# Supplementary methods

## MRI study

*MRI acquisition and image pre-processing:* All subjects were studied on a 1.5T Ingenia (Philips Healthcare, Best, The Netherlands) with eight channel head coils. Structural images were acquired using a 3D Turbo Field Echo Planar Imaging (TFEPI) sequence and parameters of echo time (TE) = 4.1ms, repetition time (TR) = 8.4ms, flip angle 8°, field of view (FOV) 230x190 matrix. A total of 145 whole-brain images per subject with thickness axial slices of 1x1x1mm³ with or without gap. The total scan time was 189.6s. The anatomical imaging data was processed and analyzed using MATLAB version R2017a (The MathWorks Inc, Natick, Mass) and Statistical Parametric software (SPM12; The Welcome Department of Imaging Neuroscience, London). Preprocessing steps involved motion correction, spatial normalization and smoothing using a Gaussian filter (FWHM 8 mm). Data were normalized to Diffeomorphic Anatomical Registration Through Exponentiated Lie (DARTEL) and resliced to a 2mm isotropic resolution in Montreal Neurological Institute (MNI) space.

*Volumetric brain analyses:* The Automated Anatomical Labeling (AAL) [1] atlas was used to obtain the volumetric information of 94 participants. Each region was orthogonalized for sex, age and total grey matter volume in MATLAB version R2017a (The Math Works Inc, Natick, MA) and subsequently entered to SPSS to investigate for differences between participants with and without obesity using independent sample t-test and for associations with the microbiota using Spearman correlation analyses and corrected for multiple comparisons using q-values [2].

**Cognitive assessment**

The *Stroop Color-Word Test (SCWT)* (Golden's version) was administered to assess cognitive flexibility, selective attention, inhibition and information processing speed. This version consists of three different parts: 1) 100 words (color names) are printed in black ink and the subject is asked to read them as fast as possible, 2) 100 "XXX" are printed in color ink (green, blue and red) and the subject is asked to name as fast as possible the ink color, and 3) 100 color names (from the first page) printed in color ink (from the second page), the color name and the ink color do not match and the subject is asked to name the ink color (and not to read the color name). The subject is given 45 seconds for each task, after the 45 seconds the last item completed is noted, obtaining three scores: one for each part of the test ("P", "C" and "PC"). The interference ("I") index was also obtained from the subtraction PC-PC' (PC'=PxC/P+C). Standard administration procedures were followed as indicated in the test manual [3].

Computerized version of the *Iowa Gambling Task (IGT)* (Bechara, A; Psychological Assessment Resources, Inc.) was used to assess decision making. Four upside down card decks are shown in the screen, each of them identified by a letter (A, B, C or D). The subject is told that he or she can freely choose cards from any deck in order to win as much money as possible. When the subject clicks on a card deck a smiley face and the amount of money won appears in the screen. Sometimes, after the smiley face is showed a sad face appears together with a message indicating the amount of money lost. In the upper left side of the screen there are a green bar and a red bar indicating the amount of money won and lost. A and B decks give bigger amounts of money but they also make important losses meanwhile C and D decks make smaller profits but they also cause less losses. On A and C decks punishment frequency raises progressively but magnitude is constant, on B and D decks punishment frequency is constant but,

magnitude raises progressively. Standard administration procedures were followed as indicated in the test manual [4] Standardized (t-score) Net total score, which results from the subtraction of the disadvantageous from the advantageous decks (CD-AB), was used for the statistical analysis.

The *Wisconsin Card Sorting Test (WCST):* computer version 4-Research Edition (Heaton, RK; Psychological Assessment Resources, Inc.) was used to assess executive function. The test consists in four stimulus cards: one with a red triangle, one with two green stars, one with three yellow crosses and another with four blue circles. The stimulus cards are always placed in the screen and different cards are shown below, one at a time. The subject is asked to match each of these cards, which have designs similar to those on the stimulus cards (varying in color, geometric form or number), with one of the four stimulus cards. No warning is provided about the sorting rule neither about changes of the rule, only feedback about the answer is given in each trial (correct or incorrect). The sorting rule (color, form or number) changes after 10 consecutive correct answers (category). Standard administration procedures were followed [5]. We analyze the "trials to complete first category" score, which is the total number of trials needed to complete 10 consecutive correct answers.

**Extraction of faecal genomic DNA and whole-genome shotgun sequencing**

Total DNA was extracted from frozen human stools using the QIAamp DNA mini stool kit (Qiagen, Courtaboeuf, France). Quantification of DNA was performed with a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Carlsbad, CA, USA), and 1 ng of each sample (0.2 ng/µl) was used for shot gun library preparation for high-throughput

sequencing, using the Nextera DNA Flex Library Prep kit (Illumina, Inc., San Diego, CA, USA) according to the manufacturers' protocol.

Sequencing was carried out on a NextSeq 500 sequencing system (Illumina) with 2 X 150-bp paired-end chemistry, at the facilities of the Sequencing and Bioinformatic Service of the FISABIO (Valencia, Spain). The obtained input fastq files were decompressed, filtered and 3· ends-trimmed by quality, using prinseq-lite-0.20.4 program [6] and overlapping pairs were joined using FLASH-1.2.11[7]. Fastq files were then converted into fast files, and human and mouse host reads were removed by mapping the reads against the GRCh38.p11, reference human genome (Dec 2013), and GRCm38.p6, reference mouse genome (Sept 2017), respectively, by using bowtie2-2.3.4.3 [8] with end-to-end and very sensitive options. Next, functional analyses were carried out by assembling the non-host reads into contigs by MEGAHIT v1.1.2 [9] and mapping those reads against the contigs with bowtie2. Reads that did not assemble were appended to the contigs. Next, the program Prodigal v2.6.342 [10] was used for predicting codifying regions. Functional annotation was carried out with HMMER [11] against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, version 2016 [12] to obtain the functional subcategory, route and annotation of the genes. The filtering of the best annotations and the assignment of the orf annotation to every read were carried out using the statistical package R 3.1.0 [13] which also was used to count the aligned reads and to add the category and its coverage, and finally to build abundance matrices. Taxonomic annotation, was implemented with Kaiju v1.6.2 [14] on the human and mouse-free reads. Addition of lineage information was added, counting of taxa and generation of an abundance matrix for all samples were performed using the package R.

**Metabolomics analyses**

For non-targeted metabolomics analysis, metabolites were extracted from plasma and faecal samples with methanol (containing phenylalanine-C13 as an internal standard) according to previously described methods [15]. Briefly, for plasma samples 30µl of cold methanol were added to 10 µl of each sample, vortexed for 1 minute and incubated for one hour at −20 °C. For faecal samples, the content of a 1.2 ml tube of Lysing Matrix E (MP biomedicals) and 600 µl of cold methanol were added to 10mg of sample. Samples were homogenized using FastPrep-24™ (MP biomedicals) and were incubated overnight in a rocker at 4°C. Then, all samples were centrifuged for three minutes at 12.000g, the supernatant was recovered and filtered with a 0.2 µm Eppendorf filter. Two µL of the extracted sample were applied onto a reversed-phase column (Zorbax SB-Aq 1.8 µm 2.1 x 50 mm; Agilent Technologies) equipped with a precolumn (Zorbax-SB-C8 Rapid Resolution Cartridge 2.1 x 30 mm 3.5 µm; Agilent Technologies) with a column temperature of 60°C. The flow rate was 0.6 mL/min. Solvent A was composed of water containing 0.2% acetic acid and solvent B was composed of methanol 0.2% acetic acid. The gradient started at 2% B and increased to 98% B in 13 min and held at 98% B for 6 min. Post-time was established in 5 min.

Data were collected in positive and negative electrospray modes time of flight operated in full-scan mode at 50–3000 m/z in an extended dynamic range (2 GHz), using N2 as the nebulizer gas (5 L/min, 350°C). The capillary voltage was 3500 V with a scan rate of 1 scan/s. The ESI source used a separate nebulizer for the continuous, low-level (10 L/min) introduction of reference mass compounds 121.050873 and 922.009798, which were used for continuous, online mass calibration. MassHunter Data Analysis Software (Agilent Technologies, Barcelona, Spain) was used to collect the results, and

MassHunter Qualitative Analysis Software (Agilent Technologies, Barcelona, Spain) to obtain the molecular features of the samples, representing different, co-migrating ionic species of a given molecular entity using the Molecular Feature Extractor algorithm (Agilent Technologies, Barcelona, Spain). We selected samples with a minimum of 2 ions. Multiple charge states were forbidden. Compounds from different samples were aligned using a retention time window of 0.1% ± 0.25 minutes and a mass window of 20.0 ppm ±2.0 mDa. We selected only those present in at least 50% of the samples of one group and corrected for individual bias.

Faecal samples for the Ironmet cohort were also analysed by Nuclear Magnetic Resonance (NMR). The preparation protocol started with around 15-20 mg of dried faecal matter that was placed in a 2 ml Eppendorf tube. Then, 500 µl of 0.05 M PBS buffer in H2O (pH=7.3) was added and vortexed vigorously, frozen and thawed twice and centrifuged (2.1000 g, 15 min, 4°C) to obtain a clear faecal water over the precipitated stool. From the upper layer, 200 µl of prepared faecal water was placed in a 2 ml Eppendorf tube and then, 400 µl of 0.05M PBS buffer in D2O (pH=7.2, TSP 0.7mM) was added. The sample was vigorously vortexed and sonicated until complete homogenization and the mixture (clear dispersion), if necessary, was centrifuged again (14.000 rpm around 14.000 g, 5 min, 4ºC). For NMR measurement the clear upper phase was placed into a 5mm o.d. NMR tube. $^{1}$H NMR spectra were recorded at 300 K on an Avance III 600 spectrometer (Bruker®, Germany) operating at a proton frequency of 600.20 or 500.13 MHz using a 5 mm PABBO gradient probe.

**Animals faecal microbiome transplantation (FMT) experiments**

All animal procedures were performed in accordance with the guidelines of the European Communities Council Directive 2010/63/EU regulating animal research and were approved by the local ethical committee (Comitè Ètic d´Experimentació Animal-Parc de Recerca Biomèdica de Barcelona, CEEA-PRBB). For each FMT study, thirty-three wild-type C57BL/6J male mice were used. Upon arrival to the animal facilities, animals were let to adapt during 5 days to housing conditions (12 hours reversed light/dark cycle, 08:00 AM lights off). Mice were housed individually in controlled laboratory conditions with temperature maintained at 21±1 °C and humidity at 55 ± 10%. Food and water were available *ad libitum* during all the experiment. Operant behavior testing was always performed during the first hours of the dark phase of the reversed light/dark cycle. Body weight gain of mice was controlled during all the experiment.

*Experimental design FMT Study 1.* After acclimatization to housing conditions, animals were divided intro Control *(n=11)* and Transplant *(n=22)* groups matched for average body weight. Transplant group mice were given *ad libitum* cocktail of antibiotics during 14 days in drinking water to deplete gut microbiota. Antibiotic cocktail consisted of ampicillin (1 g/L), metronidazole (1 g/L), vancomycin (400 mg/L), ciprofloxacin HCl (250 mg/L) and imipenem (250 mg/L). After 14 days of antibiotic intake animals were subjected to a 72 hours wash out and then colonized via daily oral gavage of donor microbiota (200 µL) for 3 days. Donor microbiota was acquired from fecal samples of non-obese (*n*=11) and obese (*n*=11) patients matched for age, sex, and education years. Booster inoculations were given twice weekly to throughout the study to reinforce donor microbiota phenotype. Control animals were subjected to the same protocol but instead of receiving donor microbiota they received oral gavage of 200 µL of saline

solution (0.9% NaCl). 10 days after the first oral gavage animals were subjected to food self-administration procedure (see below) for the next 18 days. Two days after termination of self-administration procedure animals were sacrificed and cecums were extracted, weighted and directly frozen in dry ice and stored at -80 °C.

*Food self-administration.* Mouse operant chambers (Model ENV-307A-CT, Med Associates, Georgia, VT, USA) were used for operant responding. At the start of each food self-administration session, a house ceiling light turned on during the first 3 seconds of the session to indicate the start of the session. All sessions lasted 60 min and regular-flavored pellets were used. The food self-administration session consisted of two pellet periods of 25 min and a 10 min pellet-free period in between both pellet periods (25/10/25). In the two pellet periods, animals received a pellet after an active response paired with a stimulus light (cue light). After performing an active response on the active lever, a time-out period of 10 sec was set where the cue light was off and no reward (pellet) was provided. No pellets were provided in the inactive lever. Responses on active lever, inactive lever and during the time-out period were recorded. The start of the pellet-free period was signaled by the illumination of the entire operant chamber. During this period no pellet was delivered. In the operant conditioning sessions, mice were under fixed ratio 1 (FR1) of reinforcement during 7 days (one active lever-press resulted in a delivery of one pellet). Following FR1 phase, animals were subjected to an increase FR up to 5 (5 lever-presses in order to obtain one reward) for 8 days. Criteria for the achievement of the operant responding were acquired when the following conditions were met: (1) at least 75% responding on the active lever; and (2) a minimum of 5 rewards per session (5 and 25 active lever presses in FR1 and FR5, respectively). After each session mice were returned to their home cages.

Persistence to response: Non-reinforced active responses during the pellet-free period (10 min) were measured as a persistence of food-seeking behavior.

Cognitive flexibility: after 8 days of FR5, animals were exposed to 2 sessions of reversal learning (RL). In these 2 sessions, active and inactive levers were switched. Thus, the active lever during FR1 and FR5 phases became the inactive and vice versa. Higher number of lever-presses in the inverted active lever (inactive during FR phases) indicates higher scores of cognitive flexibility.

*Statistical analysis.* All statistical analysis was performed with SPSS (IBM, version 25). Comparisons between groups were analyzed by Student t-test. ANOVA with repeated measures was used when required to test the evolution over time. For food self-administration analysis, within-subject factors were Lever (two levels: active and inactive), Day (7 levels for FR1, 9 levels for FR5 and 2 levels for RL). Between-subject factor was Transplant (2 levels: Control and Transplant). The criterion for significance (alpha) was set at 0.05.

*Experimental design FMT Study 2.* Mice were given a cocktail of ampicillin and metronidazole, vancomycin (all at 500 mg/L), ciprofloxacin HCl (200 mg/L), imipenem (250 mg/L) once daily for 14 consecutive days in drinking water, as previously described [16]. Seventy-two hours later, animals were colonized via daily oral gavage of donor microbiota (150 μL) for 3 days. Animals were orally gavaged with saline ($n$=11) and faecal material from healthy human donor's with low- ($n$=11) and high SCWT scores ($n$=11), matched for age, BMI, sex, and education years. To offset potential confounder and/or cage effects and to reinforce the donor microbiota phenotype, booster inoculations were given twice per week throughout the study. After 4 weeks, mice were sacrificed.

**Study of gene expression in the prefrontal cortex from mice in the FMT study 2**

The brains were quickly removed and the medial prefrontal cortex was dissected according to the atlas of stereotaxic coordinates of mouse brain [17]. Brain tissues were then frozen by immersion in 2-methylbutane surrounded by dry ice, and stored at -80ºC. RNA quality control performed using the RNA 6000 Nano chip (Agilent) on an Agilent Bioalyzer 2100 obtaining RIN values between 8.7 - 9.8. Libraries were prepared from 500 ng of total RNA using the TruSeq stranded mRNA library preparation kit (Illumina, #20020594) with TruSeq RNA Single Indexes (Illumina, #20020492 and #20020493) according to the manufacturer's instruction reducing the RNA fragmentation time to 4.5 minutes. Prepared libraries were analyzed on a DNA 1000 chip on the Bioanalyzer and quantified using the KAPA Library Quantification Kit (Roche, #07960204001) on an ABI 7900HT qPCR instrument (Applied Biosystems). Sequencing was performed with 2x50 bp paired-end reads on a HiSeq 2500 (Illumina) using HiSeq v4 sequencing chemistry. Raw sequencing reads in the fastq files were mapped with STAR version 2.5.3a[18] to the Gencode release 17 based on the GRCm38.p6 reference genome and the corresponding GTF file. The table of counts was obtained with FeatureCounts function in the package subread, version 1.5.1 [19]. Genes having less than 10 counts in at least 5 samples were excluded from the analysis.

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