The fecal metabolome as a functional readout of the gut microbiome

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Abstract

The human gut microbiome plays a key role in human health. However, microbiome characterization by next-generation sequencing is complex and lacks quantitative functional annotation, which is crucial for understanding the mechanisms of host-microbiome associations. The fecal metabolome provides a functional readout of microbial activity and could be used as an intermediate phenotype mediating these interactions. We present the first comprehensive description of the fecal metabolome, examining 1116 metabolites of 786 individuals from a population-based twin study (TwinsUK). We report that the fecal metabolome is modestly influenced by host genetics (h2=17.9%). We identify one replicated locus at the NAT2 gene associated with fecal metabolic traits. The fecal metabolome largely reflects gut microbial composition, which explains on average 67.7% (±18.8%) of its variance. We find strong associations with visceral fat mass that shed light on potential mechanisms underlying the well-established microbial influence on obesity. Our study highlights the potential of fecal metabolomics as a novel tool to explore mechanistic explanations for the associations of the microbiome with host phenotypes and heritable complex traits.

There is growing awareness that the gut microbiome has a beneficial role in maintaining homeostasis of host metabolism[1]. Disruption of this intricate system is implicated in human health with certain microbial species associated with diseases such as obesity[2,3] and insulin resistance[4]**.** Metabolomics and the gut microbiome are strongly related, with microbes producing many of the body's chemicals, hormones and vitamins[5]. Research has shown that the gut microbiome has an effect on circulating levels of several metabolites. For example increased microbial production of branched-chain amino acids (BCAA) together with decreased microbial uptake was shown to increase serum levels of BCAAs, potentially causing insulin resistance[4]. However, despite the advances of next generation sequencing platforms, which allow profiling of complex microbial communities using 16S sequencing, annotation is sparse at species level and virtually absent at strain level. Moreover, the microbiome only codes microbial possibilities rather than their actual activity; for instance, it cannot differentiate between alive and dead microbes[6], nor determine the transcriptional activity of the genes within each bacterial genome[7]. Fecal metabolomics, on the other hand, reports specifically on the metabolic interplay between the host, diet and the gut microbiota[8] and complements sequencing-based approaches with a functional readout of the microbiome. Here we provide the first comprehensive description of the fecal metabolome in a large population-based setting with the additional advantage of the twin model. We report (i) fecal metabolites associations with age, gender and obesity; (ii) host genetic influences; and (iii) uni- and multivariable dependencies with the gut microbiome.

We analyzed fecal samples of 786 predominantly female twins of the TwinsUK cohort, aged 65.2 (±7.6), with an average BMI of 26.1 (±4.7) (Supplemental Table S1) and we replicated our genetics results in an independent sample of 230 individuals, aged 66.9 (±8.6) with an average BMI of 27.2 (±5.2). Untargeted metabolomics profiling of the participants' fecal samples was conducted by Metabolon, Inc., using mass spectrometry, measuring a total of 1116 metabolites, 866 of them with known chemical identity. Among the metabolites identified, 570 were common and detected in at least 80% of the samples, while 345 were detected in at least 20% but in less than 80% of all samples (Fig 1a). The latter were analyzed as dichotomous traits (present/absent in a sample) and metabolites measured in less than 20% of the samples were discarded from further analysis. 647 of the 1116 measured metabolites were not detected in blood samples of the same individuals profiled on the same platform (Fig 1b). This suggests that the fecal metabolome provides complementary information to blood metabolomics. We tested 915 fecal metabolites for association with age and did not find significant associations after correcting for multiple testing. However, a multivariate partial least squares discriminant analysis incorporating all the common 570 metabolites could distinguish the oldest decile (>75 yrs.) from the youngest decile (<56 yrs.) of the study population (AUC=0.71, $p=6.8\times10^{-6}$, Fig 2b) and one metabolite, phytanate, was significantly different between the oldest and youngest deciles ($p=5.0\times10^{-3}$) (Fig 2a), suggesting non-linear association of the fecal

metabolome with age. This is in line with previous findings on the effects of age on the gut microbiome[9,10].

BMI was associated with eight metabolites at an FDR (Benjamini-Hochberg) of 5%: five fecal lipids, including *arachidonate* (β [95% CI] = 0.13 [0.07:0.19], $p=1.1\times10^{-5}$), the hemoglobin metabolite *bilirubin* (β [95% CI] = 0.13 [0.06:0.19], $p=8.9\times10^{-5}$) and two unknown metabolites (Supplemental Table S2). 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 of them passing Bonferroni correction), which together explain 28.4% of the observed total variance of visceral fat ($p<2.2\times10^{-16}$) (Supplemental Table 2). We find only 8 metabolites associated with BMI whereas 102 associate with visceral fat (after adjusting for BMI). BMI is an imprecise measure of adiposity and measures overall mass without distinction between lean and fat mass[11]. However, the gut microbiome is now known to play a major role in fatty acid metabolism and adiposity which may be better reflected by visceral fat measures[12,13]. Emerging evidence suggests a role for the intestinal microbiota in visceral development by interacting with dietary components[14]. In recent studies on this cohort we have shown strong associations between visceral fat mass and the gut microbiome composition[15,16]. The much larger number of fecal metabolite associations with visceral fat than with BMI are consistent with these findings and highlight the strong influence of metabolic processes in the gut influencing abdominal adiposity.

Visceral fat-associated metabolites were significantly enriched for amino acids (43 metabolites, enrichment $p<2\times10^{-4}$) but also included 14 fatty acids, including arachidonate (β=5.07 [2.55:7.59], $p=8.2\times10^{-5}$), 8 nucleotides, 6 sugars and 6 vitamins. The strong association we find 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. We have previously found several microbe families to be associated with lower visceral fat mass[15] and reduced weight gain in germ-free mice receiving human fecal transplants[17]. By analyzing the fecal metabolome, we found the abundance of the same families to be strongly associated with decreased abundance of amino-acids (see below), suggesting that their association with visceral fat may be mediated by the availability of amino acids (Fig 3). This may simply be due to increased utilization or decreased production of amino acids by these bacteria, or the result of more complex host-microbe interactions that influence the amino acid metabolism of one or both parties

The composition of the gut microbiome was shown to be heritable[17,18] and we found a heritable variance component for 210 OTUs, which explained on average 22.7% of the observed total variance. To test whether host genetic also influences the fecal metabolome we first estimated its

heritability, taking advantage of the twin structure in our data (148 MZ pairs, 155 DZ pairs) using structural equation modelling. For 428 metabolites the best fitting model contained a heritable variance component (A), which explained on average 17.9% (±9.7%) of the metabolite variation. We found the abundance of long chain fatty acid-containing metabolites, such as *1-palmitoyl-2-arachidonoyl-GPC* (H2=60.7% [95% CI 43.4:78.0]) and *stearoylcarnitine* (H2=54.3% [36.4:72.3]), amongst the most heritable metabolites. For 279 metabolites, including the coffee-metabolite *5-acetylamino-6-amino-3-methyluracil* (C=30.3% [20.0:40.6]), the best fitting model was the CE model, where the common environment component (C) explained on average 14.8% (±8.1%) of the variance. For the remaining 208 metabolites, the best fitting model was the E model meaning that the entire variation of the metabolite is due to individual differences such as the microbiome or individual diet (Supplemental Table 2, Fig S1). We found a significantly stronger environmental effect on lipids than other metabolites (enrichment p-value < 2×10^{-4}).

We subsequently conducted genome-wide association studies for the 428 metabolites with a heritable variance component and identified three metabolites (the amino-acid *3-phenylpropionate* and two lipids *eicosapentaenoate* and *3-hydroxyhexanoate*) to be significantly associated with genetic loci after correcting for multiple testing ($p<1.2\times10^{-10}=5\times10^{-8}/428$) (Table 1, Fig 5a). We also tested for genetic associations of metabolites ratios, which are often better proxies for chemical reactions than single metabolites[19]. After correcting for 31,226 tested ratios we found the ratio of *5-acetylamino-6-amino-3-methyluracil* and *1,3-dimethylurate* to be associated with a locus on chromosome 8 (rs35246381, $p=7.0\times10^{-21}$, p-gain=7.5×10^{9}) (Table 1, Fig 5b). We replicated our GWAS results in an independent sample of 230 individuals. Out of the 4 loci we tested, only the metabolite ratio of *5-acetylamino-6-amino-3-methyluracil* and *1,3-dimethylurate* was significantly associated in the replication cohort ($p=3.6\times10^{-10}$; meta-analysis $p=3.3\times10^{-36}$). The two metabolites *5-acetylamino-6-amino-3-methyluracil* and *1,3-dimethylurate* are products of caffeine metabolism[20]. The associated locus at the NAT2 gene codes for a N-acetyltransferase, which catalyzes the degradation of caffeine metabolites[21] (Supplementary Fig S5). Associations of this locus with other caffeine-metabolites (*1-methylxanthine*, *4-acetamidobutanoate* and *1-methylurate*) have been previously observed in blood[22] and urine[23] and likely reflect efficiency of the degradation of caffeine. We then explored if there were any eQTLs or other functional variants in strong LD with the top SNP. Although we found three eQTLs (rs11996129, rs1112005, rs1799930) for NAT2[24], these are only in weak LD ($r2<0.16$) with rs35246381[25] and the associations between these three SNPs and the metabolite ratio is weaker than that of the top SNP ($p=3.6\times10^{-10}$ vs $p=9.4\times10^{-7}$). Although the NAT2 gene is most strongly expressed in liver, the tissues where its expression is highest, after liver, are in the jejunal

and colonic mucosa, duodenum colon and small intestine[26,*]. This is consistent with polymorphisms in the *NAT2* gene being associated with the concentration of caffeine derived metabolites in feces. Thus, we explored the relationship between caffeine and the fecal metabolites *5-acetylamino-6-amino-3-methyluracil* and *1,3-dimethylurate*, and find that their ratio is indeed positively correlated with both coffee intake and serum caffeine levels in the same individuals (Supplementary Fig S5). The genetic association data shown here, therefore, illustrate how part of the complex metabolism of caffeine takes place in the intestine before reaching the liver and that the links between the host genetic makeup and xenobiotic concentrations can be captured by fecal metabolite concentrations. In addition to caffeine, the NAT2 enzyme is also involved in metabolism of xenobiotics and drugs in general and is therefore related to variance in drug response and toxicity[27]. There is, moreover, work showing that the composition of the gut microbiome regulates xenobiotic enzymes. Comparing the expression of xenobiotic enzymes, germ free animals having 1.5 higher expression of *NAT2* in the large intestine than control animals with a normal gut microbiome[28]. Taken together with our results showing a significant *NAT2* genetic effect in the level of metabolites in the human colon, these data fit with a picture of xenobiotic metabolism being regulated jointly by host genetic variation and gut microbiome composition.

We further investigated to which extent the fecal metabolome might reflect metabolic processes of the gut microbiome. To this end, we regressed metabolite levels against microbial diversity as quantified by the Shannon index, and found that more than the half of the metabolites (575 metabolites) across all pathways showed a significant association with microbial diversity at a FDR of 5%, 347 of them passing a conservative Bonferroni correction. We then estimated the proportion of variance in each metabolite explained by microbiome composition using the unweighted UniFrac beta-diversity metric, a measure of overall phylogenetic dissimilarity between individuals' microbiota[29]. We found that gut microbial composition explained a significant proportion of the observed variance of 710 metabolites, on average 67.7% (±18.8%) of the observed variance, ranging from 22.1% for *1-linolenoylglycerol* to 100% for several amino acids (Table S2). Amongst others, the microbiome explained a significant proportion of the variance of the 8 BMI-related and 101 of the visceral fat-related metabolites. Xenobiotics showed the strongest associations with microbial composition (enrichment p-value< 1×10^{-4}), which explained the entire observed variance for some of them including the B-vitamins *nicotinate* and *pantothenate*.

To explore the associations of the fecal metabolome with gut microbes at a finer taxonomic resolution we regressed each metabolite against the 581 most abundant operational taxonomic

[*] http://bgee.org/?page=gene&gene_id=ENSG00000156006

units (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 genus to phylum level. 264 metabolites were only associated with microbes at the OTU level, with the remainder also associating with broader taxonomic groupings.

Lastly, to investigate the connectivity of the fecal metabolome with microbes, we calculated a Gaussian graphical model (GGM) combining 435 common metabolites with a known chemical identity with 241 OTUs with complete taxonomy assignment to at least genus level. The resulting model consists of 2553 independent associations, 1035 of them amongst metabolites, 946 amongst microbes and 572 connecting metabolites and microbes (Supplementary Fig S6). Despite the sparsity of the graphical model, all but 9 variables form one connected component. We detect 19 clusters in the largest component, 9 of which contain both microbes and fecal metabolites and 10 consist of metabolites only. Xenobiotics have higher node degrees ($p<3\times10^{-4}$) and were more densely connected with OTUs ($p<2.4\times10^{-3}$). Our model demonstrates the high degree of interrelatedness between gut microbiome and fecal metabolome, despite the very different technologies used.

In conclusion, while next generation sequencing facilitates comprehensive profiling of the microbiome and state of the art metagenomic sequencing allows quantitative and functional annotation of species and microbial pathways[30], 16S sequencing data has limitations including the lack of quantitative functional annotations. Fecal metabolomics however, provides a complimentary functional readout of microbial metabolism as well as its interaction with the host and environmental factors. Here we focus on the relationship of fecal metabolites with host and microbial genetics, however, future studies should further investigate the influence of environmental factors, particularly nutrition. Also, future studies should consider the influence of stool frequency and type on fecal metabolite measurements, as these were previously shown to be associated with fecal microbiome composition[31,32]. We show that the fecal metabolome, while moderately influenced by host genetics, largely reflects the microbial community and thus provides a functional readout of microbial metabolism. As metabolites are the main means of communication between the host and (gut) microbiome, the fecal metabolome can be used as intermediate phenotype that promotes microbial effects on the host and vice-versa. Using as an example the associations with obesity, we demonstrate that fecal metabolomics are a useful tool to complement future genomic and microbiome studies with functional annotation to unlock mechanisms of host-microbe interactions as well as its impact on health.

Online Methods

Study population

Study participants were 786 twins from the TwinsUK cohort. TwinsUK (a national twin registry) has been recruited since 1992 through media campaigns and is representative of the population of the UK in terms of life style[33]. The study population is predominantly female (93.4% females), with an average age of 65.2 (±7.6) and an average of BMI of 26.1 (±4.7). Ethical approval by 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, aged 66.9 (±8.6) and an average BMI of 27.2 (±5.2) (Supplementary Table S1).

Data collection

Sample collection, DNA extraction, and sequencing of the samples within this study has been described previously[17,18]. Briefly, the fecal samples were collected, refrigerated and kept in ice packs until they were frozen at -80°C (mostly within 24 hours from 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, USA, using an untargeted LC/MS platform as previously described[22,34] (see supplemental methods for details). The median process variability, determined by technical replicates of pooled samples, was 12%.

A total of 1116 different metabolites were measured in the 786 fecal samples, of which 210 were observed in less than 20% of the samples and thus excluded from further analysis due to 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 vs. 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 uniform mean 0 and standard deviation 1 and analyzed quantitatively. Metabolite ratios were calculated from the run-day median normalized metabolite levels and subsequently log-transformed and scaled to a mean of 0 and standard deviation of 1.

We analyzed effects of sample storage time (i) in the fridge before samples were frozen and (ii) in the freezer before being further analyzed. To this end we regressed metabolite concentrations against storage times. After correcting for multiple testing, we found significant storage effects on 7

metabolites (FDR < 0.05) (Fig S7). We, thus, corrected all further analyses for both storage time in the fridge 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 its influence on fecal metabolites.

Microbial sequencing

16S rRNA was extracted from fecal samples, PCR amplified, barcoded per sample and sequenced using the Illumina MiSeq, as previously described[18]. Reads were clustered in operational taxonomical units (OTUs) using the Sumaclust de novo approach and taxonomy was assigned by aligning representative sequences against the Greengenes 13_8 database[35] (see supplemental methods for details). We excluded samples with less than 10,000 reads and OTUs that were observed in less than 25% of all participants, leaving 581 OTUs for further analysis. OTU counts were converted to relative abundances and then log transformed, after a pseudo count of 10^{-6} was added to account for zero counts. The transformed abundances were adjusted for sequencing run, sequencing depth, individual who extracted the DNA, individual who loaded the DNA and sample collection method as technical covariates using linear regression models. The residuals of these models were then used in downstream analysis of OTU abundances. The same normalization and control for technical effects was also carried out on taxonomic abundances collapsed at each taxonomic level. Collapsed taxonomies included counts from all OTUs. Shannon alpha diversity was also calculated from the complete OTU table. Each sample was rarefied to a depth of 10,000 reads 50 times and Shannon diversity was calculated as the average Shannon diversity across the 50 tables. Beta diversity was calculated from all OTUs excluding singletons using the unweighted UniFrac algorithm[29].

Statistical analysis

To assess the influence of age and gender on metabolite measurements, we regressed all metabolites against age and gender, correcting for family structure as random intercept. Moreover, we calculated linear and logistic regression models, respectively, to assess the relationship of the fecal metabolome with obesity, measured as BMI and visceral fat mass (measured by double X-ray absorptiometry, see supplementary methods), respectively adjusting for age, sex, storage time and family as random intercept (see supplemental methods for formulas). Visceral fat measurements were available for 647 individuals.

Heritability analysis

We used structural equation modelling to estimate the genetic (A), the common environment (C) and the unique environment (E) components of the total variance for each metabolite[36]. To this end we used the R package *mets* (version 1.1.1) to fit maximum-likelihood models, adjusting for age and

sex and storage time. For each metabolite we fitted four models, estimating (1) A, C and E components (2) A and E components, (3) C and E components and (4) E component only. The best model was selected by minimizing the Akaike Information Criterion (AIC). In case of dichotomous metabolite abundances, a liability-threshold model was fitted using the *bptwin* function of the *mets* package.

Genome-wide association study

Genetic variation was measured using whole genome sequencing, as previously described (Nature genetics, in revision). In brief, samples were sequenced using the Illumina HiSeqX sequencer with 150 base paired reads. Reads were then mapped to hg38 genome using ISIS Analysis Software (v. 2.5.26.13; Illumina) and missing genotypes were filled in with reference homozygous calls[37]. Genomes with a ratio of heterozygous to homozygous variants higher than 2.5 were excluded leaving 739 individuals for further analysis. A cohort-based high confidence region of the genome was constructed by concatenating positions with greater than 90% "PASS" call rate using data from 3 sets of 100 randomly selected genomes. Variants outside of the high confidence region and duplicated variants were removed. We moreover excluded 273,355 variants with Hardy-Weinberg $p<10^{-6}$, calculated from 420 unrelated individuals, leaving 8,208,502 biallelic SNPs and 1,408,051 InDels with minor allele frequency higher than 1% for further analysis.

We fitted linear mixed models to test for associations of heritable fecal metabolites with genetic variants, correcting for age, sex, storage time using *GEMMA*[38] 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 using the sample kinship matrix. The score test implemented in *GEMMA* was used to assess significance of the associations. We considered metabolite-associations with a p-value lower than 1.2×10^{-10} significant, which corresponds to a genome-wide significance cut-off 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 heritable variance component. We used the p-gain statistic to assess independence of the single metabolites[19]. 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 compared to individual metabolites. We considered metabolite ratios with $p < 1.6\times10^{-12}$ ($= 5\times10^{-8}$ / 31,226 metabolite ratios) and p-gain $> 3.1\times10^{5}$ ($= 10 \times 31{,}226$ metabolite ratios) significant.

Four genome-wide significant associations were replicated in 230 individuals of the TwinsUK study, adjusting for the same confounding factors. Results of discovery and replication were combined using fixed-effects inverse variance meta-analysis.

Associations of the fecal metabolome with the gut microbiome

To assess 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 using 644 individuals with both fecal metabolomics and 16S sequencing data available.

We then estimated the proportions of variance of each metabolite explained by the microbiome by fitting restricted maximum likelihood (REML) models regressing the fecal metabolite concentration against the microbial alpha-diversity using the R-package regress. This technique is commonly used to estimate heritability from genetic kinship matrices[39,40] (see supplemental methods for details).

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

Lastly, 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 in at least 80% of the samples with 241 OTUs that were assigned complete taxonomy at least to the genus level. Sparse graphical models were inferred using the GeneNet package[41] and edges with false discovery rate < 0.05 were included in the model. We used the Fruchterman-Reingold algorithm[42] to determine an unbiased graph layout and identified network modules by optimizing the modularity score as implemented in the igraph package[43] (see supplemental methods for further details).

Pathway enrichment

We used pathway annotation as provided by Metabolon for pathway enrichment using the page algorithm. Enrichment p-values were estimated using permutation tests with 10,000 random permutations as implemented in the R-package piano[44].

Data availability

16S sequencing data used for this study is deposited in the European Nucleotide Archive (ERP015317). All other TwinsUK data are available upon request on the department website (http://www.twinsuk.ac.uk/data-access/accessmanagement/).

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Acknowledgements

The study was funded by the Wellcome Trust; European Community's Seventh Framework Programme (FP7/2007-2013). The study also receives support from the National Institute for Health Research (NIHR)- funded BioResource, Clinical Research Facility and Biomedical Research Centre based at Guy's and St Thomas' NHS Foundation Trust in partnership with King's College London. HLI collaborated with KCL to produce the metabolomics data from Metabolon Inc. CM is funded by the MRC AIM HY (MR/M016560/1) project grant.

We thank Julia Goodrich and Ruth Ley for their support in sequencing of the fecal samples.

Author Contributions

Conceived and designed the experiments: AT, TDS, CM. Performed the experiments: RPM. Analyzed the data: JZ, MJ, TL, CM. Contributed reagents/materials/analysis tools: MM, GK, TL, AT, KS, CJS, JTB, AMV. Wrote the manuscript: JZ, MJ, RPM, AMV, TDS, CM. All authors revised the manuscript.

Competing financial interests

RPM is employee of Metabolon, Inc. TL and AT are employees of HLI, Inc. TDS is co-founder of MapMyGut Ltd. All other authors declare no competing financial interests.

Tables

Table 1. Genetic associations of fecal metabolites

Three metabolites and one metabolite ratio significantly associated with genetic loci in the discovery cohort. We report their respective heritabilities (H2), the associated variant along with its chromosomal position and the nearest gene, and the effect (+- its standard error) and p-value in the discovery and replication cohorts as well as the meta-analysis. The p-gain describes the strength of the association of the metabolite ratio relative to the associations of each individual metabolite.

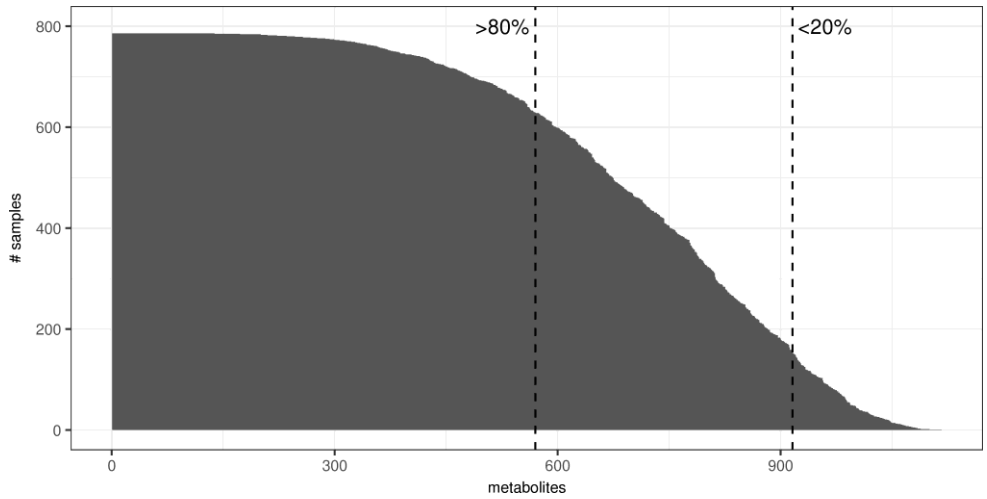
Metabolite	H2	MAF	SNP	Chr	Position	Gene	Effect	P	p-gain	Effect (Repl)	P (Repl)	Effect (meta)	P (meta)
1,3-dimethylurate/ 5-acetylamino-6-amino-3-methyluracil	40.2%	24.7%	rs35246381	8	18415025	NAT2	-0.17 (+-0.02)	7.0×10^{-21}	7.5×10^{9}	-0.22 (+-0.03)	3.5×10^{-10}	-0.18 (+-0.01)	3.3×10^{-36}
3-hydroxyhexanoate	20.7%	3.7%	rs62311177	4	92962004	GRID2	0.41 (+-0.06)	2.9×10^{-12}		0.07 (+-0.09)	0.429	0.32 (+-0.05)	3.0×10^{-11}
eicosapentaenoate (EPA; 20:5n3)	16.1%	1.4%	rs149572251	20	34322936	ITCH	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	11	28830501	AC090833.1	-1.31 (+-0.19)	3.6×10^{-11}		-0.39 (+-0.22)	0.076	-0.92 (+-0.14)	1.5×10^{-10}

Figures

Figure 1. Number of measured fecal metabolites.

1116 metabolites were detected in 786 fecal samples. (A) 570 of those were detected in at least 80% of all samples and 345 were detected in less than 80% but more than 20% of all samples. The first were analyzed continuously, while we dichotomized the latter in present/absent. 210 metabolites that were present in less than 20% of the samples were excluded from further analysis. (B) 469 metabolites where observed in both, fecal and blood samples of the sample individuals, while 647 metabolites are unique to feces. 499 of these 647 metabolites were observed in at least 20% of the fecal samples.

A) Frequencies of fecal metabolites.



B) Overlap of fecal and blood metabolome.

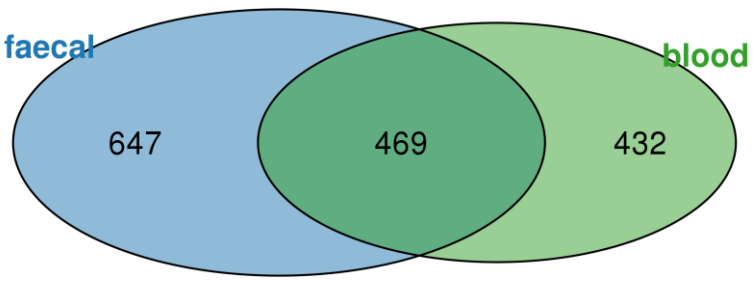
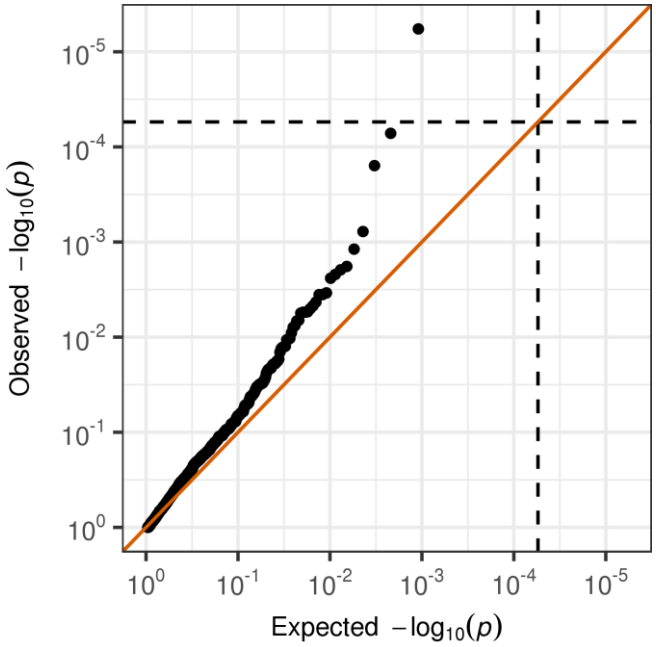


Figure 2. Association of the fecal metabolome with age.

While we found the fecal metabolome stable during adulthood, we found the oldest decile of our study population (>75 yrs.) different from the youngest decile (<56 yrs.) of the study population. We first investigated the age effect for all metabolites individually (A) and found one metabolite, *phytanate*, significantly between the youngest and the oldest decile of our data. We then fitted a multivariate PLS-DA model to distinguish the older from the younger group (B). We estimated the area under the receiver operations curve (AUC) at 0.71 ($p=6.8\times10^{-6}$) in a 10-fold cross-validation setting.

A) qq-plot of associations of metabolites and age deciles



B) PLS-DA discriminating older from younger adults

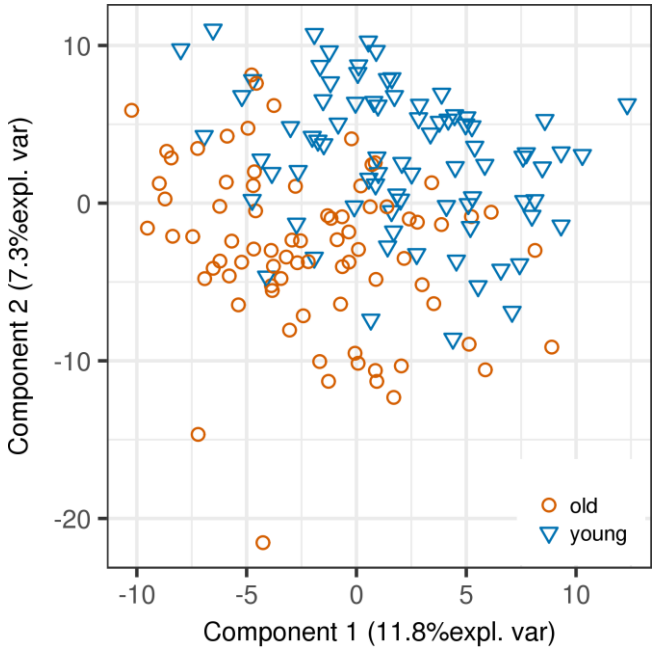


Figure 3. Associations of fecal metabolites with gut microbiome correspond to microbial effect on visceral fat.

Visceral fat mass was significantly associated with 43 fecal amino acids (all positively) and 32 OTUs (6 positively in orange, 26 negatively in green). Red tiles indicate positive associations between these metabolites and OTUs (β > 0) and blue tiles negative associations (β < 0); grey tiles indicate non-significant associations (FDR > 5 %). Microbial associations with fecal metabolites correspond to their respective associations with visceral fat, indicating that the microbial metabolic profile is more closely related to the host phenotype than taxonomy.

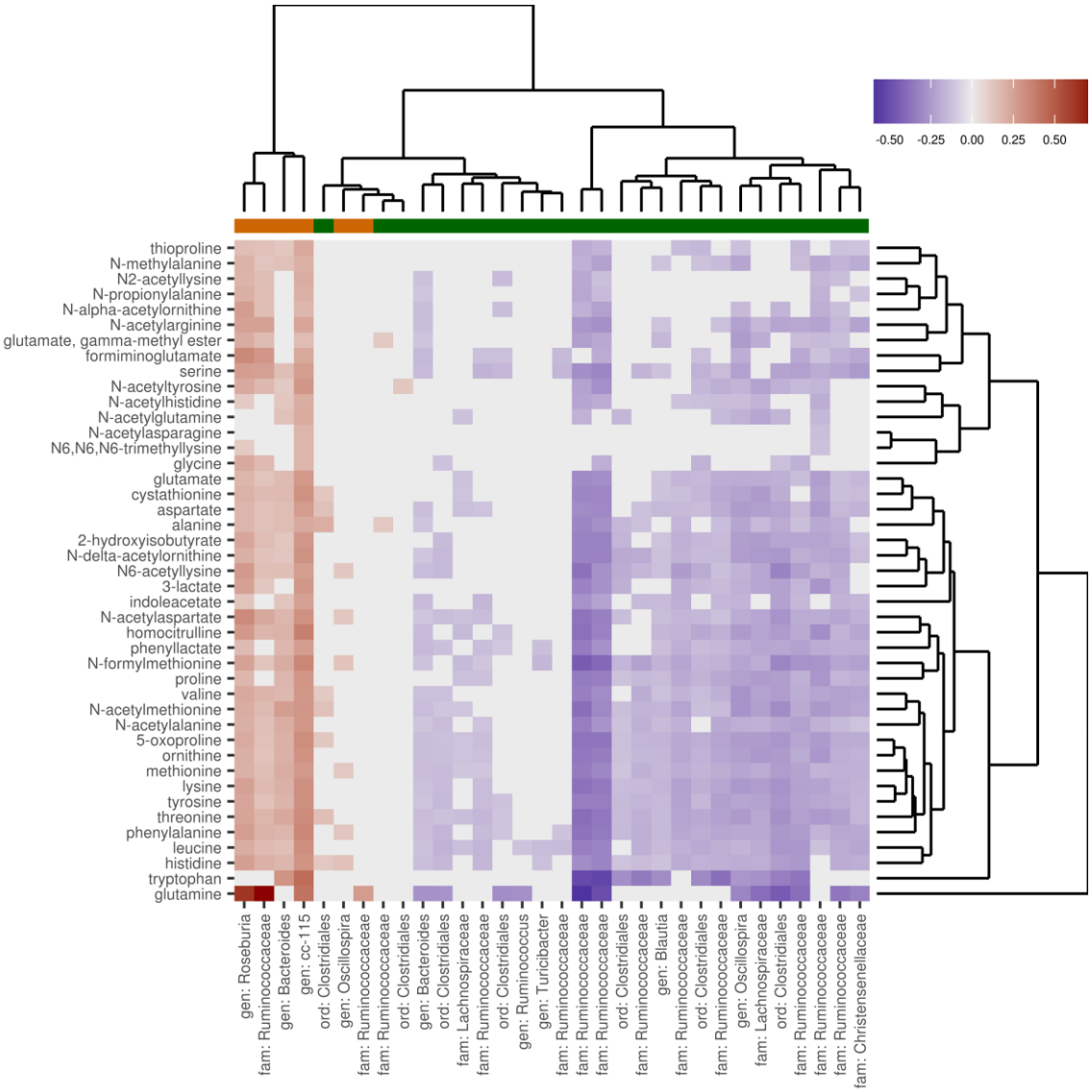


Figure 4. Intraclass correlation of fecal metabolites in MZ and DZ twins.

The intraclass correlation was calculated separately for monozygotic (MZ) and dizygotic (DZ) twins for each metabolite. Positive values of their respective differences indicate more similar metabolic profiles between MZ than DZ twins.

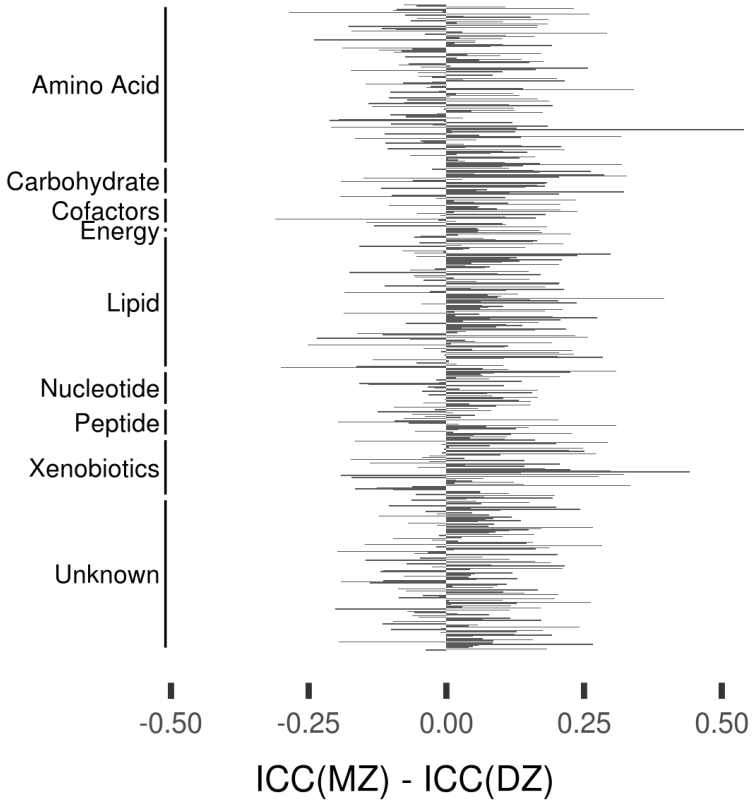
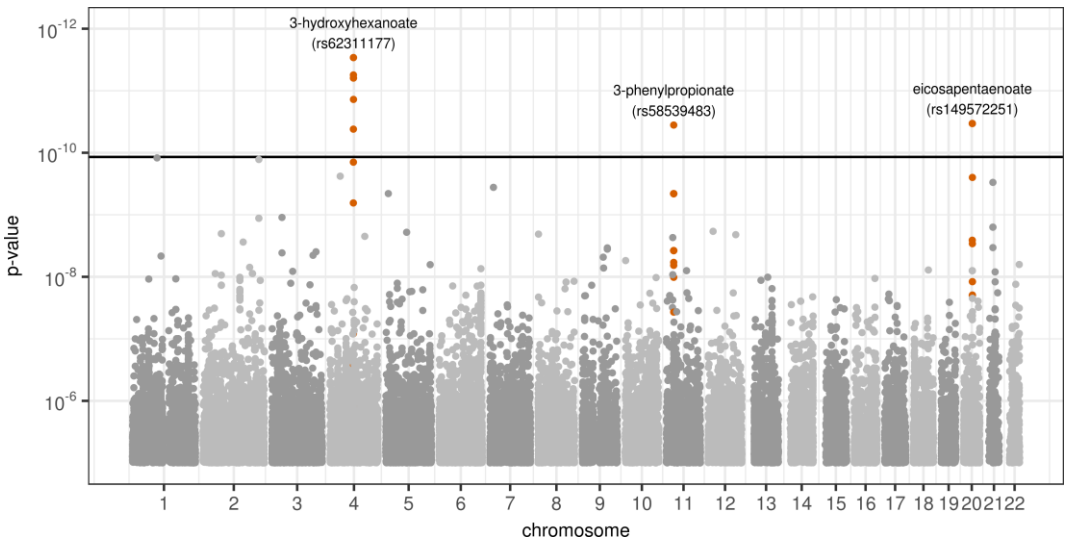


Figure 5. Host genetic influence on the fecal metabolome

(A) Manhattan plot of genome wide p-value from association analysis of fecal metabolites in the discovery sample. horizontal line indicates the Bonferroni cutoff of 1.2×10^{-10}. Three metabolites pass the Bonferroni threshold. (B) Manhattan plot of genome wide p-value from association analysis of metabolite ratios in the discovery sample. The horizontal line indicates the Bonferroni cutoff of ($p<1.6\times10^{-12}$). Two ratios pass the threshold, however only *1,3-dimethylurate / 5-acetylamino-6-amino-3-methyluracil* ($p = 6.2\times10^{-21}$) passed filtering by p-gain (p-gain > 8.9×10^{5}) and thus is considerably stronger than the association of each individual metabolite. Boxplots, QQ-plots, and regional association plots for each of the four loci are shown in Supplemental Fig S2-S4.

A)



B)