-$ mice have 67% higher epididymal fat pad LPL activity than GF littermates containing the WT *Fiaf* allele ($P < 0.01$), confirming that Fiaf is an important inhibitor of this lipase *in vivo*. Conventionalization of GF knockout mice did not produce significant changes in LPL activity in fat pads (or heart) ($P > 0.05$; $n = 10$ animals).

GF *Fiaf*$-/-$ animals have the same amount of total body fat as their age- and gender-matched CONV-D (*Fiaf*-suppressed) WT littermates (12.8 ± 1.1% of body weight versus 14.2 ± 1.9, $P > 0.05$). Moreover, a 14-d conventionalization of already *Fiaf*-deficient GF knockout animals produced only minor increases in total body fat (10 ± 8% increase versus 55 ± 16% increase in WT littermates; Fig. 4*F*). *Fiaf*$+/-$ heterozygotes had an intermediate increase (33 ± 12%). These results establish the importance of Fiaf as a prominent mediator of microbial regulation of peripheral fat storage.

The Effect of the Microbiota on *Fiaf* Expression and Fat Storage Does Not Depend on Mature Lymphocytes or PPAR-α. We found that the zebrafish homolog of mouse *Fiaf* is suppressed by the microbiota when GF fish are conventionalized, indicating that this response has been highly conserved over the course of vertebrate evolution (28). We applied two methods to identify conserved regulatory elements in the 10 kb of DNA sequence 5′ to the transcriptional start site of human, mouse, rat, zebrafish, and fugu *Fiaf* orthologs. First, we searched for novel motifs by using PHYLOCON (29). Two statistically significant motifs were identified: one overlaps with the PPAR binding site; the other is similar to the Heb binding site, which contains an E-box (Fig. 8*A*, which is published as supporting information on the PNAS web site). Second, we searched the TRANSFAC database (30) of 466 vertebrate-specific transcription factor scoring matrices with PATSER (G. Hertz and G. Stormo, personal communication, http://ural.wustl.edu) for high-scoring binding sites that appear in all five *Fiaf* orthologs and in conserved sequence blocks between the human and mouse genes. More than 40 matrices satisfied these two selection criteria (Table 4, which is published as supporting information on the PNAS web site), including sites recognized by several fork head domain-containing factors (e.g., HNF3, HNF4α, and FKH8) and an IFN-stimulated response element (Fig. 8).

Fiaf was identified during a screen for PPAR-α targets in liver (25). PPAR-α is an important regulator of energy metabolism in a variety of tissues including intestine, liver, heart, and kidney (31). We found that PPAR-α mRNA levels decrease modestly (1.7 ± 0.2-fold) in the small intestines of CONV-D compared with GF animals, but remain unchanged in their livers and fat pads ($P < 0.05$; see Fig. 9, which is published as supporting information on the PNAS web site). To directly test the role of PPAR-α in regulating the microbiota-directed change in body fat content and suppression of *Fiaf*, B6 *Ppara* knockout mice were rederived as GF. Eight- to 10-week-old male GF *Ppara*$-/-$ mice had the same amount of total body fat as their age- and gender-matched GF WT littermates (Fig. 9). Moreover, *Ppara*$-/-$ animals had no impairment in their microbiota-induced increase in body fat content (Fig. 9). Finally, qRT-PCR assays of intestinal RNAs isolated from GF and CONV-D WT and *Ppara*$-/-$ mice indicated that the absence of PPAR-α did not prevent transcriptional suppression of *Fiaf* upon conventionalization (Fig. 9). We concluded that the host fat storage response to the microbiota does not require PPAR-α. A comparable analysis of the role of PPAR-γ could not be performed because *Pparg*$-/-$ mice die at embryonic day 10 (29).

Finding a conserved IFN-stimulated response element in the orthologous *Fiaf* genes was intriguing in light of our previous GENECHIP analyses of intestinal RNAs that revealed that conventionalization of B6 GF mice regulates expression of a number of genes involved in B and T cell responses (28). Therefore, we rederived B6 *Rag1*$-/-$-deficient mice as GF to determine whether the presence or absence of mature T and B cells had an effect on the capacity of the microbiota to increase body fat content or modulate *Fiaf*. *Rag1*$+/+$ and *Rag1*$-/-$ littermates had equivalent increases in body fat content after a 14-d conventionalization (59 ± 16% versus 67 ± 16%; $P > 0.05$) and similar degrees of *Fiaf* suppression (2.8 ± 0.3- and 3.8 ± 0.3-fold, respectively; $P < 0.05$ compared with GF). Thus, it appears that

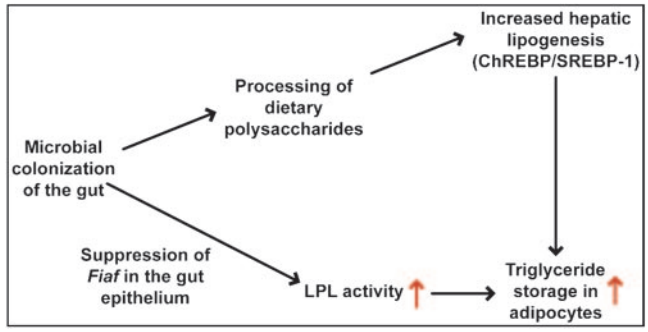

Fig. 5. Schematic view of how the gut microbiota effects host fat storage. The microbiota acts through Fiaf to coordinate increased hepatic lipogenesis with increased LPL activity in adipocytes, thereby promoting storage of calories harvested from the diet into fat. See text for further details.

these cellular components of the adaptive immune system are not required to process signals or metabolic products emanating from the gut microbiota that promote fat storage.

Prospectus: The Microbiota as an Environmental Factor That Affects Predisposition Toward Adiposity. Adult humans are composed of an estimated 10 times more resident microbial than human cells (5). Our microbial partners have coevolved with us to forge mutually beneficial (symbiotic) relationships. These relationships are typically founded on nutrient sharing. The studies described in this paper indicate that one manifestation of this symbiotic relationship is microbial processing of components of the diet and deposition the extracted energy in host fat depots. The ability to store energy would be a beneficial attribute for ancient humans who had variable access to food. However, in modern, developed societies, where there is ready access to large-portion, high-calorie diets, this "benefit" becomes a detriment.

Our finding that microbial suppression of intestinal *Fiaf* promotes adiposity, through the mechanism summarized in Fig. 5, suggests that increasing Fiaf expression and/or activity may promote leanness. We also speculate that changes in microbial ecology prompted by Western diets, and/or differences in microbial ecology between individuals living in these societies, may function as an "environmental" factor that affects predisposition toward energy storage and obesity.

We thank David O'Donnell, Maria Karlsson, Jill Manchester, Sabrina Wagoner, Trey Coleman, and Xiaoli Wu for technical assistance and John Rawls, Peter Kang, Peter Crawford, and Justin Sonnenburg for helpful advice. This work was supported in part by National Institutes of Health Grants DK30292, DK56341, and HL58427 and the Canadian Institute of Health Research. F.B. is the recipient of a postdoctoral fellowship from the Wenner-Gren Foundation.

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REVIEW

doi:10.1038/nature11552

Functional interactions between the gut microbiota and host metabolism

Valentina Tremaroli[1,2] & Fredrik Bäckhed[1,2,3]

The link between the microbes in the human gut and the development of obesity, cardiovascular disease and metabolic syndromes, such as type 2 diabetes, is becoming clearer. However, because of the complexity of the microbial community, the functional connections are less well understood. Studies in both mice and humans are helping to show what effect the gut microbiota has on host metabolism by improving energy yield from food and modulating dietary or the host-derived compounds that alter host metabolic pathways. Through increased knowledge of the mechanisms involved in the interactions between the microbiota and its host, we will be in a better position to develop treatments for metabolic disease.

Changes to lifestyle and an increase in the availability of energy-rich foods are important contributors to the worldwide obesity epidemic. The microbial inhabitants of the gut can also have an influence on metabolic processes, such as energy extraction from food, and should be considered an environmental factor that contributes to obesity and its comorbidities (such as insulin resistance, diabetes and cardiovascular disease).

Culture-independent methods to study microbial communities have advanced our knowledge of this human gut microbiota (Box 1) (see page 250 of this issue). Profiling of the common proxy for this community, the faecal microbiota, by 16S ribosomal RNA surveys and by direct sequencing of genetic material have shown that the human gut microbiota is a complex community of 100 trillion archaeal and bacterial cells distributed over more than 1,000 species[1] (Box 2). The community is dominated by bacteria, with more than 90% of the species belonging to Firmicutes and Bacteroidetes. Each person has a distinct and highly variable microbiota, but a conserved set of gut colonizers (the core gut microbiota) and genes (the core microbiome) are shared among individuals[1,2] and may be required for the correct functioning of the gut.

Germ-free mice are those born and reared without exposure to any live microbes, and they provide a powerful tool for understanding the effects of the gut microbiota on host physiology. These mice can be colonized either with selected microbial species or whole communities from mice or humans to examine the transmissibility of physiological and pathological phenotypes, and to test what role the microbiota has in a particular phenotype. The gut microbiota in these mice modulates bone-mass density[3] and promotes fat storage[4], intestinal angiogenesis[5,6] and the development of an immune response[7,8] (see page 231 of this issue). In this Review, we discuss the metagenomic and gnotobiotic-based evidence for the role of the gut microbiota in energy metabolism and the possible links with obesity.

Obesity

Gut microbiota composition is altered in people who are obese, and it can respond to changes in body weight. Genetically obese *ob*/*ob* mice[9] are hyperphagic as a result of a mutation in the gene that encodes the satiety-promoting hormone leptin. The caecal microbiota of these mice contains more Firmicutes and fewer Bacteroidetes than that of their lean wild-type littermates, even when the mice are fed the same low-fat, polysaccharide-rich diet[9]. Similar changes have also been seen in the faecal microbiota of humans who are obese[10]. Bacteroidetes levels increase when weight is reduced, either by fat- or carbohydrate-restricted diets[10], suggesting that Bacteroidetes may be responsive to calorie intake. A similar effect has also been observed in people who lost weight after a Roux-en-Y gastric bypass procedure. In these patients, increased levels of *Bacteroides* and *Prevotella* were negatively correlated with energy intake and adiposity[11]. Other studies showed no such shift in the Firmicutes–Bacteroidetes ratio[12–14], but this may be because they used different clinical criteria (such as level of obesity, age, degree of weight loss and duration of calorie restriction), geographical locations, population sizes and microbiota-profiling methodologies. Although obesity and energy intake can affect the microbial composition, whether the gut microbiota contributes to obesity in humans is unclear.

A gastric bypass promotes sustained weight reduction and diminishes the risk of diabetes and cardiovascular disease for people who are obese[15,16]. This knowledge has allowed the relationship between microbiota and obesity to be explored further. After a gastric bypass, diabetes can resolve before patients begin to lose weight, suggesting that this type of surgery has a direct antidiabetic effect. Exactly how this happens is not clear, but a shift in the composition of the faecal microbiota of humans[11,14] suggests the gut microbiota contributes to the improved metabolic phenotype after a gastric bypass. The beneficial microbe *Faecalibacterium prausnitzii*, in particular, is less abundant in patients who are obese and diabetic, but increases after surgery[11]. Levels of *F. prausnitzii* are negatively correlated with inflammatory markers, indicating that the bacterium may modulate systemic inflammation (common to diabetes and obesity) and contribute to the amelioration of diabetes. In addition, germ-free mice do not develop diet-induced obesity, and treatment of obese mice with antibiotics reduces adiposity and adipose inflammation, and improves glucose metabolism[17–19], further supporting the benefits of inducing a shift in microbiota composition.

Energy harvest

Carbohydrates are important sources of energy for human and microbial cells. Human enzymes cannot degrade most complex carbohydrates and plant polysaccharides. Instead, the non-digestible

[1]Wallenberg Laboratory for Cardiovascular and Metabolic Research, Sahlgrenska University Hospital, 413 45 Gothenburg, Sweden. [2]Department of Molecular and Clinical Medicine, University of Gothenburg, 413 45 Gothenburg, Sweden. [3]Novo Nordisk Foundation Center for Basic Metabolic Research, Faculty of Health Sciences, University of Copenhagen, Copenhagen DK-2200, Denmark.

carbohydrates, including cellulose, xylans, resistant starch and inulin, are fermented in the colon by its microbiota to yield energy for microbial growth and end products such as short-chain fatty acids (SCFAs) (Fig. 1), mainly acetate, propionate and butyrate, which have profound effects on gut health as, for example, an energy source, an inflammation modulator, a vasodilator and part of gut motility and wound healing. In addition, SCFAs are energy substrates for the colonic epithelium (butyrate) and peripheral tissues (acetate and propionate)[20]. The patterns of intestinal fermentation, and consequently the types and amount of SCFAs produced, are determined by how much carbohydrate is consumed and the composition of the gut microbiota. For example, fermentation of dietary fructans increases when gnotobiotic mice that have been colonized with *Bacteroides thetaiotaomicron*, are co-colonized with *Methanobrevibacter smithii*[21]. *B. thetaiotaomicron* produces more acetate and formate, and *M. smithii* uses formate for methanogenesis. The interactions promote more efficient carbohydrate fermentation and increased energy absorption from the gut, resulting in increased adiposity in the co-colonized mice compared with mice colonized with only *B. thetaiotaomicron*. The composition of the gut microbiota and the metabolic interactions between its species may therefore affect food digestion and energy harvest.

Direct evidence for the role of the microbiota in energy harvest and fat deposition comes from germ-free rats, which have reduced intestinal levels of SCFAs[22], and twice as much urinary and faecal excretion of calories as that of conventional rats fed the same polysaccharide-rich diet[23]. The germ-free rodents compensate for the reduced energy harvest by increasing their food intake[23]. Germ-free mice also have reduced adiposity compared with their conventional counterparts, but adiposity is normalized when they are colonized with a healthy microbiota for 14 days[4,19]. Microbial energy harvest in obesity has been investigated in conventional genetically obese *ob*/*ob* mice, which have increased amounts of SCFAs in their caecum and reduced energy content in their faeces compared with their lean littermates[24]. Metagenomic sequencing of the caecal microbiota showed an enrichment of gene functions that were related to the degradation of dietary polysaccharides in the microbiome of *ob*/*ob* mice[24]. This finding was also true of humans: the faecal microbiota of people who are obese has an increased capacity to harvest energy[2]. In mice, the obese phenotype was transmissible through microbiota transplants, and germ-free mice colonized with the microbiota from obese donors gained twice as much fat as those colonized with the microbiota from lean donors[24].

The role of the gut microbiota in promoting energy harvest from diet and fat deposition has been clearly demonstrated in mice, but most of the evidence in humans has come from indirect studies. For instance, people who are obese have higher levels of ethanol in their breath than lean people[25], indicating altered fermentation and a greater number of faecal SCFAs[13], which may suggest increased microbial energy harvest.

Diet alters the gut microbiota

Diet is known to modulate the composition of the gut microbiota in humans and mice. Long-term dietary habits have a considerable effect on the human gut microbiota. For example, children in a rural African village, who consumed high amounts of plant polysaccharides, had low levels of Firmicutes and increased levels of Bacteroidetes — mainly *Prevotella* and *Xylanibacter* — in their faecal microbiota compared with Italian children, who had high levels of Enterobacteriaceae — mainly *Shigella* and *Escherichia*[26]. *Prevotella* and *Xylanibacter* are known to degrade cellulose and xylans, and are associated with increased faecal SCFAs, suggesting that the gut microbiota of the children living in rural Africa had adapted to maximize energy extraction from a diet rich in fibre. Human gut microbiota can be divided into three discrete compositions. However, this concept is currently being challenged as enterotypes maybe more of a gradient than discrete entities. Each enterotype is dominated by a different genus — *Bacteroides*, *Prevotella* or *Ruminococcus*[27] — but not affected by gender, age or nationality[27]. Enterotypes dominated by *Bacteroides* or *Prevotella* are associated with the consumption of a diet rich in protein and animal fat, or carbohydrates, respectively[28]. The *Ruminococcus* enterotype is not well separated and is partly merged with the *Bacteroides* enterotype[28]. This division supports the association between *Prevotella* and a diet high in carbohydrates, which was seen in children from rural Africa[26]. A 10-day dietary intervention, however, was not sufficient to alter the enterotype of an individual[28],

BOX 1
Terminology

- **Enterotype** is the grouping of the microbiota of a given person into discrete configurations. But recent data have conflicted with this definition and suggest that enterotypes may be less discrete.
- **Gnotobiotics** is the study of animals living in a microbiologically defined environment, either germ-free or colonized with known bacteria.
- **Inflammasomes** are protein complexes containing an intracellular sensor (such as a nucleotide-binding oligomerization domain (NOD)-like receptor), the procaspase-1 precursor and the ASC (apoptosis-associated speck-like protein containing a caspase activation and recruitment domain) adaptor protein. These complexes recognize microbe- and the host-derived inflammatory signals, microbial-associated molecular patterns and damage-associated molecular patterns, and its activation leads to the maturation of inflammatory cytokines (such as interleukin-1β and interleukin-18). Inflammasomes participate in antimicrobial innate immune responses, but may also have a role in metabolic diseases, such as obesity, type 2 diabetes and atherosclerosis.
- **Metagenome** is the total DNA that can be extracted from an environment. The human metagenome is the aggregate of the DNA of the host and the microbiota. Metagenome and microbiome are often used interchangeably.
- **Metagenomics** is the study of the metagenome (microbiome). Metagenomics can either be targeted (usually 16S ribosomal RNA) or untargeted (shotgun sequencing).
- **Microbiota** is the collective microbial community inhabiting a specific environment. Cellular density increases along the length of the gut, and the colonic microbiota is the densest and most diverse community in the gut, and in the whole human body.
- **Microbiome** is the collective genomic content of a microbiota. It also indicates the total genetic capacity of the community.
- **Probiotics** are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit for the host[84]. Many bacterial strains in the *Lactobacillus* and *Bifidobacterium* genera are considered to be probiotic.
- **Prebiotics** are non-digestible food ingredients that, when consumed in sufficient amounts, selectively stimulate the growth, activity or both of one or a limited number of microbial genera or species in the gut microbiota that confer(s) health benefits to the host[85]. Inulin and *trans*-galacto-oligosaccharides are defined as prebiotics because they are resistant to gastric digestion and hydrolysis by human enzymes; are fermented by specific members of the gut microbiota; and induce selective growth, activity or both of beneficial intestinal bacteria[85]. Both inulin and *trans*-galacto-oligosaccharides stimulate the growth of *Bifidobacterium*, an effect defined as bifidogenic.

BOX 2

Dominant microbes

The human gut microbiota is dominated by five bacterial phyla (Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria and Verrucomicrobia) and one Archaea (Euryarchaeota). The less prevalent bacterial groups are distributed among Cyanobacteria, Fusobacteria, Lentisphaerae, Spirochaetes and TM7.

The Firmicutes phylum contains relevant genera, including *Ruminococcus, Clostridium*, *Lactobacillus* (several strains of which are probiotics), and the butyrate producers *Eubacterium*, *Faecalibacterium* and *Roseburia*.

In Bacteroidetes, *Bacteroides*, *Prevotella* and *Xylanibacter* degrade a variety of complex glycans.

The Actinobacteria phylum includes *Collinsella* and *Bifidobacterium* (which contains probiotic strains). Common Proteobacteria are *Escherichia* (from the Enterobacteriaceae family) and *Desulfovibrio* (which contains sulphate-reducing bacteria). Verrucomicrobia was recently discovered and includes *Akkermansia* (which are specialized for mucus degradation). Euryarchaeota contains the prevalent Methanobrevibacter (which is involved in the continuation of intestinal methanogenesis).

suggesting that a long-term change may be required to provoke a major shift in gut microbiota composition.

Changes in daily carbohydrate intake may affect specific groups of colonic bacteria over a short period of time. Consumption of the prebiotic inulin increases the levels of *F. prausnitzii* and *Bifidobacterium* sp. in humans[29]. Similarly, prebiotics promote a selective increase in *Bifidobacterium* sp. in diet-induced obese mice, and this increase is correlated with reduced adiposity and levels of microbe-derived inflammatory molecules, such as lipopolysaccharide, compared with mice that are fed a high-fat diet without prebiotics[30]. Human diets that are supplemented with resistant starch have increased faecal levels of *Ruminococcus bromii* and *Eubacterium rectale*, which correlates with fibre fermentation[31]. Consumption of resistant starch also improves insulin sensitivity[32], but the variation in the microbial response to changes in resistant starch between individuals suggests successful dietary interventions need to be personalized[31].

The gut microbiota also reacts to dietary fat. Mice fed on high-fat diets have reduced numbers of Bacteroidetes, and increased numbers of Firmicutes and Proteobacteria[33,34]. This change is rapid, occurring within 24 hours[35]. Transplantation of the caecal microbiota from obese mice fed on high-fat diets into germ-free recipients increases adiposity significantly more than transplantation of a lean microbiota[34]. The altered microbial community of obese mice seems to have some role in promoting diet-induced obesity, but the mechanisms that cause this are unknown. A change in diet clearly alters the gut microbiota, and these alterations may contribute to the host's metabolic phenotype. Further metatranscriptomic and proteomic studies should provide insight into the response of microbial function as a result of a dietary shift.

Microbial processing of food constituents

Products of microbial metabolism act as signalling molecules and influence the host's metabolism. Microbial products directly affect intestinal function but may also affect the liver and brain, as well as adipose and muscle tissue, which consequently may affect the level of obesity and the associated comorbidities (Fig. 2). Microbial enzymatic activities can act directly on the fermentation of polysaccharides and bile-acid metabolism, or act in conjunction with the host on the metabolism of choline (Fig. 3).

Fermentation of polysaccharides

Non-digestible carbohydrates are important sources of energy for several members of the colonic microbiota. Species such as *B. thetaiotaomicron* and *Bacteroides ovatus* contain more than twice the number of glycosidase and lyase genes than the human genome and are capable of using nearly all of the main plant and host glycans (such as mucus-associated glycoproteins)[36,37]. Of the SCFAs produced from microbial fermentation, butyrate is particularly important as an energy substrate for cellular metabolism in the colonic epithelium. The colonic epithelial cells of germ-free mice are severely energy-deprived and are characterized by increased activation of AMP-activated protein kinase (AMPK), which senses cellular energy status[38]. This is also true of the liver of germ-free mice[39]. The liver metabolism of germ-free and colonized mice differs considerably, possibly because of the increased influx of SCFAs into the liver of colonized mice (Fig. 1). Acetate and propionate are taken up by the liver and used as substrates for lipogenesis and gluconeogenesis. Colonized mice have higher levels of stored triglycerides in the liver and an increase in the synthesis of very-low-density lipoproteins[40], which transport triglycerides from the liver to other tissues. Increased triglyceride production in the liver of colonized mice is associated with reduced expression of fasting-induced adipose factor, or ANGPTL4, in the small intestine[4,39]. ANGPTL4 is a potent inhibitor of the enzyme lipoprotein lipase, which mediates cellular uptake of triglycerides. Germ-free *Angptl4*-deficient mice gained as much fat mass and body weight during high-fat feeding as colonized mice, indicating that ANGPTL4 directly mediates microbial regulation of adiposity in mice[4,39].

SCFAs also affect proliferation, differentiation and modulation of gene expression in mammalian colonic epithelial cells[41]. However, these effects have been attributed to butyrate acting as a potent histone deacetylase inhibitor and, as such, it may regulate 2% of the

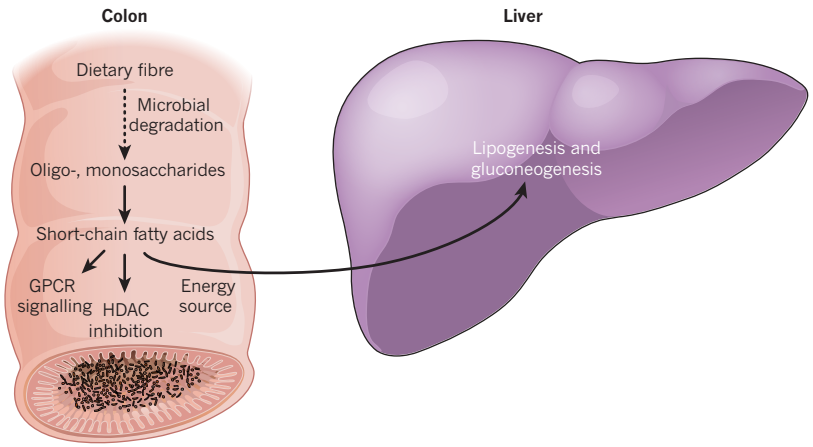

Figure 1 | Effects of colonic fermentation of dietary fibres. Complex carbohydrates, such as dietary fibre, are metabolized by the colonic microbiota to oligosaccharides and monosaccharides and then fermented to short-chain fatty acid end-products, mainly acetate, propionate and butyrate. Short-chain fatty acids are absorbed in the colon, where butyrate provides energy for colonic epithelial cells, and acetate and propionate reach the liver and peripheral organs, where they are substrates for gluconeogenesis and lipogenesis. In addition to being energy sources, short-chain fatty acids control colonic gene expression by inhibiting the enzyme histone deacetylase (HDAC) and metabolic regulation by signalling through G-protein-coupled receptors (GPCRs), such as GPR41 or GPR43.

mammalian transcriptome[41]. In addition, SCFAs can regulate gene expression by binding to the G-protein-coupled receptors (GPCRs) GPR41 (also known as FFAR3) and GPR43 (also known as FFAR2). Signalling through these receptors affects several different functions depending on the cellular type. For example, SCFAs suppress inflammation through GPR43 signalling in immune cells, such as neutrophils[42,43], and modulate secretion of the hormone GLP-1 — which improves insulin secretion and has antidiabetic effects — by enteroendocrine L-cells in the distal small intestine and colon[44]. In addition, the gut microbiota induces *Pyy* expression by L-cells through a GPR41-dependent mechanism. Conventional *Gpr41*-deficient mice have reduced adiposity compared with conventional wild-type mice, whereas germ-free wild-type and *Gpr41*-deficient mice had similar adiposity[45], indicating that the effect of the microbiota on fat deposition is dependent on this SCFA receptor.

Microbial fermentation of polysaccharides may affect host adiposity through several complementary mechanisms, so modulation of the microbiota and its fermentation capacity may provide new avenues for managing obesity.

Microbial regulation of bile-acid metabolism

The primary bile acids cholic acid and chenodeoxycholic acid are synthesized in the human liver from cholesterol, and are important for ensuring that cholesterol, dietary fats and fat-soluble vitamins from the small intestine are soluble and absorbable. Primary bile acids are conjugated to taurine in mice and to glycine in humans, and are taken up in the distal ileum for transport to the liver. However, bacteria in this part of the ileum deconjugate these bile acids, which then escape intestinal uptake and can be further metabolized by the gut microbiota into secondary bile acids. Because the gut microbiota transforms bile acids, germ-free rodents have more bile acid and a less diverse profile than their conventionally raised counterparts[46–48].

Bile acids also function as signalling molecules and bind to cellular receptors[49], such as the bile-acid-synthesis controlling nuclear receptor farnesoid X receptor (FXR)[50] and the GPCR TGR5. Both FXR and TGR5 have been implicated in the modulation of glucose metabolism in mice, but FXR impairs, whereas TGR5 promotes, glucose homeostasis[51–53]. In contrast to FXR, which is activated by primary bile acids, TGR5 binds secondary bile acids such as deoxycholic acid (formed from cholic acid) and lithocholic acid (formed from chenodeoxycholic acid). TGR5 signalling in enteroendocrine L-cells induces secretion of GLP-1, thereby improving liver and pancreatic function and enhancing glucose tolerance in obese mice[53]. Bile acids are taken up from the gut and circulated throughout the body, so activation of TGR5 and FXR in peripheral organs may contribute to overall host metabolism. Activation of TGR5 in brown adipose tissue and muscle increases energy expenditure and protects against diet-induced obesity[54]. The gut microbiota may therefore contribute to the level of obesity and type 2 diabetes by controlling lipid and glucose metabolism though the composition of bile-acid pools and the modulation of FXR and TGR5 signalling.

Microbial metabolism of choline

Choline is an important component of cell membranes and is mostly obtained from foods such as red meat and eggs, but may also be synthesized by the host[55]. Choline is also important for lipid metabolism and synthesis of very-low-density lipoprotein in the liver, and insufficient levels in the diet are associated with altered gut microbial ecology and liver steatosis in mice[56] and humans[57]. In particular, low quantities of Gamma-proteobacteria and high levels of *Erysipelotrichi* in human faecal microbiota are associated with hepatic steatosis[57]. Microbial and host enzymatic activities interact in choline's transformation into toxic methylamines, so trimethylamine that is produced by intestinal microbes can be further metabolized to trimethylamine-N-oxide in the liver[58,59]. These transformations may decrease the levels of bioavailable choline and are suggested to trigger non-alcoholic fatty liver disease (NAFLD) in mice[58]. An altered gut microbial composition and its capacity to metabolize choline may have an important role in modulating NAFLD as well as glucose homeostasis[58].

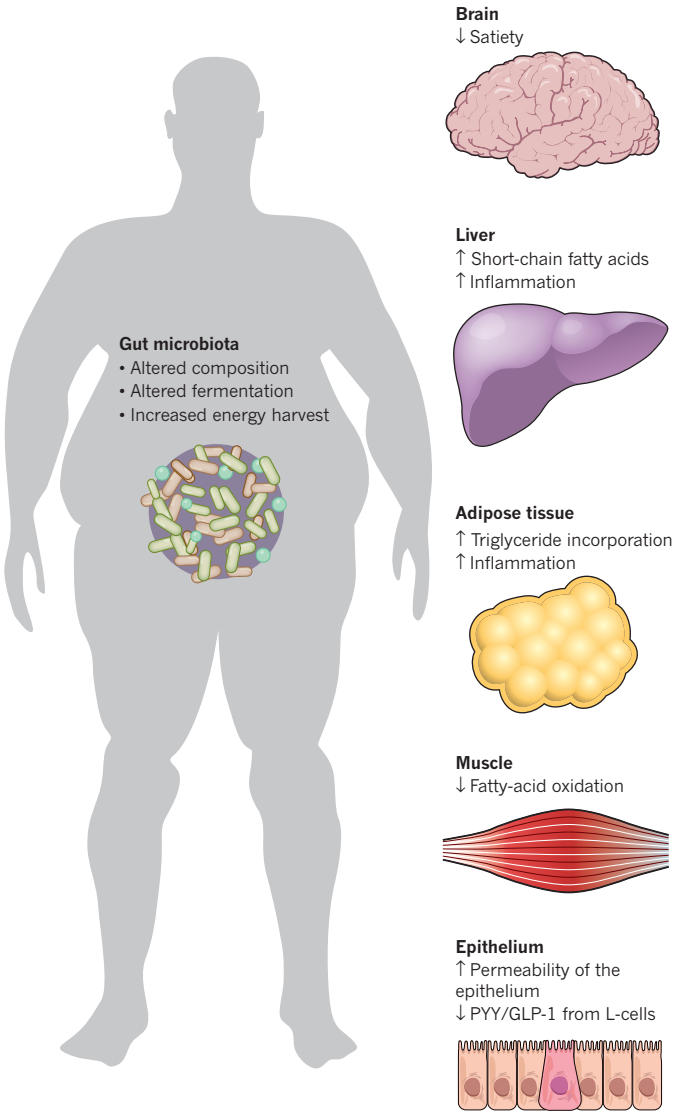

Figure 2 | Features of the gut microbiota that promote obesity and insulin resistance. Alterations to the composition and metabolic capacity of gut microbiota in obesity promote adiposity and influence metabolic processes in peripheral organs, such as the control of satiety in the brain; the release of hormones from the gut (shown as PYY and GLP-1); and the synthesis, storage or metabolism of lipids in the adipose tissue, liver and muscle. Microbial molecules also increase intestinal permeability, leading to systemic inflammation and insulin resistance.

Furthermore, plasma levels of trimethylamine-*N*-oxide and its metabolites are correlated with cardiovascular disease[60] (Fig. 3). The effect of microbial choline metabolism in cardiovascular disease is shown by the reduction of atherosclerosis in atherosclerosis-prone *Apoe*$^{-/-}$ mice treated with broad-spectrum antibiotics[60]. A gut microbiome with different capacities to process cholesterol and choline may contribute to the development of cardiovascular diseases.

Regulation of permeability and inflammation

Obesity, insulin resistance and development of type 2 diabetes are associated with systemic and adipose tissue inflammation[61]. The gut microbiota is a rich source of molecules such as lipopolysaccharide and peptidoglycan that may cause inflammation in peripheral tissues of the body. Colonization of germ-free mice with *Escherichia coli* is

sufficient to augment macrophage infiltration of adipose tissue and polarize macrophages towards the expression of pro-inflammatory cytokines[19]. Plasma lipopolysaccharide levels increase in patients with type 2 diabetes[62], and feeding lipopolysaccharide to mice for 4 weeks increase adipose tissue inflammation and reduce insulin sensitivity[63]. These findings suggest that the gut microbiota may affect host metabolism by altering adipose tissue inflammation. Higher numbers of T cells[64,65] and mast cells[66], and lower numbers of regulatory T cells[67] are also involved, but if and how the gut microbiota affects these cells and whether such interactions contribute to metabolic abnormalities is unclear.

Plasma lipopolysaccharide levels seem to rise with higher fat intake in mice[63] and humans[68,69]. Two hypotheses have been made to explain the mechanism: lipopolysaccharide is taken up with dietary fats in chylomicrons[70], or lipopolysaccharide reaches the circulation because the gut is more permeable in obese mice[63]. A connection between metabolism and the function of the epithelial barrier is thought to exist. Targeted deletion of fatty acid synthase — encoded by the *Fas* gene — in the gut epithelium of mice showed that epithelial *de novo* lipogenesis is required to maintain barrier function[71]. *Fas*-deficient epithelium has increased permeability and, as a result, increased colonic levels of proinflammatory cytokines and high serum lipopolysaccharide. These phenotypes were corrected by antibiotic treatment, suggesting a reciprocal interaction between microbiota, altered epithelial permeability and host metabolism.

A similar connection between gut permeability and type 2 diabetes in humans could also be present. Permeability is correlated with increased visceral adiposity and hepatic steatosis[72], and those with high visceral adiposity and type 2 diabetes have increased levels of bacterial DNA in their blood[73]. However, inflammation may increase permeability in the gut, and further investigation into whether increased permeability causes adipose inflammation or increased inflammation contributes to increased permeability is needed. Either way, the gut microbiota modulates permeability that may contribute to adipose inflammation and cause insulin resistance.

Lipopolysaccharide molecules bind to Toll-like receptor 4 (TLR4), and peptidoglycan to nucleotide-binding oligomerization domain (NOD) receptors, both of which activates proinflammatory signalling cascades[63,74,75]. Deletion of TLR4 in haematopoietic cells by generating bone-marrow chimaeras shows that TLR4 activation in macrophages of mice fed a high-fat diet is required for the development of fasting hyperinsulinaemia, and insulin resistance in liver and adipose tissue but not for the development of obesity[76]. The innate immune system, however, also modulates microbial composition, which may have autonomous effects on host metabolism. Mice deficient in TLR5 have an altered microbial ecology and exhibit metabolic-syndrome signs, such as obesity, insulin resistance and dyslipidaemia, which are, in part, associated with increased food consumption[77]. Transplantation of the gut microbiota from *Tlr5*-deficient and wild-type mice into germ-free recipients shows that the phenotypes are transmissible, and suggests that the gut microbiota alone can mediate disease.

Microbe-associated molecular patterns, including lipopolysaccharide and peptidoglycan, can be recognized by nucleotide-binding domain and leucine-rich-repeat-containing proteins (NLRPs), which form the inflammasome complex together with the apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC)[78]. Obesity is associated with increased adipose expression of NLRP3 in mice and ablation of NLRP3 enhances insulin signalling[79]. However, inflammasomes may be linked to gut microbiota and host metabolism (Fig. 4). NLRP3, NLRP6 and ASC are important regulators of microbial ecology in mice, and deletion of their genes increases the number of Bacteroidetes (Prevotellaceae) and TM7[56,80]. In particular, deficiency in *Nlrp6* results in altered gut microbial ecology that predisposes mice to colitis[80], and inflammasome complexes that do not contain NLRP3 or NLRP6 are associated with an altered gut microbiota and promote NAFLD and non-alcoholic steatohepatitis (NASH)[56]. Importantly, wild-type mice housed with disease-prone $Asc^{-/-}$ mice develop NAFLD or NASH, providing direct evidence that an altered gut microbiota may cause these diseases[56]. Alterations to gut microbiota composition are associated with an increased influx of TLR4 and TLR9 ligands — presumably lipopolysaccharide and bacterial DNA — respectively,

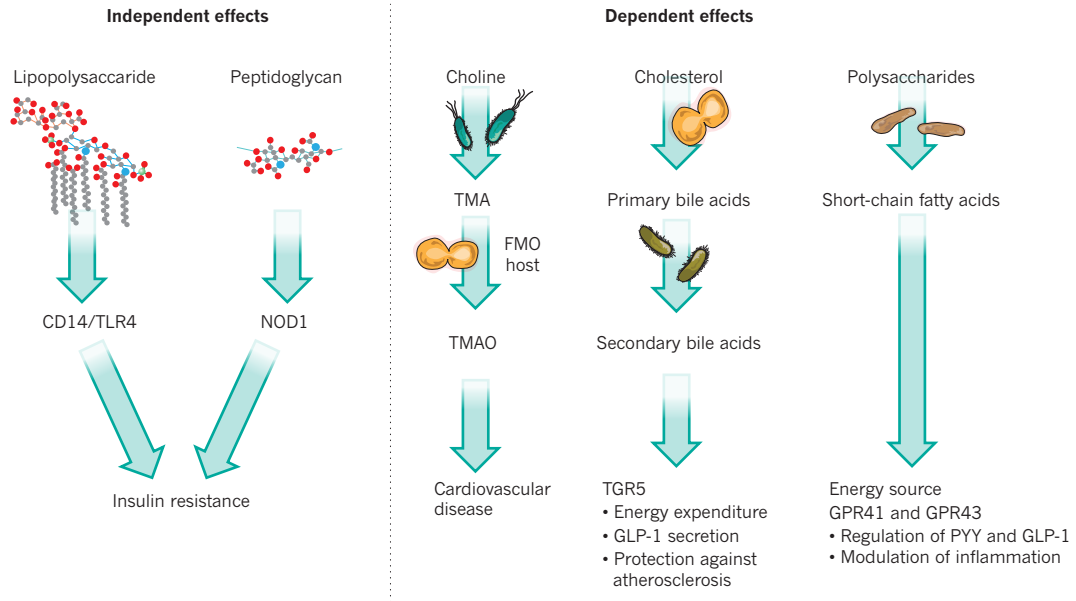

Figure 3 | Diet-independent and -dependent microbial effects on host metabolism. The gut microbiota produces pro-inflammatory molecules, such as lipopolysaccharide and peptidoglycan, which may affect host metabolism through proteins produced by the host to mediate the immune response. Choline, cholesterol and polysaccharides obtained from the diet are metabolized by the gut microbiota and either directly or through further host–microbial co-metabolization generate bioactive compounds. In the case of choline, this can lead to cardiovascular disease; for cholesterol, activation of TGR5 can increase energy expenditure and GLP-1 secretion or protection against heart disease; and for polysaccharides, short-chain fatty acids can be used as an energy source or can bind to GPR41 or GPR43 to regulate hormones and modulate inflammation. FMO, flavin-containing monooxygenase; TLR4, Toll-like receptor 4; TMA, trimethylamine; TMAO, trimethylamine-*N*-oxide.

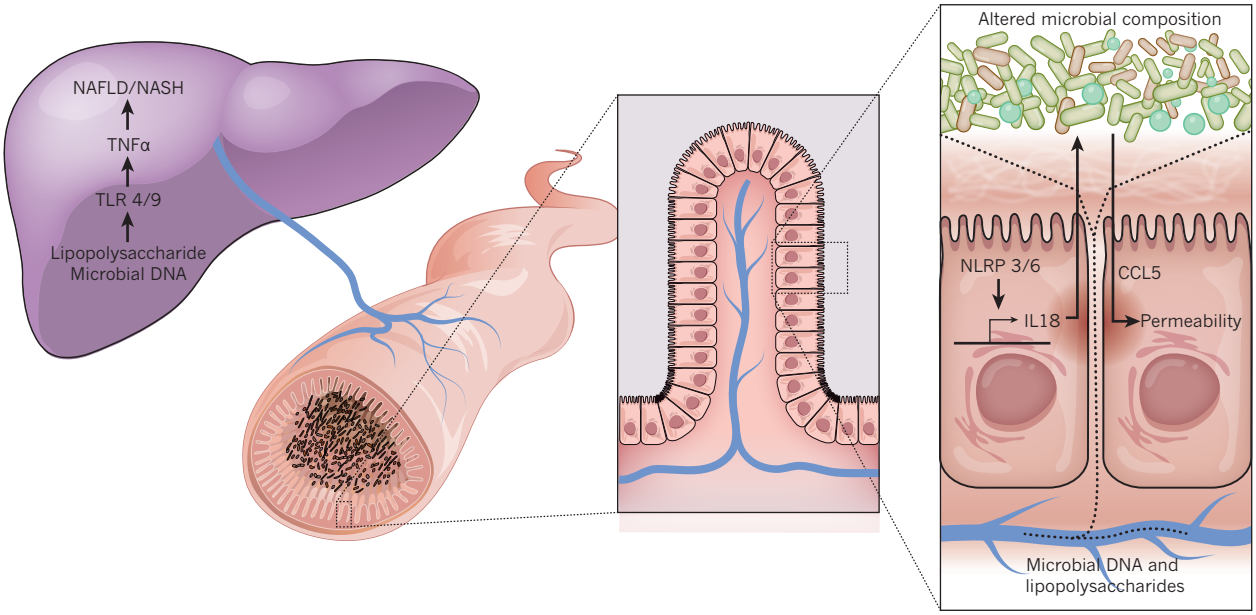

Figure 4 | Different microbial innate immune mechanisms affect host metabolism in the gut and liver. Plasma lipopolysaccharide seems to rise with higher fat intake, and those with high visceral adiposity have higher levels of microbial DNA in their blood. Both lipopolysaccharide and microbial DNA seem to be connected with gut permeability. NLRP3 and 6 are both important regulators of microbial ecology through the effector protein interleukin-18 (IL-18). The altered gut microbiota can stimulate CCL5 secretion, which can result in increased permeability and influx of microbial components. In the liver, lipopolysaccharide and bacterial DNA activate the receptors, TLR4 and 9, leading to increased tumour-necrosis-factor-α (TNFα) secretion and development of non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH).

to the liver through the portal vein[56]. Mice deficient in TLR signalling in the liver are therefore protected from developing conditions related to metabolic syndrome such as obesity, NAFLD and NASH[56] (Fig. 4). The interaction between diet, host and gut microbiota may modulate gut permeability that leads to an influx of proinflammatory molecules and subsequent activation of inflammatory signalling pathways in peripheral tissues that may cause obesity, steatosis and insulin resistance.

Future research

The gut microbiota is increasingly being accepted as an environmental factor that affects host metabolism and contributes to associated pathological conditions, such as obesity, diabetes and cardiovascular disease. However, the contribution that the gut microbiota makes to causing obesity and diabetes in humans is unclear. This is probably because the heterogeneous aetiology of obesity and diabetes can be associated with different microbes; studies are underpowered and include participants with diverse ethnic origin and food habits; the composition of the gut microbiota has large interpersonal variation; and different methods, with specific biases, have been used to profile the microbiota. Cheaper sequencing and improved bioinformatics tools for the analysis of the gut microbiota will allow more researchers to use metagenomic sequencing and avoid primer and polymerase chain reaction biases linked to 16S rRNA gene surveys. Although useful, metagenomic approaches should be complemented by metatranscriptomics and metaproteomics to assess which microbial genes and proteins are expressed in specific conditions. One of the main challenges is to obtain robust predictive biomarkers for obesity and diabetes on the basis of the gut microbiota, for which improved study designs and analytical methods are essential. Much of the focus has been on the faecal microbiota, but many metabolic functions also occur in the small intestine. Sampling intestinal specimens might contribute to the identification of microbial biomarkers for health and disease, and although challenging, more emphasis should be placed on examining its microbiota and the effects on host metabolism.

Studies in humans tend to be correlative, so the role of the microbiota in obesity and its comorbidities in humans remains to be proven. However, this role can be examined in animal studies. Germ-free mice can be 'humanized' by colonizing them with human intestinal communities, providing tools for examining the function of a specific human microbiota and testing how it interacts with specific diets. Genetically engineered germ-free mice could help to identify the molecular mechanisms by which the gut microbiota affects host metabolism. Pigs have similar gastrointestinal tracts and diets to humans, so they could be useful animal models in which to test dietary interventions and to manipulate the gut microbiota to improve health and prevent disease.

Accumulating evidence indicates that the gut microbiota may be a target for treating metabolic diseases[81]. Supplementing the diet with non-digestible food ingredients, or prebiotics, that stimulate the expansion of specific microbes to improve metabolic regulation can be a therapy. Probiotics may be an interesting approach for prevention of obesity and related diseases. But to determine the effects of both of these therapies, double-blind, placebo-controlled studies are required. Therapies that replace unhealthy with a healthy microbiota through transplantation have been used successfully since 1958 for the treatment of antibiotic-related diarrhoeal colitis[82]. Recently, transplantation of healthy lean microbiota improved insulin signalling in participants with metabolic syndrome[83]. Although a promising technique, transmission of unknown and potentially pathogenic bacteria and viruses from an unfractionated gut microbiota may have risks for the recipient. Using microbiota-based interventions to treat obesity will require probiotics that are selected for specific clinical manifestations of metabolic syndrome. ■

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Acknowledgements The authors are grateful to A. Hallén for contribution to the artwork and to R. Perkins for reading the manuscript. Work in the authors' laboratory is funded by the Swedish Research Council, the Swedish Foundation for Strategic Research, Torsten Söderberg's Foundation, Ragnar Söderberg's foundation, AFA Insurances, the Knut and Alice Wallenberg foundation, the Swedish heart lung foundation, the NovoNordisk foundation and the Swedish diabetes foundation.

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