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ADDENDUM

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Heritable components of the human fecal microbiome are associated with visceral fat

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ABSTRACT

Obesity and its associated diseases are one of the major causes of death worldwide. The gut microbiota has been identified to have essential regulatory effects on human metabolism and obesity in particular. In a recent study we provided some insights into the link between the gut microbiota (GM) and adiposity, as well as host genetic modulation of these processes. Our results identify novel evidence of association between 6 adiposity phenotypes and faecal microbial operational taxonomic units (OTUs). Accumulation of visceral fat, a key risk factor for cardio-metabolic disease, has the strongest and most pervasive signature on the gut microbiota of the factors we examined. Furthermore, we observe that the adiposity-associated OTUs were classified as heritable and in some cases were also associated with host genetic variation at obesity-associated human candidate genes *FHIT, TDRG1* and *ELAVL4*. This addendum confirms our previously published results in the TwinsUK cohort using a different approach to OTU clustering and multivariate analysis, and discusses further the importance of considering the GM as a complex ecosystem.

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Introduction

The obesity epidemic is a global health burden that concerns an increasing percentage of the population worldwide. Obesity leads to increased cancer, cardiovascular and metabolic disease risk.[1] Although overall obesity, as measured by body mass index (BMI) is generally the most commonly used phenotype to assess health implications, it is the accumulation of visceral fat that is the most significant cardio-metabolic disease risk factor.[2,3] Visceral fat is the deposition of adipose tissue around central metabolic organs such as the liver or the gastrointestinal tract, which can modify their activity. In contrast, subcutaneous fat deposition, even if not favorable for health, has fewer impacts on disease development potentially due to its deposition further away from central organs, and where it can contribute to thermoregulation.

Due to the rapid growth of the obesity epidemic and its impact on health and quality of life, much research has focused on understanding the factors that impact fat deposition and influence weight gain. Both genetic and environmental factors play a role in obesity, and identifying these effects and their dynamics may allow identification of new intervention targets.

The human body is the host of trillions of bacteria, the majority of which are concentrated within the intestinal track and specifically the colon. Gut bacteria are strongly involved in food digestion and energy expenditure,[4] but also in maintaining host metabolic homeostasis and health. For instance, obesity and associated cardio-metabolic diseases have been associated with low gut bacterial diversity,[5,6] and modifications in proportions and specific changes of individual bacteria or taxonomic groups.[7,8] In the study by Beaumont et al.[9] we investigated the association between different components of obesity, including visceral fat, and gut microbiota composition. Our findings further explored novel evidence that host genetic factors may influence the link between obesity and gut microbiota changes. In this addendum, we pursue an extended analysis of the association between gut microbiota composition and adiposity phenotypes in the TwinsUK cohort using

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multivariate statistical models and an alternate approach to cluster 16S reads to OTUs for taxonomic assignment, to evaluate the reproducibility of our results.

Questioning an ecosystem

One of the main challenges in gut microbiota studies is to grasp how the host responds to modifications of a complex bacterial ecosystem while considering inter-individual environmental variation.[10] This challenge requires integration of multiple-level interactions between the host system variables and characteristics of the bacterial taxa, and one approach to tackle this challenge is addressed by multivariate statistical tools. Indeed, bacterial processes are influenced by the surrounding environment that can impact gene expression and thereafter also impact other microorganisms. If a bacterial taxon impacts the health status of the host under specific environmental conditions, then the impacts of this bacterial unit should also be studied taking into account the status of other members of the ecosystem. An example that justifies this approach is the increasing evidence that pathogen colonization is not necessarily followed by symptom development.[11] Pathogenicity is generally observed when colonization is concomitant with dysbiosis,[12] demonstrating that colonization by a pathogen is often not sufficient to trigger disease development. Thus, pathogenicity and infection need to be considered together with potentially co-occurring modifications of the bacterial ecosystem. Furthermore, a literature search will often reveal reports of identical taxa having opposite effects on host health.[13] For instance, *Lactobacillus* that have been the object of many studies due to their important role in food production have been reported to have opposite impacts on obesity.[14] These apparently discordant findings could be explained by associated modifications of the surrounding microbial environment.

Here, we therefore explore once again the association between VFM and gut microbiota composition, but now aiming to take into account the impacts of bacterial taxa within their ecosystem as a whole using multivariate analysis in the TwinsUK cohort.[15-17] These analyses were performed in the original discovery sample of 1313 twins from Beaumont et al.,[9] who had available gut microbiota profiles and VFM phenotype estimates. The distribution of the phenotype in this data set is shown in Figure 1A. Collapsing of 16S rRNA gene sequencing (16S) data to OTUs in the data set of 1313 individuals was pursued here using an alternate approach compared with that of Beaumont et al.[9] Specifically, our previous study used open reference clustering with Greengenes v13_8 at 97% sequence similarity, while the present analysis was based on SUMACLUST De Novo clustering in QIIME at a similarity threshold of 97%, with later taxonomic assignment against the same reference database.[18] The purpose of this approach was to assess if the results are reproducible at the OTU level when changing the method for quantification, as there is evidence for differential accuracy between reference based and de novo clustering of OTUs. Several studies have compared the use of reference based and de novo clustering approaches, with some evidence that de novo clustering produces OTUs more accurately clustered by sequence identity.[19] Carrying out comparisons between methods within the TwinsUK data we also found that de novo methods, in particularly SUMACLUST, produced more heritable OTUs.[18] Beaumont et al.[9] utilized open reference clustered OTUs, combining reference and de novo clustering. We chose to use the SUMACLUST de novo OTUs in the present study given the evidence regarding the differences in clustering approaches and to demonstrate robustness of our results in respect to these differences.

Beaumont et al.[9] identified several OTUs that were significantly associated with adiposity phenotypes. All of these OTUs were classified under the 7 following bacterial genera *Ruminococcus, Blautia, Oscillospira, Clostridium, Lachnospira, Coprococcus* and *Bacteroides*. To evaluate if these results were consistent in the updated analyses, we extracted all OTUs classified under these 7 genera in the set of 1313 individuals. Out of the 582 OTUs considered in total that were observed in at least 25% of the extended TwinsUK sample (2730 individuals), 155 belonged to one of the 7 genera of interest. Scores for which relative abundance was equal to zero were adjusted to 0.000001 before log transformation. In the current study, we used the log10 transformed relative abundance of 155 OTUs (corrected for sequencing run, sequencing depth, who extracted the DNA, who loaded the DNA and sample collection method) as independent variables in an orthogonal projection to latent structure discriminant analysis (O-PLS DA) using VFM phenotype as a predictor in the 1313 individuals (Fig. 1B). O-PLS DA is a supervised analysis that allows us to determine if a phenotype (for example, VFM) can be used as a predictor of a multivariable system (for

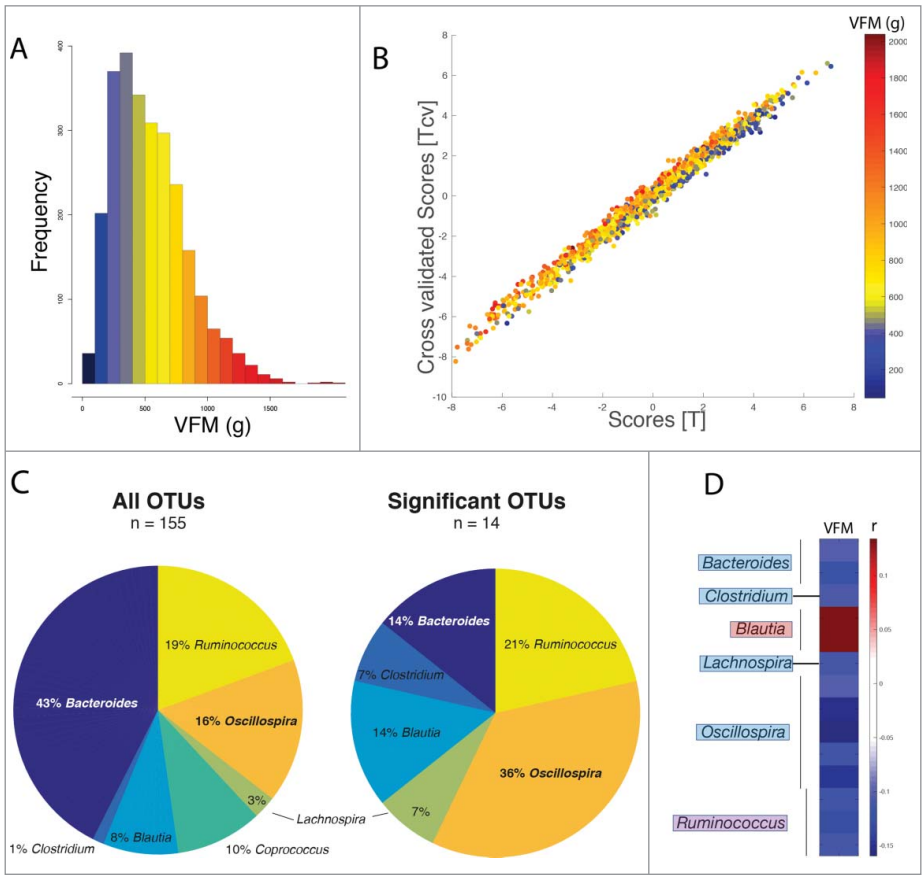

Figure 1. VFM is strongly associated with gut microbiota composition. A. VFM distribution within the extended TwinsUK data set (n = 1313). B. Plot of the observed scores against the cross-validated scores generated by the O-PLS DA calculated using VFM as a predictor and 155 OTUs (belonging to the *Bacteroides, Clostridium, Blautia, Coprococcus, Lachnospira, Oscillospira* and *Ruminococcus* genera) relative abundance as a matrix of independent responders, each score represents one of the 1313 individuals used to generate the model and is color-coded in accordance to individual VFM level. C. Diversity distribution of the 155 OTUs used to generate the O-PLS DA model and of the 26 OTUs that were considered as significantly associated to VFM from the same model. D. Association loadings between VFM and the 14 significant OTUs; genera highlighted in blue are OTUs that were negatively associated with VFM in the Beaumont et al paper, while those in red are positively associated, and purple represents a mixture of positive and negative associations.

example, the gut microbiota).[20] Model validity can be assessed by evaluating the goodness of fit and of prediction. Further, it is possible to extract from this model the variables (for example, OTUs) that are significantly contributing to the model. Here, we used an in-house algorithm (provided by Korrigan Sciences Ltd) in MatLab (version R2016b, The MathsWorks inc.), with 7 cross-validations for prediction and zero orthogonal component. To assess the fit of the model we report the observed goodness of fit ($R^2Y = 0.065$) and goodness of prediction ($Q^2Y = 0.0338$) values. We also assessed the fit of the model by permutation, where based on 10000 permutations we report significant estimates for goodness of fit (*P*-value = 0.0001). Association between specific OTUs and O-PLS DA model scores were further explored by fitting a linear mixed effects regression (LMER) model using the R package lme4. In the analyses described in this section we included several additional covariates for the 16S profiles, including sex, age and long-term summary dietary profiles (see Beaumont et al.[9]) and BMI (fixed effects), and family and zygosity (random effects). A Benjamini–Hochberg false discovery rate (FDR) correction was applied and LMER results are reported at FDR of 1%.

The central role of the Firmicutes

Altogether, 14 of the 155 OTUs used to generate the model appear to significantly contribute to VFM prediction at FDR 1% in the extended data set of 1313 individuals from TwinsUK. When considering OTU diversity of the 155 OTUs used to generate the O-PLS DA model, it appears that over a third of OTUs

belonged to the Bacteroidetes phylum and the rest were classified as Firmicutes (Fig. 1C). However, OTU taxonomic classification proportion was considerably modified when considering just the 14 OTUs that were significantly associated with VFM in Figure 1C. Within these 14 OTUs 88% belonged to the Firmicutes phylum and only 12% were Bacteroidetes. The reduction in VFM-associated proportion of Bacteroidetes in the O-PLS DA model compared with the linear regression results is somewhat surprising and suggests a potential redundancy in VFM-associated effects across some Bacteroidetes members. It has been consistently observed that an increase in Bacteroidetes results in or is associated with lean phenotypes.[5,6,21] Our results are in line with previous findings as we also observed a negative association between VFM and Bacteroidetes OTUs, similar to previously published analysis by Beaumont et al.[9]

The analyses may also in part reflect incomplete coverage of bacterial composition and diversity by 16S data, while technologies such as shot gun metagenomics may allow us to detect more subtle and accurate changes of the ecosystem.[22] However, the modification in diversity balance indicates the central role occupied by Firmicutes in energy balance and expenditure reflected by adiposity phenotypes that was also observed in Beaumont et al.[9]

In terms of considering GM deleterious or protective effects on cardio-metabolic disease risk, the overall the direction of association between OTUs and VFM were comparable to results previously published in Beaumont et al. (Fig. 1D). As previously observed, OTUs belonging to the *Bacteroides,Oscillospira, Lachnospira* and *Coprococcus* genera were negatively associated with VFM. However, none of the OTUs belonging to the *Clostridium* genus appeared to be significantly associated with VFM. Similarly, all OTUs belonging to the *Blautia* genus were positively associated with VFM both in the original and current analysis. Lastly, only negative associations between VFM and OTUs within the *Ruminococcus* genus were observed here, while both positive and negative associations were reported in the previous study.

The most associated components with VFM in the new extended analyses was a class of *Oscillospira* genus members. Of the 155 OTUs used to generate the initial linear regression model, 25 belonged to this genus (16%), demonstrating that this genus if replicated could be a key player of the host-GM mutualism in relation to fat deposition.

The potential implication of these 4 potentially "beneficial" genera (*Bacteroides, Oscillospira, Lachnospira* and *Coprococcus*) needs to be explored in further studies using animal models and human intervention. It is difficult to assess if the reported adiposity phenotype associations are a cause or a consequence of the gut microbiota modifications, and follow up work using animal and *in vitro* models may shed a light on specific mechanisms involved in the host-gut microbiota cross talk.[23] For example, germ-free mouse models have shown that a GM community associated with health status could induce specific phenotypes within recipient animals.[24] However, animal studies may also have some drawbacks in terms of translating the findings to human physiology and pathology[25] as they cannot fully recapitulate the symbiotic human host-bacterial co-evolution.[26] Nevertheless, results from the extended data set confirm the close links between the gut microbiota and fat metabolism and storage. The Beaumont et al.[9] study also explored the potential impact of host genetics on these interactions. This topic is addressed in the following section where the mechanisms by which the host might be able to modulate the microbiota will be discussed, and linked to obesity.

Potential mechanisms underlying associations between microbiota and host genetics

Several studies have demonstrated that host genetics influence gut microbiota composition.[17,27-29] A number of mechanisms may be involved in mediating these effects. First, host genetics may impact the lumen physicochemical properties, which influence the gut environment and thereby can affect bacterial growth. In this context, host immunity is a key player in shaping the gut microbial community, and it is now well established that this is a 2-way interaction between the host and its microorganisms.[30] A second mechanism of host genetic influences is linked to the secretion of host microRNAs in the intestinal lumen. MicroRNAs are small non-coding host RNA molecules involved in post-transcription regulation of host gene expression. MicroRNA expression is in part determined by host genetic factors.[31,32] Recently microRNAs

have been shown to influence the composition of the gut microbiota,[33] where specific microRNAs appear to control colonization of the gut by given bacterial taxa and contribute to global concentration of microorganisms. However, to date no study has explored if microRNA variation between individuals is associated with inter-individual variance in gut microbiota composition. Additional potential mechanisms that may in part account for host genetic impacts on gut microbiota include host genetic influences on dietary preferences, because diet is a primary factor shaping gut microbiota composition,[34,35] and related host genetic impacts previously linked to lifestyle (such as nicotine and alcohol intake,[36,37] and exercise frequency). Thus, genetic variants involved in shaping the gut microbiota of the host might be one of the factors leading to increased risk of developing adiposity phenotypes. Hence, identifying heritable microbes associated to obesity could lead to new intervention strategies that would allow weight gain reduction.

One moderately heritable genus identified within the TwinsUK cohort was *Blautia* (heritability = 0.30[16]). In Beaumont et al.,[9] and in the current study OTUs belonging to the *Blautia* genus are significantly associated with VFM. Interestingly, *Blautia* have been reported to be able to convert CO_2 into acetate,[38] which is an established lipids precursor. In 1952, shortly after the development of *ob/ob* mice, lower conversion rates of ingested acetate into CO_2 were observed in obese mice in comparison to lean animals.[39] This result hinted toward the central role of the gut microbiota in mediating energy metabolism and obesity through modulation of acetate bioavailability. Subsequently, multiple studies exploring the role of the gut microbiota in obesity using the same mouse system but applying new technologies, have confirmed this observation.[24,36] An increase in acetate content was observed in the cecum of obese animals with an enrichment of genes able to convert CO_2 into acetate compared with their lean mates.[24] The present results confirm the negative influence of this heritable genus on adiposity and energy expenditure, which could potentially be mediated by increased acetate production.

Conclusion

Our results using updated analyses of the TwinsUK cohort data set confirm the previously published association between 7 gut bacterial genera and visceral fat content. While several genera appear as protective factors in VFM, only *Blautia* stood out as a risk factor that is in part heritable. It is difficult to evaluate to which extent bacteria associated with adiposity phenotypes are a response to host genetic modulations or other environmental parameters. Still, gut bacteria are malleable parameters, for example through particular food intake, and therefore represent a potential intervention targets. Such approaches should be considered within personalized medical care strategies and might provide one avenue to tackle the obesity epidemic.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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