Longitudinal Analysis of Serum Cytokine Levels and Gut Microbial Abundance Links IL-17/IL-22 With *Clostridia* and Insulin Sensitivity in Humans

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Recent studies using mouse models suggest that interaction between the gut microbiome and IL-17/IL-22–producing cells plays a role in the development of metabolic diseases. We investigated this relationship in humans using data from the prediabetes study of the Integrated Human Microbiome Project (iHMP). Specifically, we addressed the hypothesis that early in the onset of metabolic diseases there is a decline in serum levels of IL-17/IL-22, with concomitant changes in the gut microbiome. Clustering iHMP study participants on the basis of longitudinal IL-17/IL-22 profiles identified discrete groups. Individuals distinguished by low levels of IL-17/IL-22 were linked to established markers of metabolic disease, including insulin sensitivity. These individuals also displayed gut microbiome dysbiosis, characterized by decreased diversity, and IL-17/IL-22–related declines in the phylum *Firmicutes*, class *Clostridia*, and order *Clostridiales*. This ancillary analysis of the iHMP data therefore supports a link between the gut microbiome, IL-17/IL-22, and the onset of metabolic diseases. This raises the possibility for novel, microbiome-related therapeutic targets that may effectively alleviate metabolic diseases in humans as they do in animal models.

The human gut microbiome consists of trillions of microorganisms that are known to impact host physiology. Variation in composition of the gut microbiome has been linked to metabolic disorders such as hypertension (1,2), obesity (3,4), and insulin resistance (5), as well as to type 2 diabetes (T2D) (6–10).

While much remains to be learned about the functional mechanisms underpinning this relationship, growing evidence in mouse models points to an important role for the microbially mediated immune system (11,12). Deficiencies in TLR5 (13), RORγt (14), or IL-22 (15) are associated with a variety of metabolic disorders. Additionally, high-fat diet–induced obesity results in a reduction in IL-17–producing cells in the small intestine lamina propria (SILP) (14), while induction of Th17 cells (16), or treatment with gut-homing Th17 cells (17), low-dose IL-17 (18), or IL-22 (13,15), can ameliorate the obesity-associated metabolic phenotype. Collectively, these results suggest a protective role for IL-17/IL-22–producing cells during onset of diet-related metabolic disorders in mice. While Th17 cells are a major source of IL-17 and IL-22 (16,19), other cell types also produce these cytokines (20) and may be important in this process.

In humans, low IL-22 has been associated with impaired fasting glucose and T2D (21). However, the relationship between IL-17/IL-22 production, the microbiome, and metabolic diseases remains controversial (22–24) and comparatively understudied. Large-scale integrated omics studies provide an ideal opportunity to investigate this relationship. Here, we present an ancillary study of the recently released prediabetes arm of the Integrated Human Microbiome Project (iHMP) (25), in which our goal was to investigate the relationship between IL-17/IL-22 profiles, the gut microbiome, and aspects of the metabolic syndrome. Specifically, we aimed to test hypotheses in humans that have hitherto only been convincingly demonstrated in mice.

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RESEARCH DESIGN AND METHODS

This study is an ancillary analysis of data collected as part of the iHMP. An overview of the iHMP is provided below, along with a description of the collection of the specific data sets included in this analysis. However, full details of study design, recruitment, and sample collection can be found in our iHMP flagship article (26).

iHMP Overview

The iHMP consists of 103 human participants identified as at risk for developing T2D, who were followed over a 4-year period (Supplementary Figs. 1 and 2). During this time, detailed multiomic profiling was carried out at quarterly intervals and more frequently during periods of stress or upper respiratory infection. Participants were recruited following Stanford University Institutional Review Board Protocol no. 23062.

Blood and Insulin Sensitivity Measurements

Blood samples were collected from participants following an overnight fast (26) and used for lipid and metabolic panels, as well as fasting plasma glucose (FPG) and hemoglobin A_{1c} (HbA_{1c}) tests. Measurements of FPG $\geq$126 mg/dL were classified as diabetes, while measurements of FPG between 100 and 125 mg/dL were classified as prediabetes. HbA_{1c} was assumed to be an indicator of 3-month average glucose levels, with measurements $\geq$6.5% (48mmol/mol) classified as diabetes and measurements between 5.7% and 6.5% (39–48 mmol/mol) classified as prediabetes. In addition to standard tests, a subset of study participants ($n = 65$) underwent a one-time measurement of steady-state plasma glucose (SSPG) levels via a modified insulin-suppression test (26). Briefly, individuals were infused with glucose (240 mg/m^2/min), octreotide (0.27 μg/m^2/min), and insulin (25 mU/m^2/min) for 180 min after an overnight fast. Starting from 150 min, blood was drawn at 10-min intervals. Four plasma samples (from blood drawn at 150 min, 160 min, 170 min, and 180 min) were measured for glucose and insulin concentrations. SSPG was the mean of the four plasma glucose concentrations. At these time points, insulin concentrations were at a steady state and were similar in all subjects (65 μU/mL); thus, the SSPG provides a direct measure of the relative ability of insulin to dispose of a glucose load: the higher the SSPG concentration, the more insulin resistant the individual. Individuals with SSPG $<$150 mg/dL were classified as insulin sensitive, while individuals with SSPG $\geq$150 mg/dL were classified as insulin resistant.

Microbiome Measurements

Stool samples were collected and DNA was extracted according to the Human Microbiome Project standard protocol (no. 07-001. V12.0). Bacterial relative abundance was then determined by sequencing the V1–V3 region of the bacterial 16S rRNA gene on the MiSeq platform (Illumina, San Diego, CA).

Cytokine Measurements

Cytokine data were generated from blood samples using a 63-plex Luminex antibody-conjugated bead capture assay (Affymetrix, Santa Clara, California). Raw cytokine data were normalized to median fluorescence intensity (MFI) to eliminate batch effects. Further details of approaches used to generate sequence and cytokine data can be found in our companion article (26). According to the manufacturer's protocol, CHEX1–CHEX4 are different types of background control for Luminex MFI data. Based on preliminary examination of these data, any samples with substantial background noise (determined as $>$5 SD $\pm$ mean value [mean $\pm$ 5 * SD]) for one or more CHEX measurements were removed.

Diet Data

An assessment of the frequency of consumption of 25 food items was carried out during some, but not all, sample collection visits. Details of the food items monitored as well as the results of this questionnaire can be found in Supplementary Table 1. Full details of the questionnaire design and sample collection can be found in our companion article (27).

Statistical Analysis

A two-sided Student *t* test was used for significance testing when data were normally distributed; otherwise, a two-sided Wilcoxon signed rank test or Mann-Whitney *U* test was used. A χ^2 test was used to determine whether the proportion of insulin-resistant individuals was different between high-activity (HA) and low-activity (LA) groups. Linear discriminant analysis based on effect size (LEfSe) (28) was performed to determine whether the microbial taxon abundances differed between HA and LA groups. All statistical tests were performed using R (version 3.5.0). Exploration of diet data was performed by principal components analysis using the prcomp command in R package stats. Diet scores were log transformed prior to analysis.

Data Modeling

Of the 103 iHMP study participants, not all had a sufficient number of repeated measurements for inclusion in this longitudinal study. An overview of the number of participants available for each analysis described below is provided in Supplementary Fig. 2. Key characteristics of the individuals included in the principal analyses are provided in Supplementary Table 2.

Mixture Model of Individuals Based on IL-17/IL-22

Participants with five or more longitudinal cytokine measurements ($n = 68$) were included in a general mixture model (GMM), built using the R package mclust (29). The longitudinal IL-17A, IL-17F, and IL-22 MFI data were summarized as mean value and SD for each individual and then scaled in R. For determination of the optimal number of Gaussian distributed clusters, models with 1–9 clusters were evaluated using the Bayesian information criterion, resulting in three clusters selected for further analyses (Supplementary

Fig. 3). Cluster 1 comprised 25 individuals (LA group), cluster 2 comprised 32 (indeterminate-activity [IA] group), and cluster 3 comprised 11 (HA group), and the mixing probability of each cluster was 0.3634941, 0.4749886, and 0.1615172, respectively. Participants assigned to each cluster were associated with a "confidence of assignment" probability (0%–100%); those with <99% confidence (eight individuals in total) were removed from the subsequent analyses.

Linear Mixed Models

Linear mixed models were built using the R package lme4 (30). Models were built separately to test different hypotheses, as described below.

For testing of whether metabolic profiles were different among three groups, the equation used was as follows: target of interest = group + days + sex + BMI + Adj.age + (1/subject_ID). Fixed effects included group as the categorical variable derived from the GMM model, sex was a binary categorical variable, and BMI and adjusted age (Adj.age) were continuous variables. The term days indicates a numerical measurement of how many days after the overall study start date the sample was collected. Adjusted age described the average age of an individual during the study period. Random effects included a random intercept for each participant (1 / subject_ID).

For red blood cell distribution width (RDW), for adjustment of the previous known effect of mean corpuscular hemoglobin (MCH) and mean corpuscular volume (MCV) on the readout of RDW (31), the model was built as follows: RDW = group + days + MCH + MCV + sex + BMI + Adj.age + (1 / subject_ID). For analysis related to microbiome alpha diversity and *Firmicutes*-to-*Bacteroidetes* ratio, our goal was to understand the fixed effect, group, in this mixed model, so the model is built as follows: microbiome diversity (or *Firmicutes*-to-*Bacteroidetes* ratio) = group + sex + BMI + Adj.age + (1 / subject_ID).

Bayesian Mixed-Effects Model for Taxa and Cytokine Interactions Conditional on Cluster Assignment

Participants with five or more coinciding measurements for both cytokines and microbiome ($n = 53$) were included in a Bayesian negative binomial longitudinal mixed-effects model to evaluate the relationship between individual microbes and IL-17. To account for the zero-inflated nature of microbiome abundance, we used a Bayesian framework on a sparse matrix with a negative binomial distribution (32). Cytokine-related group (HA vs. LA) and cytokine (either IL-17A or IL-17F) were scored as the interaction term and fixed effect, respectively, to test the combined effect of cytokine-related group and cytokine on microbe abundance. IL-22 was excluded from this analysis because a large proportion of IL-22 measurements appeared to be lower than the accurate detection threshold of the Luminex assay (Supplementary Fig. 4). We included the cytokine-related group–cytokine interaction term with the aim of testing the hypothesis that significant microbe-cytokine associations may be detected in HA, but not LA, subjects or vice versa.

Each microbe was modeled as the response variable with a random intercept for each participant, and with fixed effects for time, and an interaction term for the cluster identity (defined by the GMM, described above) and the cytokine of interest, thereby evaluating whether the relationship between a microbe and cytokine pair differed depending on the identity of the cluster. This followed standard matrix notation:

$$M_i = X_i\beta + Z_i b_i + \varepsilon_i$$

where M_i is a vector of microbe relative abundances for each participant i, X_i is the matrix of fixed effects, Z_i is the random effects vector of 1s denoting a random intercept, b_i is a scalar for each participant, and ε_i is a zero-centered error term. The fixed-effects matrix X_i comprised the days post–study start D_i and an interaction term for cluster (C_i) and cytokine (Y_i).

$$X_i = \begin{bmatrix} D_{i,1}\ C_i = 3\ \ Y_{i,1}\, Y_{i,1} | (C_i = 3) \\ D_{i,n} C_i = 3\ \ Y_{i,n}\, Y_{i,n} | C_i = 3 \end{bmatrix}$$

Sampling was performed with four chains with 5,000 iterations per sample and a burn-in of 1,000 iterations. Samples were drawn using the No-U-Turn Sampler implemented in the brms package (33–35). Chain convergence was confirmed by visual inspection of iteration plots and posterior predictive distributions.

Data and Resource Availability

Microbial sequence and cytokine data included in this study can be downloaded from the iHMP data depository website (https://www.hmpdacc.org/ihmp/). Diet data are included in Supplementary Table 1.

RESULTS

Overview of the iHMP Prediabetes Cohort

We used the recently released iHMP data set as the basis for a detailed longitudinal analysis of the relationship between the gut microbiome and serum levels of IL-17A, IL-17F, and IL-22. The 103 individuals in the iHMP cohort were well characterized with respect to glucose-related measures, including fasting glucose, HbA_{1c}, and insulin resistance (SSPG). Notably, however, these measures showed little concordance, suggesting they do not provide a consistent representation of the progression to T2D, which has been shown to be highly variable between individuals in this cohort (36).

Based on HbA_{1c} measurements alone, 4 individuals in the iHMP cohort had diabetes at their first time of measurement ($HbA_{1c} \geq 6.5\%$ [48mmol/mol]), while 37 had prediabetes ($5.7\% \leq HbA_{1c} < 6.5\%$ [$39 \leq HbA_{1c} < 48$ mmol/mol]). Additionally, a further four individuals came to have diabetes at one or more points during the course of the study; however, their HbA_{1c} measurements did not stay within the diabetes range (26,36). For the majority of study participants, HbA_{1c} measurements did not increase across the course of the study (Supplementary Fig. 1).

Given the complexity of T2D diagnosis, we chose to focus on SSPG, which was measured once for the majority of participants and is a robust measure of insulin resistance (37). This focus was in keeping with our central hypothesis that microbiome-mediated changes in IL-17/IL-22 may affect insulin sensitivity.

Individuals Show Discrete IL-17/IL-22 Profiles Associated With Insulin Sensitivity

From the complete iHMP cohort, we selected 68 individuals who had five or more longitudinal cytokine measurements. Using Gaussian mixture modeling based on the mean cytokine level and longitudinal variance in each individual, we observed that 60 of these individuals could be optimally separated into three groups (Supplementary Figs. 2 and 3). Individuals at one extreme were characterized by consistently low cytokine levels and variance, while individuals at the other extreme were characterized by high levels or variance of at least one cytokine (Fig. 1*A* and Supplementary Fig. 3). Henceforth, we refer to these three groups as LA ($n = 20$), IA ($n = 30$), and HA ($n = 10$) to represent differences in their temporally integrated levels of serum IL-17 and IL-22 activity (Fig. 1*A*). Importantly, identifying the three groups in this manner required estimating intraindividual variation (Supplementary Fig. 5), which is not available from cross-sectional data, highlighting the advantage of the longitudinal design. Further investigation indicated that the longitudinal IL-17/IL-22 profiles characterizing each group were not significantly impacted by periods of stress or upper respiratory infection reported during iHMP visits (see additional analyses in Supplementary Materials and Supplementary Figs. 6–8). Baseline characteristics for these 60 individuals, including blood and insulin sensitivity measurements used in subsequent analyses, are provided in Supplementary Table 2.

We next considered the possibility that discrete IL-17/IL-22 cytokine profiles may reflect different stages of metabolic disease progression. Previous studies in mice demonstrated that HFD-induced onset of metabolic disease is associated with loss of $CD4^+$ IL-17–producing cells in the SILP (14), while studies in humans have shown a negative correlation between serum IL-22 levels and physiological indicators of T2D (21). We therefore hypothesized that individuals with an LA profile would show a more severe metabolic phenotype than individuals with an HA profile. Individuals with an IA profile were excluded from this and subsequent analyses because we reasoned that, while they may reflect progression from HA to LA, they may also reflect a healthy state (i.e., pre-HA) prior to the onset of the chronic inflammation that is a characteristic of the metabolic syndrome (38).

Within the two-thirds (40 of 60) of study participants for whom an SSPG measurement was available (Supplementary Table 2), we observed that SSPG levels were significantly lower in HA subjects compared with LA subjects (two-sided Wilcoxon test, $W = 64.5$, $P = 0.021$ [Fig. 1*B*]). Accordingly, individuals in the LA group were more frequently insulin resistant (Supplementary Fig. 9*A*); however,

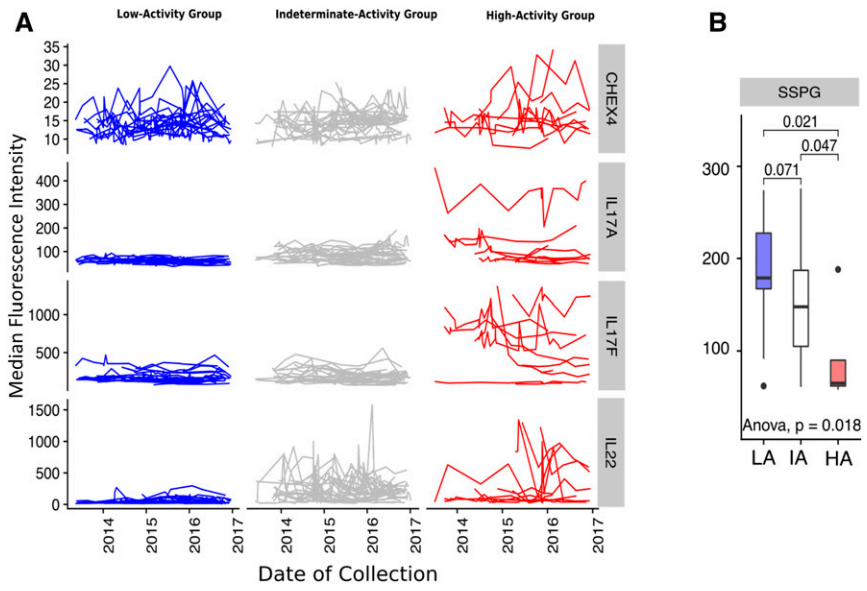

Figure 1—Participants grouped according to IL-17/IL-22 cytokines. *A*: Gaussian mixture modeling of cytokine mean abundance and variance separates study participants into three discrete groups (columns). Lines within each panel represent repeated measurements of serum cytokine abundance for one individual over the study period. Rows represent serum cytokines (IL-17A, IL-17F, IL-22). CHEX4 is a measurement of background fluorescence intensity and can be treated as a negative control. (Note: different scales on *y*-axis for each row.) *B*: SSPG (mg/dL) measurement by group. *P* values for pairwise Wilcoxon test are labeled above the bar plot, and the *P* value for a one-way ANOVA test is labeled under the bar plot. The analysis in *A* was based on 297, 371, and 112 repeated measurements for HA, IA, and LA subjects, respectively.

mean FPG levels, age, and BMI did not vary significantly across groups (Supplementary Fig. 9*A*).

IL-17/IL-22 Inactivity Is Associated With a More Severe Metabolic Phenotype

Longitudinal modeling of clinical data collected across the study period also revealed multiple markers that showed significant differences between LA compared with HA subjects (Fig. 2 and Supplementary Fig. 10). Participants classified as HA showed higher plasma HDL and lower plasma triglycerides. The RDW was higher in HA subjects, consistent with previous findings of high RDW as being associated with high HDL and low triglycerides (39). Additionally, serum sodium and chloride levels were lower in the HA group, and high insulin level has been associated with increased sodium retention in T2D (40). Established markers of T2D, including HbA_{1c} and serum glucose, did not vary significantly between groups (Fig. 2 and Supplementary Figure 10). This was not unexpected, given the limited concordance between glucose-related measurements and that the fact that the majority of participants did not develop T2D during the course of this study (36) (Supplementary Fig. 1).

IL-17/IL-22 Activity Is Associated With Variation in the Composition of the Gut Microbiome but Not Diet

The close association between IL-17/IL-22–producing cells and gut microbiota prompted us to next ask whether individuals distinguished by cytokine activity levels differed in the composition of their gut microbiome. As an individual's gut microbiome remained relatively stable throughout the course of this study (26) (Supplementary Fig. 5), we began by comparing mean microbiome abundance for each participant between HA and LA groups. We found LA subjects had a significantly lower alpha diversity (two-sided Wilcoxon test, $W = 34.0$, $P = 0.003$ [Fig. 3*A* and Supplementary Fig. 11]) and a lower *Firmicutes*-to-*Bacteroidetes* ratio (two-sided Wilcoxon test, $W = 54.0$, $P = 0.044$ [Fig. 3*B* and Supplementary Fig. 12]) compared with HA subjects. LefSE revealed differences between LA and HA groups at multiple taxonomic levels. Most notably, the classes *Bacteroidia* and *Clostridia* were more abundant in the LA and HA groups, respectively (Fig. 3*C*), indicating that members of these taxa were likely responsible for observed differences in the *Firmicutes*-to-*Bacteroidetes* ratio.

As diet profoundly influences the composition of the gut microbiome (41,42), and a high-fat diet results in a loss of IL-17–producing cells in mice (14), we next considered whether IL-17/IL-22 activity was associated with dietary habits recorded as part of the iHMP. Diet composition appeared stable and distinct between individuals (Supplementary Fig. 13*A*), which was consistent with trends observed in the microbiome (Supplementary Fig. 5A). However,

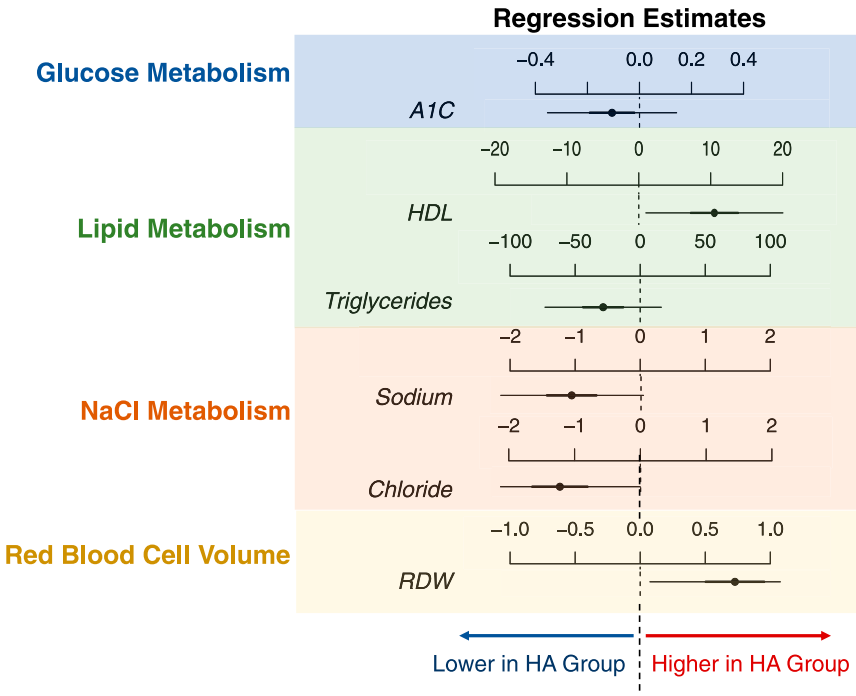

Figure 2—Linear mixed model estimates on fixed effects introduced by LA and HA group. Results for full linear mixed models are shown in Supplementary Fig. 9. The comparisons of active vs. inactive groups are presented here. Dashed lines represent the LA group, while regression estimates for the HA group are displayed as horizontal lines. The center of each horizontal line is the β-coefficient of regression, while thick lines represent 50% credible intervals or ±1 SD and thin lines represent 95% credible intervals or ±2 SD. A1C, hemoglobin A_{1c}. This analysis is based on 88 and 229 repeated measurements for HA and LA subjects, respectively.

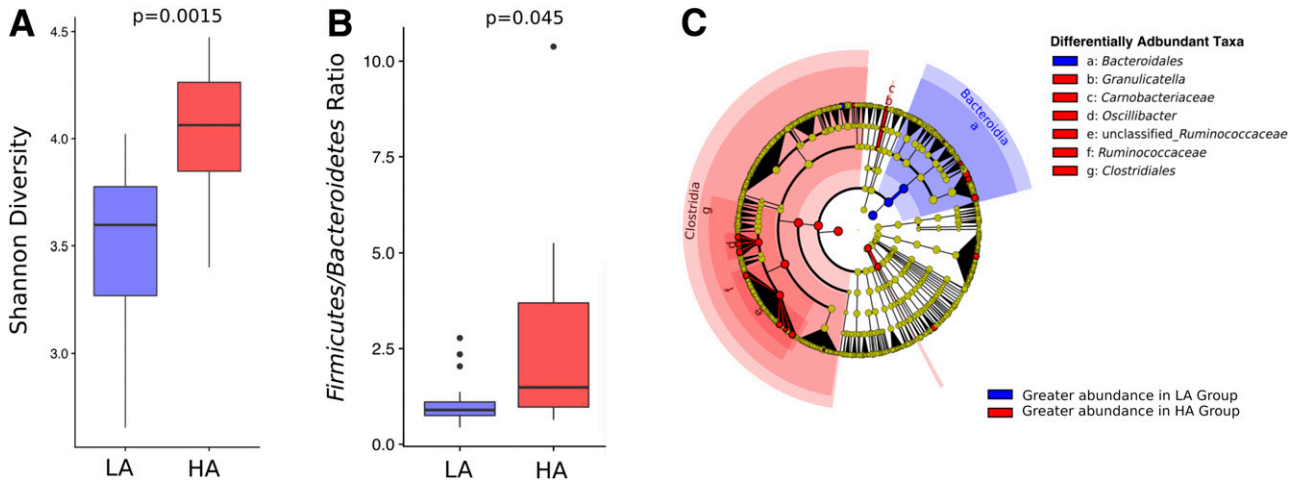

Figure 3—Differences in the gut microbiome of IL-17/IL-22 LA and HA subjects. *A*: Shannon diversity estimates for the HA and LA. Mean value of diversity for each participant across the study period is used to generate this plot. The *P* value from a Wilcoxon test is labeled above the plot. *B*: *Firmicutes*-to-*Bacteroidetes* ratio of HA and LA. Mean value of *Firmicutes*-to-*Bacteroidetes* ratio for each participant across the study period is used to generate this plot. The *P* value from a Wilcoxon test is labeled above the plot. *C*: Cladogram representing the LEfSe results for comparing taxa abundance between HA and LA groups. Circles on the cladogram represent the phylogenetic relationship of taxa that are tested, with phylum at the center and operational taxonomic unit (OTU) on the edges. Each point represents a taxonomic unit. Red color covering a dot/region indicates the taxa that are more abundant in the HA group, and blue color covering a dot/area indicates the taxa are more abundant in the LA group.

there was no evidence that diet varied significantly between LA and HA groups (Supplementary Fig. 13*B*).

Finally, we took advantage of intraindividual variation in microbe and cytokine abundance (Supplementary Fig. 5) by performing longitudinal modeling to look at pairwise relationships between individual bacterial genera and cytokine abundances within HA versus LA subjects. Previous studies identified significant associations between cytokines and gut microbes but only revealed interindividual variation (43,44) due to their cross-sectional design. The repeated and longitudinal measurements of the iHMP study allowed us to test the possibility that intraindividual variation in cytokine/bacteria abundance may be used to identify additional host-microbe associations of biological interest. To accommodate within-individual correlation, we used a mixed-effects model with random effects by individual.

We first observed the abundance of *Alistipes* was positively correlated with changes in serum IL-17F levels (Fig. 4). As models were designed to compare cytokine versus microbe interactions in the context of cytokine activity (encoded as HA vs. LA), this result indicates *Alistipes* was significantly associated with IL-17F in LA subjects. In contrast, seven bacterial taxa were significant for the activity group–cytokine interaction term (Fig. 4), indicating their relative abundance was significantly associated with IL-17F or IL-17A levels in HA subjects. Notably, six of these seven significant relationships involved taxa belonging to the class *Clostridia*, and in all eight associations, the cytokine abundance was positively correlated with taxon relative abundance. In conclusion, analyses of the taxonomic abundance of the gut microbiome both between individuals (Fig. 3) and within individuals (Fig. 4) provide evidence that members of the class *Clostridia* are positively associated with increased levels of IL-17 activity.

DISCUSSION

In this ancillary analysis of the iHMP, we present evidence that individuals at risk for developing T2D display distinct, longitudinal IL-17/IL-22 cytokine profiles, which can be associated with altered severity in a number of established markers for metabolic disorders. By subsequently providing evidence for a link between IL-17/IL-22 and the composition of the gut microbiome, we validate previous findings in mouse models and thus provide further support for the hypothesis that microbe–immune system interactions are relevant to human metabolic homeostasis.

Sustained loss of IL-17/IL-22 activity in iHMP study participants was associated with increased insulin resistance, as well as variation in metabolic markers that included lower HDL and increased triglycerides. A trend for higher HbA_{1c} in LA subjects was not statistically significant. This is consistent with the previous observation that SSPG and HbA_{1c} measures provide different perspectives on insulin resistance and glucose metabolism (36). It may also reflect the fact that few individuals were classified as having diabetes at any point during the course of this 4-year study. In spite of such inconsistencies between measures, our observations are in line with evidence that $ROR\gamma t^{-/-}$ and $IL\text{-}22^{-/-}$ mouse models show reduced insulin sensitivity on a chow diet. Furthermore, low-dose administration of IL-17 (18) or IL-22 (15) suppresses the metabolic phenotype induced by a high-fat diet. Taken together, these murine studies suggest that circulating

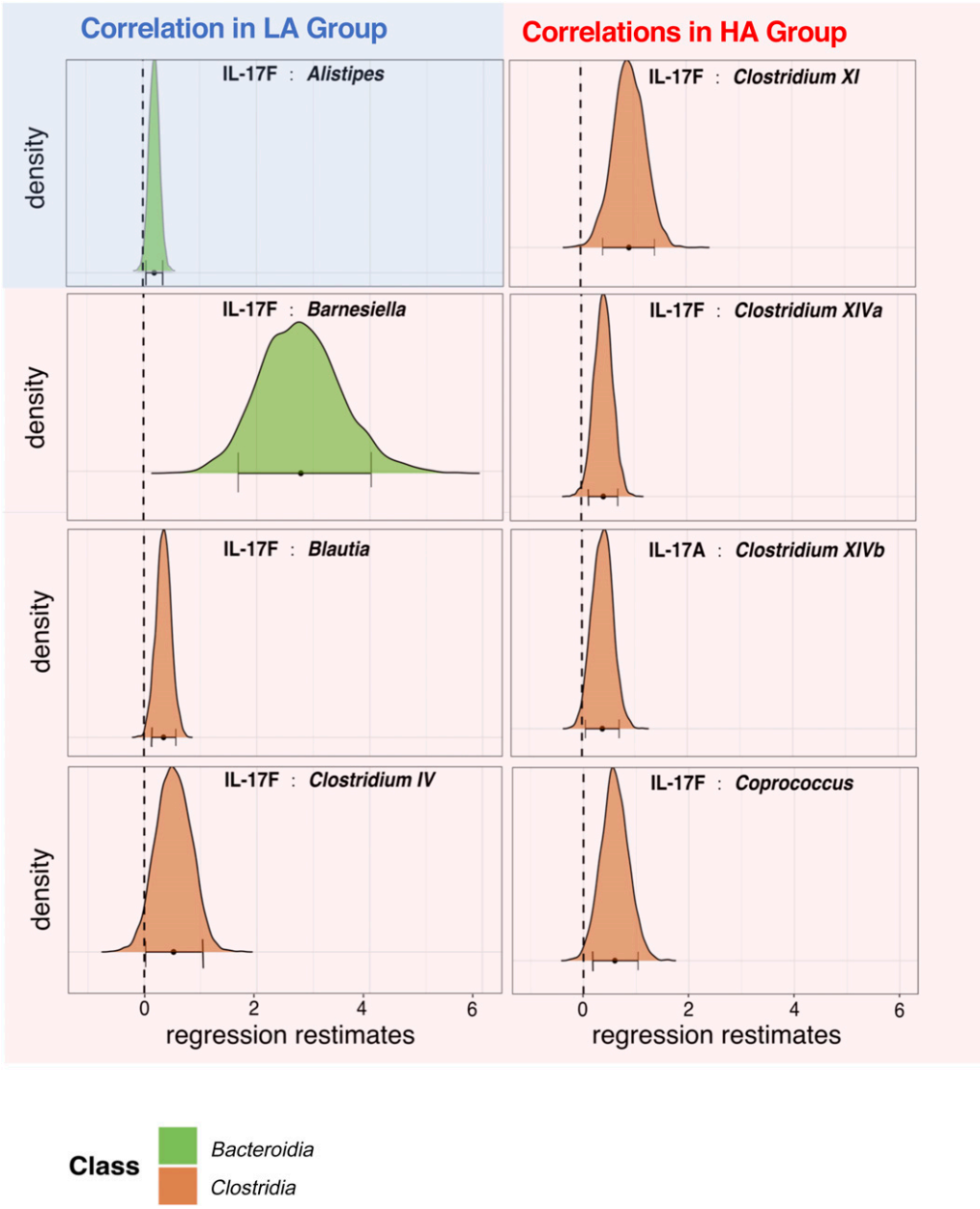

Figure 4— Bacterial genera whose abundance correlates with serum IL-17. Significant correlations between serum IL-17 and bacterial genus abundance are shown for HA subjects (red panels) and LA subjects (blue panel). Distributions show estimated effect sizes from Bayesian Markov chain Monte Carlo draws after parameter convergence. Panels show bacteria for which the estimated effect is significantly greater or less than zero (95% credible interval does not include zero). This analysis was based on 264 and 100 repeated measurements for HA and LA subjects, respectively.

levels of IL-17/IL-22 can be protective. Our work suggests that similar mechanisms may also apply to humans.

One explanation for the protective effects of IL-17/IL-22 is that these cytokines are directly, or indirectly, involved in regulating the composition of the gut microbiome, e.g., by regulating the production of antimicrobial peptides that limit the abundance of potentially pathogenic taxa (45–47). Another possibility is that IL-17/IL-22 may influence tight junction function, meaning their deficiency could result in a leaky gut (47–51). This could in turn contribute to translocation of gut bacteria to the blood, which has been associated with T2D (52). Alternatively, IL-22 may directly influence β-cell function (53), in which case changes in the gut microbiome may be correlative, rather than directly contributing to the phenotype reported here.

While IL-17/IL-22 may influence the gut microbiome, the composition of the gut microbiome may reciprocally affect metabolic diseases via the ability of certain taxa to directly, or indirectly, influence IL-17/IL-22 production. In

a cross-sectional study of the influence of the human gut microbiome on cytokine production, Schirmer et al. (44) previously demonstrated that IL-17 production from peripheral blood mononuclear cells exposed to *Staphylococcus aureus* was positively correlated with the relative abundance of *Clostridium* in the host gut microbiome. This is consistent with our observations that *1*) IL-17/IL-22 HA subjects had greater mean relative abundance of *Clostridium* in gut microbiome across the course of this study and that *2*) in longitudinal analysis, three members of the class *Clostridia* (*Clostridium IV*, *Clostridium XI*, *Clostridium XIVa*) were positively correlated with IL-17F production and one (*Clostridium XIVb*) with IL-17A production. Human isolates from three of these *Clostridium* clusters (*XIVa*, *XIVb*, *IV*) were previously found to induce Th17 cells (a major producer of IL-17 and IL-22 [54,55] in mice [56]). Notably, germ-free mice do not carry Th17 cells in the SILP (57), but inoculation with certain bacteria, including segmented filamentous bacteria, can induce Th17 cell development (58,59). While segmented filamentous bacteria remain an ambiguous clade, they may be related to the family *Clostridiales* (60). In conclusion, while our study does not characterize sources of IL-17/IL-22 production, the trends we report are consistent with previous evidence that members of the class *Clostridia* can induce development of IL-17/IL-22–producing cells. This in turn represents one plausible way in which the gut microbiome could influence human cytokine profiles and thereby influence metabolic diseases.

Establishing the causative relationship of IL-17/IL-22, the gut microbiome, and metabolic diseases is beyond the scope of these data and will benefit from a deeper understanding of the mechanisms that underpin this interaction. The role of diet in shaping both the immune environment and composition of the gut microbiome is likely to be of particular interest, given that loss of IL-17–producing cells has been linked to a high-fat diet (14). No significant variation was observed between the diets of subjects with IL-17/IL-22 activity versus inactivity in this study, which may suggest that important differences in the composition of the microbiome are influenced by factors other than diet. However, it may also be that the available diet information was not sufficient to capture differences relevant to this study.

Regardless of whether differences in the microbiome are due to diet, or other environmental and host genetic factors, the molecular mechanisms underpinning interaction between members of the *Clostridia* and the host immune system warrant further investigation. One possibility is that gut microbiome–derived aryl hydrocarbon receptor signaling is critical for maintenance of Th17 cells (61,62). Bacterial-produced aryl hydrocarbon receptor ligand is reduced after mice are switched to a high-fat diet (63). Alternatively, Qin et al. (9) established a correlational association between the loss of members of the *Clostridia* in T2D patients and reduced short-chain fatty acid (SCFA) production. A later study confirmed this observation and demonstrated that supplementing SCFA-producing bacteria strains to T2D patients can improve their clinical outcomes (64), possibly via the ability of SCFA to influence Th17 production (65).

A close link between IL-17/IL-22 and members of the *Clostridia* supports previous assertions that manipulating the relationship between cytokines and the gut microbiome presents novel therapeutic opportunities. In humans, transferring the gut microbiome from lean donors to patients with metabolic syndrome has been shown to increase the insulin sensitivity of the recipients. Notably, among 16 bacteria strains that increased in gut microbiome of recipients postprocedure, 12 strains belonged to the class *Clostridia* (66). In mice, metabolic disorders accompanied by a lack of Th17 cells and IL-22 could be rescued, either by induction or adaptive transfer of Th17 cells (16,17), low-dose IL-17 (18), and IL-22 (15) or by supplementing the gut microbiome with symbiotics that increase Th17 cell abundance (14). Our demonstration of a close relationship between IL-17/IL-22 and members of the *Clostridia* therefore provides valuable insight into the biological processes that underpin the efficacy of these approaches.

In conclusion, our analysis of the newly released iHMP data set suggests novel avenues of research and raises the possibility of therapeutic targets related to IL-17/IL-22 that may effectively alleviate metabolic diseases in humans as they do in animal models.

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