The human gut microbiome plays a key role in human health, but 16S characterization lacks quantitative functional annotation. The fecal metabolome provides a functional readout of microbial activity and can be used as an intermediate phenotype mediating host–microbiome interactions. In this comprehensive description of the fecal metabolome, examining 1,116 metabolites from 786 individuals from a population-based twin study (TwinsUK), the fecal metabolome was found to be only modestly influenced by host genetics (heritability $H^2 = 17.9\%$). One replicated locus at the NAT2 gene was associated with fecal metabolic traits. The fecal metabolome largely reflects gut microbiome composition, explaining on average 67.7% ($\pm 18.8\%$) of its variance. It is strongly associated with visceral-fat mass, thereby illustrating potential mechanisms underlying the well-established microbial influence on abdominal obesity. Fecal metabolic profiling thus is a novel tool to explore links among microbiome composition, host phenotypes, and heritable traits.

There is growing evidence that the gut microbiome contributes to maintaining homeostasis of host metabolism. Disruption of this intricate system is associated with diseases such as obesity and insulin resistance. Metabolomics and the gut microbiome are strongly related, and microbes produce many of the body’s chemicals, hormones, and vitamins. The gut microbiome has been reported to affect circulating levels of several metabolites such as branched-chain amino acids, thus potentially causing insulin resistance. However, despite the advances in next-generation-sequencing platforms, which allow for profiling of complex microbial communities through 16S sequencing, annotation is sparse. Moreover, the microbiome provides information on possible microbial entities rather than their actual activity: it cannot indicate the transcriptional activity of the genes within each bacterial genome or differentiate between alive and dead microbes. Fecal metabolomics, however, reports specifically on the metabolic interplay among the host, diet and gut microbiota, and it complements sequencing-based approaches by providing a functional readout of the microbiome. Here, we provide a comprehensive description of the fecal metabolome in a large population-based setting, by taking advantage of the twin model. We report fecal metabolite (i) associations with age, sex, and obesity; (ii) associations with host genetic influences; and (iii) uni-multivariable associations with gut microbiome composition.

We analyzed fecal samples of 786 predominantly female twins in the TwinsUK cohort, 65.2 ($\pm 7.6$) years of age, with an average body mass index (BMI) of 26.1 ($\pm 4.7$) (Supplementary Table 1), and we replicated our genetic results in an independent sample of 230 individuals, 66.9 ($\pm 8.6$) years of age, with an average BMI of 27.2 ($\pm 5.2$). Untargeted metabolomics profiling of the participants’ fecal samples was conducted through mass spectrometry performed by Metabolon, Inc. A total of 1,116 metabolites, including 866 with known chemical identity, were measured. Among the metabolites identified, 570 were common and detected in at least 80% of the samples, whereas 345 were detected in at least 20% but less than 80% of all samples (Fig. 1a). The latter 345 samples were analyzed as dichotomous traits (on the basis of presence/absence in a sample), and metabolites measured in less than 20% of the samples were discarded from further analysis. Of the 1,116 measured metabolites, 647 were not detected in blood samples of the same individuals profiled on the same platform (Fig. 1b). This result suggests that the fecal metabolome provides information complementary to that from blood metabolomics. We did not find significant associations between 915 fecal metabolites and age after correcting for multiple testing. However, a multivariate partial-least-squares discriminant analysis incorporating all the common 570 metabolites was able to distinguish the oldest decile (>75 years) from the youngest decile (<56 years) of the study population (area under the curve $= 0.71$, $P = 6.8 \times 10^{-4}$; Fig. 2a), and one metabolite, phytanate, was significantly different between the oldest and youngest deciles ($P = 5.0 \times 10^{-3}$; Fig. 2a). These results suggest nonlinear associations between the fecal metabolome and age, in line with previous reports on the effects of age on the gut microbiome.

BMI was associated with eight metabolites at a false discovery rate (FDR, Benjamini–Hochberg corrected) of 5%: five fecal lipids, including arachidonate ($\beta = 0.13$ [0.06–0.19], $P = 1.1 \times 10^{-3}$), the hemoglobin metabolite bilirubin ($\beta = 0.13$ [0.06–0.19], $P = 8.9 \times 10^{-3}$), and two unknown metabolites (Supplementary Table 2). We then looked for associations with visceral-fat mass, a measure of abdominal obesity, correcting for BMI, and found a total of 102 statistically significant associations (FDR <5%, 13 passing Bonferroni correction), which together explained 28.4% of the observed total variance in visceral fat ($P < 2.2 \times 10^{-16}$).
abundance of amino acids (described below), thus suggesting that abundance of the same families to be strongly associated with lower visceral-fat mass than with BMI is consistent with these findings. The strong influence of gut microbiota in visceral development through interaction with dietary components\(^\text{13}\). We have shown associations between visceral-fat mass and gut microbiome composition\(^\text{14,16}\). The much larger number of fecal metabolite associations with visceral fat than with BMI is consistent with these findings and highlights the strong influence of gut metabolic processes on abdominal adiposity.

Visceral-fat-associated metabolites were significantly enriched in amino acids (43 metabolites, enrichment \(P\) value \(< 2 \times 10^{-5}\)) but also included 14 fatty acids, including arachidonate (\(\beta = 5.07 [2.55–7.59]\), \(P = 8.2 \times 10^{-3}\)), 8 nucleotides, 6 sugars, and 6 vitamins. The strong association between the fecal metabolome and central obesity confirms hypotheses on the involvement of microbial amino acid metabolism in obesity and suggests new mechanisms, such as microbial vitamin B metabolism. In previous work, we have found several microbial families associated with lower visceral-fat mass\(^\text{15}\) and reduced weight gain in germ-free mice receiving human fecal transplants compared with germ-free mice not receiving human fecal transplants\(^\text{17}\). By analyzing the fecal metabolome, we found the abundance of the same families to be strongly associated with lower abundance of amino acids (described below), thus suggesting that their association with visceral fat may be mediated by amino acid availability (Fig. 3). This association may be due to increased utilization or decreased production of amino acids by these bacteria, or may be the result of more complex host–microbe interactions.

The gut microbiome is heritable\(^\text{17,18}\), and we found a heritable variance component for 210 operational taxonomic units (OTUs), which explained 22.7% of the observed total variance, on average. To test whether host genetics also influences the fecal metabolome, we first estimated its heritability, taking advantage of the twin structure in our data (Fig. 4) by using structural equation modeling (148 monozygotic (MZ) pairs and 155 dizygotic (DZ) pairs). For 428 metabolites, the best-fitting model contained a heritable variance component (A), which explained 17.9% (\(\pm 9.7\%\)) of the metabolite variation, on average. Long-chain fatty acid–containing metabolites, such as 1-palmitoyl-2-arachidonoyl-GPC (\(HF = 60.7\% [43.4–78.0]\)) and stearoylcarnitine (\(HF = 54.3\% [36.4–72.3]\)), were among the most heritable metabolites. For 279 metabolites, including the coffee metabolite 5-acetylamino-6-amino-3-methyluracil (common environment component (C) = 30.3\%\ ([20.0–40.6]), the best-fitting model was the common environment–unique environment (CE) model, in which C explained 14.8\% (\(\pm 8.1\%)\) of the variance, on average. For the remaining 208 metabolites, the best-fitting model was the unique environment (E) model, wherein the entire variation of the metabolite was due to individual differences such as the microbiome or individual diet (Supplementary Table 2). We found a significantly stronger environmental effect on lipids than other metabolites (enrichment \(P\) value \(< 2 \times 10^{-5}\)).

We subsequently conducted a genome-wide association study for the 428 metabolites with a heritable component (Supplementary Table 3) and identified three metabolites (the amino acid 3-phenylpropionate and two lipids, eicosapentaenoate and 3-hydroxyhexanooate) that were significantly associated with genetic loci after correction for multiple testing (\(P < 1.2 \times 10^{-10} = 5 \times 10^{-9}/428\)) (Table 1 and Fig. 5a). We also tested for genetic associations with metabolite ratios, which can be better proxies for chemical reactions than single metabolites\(^\text{19}\). After correcting for 31,226 tested ratios, we found that the ratio of 5-acetylamino-6-amino-3-methyluracil and 1,3-dimethyluracile was associated with a locus on chromosome 8 (rs35246381, \(P = 7.0 \times 10^{-21}\), \(P\) gain = 7.5 \(\times 10^{-9}\)) (Table 1 and Fig. 5b). We replicated the results of our genome-wide association study in an independent sample of 230 individuals. Of the four loci tested, only the metabolite ratio of 5-acetylamino-6-amino-3-methyluracil to 1,3-dimethyluracile was significantly associated in the replication cohort (\(P = 3.6 \times 10^{-26}\); meta-analysis \(P = 3.3 \times 10^{-26}\)). The two metabolites 5-acetylamino-6-amino-3-methyluracil and
1,3-dimethylurate are products of caffeine metabolism. The associated locus at the \textit{NAT2} gene encodes an N-acetyltransferase, which catalyzes the degradation of caffeine metabolites (Supplementary Fig. 3). Associations of this locus with other caffeine metabolites (1-methylxanthine, 4-acetamidobutanoate, and 1-methylurate) have been observed in blood and urine, and are likely to reflect the efficiency of caffeine degradation. We then explored whether there were any expression quantitative trait loci (eQTLs) or other functional variants in strong linkage disequilibrium (LD) with the top SNP. Although we found three eQTLs (rs11996129, rs1112005, and rs1799930) for \textit{NAT2} (ref. 23), they were in only weak LD ($r^2 < 0.16$) with rs35246381 (ref. 24), and the associations between these SNPs and the metabolite ratio were weaker than that for the top SNP ($P = 3.6 \times 10^{-10}$ versus $P = 9.4 \times 10^{-7}$). NAT2 expression is highest in the liver, then in the jejunal and colonic mucosa, duodenum, colon, and small intestine (see URLs). This tissue expression is consistent with polymorphisms in the \textit{NAT2} gene being associated with the concentration of caffeine-derived metabolites in feces. We explored the relationship between caffeine and the fecal metabolites 5-acetylamino-6-amino-3-methyluracil and 1,3-dimethylurate, and found that their ratio was positively correlated with both coffee intake and serum caffeine levels (Supplementary Fig. 3). These genetic associations capture part of the complex metabolism of caffeine that takes place in the intestine before the metabolites reach the liver, and that the links between host genetic makeup and xenobiotic concentrations can be captured in fecal metabolites. Beyond its function in caffeine metabolism, the NAT2 enzyme is involved in metabolism of various xenobiotics and is therefore related to variance in drug response and toxicity. The composition of the gut microbiome has been shown to regulate xenobiotic enzymes; for instance, the expression of \textit{NAT2} in the large intestine is 1.5 times higher in germ-free animals than control animals. These data together suggest that xenobiotic metabolism may be jointly regulated by host genetic variation and gut microbiome composition.

**Fig. 3** Associations between fecal metabolites and the gut microbiome correspond to microbial effects on visceral fat. Visceral-fat mass was significantly associated with 43 fecal amino acids (all positively) ($n = 647$) and 32 OTUs ($n = 540$) (6 positively in orange, 26 negatively in green). Red tiles indicate positive associations between these metabolites and OTUs ($P > 0$); blue tiles indicate negative associations ($P < 0$); gray tiles indicate nonsignificant associations (FDR $> 5\%$). Microbial associations with fecal metabolites correspond to their respective associations with visceral fat, thus indicating that the microbial metabolic profile is more closely related to the host phenotype than taxonomy. Gen, genus; fam, family; ord, order.
We estimated the proportion of variance in each metabolite separately for MZ (n = 148 pairs) and DZ twins (n = 155 pairs) from variance components of a one-way analysis of variance. The intraclass correlation coefficients (ICCs) were calculated from the variance of the 8 BMI-related and 101 visceral-fat-related metabolites, among others. Xenobiotics showed the strongest associations with microbial composition (enrichment P value < 1 × 10⁻⁴), which explained the entire observed variance for some associations, including the B vitamins nicotinate and pantothenate.

To explore the associations between the fecal metabolome and gut microbes at a finer taxonomic resolution, we regressed each metabolite against the 581 most abundant OTUs, adjusting for potential confounding factors including Shannon diversity. We found 42,645 significant associations of 907 different metabolites with 579 different OTUs after adjusting for multiple testing (FDR < 5%). We also calculated associations of fecal metabolites with collapsed taxonomical levels, ranging from the genus to the phylum level (Supplementary Table 4). We found that 264 metabolites were associated with microbes only at the OTU level, and the remainder were also associated with broader taxonomic groupings.

Finally, to investigate the connectivity of the fecal metabolome with microbes, we calculated a Gaussian graphical model combining 435 common metabolites with a known chemical identity with 241 OTUs with complete taxonomy assignment to at least the genus level. The resulting model consisted of 2,553 independent associations: 1,035 among metabolites, 946 among microbes, and 572 connecting metabolites and microbes (Supplementary Table 5 and Fig. 4). All but nine variables formed one connected component. We detected 19 clusters in the largest component, 9 of which contained both microbes and fecal metabolites, and 10 of which consisted of metabolites only. Xenobiotics had higher node degrees (P < 3 × 10⁻⁴) and were more densely connected with OTUs (P < 2 × 10⁻³). Our model demonstrated a high degree of interrelatedness between the gut microbiome and fecal metabolome, despite the very different technologies used.

In conclusion, although state-of-the-art metagenomic sequencing allows for quantitative and functional annotation of species and microbial pathways, 16S sequencing data have limitations, includ-

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### Table 1 | Genetic associations of fecal metabolites

| Metabolite                                      | H²     | MAF   | SNP            | Gene                  | Discovery Effect | P      | Replication Effect | P      | Meta-analysis Effect | P     |
|-------------------------------------------------|--------|-------|----------------|-----------------------|-----------------|--------|-------------------|--------|---------------------|-------|
| 1,3-Dimethylurate/5-acetylamino-6-amino-3-methyluracil | 40.2%  | 24.7% | rs35246381     | NAT2 (chr 8: 1841502) | -0.17 ± 0.02    | 7.0 × 10⁻²¹ | 7.5 × 10⁻⁹ | -0.22 ± 0.03 | 3.5 × 10⁻¹⁰ | -0.18 ± 0.01 | 3.3 × 10⁻³⁶ |
| 3-Hydroxyhexanoate                               | 20.7%  | 3.7%  | rs62311177     | GRID2 (chr 4: 92962004) | 0.41 ± 0.06    | 2.9 × 10⁻¹² | 0.07 ± 0.09 | 0.429 | 0.32 ± 0.05 | 3.0 × 10⁻¹¹ |
| Eicosapentaenoate (20:5 n3)                      | 16.1%  | 1.4%  | rs149572251    | ITCH (chr 20: 34322936) | 1.45 ± 0.21    | 3.4 × 10⁻¹¹ | -0.23 ± 0.38 | 0.544 | 1.06 ± 0.18 | 6.8 × 10⁻⁹   |
| 3-Phenylpropionate (hydrocinnamate)              | 23.9%  | 1.6%  | rs58539483     | AC090832.1 (chr 11: 28830501) | -1.31 ± 0.19   | 3.6 × 10⁻¹⁰ | -0.39 ± 0.22 | 0.076 | -0.92 ± 0.14 | 1.5 × 10⁻¹⁰ |

Three metabolites and one metabolite ratio were significantly associated with genetic loci in the discovery cohort (n = 739). We report their respective heritabilities (H²), the associated variant along with its chromosomal position and the nearest gene, and the effect (± s.e.). P-values in the discovery and replication cohorts were calculated with the two-sided score test implemented in GEMMA and combined through fixed-effects inverse-variance meta-analysis. The P gain describes the strength of the association of the metabolite ratio relative to the associations of each individual metabolite. MAF, minor allele frequency; chr, chromosome.

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Fig. 4 | Intraclass correlation of fecal metabolites in monozygotic and dizygotic twins. The intraclass correlation coefficients (ICCs) were calculated from variance components of a one-way analysis of variance separately for MZ (n = 148 pairs) and DZ (n = 155 pairs) twins for each metabolite. Positive values of their respective differences indicate more similar metabolic profiles between MZ than DZ twins.
Methods

Methods, including statements of data availability and any associated accession codes and references, are available at [https://doi.org/10.1038/s41588-018-0135-7](https://doi.org/10.1038/s41588-018-0135-7).

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Author contributions
Conceived and designed the experiments: A.T., T.D.S., and C.M. Performed the experiments: R.P.M. Analyzed the data: J.Z., M.A.J., T.L., and C.M. Contributed reagents/materials/analysis tools: M.M., G.K., T.L., A.T., K.S.S., C.J.S., J.T.B., and A.M.V. Wrote the manuscript: J.Z., M.A.J., R.P.M., A.M.V., T.D.S., and C.M. All authors revised the manuscript.

Competing interests
R.P.M. is an employee of Metabolon, Inc. T.L. and A.T. were employees of HLI, Inc. at the time this work was conducted. T.D.S is a co-founder of MapMyGut Ltd. All other authors declare no competing financial interests.

Additional information
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Methods

Study population. The study participants were 786 twins from the TwinsUK cohort. TwinsUK (a national twin registry) has recruited subjects since 1992 through media campaigns and is representative of the population of the UK in terms of lifestyle. The study population is predominantly female (98.3% females) and has an average age of 65.2 (±6.0) years and an average BMI of 26.1 (±4.7). Ethical approval was granted by the St Thomas’ Hospital ethics committee; all participants provided informed written consent.

Results of the genome-wide association study were replicated in an independent set of 230 individuals (98.3% female) from the TwinsUK study 66,9 (±6.0) years of age, with an average BMI of 27.2 (±3.5) (Supplementary Table 1).

Data collection. Sample collection, DNA extraction, and sequencing of the samples in this study has been described previously17,18. Briefly, the fecal samples were collected, refrigerated, and kept in ice packs until they were frozen at −80 °C (mostly within 24 h after collection) before further processing. A number of participants (15%) sent their samples by post.

Metabolomics profiling. Metabolite concentrations were measured from fecal samples by Metabolon, Inc., Durham, North Carolina, USA, by using an untargeted LC/MS platform as previously described17,18.

Sample preparation for global metabolomics. Samples were stored at −80 °C until processing. Sample preparation was carried out as described previously18 at Metabolon, Inc. Lyophilized fecal samples were extracted at a constant per-mass basis. Briefly, second aliquots were added as internal standards prior to any extraction process for quality-control purposes. To remove protein, disassociate small molecules bound to protein or trapped in the precipitated protein matrix, and recover chemically diverse metabolites, proteins were precipitated with methanol under vigorous shaking for 2 min (Glen Mills Genogrinder 2000), then centrifuged. The resulting extract was divided into five fractions: (i) acidic positive-ion conditions chromatographically optimized for more hydrophilic compounds; (ii) acidic positive-ion conditions chromatographically optimized for more hydrophobic compounds; (iii) basic negative-ion-optimized conditions with a separate dedicated C18 column; (iv) negative ionization after elution from a HILIC column; and (v) reserved for backup.

Three types of controls were analyzed in concert with the experimental samples: a pooled sample generated from a small portion of each experimental sample of interest served as a technical replicate throughout the platform run; extracted water samples served as process blanks; and a cocktail of standards spiked into every analyzed sample allowed for instrument performance monitoring. Instrument variability was determined by calculation of the median relative s.d. (RSD) for the standards that were added to each sample before injection into the mass spectrometers (median RSDs were determined to be 5%; n = 31 standards). Overall process variability was determined by calculating the median RSD for all endogenous metabolites (i.e., noninstrument standards) present in 90% or more of the pooled technical replicate samples (median RSD = 12%, n = 832 metabolites). Experimental samples and controls were randomized across the platform run.

Mass spectrometry analysis. Extracts were subjected to UPLC–MS/MS17. The chromatography was standardized, and no further changes were made after the method was validated. As part of Metabolon’s general practice, all columns were purchased from a single manufacturer’s lot and the out-of-expected limits. All solvents were similarly purchased in bulk from a single manufacturer’s lot in sufficient quantity to complete all related experiments. For each sample, vacuum-dried samples were dissolved in injection solvent containing eight or more injection standards at fixed concentrations, depending on the platform. The internal standards were used to ensure both injection and chromatographic consistency. Instruments were tuned and calibrated for mass resolution and mass accuracy daily.

All methods used a Waters Acuity UPLC and a Thermo Scientific Q-Exactive high-resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and an Orbitrap mass analyzer operated at 35,000 mass resolution. The sample extract was dried, then reconstituted in solvents compatible with each of the four methods. Each reconstitution solvent contained a series of standards at fixed concentrations to ensure injection and chromatographic consistency. One aliquot was analyzed by using acidic positive-ion conditions, which were chromatographically optimized for relatively hydrophilic compounds. In this method, the extract was gradient eluted from a C18 column (Waters UPLC BEH C18, 2.1 × 100 mm, 1.7 μm) with water and methanol containing 0.05% perfluoropentanoic acid and 0.1% formic acid. Another aliquot was also analyzed by using basic negative-ion-optimized conditions and a separate dedicated C18 column. The basic extracts were gradient eluted from the column with methanol and water, as well as 6.5 mM ammonium bicarbonate at pH 8. The fourth aliquot was analyzed via negative ionization after elution from a HILIC column (Waters UPLC BEH Amide 2.1 × 150 mm, 1.7 μm) with a gradient consisting of water and acetomitrile with 10 mM ammonium formate, pH 10.8. The MS analysis alternated between MS and data-dependent MSn scans using dynamic exclusion. The scan range varied slightly between methods but covered 80–1,000 m/z.

Compound identification, quantification, and data curation. Metabolites were identified by automated comparison of the ion features in the experimental samples to a reference library of chemical standard entries that included retention time, molecular weight (m/z), preferred adducts, and in-source fragments as well as associated MS spectra, and were curated by visual inspection for quality control in software developed at Metabolon17,18. Identification of known chemical entities was based on comparison to metabolomic library entries of purified standards. Commercially available purified standard compounds have been acquired and registered into LIMS for distribution to the various UPLC–MS/MS platforms for determination of their detectable characteristics. Additional mass-spectral entries have been created for structurally unnatural biochemicals, which have been identified on the basis of their recurrent nature (both chromatographic and mass spectral). These compounds have the potential to be identified by future acquisition of a matching purified standard and scaled to a median 0 and s.d. of 1.

A total of 1,116 different metabolites were measured in the 786 fecal samples, of which 210 metabolites were observed in less than 20% of the samples and thus were excluded from further analysis because of lack of power. 345 metabolites were observed in more than 20% but less than 80% of the samples and were thus analyzed qualitatively as dichotomous traits (observed in a sample versus not observed). The remaining 570 metabolites, which were observed in at least 80% of all samples, were scaled by run-day medians, log-transformed and scaled to a uniform mean 0 and s.d. 1 and analyzed quantitatively (Fig. 1). Metabolite ratios of all samples, were scaled by run-day medians, log-transformed and scaled to a median 0 and s.d. of 1.

We analyzed effects of sample storage time (i) in the refrigerator before samples were frozen and (ii) in the freezer before further analysis. To this end, we regressed metabolite concentrations against storage times. After correcting for multiple testing, we found significant storage effects on seven metabolites (FDR <0.05; Supplementary Fig. 5). We thus selected all further analyses for both storage time in the refrigerator and freezer, to avoid spurious results. Despite correcting all models for the storage time, we cannot ultimately eliminate a potential confounding effect due to storage time, and future studies should investigate the influence of storage time on fecal metabolites.

Microbial sequencing. 16S rRNA was extracted from fecal samples, PCR amplified, barcoded per sample and sequenced with the Illumina MiSeq platform, as previously described18. DNA was isolated from the samples with a PowerSoil kit. The V4 region of bacterial 16S rRNA gene sequences was PCR amplified with the 515F and 806R primers18. Reads were barcoded per sample and combined for microbial sequencing on an Illumina MiSeq instrument to generate 250-bp paired-end reads. Paired-end reads were merged with a minimal overlap of 20 nt by using fastq join within QIIME, which was also used to demultiplex the sequence data.

Preprocessing of sequences and their clustering to operational taxonomic units followed the Sumaclust de novo approach, as previously described for a subset of samples from ref. 18. In brief, 16S sequence reads for all samples were filtered to remove chimeric sequences produced during PCR, by using USEARCH for chimera identification18. The remaining reads were then collapsed to OTUs by using the Sumaclust algorithm for de novo clustering in QIIME 1.9.6; this method selected group reads at the 97% level as de novo OTUs, and Sumaclust has been found to be one of the best-performing greedy clustering algorithms in various comparisons38. Taxonomy was assigned to OTUs on the basis of alignment of representative sequences to the Greengenes 13.8 database with a 97% similarity threshold in UCLUST.

De novo clustering across all samples within the TwinsUK cohort produced ~300,000 OTUs after singleton removal; however, most of those were of low abundance and found in very few samples (table density 0.002). We subdivided the OTU table by discarding samples with fewer than 10,000 reads and OTUs that were not found in at least 25% of these samples. This procedure resulted in a table of 581 OTUs (table density 0.547) (Supplementary Table 7). OTU counts were converted to relative abundance values (over all reads in each sample), a pseudocount of 1 was added to account for very rare OTUs, and the abundance counts were log-transformed. The transformed abundance values were then used as the response in models with sequencing run, sequencing depth, individual who extracted the DNA, individual who loaded the DNA, and sample-collection method as covariates. The residuals of these models were then used in downstream analysis of OTU abundance values. The same normalization and control for technical effects were performed.
was also carried out on taxonomic abundance values collapsed at each taxonomic level. Collapsed taxonomies included counts from all OTUs. The Shannon alpha diversity was also calculated from the complete OTU table. Each sample was rarefied to a depth of 40,000 reads 50 times. Diversity metrics were calculated for each sample in each table, and the mean across all tables was taken as the final measure. Beta diversity was calculated from all OTUs, and singletons were excluded with the unweighted UniFrac algorithm42.

Visceral-fat measurements. Measurements of whole-body composition were performed with DXA fan-beam technology (Hologic QDR; Hologic, Inc.) as previously described43. Briefly, subjects with their clothes removed and wearing gowns were positioned in a standardized manner, in a supine position. The DXA machine was calibrated on a daily basis by using a spine phantom and on a weekly basis by using a step phantom, as suggested by the manufacturer. The scans were analyzed in QDR System Software, Version 12.6.

Regions of interest were defined manually by the same operator following the SOP, which was derived from the manufacturer’s guidelines. The lower horizontal margins were placed above the pelvis, just above the iliac crest, and the upper horizontal margins were placed at the half of the distance between the acromions and the iliac crest. The vertical margins were adjusted just at the external borders of the body so that all the soft tissue was included.

This DXA-based measurement of visceral fat has been validated against visceral fat measured by CT scans and shown to be reliable and reproducible42.

Statistical analysis. To assess the influence of age and sex on metabolite measurements, we regressed all metabolites against age and sex, correcting for family structure as a random intercept, in the R package lme4 (ref. 43). Moreover, we calculated linear and logistic regression models to assess the relationship of the fecal metabolome with obesity, measured as BMI and visceral-fat mass (measured by double X-ray absorptiometry), and adjusted for age, sex, storage time, and family as the random intercept. Visceral-fat measurements were available for 647 individuals.

The following regression models were used:

1. Associations of metabolites with age and sex
2. Associations with BMI
3. Associations with visceral-fat mass
4. Associations with microbial alpha diversity

Partial least-squares discriminant analysis. We used a partial least-squares discriminant analysis (PLS-DA) to investigate global differences between the metabolite profiles of the youngest and oldest deciles of our study population. To this end, all missing values were imputed in the mice package44, and metabolite levels were adjusted for storage times and family structure with linear mixed models. The residuals of these models were then used to train a PLS-DA model, as implemented in the mixOmics package45. The predictive performance was assessed with a tenfold cross validation.

Heritability analysis. We used structural equation modeling to estimate the genetic (A), common environment (C), and unique environment (E) components of the total variance for each metabolite46. To this end, we used the R package mets (version 1.1.0) to fit maximum-likelihood models, adjusted for age, sex, and storage time. For each metabolite, we fitted four models, estimating (i) A, C, and E components; (ii) A and E components; (iii) C and E components; and (iv) the E component only. The best model was selected by minimizing the Akaike information criterion. In the case of dichotomous metabolite abundance, a liability-threshold model was fitted by using the bptwin function in the mets package. Additionally, ICCs were calculated from variance components of a one-way analysis of variance for MZ and DZ twins individually in the ICC package47.

Genome-wide association study. Genetic variation was measured through whole-genome sequencing, as previously described48. In brief, samples were sequenced on an Illumina HiSeqX sequencer with 150-base paired reads. Reads were then mapped to the hg38 genome in HISIIS Analysis Software (v. 2.5.26.13; Illumina)43. Missing genotypes were filled in with reference homologous calls49. Genomes with a ratio of heterozygous to homoygous variants higher than 2.5 were excluded, thus leaving 739 individuals for further analysis. A cohort-based high-confidence region of the genome was constructed by concatenating positions with a 'pass' call rate greater than 90%, by using data from 3 sets of 100 randomly selected genomes.

Variants outside the high-confidence region and duplicated variants were removed. We moreover excluded 273,355 variants with Hardy–Weinberg P > 10−5, calculated from 420 unrelated individuals, thus leaving 8,208,502 biallelic SNPs and 1,408,051 indels.

We fitted linear mixed models to test for associations of heritable fecal metabolites with genetic variants, correcting for age, sex, and storage time, in GEMMA50 by incorporating data from 739 individuals with fecal metabolomics and sequencing data. The twin structure of our data was taken into account by adjusting for the family relatedness by using the sample kinship matrix. The score test implemented in GEMMA was used to assess the significance of the associations. We considered metabolite associations with a P value lower than 1.2 × 10−8 significant, a threshold corresponding to a genome-wide significance cutoff of 5.0 × 10−8, corrected for 428 tested metabolites. Additionally, we tested for genetic associations with all pairwise metabolite ratios of fecal metabolites with known chemical identity and a known varietal component. We used the P-gain statistic to assess the independence of the single metabolites. The P gain is defined as the minimal P value of the associations of either of the single metabolites divided by the P value of the metabolite ratio. A high P-gain statistic indicates that the ratio carries additional information beyond that of individual metabolites. We considered metabolite ratios with P < 1.6 × 10−15 (5 × 10−13; 31,226 metabolite ratios) and P gain > 3.1 × 10−10 (10 × 31,226 metabolite ratios) significant.

Four genome-wide significant associations were replicated in 230 individuals from the TwinsUK study, with adjustment for the same confounding factors. The discovery and replication results were combined through fixed-effects inverse-variance meta-analysis.

Associations of the fecal metabolome with the gut microbiome. To assess the associations of the fecal metabolome with the gut microbiome, we first regressed metabolite concentrations against the Shannon alpha diversity, adjusting for age, sex, BMI, storage time, and family structure, by using 644 individuals with both fecal metabolomics and 16S rDNA sequencing data available.

We then estimated the proportions of variance of each metabolite explained by the microbiome by regressing the fecal metabolite concentration against the microbial beta diversity. This technique is commonly used to estimate heritability from genetic kinship matrices43. To this end, we calculated a restricted maximum-likelihood model, regressing the metabolite level against the microbial similarity, adjusting for age, sex, BMI, storage time in the refrigerator and freezer, and technical covariates (sequencing run, sequencing depth, individual who extracted the DNA, individual who loaded the DNA, and sample-collection method), in the R package regres. The proportion of variance explained by microbial similarity (M) and its standard error were calculated from the variance components in the R package gap51, and P values were calculated from the ratio of M to standard error.

Next, we sought to identify microbes and taxonomical units that were associated with metabolite levels. To this end, we regressed 581 inverse-normalized OTUs against all 915 metabolites, adjusting for age, sex, BMI, sample storage times, family structure, and alpha diversity. Benjamini–Hochberg correction was applied to account for multiple testing. We further calculated associations at different taxonomical units, from the genus to the phylum level.

Finally, to assess multivariate dependencies between the fecal metabolome and the microbiome, we inferred a graphical model combining 423 metabolites with known chemical identity that were observed at least in 80% of the samples with 241 OTUs that were assigned complete taxonomy at least to the genus level. Sparse graphical models were inferred in the GeneNet package52, and edges with FDR < 0.05 were included in the model. We used the Fruchterman–Reingold algorithm53 to determine an unbiased graph layout and identified network modules by optimizing the modularity score, as implemented in the igraph package54.

Pathway enrichment. We used pathway annotation as provided by Metabolon for pathway enrichment with the page algorithm. Enrichment P values were estimated with permutation tests with 10,000 random permutations, as implemented in the R package piano55.

Graphical-model inference. To assess multivariate dependencies between the fecal metabolome and the microbiome, we inferred a graphical model combining 435 metabolites with known chemical identity that were observed in at least 80% of the samples with 241 OTUs that were annotated at least down to the genus level. To obtain a full data matrix, we first imputed missing metabolite levels in the program mice56. A sparse graphical model was inferred with the GeneNet algorithm57, by selecting edges with FDR < 0.05. We used the Fruchterman–Reingold algorithm53 to determine an unbiased graph layout and identified network modules by optimizing the modularity score, as implemented in the igraph package54.

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Data availability. 16S sequencing data used for this study have been deposited in the European Nucleotide Archive under accession code ERP015317. All other TwinsUK data are available upon reasonable request from the department website (http://www.twinsuk.ac.uk/data-access/accessmanagement/).

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Experimental design

1. Sample size
   Describe how sample size was determined.

   This is the first study to examine fecal metabolites, therefore there is no prior knowledge on the effect sizes we are to expect. However, having included 786 individuals with fecal metabolites, anthropometric, microbial and GWAS data our study has 80% power to detect a difference of 0.175 SD for the anthropometric measures adjusting for multiple testing (FDR<0.05) and 80% power to detect a difference of 0.40 for GWAS metabolites ratio adjusting for 31226 tested ratios. The largest effect size found by us in the GWAS is -0.17 for a metabolite ratio with mean=0 SD=0.30 (i.e. an effect of 0.56 SD) which is larger than the effect needed to achieve 99% power for a p< 2 x 10^{-12} needed for the GWAS of metabolite ratios (adjusting for 31226 tested ratios).

2. Data exclusions
   Describe any data exclusions.

   No data were excluded from the analysis

3. Replication
   Describe whether the experimental findings were reliably reproduced.

   We replicated our GWAS-metabolomics results in an independent sample of 230 individuals

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.

   This is not a clinical trial and so randomization is not relevant

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

   This is not a clinical trial and so blindness is not relevant

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.
6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or the Methods section if additional space is needed).

| n/a | Confirmed |
|-----|-----------|
|     | X         |

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.).
- A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly.
- A statement indicating how many times each experiment was replicated.
- The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section).
- A description of any assumptions or corrections, such as an adjustment for multiple comparisons.
- The test results (e.g. p values) given as exact values whenever possible and with confidence intervals noted.
- A summary of the descriptive statistics, including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range).
- Clearly defined error bars.

See the web collection on statistics for biologists for further resources and guidance.

Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

- R version 3.3.3
- R package mets (version 1.1.1) to estimate heritability
- GEMMA for the GWAS

For all studies, we encourage code deposition in a community repository (e.g. GitHub). Authors must make computer code available to editors and reviewers upon request. The Nature Methods guidance for providing algorithms and software for publication may be useful for any submission.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

- N/A

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

- N/A

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

- N/A

b. Describe the method of cell line authentication used.

- N/A
c. Report whether the cell lines were tested for mycoplasma contamination.

- N/A
d. If any of the cell lines used in the paper are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

- N/A

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

- N/A
12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

We included 786 individuals, predominantly females (93.4%), with an average age of 65.2 (±7.6) and an average of BMI of 26.1 (±4.7) from the TwinsUK cohort. Results of the genome-wide association study were replicated in an independent set of 230 individuals (98.3% female) from the TwinsUK study, aged 66.9 (±8.6) and an average BMI of 27.2 (±5.2). Metabolite concentrations were measured from fecal samples by Metabolon Inc., Durham, USA, using an untargeted LC/MS platform. 16S rRNA microbial sequencing was extracted from fecal samples using Illumina MiSeq. Genotypes were determined using whole-genome sequencing. Ethical approval by St Thomas’ Hospital ethics committee; all participants provided informed written consent.