Supplementary text: Materials and Methods

Clinical study protocol

This randomized clinical trial involved two research centers – The Karolinska institute in Sweden, further referred to as KI, and Helperby Therapeutics Ltd in the United Kingdom, further referred to as HP. Each study was approved by the respective institutional boards, the respective national competent authorities and was registered to the European Union clinical Trials Register (KI: Clinical Trial Centre at the Karolinska University Hospital, MPA, EudraCT Number: 2010-023889-52; HP: The Wandsworth Research Ethics Committee; MHRA, EudraCT Number: 2009-017647-34). Each center involved healthy volunteers, randomized into two test groups and one control group.

At KI, 30 volunteers (15 males and 15 females, average age 26 years, range 18-45 years) were randomly assigned to either the ciprofloxacin (Cipro), the clindamycin (Clinda) or the Placebo (Plac KI) group. The antibiotics (150 mg Clinda four times a day, 500 mg Cipro twice a day) and Placebo were administered for 10 days.

At HP, 44 volunteers (13 males, 31 females, average age 26 years, range 18-39 years) were randomly assigned to the amoxicillin (Amox, N=15), the minocycline (Minoc, N=15) or the Placebo (Plac HP, N=14) age and gender matched groups. The dosage used was that recommended by the British National Formulary for amoxicillin 250 mg (Amoxil capsules, GlaxoSmithKline, UK) three times daily for seven days and minocycline 100 mg (Aknemin capsules, Almirall) twice daily for five days. Placebo was administered in two doses in 5 days.

Saliva and faecal samples were collected on 6 occasions: immediately before administration of the antibiotic (baseline), immediately after the treatment course was completed (week 1), 1 month, 2 months, 4 months and 12 months post-dosing. Samples were collected in sterile tubes and containers and were frozen at -70°C until processed.

Sample processing, 16S rRNA gene amplicon sequencing and data processing

Frozen saliva samples were shipped to ACTA, Amsterdam, and faeces – to TNO, Zeist, both in the Netherlands, for sample processing for 16S rRNA gene amplicon sequencing. DNA was extracted as described previously (1).

Barcoded amplicon libraries of the small subunit ribosomal RNA gene hypervariable region V5-V7 were generated for each of the individual sample as described previously (2), pooled and sequenced by a Genome Sequencer FLX Titanium system (Roche, Basel, Switzerland). The sequencing data was processed using QIIME (Quantitative Insights Into Microbial Ecology)(3) version 1.5.0 as described previously (4). To allow comparisons among different samples, a randomly subsampled OTU-table at the lowest number of reads/sample was created (1730 reads/sample).

Metagenomic shotgun sequencing

Metagenomic libraries were prepared from isolated DNA using the NEB DNA Mastermix Library Prep kit (New England Biolabs https://www.neb.com/products/e6040-nebnext-dna-library-prep-master-mix-set-for-illumina), with size selection by eGel (Life Technologies https://www.lifetechnologies.com/order/catalog/product/G661002). Libraries

were sequenced 250bp paired-end using the Illumina Miseq reagent kit V2 on an Illumina MiSeq.

After quality filtering with Trimmomatic (5), the paired and unpaired forward reads were combined and reads with more than 10% ambiguous bases were removed. Next, the reads were screened for human sequences with Best Match Tagger v3.101(6) after which human reads and duplicate reads were removed.

Metagenome prediction from 16S rRNA gene amplicon data and validation with shotgun data

Microbial metagenomes were predicted from 16S rRNA gene sequences using PICRUSt (7) according to the pipeline at http://picrust.github.io/picrust/. In brief, the cleaned amplicon sequences were clustered into OTUs using the 'closed reference' OTU-picking protocol [QIIME 1.5.0] against the Greengenes database (8) v13.5, preclustered at 97% identity. The sequences from the 97% representative set were truncated to the V5-V7 region using TaxMan (5% mismatch, both primers removed) (9). Next, these sequences were clustered at 97% similarity using USEARCH v6.1.54 (10). After OTU picking, the OTU-table was randomly subsampled at 1730 reads/sample. The subsampled OTU-table was normalized by the known/predicted 16S rDNA copy number abundance using PICRUSt script normalize_by_copy_number.py. The metagenome functional predictions were created using the predict_metagenomes.py script. The obtained KEGG Pathway metadata was collapsed at the third (lowest) functional category level using categorize_by_function.py script.

The accuracy of PICRUSt predictions was compared with HUMANn (11) predictions based on the obtained metagenomic shotgun data. Shotgun data was searched against a reduced KEGG database (cf. HUMANn) using UBLAST from USEARCH v7.0.1090 (10) with a maximum of 20 hits and an E-value threshold of 1E-5. Shotgun data was not rarefied as to take all data into account for the calculation of the accuracy of PICRUSt predictions. The PICRUSt predicted KEGG Orthologs (KOs) and mapped shotgun reads were summarized with HUMANn to KEGG modules. Spearman's rank correlation coefficients between the PICRUSt and shotgun datasets were calculated for nine baseline samples (cor.test; R version 2.15.1).

Statistical analyses

For the assessment of sample inter-relationships the subsampled OTU-table was mapped into an ordination by Principal Component Analysis in PAST software (12). The OTU-data was log-2 transformed before the ordination to normalize for highly abundant taxa. One-way PERMANOVA with Bray-Curtis similarity (13) and SIMPER with Bray-Curtis similarity, both in PAST, were used to assess the differences in microbiomes between groups. The diversity statistics (Shannon Diversity Index) was performed using PAST. Diversity index was compared using the General Lineal Model Repeated Measures (GLM RM) test (for related samples) and one-way ANOVA with the Games-Howell post-hoc test (for unrelated samples) in SPSS (SPSS, version 20.0). Bray-Curtis similarity between the microbiome profiles of the baseline and the other time point sample from the respective individual were calculated and the similarity of the respective visit pairs from different treatment groups were compared using one-way ANOVA with the Games-Howell post-hoc test.

Within each of the treatment groups, the similarity between the baseline visit and the last visit (month 12) was compared with the similarity of the baseline and each of the other visit pairs (baseline – week 1, baseline – month 1, baseline – month 2 and baseline – month 4) using GLM RM test. The difference in predominant genera (present in at least 20% of samples) between the treatment and control groups was assessed using the Mann-Whitney test and corrected for multiple comparisons using Bonferroni correction.

Predicted metagenomes were analyzed using STAMP – Statistical Analysis of Taxonomic and Functional Profiles (14) software version 2.0.8. The relative frequencies of functional categories and KEGG ortholog groups (KO system) were compared between the sample groups using a two-sided Welch's t-test with the Welch's inverted CI method and Storey FDR correction for multiple comparisons.

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