COGNITIVE FUNCTION IN OBESITY: INTERACTIONS WITH THE GUT MICROBIOME

María Arnoriaga Rodríguez

Universitat de Girona

DOCTORAL THESIS

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Doctoral program in Molecular Biology, Biomedicine and Health

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WE DECLARE:

That the thesis titles "Cognitive function in obesity: Interactions with the gut microbiome", presented by María Arnoriaga Rodríguez to obtain a doctoral degree in Molecular Biology, Biomedicine and Health at the *Universitat de Girona* has been completed under our supervision and meets all the requirements to opt for an International Doctorate.

For all intents and purposes, we hereby sign this document.

José Manuel Fernández-Real Lemos Rémy Burcelin Wifredo Ricart Engel

Girona, December 10, 2021

No hay ninguna sensación comparable a la emoción del descubrimiento.

«...la emoción que un científico siente cuando encuentra algo nuevo, algo que es él el primero en ver. Para mí no hay emoción o satisfacción comparable a la que produce la actividad creadora, tanto en ciencia como en el arte, literatura u otras ocupaciones del intelecto humano».

Severo Ochoa de Albornoz

A mis padres,

no tengo palabras.

Acknowledgements

Quiero mostrar mi más sincero agradecimiento a todas las personas, que, en mayor o menor medida, han hecho posible esta tesis:

A mis directores, por darme la oportunidad de trabajar en este proyecto y poder aprender de ellos. Mención especial a José Manuel, por ser la pieza clave en la elaboración de este trabajo. A Wifredo, por su confianza. A Rémy, por su accesibilidad.

A todos los grupos de investigación de Barcelona, Valencia y Lleida, implicados en el abordaje multidisciplinar de este estudio. A Bruno, Sébastien y Margot, por hacer agradable mi estancia en Toulouse.

A los participantes de los estudios incluidos en esta tesis, por su ayuda desinteresada, su tiempo y disponibilidad. Esto es vuestro.

A toda la UDEN y en particular al grupo de recerca, por todos los conocimientos compartidos estos años: José, Paco, Jèssica, Ferran, Aina, Estefanía, Anna, Núria; especialmente a aquellos que me han acompañado en el día a día: Yenny, Isma, Mònica, Marzena, Marien, Ylenia, Agustina, Kenia, Anna, Marisel, Elena; y los que muy activamente han contribuido a este proyecto: Jordi, por el trabajo estadístico y Emili, Judit, Irene y Oscar, por la organización de los estudios clínicos. Mi agradecimiento también a otros grupos del IDIBGI: a Rocío, Marta e Iona; a Clàudia, por la evaluación neuropsicológica de los sujetos; a Oren y Carles, por el análisis de neuroimagen.

A mis maestros: Los Marañetas, por darme el privilegio de compartir la residencia en familia. A Susana, por su cercanía y ayuda. A Pedro, por facilitarme compaginar el Piano y la Medicina, como sólo lo entiende quién lo ha vivido. A Javier y José Antonio, por haber tenido la mejor base y el lujo de iniciarme en la ciencia con vosotros.

A mis mejores amigas: Natalia, Alba, Cris, Isabel, Nadia, por escogerme para crecer juntas desde el colegio, instituto, universidad o residencia. Gracias por aparecer en mi vida, por todas las vivencias vividas juntas, pero, sobre todo, por vuestro apoyo cuando más lo he necesitado.

Finalmente, gracias a mi familia. En especial, a mi abuelo Pelayo, por inculcarnos el valor del trabajo y a mi tía Camino, por su apoyo, por estar siempre ahí.

Gracias a Javiño, compañero, amigo, amor; por la logística, optimismo y la impaciente paciencia.

Gracias a mis padres, por darme las más completa educación con los mejores valores. A mi padre, por transmitirme su generosidad, curiosidad e inquietud por descubrir cosas nuevas. A mi madre, por su paciencia infinita, escucha activa y un sinfín de cualidades que no cabrían en estas páginas. El pilar de mi vida, el amor sin condiciones.

This thesis was carried out within the framework of the international research project:

THINKGUT – Personalized prediction of cognition through the human microbiota (EFA345/19)

Action 5: Training of inter/multidisciplinary specialists in microbiota – cognition relationships in the cross-border territory.

Activity 5.1: Doctoral and postdoctoral management and stays of the thesis co-direction.

POCTEFA Statement:

Le projet a été cofinancé à hauteur de 65% par le Fonds Européen de Développement Régional (FEDER) au travers du Programme Interreg V-A Espagne-France-Andorre (POCTEFA 2014-2020). L'objectif du POCTEFA est de renforcer l'intégration économique et sociale de l'espace frontalier Espagne-France-Andorre. Son aide est concentrée sur le développement d'activités économiques, sociales et environnementales transfrontalières au travers de stratégies conjointes qui favorisent le développement durable du territoire.

El proyecto Thinkgut ha sido cofinanciado al 65% por el Fondo Europeo de Desarrollo Regional (FEDER) a través del Programa Interreg V-A España-Francia-Andorra (POCTEFA 2014-2020). El objetivo del POCTEFA es reforzar la integración económica y social de la zona fronteriza España-Francia-Andorra. Su ayuda se concentra en el desarrollo de actividades económicas, sociales y medioambientales transfronterizas a través de estrategias conjuntas a favor del desarrollo territorial sostenible.

List of Publications

This thesis is presented as a compendium of publications.

Original Paper I

Title: Obesity impairs short-term and working memory through gut microbial metabolism of aromatic amino acids.

Authors: Arnoriaga-Rodríguez M, Mayneris-Perxachs J, Burokas A, Contreras-Rodríguez O, Blasco G, Coll C, Biarnés C, Miranda-Olivos R, Latorre J, Moreno-Navarrete JM, Castells-Nobau A, Sabater M, Palomo-Buitrago ME, Puig J, Pedraza S, Gich J, Pérez-Brocal V, Ricart W, Moya A, Fernández-Real X, Ramió-Torrentà L, Pamplona R, Sol J, Jové M, Portero-Otin M, Maldonado R, Fernández-Real JM.

Journal: *Cell Metabolism*. Online ISSN: 1932-7420.

Year: 2020. Volume: 32. Issue: 4. Pages: 548-560.e7.

Impact factor (JCR 2020): 27.287 (D1, 3/145 Endocrinology & Metabolism).

doi: 10.1016/j.cmet.2020.09.002.

Original paper II

Title: Obesity-associated deficits in inhibitory control are phenocopied to mice through gut microbiota changes in one-carbon and aromatic amino acids metabolic pathways.

Authors: Arnoriaga-Rodríguez M, Mayneris-Perxachs J, Contreras-Rodríguez O, Burokas A, Ortega-Sánchez JA, Blasco G, Coll C, Biarnés C, Castells-Nobau A, Puig J, Garre-Olmo J, Ramos R, Pedraza S, Brugada R, Vilanova JC, Serena J, Barretina J, Gich J, Pérez-Brocal V, Moya A, Fernández-Real X, Ramió-Torrentà L, Pamplona R, Sol J, Jové M, Ricart W, Portero-Otin M, Maldonado R, Fernández-Real JM.

Journal: *Gut*. Online ISSN: 1468-3288.

Year: 2021. Volume: 70. Issue: 12. Pages: 2283-2296.

Impact factor (JCR 2020): 23.059 (D1, 3/90 Gastroenterology & Hepatology).

doi: 10.1136/gutjnl-2020-323371.

Additional publications generated by this thesis topic during the PhD are shown in **Appendix A.**

List of Abbreviations

1C, One-carbon.
3-IAAld, Indole-3-acetaldehyde.
3-IPA, Indole-3-propionic acid.
4-HPLA, 4-hydroxyphenyllactic acid.
5-HIAA, 5-hydroxyindoleacetic acid.
α-MSH, Alpha-melanocyte stimulating hormone.

AAA, Aromatic amino acid.
AACE, American Association of Clinical Endocrinology.
AAL, Automated anatomical labeling.
ABCD, Adiposity-Based Chronic Disease.
ACC, Anterior cingulate cortex.
ACE, American College of Endocrinology.
AD, Alzheimer's disease.
AEA, Arachidonoylethanolamide.
AGEs, Advanced glycation end products.
AhR, Aryl hydrocarbon receptor.

BBB, Blood-brain barrier.
BIC, Borderline intellectual capacity.
BMI, Body mass index.

CCK, Cholecystokinin.
CT, Computed tomography.
CVLT, California Verbal Learning Test.
CVLT-IR, California Verbal Learning Test Immediate Recall.
CVLT-SDCR, California Verbal Learning Test Short Delayed Cued Recall.
CVLT-SDFR, California Verbal Learning Test Short Delayed Free Recall.
CVPVI, Cross-validated permutation variable importance.

DARTEL, Diffeomorphic Anatomical Registration Through Exponentiated Lie Algebra.
DHF, Dihydrofolate.
DMA, Dimethylamine.
DMG, Dimethylglycine.
DMSO2, Dimethyl sulfone.
DSM-5, Diagnostic and Statistical Manual of Mental Disorders, fifth edition.
DTI, Diffusion tensor imaging.
DXA, Dual-energy X-ray absorptiometry.

EASO, European Association for the Study of Obesity.
EDCs, Endocrine-disrupting chemicals.
ER, Endoplasmic reticulum.

FDR, False discovery rate.
FGAR, 5'-phosphoribosyl-N-formylglycineamide.
FIO, Frontal inferior orbital.
FMT, Fecal microbiota transplantation.
FOV, Field of view.
FR, Fixed ratio.

GABA, Gamma-aminobutyric acid.
GAR, 5'-phosphoribosylglycineamide.
GLP-1, Glucagon-like peptide-1.
GO, Gene ontology.

HPA, Hypothalamic-pituitary-adrenal axis.
HPLC-ESI-MS/MS, High-performance liquid chromatography-electrospray ionization tandem mass spectrometry.

IDO, Indole-amine-2,3-dioxygenase.
IGT, Iowa Gambling Task.
IPAM, Indolepropionamide.

KEGG, Kyoto Encyclopedia of Genes and Genomes.

LBP, Lipopolysaccharide binding protein.
LPS, Lipopolysaccharide.

MA, Methylamine.
MC4R, Melanocortin-4 receptor.
MNI, Montreal Neurological Institute.
MRI, Magnetic resonance imaging.

NAPEPLD, N-acyl phosphatidylethanolamine-hydrolyzing phospholipase D.
NMR, Nuclear magnetic resonance.
NOR, Novel Object Recognition.
NPY/AgRP, Neuropeptide Y/Agouti-related protein.

OSA, Obstructive sleep apnea.
OTU, Operational taxonomic unit.

PAGIn, Phenylacetylglutamine.
PAGly, Phenylacetylglycine.
PFC, Prefrontal cortex.
PHQ-9, Patient Health Questionnaire-9.
POMC/CART, Proopiomelanocortin/Cocaine- and amphetamine-regulated transcript.
PRIME-MD, Primary Care Evaluation of Mental Disorders.
PYY, Peptide YY.

RAGE, Receptor for advanced glycation end products.
RF, Random Forest.
RL, Reversal learning.
RNA-seq, RNA sequencing.
ROCFT, Rey-Osterrieth Complex Figure Test.
ROI, Region of interest.
ROS, Reactive oxygen species.

SAH, 5-adenosylhomocysteine.
SAM, 5-adenosylmethionine.
SCFAs, Short-chain fatty acids.
SCWT, Stroop Color and Word Test.
SEEN, Sociedad Española de Endocrinología y Nutrición.

T2D, Type 2 diabetes.
TDS, Total Digit Span.
TE, Echo time.
TFEPI, Turbo field echo planar imaging.
THF, Tetrahydrofolate.
TMA, Trimethylamine.
TMT, Trail Making Test.
TR, Repetition time.

VIM, Variable Importance Measure.
VITA, Variable Importance Testing Approach.

XOS, Xylooligosaccharide.

WCST, Wisconsin Card Sorting Test.
WHO, World Health Organization.

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Summary

Obesity is considered a modifiable risk factor for cognitive impairment that may contribute to perpetuating the vicious cycle of overeating and weight gain. Compelling evidence in animal models has demonstrated the role of the gut microbiome on cognition. In particular, the learning and memory domain has recently been associated with specific gut microbiota composition and metabolites in mice. Nevertheless, while the influence of the gut microbiome in metabolic health and disease is increasingly recognized, evidence regarding its impact on obesity-associated cognitive impairment in humans is still scarce.

This thesis aims to describe the impact of the gut microbiome on cognitive function in middle-aged subjects with obesity. For this purpose, a prospective longitudinal case-control study (n=114) of subjects with and without obesity was conducted. Brain function was assessed through neurocognitive testing, including the California Verbal Learning Test (CVLT) and Total Digit Span (TDS) for short-term and working memory, respectively, and the Stroop Color and Word Test (SCWT) for inhibitory control, a key subdomain of executive function. Brain structure was studied through the analysis of gray matter volume by magnetic resonance imaging. Fecal metagenomics were explored through shotgun sequencing and fecal/plasma metabolomics by high-performance liquid chromatography-electrospray ionization tandem mass spectrometry and nuclear magnetic resonance. Some of these parameters were also measured in two independent cohorts (n=24, n=970). Fecal microbiota transplantation from humans to mice were performed. Subsequent neuropsychological assessment and study of prefrontal cortex gene expression were conducted in mice, in an attempt to identify transmissible factors that impact the mouse brain's transcriptome.

The results point to the existence of an ecosystem of gut bacteria that is differentially linked to memory and inhibitory control in subjects with and without obesity. Firstly, subjects with obesity presented deficits in short-term memory, working memory and inhibitory control as indicated by lower scores in the CVLT, TDS and SCWT than their counterparts without obesity. Secondly, a characteristic microbiome profile was associated with these cognitive scores after adjusting for the main confounding factors. Convergent and divergent patterns of bacterial species, functions and circulating metabolites were identified at baseline and one-year follow-up and were mostly replicated in independent cohorts. Overall, altered plasma and fecal levels of tryptophan, tyrosine and phenylalanine aromatic amino acids (AAA) and their catabolites, circulating metabolites involved in one-carbon (1C) metabolism and bacterial functions related to these pathways were linked to memory and inhibitory control scores. In particular, the alterations of memory and inhibitory control and tryptophan-related metagenomic functions and levels were

only observed in subjects with obesity, whereas disturbances in methionine and betaine pathways involved in 1C metabolism were observed in individuals without obesity. Thirdly, the gray matter volume of brain areas involved in memory and inhibitory control such as the hippocampus, the frontal inferior orbital or the anterior cingulate cortex were longitudinally associated with the CVLT, TDS and SCWT scores, respectively, and concordantly with the cognition-gut microbiome relationships. Notably, in individuals with obesity, no significant associations among memory domain, brain volumes and metagenomic functions were observed. Finally, cognitive deficits from human donors with obesity were phenocopied in recipient mice through fecal microbiota transplantation, leading to decreased memory and inhibition-like behavior scores in mice. Donors' metagenomic species and functions associated with human cognitive tests were also linked to the cognition-like performance in mice. The mice RNA sequencing of the prefrontal cortex revealed AAA- and 1C-related genes simultaneously associated in the same direction with the mice cognitive tests and different bacterial clusters.

Altogether, these innovative findings suggest bidirectional host-microbiome networks that may impact brain physiology and highlight the potential diagnostic and therapeutic value of targeting the gut microbiome for memory and inhibitory control impairment, particularly in subjects with obesity. Further studies to confirm these results and assess their clinical implications are warranted.

Resum

L'obesitat es considera un factor de risc modificable per al deteriorament cognitiu que pot contribuir a perpetuar el cercle viciós del menjar en excés i l'augment de pes. Existeixen evidències convincents en models animals que han demostrat un paper del microbioma intestinal en la cognició. En particular, el domini d'aprenentatge i memòria s'ha associat recentment amb la composició i metabòlits específics de la microbiota intestinal en ratolins. No obstant això, en humans, tot i que la influència del microbioma intestinal en la salut metabòlica i la malaltia és cada cop més reconeguda, el seu impacte en el deteriorament cognitiu associat a l'obesitat és encara escàs.

Aquesta tesi té com a objectiu descriure l'impacte del microbioma intestinal en la funció cognitiva en subjectes de mitjana edat amb obesitat. Amb aquesta finalitat, es va realitzar un estudi longitudinal prospectiu de casos i controls (n=114) de subjectes amb i sense obesitat. La funció cerebral es va avaluar mitjançant proves neurocognitives, incloent el *California Verbal Learning Test* (CVLT) y el *Total Digit Span* (TDS) per a la memòria a curt termini i la memòria de treball, respectivament, y el *Stroop Color and Word Test* (SCWT) per al control inhibitori, un subdomini clau de la funció executiva. Es va estudiar l'estructura del cervell analitzant el volum de matèria grisa mitjançant imatges de ressonància magnètica. La metagenòmica fecal es va explorar mitjançant la seqüenciació *shotgun* i la metabolòmica fecal/plasma mitjançant espectrometria de masses en tàndem de cromatografia líquida d'alt rendiment amb ionització d'electrospray i també per ressonància magnètica nuclear. Alguns d'aquests paràmetres també es van mesurar en dues cohorts independents (n=24, n=970). Es van realitzar experiments de trasplantament de microbiota fecal i, posteriorment, una avaluació neuropsicològica i un estudi de l'expressió gènica de l'escorça prefrontal en ratolins, en un intent d'identificar els factors transmissibles que afecten el transcriptoma del cervell del ratolí.

Els resultats apunten a l'existència d'un ecosistema de bacteris intestinals relacionat amb la memòria i el control inhibitori de manera diferencial en subjectes amb i sense obesitat. En primer lloc, els subjectes amb obesitat presentaven dèficits en la memòria a curt termini, la memòria de treball i el control inhibitori que es mostraven com a puntuacions més baixes en CVLT, TDS i SCWT que les persones sense obesitat. En segon lloc, es va associar un perfil de microbioma característic amb aquestes puntuacions cognitives després d'ajustar-se als principals factors de confusió. Es va identificar un ecosistema d'espècies bacterianes, funcions metabòliques i metabòlits circulants que es trobaven associats als tests cognitius, tant al principi de l'estudi a nivell basal com després de seguiment d'un any. Aquestes observacions es van replicar majoritàriament en cohorts independents. En general, els nivells plasmàtics i fecals alterats

d'aminoàcids aromàtics (AAA) triptòfan, tirosina i fenilalanina i els seus catabòlits, així com metabòlits implicats en el metabolisme d'un carboni (1C) i funcions bacterianes relacionades amb aquestes vies es van relacionar amb la memòria i les puntuacions de control inhibitori. En particular, les alteracions relacionades amb la memòria i el control inhibitori i diferents funcions metagenòmiques relacionades amb el triptòfan només es van observar en subjectes amb obesitat, mentre que es van observar alteracions en les vies de la metionina i la betaïna implicades en el metabolisme 1C en individus sense obesitat. En tercer lloc, el volum de matèria grisa de les àrees cerebrals implicades en la memòria i el control inhibitori com l'hipocamp, l'orbital frontal inferior o l'escorça cingulada anterior es van associar longitudinalment amb les puntuacions CVLT, TDS i SCWT, respectivament i en concordança amb les relacions cognitives-microbioma intestinal. En particular, no es van observar associacions significatives entre les puntuacions de memòria, els volums cerebrals i les funcions metagenòmiques en individus amb obesitat. Finalment, els dèficits cognitius de donants humans amb obesitat es van fenocopiar en ratolins receptors mitjançant experiments de trasplantament de microbiota fecal, donant lloc a una disminució de les puntuacions de comportament semblants a la inhibició i memòria en els ratolins. Les espècies bacterianes del donant i les funcions metagenòmiques associades a les proves cognitives humanes també es van relacionar amb un rendiment cognitiu similar dels ratolins. La seqüenciació de l'ARN del ratolí de l'escorça prefrontal va revelar gens relacionats amb AAA i 1C associats simultàniament amb les tasques cognitives del ratolí i diferents grups de bacteris de la microbiota en la mateixa direcció.

En conjunt, aquestes troballes innovadores suggereixen xarxes hoste-microbioma bidireccionals que poden afectar la fisiologia del cervell i destaquen el valor potencial diagnòstic i terapèutic de la microbiota intestinal com una eina d'estudi per al deteriorament de la memòria i el control inhibitori, especialment en subjectes amb obesitat. Es requereixen estudis addicionals per confirmar aquests resultats i avaluar les seves implicacions clíniques.

Resumen

La obesidad se considera un factor de riesgo modificable de deterioro cognitivo, el cual parece contribuir a perpetuar el círculo vicioso de la sobrealimentación y finalmente, el aumento de peso. Evidencia convincente en modelos animales ha demostrado el papel del microbioma intestinal en la cognición. En particular, el dominio del aprendizaje y la memoria se ha asociado recientemente con la composición y metabolitos específicos del microbioma en ratones. Sin embargo, aunque la influencia del microbioma intestinal tanto en los estados fisiológicos como patológicos metabólicos, es cada vez más reconocida, la evidencia disponible acerca del impacto en el deterioro cognitivo asociado a la obesidad en humanos, sigue siendo escasa.

Esta tesis tiene como objetivo describir el impacto del microbioma intestinal en la función cognitiva de sujetos de mediana edad con obesidad. Para ello, se realizó un estudio prospectivo longitudinal de casos y controles (n=114) incluyendo sujetos con y sin obesidad. La función cerebral se evaluó mediante pruebas neurocognitivas, incluyendo el *California Verbal Learning Test* (CVLT) y el *Total Digit Span* (TDS) para la memoria a corto plazo y de trabajo, respectivamente, y el *Stroop Color and Word Test* (SCWT) para el control inhibitorio, un subdominio clave de la función ejecutiva. La estructura del cerebro se estudió mediante el análisis del volumen de materia gris mediante resonancia magnética. La metagenómica fecal se exploró mediante secuenciación *shotgun* y la metabolómica fecal/plasmática mediante cromatografía líquida de alto rendimiento con espectrometría de masas en tándem de ionización por electropulverización y resonancia magnética nuclear. Algunos de los parámetros anteriores también fueron medidos en dos cohortes independientes (n=24, n=970). El diseño experimental incluyó modelos de trasplante de microbiota fecal de humanos a ratones. Posteriormente, estos ratones fueron sometidos a una evaluación neuropsicológica y al estudio de la expresión génica de la corteza prefrontal, en un intento de identificar factores transmisibles que pudiesen afectan el transcriptoma del cerebro del ratón.

Los resultados apuntan a la existencia de un ecosistema de bacterias intestinales vinculadas a la memoria y al control inhibitorio de forma diferente en sujetos con y sin obesidad. En primer lugar, los sujetos con obesidad presentaron déficits en la memoria a corto plazo, la memoria de trabajo y el control inhibitorio mostrando puntuaciones más bajas en el CVLT, TDS y SCWT que los sujetos sin obesidad. En segundo lugar, un perfil de microbioma característico se asoció con estas puntuaciones cognitivas después de ajustar por los principales factores de confusión. Se identificaron patrones convergentes y divergentes de especies bacterianas, funciones y metabolitos circulantes al inicio del estudio y al año de seguimiento, que en su mayoría se replicaron en cohortes independientes. En general, niveles alterados en plasma y heces de los

aminoácidos aromáticos (AAA) triptófano, tirosina y fenilalanina y sus catabolitos, metabolitos involucrados en el metabolismo de un carbono (1C) y las funciones bacterianas relacionadas con estas vías se relacionaron con las puntuaciones de memoria y de control inhibitorio. En particular, las alteraciones relacionadas con la memoria y el control inhibitorio y los niveles circulantes y funciones metagenómicas relacionadas con el triptófano sólo se observaron en individuos con obesidad, mientras que las alteraciones en las vías de la metionina y betaína involucradas en el metabolismo del 1C, se observaron en individuos sin obesidad. En tercer lugar, el volumen de materia gris de las áreas cerebrales involucradas en la memoria y el control inhibitorio, como el hipocampo, la corteza orbitofrontal inferior o la corteza cingulada anterior, se asociaron longitudinalmente con las puntuaciones CVLT, TDS y SCWT, respectivamente, y de forma concordante con las relaciones cognición-microbioma intestinal. En particular, no se observaron asociaciones significativas entre las puntuaciones de memoria, los volúmenes cerebrales y las funciones metagenómicas en individuos con obesidad. Finalmente, los déficits cognitivos de donantes humanos con obesidad fueron replicados en ratones receptores a través del trasplante de microbiota fecal, lo que condujo a una disminución en las puntuaciones similares a memoria y control inhibitorio en ratones. Las especies y funciones bacterianas del donante asociadas con las puntuaciones cognitivas en humanos también se relacionaron con el desempeño cognitivo en ratones. Por último, la secuenciación del ARN de la corteza prefrontal en estos ratones reveló genes relacionados con el metabolismo de AAA y 1C asociados simultáneamente y en la misma dirección con las tareas cognitivas de los ratones y diferentes grupos bacterianos del microbioma intestinal.

En conjunto, estos hallazgos innovadores sugieren la existencia de redes bidireccionales huésped-microbioma que podrían jugar un papel en la fisiología cerebral. Además, estos resultados resaltan el valor diagnóstico y terapéutico potencial del microbioma intestinal en el deterioro de la memoria y el control inhibitorio, particularmente en sujetos con obesidad. No obstante, se necesitan más estudios para confirmar estos resultados y evaluar sus implicaciones clínicas.

1. INTRODUCTION

1.1. Obesity

1.1.1. Definition and classification

Obesity is a multifactorial chronic disease in which abnormal or excess body fat impairs physical and mental health, increases the risk of long-term medical complications[1] and reduces lifespan.[2]

Classically, body mass index (BMI) has been a simple and an available index widely used to classify overweight and obesity in adults. It is defined as body weight in kilograms divided by height in square meters (kg/m^2). Based on the BMI, the World Health Organization (WHO)[3] defined obesity as a BMI equal or higher than 30 kg/m^2 (Table 1).

Table 1. Nutritional status based on BMI according to WHO (adapted from WHO, 2021).[3]

Nutritional Status	Body mass index (kg/m^2)
Underweight	< 18.5
Normal weight	18.5 – 24.9
Overweight, pre-obesity	25.0 – 29.9
Obesity:	≥ 30
Class I	30.0 – 34.9
Class II	35.0 – 39.9
Class III	≥ 40

BMI is fairly well correlated to adiposity, adverse health outcomes and overall mortality. Globally, high BMI accounted for 4 million deaths worldwide, mainly due to cardiovascular disease.[4] Moreover, figures get worst in parallel to the BMI. Thus, it is estimated[2] a median reduction of survival of 2-4 years at BMI between 30-35 kg/m^2 and 8-10 years at BMI of 40-45 kg/m^2.

1.1.2. Epidemiology

The global prevalence of obesity was 13% among adults in 2016.[5] In Europe, obesity was present in roughly 23% of women and 20% of men[6] whereas in Spain, obesity was observed in 21.6% of Spanish adult population; more specifically, 22.8% among men and 20.5% among women.[7]

Unfortunately, the evolution of data shows alarming figures. According to the WHO, worldwide obesity has nearly tripled since 1975. In 2016, more than 1.9 billion adults were overweight and of these, 650 million had obesity.[5] A similar tendency is shown in Spain. The prevalence of obesity is steadily and progressively rising in all groups of ages and both sexes. During the period 1993-2006, class I obesity, class II obesity and class III obesity exhibited a relative increment of 50%, 110% and 240% of their previous prevalence, respectively.[8] It is important to note the alarming rate of class III obesity which involves more serious difficulties and a therapeutic challenge.

In addition to the adverse health consequences; obesity and its associated diseases have a negative economic impact on the health care system. In the United States, obesity-related medical care costs were estimated to be $78.5 billion in 1998, rising to $147 billion per year by 2008.[9] An increase of medical costs by $48-66 billion per year in parallel to the boost of obesity and combined-entities are predicted by 2030.[10] In Spain, direct costs related to overweight already account for 9.7% of healthcare budget.[11]

Therefore, preventive and therapeutic strategies are needed to reverse this trend, improve the adverse health consequences of obesity and cut down the medical expenses.

1.1.3. Pathophysiology and etiology

Energy homeostasis is the biological process responsible for the maintenance of a balance between energy intake and energy expenditure over time. The central nervous system, especially the neurons in the hypothalamic arcuate nucleus and nucleus of the solitary tract, play a key role in this homeostatic system.[12] Moreover, other brain areas are involved, such as the cortico-limbic system and the hindbrain.[13] The central nervous system integrates inputs from long-term energy storage from adipose tissue, such as leptin and short-term meal-related signal, like nutrients and gut-derived molecules; developing behavioral, autonomic and neuroendocrine responses to maintain energy homeostasis.[14] Any dysfunction of this homeostatic control system might develop obesity.

Obesity, by definition, results from consumption of calories in excess of ongoing requirements;[15] resulting in energy storage in the form of lipids in adipose tissue, which expands to accommodate this storage.[16] The expansion of adipose tissue comprises two features: the ability to increase in size, hypertrophy, and in number, hyperplasia.[17] As a result, ectopic locations of adipocytes in organs and tissues where they are not normally found, like skeletal muscle, liver, heart and pancreas determine an important lipid overload, called lipotoxicity, which finally impairs the functions of these tissues and organs.[18]

Furthermore, adipose tissue produces factors with endocrine and paracrine functions, such as leptin, adiponectin or pro-inflammatory cytokines, which interact with the central nervous system and peripheral organs and contribute to insulin resistance and chronic inflammation underlying obesity and related-disorders.[19,20]

Moreover, the adipose tissue physiology is even more complex.[21] Apart from white adipose tissue, the classically known as adipose tissue, specialized for energy storage and mobilization;[22] brown adipose tissue has been identified with opposite action to the former. Brown adipose tissue is implicated in adaptative thermogenesis and may prevent obesity modulating energy expenditure. It also has secretory function. Brown adipose tissue activity seems to be altered in obesity.[23] Finally, beige adipocytes, the latest identified, appear to be bifunctional, having the capacity to switch between energy storage and energy dissipation phenotype.[24]

Genetic, developmental and environmental forces affect this energy homeostasis system promoting obesity[25] (Figure 1):

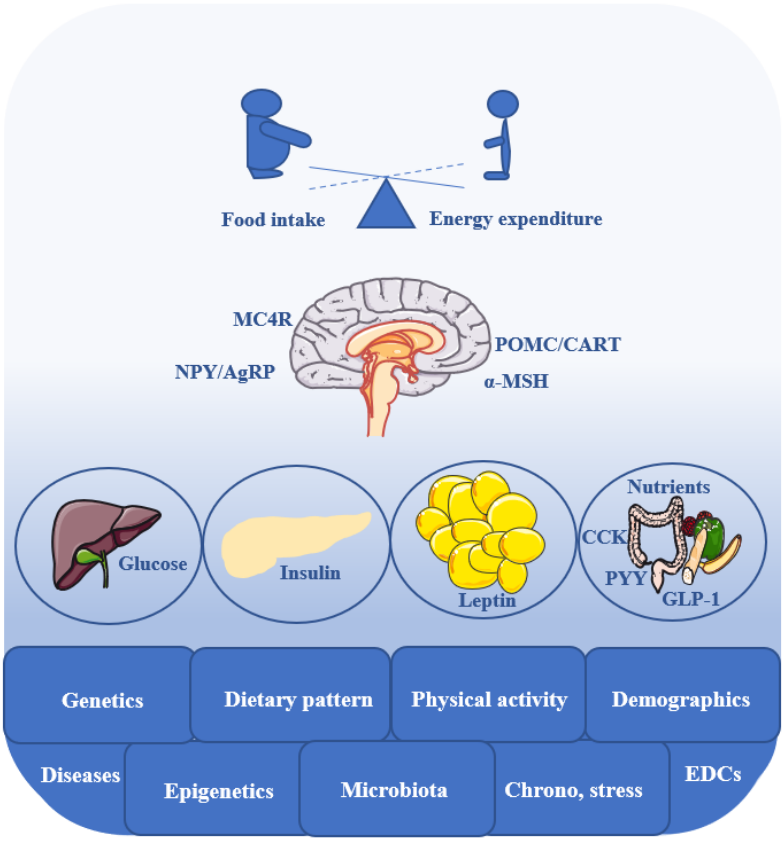


Figure 1. Pathophysiology and etiology of obesity. Genetic and environmental factors could impact energy homeostasis promoting endocrine, autonomic and behavioral responses converging on a positive or negative energy balance. α-MSH, α-Melanocyte-stimulating hormone; CCK, cholecystokinin; GLP-1 glucagon-like peptide-1; EDCs, endocrine-disrupting chemicals; MC4R, melanocortin-4 receptor; NPY/AgRP, neuropeptide Y/agouti-related protein; POMC/CART proopiomelanocortin/cocaine-and amphetamine-regulated transcript; PYY, peptide YY (original illustration by Arnoriaga-Rodríguez, 2021).

Genetic factors: Obesity is mainly considered a polygenic disease. More than 140 genetic regions have been related to different adiposity measures.[26] Monogenic obesity is rare; mutations in the melanocortin-4 receptor (MC4R),[27] leptin receptor[28] or leptin deficiency[29] have been identified, to name just a few.

Epigenetic modifications: Genetic predisposition, ageing and environmental factors can interact with the human epigenome contributing to obesity through DNA methylation, histone modifications and RNA-mediated processes.[30]

Obesogenic environment*:* It refers to the influences that the environment, opportunities and living conditions exert in the development of obesity.[31] It includes dietary patterns,[32] sedentary lifestyle,[33] overeating and stress,[34] sleep disturbances[35] and socio-demographic indicators,[36] among others.

Several diseases and treatments: Endocrine disorders[37] and medications, such as insulin, secretagogues, thiazolidinediones can promote obesity. This is also the case of some antipsychotic, antidepressants and antiepileptic drugs.[38]

In addition, there are other novel factors involved in obesity development that have had a great impact in the last decade, including:

Gut microbiota: The community of microorganism that live in the digestive tract acts through an integrated host signaling pathway to regulate energy storage.[39] Specific bacteria profiles have been identified in obesity[40] and this hypothesis has been supported by the transmission of obesity phenotype via fecal microbiota transplant from obese to lean mice.[41]

Endocrine-disrupting chemicals (EDCs): Exogenous chemicals such as tributyltin, bisphenol A, flame retardants, polychlorinated biphenyls, phthalates or perfluorinated, can mimic or block actions of hormones interfering with their receptors, particularly important during the early human development.[42]

Chronodisruption: Alterations in the physiological circadian rhythm impairing the internal clock or late timings of food intake have been related to obesity pathogenesis.[43]

1.1.4. Diagnosis

BMI is the measurement used in clinical practice to tackle obesity. A BMI of 30 kg/m^2 or higher is considered obesity (Table 1). Although fairly correlated to adiposity and negative health outcomes; it also has limitations.

For example, it does not report body fat distribution; does not discriminate between fat mass and fat-free mass and varies between races and ethnicities; being less accurate in pregnancy, short stature, advanced age and pathologies with impaired hydrosaline balance.[44]

Waist circumference might complement BMI assessing visceral fat.[45] In Caucasic population, abdominal or central obesity is considered with waist circumference equal or greater than 88 cm in women and 102 cm in men. These cut-off values strongly correlate with increased cardiovascular disease.[46] Skin thickness or waist to hip ratio, are other indices which estimate obesity, less used in clinical practice.

Nevertheless, obesity by definition implies an excess of fat mass and neither of those indices are perfect indicators of adiposity, the true gold-standard measure in obesity assessment. A percentage of fat greater than 25% in men and 33% in women defines obesity.[47] Dual-energy X-ray absorptiometry (DXA), magnetic resonance imaging (MRI), computed tomography (CT) scan, bioelectrical impedance, plethysmography and abdominal ultrasonography, although less available in clinical settings, provide a complete evaluation of body composition.[48]

In fact, the latest position statement for the American Association of Clinical Endocrinology (AACE) and American College of Endocrinology (ACE) advocate for the concept of obesity as an adiposity-based chronic disease (ABCD). This term considers obesity as a chronic disease with characteristic adiposity-based complications and evaluate quantity, distribution and function of adipose tissue moving beyond BMI as the key tool.[49] This declaration has also been adopted by the European Association for the Study of Obesity (EASO)[50] and the *Sociedad Española de Endocrinología y Nutrición* (SEEN)[44] (Table 2).

Table 2. Obesity as an Adiposity-Based Chronic Disease (Arnoriaga-Rodríguez, 2021).

Adiposity-Based Chronic Disease (ABCD)		
AACE/ACE[49,51]	**EASO[50]**	**SEEN[44]**
Pathophysiology (A)	Etiology	Fisiopatología
BMI classification (B)	Degree of adiposity	Grado de adiposidad
Complications (C)	Health risks	Riesgo para la salud
Degree of severity of complications (D)		Gravedad de las complicaciones

The coding system of ABCD[49,51] propose the evaluation of obesity not only based on the BMI classification but also on its pathophysiology and the number and severity of the complications. This approach has also been adopted by the EASO[50] and the SEEN[44]. AACE, American Association of Clinical Endocrinology; ACE, American College of Endocrinology; EASO, European Association for the Study of Obesity; SEEN, *Sociedad Española de Endocrinología y Nutrición.*

1.1.5. Obesity and related disorders

Obesity is associated with countless conditions contributing to a decline in quality of life and life expectancy; as well as unemployment and social disadvantage.[52] To note:

Metabolic syndrome and cardiovascular disease: One of the main consequences of obesity is atherosclerotic cardiovascular disease. Adiposity is significantly associated with increased risk for stroke[53] and ischemic heart disease.[54] Insulin resistance underlying obesity leads to atherogenic dyslipidemia, elevated triglycerides and reduced HDL-cholesterol levels; endothelial dysfunction and inflammation; impaired glucose tolerance and type 2 diabetes (T2D) and hypertension. All these alterations added to abdominal obesity define the metabolic syndrome.[55]

Obstructive sleep apnea (OSA): The prevalence of moderate to severe OSA is very high, roughly 60% in metabolic syndrome.[56] Obesity hypoventilation syndrome can also appear in association with OSA and other metabolic entities.[57]

Cancer: Obesity is estimated to contribute up to 14% and 20% of all deaths from cancer in men and women, respectively.[58] Epidemiological evidence of obesity-associated tumors includes mammary, renal, esophageal, gastrointestinal (liver, gallbladder, pancreas, colon), hematopoietic, and reproductive cancers (endometrial, aggressive prostate cancer) (etc.).[59, 60]

Gastrointestinal conditions: Non-alcoholic fatty liver disease is prevalent in obesity, progressing to steatohepatitis and cirrhosis in roughly 20% of cases.[61] Obesity also increases the risk of gastroesophageal reflux disease, erosive esophagitis, Barrett's esophagus, diarrhea, colonic diverticular disease, polyps, gallstones and acute pancreatitis.[62]

Neurological manifestations: Obesity is associated with central and peripheral nervous system impairments such as mild cognitive impairment and dementia or polyneuropathy.[63] These alterations of cognitive function related to obesity are one of the reasons that motivated this thesis topic.

Psychiatric conditions: Binge eating disorders and night eating syndrome are more prevalent in obesity.[64] Increased odds of anxiety (generalized, panic without agoraphobia, specific phobia), mood (major depression, dysthymia, manic episode) and personality (antisocial, avoidant, schizoid, paranoid and obsessive-compulsive) and alcohol abuse disorders have been observed in obesity.[65]

Polycystic ovary syndrome and infertility: Polycystic ovary syndrome,[66] menstrual irregularities and pregnancy complications are some of the reproductive concerns linked to obesity. Infertility is also prevalent due to mainly anovulation in women or reduced testosterone levels and sperm count in men.[67]

Osteoarthritis: Obesity is a risk factor for both osteoarthritis incidence and progression, leading to negative weight loss outcomes and impaired quality of life.[68]

Nutritional deficiencies: Deficiency of iron, calcium, magnesium, zinc, copper, thiamine, folate, retinol acid[69] and vitamin D[70] have been identified in obesity; due to inadequate intake with high-calorie low-nutrient processed food and altered pharmacokinetics.

Dermatological entities: Acanthosis nigricans, acrochordons, keratosis pilaris, striae distensae, cellulite and plantar hyperkeratosis are the most common skin manifestations of obesity. There is also an increase of cutaneous bacterial and Candida infections including inflammatory skin diseases, onychomycosis and chronic dermatosis.[71]

1.1.6. Management

The ABCD approach proposed by the AACE/ACE focuses on the lifestyle medicine as the central therapeutic intervention.[49] The components of lifestyle medicine that should be addressed include establishing a healthy eating pattern and physical activity; evaluating percentage and distribution of fat mass by body composition; improving sleep hygiene; reducing stress; quitting smoking; moderate drinking; counseling about substance abuse, mood and behavior and providing community engagement and transculturalization.[49]

In addition to adopting a healthy lifestyle, pharmacotherapy is indicated in patients with BMI $\geq$ 30 kg/m^2 or BMI $\geq$ 27 kg/m^2 with other related disorders. Medications approved for weight management and long-term use in the United States are orlistat, naltrexone/bupropion, liraglutide, semaglutide and phentermine/topiramate, the three first ones also approved in Europe. In addition, drugs associated with weight gain should be replaced by others with neutral or weight loss effect.[72] Pharmacological therapeutics are linked to achieving at least 5% weight loss at 52 weeks of treatment.[73]

Bariatric surgery might be considered in BMI $\geq$ 40 kg/m^2 or BMI $\geq$ 35 kg/m^2 with related disorders. Sleeve gastrectomy and Roux-en-Y gastric bypass are two of the most widely used techniques.[72] Bariatric surgery is associated with long-term weight loss and decreased overall mortality[74] and the best option for extreme obesity.[75]

In general, despite the fact that the more weight loss the greater benefits, modest weight loss of 5 to 10% are associated with significant cardiovascular improvements.[76]

1.1.7. Long-term weight loss maintenance

The real challenge is not to lose weight but to maintain it long-term. Durable benefits are frequently elusive. Successful weight loss maintenance considered as losing at least 10% of initial body weight and maintaining the loss for at least 1 year, is observed in just about 20% of cases.[77]

The usual pattern of weight loss in interventions based on lifestyle changes reaches a peak at 6 months after the initiation of treatment, followed by a plateau and gradual recovery.[78] If pharmacological or surgical treatments are added, weight regain can be delayed over time. Unfortunately, the percentage of subjects who regain lost weight is high because of appetite changes[12] and reduced energy expenditure[79] after weight loss can promote weight recovery. Suggested strategies for long-term weight loss success are:[77, 80]

Greater initial weight loss: Higher weight loss in the 1-2 first months seems to predict weight loss at 4-8 years.[81]

Healthy lifestyle: Adherence to a healthy eating pattern (consumption of fruits and vegetables and low intake of fats and sugars; eating breakfast; maintaining a consistent eating pattern across weekdays and weekends) and increased physical activity (preferably more than 1 hour a day or 300 min/week) are the keys of the weight loss maintenance. Regular physical activity has been shown to be an integral component of sustained weight loss in the long-term.

Monitoring: Continuous monitoring by professionals in addition to self-monitoring. Longer duration of the treatment and follow-up gives participants more time to engage in consistent habits related to physical activity and healthy eating in all settings.

Behavioral modifications: Training inhibitory control, developing problem-solving skills resulting in reduced food intake, has been beneficial. In fact, low disinhibition and food addition scores at the end of the weight loss intervention are related to greater odds of success.[82] The importance of the inhibitory control motivated us to include it as a priority element of cognitive function to analyze in the interaction between obesity and cognitive function.

1.2. Obesity and the brain

1.2.1. Introduction

Obesity is associated with different neurological disorders,[63] being cognitive impairment one of the major health concerns nowadays due to both increased life expectancy[83] and the growing prevalence of obesity and metabolic disorders.[84]

The fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5)[85] defines six key domains of cognitive function (Table 3) whose impairment lies in the main neurocognitive syndromes: delirium, mild neurocognitive disorder and major neurocognitive disorder.[86] Furthermore, particular domains and subdomains can be predominantly affected in different neurodegenerative diseases, for example Alzheimer's disease (AD), frontotemporal lobar degeneration, vascular disease or Parkinson's disease.[85]

Table 3. Neurocognitive domains and subdomains defined by DSM-5 (adapted from American Psychiatric Association, 2013[85] and Sachdev et al., 2014).[86]

Neurocognitive domains (DSM-5)	
Learning and memory	**Language**
Free recall	Object naming
Cued recall	Word finding
Recognition memory	Fluency
Semantic and autobiographical long-term memory	Grammar and syntax
Implicit learning	Receptive language
Perceptual-motor function	**Social cognition**
Visual perception	Recognition of emotions
Visuoconstructional reasoning	Theory of mind
Perceptual-motor coordination	Insight
Executive function	**Complex attention**
Planning	Sustained attention
Decision-making	Divided attention
Working memory	Selective attention
Responding to feedback	Processing speed
Inhibition	
Flexibility	

DSM-5 sets up six principal domains: learning and memory, perceptual-motor functions, executive function, language, social cognition and complex attention. These domains are divided into different subdomains as they can be seen in Table 3. DSM-5, Diagnostics and Statistical Manual of Mental Disorders 5th edition.

Mild neurocognitive disorder is the evidence of cognitive decline from a previous level of performance in one or more cognitive domains, which could not be explained by another mental disorder and does not interfere with the capacity for independence. Otherwise, if this capacity is affected, a major neurocognitive disorder, classically known as dementia, is considered.[85] Thus, mild neurocognitive disorder is an intermediate stage in the spectrum from normal to severe cognitive impairment; although it is not always a precursor of major neurocognitive disorder.[86]

Rates of progression from mild to major neurocognitive disorder have been estimated from 3% and 13% per year, in community and clinic settings, respectively.[87] Percentages might vary relating to sample selection and inconsistency of diagnostic criteria, but are higher than in age-matched subjects without mild neurocognitive disorder.[88] A larger initial degree of functional impairment, generally shown in those who seek medical evaluation, has been identified as an important predictor of conversion to major neurocognitive disorder.[87] Involvement of two or more domains, one of them being verbal learning and memory with impairments in psychomotor speed or executive function, have also strongly predicted progression to a specific major condition, AD.[89]

On the contrary, mild cognitive impairment could remain stable in case of, for example, traumatic brain injury; or even improve if causes are potentially reversible, such as in pharmacologic, metabolic, vascular or systemic etiologies.[88] In this line, atrial fibrillation, hypertension, diabetes mellitus, obesity and metabolic syndrome are some of the recognized risk factors for vascular neurocognitive disorders but also for AD.[90] Indeed, 40% of dementia cases have been attributable to a combination of twelve potentially modifiable risk factors: low educational attainment, physical inactivity, smoking, diabetes, midlife obesity, midlife hypertension, late-life depression, hearing loss, social isolation,[91] air pollution, excessive alcohol consumption and head injury.[92] Focusing on obesity, a reduction of 4-5% in 2035 and 9-10% in 2050 in dementia cases is estimated if obesity is diminished by 20%; in a projection model of an Australian cohort.[93]

Although subjective cognitive complaints alone are insufficient to diagnose mild neurocognitive disorder; they might reflect a change in cognitive function, making these subjects eligible to close follow-up and study to assess for reversible causes.[88] Impairments in cognitive function should be documented objectively through standardized neuropsychological testing to determine the severity and subtype of disorder. Accordingly, in mild neurocognitive disorder the level of cognitive decline is modest, in the range of 1-2 SD or between the 3rd and 16th percentiles, below the normative data for age, educational attainment and cultural-linguistic background; larger involvements are attributed to major neurocognitive disorders.[86]

Nevertheless, these measures are not an absolute threshold and should be taken into account in the context of each subject. Moreover, mood and behavioral symptoms are more common in mild and major neurocognitive disorders than in healthy counterparts.[94] To note, the prevalence of depression in mild neurocognitive disorder ranges from 25 to 40% in community- and clinic-based samples, respectively.[95] Remarkably, the presence of neuropsychiatric symptoms in turn appears to be a marker of mild neurocognitive disorder severity,[96] since it has been associated with greater cognitive impairment.

In addition to neuropsychological testing, structural neuroimaging using MRI or CT scan can support the suspicion of cognitive decline as well as fluid biomarkers. However, it is the medical judgement what ultimately establishes the diagnosis.[88]

Regarding the management, apart from reversible and treatable causes and control of modifiable risk factors during midlife (age 45 to 65 years); no specific medications or dietary agents are approved for mild neurocognitive disorders that improve cognition or delay progression to major neurocognitive disorders.[88] Therefore, more studies are needed to identify therapeutic targets.

1.2.2. Obesity and the brain function

Growing evidence in systematic reviews and meta-analyses supports the association between obesity in middle-ages and an increased risk of later-life dementia;[97-99] although not all the studies have found this relationship.[100] Whether the link between obesity and cognitive dysfunction is independent of other obesity comorbidities is more controversial.[101] Nevertheless, the independency of this relation is gradually becoming clearer, since more and more research demonstrates that these effects are not solely mediated by obesity-related diseases.[102-105] For instance, in a large cohort of more than 10.000 individuals and 36-years follow-up, obesity in middle-aged (40-45 years) subjects was independently associated with a 3.10- and 5-fold increase in risk of AD and vascular dementia respectively, after adjusted for main confounding factors.[106] In addition to obesity as a modifiable risk factor for cognitive impairment;[107] cognitive dysfunction seems to be a predisposing factor for overeating and obesity;[103,108] perpetuating the cycle and making this association even more complex.

The optimal therapy of obesity-related cognitive dysfunction may target obesity itself.[63] Weight loss through lifestyle interventions and bariatric surgery[109] has proved short-term neurocognitive improvements, although there is a lack of evidence of long-term effects. Therefore, the ideal intervention to tackle cognitive decline in obesity is currently not known.

Focusing on neurocognitive domains and subdomains, deficits in verbal learning and memory, attention and executive function are the most common cognitive impairments observed in obesity. These alterations were also linked to physiological and structural changes in the brain (Figure 2).[63]

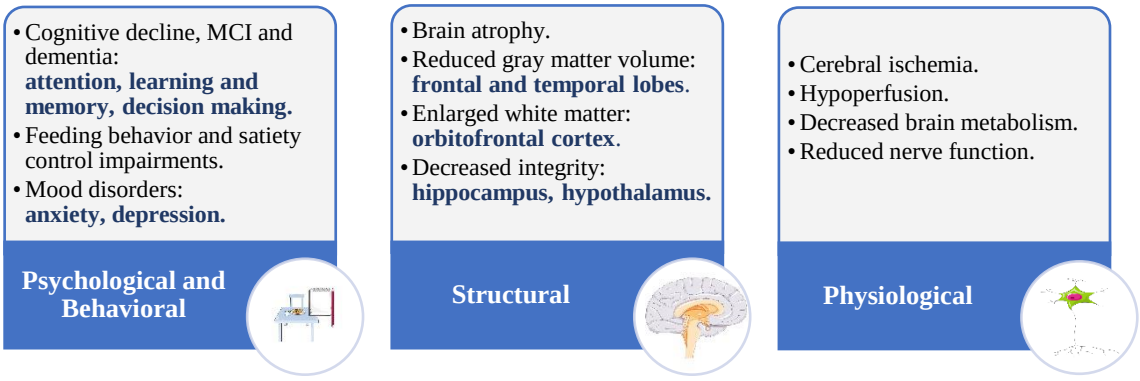


Figure 2. Neurological changes of the central nervous system in obesity. Obesity is associated with psychological and behavioral, structural and physiological brain modifications which finally lead to cognitive dysfunction. MCI, mild cognitive impairment (adapted from O'Brien et al., 2017).[63]

1.2.2.1. Verbal learning and memory function in obesity

Memory refers to the complex processes in a multicomponent system with a variety of different subtypes, mediating by several factors and neural mechanisms.[110] Theoretically, different models have been proposed based on its major components:[111]

Long-term memory: Storage of stable or permanent memories.

Short-term memory: The limited-capacity storage over seconds to minutes.

Working memory: It includes short-term storage components but also central executive processes that manipulate stored information and help to make use of short-term memory. Thus, other authors consider working memory as the set that includes immediate and short-term memory.[110]

Clinically, data from molecular biology, neuropsychology, clinical neurology and neuroimaging support the concept of distinct memory systems: episodic, semantic, working and procedural memory.[112] This model of multiple memory systems that are distinct yet complementary can compensate for each other when other parts fail.[112]

Focusing on the memory systems evaluated in this thesis:

Episodic memory: The ability to recall personal experiences. It is the subtype of memory most often perceived as dysfunctional. It involves the process of encoding, the direction of cerebral resources to process information from attentional mechanisms; consolidation, storage of information accessible in the future; and retrieval, the act of remembering that information.[112] Clinical measures of learning and memory are commonly assessed episodic memory and typically involve free recall, cued recall and recognition of lists of items (words, pictures, faces).[110]

Working memory: It refers to the active maintenance of information for potential manipulation to complete goal-directed tasks and behaviors. Although working memory is a memory subtype, it is generally considered a component of executive function.[112,113]

At a structural level, medial temporal lobe structures, in particular the hippocampal formation and associated cortical and subcortical structures (diencephalon, limbic system, posterior cingulate and precuneus) are most often associated with episodic memory loss.[112] Working memory is mainly linked to prefrontal cortex (PFC), subcortical structures and parietal association cortex.[112]

Episodic and working memory are core cognitive processes that are critical for food-related decision-making. In fact, disruptions of memory systems are linked to appetite dysregulation and weight gain.[114]

Obesity has been associated with impaired cognitive function, especially executive function.[115] What is less explored is the impact of obesity on learning and memory. Compared with executive function, relatively fewer studies in humans have evaluated this cognitive domain showing contradictory results. Memory has been negatively and independently associated with body mass index (BMI) across adult lifespan[116, 117] and improvements on memory function have been identified three years after bariatric surgery.[118] Conversely, other studies did not find differences in memory tasks between subjects with and without obesity.[119,120] More recently, in a systematic review that included fourteen studies evaluating the effects of obesity on episodic memory in mainly middle-aged subjects, ten found an inverse association of weight status and memory function whereas four did not observe a direct effect of obesity on memory.[121]

1.2.2.2. Executive function in obesity

Executive function represents a constellation of cognitive abilities that drive goal-oriented behavior and are critical to the ability to adapt to an ever-changing world.[122] From a clinical perspective, executive function can be split into four distinct components:

Working memory: A limited capacity system that enables us to temporarily process, store and manipulate information. It is critical for planning and decision-making tasks.

Inhibition: The ability to hold back a predominant, automatic, impulsive or previously learned response that might be inappropriate or irrelevant.

Set shifting: The capacity to modify attention and behavior in response to changing circumstances and demands. Also known as cognitive or mental flexibility.[123]

Fluency: The ability to maximize the production of verbal or visual information in a specific time period, whilst avoiding repeating responses.

Executive dysfunction relies on PFC, parietal cortex, basal ganglia, thalamus and cerebellum as well as their matter connections and neurotransmitter systems.[122] Roughly on working memory tasks, the dorsolateral PFC is active in executive processing, active manipulation or updating of information, whereas the ventrolateral prefrontal regions are responsible for storage-related processes.[124] In addition, inferior frontal regions may be critical for inhibiting inappropriate responses[125,126.]

Executive dysfunction may manifest in many problems in everyday life, such as inappropriate social behavior; problems with decision making and organization; difficulties following and shifting plans or in situations involving memory aspects and distractibility.[110] Remarkably, impaired executive abilities such as reduced self-control, low inhibitory control and mental flexibility might lead to poorer food choices, disordered eating, overeating and weight gain;[127] entering in a vicious cycle of positive energy balance perpetuating obesity.[115] On the other hand, improving elements of executive function through inhibitory control training has shown a moderate reduction of unhealthy behaviors in the short-term.[128]

Consistent findings linked obesity to impaired executive function, in most of the studies as a direct association.[102,127,129-131] The most promising executive function subdomains correlating obesity and eating behavior seems to be inhibitory control and working memory.[132,133] Impaired inhibitory control[131,134-136] and working memory[115,131] have been identified in obesity, being altered even since the overweight status.[131] Furthermore, in severe obesity, namely eligible bariatric surgery patients, executive dysfunction is common[102,137,138] and seems to play an important role on weight loss maintenance and treatment adherence in both behavioral programs[139] and after bariatric surgery at short-term.[140,141]

Overall, most studies support the associations between obesity and worse memory and executive function. Moreover, more and more data reinforce its independent role in this interplay, in particular in executive function. However, the existing evidence is somewhat inconsistent in case of the impairment of learning and memory domain in middle-aged subjects with obesity.

1.2.3. Obesity and the brain architecture

1.2.3.1. Morphometric changes in obesity

Impairments on cognitive function in subjects with obesity have been associated with morphometric and functional changes in the brain identifying by different MRI techniques. Structural MRI is used to obtain brain morphometry that includes indices of volume and integrity, thickness and surface areas.

Brain volume and integrity: Gray matter volume which contains neurons and nerve fibers and white matter volume, myelinated nerve fibers, may be obtained in anatomical detail based on the different paramagnetic properties of the brain tissues.[142]

Lower gray matter volume has been found in association with BMI, waist-to hip ratio and total fat mass, even after adjustment for covariates.[143] Moreover, greater gray matter atrophy has been observed when high BMI and central obesity were simultaneously presented.[143] A meta-analysis revealed the largest cluster of reduced gray matter volume in the left, middle and right inferior frontal gyrus, the left middle temporal cortex, the left precentral gyrus and the cerebellum in subjects with obesity.[144] Similar findings have been observed in other studies, showing lower gray matter volume in the medial prefrontal cortex, cerebellum and left temporal pole.[145] On the other hand, increased gray matter volume has also been identified in subjects with obesity, in particular in the left cuneus, left middle frontal gyrus, left inferior occipital gyrus[144] as well as the subcortical volumes of the amygdala, thalamus and nucleus.[146] Conversely, other studies revealed no differences in brain gray matter volume between subjects with and without obesity.[147]

White matter refers to myelinated tracts connecting gray matter structures throughout the brain.[148] Albeit, there is less conclusive evidence of measures of white matter with respect to gray matter[149]; increased in the frontal, temporal and parietal lobes[150] and reduced in the basal ganglia and corona radiata white matter volumes were found in association with higher BMI.[151] Microstructural composition and architecture of the white matter have also been studied revealing global reductions in white matter integrity in parallel to higher BMI,[152] in form of demyelination or axonal loss.

This loss of integrity has been described in some tracts within the limbic system and those connecting the temporal and frontal lobe,[153] among others. In addition, white matter hyperintensities have been identified, ranging from slight disentanglement to varying degrees of myelin and axonal loss.[154]

Fortunately, both gray matter and white matter alterations appear to be recovered after weight loss, particularly after bariatric surgery techniques, at least in the short-term, at six months follow-up.[149,155]

Thickness: Cortical thickness reductions in the temporal and frontal cortex have been identified in obesity, with a similar effect size to thickness decrements observed in neuropsychiatric disorders, such as major depressive and bipolar disorders.[146]

Surface: Both higher surface cortical area, as for example, in the isthmus cingulate cortex, paracentral lobule or left transverse temporal gyrus and lower surface area in the inferior temporal gyrus, right rostral middle frontal gyrus and left lingual gyrus have been found in subjects with obesity.[146]

In addition, MRI can determine brain iron load. Iron accumulates in the brain during ageing resulting in cognitive impairment and neurological disorders.[156] Higher brain iron accumulation has been observed in subjects with obesity.[157,158] Particularly, in the caudate nucleus, lenticular nucleus, hypothalamus and hippocampus,[157] in association with worse cognitive performance.[158]

1.2.3.2. Functional changes in obesity

In addition to structural and morphological alterations in the obese brain, neural imaging studies have shown altered brain activation and functional connectivity.

Functional MRI measures differences in brain activity through the detection of changes in cerebral blood flow in resting state or doing a task.[159] Roughly, an increase of the activity relating to food in brain areas associated with reward, emotion/memory and sensory/motor processing, in parallel to a decrease in areas associated with homeostatic satiety and cognitive control/attention have been identified in obesity.[142] Notably, greater hypothalamic connectivity with regions implicated in motivation feeding, as the insula or the thalamus, was associated with higher BMI. On the contrary, reduced connectivity was found with the superior parietal lobe, involved in the cognitive control of food intake.[160]

Neuroimaging studies using positron emission tomography also evidenced lower prefrontal metabolism linked to higher BMI.[161] Accordingly, decreased blood flow in the prefrontal cortex and thus, prefrontal function was found in those subjects with higher BMI, using single photon emission computed tomography imaging.[162]

1.2.4. Mechanisms underlying obesity-associated cognitive dysfunction

Although the mechanisms involved are still largely unclear and need to be fully elucidated; it is speculated that multiple pathways may interact and stablish bidirectional communications and a feed-forward cycle which ultimately culminates into cognitive decline. Some of the proposed factors are briefly summarized below:[149,163-166]

Inflammation: Chronic or low-grade inflammation is a key feature of obesity characterized by high circulating levels of inflammatory markers such as cytokines, hormones, proteins and other mediators.[167] Some of these markers, such as lipopolysaccharide binding protein (LBP), have been associated with either cognitive dysfunction and brain microstructure and integrity.[168] In fact, obesity and neurodegenerative disorders, even since initial stages, share some patterns of inflammatory markers.[169] Therefore, some authors consider peripheral inflammation as a potential prodromal indicator of dementia.[170] Apart from peripheral inflammation, inflammation in the central nervous system or neuroinflammation coexists in obesity.[171] They are not isolated since different communication pathways participate in the cross-talk between the periphery and the central nervous system such as the vagus nerve, blood-brain barrier (BBB) and choroid plexus.[172]

Insulin resistance: Insulin is released from the pancreas, crosses the BBB and activates the insulin receptors in the central nervous system inducing different signaling pathways. Impaired insulin signaling contributes to cerebral insulin resistance[154] and thus, a reduction of brain insulin sensitivity.

Advanced glycation end products (AGEs): Chronic hyperglycemia also leads to an increase of the formation of AGEs and the expression of the AGEs receptor (RAGE) in the endothelium and the brain.[173] It enhances oxidative stress, tissue damage and cellular dysfunction;[174] affects BBB integrity and promotes amyloid deposition.[175] Apart from hyperglycemia, high levels of exogenous AGEs from high-fat and processed food may impact cognition.[176] AGEs have also been related to the severity of cognitive impairment, since the presence of AGEs in cortical neurons and cerebral vessels in subjects with cerebrovascular disease were associated with larger cognitive decline.[177]

Bioactive lipids: Triglycerides and free fatty acids might mediate changes of the central nervous system in obesity.[163] The injection of triglycerides into the brain impaired cognitive function in mice. Conversely, the administration of a fibrate to reduce triglycerides improved cognition.[178] Triglycerides also impact leptin transport across the BBB contributing to leptin resistance seen in obesity.[179] Free fatty acids are the result of the breakdown of triglycerides with are linked to both metabolic[180] and brain dysfunction.[181] Elevation of circulating free fatty acids causes insulin resistance and peripheral[182] and central inflammation.[183]

Glucocorticoids: Stress might contribute to the relation between obesity and cognitive function. The stress response is mediated by the sympathetic adrenal-medullary system and the hypothalamic-pituitary-adrenal (HPA) axis. Their activation induces the release of corticosteroids and glucocorticoids, respectively.[184] Glucocorticoids are the primary moderators of the acute effects of stress on cognition and also impair neural integrity in the long-term.[184] Chronic elevated glucocorticoid levels are associated with reduced hippocampal volume and hippocampus-dependent memory deficits.[185] In addition, stress may promote central obesity through energy homeostasis and feeding behavior disbalance[184] and, in turn, subjects with obesity seem to be more vulnerable to cognitive impairments due to basal and reactive raised glucocorticoid levels.[184]

Mitochondrial dysfunction and endoplasmic reticulum (ER) stress: Mitochondrial dysfunction and ER stress are responsible for increased oxidative stress and inflammation in the brain.[186] In fact, oxidative stress and mitochondrial dysfunction are some of the common mechanisms observed in obesity, diabetes and AD.[182] Diet-induced obesity mice exhibited higher levels of reactive oxygen species (ROS) in the brain in association with cognitive impairment.[187] Ectopic fat deposition in obesity induced the production of ROS promoting mitochondrial dysfunction and insulin resistance.[188] ER stress is also associated with insulin[189] and leptin resistance[190] in obesity.

Blood-brain barrier (BBB) disturbances: The BBB is a continuous endothelial membrane within brain microvessels whose integrity is necessary for proper synaptic and neural functioning. BBB breakdown, which causes infiltration of immune cells into the brain, has been identified in neurodegenerative disorders.[191] Obesity is also linked to macro and microvascular endothelial dysfunction.[164] Microvascular dysfunction is related to increased oxidative stress, inflammatory and immune responses and BBB permeability; leading to perfusion defects, hypoxia, increased angiogenesis and cognitive dysfunction.[192]

Gut microbiome: In the last twenty years, there has been a massive gain of knowledge on the impact of gut microbiome in health as well as in the pathophysiology of common metabolic disorders including obesity.[193] Changes in microbiota composition and diversity have been observed in obesity[194] promoting metabolic inflammation.[195] In addition, rodent models provide compelling evidence on the effects of gut microbiota in brain function and structure through the gut-brain axis communication.[196] Nevertheless, further investigations are needed to elucidate the role of gut-brain axis on obesity-associated cognitive dysfunction in humans.

The picture illustrates the proposed factors that may be involved in the pathophysiology of cognitive decline in obesity (Figure 3).

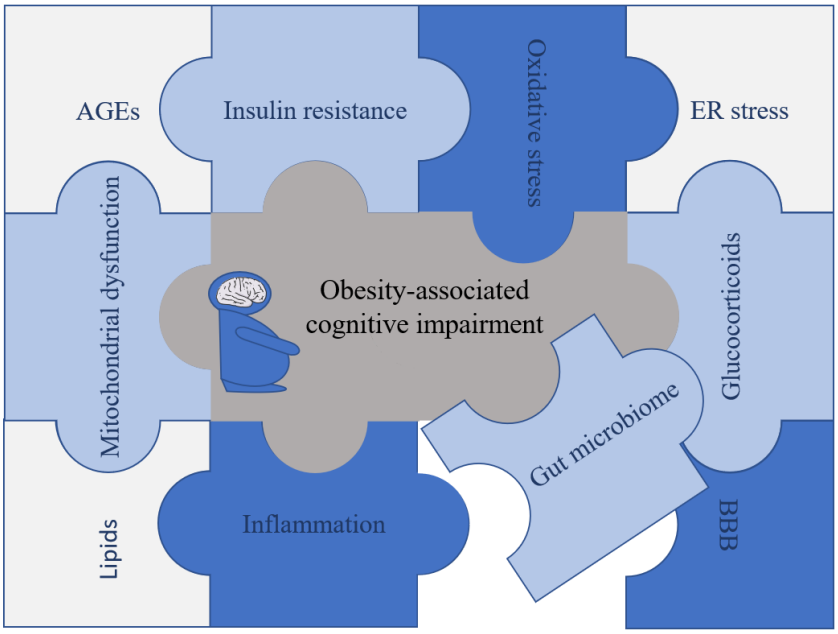


Figure 3. Overview of the mechanisms underlying obesity-associated cognitive decline. Inflammation, insulin resistance, advanced glycation end products (AGEs), glucocorticoids, lipids, mitochondrial dysfunction, endoplasmic reticulum (ER) stress, oxidative stress and blood-brain barrier (BBB) dysfunction are the mechanisms widely proposed to explain the pathophysiology of cognitive impairment in obesity. The missing piece of the puzzle may be the gut microbiome, whose broad role needs to be further elucidated (original illustration by Arnoriaga-Rodríguez, 2021).

1.3. Obesity, cognitive dysfunction and the gut microbiome

1.3.1. The gut microbiome

The microbiome constitutes a complex and immense ecosystem. Microbiota includes bacteria, viruses and bacteriophages, fungi, protozoa and archaea. Firmicutes and Bacteroidetes and, to a lesser extent, Actinobacteria and Proteobacteria, are the dominant bacteria phyla in the gut.[197] This microbiota and its collective genome is referred to as the microbiome.[198] The microbiome is essential for human health, since host-microbial relationships are established for mutual benefit.[199]

Gut microbiota composition is characterized by a large interindividual variability and heterogeneity due to constant interactions with host elements.[200] Exogenous and endogenous factors such as diet, drugs, the intestinal mucosa, the immune system and the microbiota itself may affect the gut microbiota.[201] Disturbances in gut microbiota, termed dysbiosis, comprise decreased microbial diversity or expansion of specific bacteria taxa, with a loss of beneficial and an overgrowth of detrimental bacteria.[201]

Dysbiosis is involved in the pathophysiology of several disorders.[202] In obesity, a dysbiotic microbiota has been identified as, for example, an increase in the ratio of Firmicutes/Bacteroidetes,[40] although not consistent in all the studies;[203] or a reduction in bacterial diversity;[204] in association with adiposity, insulin resistance and dyslipidemia.[205] In turn, as a vicious cycle, dysbiosis may trigger intestinal barrier dysfunction, promoting the translocation of bacterial metabolites[195] and low-grade inflammation.[206] Dysbiosis and obesity phenotype can be transmissible; fecal microbiota transplantation (FMT) from obese to germ-free mice leads to obesity.[41] By contrast, dysbiosis may also be amended; an increase of gene richness and bacterial diversity has been described in bariatric surgery-induced weight loss in parallel to improvements in metabolic and inflammatory profiles.[207]

1.3.2. The gut microbiome and obesity-associated cognitive dysfunction

The cross-talk between the microbes and the host involves different immune-mediated signalling pathways, establishing host-microbiota axis.[208] Compelling evidence demonstrates that there is a link between the gut and the brain, the gut-brain axis (Figure 4), in which microbiota plays a crucial role;[209] influencing neural development, cognition and behaviour.[210,211]

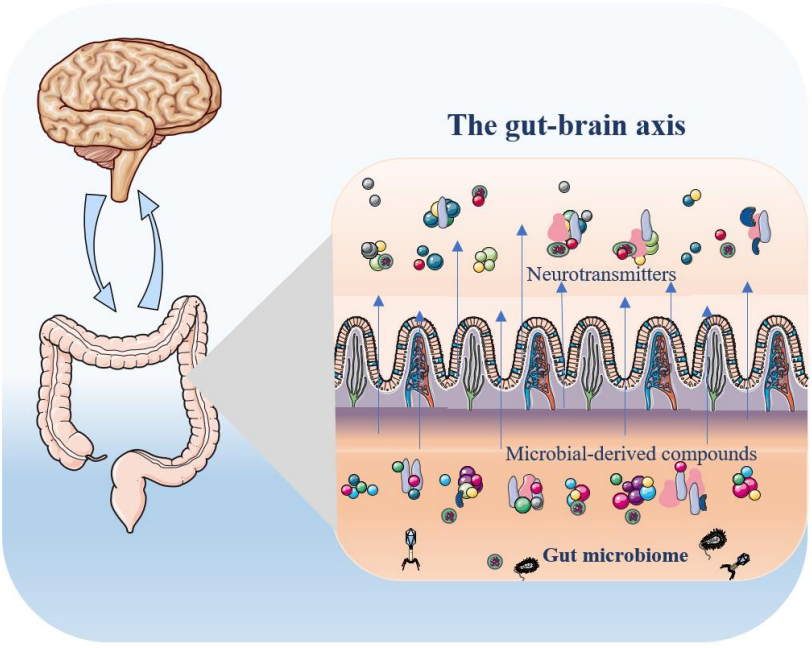


Figure 4. The gut-brain axis. Bidirectional pathways communicate the gut and the brain such as the hypothalamic-pituitary axis, the vagal afferent neurons, inflammatory markers, microbial metabolites, neurotransmitters or gut peptides, to name a few (original illustration by Arnoriaga-Rodríguez, 2021).

Multiple pathways can communicate the brain and the gastrointestinal tract, including the HPA axis, the sympathetic-adrenal axis, descending monoaminergic pathways or the autonomic nervous system such as the vagus nerve and the enteric nervous system.[212] Moreover, a gut to brain communication is mediated through vagal and spinal afferents.[212] Dysbiosis can alter this bidirectional crosstalk between the gut and the brain[213] impacting on its function.

Different mechanisms may shift the microbiome and promote cognitive dysfunction, as listed below:[214-216]

Immune signalling pathways: The microbiome is deeply linked to the immune system and can impair peripheral insulin sensitivity[215,217] Over the past century, the improvements in household amenities and standards of personal cleanliness with a decline of family size resulted in a higher prevalence of chronic inflammatory disorders.[218] In this line, changes in the environment also influenced the microbiome, leading to an enhancement of inflammatory responses which may also be extended to brain diseases.[219]

Neural pathways: Vagal afferent neurons influence gastrointestinal function, glucose homeostasis, food intake and body weight[220] and are one of the main pathways to connect the gut and the brain. Microbiota can also modulate the HPA axis in response to stress, especially important during early development.[221]

Intestinal and blood-brain barriers: The gut microbiome can modulate intestinal permeability.[222] Impaired intestinal barrier allows translocation of gram-negative bacteria-derived lipopolysaccharide (LPS) into circulation, promoting endotoxemia and inflammation. In parallel, systemic inflammation may increase intestinal barrier permeability, allowing translocation of commensal bacteria with implications for systemic inflammation and the brain.[223] Preclinical evidence from rodent models suggests that the microbiota and microbial-derived compounds can impact on the BBB.[224] For instance, a reduction in gut microbiota richness and diversity, in association with reduced tight junction proteins, increased plasma endotoxin LPS and impaired recognition and spatial memory was observed in high-fat diet-induced obesity mice.[225]

Microbial products: Metabolites: The microbiota can produce short-chain fatty acids (SCFAs): including butyrate, propionate and acetate, which may exert central effects[226] and influence the production of neurotransmitters or the release of gut peptides from enteroendocrine cells. Neurometabolites: The microbiota can synthetize neurotransmitters including serotonin, dopamine, noradrenaline, gamma-aminobutyric acid (GABA), acetylcholine.[226] Interestingly, serotonin and its precursor tryptophan are key systems in the gut-brain axis.[227] Enterosynes: intestinal bioactive molecules that can modulate the enteric nervous system to modify duodenal contraction.[228]

The factors briefly summarized above are some of the plausible mechanisms through the gut microbiome may impact on cognition. Available evidence is based mainly on preclinical studies. However, more studies are needed to elucidate the involved pathways and their effects in humans.

1.3.2.1. Evidence in preclinical models

As it has been previously described, dysbiosis is involved in the pathophysiology of neurodegenerative[229] and psychiatric disorders.[230] More recently, the disbalance of the gut-brain axis has been associated with cognitive decline in obesity and other metabolic disorders.[196,211] Most of the research supporting the role of the gut microbiome in obesity-associated cognitive dysfunction have been conducted in mice.[196,211]

Rodent models of obesity: Mice fed a high-sucrose diet showed impaired short and long-term memory and reversal training in association with changes in the gut microbiota. Higher and lower expression of Clostridiales and Bacteroidales, respectively, were linked to decreased cognitive flexibility.[231] Mice which developed diet-induced obesity exhibited a reduction in gut microbiota richness and diversity in parallel to diminished recognition and spatial memory.[225] Obesity, memory impairment, modifications of the expression of hippocampal markers and an increase of Proteobacteria phylum have been observed by high-fat diet feeding.[232]

Fecal microbiota transplantation (FMT): Evidence of microbial influence on cognitive dysfunction-associated obesity derives from the effects observed from FMT. Transplantation of high-fat diet microbiota to mice which had previously been fed a chow diet led to learning and memory deficits.[233]

Prebiotics: Administration of prebiotics, non-digestible dietary substances, has shown beneficial effects in cognition. The prebiotic xylooligosaccharide (XOS) restored cognitive function in high-fat diet rats through improving hippocampal plasticity, brain mitochondrial dysfunction and decreasing microglial activation.[234]

Curdlan, a bacterial polysaccharide produced by some species of Agrobacterium and Alcaligenes, may exert beneficial effects on cognitive dysfunction linked to obesity. Acute curdlan supplementation for 7 days prevented shifts induced by high-fat diet in the gut microbiota composition. In a chronic period of 15 weeks, curdlan also improved cognitive dysfunction using the Temporal Order Memory Test, the Novel Object Recognition (NOR) Test and the Y-maze test and mitigated microgliosis, neuroinflammation and synaptic impairments in the prefrontal cortex and hippocampus. In parallel, prebiotic supplementation attenuated hyperendotoxemia and colonic permeability and inflammation.[235]

Recently, two studies have demonstrated a favorable effect of the β-glucan.[236,237] In high-fat and fiber-deficient diet rodents, long-supplementation for 15 weeks with the β-glucan prevented cognitive dysfunction assessed by Object Location, NOR and Nesting Building. In addition, it ameliorated gut microbiota dysbiosis, impairment of intestinal barrier and hippocampal neuroinflammation. Short-term β-glucan supplementation for 7 days produced microbiota changes before establishing cognitive dysfunction. Furthermore, the beneficial effects of the supplementation disappeared after a broad-spectrum antibiotic treatment and thus, the ablation of the microbiota.[236]

Probiotics: Supplementation with live microorganisms seems to have a positive impact on cognitive dysfunction related to obesity. *Lactobacillus helveticus* improved deficits in spatial memory and anxiety in mice on a Western diet.[238] A probiotic, which contained a combination of different strains of bacteria species belonging to *Bifidobacterium*, *Lactobacillus* and *Streptococcus* genera, prevented the diet-induced memory deficits in rats on Western diet; increasing some bacteria taxa and reversing alterations in hippocampal genes.[239] In addition, *Akkermansia muciniphila* improved learning and memory in mice which had been fed a high-fat diet, reducing hippocampal microgliosis and proinflammatory cytokines and restoring neuronal development and synapse plasticity.[233]

1.3.2.2. Evidence in clinical models

In contrast to the emergence of a more and more robust preclinical literature on obesity and microbiota-neurocognition interactions, limited information is available from human studies.[240] To the best of our knowledge, only two cross-sectional case-control studies;[241,242] one longitudinal case-control study[243] and one placebo-controlled trail[244] have been published on this topic.

The earliest evidence of the relationship between the gut microbiota and obesity-associated cognitive dysfunction in humans was published in 2015.[241] The study included 20 and 19 middle-aged subjects with and without obesity, respectively. A specific microbiota profile was linked to disturbances of brain microstructure and cognition. Remarkably, the higher the gut microbiota diversity, the lower the MRI R2* values, an in vivo measure of iron brain content, in the hypothalamus, hippocampus and caudate nucleus; in association with cognitive impairment.[157] At phylum level, relative abundance of Actinobacteria was linked to well-organized brain microstructure, through fractional anisotropy values and better motor speed, attention and cognitive flexibility reflected in the Trail Making Test score, whereas the genus *Prevotella* was linked to worse cognitive traits. Bacterial diversity was also negatively associated with total fat mass and obesity-associated inflammatory markers.

Later, a study described the impact of gut microbiota on metabolites and neurotransmitters involved in cognition. Particularly, the glutamate, one of the key neurotransmitters of the central nervous system. The study[242] included 19 participants with obesity and 16 paired controls. The cognitive assessment encompassed the Trail Making Test to measure executive function and focused on different determinations of glutamate. In this research, some bacterial families were associated with processing speed and mental flexibility, modulating fecal and plasma glutamate and glutamate-derived indices related to its production or degradation. Some microorganisms that appeared to influence glutamate levels included the families of *Corynebacteriaceae*, *Coriobacteriaceae* and *Burkholderiaceae*. Moreover, bacterial ratios such as *Coriobacteriaceae/Streptococcaceae* and *Corynebacteriaceae/Streptococcaceae* were linked simultaneously to cognitive scores and fecal glutamate/glutamine ratio.

Longitudinal evidence is even sparser, since only one study has evaluated the gut microbiota and obesity-associated cognitive dysfunction at 2-year follow-up.[243] The research comprised 35 participants, 17 of them with obesity. Cognitive function assessment included immediate and delayed visual memories; visual-spatial constructional ability and executive function. During that period, changes in the gut microbiota were longitudinally linked to cognitive function and brain iron deposition.

In particular, shifts in the relative abundance of the Gemmatimonadetes, Bacteroidetes, Proteobacteria, Caldiserica, Tenericutes, Thermodesulfobacteria and Chlorobi phyla were associated with increased percentage change of R2* at the striatum, superficial amygdala and hippocampus, as a measure of brain iron load, but also beta-amyloid deposition. Moreover, the increase of waist circumference was associated with higher R2* brain values in parallel to worse visual-spatial constructional ability and circulating beta-amyloid levels.

Recently, a multicentric, single-blinded, placebo-controlled trial has evaluated the effects of a prebiotic in biochemical and psychological parameters, in parallel to gut microbiota changes. The study included 106 patients with obesity who received a prebiotic, inulin, or placebo, maltodextrin; combined with inulin-rich or poor food, respectively, dietary advise and a restriction of caloric intake for three months. Cognitive assessment included flexibility, working memory and inhibition. Prebiotic intervention led to moderate improvements of mood and cognitive flexibility. Moreover, baseline microbiota could identify those individuals who may experiment more beneficial for inulin intervention in case of mood but not cognition. Particularly, responders in terms of mood had higher levels of basal *Coprococcus* genus in association with a more deleterious metabolic and inflammatory profile.[244]

An overview of the studies previously presented is compiled in the next page (Table 4).

Table 4. Summary of the human studies related to obesity-associated cognitive dysfunction (Arnoriaga-Rodríguez, 2021).

Authors (Year)	Study design	Population	Age	Obesity measurements	Cognitive assessment	Brain imaging	Microbiota	Results
Fernández-Real et al.[241] (2015)	Cross-sectional	20 Obesity 19 Controls	48.7	BMI, Waist, Fat mass	TMT	R2*, DTI	Diversity; phylum, genus	A specific microbiota-brain map in obesity. p_Actinobacteria (+) g_*Prevotella* (-)
Blasco et al.[243] (2017)	Longitudinal (2 years)	17 Obesity 18 Controls	51.5	BMI, Waist	TMT, ROCFT, Verbal fluency task	R2*	Phylum	Changes in gut metagenome were inked to brain iron and cognitive function at 2-years follow-up. p_Tenericutes (+) p_Thermodesulfobacteria (+) p_Bacteroidetes (-) p_Proteobateria (-) p_Chlorobi (-)
Palomo-Buitrago et al.[242] (2019)	Cross-sectional	19 Obesity 16 Controls	52	BMI	TMT	---	Family	Gut microbiota may modulate fecal glutamate improving cognitive function. f_*Coriobacteriaceae* (+) f_*Corynebacteriaceae* (+) f_*Burkholderiaceae* (+) f_*Streptococcaceae* (-)
Leyrolle et al.[244] (2021)	Randomized, single-blinded, multicentric, inulin vs placebo trail (3 months)	106 Obesity	47	BMI, Waist/Hip, Fat mass	Number-letter, Mental Counters, Stop-Signal	---	Genus	Inulin supplementation in obesity led to moderate improvements in cognitive flexibility.

Obesity-associated cognitive impairment: Evidence in humans. Age represents the mean age of study population; BMI, body mass index; DTI, diffusion tensor imaging; g_, genus; f_, family; p_, phylum; TMT, Trail Making Test; R2*, brain relaxometry as a measure of iron brain content; ROCFT, Rey-Osterrieth Complex Figure Test.

Overall, based on existing evidence, more data in humans are needed to further confirm and translate the animal findings. Furthermore, the pathways through the gut microbiome which may impact on cognitive dysfunction in obesity remain largely unknown and, to date, only associations and plausible speculations have been proposed to be involved. Therefore, it might be interesting to deeply explore molecular mechanisms underlying this relationship. All these gaps of knowledge motivated the aim of the studies included in the present thesis.

2. JUSTIFICATION

The prevalence of obesity has dramatically increased in the last forty years, reaching epidemic proportions. Obesity has nearly tripled since 1975 and a 13% of adults with obesity was estimated in 2016.[5] Unfortunately, this trend appears to continue, owing to changes in society, behavioural patterns[245] and the low effectiveness of the conservative treatments in the long-term weight loss maintenance.[77]

Obesity is associated with multiple disorders including cognitive impairment.[63] Cognitive decline and dementia are nowadays major health concerns due to larger life expectancy and greater rates of metabolic disorders. More than 55 million people worldwide live with dementia with almost 10 million new diagnostics annually,[246] which implies social and economic costs in terms of carers, families and health-care systems.[247] Regrettably, there are no curative treatments except the control of modifiable risk factors.[88] Midlife obesity is one of these potentially factors.[91,92] In fact, a projection model estimated a reduction of 9-10% of dementia cases in 2050 with the decline of 20% of obesity cases.[93]

The gut microbiome has revolutionized the concept of health and disease,[193] in particular in obesity pathophysiology. Gut microbiome may impact on metabolic inflammation,[195] identify subjects with obesity[248] and even predict weight loss.[249] Changes in the gut microbiota, towards a more beneficial profile, have been observed after weight loss interventions.[207] In addition, different rodent models have also demonstrated the role of microbiota in obesity-related cognitive impairment.[225,231-239] Nevertheless, data in humans are limited.[240] The evidence available is based on preliminary studies[241-243] with small sample sizes and simple associations. In addition, underlying mechanisms remain largely unknown.

Therefore, more studies assessing the role of microbiota in obesity-related cognitive impairment in humans are needed. These should include large populations, optimal standardized approaches and a combination of clinical and preclinical models.

Gaining further insight into potential metabolic pathways may provide a major breakthrough in the management of obesity and cognitive impairment and could identify new targets to tackle and even to prevent obesity and related disorders.

3. HYPOTHESIS AND OBJECTIVES

Hypothesis

It is hypothesized that the gut microbiome may differently influence cognition, at both functional and structural levels, in subjects without and with obesity, in whom impaired cognitive function could be expected.

General objective

The aim of the studies included in this thesis was to evaluate the impact of the gut microbiome on cognitive function in midlife obesity.

Specific objectives

1) To assess cognitive function in middle-aged subjects with obesity compared to their counterparts without obesity, focused on memory and inhibitory control.

2) To investigate the influence of the gut microbiome on cognitive function, combining OMICS' approaches:
    2.1. Metagenomics.
    2.2. Metabolomics.

3) To determine the links between brain structure, cognitive function and the gut microbiome.

4) To explore the neurocognitive effects of fecal microbiota transplantation from human donors to recipient mice.

4. METHODS

4.1. Clinical and experimental design

4.1.1. Clinical discovery and replication cohorts

Discovery cohort: The main results of the original papers I and II included in this thesis were based on the Ironmet cohort. The aim of the Ironmet project (PI15/01934) was to evaluate adipose tissue, muscle and brain iron and volume in subjects with obesity, as well as their relationships with the gut microbiota and the effects of weight loss. To address the hypothesis of the present thesis a subset of variables was examined including clinical parameters, cognitive assessment, brain gray matter volume and the gut microbiome.

Study design: A pilot prospective 1-year case-control study including subjects with (BMI ≥ 30 kg/m^2) and without (BMI < 30 kg/m^2) obesity matched by age and sex. Due to a lack of standardized studies evaluating cognition and microbiota in middle-aged subjects with and without obesity; we established a number of 90 subjects in each arm (30 men, 30 pre- and 30 postmenopausal women).

Eligible participants: The inclusion and exclusion criteria are listed below (Table 5).

Table 5. Discovery cohort: Inclusion and exclusion criteria (Arnoriaga-Rodríguez, 2021).

Inclusion criteria	
Aged 25-67 years.	
Provide informed consent prior to any study procedure.	
Available to complete the follow-up.	
Exclusion criteria	
Presence of concomitant diseases:	Type 1 or 2 diabetes
	Malignant condition
	Chronic active inflammatory disease
	Chronic kidney or liver conditions.
Neuropsychiatric conditions:	Neurological or psychiatric diseases
	History of traumatic brain injury or language disorders
	Severe eating disorders.
Infection in the previous month.	
Treatment with antibiotics, antifungal, antiviral, proton pump inhibitors, anti-inflammatory drugs in the last 3 months.	
Use of drugs or alcohol abuse (40 g/d men, 20 g/d women).	
Pregnancy or lactation.	

The graph (Figure 5) shows the flowchart and an overview of the timeline of the study.

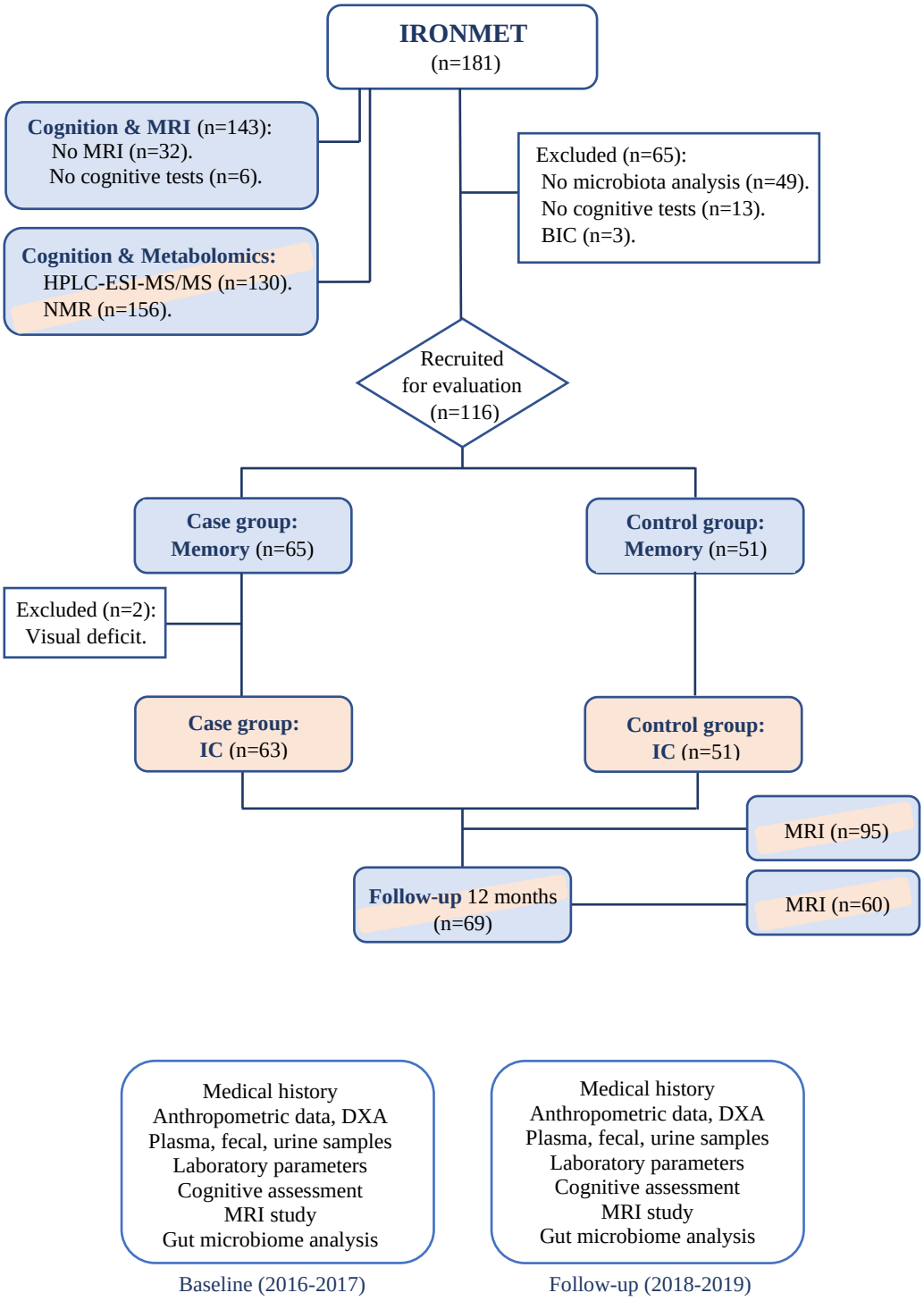


Figure 5. Discovery cohort: Flowchart and timeline of the study. Blue, salmon and blue-salmon indicate the selection included in paper I, paper II and both papers, respectively. BIC, borderline intellectual capacity; DXA, dual-energy X-ray absorptiometry; HPLC-ESI-MS/MS, high-performance liquid chromatography-electrospray ionization tandem mass spectrometry; IC, inhibitory control; MRI, magnetic resonance imaging; NMR, nuclear magnetic resonance (original illustration by Arnoriaga-Rodríguez, 2021).

Period of recruitment: From January 2016 to October 2017 and from February to April 2019 for the cross-sectional and longitudinal study, respectively.

Setting: Department of Endocrinology, *Hospital Universitari Dr. Josep Trueta* (Girona).

Data collection: Participants were recruited from both clinical (cases) and community-based (controls) sources through word-of-mouth or announcements. Selection of participants, ascertainment of inclusion and exclusion criteria, medical history and physical examination were conducted by an endocrinologist in a personal interview. Blood sample testing was performed by a trained nurse. Urine and fecal samples were provided by the participants. Data were stored in a confidential database whose content was encrypted.

Funding: This work was partially supported by research grant (PI15/01934) from the *Instituto de Salud Carlos III* from Spain and the Project ThinkGut (EFA345/19) 65% co-financed by the European Regional Development Fund (ERDF) through the Interreg V-A Spain-France-Andorra program (POCTEFA 2014-2020).

Ethical considerations: The Institutional review board - Ethics Committee and the Committee for Clinical Research (CEIC) of Hospital Universitari Dr. Josep Trueta (Girona) approved the study protocol and informed written consent was obtained from all participants.

Replication cohorts: Findings from the discovery study Ironmet were validated in two different cohorts. The main features of these studies are summarized below (Table 6).

Table 6. Replication cohorts: Main characteristics (Arnoriaga-Rodríguez, 2021).

Features	Florinash-MRI (n=24)	Imageomics (n=970)[250]
Aim	To evaluate the role of intestinal microflora in non-alcoholic fatty liver disease. Florinash-MRI is a sub-study of the entire project, in a cohort from Girona.	To identify biomarkers of human aging by analyzing imaging, biopsychosocial, cardiovascular, metabolomic, lipidomic and microbiome variables.
Design	Cross-sectional case-control.	Population-based study.
Recruitment	2010-2012.	2017-2019.
Participants	Age 30-60 years.	Age ≥ 50 years, dwelling in the community.
Exclusion criteria	Infection in the previous month. Major systemic diseases, including malignancy. History of drug or alcohol abuse. Medication interfering with insulin action. Acute cardiovascular event or illness (6 months). Mental illness.	Infection during the previous 15 days. Contraindications for MRI.
Setting	*Hospital Universitari Dr. Josep Trueta.*	Province of Girona.

All subjects gave written informed consent, validated and approved by the Ethics Committee and the Committee for Clinical Research (CEIC) of *Hospital Universitari Dr. Josep Trueta* (Girona).

4.1.2. Experimental design

Preclinical research included different mouse models of fecal microbiota transplantation and subsequent assessments of cognitive-like behavior. They were designed in an attempt to validate the results and to establish a causal relationship of the observations identified in humans.

All the experiments were conducted in wild-type C57BL/6J male mice (Charles River, France) with an initial weight of 23–26 g and a number of 11 mice for each group. Mice were housed individually in controlled laboratory conditions with the temperature maintained at 21 ± 1ºC, humidity at 55 ± 10% and 7:30h-19:30h light/dark cycle, local time for memory-like behavior or 12 hours reversed light/dark cycle, lights off at 8:00h for operant behavior. In the latter, mice were tested during the first hours of the dark phase of the reversed light/dark cycle. Animals were fed a standard chow diet RM1 (Irradiated Vacuum packed, Dietex International Ltd.) with food and water *ad libitum*. Health status of each mouse was checked every day before the experimental sessions and recorded in the protocol notebook. It included assessments of body weight, physical aspect, behavior and clinical signs.

Fecal microbiota transplantation (FMT) experiments: After acclimatization to housing conditions, animals were divided into groups matched by average body weight. Mice were treated with a cocktail of antibiotics[251] 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 72 hours wash out and then colonized daily via oral gavage of donor microbiota (200 µL) for 3 days. Donor microbiota was acquired from fecal samples of selected human subjects. Booster inoculations were given twice weekly 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). After 4 weeks, animals were exposed to a series of behavioral testing. At the end of the study, mice were sacrificed and cecums were extracted, weighted and directly frozen in dry ice and stored at -80ºC. Mice brains were also removed to further study the prefrontal cortex (PFC) gene expression (Figure 6).

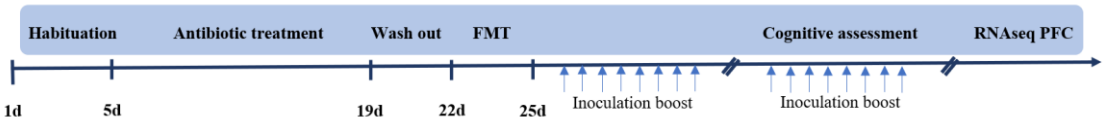


Figure 6. Timeline of the fecal microbiota transplantation experiments. FMT, fecal microbiota transplantation; PFC, prefrontal cortex; RNA-seq, RNA sequencing (original illustration by Arnoriaga-Rodríguez, 2021).

Experimental design for FMT – Memory (Paper I): Microbiota from 22 human subjects (11 with low and 11 with high memory scores matched for age, sex, BMI, and depression scores) was orally delivered to individual mice in a blinded fashion. Control mice (n=11) received saline. Cognitive assessment including the Novel Object Recognition (NOR) and Fear Conditioning Tests. Finally, RNA sequencing of the PFC was performed. Analysis: i) The effects of FMT on memory-like behavior in mice compared with control mice receiving saline. ii) The effect of FMT from human donor with and without obesity compared to control mice. iii) Study of the PFC gene expression.

Experimental design for FMT - Inhibitory control. Study I (Paper II): The microbiota from 22 human donors with (BMI ≥ 30 kg/m2, n=11) and without obesity (BMI < 30 kg/m2, n=11) matched for age, sex and education years was orally delivered to recipient mice. Reversal learning was conducted. Then, rodent performance of mice receiving human microbiota from donors with obesity was compared to those from human donors without obesity.

Experimental design for FMT - Inhibitory control. Study II (Paper II): The microbiota from human donors with low (n=11) and high (n=11) Stoop Color and Word Test (SCWT) scores matched for age, BMI, gender and education years was delivered to recipient mice. RNA sequencing of the PFC was performed.

The overview of the FMT designs is presented below (Figure 7).

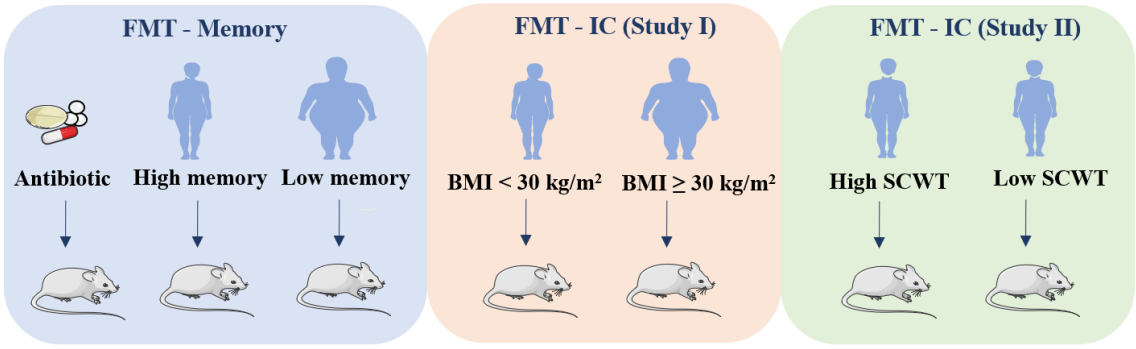


Figure 7. Experimental design for the fecal microbiota transplantation experiments. FMT-Memory (Paper I), mice were subjected to the Novel Object Recognition and Fear Conditioning Tests. FMT-IC Study I (Paper II), Reversal learning was conducted after FMT. FMT-IC Study II (Paper II), RNA sequencing of the mice PFC was performed. BMI, body mass index; FMT, fecal microbiota transplantation; IC, inhibitory control; SCWT, Stroop Color and Word Test (original illustration by Arnoriaga-Rodríguez, 2021).

Ethical considerations: Animal procedures were conducted 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*).

4.2. Method details

4.2.1. Clinical measurements

4.2.1.1. History and physical examination

Family, social and medical history was assessed in a personal interview by an Endocrinologist as well as a physical examination. Nutritional history was collected using a validated food-frequency questionnaire.[252]

4.2.1.2. Measures of obesity

BMI was calculated by the kilograms of body weight divided by the square of the height in meters (kg/m^2).[3] Waist circumference was measured using a tape around the highest point of the upper margin of the iliac crest in parallel to the ground without compressing the skin at the end of a non-forced expiration. Total percentage of fat mass was assessed with dual-energy x-ray absorptiometry (DXA, GE lunar, Madison, Wisconsin).

4.2.1.3. Biochemical parameters

After fasting for a minimum of eight hours, blood sample testing was performed. As routine measurements in clinical practice, the biochemical parameters were analyzed with the same biochemical procedures. Plasma glucose, lipids profile and high-sensitivity C-reactive protein concentrations were measured using an analyzer (Cobas 8000 c702, Roche Diagnostics, Basel, Switzerland). Glycated hemoglobin was determined by performance liquid chromatography (ADAMA1c HA-8180V, ARKRAY, Kyoto, Japan).

4.2.1.4. Hyperinsulinemic-euglycemic clamp

Insulin sensitivity was determined by the hyperinsulinemic-euglycemic clamp. The procedure consists in creating in fasting conditions, a hyperinsulinemic state with an insulin infusion of predetermined fixed dosage and a variable rate glucose infusion.[253] Glucose levels should be maintained constant at normal fasting or any pre-existing (isoglycemic) level adjusting the infusion rate of a 20% glucose solution. A steady state is usually reached in the last 40 minutes after 2 hours. Under these conditions the glucose infusion rates equal the glucose disposal rate, M ($mg \cdot kg^{-1} \cdot min^{-1}$), a measurement of overall insulin sensitivity (Paper II).

4.2.2. Neuropsychological assessment

4.2.2.1. The California Verbal Learning Test

Episodic verbal learning and memory was measured by the California Verbal Learning Test (CVLT) - second edition.[254] It consisted of 5 learning tests in which a list of words (list A) was presented. The subject was immediately asked to recall as many words as possible after each presentation. The result of these first five tests, CVLT Immediate Recall (CVLT-IR) provided information about the learning process. Second, an interference list (list B) was introduced and the subject was requested to repeat the same task. Then, in the short delay test, the patient was asked to recall list A, free, CVLT Short Delayed Free Recall (CVLT-SDFR) or with semantic facilitation, CVLT Short Delayed Cued Recall (CVLT-SDCR). The higher the score, the better the memory function. About 30 minutes were necessary to administrate the test. The reliability of the CVLT-II ranges from 0.78 to 0.94.[254] (Paper I).

4.2.2.2. The Digit Span

Working memory was assessed by the Digit Span, a subtest of the Wechsler Adult Intelligence Scale-III (WAIS-III)[255] a measure of general intellectual functioning. It is based on numbers and includes the Forward and Backward Digit Span. In the Forward Digit Span, the examinee repeated a sequence of numbers in the same order as presented; a measure of working memory but also attention. Sequence length is increased until the participant cannot repeat the sequence properly. In the Backward Digit Span, the examinee repeated the number sequence in reverse order. Total Digit Span (TDS) represented the total score of the two previous tests. A higher score reflected better memory function. In a standardization sample of 394 participants (aged 16-89 years), the reliability coefficient was high, in the 0.80s-0.90s.[110] (Paper I).

4.2.2.3. The Stroop Color and Word Test

The Golden's version of the Stroop Color and Word Test (SCWT)[256] assesses inhibition, but also cognitive flexibility, selective attention and information processing speed. Subjects are required to read three tables as fast as possible: 1) 100 words (color names) printed in black ink; 2) 100 "XXX" printed in color ink (green, blue and red); 3) 100 color names (from the first page) printed in color ink (from the second page) which did not match and the subject was asked to name the ink color (and not to read the color name). Forty-five seconds were given for each task and after that, the last item completed was noted, obtaining three scores W, C and CW, respectively.

The interference (I) index was also obtained from the subtraction CW-CW' (CW'=WxC/W+C). Reliability coefficients have been reported around 0.7-0.8. Standard administration procedures were followed as indicated in the test manual.[256] (Paper II).

4.2.2.4. The Iowa Gambling Task

The computerized version of the Iowa Gambling Task (IGT) (Bechara A; Psychological Assessment Resources, Inc.) measured decision making. Four upside down card decks were shown in the screen, each of them identified by a letter (A, B, C or D). Participants could freely choose cards from any deck in order to win as much money as possible. When the subject clicked on a card deck a smiley face and the amount of money won appeared in the screen. Sometimes, after the smiley face was shown a sad face appeared 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 gave bigger amounts of money but they also made important losses whereas C and D decks made smaller profits but they caused fewer losses. On the A and C decks the punishment frequency raised progressively but magnitude was constant, whereas on the B and D decks the punishment frequency was constant but magnitude raised progressively. Standard administration procedures were followed as indicated in the test manual.[257] Standardized (t-score) Net Total Score, resulted from the subtraction of the disadvantageous from the advantageous decks (CD-AB), was used for the statistical analysis (Paper II).

4.2.2.5. The Wisconsin Card Sorting Test

The computer version 4-Research Edition (Heaton RK; Psychological Assessment Resources, Inc.) of Wisconsin Card Sorting Test (WCST) was used to assess executive function. The test consisted of four stimulus cards: a red triangle, two green stars, three yellow crosses and four blue circles. The stimulus cards were always placed in the screen and different cards were shown below, one at a time. The subject was 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 was provided about the sorting rule nor about changes of the rule; only feedback about the answer was given in each trial (correct or incorrect). The sorting rule (color, form or number) changed after 10 consecutive correct answers (category). Standard administration procedures were followed.[258] We analyzed the trials to complete first category score, which was the total number of trials needed to complete 10 consecutive correct answers (Paper II).

4.2.2.6. The Patient Health Questionnaire-9

The Patient Health Questionnaire-9 (PHQ-9) is the depression module of the Primary Care Evaluation of Mental Disorders (PRIME-MD).[259] It encompasses 9 items of depression symptoms plus a question about functional impairment and can be scored as a depression severity rating (scores of 10-14 moderate, 15-19 moderately severe and 20-27 severe depressive symptoms) or with an algorithm based on the DSM-IV criteria (major and minor episode). Scores of 10 or more have an 88% sensitivity and specificity. PHQ-9 score was considered as a possible confounding factor in the analyses.

4.2.3. Magnetic resonance imaging (MRI)

4.2.3.1. MRI acquisition and image pre-processing

All subjects were studied on a 1.5 T 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) = 4.1 ms, repetition time (TR) = 8.4 ms, flip angle 8, field of view (FOV) 230 x 190 matrix. A total of 145 whole-brain images per subject with thickness axial slices of 1 x 1 x 1 mm^3 with or without gap. The total scan time was 189.6 s. The anatomical imaging data was processed and analyzed using MATLAB version R2017a (The MathWorks Inc, Natick, Mass) and Statistical Parametric Mapping 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 Algebra (DARTEL) and resliced to a 2 mm isotropic resolution in Montreal Neurological Institute (MNI) space.

4.2.3.2. Volumetric brain analysis

The Automated Anatomical Labeling (AAL)[260] atlas was used to obtain the volumetric information of the right and left hippocampus, opercula (orbitalis, tringularis, opercularis), and middle and superior frontal gyri as informed by the involvement of these brain regions in verbal memory[261-263] in 14394 participants as well as the anterior cingulate cortex in the Stroop task.[264] Each region was orthogonalized for sex, age and total gray matter volume in MATLAB version R2017a (The Math Works Inc, Natick, MA).

4.2.4. Metagenomics

4.2.4.1. Extraction of fecal genomic DNA

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/mL) was used for shotgun 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.

4.2.4.2. Whole-genome shotgun sequencing

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). Metagenome sequences were trimmed and quality controlled using the PRINSEQ-lite-0.20.4 program[265] and overlapping pairs were joined using FLASH-1.2.11.[266] Host reads were removed by mapping the reads against the host reference genome, by using bowtie2-2.3.4.3[267] with end-to-end and very sensitive options.

Functional analyses were performed by assembling the host-free reads into contigs using MEGAHIT v1.1.2[268] 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[269] was used for predicting codifying regions. The functional annotation was carried out with HMMER[270] against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database[271] to obtain the functional subcategory, pathway 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,[272] which was also 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[273] on the host-free reads. Addition of lineage information, counting of taxa and generation of the operational taxonomic unit (OTU) absolute and relative abundance matrices for all samples were performed using the package R.[272]

4.2.5. Metabolomics

For non-targeted metabolomics analysis, metabolites were extracted from fecal and plasma samples with methanol (containing phenylalanine-C13 as an internal standard) according to previously described methods.[274] Briefly, for plasma samples 30 µl of cold methanol were added to 10 µl of each sample, vortexed for 1 min and incubated for one hour at -20ºC. For fecal samples, the content of a 1.2 mL tube of Lysing Matrix E (MP biomedicals) and 600 µl of cold methanol were added to 10 mg of sample. Samples were homogenized using FastPrep-24™ (MP biomedicals) and were incubated overnight in a rocker at 4ºC. Then, all samples were centrifuged for three minutes at 12,000 g, 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 60ºC. 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 at 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, 350ºC). 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.

Fecal samples for the Ironmet cohort were also analyzed by Nuclear Magnetic Resonance (NMR) (Paper II). The preparation protocol started with around 15-20 mg of dried fecal 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 fecal water over the precipitated stool.

From the upper layer, 200 µl of prepared fecal water was placed in a 2 mL Eppendorf tube and then, 400 µl of 0.05 M PBS buffer in D2O (pH = 7.2, TSP 0.7 mM) 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 5 mm o.d. NMR tube. 1H 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.

4.2.6. Behavioral testing in mice

4.2.6.1. The Novel Object Recognition

The Novel Object Recognition (NOR) Test was performed in a V-maze as previously published.[275] Three phases of 9 minutes were performed on consecutive days. Mice were first habituated to the V-maze. On the second day, two identical objects (chess pieces) were presented to the mice and the time that they spent exploring each object was recorded. In the test phase, 3 hours later for short-term memory (NOR3h) or 24 hours later for long-term memory (NOR24h), one of the familiar objects was replaced with a novel object (a different chess piece) and the time spent exploring each object, novel and familiar, was computed. A discrimination index was calculated as the difference between the time that the animal spent exploring the novel (Tn) and familiar (Tf) object divided by the total time of object exploration: (Tn-Tf)/(Tn + Tf). (Paper I).

4.2.6.2. Fear Conditioning

Fear Conditioning was conducted as previously described with some modifications.[276,277] Mice were individually placed in a shuttle chamber (LE918, Panlab, Barcelona) surrounded by a sound-attenuating cabinet. The chamber floor was formed by parallel stainless-steel bars connected to a scrambled shock generator. On the training day, mice were habituated to the chamber for 180 seconds before the exposure to an acute 30 second beeping sound (80 dB). Each animal received an unconditioned stimulus (0.6 mA footshock for 2 seconds) paired with the end of the sound (conditioned stimulus). After the shock, the animal remained in the shuttle chamber for 60 seconds. To evaluate Cued Fear Conditioning, mice were re-exposed to the conditioned stimulus in a novel environment (a wide white cylinder in the chamber) 24 hours after the conditioning session. Mice were allowed to adapt for 180 seconds to the new environment which was followed by 30 seconds of the sound used in the training day.

After the last sound trial, mice remained in the cylinder for 60 seconds. Fear memory was assessed as the percentage of time that mice spent freezing during the session. Freezing response, a rodent's natural response to fear, was evaluated by direct observation and defined as complete lack of movement, except for respiration for more than 1 second. The procedure was performed between 8.00 and 12.00h in an experimental room different to the housing room.

4.2.6.3. Operant Conditioning

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 for the first 3 seconds of the session to indicate the start of the session. All sessions lasted 60 minutes and regular-flavored pellets were used. The food self-administration session consisted of two pellet periods of 25 minutes and a 10-minute 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 seconds 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 for 7 days (one active lever-press resulted in a delivery of one pellet). Following FR1 phase, animals were subjected to an increase fixed ratio up to 5 (5 lever-presses in order to obtain one reward) (FR5) for 8 days. Criteria for the achievement of the operant responding were acquired when the following conditions were met: (i) at least 75% responding on the active lever; and (ii) 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.

4.2.7. Study of the gene expression in the prefrontal cortex of mice

The brains of mice were quickly removed and the medial prefrontal cortex (PFC) was dissected according to the atlas of stereotaxic coordinates of mouse brain.[278] Brain tissues were then frozen by immersion in 2-methylbutane surrounded by dry ice and stored at -80ºC. For RNA preparation, each brain sample was treated individually. Total RNA was isolated from the brains using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen Düsseldorf, Germany) according to the manufacturer's protocol.

Quality control of the RNA was 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[279] 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.[280] The differential expression gene analysis (DEG) was assessed with voom+limma in the limma package version 3.30.13[281] and R version 3.4.3. Genes having less than 10 counts in at least 5 samples were excluded from the analysis. Raw library size differences between samples were treated with the weighted ''trimmed mean method'' TMM[282] implemented in the edgeR package.[283] The normalized counts were used in order to make unsupervised analysis, PCA and clusters. For the differential expression (DE) analysis, read counts were converted to log2 counts per million (logCPM) and the mean-variance relationship was modeled with precision weights using voom approach in limma package.

4.3. Statistical analysis

4.3.1. General statistical analysis

First, normal distribution and homogeneity of variances were tested. Results were expressed as number and frequencies for categorical variables, mean and standard deviation for normal distributed continuous variables and median and interquartile range for non-normal distributed continuous variables. To determine differences between study groups, we used Chi-Square for categorical variables, unpaired T-Test in normal quantitative and Mann-Whitney U test for non-normal quantitative variables. Spearman or Pearson analysis was used to determine the correlation between quantitative variables. ANOVA with repeated measures was used when required to test the evolution over time. In particular, for food self-administration analysis in mice, 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. These statistical analyses were performed with SPSS, version 19 (SPSS Inc., Chicago, IL).

4.3.2. Specific statistical procedures

4.3.2.1. MRI data analysis

Brain regions of interest (ROI) were orthogonalized for sex, age and total gray matter volume in MATLAB version R2017a (The Math Works Inc., Natick, MA). Spearman or Pearson analysis was used to determine the correlation between quantitative variables, corrected for multiple comparisons using q-values.[284]

4.3.2.2. Metagenomic analysis

Differential abundance analyses for taxa and KEGG-based metagenome functions associated to cognitive tests and brain volumes were performed using the DESeq2 R package,[285] controlling for i) age, sex, BMI, education years and PHQ-9 scores in memory; ii) age, sex and education years or iii) age, sex, education years, insulin sensitivity and high-sensitivity C-reactive protein in inhibitory control. Fold change with a unit change in the corresponding variable and Benjamin-Hochberg adjusted p-values[286] were plotted for each taxon. Significantly different taxa were colored according to phylum. Taxa and bacterial functions were previously filtered so that only those with more than 10 reads in at least two samples were selected.

Manhattan-like plot were used to show significantly expressed KEGG metagenome functions. The −log10(*p*FDR) values were multiplied by the fold change sign to take into account the direction of the association. Bars were colored according to the *p*FDR. A significance <0.05 was established unless otherwise indicated.

To take into account the compositional structure of the microbiome and rule out possible spurious associations, microbiome data were also analyzed using a compositional approach with the ALDEx2 R package,[287] controlling for age, sex, BMI, education years and depression scores and adjusting for multiple comparisons using q-values[284] and with a multivariate machine learning feature selection algorithm applied to the centered log ratio transformed data using the Variable Importance Testing Approach (VITA) algorithm.[288]

4.3.2.3. Metabolomic analysis

Metabolomic data were analyzed using machine learning methods. In particular, an all-relevant machine learning variable selection strategy was adopted, applying two random forest (RF)-based methods as implemented in the Boruta algorithm[289] and the VITA method.[288]

The Boruta algorithm performs feature selection based on the learning performance of the model.[289] It performs variables selection in different steps: (a) randomization, to create a duplicate copy of the original features randomly permutate across the observations; (b) model building, to compute the normalized permutation Variable Importance Measure (VIM) scores; (c) statistical testing, to find those relevant features with a VIM higher than the best randomly permutate variable using a Bonferroni corrected two-tailed binomial test and (d) iteration, until the status of all features is decided. We run the Boruta algorithm with 500 iterations, a confidence level cut-off of 0.005 for the Bonferroni adjusted p values, 5000 trees to grow the forest (*ntree*) and a number of features randomly sampled at each split given by the rounded down number of features/3 (the *mtry* recommended for regression).

The VITA algorithm[288] uses a strategy inspired in the cross-validation procedure to obtain the cross-validated permutation variable importance (CVPVI). The method randomly splits the data in a total of k-folds of equal size. For each i-fold, a RF is trained using all samples that are not part of the i-test set and the response variable is predicted for the samples in the i-test set. The procedure is repeated after permutating n times the values of the predictor variables. The permutation variable importance is calculated as the average difference in the prediction errors between the original data and the permutations and the CVPVI is the average over all k-fold-specific permutation variable importance.

Second, taking into account that for non-relevant features the change in accuracy is only due to random variations and thus it does not change (zero CVPVI) or slightly increases (negative CVPVI) when not using the variable for prediction, the non-positive CVPVI values are used to compute a symmetric null distribution of CVPVI scores around zero for non-relevant features by mirroring them on the y axis. From this approximated null distribution, p-values can be calculated. As the null distribution is obtained from non-relevant features, this testing approach is only suitable for datasets with a large number of variables without effect. In our calculations we used 5000 trees, a 7-fold CV and 10 permutations. P-values were then corrected using the Benjamini-Hochberg procedure for false discovery rate (FDR).[286]

4.3.2.4. Prefrontal cortex gene expression analysis

DESeq2[285] was also used to identify the medial PFC genes of recipient mice associated with metagenomic functions of human donors linked to cognitive tests controlling for donor's age, sex and education years.

Gene Ontology (GO) and Reactome pathway analyses of differentially expressed genes were performed using the clusterProfiler R package[290] and the ConsensusPathDB[291] respectively. The p value of each term was assessed using a hypergeometric test and significantly enriched terms were determined based on a q value (Storey correction)[284] cut-off of 0.1, to account for multiple testing. GO terms were visualized using the goplot function from the enrichplot R package, and significant reactome pathways were visualized using a gene overlap plot.

5. RESULTS

The results of this thesis are included in the following publications:

Original Paper I

Arnoriaga-Rodríguez M, Mayneris-Perxachs J, Burokas A, Contreras-Rodríguez O, Blasco G, Coll C, Biarnés C, Miranda-Olivos R, Latorre J, Moreno-Navarrete JM, Castells-Nobau A, Sabater M, Palomo-Buitrago ME, Puig J, Pedraza S, Gich J, Pérez-Brocal V, Ricart W, Moya A, Fernández-Real X, Ramió-Torrentà L, Pamplona R, Sol J, Jové M, Portero-Otin M, Maldonado R, Fernández-Real JM. **Obesity Impairs Short-Term and Working Memory through Gut Microbial Metabolism of Aromatic Amino Acids.** Cell Metab. 2020 Oct 6;32(4):548-560.e7. doi: 10.1016/j.cmet.2020.09.002.
Impact factor (JCR 2020): 27.287 (D1, 3/145 Endocrinology & Metabolism).

Original paper II

Arnoriaga-Rodríguez M, Mayneris-Perxachs J, Contreras-Rodríguez O, Burokas A, Ortega-Sanchez JA, Blasco G, Coll C, Biarnés C, Castells-Nobau A, Puig J, Garre-Olmo J, Ramos R, Pedraza S, Brugada R, Vilanova JC, Serena J, Barretina J, Gich J, Pérez-Brocal V, Moya A, Fernández-Real X, Ramio-Torrentà L, Pamplona R, Sol J, Jové M, Ricart W, Portero-Otin M, Maldonado R, Fernández-Real JM. **Obesity-associated deficits in inhibitory control are phenocopied to mice through gut microbiota changes in one-carbon and aromatic amino acids metabolic pathways**. Gut. 2021 Dec;70(12):2283-2296. doi: 10.1136/gutjnl-2020-323371.
Impact factor (JCR 2020): 23.059 (D1, 3/90 Gastroenterology & Hepatology).

5.1. Original paper I

Obesity impairs short-term and working memory through gut microbial metabolism of aromatic amino acids

Arnoriaga-Rodríguez M, Mayneris-Perxachs J, Burokas A, Contreras-Rodríguez O, Blasco G, Coll C, Biarnés C, Miranda-Olivos R, Latorre J, Moreno-Navarrete JM, Castells-Nobau A, Sabater M, Palomo-Buitrago ME, Puig J, Pedraza S, Gich J, Pérez-Brocal V, Ricart W, Moya A, Fernández-Real X, Ramió-Torrentà L, Pamplona R, Sol J, Jové M, Portero-Otin M, Maldonado R, Fernández-Real JM.

Cell Metab. 2020 Oct 6;32(4):548-560.e7.
doi: 10.1016/j.cmet.2020.09.002.

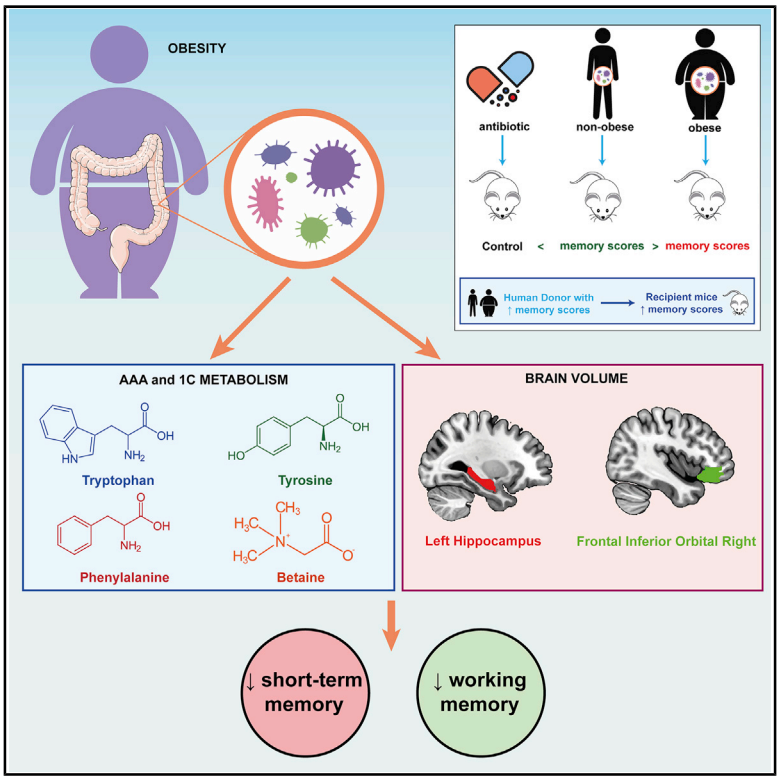
Highlights

- Metagenomic data associate aromatic amino acids (AAA) and one-carbon (1-C) metabolism with memory and brain region volumes.
- Memory scores are associated with altered plasma levels of AAA and betaine.
- Obesity modulates these relationships and is associated with impaired memory.
- FMTs from obese subject lead to decreased memory scores in recipient mice.

Cell Metabolism
Clinical and Translational Report

Obesity Impairs Short-Term and Working Memory through Gut Microbial Metabolism of Aromatic Amino Acids

Graphical Abstract



Highlights

- Metagenomic data associate AAA and 1-C metabolism with memory and brain region volumes
- Memory scores are associated with altered plasma levels of AAA and betaine
- Obesity modulates these relationships and is associated with impaired memory
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In Brief

Learning and memory have recently been associated with specific microorganisms and metabolites. Here, Arnoriaga-Rodríguez et al. reveal a unique microbiota profile associated with memory through pathways involving aromatic amino acid and one-carbon metabolism. Importantly, these relationships are modulated by obesity; fecal microbiota transplantation from human subjects with obesity decreases the memory score of recipient mice.

Arnoriaga-Rodríguez et al., 2020, Cell Metabolism *32*, 548–560
October 6, 2020
https://doi.org/10.1016/j.cmet.2020.09.002

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Clinical and Translational Report

Obesity Impairs Short-Term and Working Memory through Gut Microbial Metabolism of Aromatic Amino Acids

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https://doi.org/10.1016/j.cmet.2020.09.002

SUMMARY

The gut microbiome has been linked to fear extinction learning in animal models. Here, we aimed to explore the gut microbiome and memory domains according to obesity status. A specific microbiome profile associated with short-term memory, working memory, and the volume of the hippocampus and frontal regions of the brain differentially in human subjects with and without obesity. Plasma and fecal levels of aromatic amino acids, their catabolites, and vegetable-derived compounds were longitudinally associated with short-term and working memory. Functionally, microbiota transplantation from human subjects with obesity led to decreased memory scores in mice, aligning this trait from humans with that of recipient mice. RNA sequencing of the medial prefrontal cortex of mice revealed that short-term memory associated with aromatic amino acid pathways, inflammatory genes, and clusters of bacterial species. These results highlight the potential therapeutic value of targeting the gut microbiota for memory impairment, specifically in subjects with obesity.

INTRODUCTION

The decline of cognitive function is rising worldwide due to longer life expectancy (Larson et al., 2013) and increased prevalence of obesity and related metabolic disorders (Ward et al., 2019). Obesity has been identified as a modifiable risk factor for cognitive impairment (Kivipelto et al., 2018), but in turn, cognitive dysfunction is a predisposing factor for overeating and

Table 1. Clinical and Neuropsychological Data of the Human Discovery Cohort

	Total Population (n = 116)	Without Obesity (n = 51)	With Obesity (n = 65)	*p*
Age (years)	50.4 [41.8-58.5]	53.9 [44.4-59.0]	48.6 [41.1-57.1]	0.097
Females n (%)	81 (69.8)	34 (66.7)	47 (72.3)	0.511
Education (years)	12 [11-16.8]	15 [12-17]	12 [9-14]	$9.0x10^{-6}$
BMI (kg/m^2)	34.8 [25.3-43.3]	24.6 (2.6)	43.2 (6.7)	$3.3x10^{-34}$
Waist (cm)	110 [92-126]	89.8 (9.6)	125.2 (13.9)	$3.6x10^{-29}$
Fat mass (%)	43.6 [34-50.5]	32.4 (7.2)	49.9 (5.5)	$2.7x10^{-27}$
SBP (mmHg)	132.8 (20.0)	124.3 (15.8)	139.3 (20.6)	$2.3x10^{-5}$
DBL (mmHg)	74.8 (11.5)	71.2 (10.9)	77.6 (11.3)	0.003
HDL-C (mg/dL)	56 [45-68]	66.0 (17.0)	50.8 (12.7)	$2.1x10^{-7}$
Triglycerides (mg/dL)	90 [65.3-134.8]	79 [58-96]	123 [81.5-156]	$7.1x10^{-5}$
FPG (mg/dL)	96 [90-102.8]	95 [89-101]	97 [92.5-104.5]	0.196
HbA1c (%)	5.5 (0.3)	5.5 (0.3)	5.6 (0.3)	0.035
hsCRP (mg/dL)	2.4 [0.7-5.9]	0.7 [0.4-1.4]	5.0 [2.7-9.5]	$8.1x10^{-14}$
CVLT-IR (score)	61 [55-67.8]	65 [56-70]	59 [52.5-65]	0.003
CVLT-SDFR (score)	14 [12-15]	14 [12-16]	13 [11-14]	0.002
Total Digit Span (score)	14 [11.3-17]	15 [13-18]	13 [11-16]	0.003
PHQ-9 (score)	5.5 [3-9]	4 [2-6]	7 [4-10]	$1.8x10^{-4}$

Results are expressed as number and frequencies for categorical variables, mean and standard deviation (SD) for normal distributed continuous variables, and median and interquartile range [IQ] for non-normal distributed continuous variables. To determine differences between study groups, we used $\chi 2$ for categorical variables, unpaired Student's t test in normal quantitative, and Mann-Whitney U test for non-normal quantitative variables. p values for the difference between subjects with obesity (BMI > 30 kg/m^2) and without obesity (BMI between 18.5–30 kg/m^2). SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL-C, high density lipoprotein cholesterol; FPG, fasting plasma glucose; HbA1c, glycated hemoglobin; hsCRP, high-sensitive C-reactive protein; CVLT, California Verbal Learning Test; IR, Immediate Recall; SDFR, Short Delayed Free Recall; PHQ-9, Patient Health Questionnaire.

obesity (Gunstad et al., 2020). One of the core cognitive domains that is impaired first is learning and memory (Petersen et al., 1999). Subjects with obesity have shown memory deficits, with body mass index (BMI) being negatively associated with memory traits across adult lifespan (Cournot et al., 2006; Gunstad et al., 2006).

The link between obesity and altered gut microbiota is clearly recognized (Ley et al., 2006). Increasing evidence supports the role of microbiota in cognitive disorders (Rogers et al., 2016; Sarkar et al., 2018). Learning and memory have been associated with specific microorganisms and metabolites (Mao et al., 2020). For example, the lack of microbiota produced fear extinction learning deficits in germ-free mice (Chu et al., 2019) and the administration of *Lactobacillus helveticus* prevented the memory impairment induced by a western diet (Ohland et al., 2013). *Bifidobacterium longum* also led to a beneficial effect in the object recognition tasks (Savignac et al., 2015). However, it is important to note that all of these studies have been performed in mice.

Evidence in humans is still scarce. Preliminary findings have shown impaired cognitive traits and detrimental metabolic profiles linked to some bacterial families in subjects with obesity (Arnoriaga-Rodríguez and Fernández-Real, 2019; Blasco et al., 2017; Fernandez-Real et al., 2015; Palomo-Buitrago et al., 2019). In fact, interventions that delay or prevent cognitive impairment, such as weight loss and treatment with some antidiabetic drugs, are well known to be associated with microbiota shifts (Brunkwall and Orho-Melander, 2017; Livingston et al., 2017; Maruvada et al., 2017).

Herein, we hypothesized that memory impairment is associated with both obesity status and a specific gut microbiome profile. We evaluated brain structure (through magnetic resonance imaging [MRI]) and function (using validated neuropsychological tests) in subjects with and without obesity and determined how these measurements associated with the gut microbiota and the plasma and fecal metabolome. We also tested whether fecal microbiota transplantation (FMT) from humans into mice could help identify transmissible factors that impact the brain's transcriptome. The results showed that a specific gut microbiome profile was linked to several memory domains and to the volume of hippocampus and prefrontal regions differentially in subjects with and without obesity. A plasma and fecal metabolomics signature associated with these traits was also identified. Importantly, the microbiota from obese subjects led to decreased short-term memory scores in recipient mice, which had shifts in aromatic amino acid (AAA) pathways and inflammatory genes in the prefrontal cortex (PFC) linked to clusters of bacterial species.

RESULTS AND DISCUSSION

Analysis of the Gut Metagenome Reveals Bacterial Gene Functions and Species Associated with Memory Scores

Memory function was evaluated in a cohort of 116 middle-aged subjects (n = 65 with obesity, n = 51 without obesity; Table 1). Impairments in learning, immediate recall, short delayed recall, and working memory were observed among subjects with obesity, based on the scores of California Verbal Learning Test

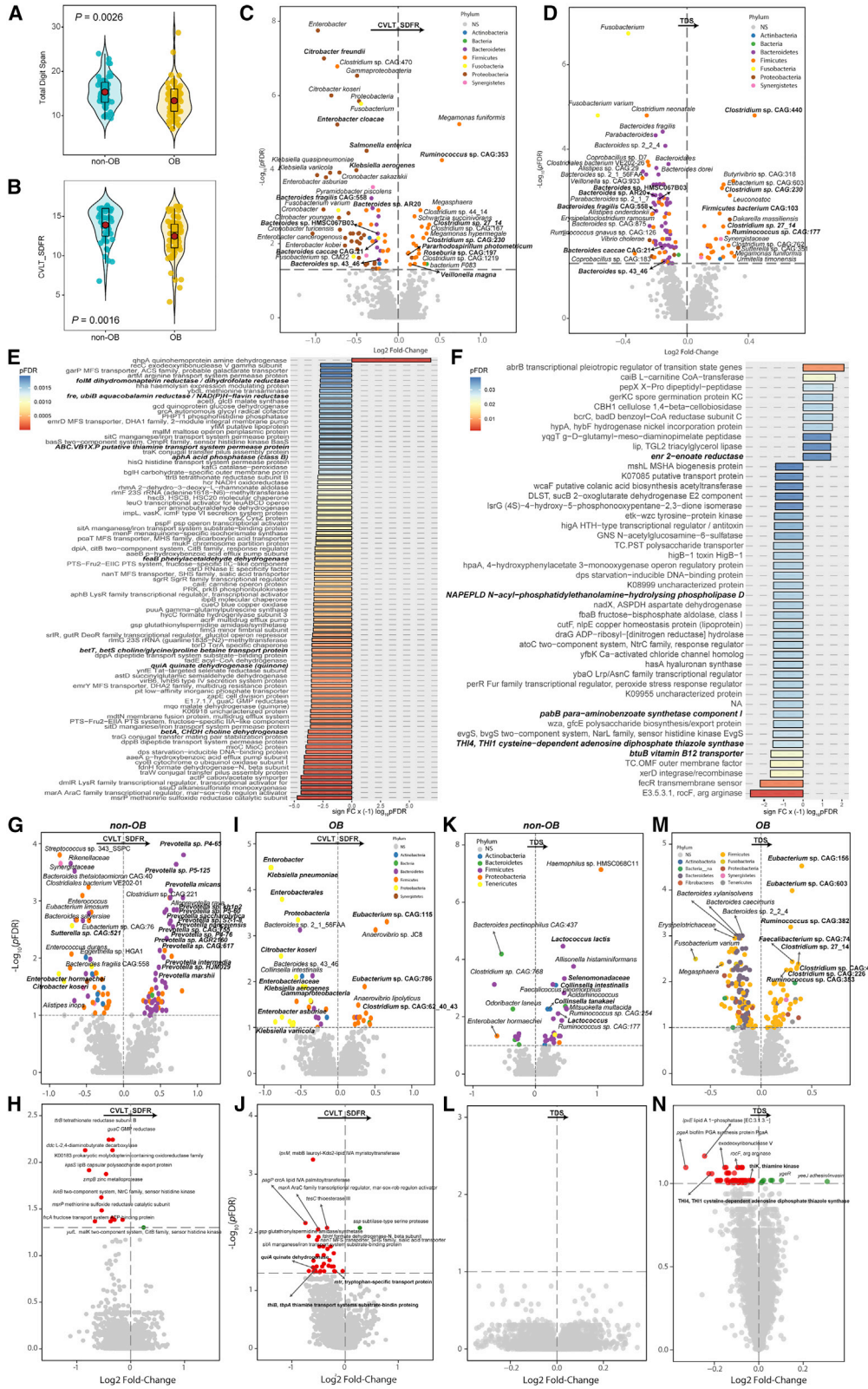

Figure 1. A Characteristic Microbiota Taxonomic and Functional Profile Is Associated with Memory Scores and Modulated by Obesity Status

(A and B) Boxplot for the total digit span (TDS) (A) and California Verbal Learning Short Delayed Free Recall (CVLT_SDFR) (B) in subjects with and without obesity. Differences between groups were analyzed by a Wilcoxon tests.

(legend continued on next page)

Immediate Recall (CVLT-IR), California Verbal Learning Test Short Delayed Free Recall (CVLT-SDFR), and Total Digit Span (TDS), respectively (Figures 1A, 1B, and S1A; Table 1).

A characteristic microbiome ecosystem was associated with cognitive scores using DESeq2 (Love et al., 2014) after adjusting for age, sex, BMI, years of education, and depression scores assessed using the Patient Health Questionnaire (PHQ)-9 (Figures 1C–1F, S1B, and S1C; Tables S1A–S1F). To take into account the compositional structure of the microbiome data and rule out possible spurious associations, we further analyzed the data using a compositional univariate approach (Table S2) with the ALDEx2 R package (Fernandes et al., 2014), as well as a multivariate machine learning feature selection strategy on the centered log-ratio transformed data (Table S3). Common species were positively associated with learning and verbal memory (CVLT-SDFR [Figure 1C; Tables S1A, S2A, and S3A]; CVLT-IR [Figure S1B; Table S1B]) and working memory (TDS [Figure 1D; Tables S1C, S2B, and S3B]), such as *Clostridium* sp. 27_14 or *Clostridium* sp. CAG:230, all of them belonging to Firmicutes phylum. On the contrary, negative associations between the gut microbiota and memory scores were identified within the phylum Bacteroides (*Bacteroides fragilis* CAG:558, *Bacteroides* sp. 43_46, *Bacteroides caccae* CAG:21, *Bacteroides* sp. HMSC067B03, and *Bacteroides* sp. AR20) and phylum Proteobacteria (*Citrobacter freundii*, *Enterobacter cloacae*, *Salmonella enterica*, and *Klebsiella aerogenes*).

Of note, while some species were positively and specifically associated with verbal learning, such as *Ruminococcus* sp. CAG:353, *Roseburia* sp. CAG:197, *Pararhodospirillum photometricum*, and *Veillonella magna* (Figures 1C and S1B; Tables S1A, S1B, S2A, and S3A), others were positively linked to working memory but not with learning or verbal memory (*Clostridium* sp. CAG:440, *Ruminococcus* sp. CAG:177, and *Firmicutes bacterium* CAG:103) (Figure 1D; Tables S1C, S2B, and S3B), suggesting divergent memory domains. Remarkably, several of the identified bacterial species were also longitudinally associated with the several memory domains measured one year later (Figure S2). The characteristics of these subjects are shown in Table 2.

Not only did the microbiota composition associate with memory, but also the metagenome functions were linked to this cognitive trait (Figures 1E, 1F, and S1C; Tables S1D–S1F, S2G, S2H, S3G, and S3H). Bacterial functions related to vitamin B metabolism, such as riboflavin (*ribBA*, *aphA*, *fre*, and *ubiB*), vitamin B6 (*pdxA*), folic acid (*pabB*, *queE*, *pabC*, *folM*, and *folX*), and vitamin B12 (*btuB*), were negatively associated with all memory domains (highlighted in black in Figures 1E, 1F, and S1 and in Tables S1D–S1F). Of note, all these vitamins are essential for one-carbon metabolism. There is convincing data for the association between B vitamins and cognition (Mendonça et al., 2017; Obeid et al., 2007; Smith et al., 2010). In particular, it is well known that thiamine and folate impact memory (Matté et al., 2009; Witt and Goldman-Rakic, 1983). Bacterial functions involved in thiamine (vitamin B1) metabolism (*thiB*, *thiK*, and *ABC.VB1X.P*) were also associated with low memory scores. We hypothesized that these functions would result in preferential uptake or catabolism of thiamine by intestinal bacteria, resulting in decreased thiamine uptake by the host. Concordantly, significantly low plasma thiamine levels were found in subjects with lower memory scores (34.5 [27.2–45.3] versus 44.3 [32.3–64.6] ng/mL, p = 0.016). Other relevant metagenomic functions associated with several memory domains included those related to the AAA metabolism, one-carbon metabolism, and endocannabinoid signaling (highlighted in Figures 1E, 1F, and S1C and in Tables S1D–S1F) and are further discussed below.

When we evaluated the associations separately in subjects who were obese and non-obese (Figures 1G–1N and S1D–S1G; Tables S1G–S1R, S2C–S2F, and S3C–S3F), we found that several *Prevotella* sp. were positively associated with verbal memory among non-obese subjects (Figures 1G and S1D; Tables S1G, S1O, S2C, and S3C) while *Eubacterium* and *Clostridium* sp. showed similar associations within subjects with obesity (Figure 1I and S1E; Tables S1I, S1Q and S2D). Bacteria belonging to Proteobacteria phylum were similarly and negatively associated in subjects without and with obesity, but preferentially in the latter (Figures 1G and 1I; Tables S1I and S3D). Regarding working memory, we observed positive associations of *Selenomonadaceae*, *Lactococcus* sp., and *Colinsella* sp. in non-obese subjects (Figure 1K; Tables S1K, S2E, and S3E) and *Eubacterium sp.*, *Ruminococcus sp.*, *Clostridium sp.*, *and Faecalibacterium sp. CAG:74* in subjects with obesity (Figure 1M; Tables S1M, S2F, and S3F). The associations of bacterial functions related to thiamine were more marked among subjects with obesity (Figures 1J and 1N; Tables S1J, S1N, S2J, S2L, S3J, and S3L) who have been described to be particularly susceptible to thiamine deficits (Maguire et al., 2018).

In summary, several species of the phylum Firmicutes (belonging to *Clostridium, Ruminococcus*, and *Eubacterium* genera, and Selenomonadaceae family) were positively associated with memory scores. Species from the phyla Bacteroidetes and Proteobacteria mainly presented negative associations with memory scores.

To our knowledge, there are no previous descriptions of gut microbiota linked to the different memory domains in humans. Current results are in line with those identifying a higher prevalence of Bacteroidetes in patients with mild cognitive impairment (Saji et al., 2019). Species of the Enterobacteriaceae family such

(C and D) Volcano plots of differential bacterial abundance associated with the CVLT_SDFR (C) and the TDS (D), as calculated by DESeq2 from shotgun metagenomic sequencing in the IRONMET cohort, adjusting for age, BMI, sex, education years, and Patient Health Questionnaire (PHQ)-9 scores. Fold change (FC) associated with a unit change in the corresponding test and Benjamini-Hochberg-adjusted p values (*p*FDR) are plotted for each taxon. Significantly different taxa are colored according to phylum.

(E and F) Manhattan-like plot of significantly expressed KEGG bacterial genes associated with the CVLT_SDFR (*p*FDR < 0.002) (E) and TDS (*p*FDR < 0.04) (F), identified from DESeq2 analysis adjusted for age, BMI, sex, educations years, and PHQ-9. The $-\log_{10}$(*p*FDR) values are multiplied by the FC sign to take into account the direction of the association. Bars are colored according to the *p*FDR. Those functions related to B vitamin metabolism, one-carbon metabolism, phenylalanine, tryptophan, and endocannabinoid metabolism are highlighted in black.

(G–N) Taxonomic and functional associations for the CVLT_SDFR and TDS tests in subjects with and without obesity. The complete list of significantly associated species and metagenomic functions can be found in Table S1.

Table 2. Clinical and Neuropsychological Data of the Human Follow-up Cohort

Total Population (Female n = 47, 68.1%)	Baseline (n = 69)	Follow-up (n = 69)	*p*
Age (years)	51.9 [44.3-59]	53 [45.4-60.2]	$5.2x10^{-13}$
BMI (kg/m^2)	28.2 [24.7-40.0]	28 [24.9-36.4]	0.192
Waist (cm)	103 [86.3-121.3]	97 [87-119]	0.044
Fat mass (%)	40.2 [32.7-49.7]	36.9 [31.8-46.9]	0.158
SBP (mmHg)	128 [118-141.8]	128 [118-138.3]	0.278
DBL (mmHg)	72.5 [67-82]	74 [67.8-80]	0.817
HDL-C (mg/dL)	58 [47-70.5]	57 [49-68.5]	0.601
Triglycerides (mg/dL)	86 [59-122]	88 [64-122]	0.796
FPG (mg/dL)	96 [89-102]	95 [90-102]	0.820
HbA1c (%)	5.5 [5.3-5.6]	5.5 [5.3-5.7]	0.317
hsCRP (mg/dL)	1.5 [0.6-5.1]	1.9 [0.7-3.3]	0.335
CVLT IR (score)	63 [56-70]	65 [60.5-72]	$1.8x10^{-4}$
CVLT SDFR (score)	14 [12-16]	15 [13.5-16]	0.005
Total Digit Span (score)	15 [12-17]	15 [12.5-17]	0.169
PHQ-9 (score)	5 [3-9]	4 [2-8]	0.209

Results are expressed as median and interquartile range [IQ]. To determine differences between study groups, we used paired Mann-Whitney U test. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL-C, high density lipoprotein cholesterol; FPG, fasting plasma glucose; HbA1c, glycated hemoglobin; hsCRP, high-sensitive C-reactive protein; CVLT, California Verbal Learning Test; IR, Immediate Recall; SDFR, Short Delayed Free Recall; PHQ-9, Patient Health Questionnaire.

as *Citrobacter rodentium* (phylum Proteobacteria) were associated with impaired memory in acute stress (Gareau et al., 2011). *Ruminococcus gnavus* and different Bacteroidetes and Enterobacter species were increased in subjects with insulin resistance and obesity (Ley et al., 2006; Org et al., 2015) and associated with a worse cognitive profile (Tables S1A–S1C). Conversely, taxa of the phylum Firmicutes such as Clostridiales and *Roseburia* linked to higher memory score had a decreased relative abundance in subjects with type 2 diabetes (Tilg et al., 2020). In mice, the combined administration of *Lactobacillus rhamnosus* and *helveticus* led to increased non-spatial memory, improving c-Fos expression in the hippocampus (Gareau et al., 2011; Smith et al., 2014).

Brain Structure Differentially Associates with the Gut Microbiome and Bacterial Functions in Subjects Who Are Obese versus Non-obese

We evaluated the volume of different brain areas involved in verbal and working memory in 143 subjects using MRI (Table S4). Verbal and learning memory were associated with the volumes of the right and left hippocampus, and working memory with the right frontal inferior orbital (FIO) volume in all subjects after adjustment for age, BMI, sex, total intracranial volume (TIV), and PHQ-9 (from now on the term ''adjusted'' will refer to these adjustments) (Figure 2A). The hippocampal associations were also significant and positive within non-obese subjects, although no significant associations were found with the frontal areas (Figures 2B–2D). Conversely, working memory (TDS) was positively associated with the left FIO volume in all subjects (Figure 2A) and with other frontal areas within non-obese subjects (Figures 2B, 2E, and 2F). Notably, no significant associations among these memory domains and brain volumes were observed in individuals with obesity. The adjusted relationships between the baseline verbal and learning memory (free retrieval of words in CVLT tests) and the volumes of the right and left hippocampus as assessed one year later in 69 of the participants were also significant. These findings highlight different brain structures involved in verbal and working memory and are in line with previous reports linking verbal memory performance with prefrontal and temporal brain features, such as the hippocampus (Aslaksen et al., 2018; Colom et al., 2007; Gross et al., 2018; Yu et al., 2018). Interestingly, we found several *Roseburia sp*. positively associated with verbal memory that were directly associated with the adjusted volume of the left hippocampus, and also concordant negative associations among *Bacteroides sp*., verbal memory scores, and the adjusted volume of left hippocampus (Figure 2G; Table S5A). Other concordant associations are shown in bold in Figure 2G.

On the other hand, *Acetitomaculum ruminis* was concomitantly associated with working memory and the adjusted volume of the right FIO area (Figure 2H; Table S5D) while several *Bacteroides sp*. appeared negatively and concordantly associated with both verbal and working memory and the adjusted volume of both the left hippocampus and right FIO area (Figure 2H; Tables S5A and S5D). We also found several bacterial functions concordantly associated with memory scores and adjusted volumes (both positively and negatively), shown in bold in Figure 2I and Tables S5B and S5E. Of note, a function related to thiamine metabolism was associated with the adjusted right FIO volume.

Notably, the metagenomic functions found to be associated with the volume of the hippocampus were also associated with verbal memory, while those associated with the FIO volume were also concordantly linked to working memory. In addition, the bacterial taxonomy and metagenomic functions were associated with the volume of brain areas and memory domains not only at baseline but also at follow up (Figures S3A–S3D; Tables S5G–S5J).

When subjects with and without obesity were evaluated separately, several bacterial functions that were found to be significantly linked with verbal and working memory were also associated with the adjusted left hippocampus (Figure 2K) and right FIO volumes (Figure 2L), respectively, in subjects without obesity (shown in bold). Remarkably, no associations were found between metagenomic functions and these brain volumes in subjects with obesity, which is in line with the lack of significant associations between memory tests and selected brain volumes in subjects with obesity.

There is preliminary evidence that commensal bacteria are associated with morphological brain features in animal models (Lu et al., 2018; Luczynski et al., 2016). In addition, the gut microbiota composition at a single timepoint was associated with several brain features in humans (Labus et al., 2017; Tillisch

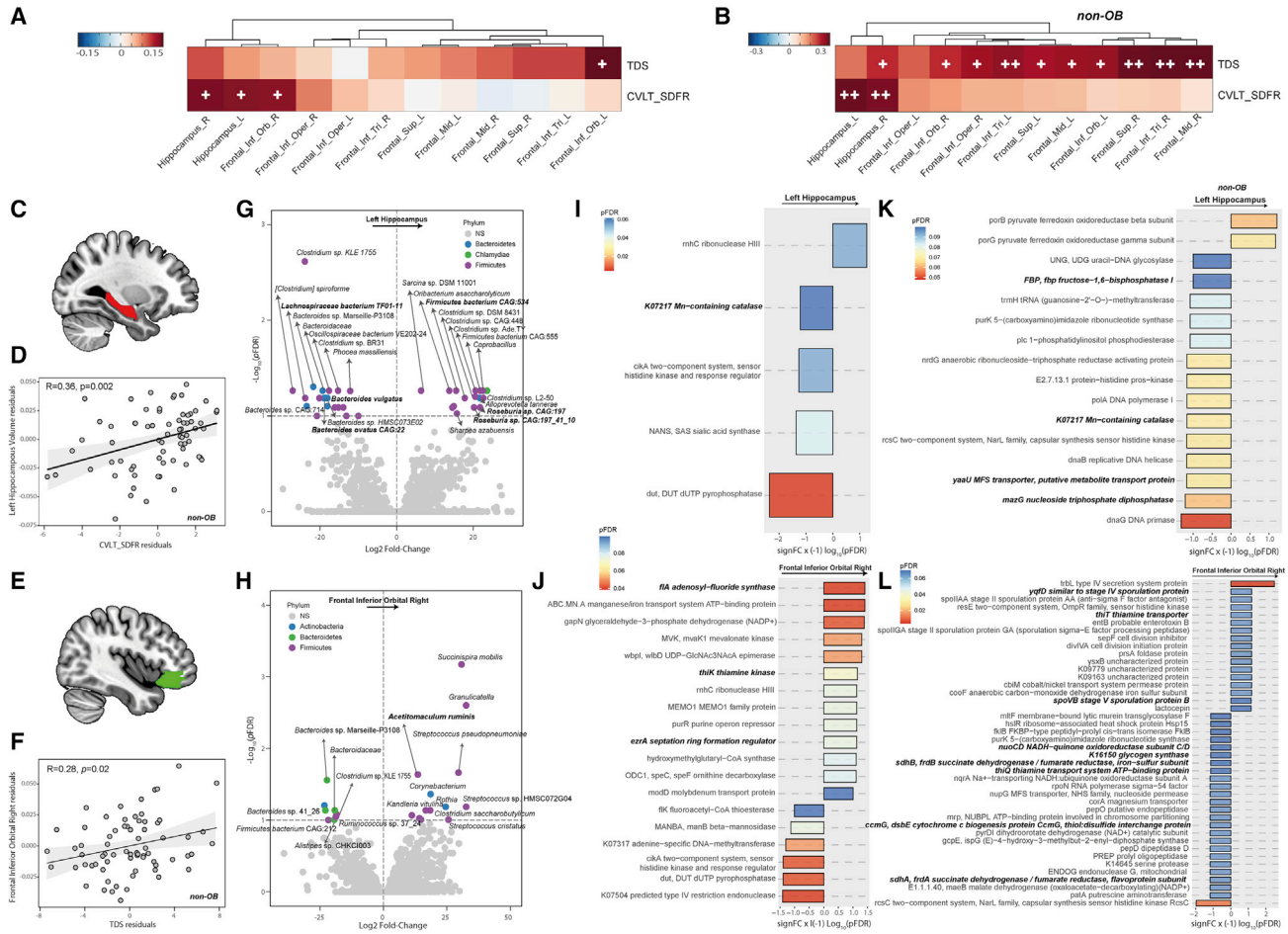

Figure 2. The Gut Microbiota Is Associated with Brain Structure

(A and B) Heatmap showing the partial correlations (adjusted by age, sex, BMI, education years, PHQ-9, and total intracranial volume [TIV]) between the TDS and CVLT_SDFR tests and selected brain volumes in all subjects with and without obesity (A) and subjects without obesity (B). Significant associations are shown with a cross: +, $p < 0.05$; ++ $p < 0.01$. No statistically significant associations were found in individuals with obesity and are not shown.

(C, D, E, and F) After controlling for the above covariates, the left hippocampus volume had a positive association with the CVLT_SDFR (C and D), whereas the right frontal inferior orbital volume was positively associated with the TDS (E and F). Both associations were more marked when only individuals without obesity were considered.

(G and H) Volcano plots of differential bacterial abundance associated with the left hippocampus volume (G) and right frontal inferior orbital volume (H), as calculated by DESeq2, controlling for covariates. Fold change (FC) associated with a unit change in the corresponding volumes and Benjamini-Hochberg-adjusted p values (*p*FDR) are plotted for each taxon. Significantly different taxa are colored according to phylum. Taxa that were also associated with the memory domains are highlighted in bold.

(I and J) Manhattan-like plot of significantly expressed KEGG bacterial genes associated with the left hippocampus volume (I) and right frontal inferior orbital volume (J), identified from covariate-adjusted DESeq2 analysis. The -$\log_{10}$(*p*FDR) values are multiplied by the FC sign to take into account the direction of the association. Bars are colored according to the *p*FDR. Metagenomic functions that were also associated with the several cognitive domains are highlighted in bold.

(K and L) The results of the same functional analysis for the left hippocampus volume (K) and right frontal inferior orbital volume (L) in individuals without obesity. The complete list of associated functions can be found in Table S5. No significant functional associations were found in individuals with obesity for these brain volumes.

et al., 2017). For instance, in agreement with our findings, Tillisch and colleagues (2017) found greater Bacteroides abundance to be associated to larger gray matter volume in the hippocampus of healthy women. In patients with irritable bowel syndrome, the relative abundance of Firmicutes and Bacteroidetes showed a relationship with the gray matter volume of the opercula (orbital and triangularis sections) as well as with the temporal cortex (Labus et al., 2017).

Memory Scores Differentially Associate with Plasma/Fecal Metabolomics and Bacterial Functions in Subjects Who Are Obese versus Non-obese

We then performed metabolome-wide association studies (MWASs) using random forest-based machine learning variable selection techniques to identify plasma (Figures 3A–3H, S4A–S4H, and S5A–S5H) and fecal (Figures 3I–3P, S4I–S4P, and S5I–S4P) metabolites associated with the memory tests. Remarkably,

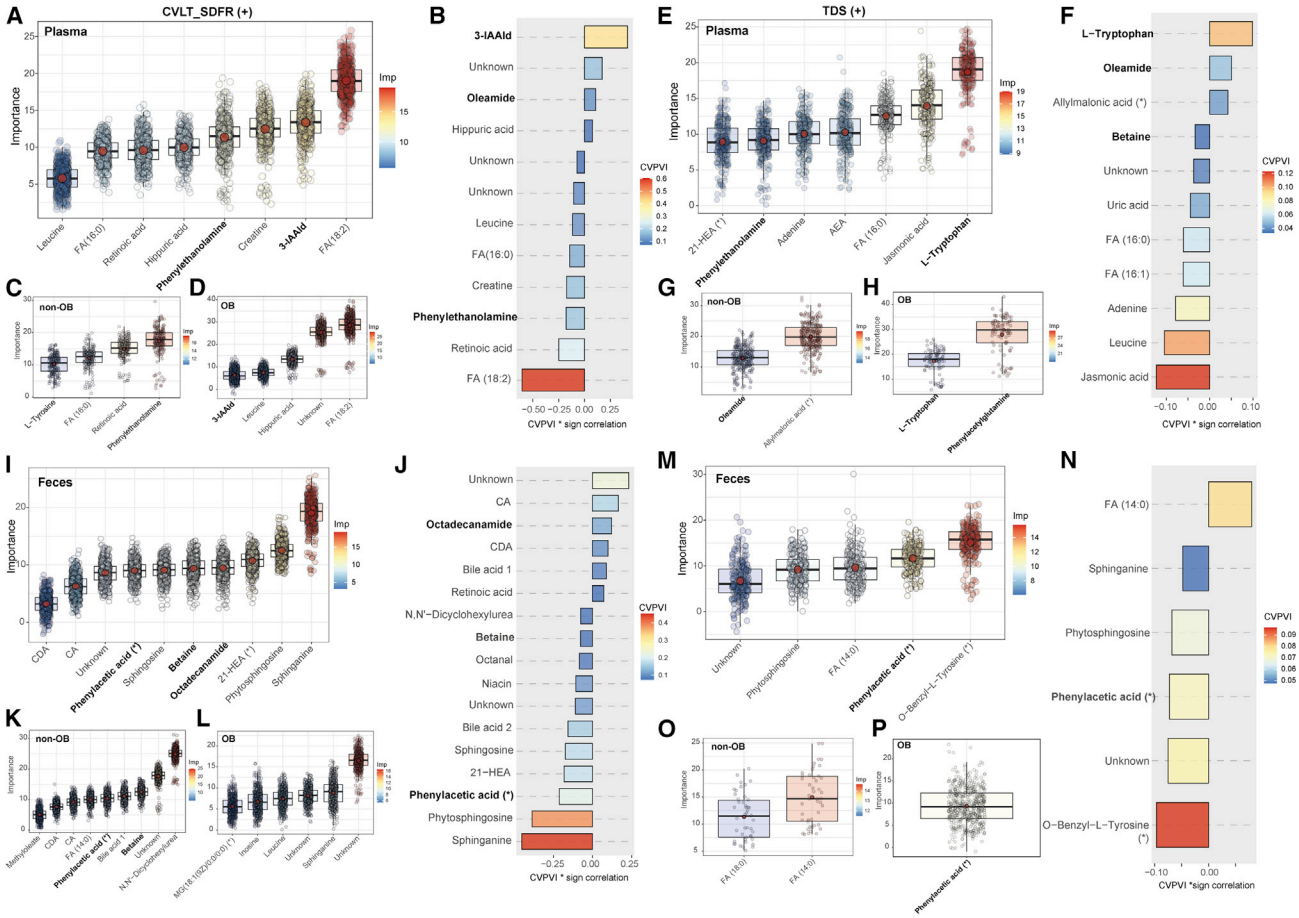

Figure 3. Plasma and Fecal Metabolomics in Electrospray Ionization (ESI) Positive Mode Linked to Memory Domains

(A, E, I, and M) Boxplots of the normalized permutation importance measure for the metabolites associated to the to the CVLT_SDFR in plasma (A), the TDS in plasma (E), the CVLT_SDFR in feces (I), and the TDS in feces (M), identified by machine learning thorough the random forest-based Boruta feature selection algorithm at each of the 500 iterations.

(B, F, J, and N) Cross-validated permutation variable importance (CVPVI) measure × sign of the correlation between each metabolite associated to the CVLT_SDFR test in plasma (B), the TDS in plasma (F), the CVLT_SDFR in feces (J), and the TDS in feces (N), identified by machine learning using the random forest-based Vita method.

(C, D, G, H, K, L, O, and P) Normalized permutation importance measure for Boruta selected metabolites associated to the CVLT_SDFR in plasma (C and D), the TDS in plasma (G and H), the CVLT_SDFR in plasma (K and L), and the TDS in feces (O and P), in individuals with and without obesity, respectively. All metabolites were identified based on exact mass, retention time and MS/MS spectrum, except those with (*) that were only identified based on exact mass and retention time.

3-IAAld, Indole-3-acetaldehyde; AEA, arachidonoylethanolamide; CA, cholic acid; CDA, chenodeoxycholic acid; FA, fatty acid.

the scores of all memory domains were associated with altered plasma levels of the AAAs tryptophan, tyrosine, and phenylalanine and their catabolites (Tryptophan catabolites: Indole-3-acetaldehyde [3-IAAld], Indole-3-propionic acid [3-IPA]; Tyrosine catabolites: 4-hydroxyphenyllactic acid [4-HPLA]; Phenylalanine catabolites: Phenylacetylglutamine and Phenylacetylglycine). These AAAs are the precursor amino acids of serotonin and dopamine, two neurotransmitters that play a key role in the central nervous system. Brain regions implicated in cognition, such as the hippocampus and the PFC, are vastly innervated by dopaminergic and serotonergic afferents, and alterations in both the serotonergic and dopaminergic neurotransmission are associated with impaired learning and memory (González-Burgos and Feria-Velasco, 2008; Švob Štrac et al., 2016). Both tryptophan and tyrosine positively associated with memory scores. This finding is in line with past work where the oral administration of tryptophan led to improved memory acquisition, consolidation, and storage in rodents (Haider et al., 2007; Noristani et al., 2012).

Previous studies have shown that alterations of the microbiota due to antibiotic treatment resulted in decreased AAA concentrations and serotonin and dopamine levels in the porcine hypothalamus (Gao et al., 2018). The gut microbiota has also shown to directly metabolize tryptophan into several indole derivatives, which are potent ligands of the aryl hydrocarbon receptor (AhR). Deletion of the AhR alters adult hippocampal neurogenesis and contextual fear memory (de la Parra et al., 2018; Latchney et al., 2013). Consistently, we found several indole derivatives positively associated with memory scores. In addition, we also identified several bacterial functions involved in tryptophan and phenylalanine metabolism that negatively associated with the

different memory domains (Figures S1H and S1I; Tables S1D–S1F). In particular, functions related to tryptophan transporters such as tryptophan-specific transporter (*mtr*) and low-affinity tryptophan permease (*tnaB*) had negative associations with the CVLT-SDFR. Quinate dehydrogenase (*quiA*), involved in tryptophan, tyrosine, and phenylalanine metabolism, had the strongest negative association with CVLT. Notably, fecal quinic acid had, by far, the strongest negative association with the TDS scores, followed by tryptophan (Figures S4I and S4J). The negative association between fecal tryptophan and memory scores might be related either to its transformation into tryptophan metabolites (see below) or to its increased systemic absorption.

Interestingly, the memory-related alterations in tryptophan metabolism were only observed in individuals with obesity, aligning with associations of tryptophan-related metagenomic functions and memory domains in subjects with obesity, but not in subjects without obesity. Chronic low-grade inflammation is a hallmark of obesity, and the association between obesity and cognitive decline has recently been shown to be mediated by inflammation (Bourassa and Sbarra, 2017; Yang et al., 2020). Consistently, we found a strong positive association between BMI and hs-CRP ($R = 0.71$, $p < 1 \times 10^{-16}$). Notably, more than 90% of tryptophan is metabolized through the kynurenine pathway, which is activated under inflammatory conditions (Wang et al., 2015). In line with this, plasma tryptophan levels had a negative correlation with the hs-CRP ($R = -0.34$, $p < 3.3 \times 10^{-4}$). Importantly, microbial-derived products, including indoles, play a key role in the activation of indole-amine 2,3-dioxygenase (IDO), the rate-limiting enzyme in the kynurenine pathway (Gao et al., 2020). There is previous evidence that these metabolites have an effect on astrocytes to limit inflammation of the central nervous system in experimental models (Rothhammer et al., 2016). The current observations are the first in humans, to our knowledge, linking tryptophan and its metabolites to cognition.

Cholinergic systems have also been linked to cognitive processes such as attention and memory (Jeltsch-David et al., 2008). Hence, choline is the precursor of the neurotransmitter acetylcholine, but it can also be metabolized to betaine, a key methyl donor in the one-carbon metabolism and modulator of homocysteine status, whose elevated plasma levels have been implicated in learning and memory deficits (Mendonça et al., 2017). Thus, betaine supplementation has shown to prevent homocysteine-induced memory impairment via changes in the activity of MMP-9 in the frontal cortex (Kunisawa et al., 2015). In agreement, we found circulating betaine levels associated with memory scores. The changes in betaine levels are in line with the associations between cognitive domains and several metagenomic functions involved in choline and betaine transporters, such as choline/betaine transport protein (betT and betS), betaine/proline transport systems ATP-binding protein (proV), and betaine/proline transport systems substrate-binding protein (proX) (Tables S1D–S1F). Additionally, one of the functions most associated with short and immediate memory implicated the choline dehydrogenase (betA) gene (Tables S1D and S1E), responsible for the conversion of choline to betaine. Interestingly, we also found several alterations in metagenomic functions related to the metabolism of B vitamins involved in one-carbon metabolism, homocysteine levels, and cognition (Mendonça et al., 2017; Obeid et al., 2007; Smith et al., 2010), mainly B2, B6, B9, and B12.

Other metabolites that had positive associations with the different memory domains were the endocannabinoids oleamide and arachidonoylethanolamide (AEA, anandamide). The endocannabinoids are lipid-derived mediators that play a key role in neurotransmission. Consequently, extensive evidence indicates a role of the endocannabinoid system in the modulation of cognition, particularly in learning and memory functioning (Maroso et al., 2016; Morena and Campolongo, 2014). Anandamide has been reported to reverse hippocampal damage and memory impairment in rodents and protect neurons from amyloid-β cytotoxic effects (van der Stelt et al., 2006). Similarly, oleamide administration significantly reversed memory and cognitive impairment in mice (Heo et al., 2003). Interestingly, we found that microbial N-acetyl Phosphatidylethanolamine Phospholipase D (NAPEPLD) (Figures 1F and 4H; Tables S1D and S1F), which is necessary for the biosynthesis of fatty acid ethanolamides, including the endocannabinoids (Basavarajappa, 2007), had one of the strongest associations with the cognitive domains of both humans and mice.

Effects of Microbiota Transplantation from Humans to Mice

We then tested the possible effects of the microbiota on memory scores in mice. The mouse behavioral models used in this study evaluated two different memory tasks. The cue-induced fear conditioning is a well-recognized model of emotional memories (Sun et al., 2020), whereas the novel object recognition paradigm is a widely used model of memories with a different neurobiological substrate (Puighermanal et al., 2009). Specifically, cue-induced fear conditioning evaluates emotional memory by assessing mice ability to associate neutral cues with an aversive experience, in which behavioral responses are mainly mediated by the amygdala (Barsy et al., 2020). The subsequent presentation of the cue retrieves the memory trace and initiates a conditioned response; freezing, driven by the central amygdala (Sun et al., 2020). In contrast, the hippocampus plays a crucial role in the memory responses evaluated in the novel object recognition paradigm. The long-term memory traces evaluated in this paradigm are related to spatial memories not related to emotional aspects (Puighermanal et al., 2009).

The novel object recognition cognitive task was performed using a V maze, since the accuracy and reliability of the behavioral response is improved when compared to the use of an open field for this task. In this task, the exploration of the mouse is directed to the two different objects located in the extremes of the V maze (Puighermanal et al., 2009; Busquets-Garcia et al., 2013).

Microbiota from 22 human subjects (11 with low and 11 with high memory scores matched for age, sex, BMI, and PHQ-9 scores) (Table S6) was orally delivered to individual mice in a blinded fashion (the investigator who performed the experiment was blinded regarding the origin of feces). The effects on memory were compared with those of saline in 11 control mice. All mice were pretreated with antibiotics for 14 days (Figure 4A). Mice receiving FMT had higher scores in the Novel Object Recognition test at 24 h (NOR24 h) and lower Freezing Total scores than control mice (Figures 4B and S6A). Interestingly, microbiota from non-obese donors led to significantly increased NORII24 h scores compared with both obese donors ($p = 0.026$)

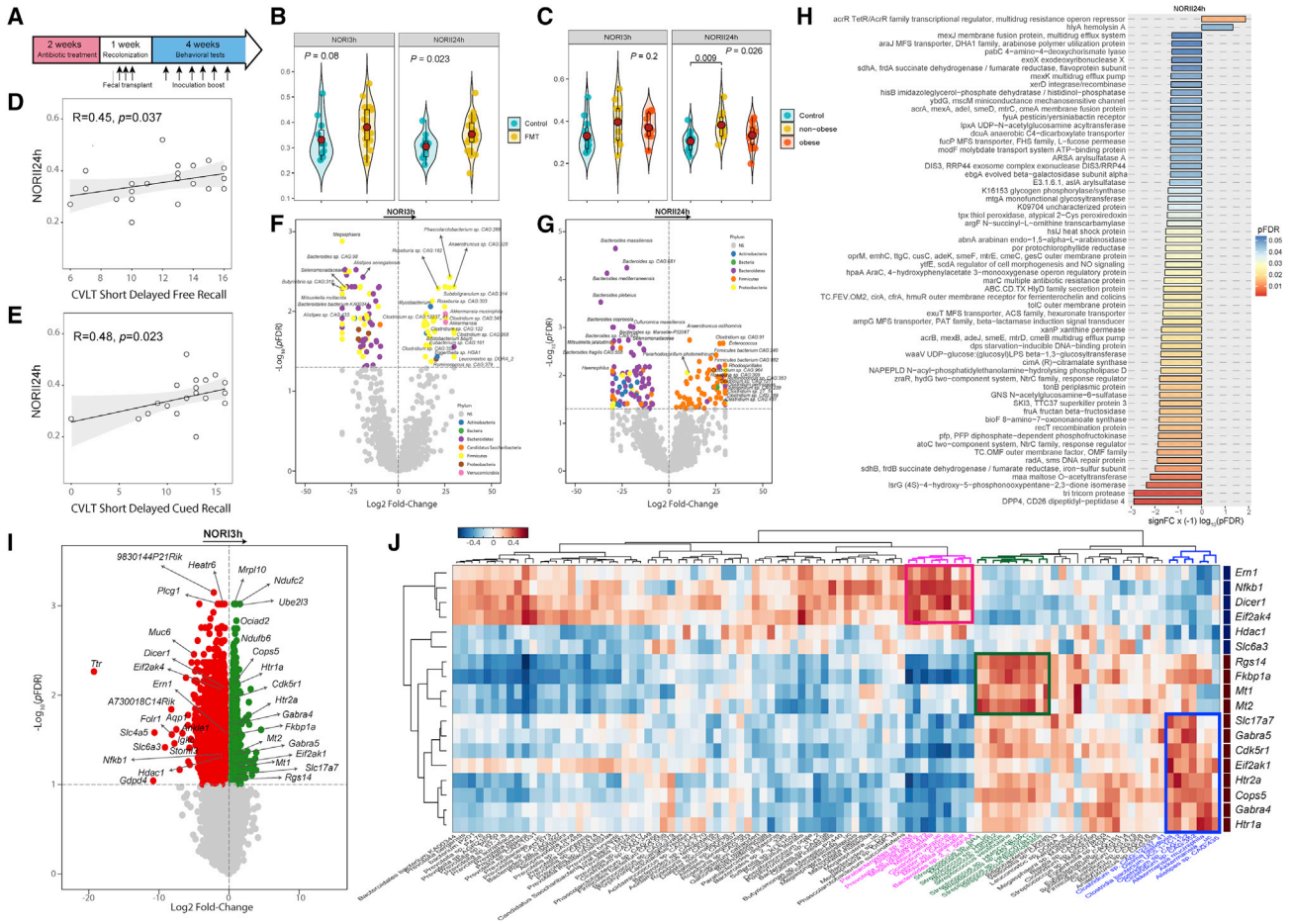

Figure 4. Human Donor's and Recipient's Mice Memory Became Aligned through the Microbiota

(A) Experimental design for the fecal microbiota transplantation (FMT) study. The microbiota from low-memory (n = 11) and high-memory (n = 11) human donors were delivered to recipient mice pre-treated with antibiotics for 14 days. n = 11 control mice were treated with saline. Cognitive tests were performed after 4 weeks.

(B and C) Violin plots for the Novel Object Recognition tests comparing the control group and the FMT group (t test) (B), and comparing the control group to the groups receiving microbiota from human donors with and without obesity (one-way ANOVA) (C).

(D and E) Spearman correlation between the California Verbal Learning tests (CVLTs) in humans and the NOR24 h in mice.

(F and G) Volcano plots of differential human donor bacterial abundance associated with the recipient's mice NOR3 h (F) and the NOR24 h (G), from DESeq2 analysis. Fold change (FC) associated with a unit change in the corresponding memory test and Benjamini-Hochberg-adjusted p values (*p*FDR) are plotted for each taxon. Significantly different taxa are colored according to phylum.

(H) Manhattan-like plot showing only the significantly expressed KEGG bacterial genes associated with the mice NOR 24 h test (*p*FDR < 0.05) that were also associated to the total digit span score in humans. The $-\log_{10}$(*p*FDR) values are multiplied by the FC sign to take into account the direction of the association. Bars are colored according to the *p*FDR. A complete list of significantly associated bacterial genes can be found in Table S5C.

(I) Volcano plot of differential prefrontal cortex (PFC) genes associated with the NOR3 h. FC associated with a unit change in the NOR3 h test and Benjamini-Hochberg-adjusted p values (*p*FDR) are plotted for each gene. Those genes with the highest FC and the lowest *p*FDR values are highlighted. Genes with a possible role in memory based on the literature are also highlighted.

(J) Correlation heatmap among mice bacterial species and selected PFC genes associated with NOR3 h. Clustering was performed using Euclidean distances and Ward linkage. Three bacterial clusters with strong correlations were identified and highlighted. These involve bacterial species positively linked to both the NOR3 h and PFC genes positively associated with the NOR3 h, and bacterial species negatively associated to the NOR3 h and at the same negatively associated to PFC genes positively associated with the NOR3 h and positively associated to genes negatively associated with the NOR3 h.

and control mice (p = 0.009) (Figure 4C). Of note, both donor's CVLT-SDFR and CVLT-Short Delayed Cued Recall scores were significantly correlated with NOR24 h scores in recipient mice (Figures 4D and 4E). Bacterial species from the donor's microbiota, including *Akkermansia sp*. and *Subdoligranulum sp*. (NOR3 h) (Figure 4F; Table S7A), and *Clostridium*, *Ruminococcus*, and *Roseburia sp*. (NOR24 h) (Figure 4G; Table S7B), were associated with increased memory scores of recipient mice, while several *Bacteroides sp*. were negatively associated with this score. Accordingly, the same *Bacteroides* sp. were positively associated with the Freezing Total scores (Figure S6C). Notably, several donors' metagenomic functions, including the *NAPEPLD,* associated with the TDS memory domains of the donor and with the NOR24 h scores of recipient mice (Figure 4H).

Further, in line with the human results, other associated functions included those related to vitamin B6 (*pdxJ* and *pdxB*), B12 (*btuB*), and tryptophan metabolism (*trpA* and *trpB*) (Table S7C).

Finally, an RNA sequencing of the PFC of the mice highlighted several significant genes associated with the NOR3 h score (Figure 4I; Table S7D). In the test phase, different memory scores were recorded: mice were studied 3 h later for short-term memory (NOR3 h) and 24 h later for long-term memory (NOR24 h). Notably, the gene with the highest negative fold change was transthyretin (*ttr*), which has been shown to have altered hippocampal expression associated with memory deficits in aged animals (Brouillette and Quirion, 2008). The gene with the second strongest fold change was *slc6a3,* which encodes a dopamine transporter. In addition, there was a direct association between NORI3 h and the 5HT receptor genes *htr1a* and *htr2a*, as well as the folate receptor gene *folr1*, further emphasizing the connection between AAAs, folate metabolism, and memory. The nuclear factor gene *nfkb1*, known to be crucial in the inflammatory cascade and in memory consolidation (Snow et al., 2014), was also directly associated to short-term memory; whereas *dicer1* was negatively associated with this memory trait. Relatedly, the knockout of *dicer1* has been previously reported to enhance memory (Konopka et al., 2010). Finally, *acss2* and *hdac1* were directly associated to short-term memory, confirming recent observations of brain histone acetylation relationships with associative learning (Mews et al., 2017). Interestingly, the expression of the memory genes associated to the NORI3 h was simultaneously associated with different bacterial clusters and in the same direction (Figure 4J).

Altogether, the current findings point to the existence of an ecosystem of bacteria that are simultaneously linked to verbal and working memory, the volume of brain areas involved in these traits, plasma/fecal tryptophan, microbiota-driven tryptophan metabolites, and 5HT receptor expression in the PFC. Several of the species identified here have been previously linked with positive (Roseburia, Subdoligranulum, and Faecalibacterium) and negative (Fusobacterium and Bacteroides) healthy eating scores (Liu et al., 2019) in the same direction as the increased and decreased memory scores described here. These findings suggest a bidirectional host/microbe ecosystem that impacts brain physiology. In this sense, the gut microbiota phenocopied memory traits from humans to mice.

Limitations of Study

The current study presents some limitations. Although the sample size of the different cohorts seems appropriate, population-based studies including subjects with different classes of obesity and ethnic groups would be more representative of this condition. In addition, although our conclusions are based on the findings of cross-sectional and one-year longitudinal studies, longer term follow-up would be necessary to better understand the strength of our conclusions. Finally, regarding the mouse models, despite being widely used and validated to infer cognitive function in real settings, they cannot be exactly comparable with cognitive evaluation and brain morphology in humans.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cmet.2020.09.002.

ACKNOWLEDGMENTS

We are indebted to the subjects involved in this project. We also thank Emili LosHuertos and Oscar Rovira for their help in the recruitment of the subjects. This work was partially supported by FIS research grants (PI15/01934 and PI18/01022) from the Instituto de Salud Carlos III, Spain; SAF2015-65878-R and #AEI-SAF2017-84060-R FEDER from Ministry of Economy and Competitiveness; Prometeo/2018/A/133 from Generalitat Valenciana, Spain; and also by Fondo Europeo de Desarrollo Regional (FEDER) funds; European Commission (FP7, NeuroPain #2013-602891); the Catalan Government (AGAUR, #SGR2017-669, ICREA Academia Award 2015); the Spanish Instituto de Salud Carlos III (RTA, #RD16/0017/0020); the Spanish Ministry of Science, Innovation and Universities (RTI2018-099200-B-I00); the Catalan Government (Agency for Management of University and Research Grants [2017SGR696] and Department of Health [SLT002/16/00250]); the European Regional Development Fund (project no. 1.2.2-LMT-K-718-02-0014) under grant agreement with the Research Council of Lithuania (LMTLT); and *Fondo Europeo de Desarrollo Regional* (FEDER) through the Programa Interreg V-A España-Francia-Andorra (POCTEFA 2014-2020). M.A.-R. is funded by Instituto de Salud Carlos III, Río Hortega (CM19/00190, co-funded by European Social Fund "*Investing in your future*"). O.C.-R. is funded by a postdoctoral "PERIS" contract (SLT006/17/00236) from the Catalan Government, 2017, Spain. J.M.-P. is funded by the Miguel Servet Program from the Instituto de Salud Carlos III (ISCIII, CP18/00009, co-funded by the European Social Fund "*Investing in your future*"). J.S. is funded by a predoctoral PERIS contract (SLT002/16/00250) from the Catalan Government. M.J. is a professor under the Serra Hunter program (Generalitat de Catalunya).

AUTHOR CONTRIBUTIONS

M.A.-R. and J.M.-P. researched the data, performed part of the statistical analysis, and wrote the manuscript. X.F.-R. performed part of the statistical analysis. G.B., C.C., C.B., R.M.-O., M.S., M.E.P.-B., O.C.-R., and J.-M.M.-N. researched the data. C.C. performed the neuropsychological examination. J.L. determined the mice gene expression in hippocampus. A.C.-N. performed the prefrontal gene expression analysis. V.P.-B. and A.M. contributed with the determination and analysis of the microbiota. A.B., A.C.-N., and R.M. performed or analyzed the experiments in mice and contributed in writing the corresponding parts of the paper associated with the mice data. J.P., S.P., J.G., L.R.-T., and W.R. contributed to the discussion and

reviewed the manuscript. M.J., J.S., R.P., and M.P.-O. performed the metabolomics analyses and contributed in writing the corresponding parts of the paper associated with the metabolomics data. J.M.F.-R. carried out the conception and coordination of the study, performed the statistical analysis, and wrote the manuscript. All authors participated in final approval of the version to be published. J.M.F.-R. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: January 17, 2020
Revised: May 12, 2020
Accepted: August 31, 2020
Published: October 6, 2020

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological Samples		
Human body fluids (feces, plasma)	This paper	N/A
Mice feces	This paper	N/A
Mice prefrontal cortex	This paper	N/A
Chemicals, Peptides, and Recombinant Proteins		
Methanol LC-MS	Scharlau	Cat#ME03262500
Lysing Matrix E	MP biomedicals	Cat#SKU116914050-CF
Acetic acid LC-MS	Scharlau	Cat#AC03470050
Critical Commercial Assays		
QIAamp DNA mini stool kit	QIAGEN	Cat#51504
Nextera DNA Flex Library Preparation kit	Illumina	Cat#20018705
TrueSeq stranded mRNA library preparation kit	Illumina	Cat#20020594
Truseq RNA Single Indexes	Illumina	Cat#20020492
Truseq RNA Single Indexes	Illumina	Cat#20020493
RNA 6000 Nano chip	Agilent	Cat#5067-1511
DNA 1000 chip	Agilent	Cat#5067-1504
KAPA Library Quantification Kit	Roche	Cat#07960204001
Deposited Data		
Metagenome Sequencing Data of Fecal Samples from Human subjects and Mice	European Nucleotide Archive (ENA)	Project number: PRJEB39631 Human samples accession numbers: ERS4859818-ERS4859933
Experimental Models: Organisms/Strains		
Mouse C57BL/6J	Charles River	N/A
Software and Algorithms		
SPSS software (version 19)	IBM	https://www.ibm.com/analytics/spss-statistics-software
Rstudio (version 1.3.959)	Rstudio Team	https://rstudio.com/
R (version 3.6)	R	https://www.r-project.org/
MATLAB (version R20217a)	Mathworks	https://www.mathworks.com/products/matlab.html
Statistical Parametric Mapping software (SPM12)	UCL Queen Square Institute of Neurology	https://www.fil.ion.ucl.ac.uk/spm/software/
MassHunter Data Analysis software	Agilent Technologies	https://www.agilent.com/en/products/software-informatics/mass-spectrometry-software
Prinseq-lite-0.20.4	(Schmieder and Edwards, 2011)	http://prinseq.sourceforge.net/
FLASh 1.2.11	(Magoč and Salzberg, 2011)	https://ccb.jhu.edu/software/FLASH/
Bowtie2-2.3.4.3	(Langmead and Salzberg, 2012)	http://bowtie-bio.sourceforge.net/bowtie2/index.shtml
MEGAHIT v1.1.2	(Li et al., 2015)	https://github.com/voutcn/megahit
Prodigal v2.6.342	(Hyatt et al., 2010)	https://github.com/hyattpd/Prodigal
HMMER	(Durbin et al., 1998)	http://hmmer.org/
Kaiju v1.6.2	(Menzel et al., 2016)	https://github.com/bioinformatics-centre/kaiju
STAR software (version 2.5.3a)	(Dobin et al., 2013)	https://github.com/alexdobin/STAR
Subread version (1.5.1)	(Liao et al., 2014)	http://subread.sourceforge.net/
Limma (version 3.30.13)	(Smyth, 2005)	https://bioconductor.org/packages/release/bioc/html/limma.html

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
edgeR (version 3.26.8)	(Robinson et al., 2010)	https://bioconductor.org/packages/release/bioc/html/edgeR.html
DESeq2 (version 1.26.0)	(Love et al., 2014)	https://bioconductor.org/packages/release/bioc/html/DESeq2.html
ALDEx2 (version 1.18.0)	(Fernandes et al., 2014)	https://www.bioconductor.org/packages/release/bioc/html/ALDEx2.html
VITA (version 1.0.0)	(Janitza et al., 2018)	https://cran.r-project.org/web/packages/vita/index.html
Boruta (version 6.0.0)	(Kursa and Rudnicki, 2010)	https://cran.r-project.org/web/packages/Boruta/
Other		
1.5T Ingenia	Philips Healthcare	N/A
Dual energy X-ray absorptiometry	GE Healthcare	N/A
Cobas 8000 c702 analyzer	Roche Diagnostics	N/A
ADAM®A1c HA-8180V	ARKRAY, Inc	N/A
FastPrep-24™	MP biomedicals	N/A
Reversed-phase column (Zorbax SB-Aq 1.8 μm 2.1 × 50 mm)	Agilent Technologies	Cat#AG827700-914
Precolumn (Zorbax-SB-C8 Rapid Resolution Cartridge 2.1 × 30 mm 3.5 μm)	Agilent Technologies	Cat#AG873700-906
Shuttle chamber LE918	Panlab	N/A
Bioanalyzer 2100	Agilent	N/A
ABI 7900HT qPCR	Applied Biosystems	N/A
HiSeq 2500	Illumina	N/A
Qubit 3.0 fluorometer	Thermo Fisher Scientific	N/A
NextSeq 500	Illumina	N/A

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact José Manuel Fernández-Real (jmfreal@idibgi.org).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

The data that support the findings of this study are available from the lead contact (jmfreal@idibgi.org) upon reasonable request. The accession numbers for the raw metagenomic sequence data of the 116 humans subjects reported in this paper are [European Nucleotide Archie]: ERS4859818-ERS4859933.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Clinical Study

Recruitment of Study Subjects

From January 2016 to October 2017, a cross-sectional case-control study was undertaken in the Endocrinology Department of Josep Trueta University Hospital. We included consecutive subjects with obesity (body mass index, BMI^{3}30kg/m^2) and age- and sex-matched nonobese subjects (BMI 18.5-<30kg/m^2), with an age range of 27.2-66.6 years. The sex Distribution and age range is reported in Table 1. All analysis were adjusted by gender to remove the influence of gender on the results. Exclusion criteria were: type 2 diabetes mellitus, chronic inflammatory systemic diseases, acute or chronic infections in the previous month; use of antibiotic, antifungal, antiviral or treatment with proton-pump inhibitors; severe disorders of eating behavior or major psychiatric antecedents; neurological diseases, history of trauma or injured brain, language disorders; and excessive alcohol intake (^{3} 40 g OH/day

in women or 80 g OH/day in men). The Institutional review board - Ethics Committee and the Committee for Clinical Research (CEIC) of Dr. Josep Trueta University Hospital (Girona, Spain) approved the study protocol and informed written consent was obtained from all participants.

Longitudinal Cohort

Cognitive tests and MRI variables were collected again in 93 consecutive subjects after 1 year of follow up. The sex distribution and age range is reported in Table 2. All analyses were adjusted by gender to remove the influence of gender on the results.

Animal Study

Male C57BL/6J mice (Charles River, France), weighing 23–26 g at the beginning of the experiment were used in this study. Mice were housed individually in controlled laboratory conditions with the temperature maintained at 21 ± 1°C, humidity at 55 ± 10%, and 7 h30/19 h30 light/dark cyles. All animals were fed a standard chow diet RM1 (Irradiated Vacuum packed, Dietex International Ltd.). The health status of each mouse included in the experimental schedule was checked every day before the experimental sessions and recorded in the experimenter protocol notebook. Health status checks included body weight, physical aspect, behavior, and clinical signs. No abnormalities were recorded in the animals included in this study. Animal procedures were conducted in strict accordance with the guidelines of the European Communities Directive 86/609/EEC regulating animal research and were approved by the local ethical committee (CEEA-PRBB). All the experiments were performed under blinded conditions (the researcher who administered the microbiota was blinded in relation to the memory scores of the subjects who provided the feces). 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 (Kelly et al., 2016). Seventy-two h 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 fecal material from healthy volunteers' samples from humans with better cognitive scores (n = 11) and humans with decreased cognitive scores (n = 11)). No differences were found related to BMI, age, years, sex within these two groups. 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. Animals were exposed to a series of behavioral testing including novel object recognition (NOR) test and fear conditioning with nociception assessed by the hot plate test to ensure specificity.

At the end of the study the animals were consecutively sacrificed. The cecum was removed, weighted and stored, and the feces collected and stored at −80°C for further microbiota analysis.

METHOD DETAILS

Clinical and Laboratory Parameters

Body composition was assessed using a dual energy X-ray absorptiometry (DEXA, GE lunar, Madison, Wisconsin). Fasting plasma glucose (FPG), lipids profile and high-sensitivity C-reactive protein (hsCRP) levels were measured using an analyzer (Cobas® 8000 c702, Roche Diagnostics, Basel, Switzerland). Glycated hemoglobin (HbA1c) was determined by performance liquid chromatography (ADAM®A1c HA-8180V, ARKRAY, Inc., Kyoto, Japan). *Dietary pattern:* The dietary characteristics of the subjects were collected in a personal interview using a validated food-frequency questionnaire (Vioque et al., 2013).

Magnetic Resonance Imaging (MRI)

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 1x1x1mm^3 with or without gap. The total scan time was 189.6 s. The anatomical imaging data was processed and analyzed using MATLAB version R2017a (The MathWorks Inc, Natick, Mass) and Statistical Parametric Mapping 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) (Tzourio-Mazoyer et al., 2002) atlas was used to obtain the volumetric information of the right and left hippocampus, opercula (orbitalis, tringularis, opercularis), and middle and superior frontal gyri as informed by the involvement of these brain regions in verbal memory (Aslaksen et al., 2018; Colom et al., 2007; Gross et al., 2018; Yu et al., 2018) in 14394 participants. Volumetric differences for these targeted regions between participants with and without obesity were explored using independent sample t tests, and we used Pearson Partial correlations to explore for Each region was orthogonalized for sex, age and total gray matter volume in MATLAB version R2017a (The Math Works Inc, Natick, MA) and subsequently entered to SPSS to investigate associations between the gray matter volumes and the performance in the CVLT and the digit tasks controlling for age, sex, education, depressive symptoms, BMI and total intracranial volume in the whole sample, and within the obese and non-obese

groups. Finally, we investigated the associations and between the volume in the selected brain regions and the microbiota using Spearman correlation analyses corrected for multiple comparisons using q-values (Storey, 2002).

Neuropsychological Assessment in Humans

California Verbal Learning Test-II (CVLT)

CVLT is used to assess verbal learning and memory (Delis et al., 2000). It consists of 5 learning tests in which a list of words (list A) is presented and the subject is asked, immediately after each presentation, to recall as much words as possible. Then an interference list (list B) is presented, and the subject is asked to repeat the same task. CVLT Immediate Recall score is a result of the first five tests and provides information about the learning process. In the short delay test, the patient is asked to recall list A, free (CVLT Short Delayed Free Recall) or with semantic facilitation (CVLT Short Delayed Cued Recall). A higher score reflects a better memory function. About 30 min are necessary to administrate this test and its reliability ranges from 0.78-0.94 (Paolo et al., 1997).

Total Digit Span (TDS)

Working memory was assessed by the Digit Span, a subtest of the Wechsler Adult Intelligence Scale-III (WAIS-III) (Wechsler, 2012) a measure of general intellectual function. It is based on numbers and includes the Forward and Backward Digit Span tests. In the Forward Digit Span test, the examinee repeats a number sequence in the same order as presented. This constitutes a measure of working memory but also of attention. In the Backward Digit Span, the examinee repeats the number sequence in reverse order. Total Digit Span represents the total score of the two previous tests. A higher score reflects a better memory function. In a standardization sample of 394 participants (aged 16-89 years), the reliability coefficient was very high, ranging from 0.94-0.97 (Strauss et al., 2006).

The Patient Health Questionnaire-9 (PHQ-9)

Is a depression module of the PRIME-MD diagnostic instrument for mental disorders (Spitzer et al., 1999). It encompasses 9 items of depression symptoms plus a question about functional impairment and can be scored as a depression severity rating (scores of 10-14 moderate, 15-19 moderately severe and 20-27 severe depressive symptoms) or with an algorithm based on the DSM-IV criteria (major and minor episode). Scores of 10 or more have an 88% sensitivity and specificity. PHQ-9 score was considered as a possible confounding factor in the analyses.

Extraction of Fecal 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 (Schmieder and Edwards, 2011) and overlapping pairs were joined using FLASH-1.2.11 (Magoč and Salzberg, 2011). 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 (Langmead and Salzberg, 2012) 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 (Li et al., 2015) 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 (Hyatt et al., 2010) was used for predicting codifying regions. Functional annotation was carried out with HMMER (Durbin et al., 1998) against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, version 2016 (Kanehisa and Goto, 2000) 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 (R Development Core Team, 2013) 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 (Menzel et al., 2016) 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 (R Development Core Team, 2013). Fecal microbiota composition from mice was also analyzed following the same procedures as humans.

Metabolomics Analyses

For non-targeted metabolomics analysis, metabolites were extracted from fecal and plasma samples with methanol (containing phenylalanine-C13 as an internal standard) according to previously described methods (Wikoff et al., 2008). Briefly, for plasma samples 30μl of cold methanol were added to 10 μl of each sample, vortexed for 1 min and incubated for one h 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 12,000 g, 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 × 50 mm; Agilent Technologies) equipped with a precolumn (Zorbax-SB-C8 Rapid Resolution Cartridge 2.1 × 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), as described 5,6. 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 min 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.

Behavioral Testing in Mice

The NOR was performed in a V-maze as previously published (Burokas et al., 2014). Three phases of 9-min were performed on consecutive days. Mice were first habituated to the V-maze. On the second day, 2 identical objects (chess pieces) were presented to the mice, and the time that they spent exploring each object was recorded. In the test phase (3 h later for short-term memory or 24 h later for long-term memory), 1 of the familiar objects was replaced with a novel object (a different chess piece), and the time spent exploring each object (novel and familiar) was computed. A discrimination index was calculated as the difference between the times that the animal spent exploring the novel (Tn) and familiar (Tf) object divided by the total time of object exploration: (Tn-Tf)/(Tn + Tf).

Fear conditioning was conducted as described previously with some modifications (Burokas et al., 2017; Saravia et al., 2019). Mice were individually placed in a shuttle chamber (LE918, Panlab, Barcelona) surrounded by a sound-attenuating cabinet. The chamber floor was formed by parallel stainless-steel bars connected to a scrambled shock generator. On the training day, mice were habituated to the chamber during 180 s before the exposure to an acute beeping 30 s sound (80 dB). Each animal received an unconditioned stimulus (US) (0.6 mA footshock during 2 s) paired with the end of the sound (conditioned stimulus, CS). After the shock, the animal remained for 60 s in the shuttle chamber. To evaluate cued fear conditioning, mice were re-exposed to the CS in a novel environment (a wide white cylinder in the chamber) 24 h after the conditioning session. Mice were allowed to adapt for 180 s to the new environment which was followed by 30 s of the sound used in the training day. After the last sound trial, mice remained in the cylinder for 60 s. Fear memory was assessed as the percentage of time that mice spent freezing during the session. Freezing response, a rodent's natural response to fear, was evaluated by direct observation and defined as complete lack of movement, except for respiration for more than 1 s. The procedure was performed between 8.00 and 12.00 h in an experimental room different to the housing room.

Study of Gene Expression in Prefrontal Cortex

Sample Preparation

The mice brains were quickly removed and the medial prefrontal cortex was dissected according to the atlas of stereotaxic coordinates of mouse brain (Paxinos and Franklin, 1997). Brain tissues were then frozen by immersion in 2-methylbutane surrounded by dry ice, and stored at −80°C.

RNA Quality Control

Quality control of the RNA was performed using the RNA 6000 Nano chip (Agilent) on an Agilent Bioalyzer 2100 obtaining RIN values between 8.7 - 9.8.

RNA Libraries

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 min. 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.

Bioinformatic Analysis

Raw sequencing reads in the fastq files were mapped with STAR version 2.5.3a (Dobin et al., 2013) 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. (Liao et al., 2014). The differential expression gene analysis (DEG) was assessed with voom+limma in the limma package version 3.30.13 (Smyth, 2005) and R version 3.4.3. Genes having less than 10 counts in at least 5 samples were excluded from the analysis. Raw library size differences between samples were treated with the weighted "trimmed mean method" TMM (Robinson and Oshlack, 2010) implemented in the edgeR package (Robinson et al., 2010). The normalized counts were used in order to make unsupervised analysis, PCA and clusters. For the differential expression (DE) analysis, read counts were converted to log2-counts-per-million (logCPM) and the mean-variance relationship was modeled with precision weights using voom approach in limma package.

QUANTIFICATION AND STATISTICAL ANALYSIS

First, normal distribution and homogeneity of variances were tested. Results are expressed as number and frequencies for categorical variables, mean and standard deviation (SD) for normal distributed continuous variables and median and interquartile range [IQ] for non-normal distributed continuous variables. To determine differences between study groups, we used χ^2 for categorical variables, unpaired Student's t test in normal quantitative and Mann-Whitney U test for non-normal quantitative variables. Spearman or Pearson analysis was used to determine the correlation between quantitative variables. Theses statistical analyses were performed with SPSS, version 19 (SPSS, Inc, Chicago, IL). Statistics can be found in the figures and legends.

Differential abundance analyses for taxa and functions associated to the memory tests and brain areas volumes were performed using the DESeq2 R package (Love et al., 2014), adjusting for age, body mass index, sex, education years, and Patient Health Questionnaire (PHQ)-9 scores. Fold change associated with a unit change in the corresponding test and adjusted *p-value*s are plotted for each taxon. Significantly different taxa are colored according to phylum. OTUs and bacterial functions were previously filtered so that only those with more than 10 reads in at least two samples were selected. To take into account the compositional structure of the microbiome data and rule out possible spurious associations microbiome data were also analyzed using a compositional approach with the ALDEx2 R package (Fernandes et al., 2014). ALDEx2 uses a Dirichlet-multinomial model to inter abundance from read counts. We used 128 Dirichlet Monte Carlo instances in the aldex.clr function, and then applied a generalized linear model with the aldex.glm function controlling for age, BMI, sex, education years and depression scores. The p values were then adjusted for multiple comparisons using q-values (Storey, 2002). We further analyzed the microbiome data adopting a multivariate machine learning feature selection strategy after transforming the data to take into account the compositional nature. Specifically, first we imputed the zero values with a Geometric Bayesian multiplicative replacement using the zcompositions R package. Then, we applied a clr transformation using the clr function from the compositions R package. Finally, we applied an all-relevant machine learning variable selection strategy to the clr-transformed data using the VITA algorithm (describe below).

Metabolomics data were also analyzed using machine learning (ML) methods. Omics datasets are usually composed of high-dimensional data with many redundant, non-informative and noisy features, i.e., not related to the outcome, with complex correlation patterns. Therefore, feature selection, plays a crucial role in omics data analysis. In this context, ML methods, such as random forest (RF), are promising computational approaches for feature selection in high-dimensional omics datasets. ML tree-based algorithms are particularly well-suited to this aim. Thus, variable selection tree-based methods have shown to perform better than classic regression-based methods in large datasets (Sanchez-Pinto et al., 2018).

When the main goal is building a predictive model, variable selection techniques designed to identify a minimal set of strongest predictors associated with the outcome are used (minimal-optimal problem). However, if the objective involves providing a more holistic pictures of the underlying mechanisms, networks and pathways involved in pathophysiological or metabolic processes, all-relevant variable selection methods, which include weak, correlated and redundant features, but avoid inclusion of uninformative variables, are preferred (Shi et al., 2019). Therefore, we adopted an all-relevant machine learning variable selection strategy applying two random forest-based methods, the Boruta algorithm (Kursa and Rudnicki, 2010) and the Variable Importance Testing Approach (VITA) method (Janitza et al., 2018). The Boruta and Vita approaches have been recently proposed as the two best-performing variable selection methods making use of RF for high-dimensional omics datasets (Degenhardt et al., 2019).

RF is an ensemble machine learning method based on "growing" many classification or regression trees. The advantage of the RF is that the observations not used for the construction of a specific tree (termed *out-of-bag* (OOB) observations) may be used to estimate the variable importance measure (VIM). Among the several VIMs, the permutation variable importance has shown to be the most reliable. However, a drawback of VIMs in RF is that they are not directly related to the statistical significance and there is no statistical test that discriminates between relevant and non-relevant features. Boruta and Vita are two RF-based approaches that deal with this issue. The **Boruta algorithm** is a wrapper algorithm that performs feature selection based on the learning performance of the model (Kursa and Rudnicki, 2010). The main idea behind this approach consists in: **a) Randomization.** Create a duplicate copy of the original features randomly permutate across the observations (the so-called shadow features) to remove their correlation with the response; **b) Model building.** Add the shadow feature to the original predictor feature dataset, built a RF with the extended dataset, and compute the normalized permutation importance (*Z*) scores for each predictor and shadow feature; **c) Statistical testing.** Find the maximum normalized importance among the shadow attributes (MZSA) and compare it with each original predictor feature using a Bonferroni corrected two-tailed binomial test. Predictor features with significantly higher, significantly lower, or non-significantly different *Z* scores than expected at random compared to the MZSA are deemed important, unimportant, or tentative, respectively. **d) Iteration**. Unimportant and shadow features are removed and the previous steps are repeated until the status of all features is decided or a predefined number of iterations has been performed. We run the Boruta algorithm with 500 iterations, a confidence level cut-off of 0.005 for the Bonferroni adjusted *p-value*s, 5000 trees to grow the forest (*ntree*), and a number of features randomly sampled at each split given by the rounded down number of features/3 (the *mtry* recommended for regression).

The **Vita algorithm** is based on the assumption that most variables in omics datasets are non-relevant for the biological question and can be used to approximate the unknown null distribution of variable importance scores to be able to select relevant variables based on *p-value*s (Janitza et al., 2018). First, the VIM for all features are obtained. The importance measure in the vita algorithm is not based on the "standard" permutation variable importance calculated using the OOB samples, but uses a strategy inspired in the cross-validation (CV) procedure, which is not based on the OOB observations, to obtain the CV permutation variable importance (CVPVI). The method randomly splits the data in a total of *k*-folds of equal size. For each *i*-fold, a RF is trained using all samples

that are not part of the *i*-test set, and the response variable is predicted for the samples in the *i*-test set. The procedure is repeated after permutating *n* times the values of the predictor variables. The permutation variable importance is calculated as the average difference in the prediction errors between the original data and the permutations, and the CVPVI is the average over all *k*-fold-specific permutation variable importance. Second, taking into account that for non-relevant features the change in accuracy is only due to random variations and thus it does not change (zero CVPVI) or slightly increases (negative CVPVI) when not using the variable for prediction, the non-positive CVPVI values are used compute the a symmetric null distribution of CVPVI scores around zero for non-relevant features by mirroring them on the y axis. From this approximated null distribution, *p-value*s can be calculated. As the null distribution is obtained from non-relevant features, this testing approach is only suitable for datasets with a large number of variables without effect. In our calculations we used 5000 trees, a 7-fold CV, and 10 permutations. *P*-values were then corrected using the Benjamini-Hochberg procedure for FDR.

Cell Metabolism, Volume *32*

Supplemental Information

Obesity Impairs Short-Term and Working Memory through Gut Microbial Metabolism of Aromatic Amino Acids

María Arnoriaga-Rodríguez, Jordi Mayneris-Perxachs, Aurelijus Burokas, Oren Contreras-Rodríguez, Gerard Blasco, Clàudia Coll, Carles Biarnés, Romina Miranda-Olivos, Jèssica Latorre, José-Maria Moreno-Navarrete, Anna Castells-Nobau, Mònica Sabater, María Encarnación Palomo-Buitrago, Josep Puig, Salvador Pedraza, Jordi Gich, Vicente Pérez-Brocal, Wifredo Ricart, Andrés Moya, Xavier Fernández-Real, Lluís Ramió-Torrentà, Reinald Pamplona, Joaquim Sol, Mariona Jové, Manuel Portero-Otin, Rafael Maldonado, and José Manuel Fernández-Real

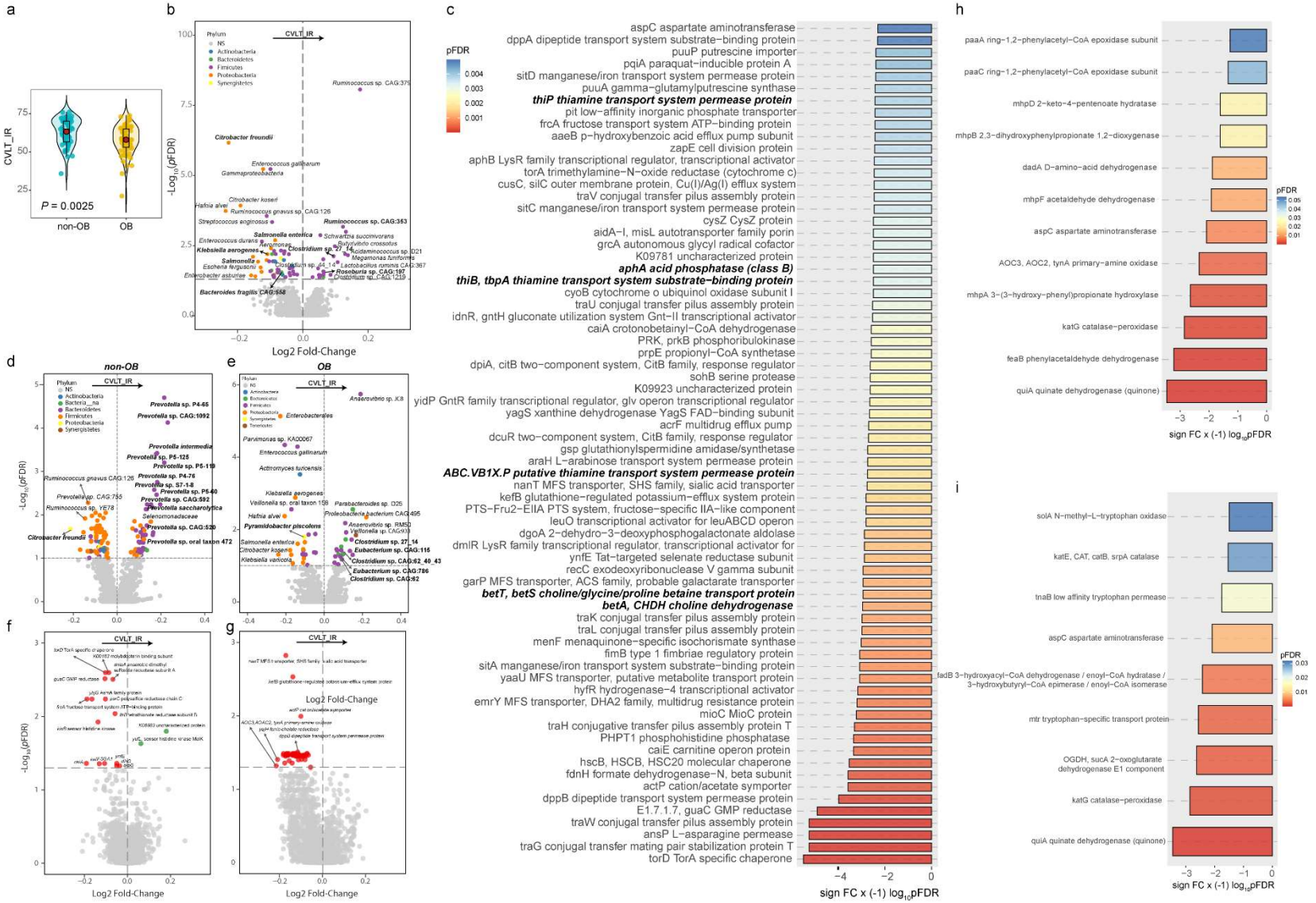

a
b
c
h
i
CVLT_IR
P = 0.0025
non-OB
OB
Phylum
NS
Actinobacteria
Bacteroidetes
Firmicutes
Proteobacteria
Synergistetes
-Log10(pFDR)
Log2 Fold-Change
Ruminococcus sp. CAG:375
Citrobacter freundii
Enterococcus gallinarum
Gammaproteobacteria
Citrobacter koseri
Hafnia alvei
Ruminococcus gnavus sp. CAG:126
Streptococcus anginosus
Ruminococcus sp. CAG:353
Salmonella enterica
Schwartzia succinivorans
Enterococcus durans
Butyrivibrio crossotus
Klebsiella aerogenes
Clostridium sp. 27_14
Acidaminococcus sp. D21
Megamonas funiformis
Salmonella
Eschena fergusonii
Clostridium sp. 64_14
Lactobacillus ruminis CAG:367
Roseburia sp. CAG:197
Enterobacter asburiae
Clostridium sp. CAG:1219
Bacteroides fragilis CAG:558
pFDR
0.004
0.003
0.002
0.001
aspC aspartate aminotransferase
dppA dipeptide transport system substrate−binding protein
puuP putrescine importer
pqiA paraquat−inducible protein A
sitD manganese/iron transport system permease protein
puuA gamma−glutamylputrescine synthase
thiP thiamine transport system permease protein
pit low−affinity inorganic phosphate transporter
frcA fructose transport system ATP−binding protein
aaeB p−hydroxybenzoic acid efflux pump subunit
zapE cell division protein
aphB LysR family transcriptional regulator, transcriptional activator
torA trimethylamine−N−oxide reductase (cytochrome c)
cusC, silC outer membrane protein, Cu(I)/Ag(I) efflux system
traV conjugal transfer pilus assembly protein
sitC manganese/iron transport system permease protein
cysZ CysZ protein
aidA−I, misL autotransporter family porin
grcA autonomous glycyl radical cofactor
K09781 uncharacterized protein
aphA acid phosphatase (class B)
thiB, tbpA thiamine transport system substrate−binding protein
cyoB cytochrome o ubiquinol oxidase subunit I
traU conjugal transfer pilus assembly protein
idnR, gntH gluconate utilization system Gnt−II transcriptional activator
caiA crotonobetainyl−CoA dehydrogenase
PRK, prkB phosphoribulokinase
prpE propionyl−CoA synthetase
dpiA, citB two−component system, CitB family, response regulator
sohB serine protease
K09923 uncharacterized protein
yidP GntR family transcriptional regulator, glv operon transcriptional regulator
yagS xanthine dehydrogenase YagS FAD−binding subunit
acrF multidrug efflux pump
dcuR two−component system, CitB family, response regulator
gsp glutathionylspermidine amidase/synthetase
araH L−arabinose transport system permease protein
ABC.VB1X.P putative thiamine transport system permease protein
nanT MFS transporter, SHS family, sialic acid transporter
kefB glutathione−regulated potassium−efflux system protein
PTS−Fru2−EIIA PTS system, fructose−specific IIA−like component
leuO transcriptional activator for leuABCD operon
dgoA 2−dehydro−3−deoxyphosphogalactonate aldolase
dmlR LysR family transcriptional regulator, transcriptional activator for
ynfE Tat−targeted selenate reductase subunit
recC exodeoxyribonuclease V gamma subunit
garP MFS transporter, ACS family, probable galactarate transporter
betT, betS choline/glycine/proline betaine transport protein
betA, CHDH choline dehydrogenase
traK conjugal transfer pilus assembly protein
traL conjugal transfer pilus assembly protein
menF menaquinone−specific isochorismate synthase
fimB type 1 fimbriae regulatory protein
sitA manganese/iron transport system substrate−binding protein
yaaU MFS transporter, putative metabolite transport protein
hyfR hydrogenase−4 transcriptional activator
emrY MFS transporter, DHA2 family, multidrug resistance protein
mioC MioC protein
traH conjugative transfer pilus assembly protein T
PHPT1 phosphohistidine phosphatase
caiE carnitine operon protein
hscB, HSCB, HSC20 molecular chaperone
fdnH formate dehydrogenase−N, beta subunit
actP cation/acetate symporter
dppB dipeptide transport system permease protein
E1.7.1.7, guaC GMP reductase
traW conjugal transfer pilus assembly protein
ansP L−asparagine permease
traG conjugal transfer mating pair stabilization protein T
torD TorA specific chaperone
sign FC x (-1) log10pFDR
paaA ring−1,2−phenylacetyl−CoA epoxidase subunit
paaC ring−1,2−phenylacetyl−CoA epoxidase subunit
mhpD 2−keto−4−pentenoate hydratase
mhpB 2,3−dihydroxyphenylpropionate 1,2−dioxygenase
dadA D−amino−acid dehydrogenase
mhpF acetaldehyde dehydrogenase
aspC aspartate aminotransferase
AOC3, AOC2, tynA primary−amine oxidase
mhpA 3−(3−hydroxy−phenyl)propionate hydroxylase
katG catalase−peroxidase
feaB phenylacetaldehyde dehydrogenase
quiA quinate dehydrogenase (quinone)
solA N−methyl−L−tryptophan oxidase
katE, CAT, catB, srpA catalase
tnaB low affinity tryptophan permease
fadB 3−hydroxyacyl−CoA dehydrogenase / enoyl−CoA hydratase / 3−hydroxybutyryl−CoA epimerase / enoyl−CoA isomerase
mtr tryptophan−specific transport protein
OGDH, sucA 2−oxoglutarate dehydrogenase E1 component
non-OB
OB

Figure S1. Microbiota taxonomic and functional profiles associated to the California Verbal Learning Immediate Recall (CVLT_IR). Related to Figure 1. a) Boxplot for the CVLT_IR in non-obese and obese patients. Differences between groups were analysed by a Wilcoxon tests. **b)** Volcano plots of differential bacterial abundance associated with the CVLT_IR calculated by DESeq2 controlling for age, BMI, sex, education years, and Patient Health Questionnaire (PHQ)-9 scores. Fold change associated with a unit change in the CVLT_IR and Benjamini-Hochberg adjusted *p*-values (*p*FDR) are plotted for each taxon. Significantly different taxa are coloured according to phylum. **c)** Manhattan-like plot of significantly expressed KEGG bacterial genes associated with the CVLT_IR (*p*FDR <0.005) identified from DESeq2 controlled for the previous covariates. The $-\log_{10}$(*p*FDR) values are multiplied by the fold change (FC) sign to take into account the direction of the association. Bars are coloured according to the *p*FDR. Those functions related to B vitamins metabolism, one-carbon metabolism, phenylalanine, tryptophan, and endocannabinoid metabolism are highlighted in black. The complete list of significantly associated metagenomic functions can be found in Table S1. **d-g)** Taxonomic and functional associations for the CVLT_IR in non-obese and obese patients. **h)** Manhattan-like plot of significantly expressed KEGG bacterial genes related to the phenylalanine metabolism and **i)** the tryptophan metabolism associated with the CVLT Short Delayed Free Recall.

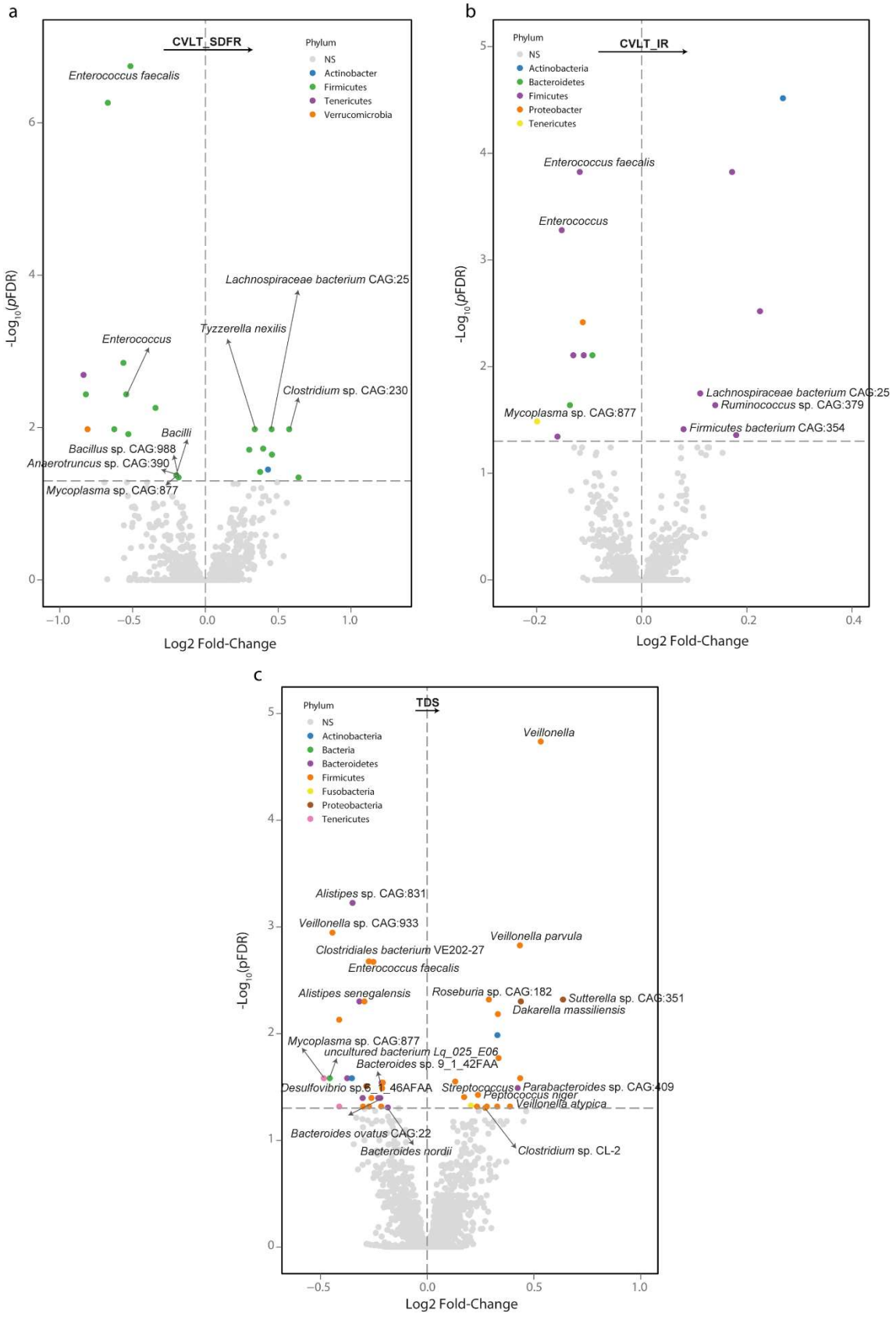


Figure S2. Microbiota composition associated with memory domains in the replication longitudinal cohort. Related to Figure 1. **a)** Volcano plots of differential bacterial abundance

associated with the California Verbal Learning Tests Short Delayed Free Recall (CVLT_SDFR), **b)** the CVLT Immediate Recall (IR), and **c)** the Total Digit Span (TDS) in 69 consecutive subjects after 1 year of follow-up as calculated by DESeq2 analysis controlling for age, body mass index, sex, education years, and Patient Health Questionnaire (PHQ)-9 scores. Fold change associated with a unit change in the corresponding test and Benjamini-Hochberg-adjusted *p*-values (*p*FDR) are plotted for each taxon. Significantly different taxa are coloured according to phylum. Only the bacterial species that were also significantly associated with the memory domains at baseline and in the same direction are highlighted.

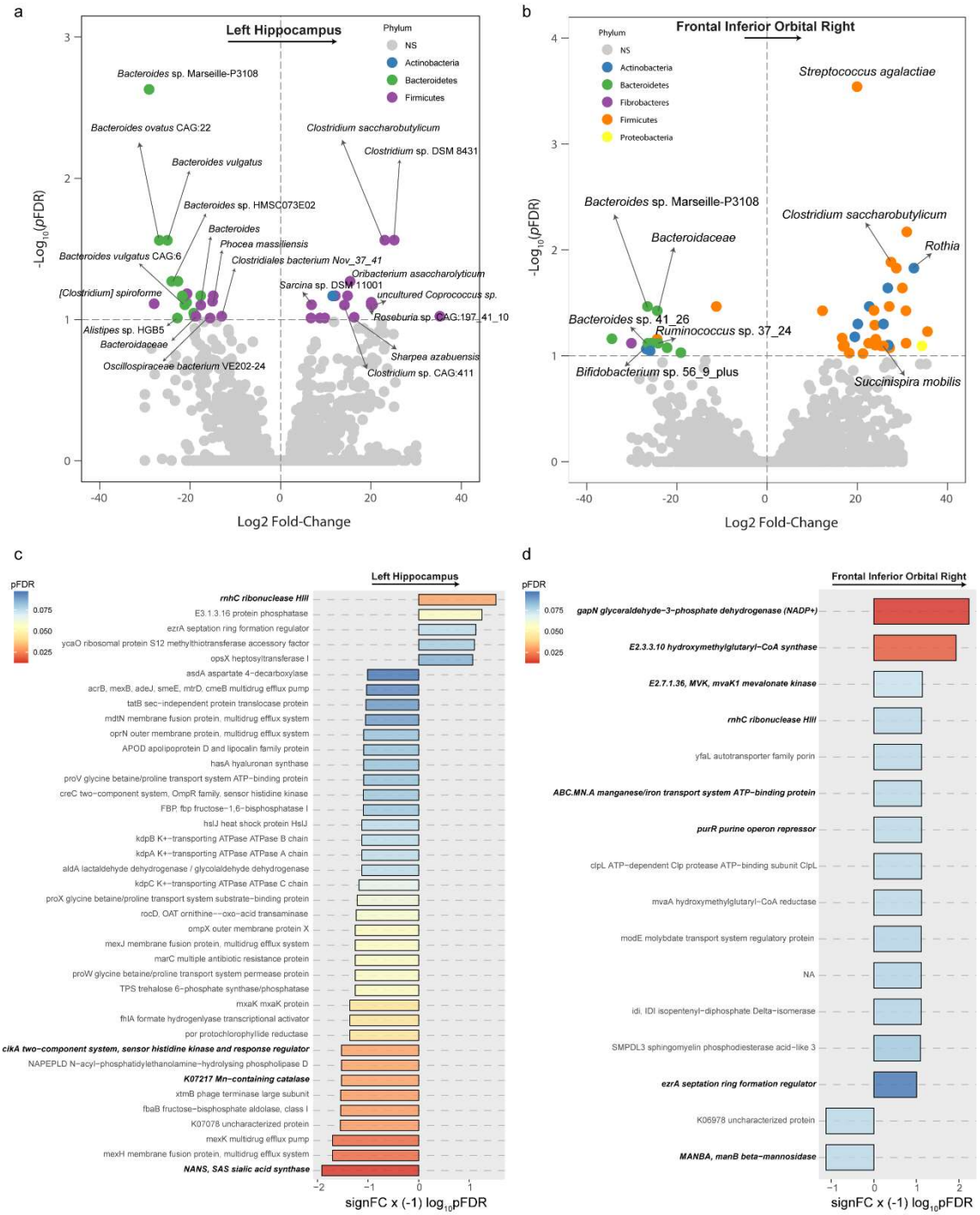
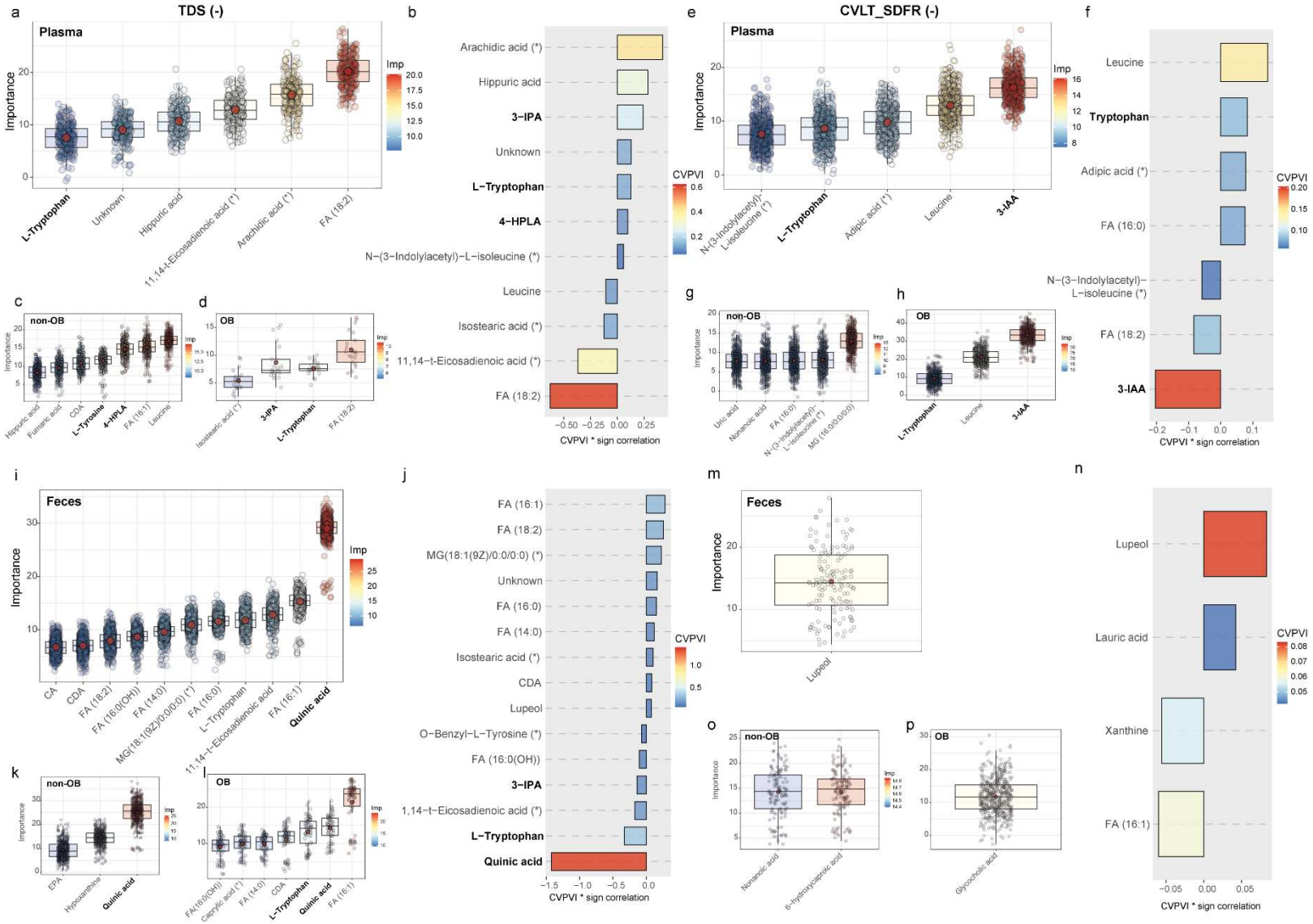


Figure S3. Microbiota taxonomic and functional associations the brain structure in the replication longitudinal cohort. Related to Figure 2. a) Volcano plots of differential bacterial abundance associated with the left hippocampus volume and **b)** right frontal inferior orbital volume in the replication cohort after 1 year of follow-up, as calculated by DESeq2 controlling for age, BMI, sex, education years, PHQ-9 and total intracranial volume (TIV). Fold change associated with a unit change in the corresponding volumes and Benjamini-Hochberg adjusted

p-values (*p*FDR) are plotted for each taxon. Significantly different taxa are coloured according to phylum. Only those bacterial species that were also significantly associated with the corresponding brain volumes at baseline and in the same direction are highlighted. **c)** Manhattan-like plot of significantly expressed KEGG bacterial genes associated with the left hippocampus volume and **k)** the right frontal inferior orbital in the replication cohort after 1 year of follow-up, identified from covariate-adjusted DESeq2 analysis. For the left hippocampus, only those bacterial functions also associated to the memory domains are represented. The $-\log_{10}$(pFDR) values are multiplied by the fold change (FC) sign to take into account the direction of the association. Bars are coloured according to the pFDR. Metagenomic functions that were also associated with the corresponding brain volumes at baseline are highlighted in bold. The complete list of significantly associated functions can be found in Supplemental Table S5g-j.


a
TDS (-)
Plasma
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L-Tryptophan
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Hippuric acid
11,14-t-Eicosadienoic acid (*)
Arachidic acid (*)
FA (18:2)
b
Arachidic acid (*)
Hippuric acid
3-IPA
Unknown
L-Tryptophan
4-HPLA
N-(3-Indolylacetyl)-L-isoleucine (*)
Leucine
Isostearic acid (*)
11,14-t-Eicosadienoic acid (*)
FA (18:2)
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CVPVI * sign correlation
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Hippuric acid
Fumaric acid
CDA
L-Tyrosine
4-HPLA
FA (16:1)
Leucine
d
OB
Isostearic acid (*)
3-IPA
L-Tryptophan
FA (18:2)
e
CVLT_SDFR (-)
Plasma
N-(3-Indolylacetyl)-L-isoleucine (*)
L-Tryptophan
Adipic acid (*)
Leucine
3-IAA
f
Leucine
Tryptophan
Adipic acid (*)
FA (16:0)
N-(3-Indolylacetyl)-L-isoleucine (*)
FA (18:2)
3-IAA
g
non-OB
Uric acid
Nonanoic acid
FA (16:0)
N-(3-Indolylacetyl)-L-isoleucine (*)
MG (16:0/0:0/0:0)
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OB
L-Tryptophan
Leucine
3-IAA
i
Feces
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CDA
FA (18:2)
FA (16:0(OH))
FA (14:0)
MG(18:1(9Z)/0:0/0:0) (*)
FA (16:0)
L-Tryptophan
11,14-t-Eicosadienoic acid
FA (16:1)
Quinic acid
j
FA (16:1)
FA (18:2)
MG(18:1(9Z)/0:0/0:0) (*)
Unknown
FA (16:0)
FA (14:0)
Isostearic acid (*)
CDA
Lupeol
O-Benzyl-L-Tyrosine (*)
FA (16:0(OH))
3-IPA
1,14-t-Eicosadienoic acid (*)
L-Tryptophan
Quinic acid
CVPVI * sign correlation
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EPA
Hypoxanthine
Quinic acid
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OB
FA(16:0(OH))
Caprylic acid (*)
FA (14:0)
CDA
L-Tryptophan
Quinic acid
FA (16:1)
m
Feces
Lupeol
o
non-OB
Nonanoic acid
6-hydroxycaproic acid
p
OB
Glycocholic acid
n
Lupeol
Lauric acid
Xanthine
FA (16:1)
CVPVI * sign correlation

Figure S4. Plasma and fecal metabolomics in electrospray ionization (ESI) negative mode linked to memory domains. Related to Figure 3. a) Boxplots of the normalized permutation importance measure for the metabolites associated to the to the TDS in plasma, **e)** the CVLT_SDFR in plasma, **i)** the TDS in faeces, and **m**) the CVLT_SDFR in faeces, identified by machine learning thorough the random forest-based Boruta feature selection algorithm at each of the 500 iterations. **b)** Cross-validated permutation variable importance (CVPVI) measure × sign of the correlation between each metabolite associated to the TDS test in plasma, **f)** the CVLT_SDFR in plasma, **j)** the TDS in faeces, and **n)** the CVLT_SDFR in faeces, identified by machine learning using the random forest-based Vita method. **c-d)** Normalized permutation importance measure for Boruta selected metabolites associated to the TDS in plasma, **g-h)** the CVLT_SDFR in plasma, **k-l)** the TDS in plasma, and **o-p)** the CVLT_SDFR in faeces. All metabolites were identified based on exact mass, retention time and MS/MS spectrum, except those with (*) that were only identified based on exact mass and retention time. 3-IPA, Indole-3-propionic acid; CA, cholic acid; CDA, chenodeoxycholic acid; FA, fatty acid; 4-HPLA, 4-hydroxyphenyllactic acid; MG, monoglyceride.

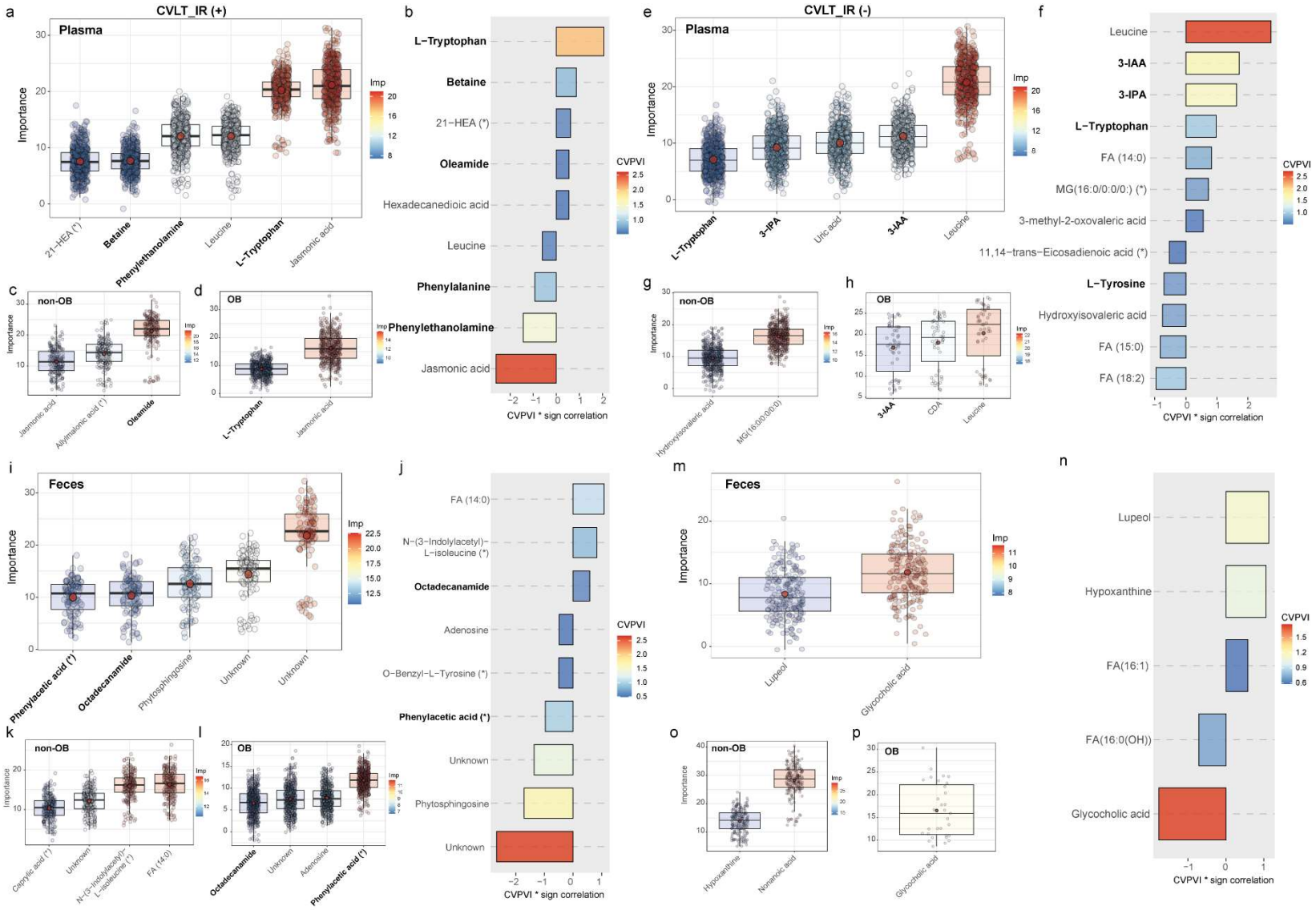
a
CVLT_IR (+)
Plasma
Importance
Imp
21−HEA (*)
Betaine
Phenylethanolamine
Leucine
L−Tryptophan
Jasmonic acid
b
L−Tryptophan
Betaine
21−HEA (*)
Oleamide
Hexadecanedioic acid
Leucine
Phenylalanine
Phenylethanolamine
Jasmonic acid
CVPVI
CVPVI * sign correlation
c
non-OB
Jasmonic acid
Allylmalonic acid (*)
Oleamide
d
OB
L−Tryptophan
Jasmonic acid
e
CVLT_IR (-)
Plasma
L−Tryptophan
3-IPA
Uric acid
3-IAA
Leucine
f
Leucine
3-IAA
3-IPA
L−Tryptophan
FA (14:0)
MG(16:0/0:0/0:) (*)
3-methyl-2-oxovaleric acid
11,14−trans−Eicosadienoic acid (*)
L−Tyrosine
Hydroxyisovaleric acid
FA (15:0)
FA (18:2)
g
non-OB
Hydroxyisovaleric acid
MG(16:0/0:0/0:)
h
OB
3-IAA
CDA
Leucine
i
Feces
Phenylacetic acid (*)
Octadecanamide
Phytosphingosine
Unknown
Unknown
j
FA (14:0)
N−(3−Indolylacetyl)−L−isoleucine (*)
Octadecanamide
Adenosine
O−Benzyl−L−Tyrosine (*)
Phenylacetic acid (*)
Unknown
Phytosphingosine
Unknown
k
non-OB
Caprylic acid (*)
Unknown
N−(3−Indolylacetyl)−L−isoleucine (*)
FA (14:0)
l
OB
Octadecanamide
Unknown
Adenosine
Phenylacetic acid (*)
m
Feces
Lupeol
Glycocholic acid
n
Lupeol
Hypoxanthine
FA(16:1)
FA(16:0(OH))
Glycocholic acid
o
non-OB
Hypoxanthine
Noranic acid
p
OB
Glycocholic acid

Figure S5. Plasma and fecal metabolomics linked to the California Verbal Learning Tests Immediate Recall (CVLT_IR). Related to Figure 3. a) Boxplots of the normalized permutation importance measure for the metabolites associated to the to the CVLT_IR in positive ESI mode in plasma, **e)** negative ESI mode in plasma, **i)** positive ESI mode in faeces, and **m**) negative ESI mode in faeces, identified by machine learning thorough the random forest-based Boruta feature selection algorithm at each of the 500 iterations. **b)** Cross-validated permutation variable importance (CVPVI) measure × sign of the correlation between each metabolite associated to the CVLT_IR in ESI positive mode in plasma, **f)** negative ESI mode in plasma, **j)** positive ESI mode in faeces, and **n)** negative ESI model in faeces, identified by machine learning using the random forest-based Vita method. **c-d)** Normalized permutation importance measure for Boruta selected metabolites associated to the CVLT_IR in positive ESI mode in, **g-h)** negative ESI mode in plasma, **k-l)** positive ESI model in faeces, and **o-p)** negative ESI model in faeces, for non-obese and obese patients, respectively. All metabolites were identified based on exact mass, retention time and MS/MS spectrum, except those with (*) that were only identified based on exact mass and retention time. 21-HEA, 21-hydroxyheneicosanoic acid; 3-IAA, Indoe-3-acetic acid; 3-IPA, Indole-3-propionic acid; CA, cholic acid; CDA, chenodeoxycholic acid; FA, fatty acid; MG, monoglyceride.

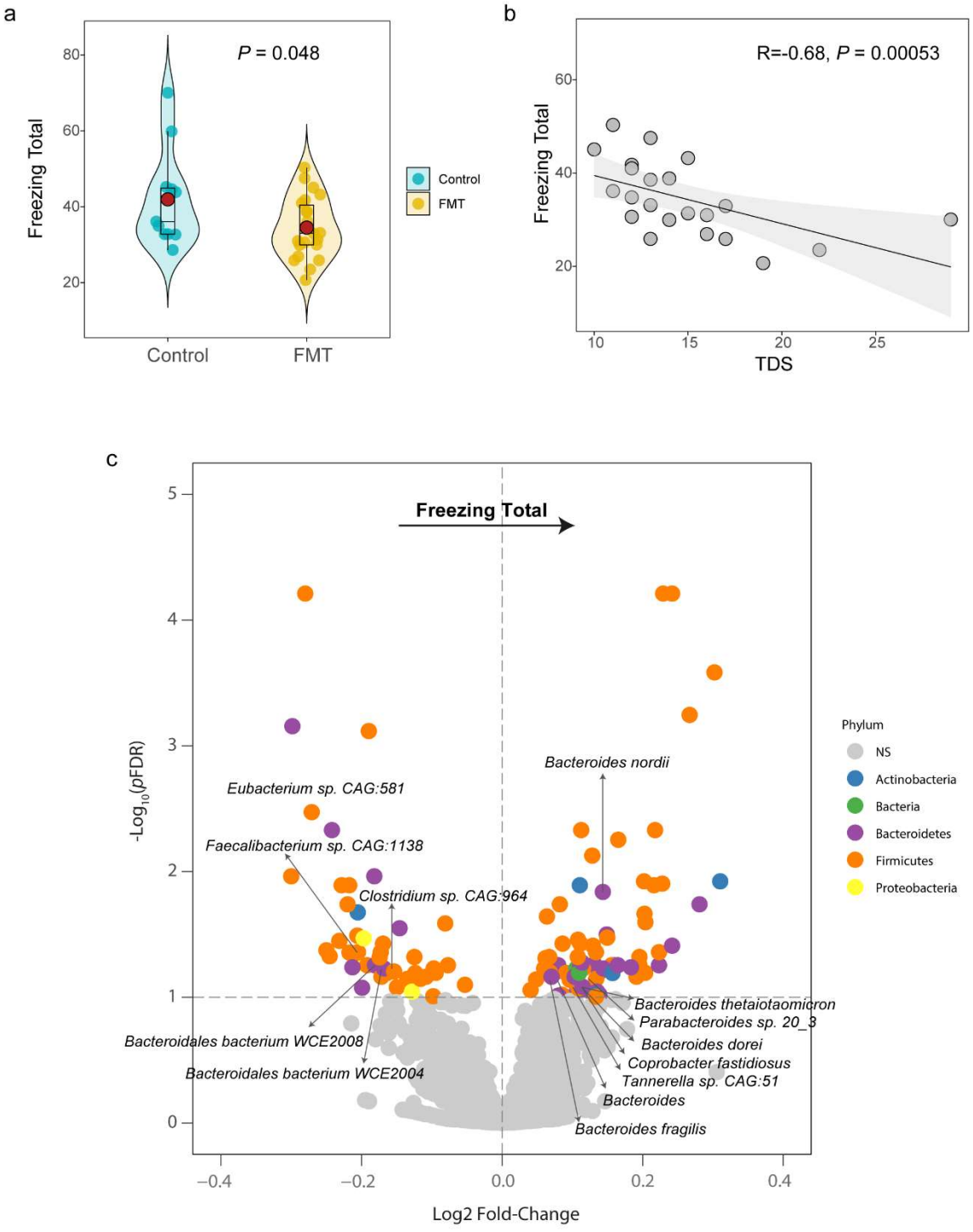

Figure S6. Results for the freezing total test in the faecal microbiota transplantation (FMT) experiment. Related to Figure 4. a) Violin plots for the Freezing total test scores comparing the

control group and the FMT group (t-test). **b)** Spearman correlation between the freezing total test in recipient's mice and the total digit span (TDS) test in human donors. **c)** Volcano plots of differential human donor bacterial abundance associated with the recipient's mice freezing total test from DESeq2 analysis. Fold change associated with a unit change in the freezing total test and Benjamini-Hochberg adjusted *p*-values (*p*FDR) are plotted for each taxon. Significantly different taxa are coloured according to phylum. Only significant species that were also associated inversely in the NORII24h test are highlighted.

	Total population (n=143)	Without obesity (n=71)	With obesity (n=72)	*p*
Age (years)	48.6 [40.3-57.0]	50.6 [40.6-59.0]	47.9 [37.0-53.2]	0.095
Females n (%)	97 (67.8)	46 (64.8)	51 (70.8)	0.439
Education (years)	14 [11-17]	15 [12-17]	12 [9.5-15]	$6.0x10^{-5}$
Smoking n (%)	19 (13.3)	7 (9.9)	12 (16.7)	0.471
Alcohol intake (g/d)	1.4 [0-4.3]	2.3 [0.6-7.9]	0 [0-2.3]	1.5x10-5
BMI (kg/m^2)	30 [24.8-43.3]	24.8 [22.4-26.8]	43.3 [39.9-47.3]	$5.7x10^{-25}$
Waist (cm)	105 [88-125]	88 [83-97.3]	125 [116-138]	$1.9x10^{-23}$
Fat mass (%)	41.8 [32.3-50.5]	32.5 [26.4-37.8]	50.5 [47.8-53.5]	$8.0x10^{-23}$
SBP (mmHg)	129 [117.8-143.3]	124 [111-135]	134.5 [123.3-151.5]	$1.3x10^{-4}$
DBL (mmHg)	74.2 (11.3)	71.9 (10.9)	76.5 (11.3)	0.014
HDL-C (mg/dL)	56 [46-68]	61 [54-77]	49 [43-59.8]	$6.3x10^{-7}$
Triglycerides (mg/dL)	86 [64-129]	77 [58-98]	109.5 [69.5-146.5]	0.001
FPG (mg/dL)	94 [89-102]	93 [89-100]	96 [89.5-103.8]	0.211
HbA1c (%)	5.5 [5.3-5.6]	5.4 [5.2-5.5]	5.5 [5.3-5.8]	0.010
hsCRP (mg/dL)	2.0 [0.7-5.5]	0.7 [0.4-1.6]	4.6 [2.8-8.2]	$8.6x10^{-16}$
CVLT IR (score)	61 [55-67]	63 [55-70]	59 [54.5-65]	0.020
CVLT SDFR (score)	14 [12-15]	14 [12-15]	13 [11-15]	0.087
Total Digit Span (score)	15 [12-17]	16 [13-19]	14 [11-16]	0.001
PHQ-9 (score)	5 [3-9]	3 [2-6]	8 [4-11]	$3.0x10^{-6}$
Hippocampus L (mm3)	0.37 [0.34-0.39]	0.37 [0.35-0.40]	0.36 [0.34-039]	0.046
Hippocampus R (mm3)	0.36 [0.34-0.39]	0.36 [0.34-0.39]	0.36 [0.34-0.38]	0.012
Frontal Sup L (mm3)	0.28 (0.03)	0.28 (0.04)	0.27 (0.02)	0.015
Frontal Sup R (mm3)	0.29 (0.03)	0.30 (0.04)	0.28 (0.03)	0.004
Frontal Mid L (mm3)	0.31 (0.04)	0.32 (0.04)	0.30 (0.03)	0.008
Frontal Mid R (mm3)	0.32 (0.04)	0.33 (0.04)	0.32 (0.03)	0.006
Frontal Inf Oper L (mm3)	0.33 (0.04)	0.33 (0.04)	0.32 (0.03)	0.008
Frontal Inf Oper R (mm3)	0.33 (0.04)	0.34 (0.04)	0.32 (0.03)	0.003
Frontal Inf Orb L (mm3)	0.37 (0.04)	0.38 (0.05)	0.37 (0.04)	0.186
Frontal Inf Orb R (mm3)	0.35 (0.04)	0.35 (0.04)	0.35 (0.04)	0.941
Frontal Inf Tri L (mm3)	0.33 (0.04)	0.33 (0.04)	0.32 (0.03)	0.032
Frontal Inf Tri R (mm3)	0.31 (0.03)	0.31 (0.03)	0.30 (0.03)	0.116

Results are expressed as number and frequencies for categorical variables, mean and standard deviation (SD) for normal distributed continuous variables and median and interquartile range [IQ] for non-normal distributed continuous variables. To determine differences between study groups, we used $\chi 2$ for categorical variables, unpaired Student's t-test in normal quantitative and Mann-Whitney U test for non-normal quantitative variables. P value determinates differences between subjects with obesity (Body mass index, BMI ≥ 30 kg/m^2) and without obesity (BMI 18.5-30 kg/m^2). SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL-C, high density lipoprotein cholesterol; FPG, fasting plasma glucose; HbA1c, glycated hemoglobin; hsCRP, high-sensitive C-reactive protein; CVLT, California Verbal Learning Test; IR, Immediate Recall; SDFR, Short Delayed Free Recall; PHQ-9, Patient Health Questionnaire; L, left; R, right; Sup, superior; Mid, middle; Inf, inferior; Oper, opercularis; Orb, orbital; Tri, triangularis.

Table S4. Clinical and neuropsychological data of the human MRI cohort. Related to Figure 2.

	Total population (n=22)	Without obesity (n=9)	With obesity (n=13)	*p*
Age (years)	51.5 [46.3-55.8]	55 [50-59]	48 [41-52]	0.030
Females n (%)	12 (54.5)	4 (44.4)	8 (61.5)	0.429
Education (years)	12 [10-17]	17 [11-17.5]	11 [9-13.5]	0.060
BMI (kg/m^2)	36.5 [24.9-42.0]	24.8 [22.8-25.3]	41 [39.0-44.7]	$4x10^{-6}$
Waist (cm)	118.5 [90.8-128.0]	87 [83.5-92.5]	127 [121.5-139]	$4x10^{-5}$
Fat mass (%)	46.9 [24.6-51.1]	23.7 [20.1-35.8]	49.4 [47.1-52.6]	$5.3x10^{-5}$
SBP (mmHg)	131 [118.5-139.5	123 [105.5-129.5]	135 [129.5-145]	0.025
DBL (mmHg)	75 [67.8-81.5]	64 [57-73]	81 [74.5-84]	0.001
HDL-C (mg/dL)	52.5 [45.8-65.5]	61 [53.5-77]	48 [39.5-57.5]	0.021
Triglycerides (mg/dL)	111 [85.8-136.5]	81 [83-117]	128 [92.5-158]	0.096
FPG (mg/dL)	98 [91.8-110.8]	99 [89.5-110]	97 [91.5-114]	0.948
HbA1c (%)	5.5 [5.3-5.7]	5.5 [5.4-5.7]	5.5 [5.3-5.7]	0.896
hsCRP (mg/dL)	1.6 [0.5-5.0]	0.5 [0.4-0.9]	3.9 [1.6-6.5]	0.003
CVLT IR (score)	49.5 [47-66]	57 [47-68]	48 [46.5-65]	0.512
CVLT SDFR (score)	12.5 [10-14.3]	13 [10.5-14.5]	11 [10-14]	0.324
Total Digit Span (score)	13.5 [12-16.3]	15 [13-18]	13 [12-15.5]	0.164
PHQ-9 (score)	6 [2.8-9]	4 [1.5-5.5]	8 [5.5-10.5]	0.011

Table 4. Results are expressed as number and frequencies for categorical variables and median and interquartile range [IQ] for non-normal distributed continuous variables. To determine differences between study groups, we used $\chi 2$ for categorical variables and Mann-Whitney U test for non-normal quantitative variables. P value determinates differences between subjects with obesity (Body mass index, BMI ≥ 30 kg/m^2) and without obesity (BMI 18.5-30 kg/m^2). SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL-C, high density lipoprotein cholesterol; FPG, fasting plasma glucose; HbA1c, glycated hemoglobin; hsCRP, high-sensitive C-reactive protein; CVLT, California Verbal Learning Test; IR, Immediate Recall; SDFR, Short Delayed Free Recall; PHQ-9, Patient Health Questionnaire.

Table S6. Clinical and neuropsychological data of the human donors' cohort. Related to Figure 4.

5.2. Original paper II

Obesity-associated deficits in inhibitory control are phenocopied to mice through gut microbiota changes in one-carbon and aromatic amino acids metabolic pathways

Arnoriaga-Rodríguez M, Mayneris-Perxachs J, Contreras-Rodríguez O, Burokas A, Ortega-Sanchez JA, Blasco G, Coll C, Biarnés C, Castells-Nobau A, Puig J, Garre-Olmo J, Ramos R, Pedraza S, Brugada R, Vilanova JC, Serena J, Barretina J, Gich J, Pérez-Brocal V, Moya A, Fernández-Real X, Ramio-Torrentà L, Pamplona R, Sol J, Jové M, Ricart W, Portero-Otin M, Maldonado R, Fernández-Real JM.

Gut. 2021 Dec;70(12):2283-2296.
doi: 10.1136/gutjnl-2020-323371.

Highlights

- Gut microbiome composition and functionality was linked to several tests evaluating inhibitory control in subjects with and without obesity.
- Brain structures associated with this cognitive domain were also associated with gut microbiome alterations.
- The impairment of inhibitory control from the donors was phenocopied in recipient mice through a fecal microbiota transplantation, resulting in alterations of reversal learning and changes in brain transcriptomics.

OPEN ACCESS

Original research

Obesity-associated deficits in inhibitory control are phenocopied to mice through gut microbiota changes in one-carbon and aromatic amino acids metabolic pathways

María Arnoriaga-Rodríguez ,[1,2,3,4] Jordi Mayneris-Perxachs ,[1,2,3] Oren Contreras-Rodríguez ,[2,5] Aurelijus Burokas ,[6,7] Juan-Antonio Ortega-Sanchez,[6] Gerard Blasco,[8,9] Claudia Coll,[10] Carles Biarnés,[9] Anna Castells-Nobau,[1,2,3] Josep Puig,[4,8,9] Josep Garre-Olmo,[4,11] Rafel Ramos ,[4,12] Salvador Pedraza,[4,9,13] Ramon Brugada ,[4,14,15,16] Joan C Vilanova ,[4,9,13] Joaquín Serena,[4,17] Jordi Barretina,[18] Jordi Gich,[4,19] Vicente Pérez-Brocal,[20,21] Andrés Moya ,[20,21] Xavier Fernández-Real,[22] Lluis Ramio-Torrentà ,[4,10,17,19,23] Reinald Pamplona,[24] Joaquim Sol,[24,25,26] Mariona Jové ,[24] Wifredo Ricart,[1,2,3,4] Manuel Portero-Otin,[24] Rafael Maldonado,[6,27] Jose Manuel Fernández-Real [1,2,3,4]

► Additional material is published online only. To view, please visit the journal online (http://dx.doi.org/10.1136/gutjnl-2020-323371).

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Received 14 October 2020
Revised 16 December 2020
Accepted 8 January 2021
Published Online First 29 January 2021

Check for updates

To cite: Arnoriaga-Rodríguez M, Mayneris-Perxachs J, Contreras-Rodríguez O, *et al*. *Gut* 2021;**70**:2283–2296.

ABSTRACT

Background Inhibitory control (IC) is critical to keep long-term goals in everyday life. Bidirectional relationships between IC deficits and obesity are behind unhealthy eating and physical exercise habits.

Methods We studied gut microbiome composition and functionality, and plasma and faecal metabolomics in association with cognitive tests evaluating inhibitory control (Stroop test) and brain structure in a discovery (n=156), both cross-sectionally and longitudinally, and in an independent replication cohort (n=970). Faecal microbiota transplantation (FMT) in mice evaluated the impact on reversal learning and medial prefrontal cortex (mPFC) transcriptomics.

Results An interplay among IC, brain structure (in humans) and mPFC transcriptomics (in mice), plasma/faecal metabolomics and the gut metagenome was found. Obesity-dependent alterations in one-carbon metabolism, tryptophan and histidine pathways were associated with IC in the two independent cohorts. Bacterial functions linked to one-carbon metabolism (*thyX,dut,* exodeoxyribonuclease V), and the anterior cingulate cortex volume were associated with IC, cross-sectionally and longitudinally. FMT from individuals with obesity led to alterations in mice reversal learning. In an independent FMT experiment, human donor's bacterial functions related to IC deficits were associated with mPFC expression of one-carbon metabolism-related genes of recipient's mice.

Conclusion These results highlight the importance of targeting obesity-related impulsive behaviour through the induction of gut microbiota shifts.

Summary box

What is already known on this subject?
- ► Inhibitory control is fundamental to keep long-term goals in everyday life.
- ► In subjects with obesity, this cognitive domain is impaired.

What are the new findings?
- ► Gut microbiome composition and functionality was linked to several tests evaluating inhibitory control in subjects with and without obesity.
- ► Brain structures associated with this cognitive domain was also associated with gut microbiome alterations.
- ► The impairment of inhibitory control from the donors was phenocopied in recipient mice through a faecal microbiota transplantation, resulting in alterations of reversal learning and changes in brain transcriptomics.

How might it impact on clinical practice in the foreseeable future?
- ► The adherence to diet could be improved by modifications in the gut microbiome.

INTRODUCTION

Executive function constitutes one of the six key domains of cognition and mainly comprises reasoning, problem solving and component skills management, required for real-world adaptive success.[1] Executive functions are critical to keep long-term goals in everyday life.[2] Detrimental effects of excess weight on executive functions determine the individual's ability to break ingrained actions such as unhealthy eating and physical exercise habits in obese conditions.[3] Very preliminary small studies showed impaired executive function linked to the gut microbiota composition, suggesting that

the deleterious effects of adiposity on cognition are not merely mediated by the metabolic complications.[4]

We here aimed to study the interplay among IC, brain structure (in humans) and medial prefrontal cortex (mPFC) transcriptomics (in mice), plasma/faecal metabolomics and the gut metagenome and their transmission to mice through microbiota transplantation.

MATERIALS AND METHODS

Clinical study

Discovery cohort, cohort 1 (Ironmet): this is a cross-sectional case-control study setting at the Endocrinology Department of Josep Trueta University Hospital. The recruitment of subjects started in January 2016 and finished in October 2017. Consecutive middle-aged subjects, 27.2–66.6 years, were included. Patients with obesity (body mass index (BMI) ≥30 kg/m^2) and age-matched and sex-matched subjects without obesity (BMI 18.5–<30 kg/m^2), were eligible. Exclusion criteria were type 2 diabetes mellitus, chronic inflammatory systemic diseases, acute or chronic infections in the previous month; use of antibiotic, antifungal, antiviral or treatment with proton pump inhibitors; severe disorders of eating behaviour or major psychiatric antecedents; neurological diseases, history of trauma or injured brain, language disorders and excessive alcohol intake (≥40 g OH/day in women or 80 g OH/day in men).

Longitudinal cohort (Ironmet study): cognitive tests and MRI variables were collected again in 69 consecutive subjects after 1 year of follow-up.

Replication cohort, cohort 2: the study participants were recruited to evaluate the role of intestinal microflora in non-alcoholic fatty liver disease. The cohort included 24 subjects, 12 participants with obesity (BMI ≥30 kg/m^2) and 12 without obesity (BMI <30 kg/m^2). The exclusion criteria were systemic diseases, infection in the previous month, serious chronic illness, ethanol intake >20 g/day or use of medications that might interfere with insulin action. All control subjects were normotensive and were selected on the basis of similarity in age and sex compared with subjects with obesity and the absence of a personal history of inflammatory diseases or current drug treatment.

Replication cohort, cohort 3 (Imageomics): the Ageing Imageomics Study is an observational study including participants from two independent cohort studies (MESGI50 and MARK). Detailed description of the cohorts can be found elsewhere.[5] Briefly, the MESGI50 cohort included a population aged ≥50 years, while the MARK cohort included a random sample of patients aged 35–74 years with intermediate cardiovascular risk. Elegibility criteria included age ≥50 years, dwelling in the community, no history of infection during the last 15 days, no contraindications for MRI and consent to be informed of potential incidental findings.

Clinical and laboratory parameters: body composition was assessed using a dual energy X-ray absorptiometry (GE lunar, Madison, Wisconsin). Fasting plasma glucose, lipids profile and high-sensitivity C reactive protein (hsCRP) levels were measured using an analyzer (Cobas 8000 c702, Roche Diagnostics, Basel, Switzerland). Glycated haemoglobin was determined by performance liquid chromatography (ADAMA1c HA-8180V, ARKRAY, Kyoto, Japan).

Study of insulin sensitivity: Insulin sensitivity was determined by the hyperinsulinemic euglycemic clamp. The procedure consists in create in fasting conditions, a hyperinsulinemic state with an insulin infusion of predetermined fixed dosage and a variable rate glucose infusion. Glucose levels should be maintained constant at normal fasting (5 mmol/L) or any pre-existing (isoglycaemic) level adjusting the infusion rate of a 20% glucose solution. A steady state is usually reached in the last 40 minutes after 2 hours. Under these conditions the glucose infusion rates equal the glucose disposal rate, M (µmol· kg-1· min-1), a measurement of overall insulin sensitivity.

Dietary pattern: the dietary characteristics of the subjects were collected in a personal interview using a validated food-frequency questionnaire.[6]

The MRI acquisition and image preprocessing, the cognitive assessment (through the Stroop Color and Word Test (SCWT), Iowa Gambling Task (IGT) and the Wisconsin Card Sorting Test (WCST)), the extraction of faecal genomic DNA and whole-genome shotgun sequencing, plasma metabolomics analyses and animal experiments, including transcriptomics of the mPFC are described as online supplemental methods.

Statistical analyses

First, normal distribution and homogeneity of variances were tested. Results are expressed as number and frequencies for categorical variables, mean and SD for normal distributed continuous variables and median and IQR for non-normal distributed continuous variables. To determine differences between study groups, we used χ^2 for categorical variables, unpaired Student's t-test in normal quantitative and Mann-Whitney U test for non-normal quantitative variables. Spearman's analysis was used to determine the correlation between quantitative variables. All statistical analyses were performed with SPSS, V.19 (SPSS, Chicago, Illinois, USA).

Differential abundance analyses for taxa and Kyoto Encyclopedia of Genes and Genomes (KEGG)-based metagenome functions associated with the SCWT, the anterior cingulate cortex (ACC) volume and the recipient's mice mPFC gene transcripts were performed using the DESeq2 R packages, controlling for age, sex and education years. Fold change (FC) associated with a unit change in the corresponding variable and Benjamin-Hochberg adjusted p values were plotted for each taxon. Significantly different taxa were coloured according to phylum. Taxa and bacterial functions were previously filtered so that only those with >10 reads in at least two samples were selected. Manhattan-like plot were used to show significantly expressed KEGG metagenome functions. The $-\log_{10}$(*p*FDR) values were multiplied by the FC sign to take into account the direction of the association. Bars were coloured according to the *p*FDR. A significance <0.05 was established unless otherwise indicated. DESeq2 was also used to identify recipient's mice mPFC genes associated with donor's metagenomic functions linked to the SCWT test controlling for donor's age, sex and education years. Gene Ontology (GO) and Reactome pathway analyses of differentially expressed genes were performed using the clusterProfiler R package[7] and the ConsensusPathDB,[8] respectively. The p value of each term was assessed using an hypergeometric test and significantly enriched terms were determined based on a q value (Storey correction) cut-off of 0.1, to account for multiple testing. GO terms were visualised using the goplot function from the enrichplot R package, and significant reactome pathways were visualised using a gene overlap plot.

Metabolomics data were analysed using machine learning (ML) methods. In particular, we adopted an all-relevant ML variable selection strategy applying a multiple random forest (RF)-based method as implemented in the Boruta algorithm.[9] It has been

Table 1 Clinical and neuropsychological data of the human discovery cohort

	Total population (n=114)	Without obesity (n=51)	With obesity (n=63)	P value
Age (years)	50.4 (41.8–58.6)	53.9 (44.4–59.0)	48.6 (40.7–57.5)	0.096
Females n (%)	79 (69.3)	34 (66.7)	45 (71.4)	0.584
Education (years)	12.5 (11–17)	15 (12–17)	12 (9–14)	1.9×10^{-5}
BMI (kg/m^2)	34.6 (25.3–43.3)	24.9 (2.6)	43.1 (6.7)	6.8×10^{-33}
Waist (cm)	110 (92–126)	89.8 (9.6)	125.5 (14.0)	5.8×10^{-29}
Fat mass (%)	43.5 (33.8–50.2)	32.4 (7.2)	49.7 (5.5)	2.7×10^{-24}
SBP (mmHg)	132.8 (20.2)	124.3 (15.8)	139.5 (20.9)	2.6×10^{-5}
DBP (mmHg)	74.8 (11.6)	71.2 (10.9)	77.7 (11.4)	0.003
HDL-C (mg/dL)	56 (45–68)	66.0 (17.0)	51.0 (12.9)	4.1×10^{-7}
Triglycerides (mg/dL)	90.5 (65.8–135.3)	79 (58–96)	124 (82–156)	4.0×10^{-5}
FPG (mg/dL)	96 (90–103)	95 (89–101)	97 (93–105)	0.155
HbA1c (%)	5.5 (0.3)	5.5 (5.3–5.6)	5.6 (5.3–5.8)	0.021
hsCRP (mg/dL)	1.8 (0.7–5.9)	0.7 (0.4–1.3)	4.9 (2.7–9.5)	1.6×10^{-13}
SCWT (score)	43.1 (10.1)	45.9 (9.6)	40.8 (10.0)	0.006

Results are expressed as number and frequencies for categorical variables, mean and SD for normal distributed continuous variables and median and IQR for non-normal distributed continuous variables. To determine differences between study groups, we used χ^2 for categorical variables, unpaired Student's t-test in normal quantitative and Mann-Whitney U test for non-normal quantitative variables. P value determines differences between subjects with obesity (BMI ≥30 kg/m^2) and without obesity (BMI 18.5–30 kg/m^2). BMI, body mass index; DBP, diastolic blood pressure; FPG, fasting plasma glucose; HbA1c, glycated haemoglobin; HDL-C, high-density lipoprotein cholesterol; hsCRP, high-sensitivity C reactive protein; SBP, systolic blood pressure; SCWT, Stroop Color and Word Test.

recently proposed as one of the two best-performing variable selection methods making use of RF for high-dimensional omics datasets.[10] The Boruta algorithm is a wrapper algorithm that performs feature selection based on the learning performance of the model.[9] It performs variables selection in three steps: (a) randomisation, which is based on creating a duplicate copy of the original features randomly permutate across the observations; (b) model building, based on RF with the extended data set to compute the normalised permutation variable importance measure (VIM) scores; (c) statistical testing, to find those relevant features with a VIM higher than the best randomly permutate variable using a Bonferroni corrected two-tailed binomial test and (d) iteration, until the status of all features is decided. We run the Boruta algorithm with 500 iterations, a confidence level cut-off of 0.005 for the Bonferroni adjusted p values, 5000 trees to grow the forest (*ntree*) and a number of features randomly sampled at each split given by the rounded down number of features/3 (the *mtry* recommended for regression).

RESULTS AND DISCUSSION

We initially evaluated the SCWT in a cohort of subjects with and without obesity. As expected, lower SCWT scores, indicative of impaired inhibitory control, were found in subjects with obesity (table 1, figure 1A). Inhibitory control was associated with the gut microbiota composition. Differential abundance of species associated with the SCWT were identified from raw read count data adjusted by age, sex and education years using the DESeq2 package in R. We identified 297 species (*p*FDR <0.1) associated with the SCWT (figure 1A; online supplemental table S1). In all subjects as a whole, positive associations with executive performance were found with *Eubacterium* sp *CAG:603* and *Firmicutes bacterium CAG:238*; whereas most of the species negatively linked to SCWT scores belonged to the Bacteroidetes phylum: *Bacteroides plebeius*, *Bacteroides gallinarum*, *Bacteroides mediterranensis*, *Desulfovibrio fairfieldensis*, *Lachnospiraceae bacterium 5_1_57FAA* and *Lachnospiraceae bacterium 6_1_37FAA*. As both inflammation and insulin resistance may play a role in cognitive function and neurodegenerative disorders, we further analysed the data controlling for hsCRP, a marker of inflammation, and the hyperinsulinaemic-euglycaemic clamp (he-clamp), the gold-standard method to assess insulin sensitivity. Notably, ~85% of the species associated with the SCWT were still significant after adjusting for both parameters. We could reproduce these results in an independent cohort (n=24) (table 2) using two measures of executive function (the IGT and the WCST): several *Bacteroides* sp and *Alistipes* sp were also associated with the scores of these tests (figure 1B, online supplemental figure S1A, online supplemental tables S2 and S3).

Metagenome functional analyses based on KEGG pathways controlling for age, sex and education years also revealed significant associations of several bacterial pathways with the SCWT (figure 1C, online supplemental table S6), which were also replicated in an independent cohort (figure 1D). Further analysis controlling for hs-CRP and he-clamp revealed no effect of either inflammation or insulin sensitivity. Thus, >98% of the species associated with the SCWT were still significant after controlling for these additional variables. Notably, three bacterial functions related to nucleotide metabolism (dUTP pyrophosphatase, *dut*; thymidylate synthase, *thyX* and exodeoxyribonuclease V) had the strongest negative associations with the SCWT. Both *dut* and *thyX* participate in folate-mediated one-carbon metabolism. Consistently, *dut* correlated significantly with the plasma folic acid concentration (R=0.32, $p=7\times10^{-4}$, online supplemental figure S1B). It could seem counterintuitive that plasma folic acid was positively associated with a function linked to worse cognitive function. However, circulating unmetabolised folic acid implies that the body's capacity to convert folic acid to the metabolically active 5-methyltetrahydrofolate has been overwhelmed and that folic acid has passively diffused intact into the circulation.[11] In addition, *thyX* had a negative correlation with plasma uric acid levels (R=−0.20, p=0.034, online supplemental figure S1C). Alterations in folate-mediated one-carbon metabolism have been associated with increased risk for cognitive decline.[11] In line with these findings, other functions related to folate-mediated one-carbon metabolism (phosphoribosylglycinamideformyltransferase 2, *purT*) or folate biosynthesis (2-amino-4-hydroxy-6-hydroxymethyldihydropteridine diphosphokinase, *folK*; dihydroneopterin triphosphate diphosphatase, *nudB*) were also negatively

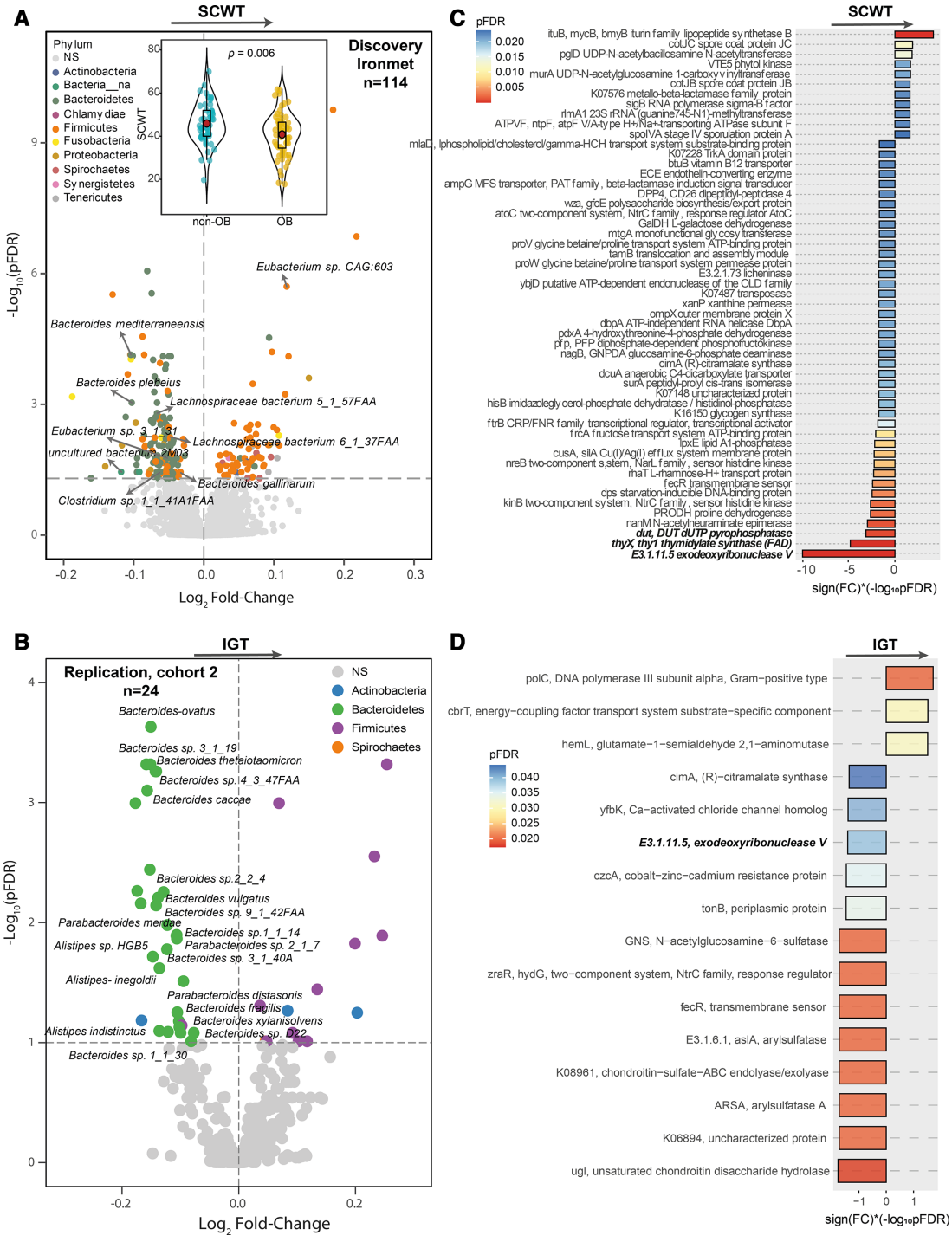

Figure 1 A microbiota taxonomic and functional signature is associated with inhibitory control. (A) Volcano plot of differential bacterial abundance (*p*FDR <0.05) associated with the Stroop Color Word Test (SCWT) as calculated by DESeq2 from shotgun metagenomic sequencing in the IRONMET cohort (n=114), adjusting for age, sex and education years. Fold change (FC) associated with a unit change in the SCWT and Benjamini-Hochberg adjusted p values (*p*FDR) are plotted for each taxon. Significantly different taxa are coloured according to phylum. In the same graph, the violin plots for the SCWT scores in patients with and without obesity are also shown. Differences between groups were analysed by a Wilcoxon tests. (B) Volcano plot of differential bacterial abundance (*p*FDR <0.1) associated with Iowa Gambling Test (IGT) as calculated by DESeq2 from shotgun metagenomic sequencing in an independent cohort (n=24), adjusting for age, sex and education years. (C) Manhattan-like plot of significantly expressed KEGG metagenome functions associated with the SCWT (*p*FDR <0.020) identified from DESeq2 analysis in the IRONMET cohort (n=114) adjusted for age, sex and educations. The $-\log_{10}$(*p*FDR) values are multiplied by the FC sign to take into account the direction of the association. Bars are coloured according to the *p*FDR. (D) Manhattan-like plot of significantly expressed KEGG metagenome functions associated with the IGT (*p*FDR <0.05) identified from DESeq2 analysis in an independent cohort (n=24) adjusted for age, sex and educations.

Table 2 Clinical and neuropsychological data of the human replication cohort

	Total population (n=24)	Without obesity (n=12)	With obesity (n=12)	P value
Age (years)	53.5 (44.3–57.8)	52 (39–58.3)	53.5 (48.5–57.8)	0.478
Females n (%)	15 (62.5)	7 (58.3)	8 (66.7)	0.673
Education (years)	16 (15–17)	17 (14–17)	16 (15–17)	1.9×10^{-5}
BMI (kg/m^2)	29.9 (23.2–45.1)	23.2 (21.3–25.5)	44.3 (38.6–47.7)	3.2×10^{-5}
Waist (cm)	96 (81–127)	82 (72.8–87.8)	127 (121–136)	7.1×10^{-5}
Fat mass (%)	37.4 (29.2–45.3)	29.6 (21.3–34.9)	45.2 (39.6–51.3)	2.8×10^{-4}
SBP (mmHg)	123.5 (115–136.8)	116 (108–121.3)	135.5 (124.5–148.3)	0.001
DBP (mmHg)	69.5 (64.3–78.8)	64.5 (59.5–69.5)	78 (69.3–150.8)	0.003
HDL-C (mg/dL)	56 (43–76)	67 (58.3–77)	44.5 (40–52.8)	0.043
Triglycerides (mg/dL)	73 (48.8–117.5)	57.5 (42.3–98.5)	93 (68.5–150.8)	0.069
FPG (mg/dL)	94.5 (84–103.8)	92 (84–102)	99 (85.3–103.8)	0.418
HbA1c (%)	5.5 (5.3–5.8)	5.5 (5.3–5.7)	5.7 (5.3–6.3)	0.147
hsCRP (mg/dL)	0.2 (0–0.5)	0.1 (0–0.2)	0.5 (0.2–0.8)	0.009
IGT (score)	45 (42–49)	44.5 (39–48.3)	45.5 (43.3–49.8)	0.271
WCST (score)	11 (11–12)	11 (10.5–12.5)	11 (11–12)	0.918

Results are expressed as number and frequencies for categorical variables, mean and SD for normal distributed continuous variables and median and IQR for non-normal distributed continuous variables. To determine differences between study groups, we used χ^2 for categorical variables, unpaired Student's t-test in normal quantitative and Mann-Whitney U test for non-normal quantitative variables. P value determines differences between subjects with obesity (BMI ≥30 kg/m^2) and without obesity (BMI 18.5–30 kg/m^2). BMI, body mass index; DBP, diastolic blood pressure; FPG, fasting plasma glucose; HbA1c, glycated haemoglobin; HDL-C, high-density lipoprotein cholesterol; hsCRP, high-sensitivity C reactive protein; IGT, Iowa Gambling Test; SBP, systolic blood pressure; WCST, Wisconsin Card Sorting Test.

associated with the SCWT. In addition, several functions related to vitamins involved in the folate and one-carbon metabolism, specifically vitamin B_6 (4-hydroxythreonine-4-phosphate dehydrogenase, *pdxA*), vitamin B_{12} (vitamin B_{12} transporter, *btuB*) and vitamin B_2 (5,6-dimethylbenzimidazole synthase, *bluB*; 3,4-dihydroxy 2-butanone 4-phosphate synthase, *ribBA*; riboflavin synthase, *ribE*), were also negatively associated with the SCWT.

Notably, the associations between the SCWT and bacterial composition were different in subjects with and without obesity. Negative associations with *Bacteroides nordii*, *Fusobacterium varium*, *Prevotella* sp CAG:279 and *Prevotella timonensis* were observed in subjects with obesity, while *Bacteroides ovatus* CAG:22, *Bacteroides* sp CAG:462, *Alistipes* sp CAG:157, *Rikenellaceae* sp and *Acidaminococcus* sp were associated negatively in subjects without obesity (figure 2A,B, online supplemental tables S4 and S5). In the latter, we also observed positive associations with *Roseburia* sp CAG:471, *Clostridum* sp CAG:632, *Pasteurellaceae* sp and *Butyrivibrio* sp CAG:318. Similarly, the associations at the functional level were also different in subjects with and without obesity. In particular, the association of the SCWT with the one-carbon metabolism-related functions *thyX* and *dut* was specifically significant among individuals without obesity (figure 2C, online supplemental table S7), white the link with exodeoxyribonuclease V was prominent among subjects with obesity (figure 2D, online supplemental table S8). Remarkably, most of these metagenomic composition and functional associations were replicated longitudinally after 1 year of follow-up (online supplemental figure S1D,E and tables S9 and S10). In addition to exodeoxyribonuclease V, *dut*, *thyX* and *kinB*, the SCWT performance at follow-up had a relatively strong negative association with nicotinamide phosphoribosyltransferase. The knockdown of this gene in mice has shown to recapitulate hippocampal cognitive phenotypes in old mice.[12]

We then used a multiple RF models-based ML variable selection strategy, as implemented in the Boruta R package, to identify plasma and faecal metabolites predictive of the SCWT. Among the plasma metabolites positively associated with SCWT performance, tryptophan and 4-hydroxyphenyllactic acid (4-HPLA, a tyrosine catabolite) were the most important (figure 3A,B). Tryptophan and tyrosine are the precursors for the synthesis of the neurotransmitters serotonin and dopamine, respectively. In healthy adults, low tryptophan levels were associated with a decrease in the Stroop interference effect,[13 14] although the results are somewhat inconsistent.[15 16] Previous research has also suggested that inhibitory control also relies on dopaminergic signalling.[17] Stimulation of dopamine D_2 receptors has shown to decrease Stroop interference.[18] A higher dopaminergic uptake was also linked to less WCST-related activation in the PFC.[19] Increased 4-HPLA acid levels may indicated that tyrosine is diverted from dopamine synthesis. Interestingly, 4-HPLA is a microbial-derived tyrosine catabolite that has shown to decrease reactive oxygen species production in both mitochondria and neutrophils.[20] Alterations in tryptophan and tyrosine metabolism in relation to SCWT performance were also observed in faecal samples (figure 3C,D). Hence, 5-hydroxyindoleacetic acid (5-HIAA), the end-product of serotonin metabolism, tyrosine itself and some microbial-derived tyrosine metabolites (2-phenylpropanoic acid) had consistent associations with the SCWT. The associations between the SCWT and alterations in tryptophan metabolism were replicated in the Imageomics cohort (n=970), where plasma levels of tryptophan and some microbial-derived tryptophan catabolites (indolepropionamide) were positively associated with the SCWT performance (figure 3F,G). Remarkably, these alterations in tryptophan metabolism were only observed in individuals with obesity in both the Ironmet (figure 4A-J) and Imageomics cohorts (figure 4K-M). This is in line with recent studies reporting alterations in tryptophan metabolic pathways in obesity in association with systemic inflammation.[21] A summary of these findings can be found in figure 5A.

The purine, thymidylate and methionine cycles encompass the one-carbon metabolism in the cytosol, which largely rely on B vitamins, specifically vitamins B_2, B_6, folate (B9) and vitamin B_{12}.[22] In agreement with alterations in the metagenomic functions involved in the two former cycles and one-carbon

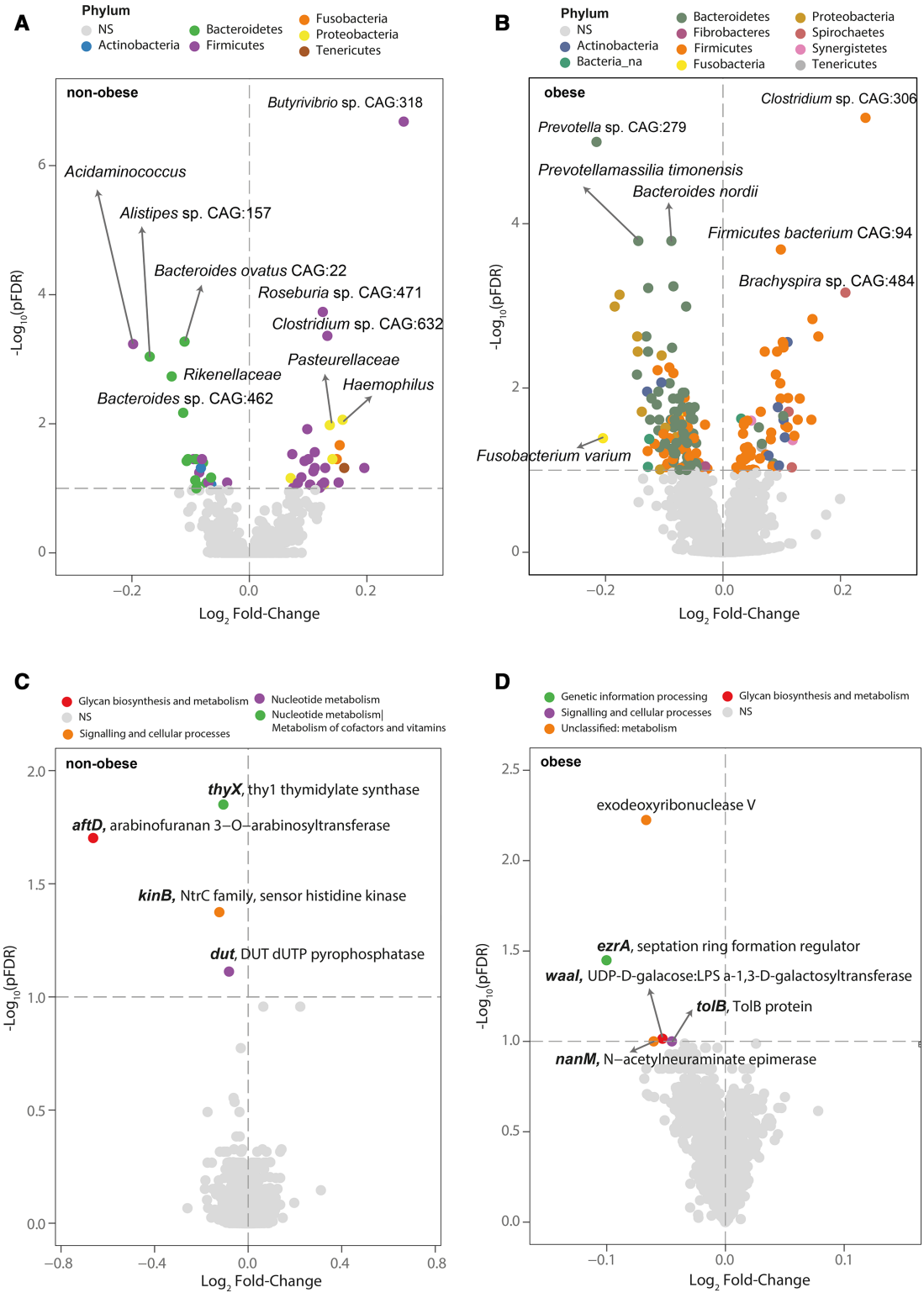

Figure 2 The microbiota taxonomic and functional signature linked to inhibitory control is modulated by obesity. (A, B) Volcano plot of differential expressed (*p*FDR <0.1) bacterial abundance and (C, D) bacterial functions associated with the Stroop Color and Word Test (SCWT) as calculated by DESeq2 from shotgun metagenomic sequencing in the patients without and with obesity from the IRONMET cohort, respectively, controlling for age, sex and education years. Fold change associated with a unit change in the SCWT and Benjamini-Hochberg adjusted p values (*p*FDR) are plotted for each taxon. Significantly different taxa are coloured according to phylum.

metabolism-related vitamins in relation to the SCWT scores, we found negative associations between the faecal levels of methionine and microbial-derived methionine catabolites (dimethyl sulfone)[23] in the Ironmet cohort (figures 3D,E and 5B). Interestingly, these alterations in the methionine cycle were only observed in individuals without obesity (figure 4E-I), who also had alterations in betaine (figure 4E), which serves as a methyl donor in the reaction converting homocysteine to methionine

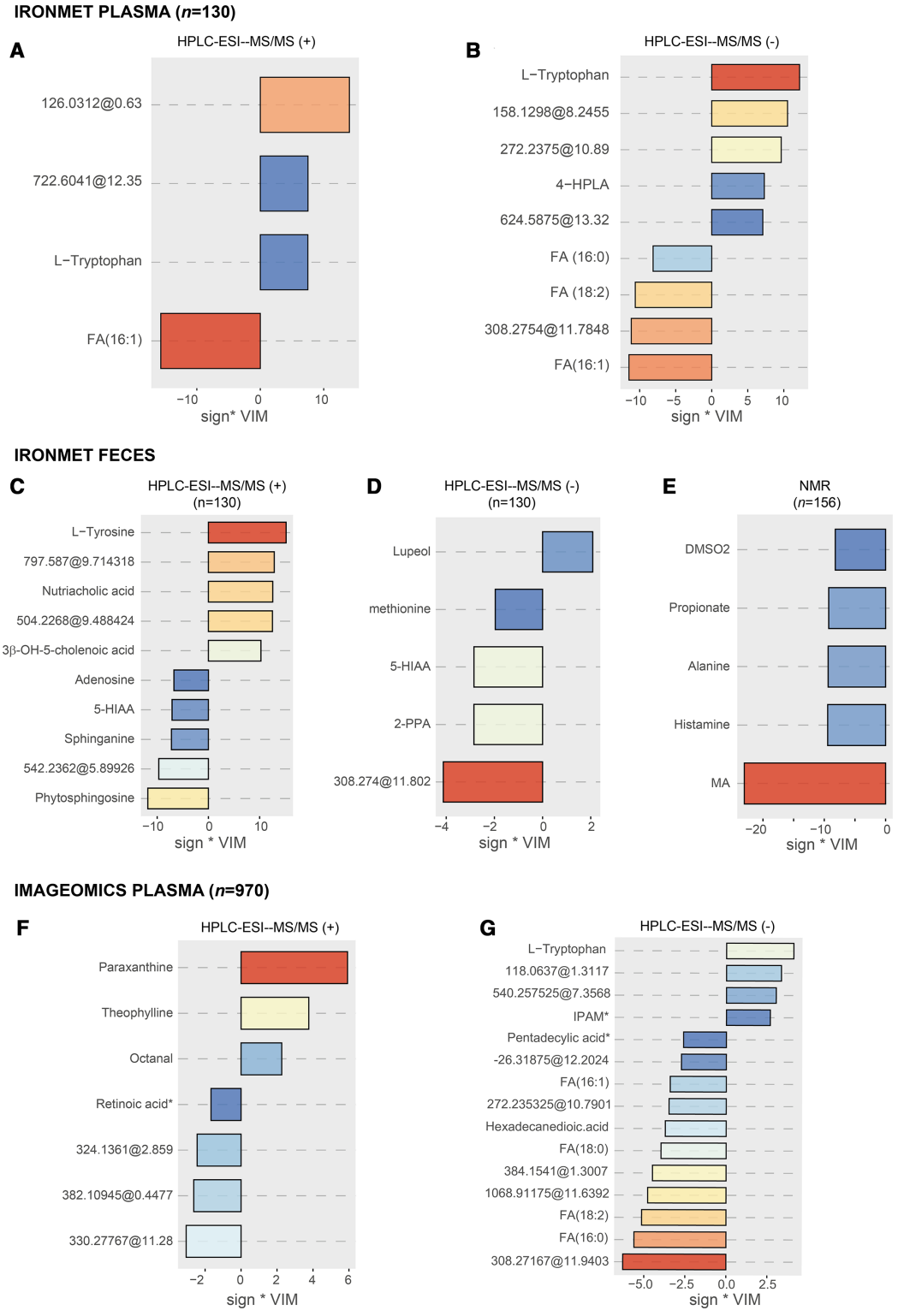

Figure 3 Plasma and faecal metabolomics linked to inhibitory control in the Ironmet and Imageomics cohorts. Bar plots of normalised variable importance measure (VIM) for the metabolites associated with the Stroop Color and Word Test (SCWT) in (A, B) plasma and (C–E) faecal samples identified by HPLC-ESI-MS/MS in positive mode (n=130), negative mode (n=130) and NMR (n=156), respectively, in the Ironmet cohort. Bar plots of VIM for the metabolites associated with the SCWT in plasma samples of the Imageomics cohort (n=970) identified by HPCL-ESI-MS/MS in (F) positive and (G) negative mode. In all cases, metabolites were identified using a multiple random forest-based machine learning variable selection strategy using the Boruta algorithm with 5000 trees and 500 iterations. All metabolites were identified based on exact mass, retention time and MS/MS spectrum, except those with (*) that were only identified based on exact mass and retention time. Unidentified metabolites are shown as exact mass at retention time. 2-PPA, 2-phenylpropanoic acid; 4-HPLA, 4-hydroxyphenyllactic acid; 5-HIAA, 5-hydroxyindoleacetic acid; DMSO2, dimethyl sulfone; FA, fatty acid; IPAM, indolepropionamide; MA, methylamine; TMA, trimethylamine.

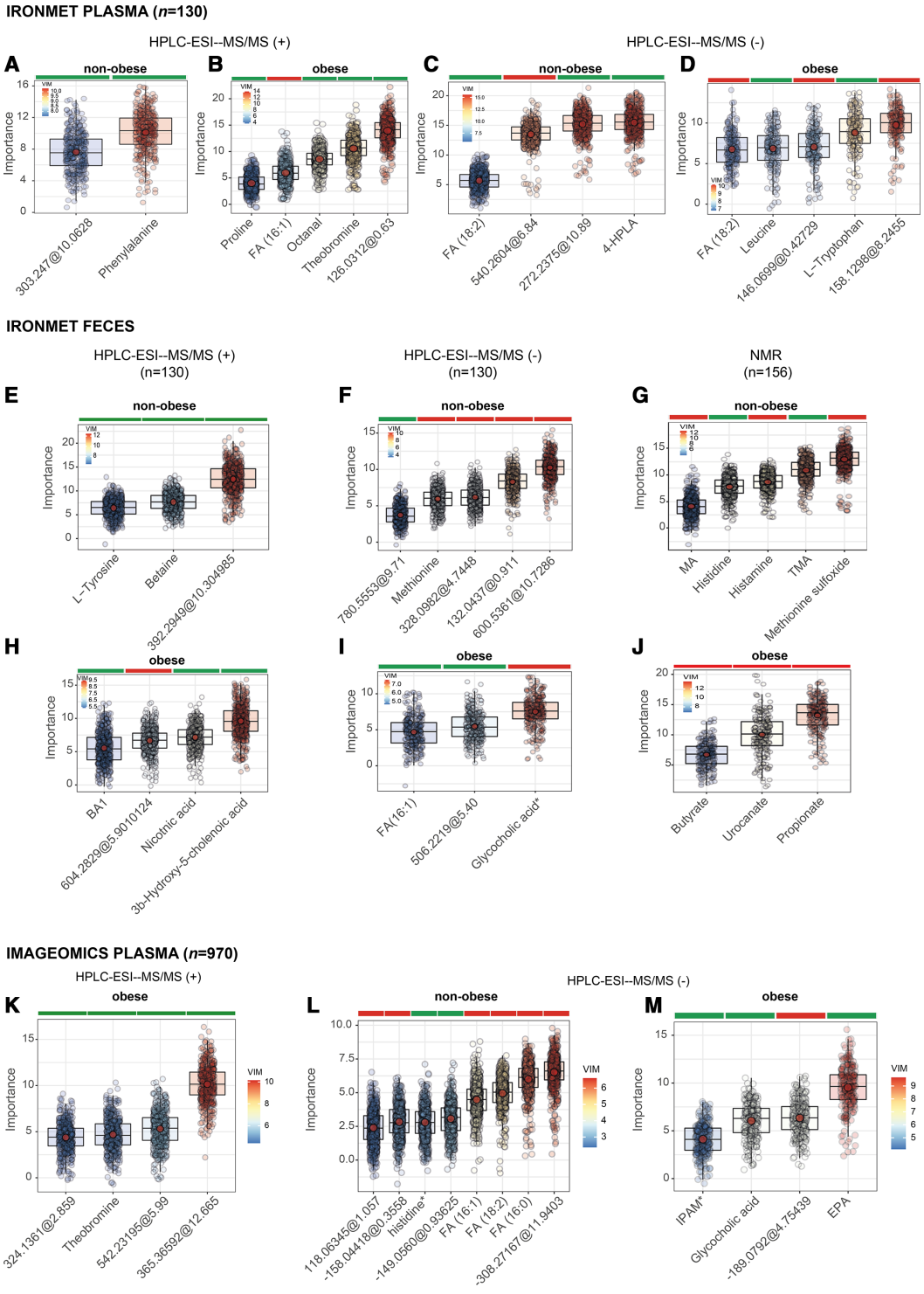

Figure 4 Plasma and faecal metabolomics linked to inhibitory control in the Ironmet and Imageomics cohorts according to obesity status. Bar plots of normalised variable importance measure (VIM) for the metabolites associated with the Stroop Color and Word Test (SCWT) in (A–D) plasma and (E–J) faecal samples identified by HPLC-ESI-MS/MS in positive mode (n=130), negative mode (n=130) and NMR (n=156), respectively, in the Ironmet cohort in patients with and without obesity. Bar plots of VIM for the metabolites associated with the SCWT in plasma samples of the Imageomics cohort (n=970) identified by HPCL-ESI-MS/MS in (K) positive and (L, M) negative mode in patients with and without obesity. The above colour bar indicates the sign of the association among the metabolites and the SCWT, with red indicating negative correlation and green positive correlation. In all cases, metabolites were identified using a multiple random forest-based machine learning variable selection strategy using the Boruta algorithm with 5000 trees and 500 iterations. All metabolites were identified based on exact mass, retention time and MS/MS spectrum, except those with (*) that were only identified based on exact mass and retention time. Unidentified metabolites are shown as exact mass at retention time. 4-HPLA, 4-hydroxyphenyllactic acid; BA1, Bile acid1: 4,4-dimethyl-5-α-cholesta-8,14-dien-3β-ol; EPA, eicosapentaenoic acid; FA, fatty acid; IPAM, indolepropionamide; MA, methylamine; TMA, trimethylamine.

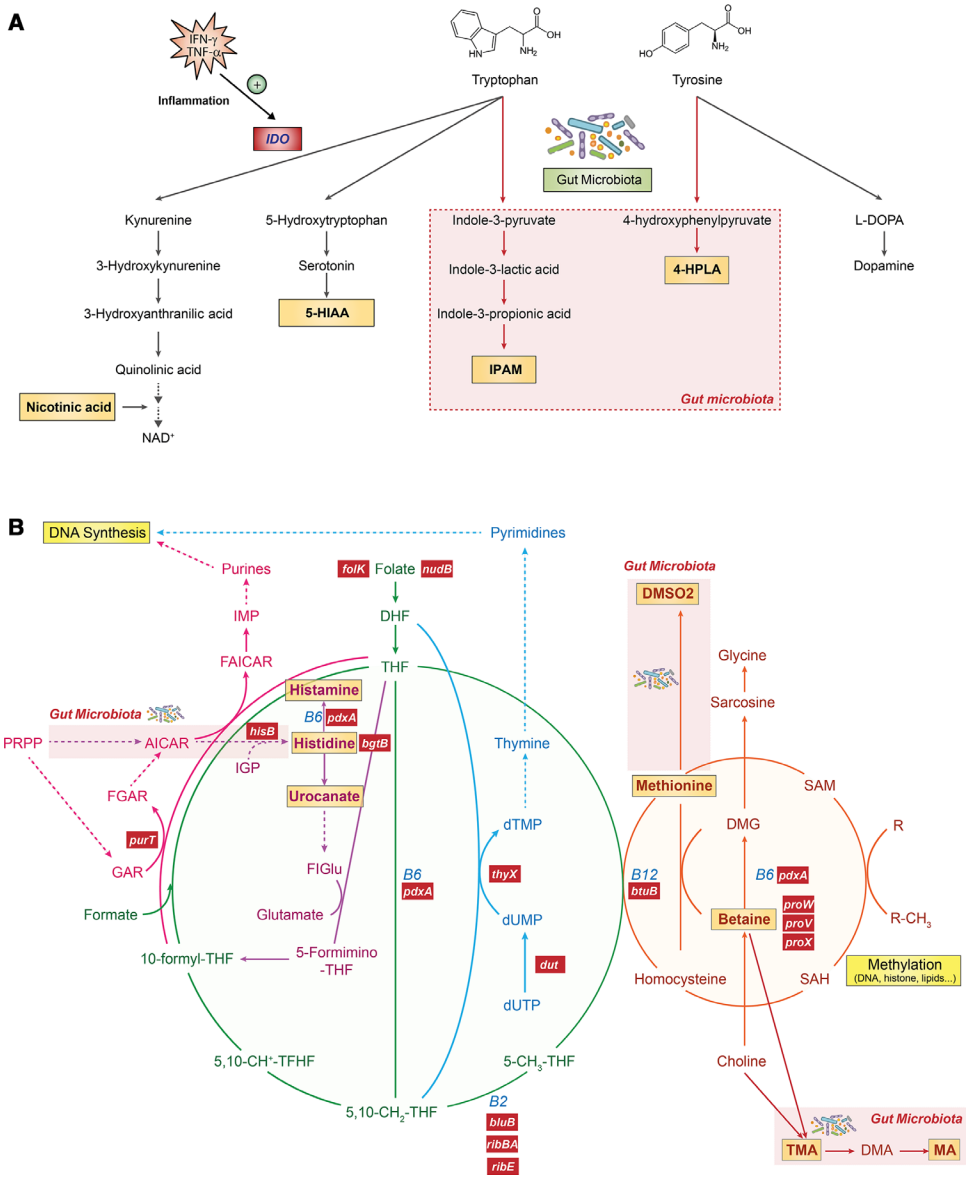

Figure 5 Main metabolic pathways involved in the associations among metagenomics, metabolomics and the Stroop Color and Word Test (SCWT). (A) Overview of the main catabolic pathways of tryptophan and tyrosine. Tryptophan and tyrosine are the precursors for the synthesis of the neurotransmitters serotonin and dopamine, respectively. The gut microbiota can also metabolise tryptophan and tyrosine to indoles and hydroxyphenolic acids, respectively. Dietary tryptophan is mostly metabolised via the Kynurenine pathway, which is activated by inflammation. (B) Overview of the folate-mediated one-carbon metabolism. The folate cycle (green) is required for the synthesis of DNA (pink and blue) as well as methylation reaction (DNA, proteins and lipids) through the methionine cycle (orange). Histidine (purple), choline and betaine are two sources of 1C units feeding into the one-carbon metabolism. Bacterial pathways have been shaded in red. Metabolites involved in the one-carbon metabolism and significantly associated with the SCWT are highlighted in bold in a yellow box. Bacterial functions participating in the one-carbon metabolism and significantly associated with the SCWT are highlighted in bold in a red box. AICAR, 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside; *bgtB*, arginine/lysine/histidine/glutamine transport system substrate-binding and permease protein; *bluB,* 5,6-dimethylbenzimidazole synthase; *btuB,* vitamin B_{12} transporter; DHF, dihydrofolate; DMA, dimethylamine; DMSO2, dimethylsulfone; *dut*, dUTP pyrophosphatase; FAICAR, 5-formamidoimidazole-4-carboxamide ribotide; FGAR, 5′-phosphoribosyl-N-formylglycineamide; FIGlu, N-Formimino-glutamate; *folK*, 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine diphosphokinase; GAR, 5′-phosphoribosylglycineamide; *hisB*, imidazoleglycerol-phosphate dehydratase; IGP, imidazole glycerol-phosphate; IMP, inosine 5′-monophosphate; MA, methylamine; *nudB*, dihydroneopterin triphosphate diphosphatase; *pdxA*, 4-hydroxythreonine-4-phosphate dehydrogenase; *proV*, glycine betaine/proline transport system ATP-binding protein; *proW*, glycine betaine/proline transport system permease protein; *proX*, glycine betaine/proline transport system substrate-binding protein; *purT*, phosphoribosylglycinamide formyltransferase 2; *ribBA*, 3,4-dihydroxy 2-butanone 4-phosphate synthase/GTP cyclohydrolase II; *ribE*, riboflavin synthase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; THF, tetrahydrofolate; *thyX*, thymidylate synthase; TMA, trimethylamine.

(figure 5B). These results are in agreement with the observed associations among one-carbon metabolism related metagenomic functions (*thyX* and *dut*) and SCWT only in individuals without obesity. These alterations in betaine levels are also consistent with the significant associations between the SCWT and bacterial betaine transport functions (*proW*, betaine/proline transport system permease protein; *proV* betaine/proline transport system ATP-binding protein; *proX*, betaine/proline transport

system substrate-binding protein) (online supplemental table S6, figure 5B). It is also worth noting that both betaine and its precursor (choline) can be metabolised by the gut microbiota to trimethylamine (TMA) and eventually methylamine (MA), which were also only associated with the SCWT in individuals without obesity (figure 4G).

Another one-carbon donor that contributes to the pool of 1C units in the folate-bound one-carbon metabolism is histidine (figure 5B).[24] Again, in agreement with the above findings, plasma histidine was positively associated with SCWT in individuals without obesity in the Imageomics cohort (figure 4L). Histidine is an important precursor of histamine, which acts as a neurotransmitter in the brain and has been involved in anxiety, stress response, learning and memory.[25] Consistently, we also found negative associations between faecal histamine and SCWT (figure 4G). Notably, vitamin B_6 acts as a cofactor for histidine decarboxylase, the sole enzyme responsible for the conversion of histidine to histamine. Histidine can alternatively be converted to urocanic acid, which was significantly associated with the SCWT performance in individuals with obesity (figure 4J). Urocanate has recently shown to cross the blood-brain barrier and promote glutamate biosynthesis and release in various brain regions, thereby enhancing learning and memory.[26] Therefore, histidine might be metabolised differently in obesity.

We further analysed the associations among the metabolome and those bacterial functions most negatively associated with the SCWT. ML analyses revealed consistent associations with tryptophan, 4-HPLA, 3-methyl-2-oxovalerate, FA(16:1) and FA(18:2) (online supplemental figure S2 A–C). These functions also had positive associations with betaine, reflecting the involvement of these functions in the one-carbon metabolism.

We next questioned whether these associations could have a structural correlate. Both the anterior cingulate and the PFC are thought to be critically involved in the performance of the Stroop task. In agreement with previous findings,[27] we found that the grey matter volume of the ACC was positively linked to SCWT scores (figure 6A,B). Interestingly, in line with inhibitory control-bacterial relationships, we found negative associations of different *Bacteroides* sp, *Anaerovibrio* sp RM50 and *Selenomonas* sp *Oral taxon 478* with ACC volume (figure 6C, online supplemental table S11). Conversely, bacterial species positively associated with better inhibitory control were also directly linked to ACC volume (*Clostridium* sp CAG:226, *Roseburia* sp CAG:182 and *Ruminococcus* sp CAG:417). The strongest negative associations between the bacterial functions and the ACC volume were with *kinB*, *dut* and *thyX*, that were precisely the bacterial function negatively associated with SCWT in subjects without obesity (figure 6D). Other bacterial functions involved in pyrimidine metabolism were positively linked (pseudouridylate synthase, *psuG*; TYMP thymidine phosphorylase, *deoA*; 5'-deoxynucleotidase, *yfbR*) (figure 6D,E, online supplemental table S12). An increased frequency of red meat consumption was associated with those bacterial functions negatively related to inhibitory control (online supplemental figure S3A). Of note, *dut* and *kinB* had the strongest negative associations with the ACC volume at baseline, and after 1 year of follow-up (online supplemental figure S3B).

The correlates of cognitive flexibility of humans and mice are often measured using reversal learning (RL) paradigm experiments, in which subjects need to overcome established associations and learn new ones based on feedback.[28] RL also provides a measure of inhibition and impulsiveness.[29] In a faecal microbiota transplantation experiment, microbiota from n=22 humans donors (n=11 with BMI <30 kg/m^2 and n=11 with BMI ≥30 kg/m^2 matched for age, sex and education years) was orally delivered to recipient mice (figure 7A, online supplemental table S13). Mice receiving the microbiota from the subjects with obesity with lower inhibitory control had significantly lower RL performance at day 18 evaluated as the number of lever-presses in the inverted active lever (figure 7A). Remarkably, several human donor's bacterial species (figure 7B, online supplemental table S1) and functions (figure 7C, online supplemental table S6) associated with the SCWT in humans were also associated with the recipient's mice RL task performance.

PFC activity is known to affect inhibitory control, and inhibitory control-related activity in regions of the PFC have been found to correlate inversely with BMI and weight increase.[30 31] Therefore, in an independent faecal microbiota transplantation experiment, we performed an RNA sequencing of the mPFC of mice that received microbiota from human donor's with either low or high SCWT scores matched for age, gender and education years (figure 7D, online supplemental table S14). DESeq2 analyses adjusted for donor's age, sex and education years, revealed several mPFC genes from recipient's mice associated with the SCWT-related human's donor bacterial functions (figure 7E,F, online supplemental table S15 and S16). In particular, *dut* had significative positive associations with the expression of *Kcne2*, *Prlr*, *Folr1*, *Cldn2*, *Slc4a5*, *Sostdc1* and borderline associations (*p*FDR <0.11) with *Tmem72, F5* and *Ttr* (figure 7E). Remarkably, all these genes were found among the top 25 genes changing the hippocampal expression after contextual fear conditioning.[32] Enrichment analysis of differentially expressed genes based on GO revealed over-representation of biological processes related to neuron development and histone methylation (online supplemental figure S4A). The genes involved in these processes included *Folr1, Mecp2*, *Auts2, Mfrp* and *Hipk1*. It is particularly noticeable, the significant association between this donor's bacterial function involved in folate-mediated one-carbon metabolism and the expression of the folate receptor 1 (*Folr1*) and methyl-CpG binding protein 2 (*Mecp2*) in the recipient mice mPFC. This is in agreement with our previous findings and further highlights the key role of the one-carbon metabolism and its involvement in DNA methylation. The *Mecp2* has a well-established function in neurodevelopment[33] and has also been linked to autism and Alzheimer's disease.[34] On the other hand, exodeoxyribonuclase V was significantly associated with the expression of 152 PFC genes, with transthyretin having the strongest FC by far (figure 7F). Ttr is one of the major amyloid β peptide-binding proteins acting as a neuroprotector in Alzheimer's disease.[35] This bacterial function also had a significant association with methylenetetrahydrofolate dehydrogenase 1-like (*Mthfd1l*) gene, which encodes for an enzyme that has an important role in folate-mediated one-carbon metabolism. Deletion of one allele of *Mthfd1* resulted in impaired cue-conditional learning in mice.[36] Finally, enrichment analyses based on reactome pathways revealed a network of pathways related to netrin signalling, which has recently shown to play an important role in synaptic plasticity and memory formation.[37]

Next, we also searched for relevant genes in the PFC of recipient's mice that were able to predict the human's donor SCWT using an ML-based variable selection strategy. After application of the Boruta algorithm, we identified *Ms4a4a* and the monocarboxylic acid transporter 12 (*Slc16a12*) as the main predictors of SCWT (figure 7G). Interestingly, the later gene has been recently described to be linked to folate status.[38] Monocarboxylate transporter transports lactate to the brain and promotes neurogenesis.[39] In addition, this is the transporter for creatine. Spontaneous mutations in creatine transporters and creatine

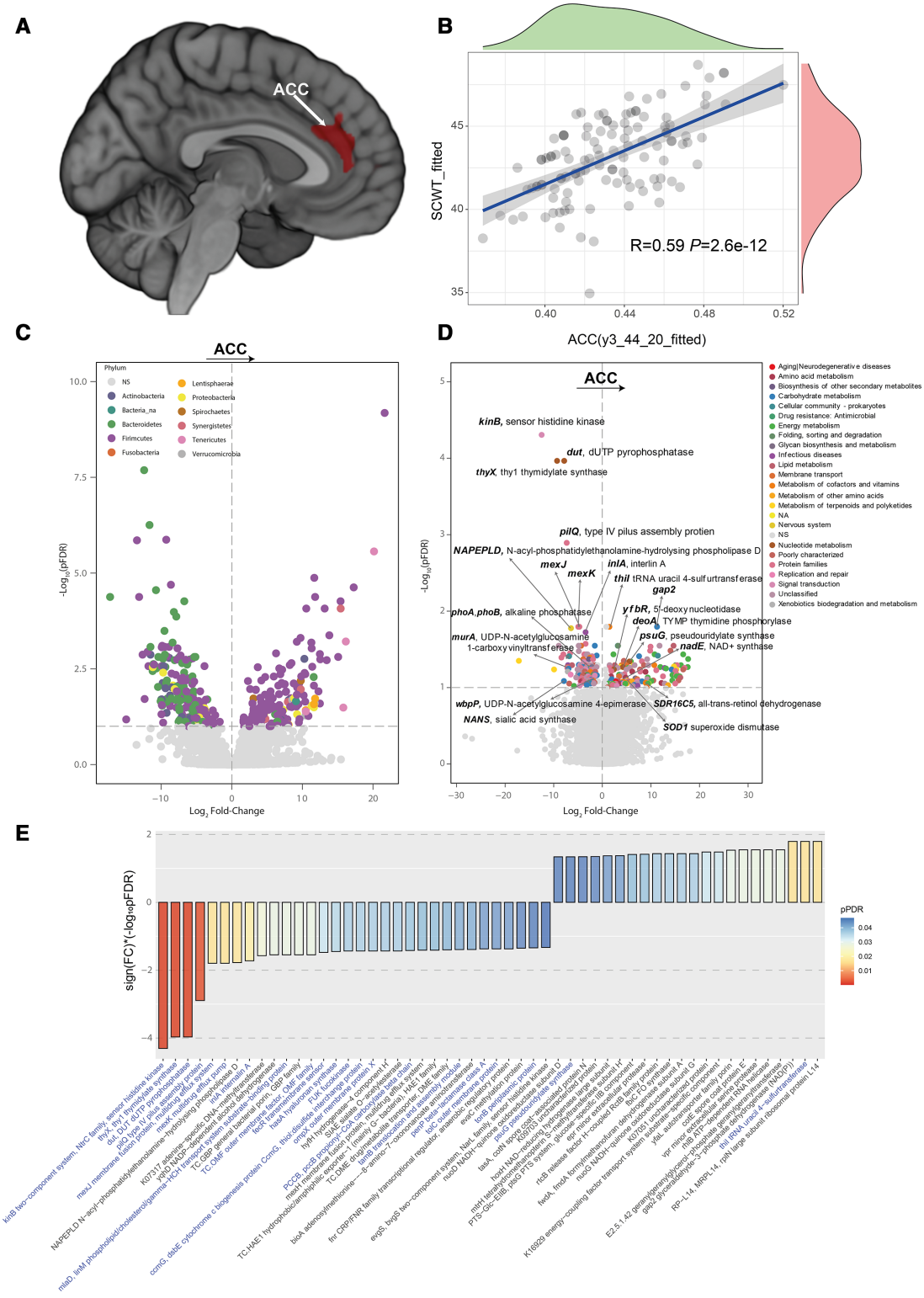

Figure 6 The gut microbiota is associated with the brain area involved in inhibitory control. (A, B) The anterior cingulate cortex (ACC) volume was positively associated with the Stroop Color and Word Test (SCWT) in the Ironmet cohort (n=95) after controlling for age, sex, education years and total intracranial volume. (C) Volcano plots of differential bacterial abundance and (D) KEGG metagenome functions associated with the ACC volume as calculated by DESeq2 controlling for previous covariates. Fold change (FC) associated with a unit change in the corresponding volumes and Benjamini-Hochberg adjusted p values (*p*FDR) are plotted for each taxon. (E) Manhattan-like plot of significantly expressed KEGG metagenome functions associated with the ACC volume highlighting those bacterial functions also associated with the SCWT in blue. The $-\log_{10}$(pFDR) values are multiplied by the FC sign to take into account the direction of the association. Bars are coloured according to the *p*FDR.

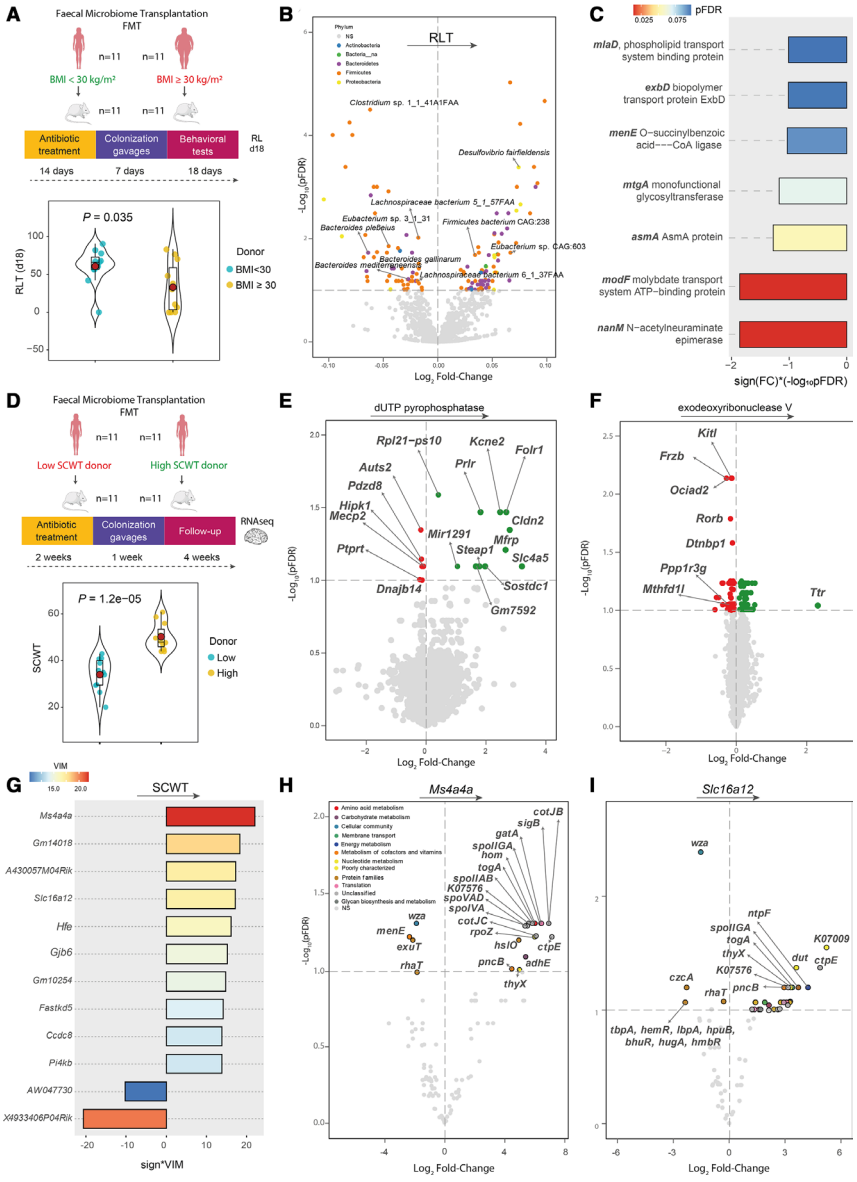

Figure 7 Faecal microbiota transplantation (FMT) mice studies. (A) Experimental design for the first FMT study. The microbiota from human donors without obesity (body mass index (BMI) <30 kg/m^2, n=11) and with obesity (BMI ≥30 kg/m^2, n=11) was delivered to recipient mice pretreated with antibiotics for 14 days. Reversal learning tests (RLT) were performed after 18 days. Violin plot for the recipient's mice RLT scores at 18 days based on human donor obesity status. (B) Volcano plot of differential human donor bacterial abundance associated with the recipient's mice RLT at day 18, identified from DESeq2 analysis controlling for donor's age, sex and education years. Fold change (FC) associated with a unit change in the corresponding memory test and Benjamini-Hochberg adjusted p values (*p*FDR) are plotted for each taxon. Significantly different taxa are coloured according to phylum. (C) Manhattan-like plot showing only the significantly expressed KEGG metagenome functions associated with the recipient's mice RLT (*p*FDR <0.1) that were also associated with the Stroop Color and Word Test (SCWT) in humans. The $-\log_{10}$(pFDR) values are multiplied by the FC sign to take into account the direction of the association. Bars are coloured according to the *p*FDR. (D) Experimental design for the second FMT study. The microbiota from human donors with low (n=11) and high (n=11) SCWT scores was delivered to recipient mice pretreated with antibiotics for 14 days. RNA sequencing of the medial prefrontal cortex (mPFC) was performed after 4 weeks. Violin plots for the SCWT according to the human donor scores. (E) Volcano plots of recipient's mice differential mPFC genes associated (*p*FDR <0.1) with the human's donor metagenome functions dUTP pyrophosphatase and (F) exodeoxyribonuclease V controlling for donor's age, sex and education years. FC associated with a unit change in the expression of the corresponding bacterial function and the Benjamini-Hochberg adjusted p values (*p*FDR) are plotted for each gene. (G) Bar plot of the normalised variable importance measure (VIM) for the recipient's mice mPFC genes associated with the human donor's SCWT identified by machine learning using multiple random forest-based variable selection strategy with the Boruta algorithm with 5000 trees and 500 iterations. (H) Volcano plot of differential human donor's KEGG metagenome functions associated with recipient's mice *Ms4a4a* and (I) *Slc16a12* genes. FC associated with a unit change in the expression of both genes and Benjamini-Hochberg adjusted p values (*p*FDR) are plotted for each metagenome function.

transporter knockout mice show impairments of short-term and long-term memory,[40] and severe deficits in cognitive function.[41] Recently, *Ms4a4a* has also been identified as a key modulator of soluble TREM2 and Alzheimer's disease risk.[42] We then used DESeq2 to identify those SCWT-related bacterial functions that were associated with the expression of these two genes (figure 7H,I, online supplemental tables S17 and S18). Interestingly, both genes were significantly associated with *dut*

and *thyX*, which we had identified as the main contributors the SCWT.

Inhibitory control, a fundamental component of executive function,[2] overrules automatic intentions to directly respond to stimuli without thought.[3] We here describe multiple interactions among the gut microbiota taxonomy and functionality, and plasma and faecal-microbiota metabolites, with inhibitory control in humans with obesity that were partially transmissible to mice. This may have therapeutic implications for the disengagement of ongoing behaviours, including the suppression of impulsive food reward-related choices in subjects with obesity.[3]

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Acknowledgements We are indebted to the subjects involved in this project. We also thank Emili LosHuertos and Oscar Rovira for their help in the recruitment of the subjects.

Contributors MA-R and JM-P researched the data, performed part of the statistical analysis and wrote the manuscript; OC-R wrote and performed the statistical analysis of the MRI and neuropsychological data; JAO-S, AB and RM performed or analysed the experiments in mice and contributed to write the corresponding parts of the paper that are associated with the mice data; GB and CB researched the MRI data; CC performed the neuropsychological examination. AC-N determined the mice gene expression in hippocampus and performed the prefrontal gene expression analysis. VP-B and AM contributed with the determination and analysis of the microbiota; XF-R performed part of the statistical analysis; JP, JG-O, RR, SP, RB, JC-V, JS, JB, JG, LR-T and WR contributed to the discussion and reviewed the manuscript. MJ, RP, JS and MP-O performed the metabolomics analyses and contributed to write the corresponding parts of the paper that are associated with the metabolomics data. JMF-R carried out the conception and coordination of the study, performed the statistical analysis and wrote the manuscript. All authors participated in final approval of the version to be published. JMF-R is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data.

Funding This work was partially supported by research grants FIS (PI15/01934 and PI18/01022) from the Instituto de Salud Carlos III from Spain, SAF2015-65878-R and #AEI-SAF2017-84060-R-FEDER from Ministry of Economy and Competitiveness, Prometeo/2018/A/133 from Generalitat Valenciana, Spain; and also by Fondo Europeo de Desarrollo Regional (FEDER) funds, European Commission (FP7, NeuroPain #2013-602891), the Catalan Government (AGAUR, #SGR2017-669, ICREA Academia Award 2015), the Spanish Instituto de Salud Carlos III (RTA, #RD16/0017/0020), the Spanish Ministry of Science, Innovation and Universities (RTI2018-099200-B-I00), the Catalan Goverment (Agency for Management of University and Research Grants [2017SGR696] and Department of Health [STL002/16/00250]; the European Regional Development Fund (project No. 01.2.2-LMT-K-718-02-0014) under grant agreement with the Research Council of Lithuania (LMTLT); and the Project ThinkGut (EFA345/19) 65% co-financed by the European Regional Development Fund (ERDF) through the Interreg V-A Spain-France-Andorra programme (POCTEFA 2014-2020). MA-R is funded by a predoctoral Río Hortega contract from the Instituto de Salud Carlos III (ISCIII, CM19/00190), co-funded by the European Social Fund "Investing in your future". OC-R is funded by the Miguel Servet Program from the Instituto de Salud Carlos III (ISCIII CP20/00165), co-funded by the Europeran Social Fund "Investing in your future". JM-P is funded by the Miguel Servet Program from the Instituto de Salud Carlos III (ISCIII CP18/00009), co-funded by the European Social Fund "Investing in your future". JS is funded by a predoctoral PERIS contract (SLT002/16/00250) from the Catalan Government. MJ is a professor under the "Serra Hunter" programme (Generalitat de Catalunya).

Competing interests None declared.

Patient consent for publication Not required.

Ethics approval The institutional review board—Ethics Committee and the Committee for Clinical Research (CEIC) of Dr Josep Trueta University Hospital (Girona, Spain) approved the study protocol and informed written consent was obtained from all participants. The Ageing Imageomics Study protocol was approved by the ethics committee of the Dr Josep Trueta University Hospital.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement All data relevant to the study are included in the article or uploaded as supplementary information.

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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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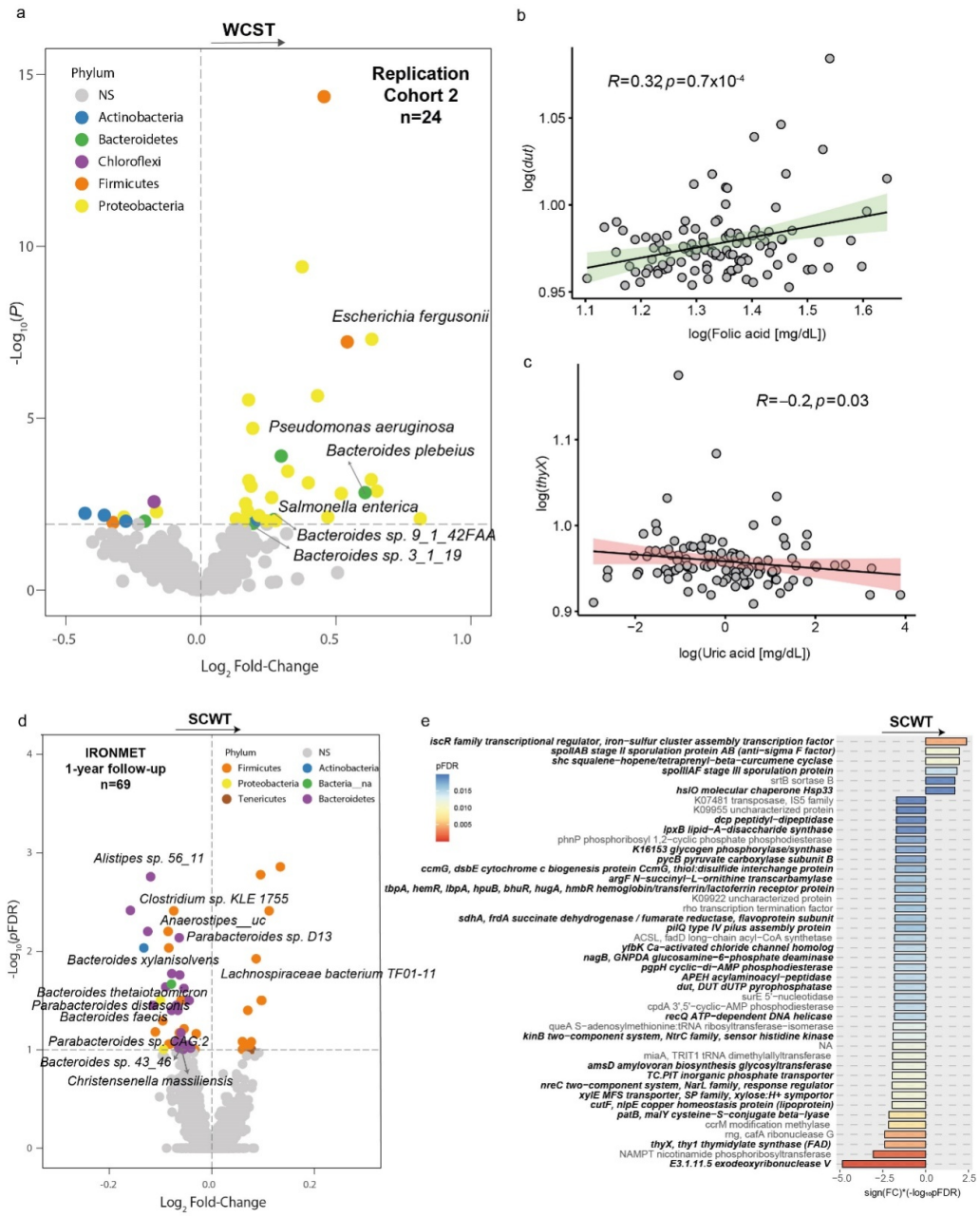


Fig. S1. a) Volcano plot of differential bacterial abundance associated with the Wisconsin Card Sorting Test (WCST) as calculated by DESeq2 from shotgun metagenomic sequencing in an independent cohort (*n*=24), controlling for age, sex, and education years. Fold change associated with a unit change in the WCST and Benjamini-Hochberg adjusted *p*-values (*p*FDR) are plotted for each taxon. Significantly different taxa are coloured according to phylum. **b)** Partial Spearman correlation controlling for age, sex, and education years, between the plasma levels of folic acid

and the KEGG metagenome function *dut.* **c)** Partial Spearman correlation controlling for age, sex, and education years, between the plasma levels of uric acid and the KEGG metagenome function *thyX.* **d)** Volcano plot of differential bacterial abundance associated with the Stroop Colour Word Test (SCWT) as calculated by DESeq2 from shotgun metagenomic sequencing in the IRONMET cohort after 1-year of follow-up (n=69), controlling for age, sex, and education years. Fold change associated with a unit change in the SCWT and Benjamini-Hochberg adjusted p-values (pFDR) are plotted for each taxon. Significantly different taxa are coloured according to phylum. Only bacterial species that were also significantly associated with the SCWT at baseline and in the same direction are highlighted. **e)** Manhattan-like plot of significantly expressed KEGG metagenome functions associated with the SCWT identified from DESeq2 analysis in the IRONMET cohort after 1-year follow-up (n=69) adjusted for age, sex, and educations. The $-\log_{10}(p\text{FDR})$ values are multiplied by the fold change (FC) sign to take into account the direction of the association. Bars are coloured according to the pFDR. Metagenomic functions that were also associated with the SCWT at baseline are highlighted in bold.

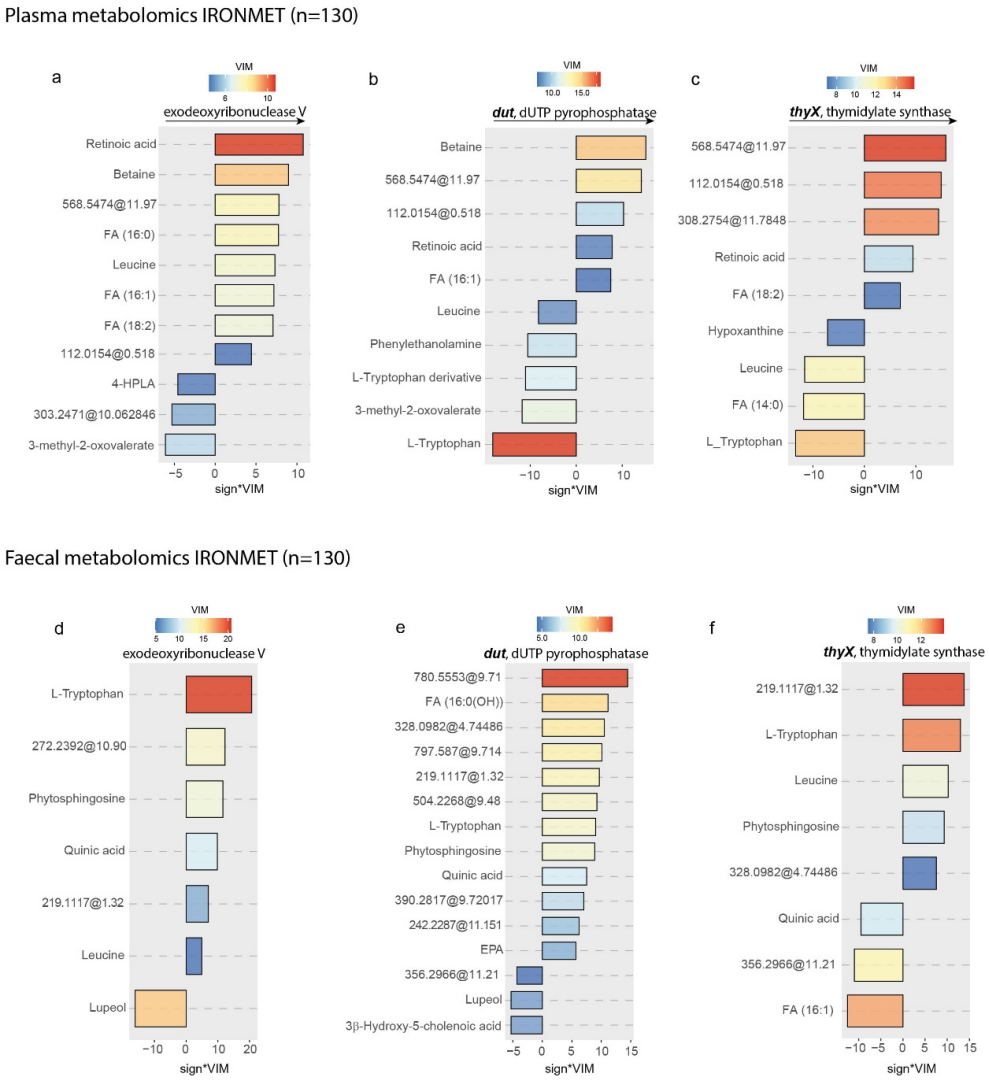
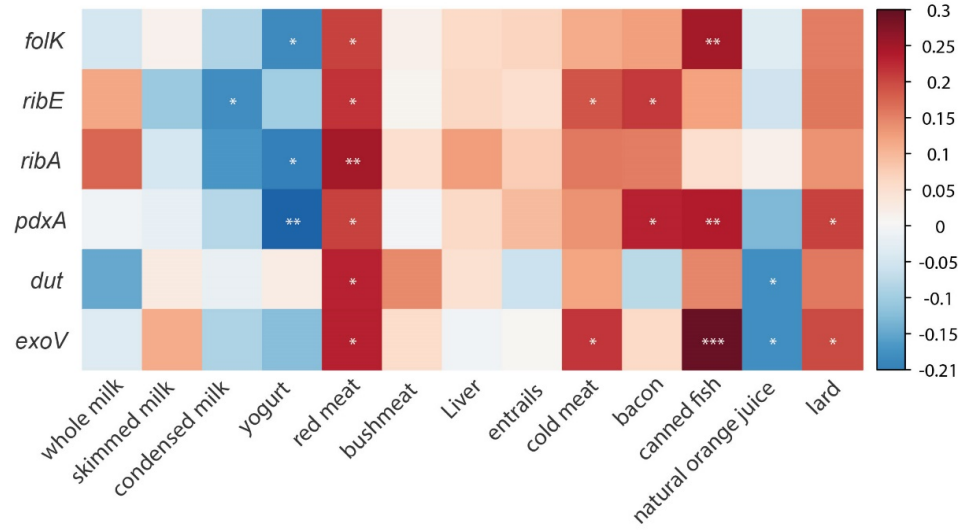


Fig. S2. Plasma and faecal metabolomics linked to metagenome functions associated with the SCWT in the Ironmet cohort. Barplots of the normalized variable importance measure (VIM) for the metabolites associated with the KEGG metagenome functions exodeoxyribonuclease V, dUTP pyrophosphatase (*dut*), and thymidylate synthase (*thyX*) in **a-c)** plasma and **d-f)** faecal samples, respectively. Significant metabolites were identified through a multiple random forest-based machine learning variable selection strategy using the Boruta algorithm with 5000 trees and 500 iterations. All metabolites were identified based on exact mass, retention time and MS/MS spectrum, except those with (*) that were only identified based on exact mass and retention time. Unidentified

metabolites are shown as exact mass@retention time. 4-HPLA, 4-hydroxyphenyllactic acid; EPA, eicosapentaenoic acid; FA, fatty acid.

a


folK
ribE
ribA
pdxA
dut
exoV
whole milk
skimmed milk
condensed milk
yogurt
red meat
bushmeat
Liver
entrails
cold meat
bacon
canned fish
natural orange juice
lard
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
-0.1
-0.15
-0.21

b

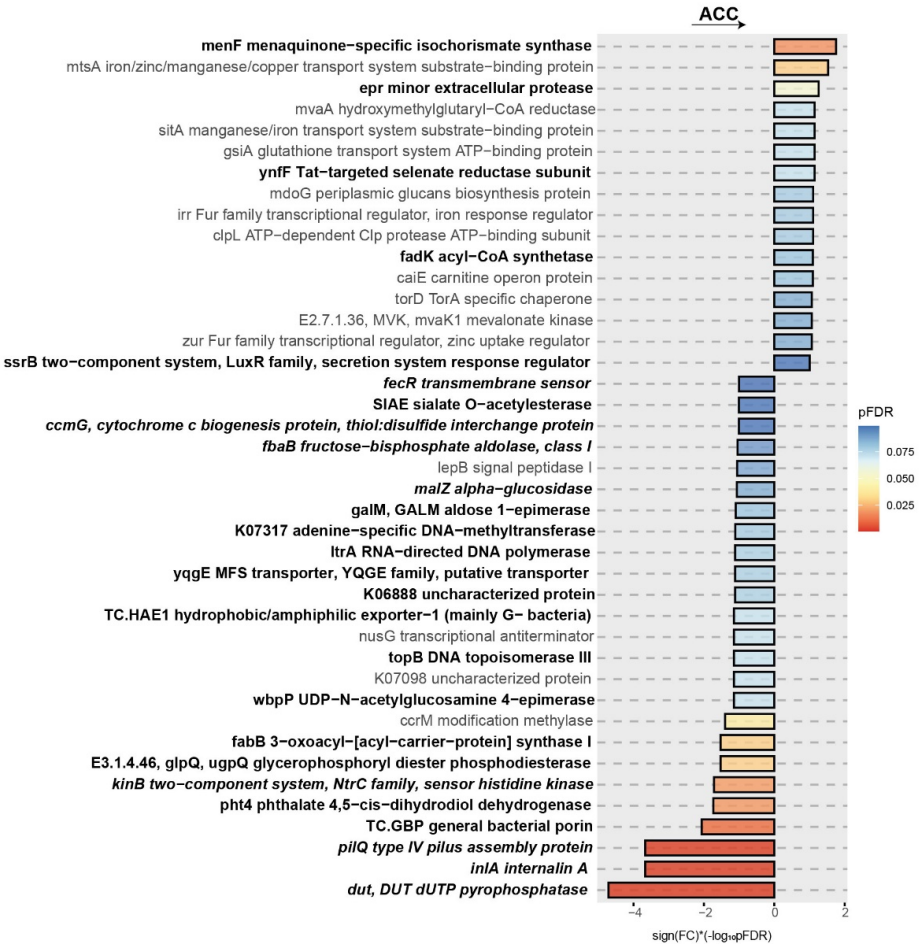

ACC
menF menaquinone−specific isochorismate synthase
mtsA iron/zinc/manganese/copper transport system substrate−binding protein
epr minor extracellular protease
mvaA hydroxymethylglutaryl−CoA reductase
sitA manganese/iron transport system substrate−binding protein
gsiA glutathione transport system ATP−binding protein
ynfF Tat−targeted selenate reductase subunit
mdoG periplasmic glucans biosynthesis protein
irr Fur family transcriptional regulator, iron response regulator
clpL ATP−dependent Clp protease ATP−binding subunit
fadK acyl−CoA synthetase
caiE carnitine operon protein
torD TorA specific chaperone
E2.7.1.36, MVK, mvaK1 mevalonate kinase
zur Fur family transcriptional regulator, zinc uptake regulator
ssrB two−component system, LuxR family, secretion system response regulator
fecR transmembrane sensor
SIAE sialate O−acetylesterase
ccmG, cytochrome c biogenesis protein, thiol:disulfide interchange protein
fbaB fructose−bisphosphate aldolase, class I
lepB signal peptidase I
malZ alpha−glucosidase
galM, GALM aldose 1−epimerase
K07317 adenine−specific DNA−methyltransferase
ltrA RNA−directed DNA polymerase
yqgE MFS transporter, YQGE family, putative transporter
K06888 uncharacterized protein
TC.HAE1 hydrophobic/amphiphilic exporter−1 (mainly G− bacteria)
nusG transcriptional antiterminator
topB DNA topoisomerase III
K07098 uncharacterized protein
wbpP UDP−N−acetylglucosamine 4−epimerase
ccrM modification methylase
fabB 3−oxoacyl−[acyl−carrier−protein] synthase I
E3.1.4.46, glpQ, ugpQ glycerophosphoryl diester phosphodiesterase
kinB two−component system, NtrC family, sensor histidine kinase
pht4 phthalate 4,5−cis−dihydrodiol dehydrogenase
TC.GBP general bacterial porin
pilQ type IV pilus assembly protein
inlA internalin A
dut, DUT dUTP pyrophosphatase
pFDR
0.075
0.050
0.025
−4
−2
0
2
sign(FC)*(-log10pFDR)

Fig. S3. a) Correlations among selected KEGG metagenomes functions and dietary items. **b)**Manhattan-like plot of significantly expressed KEGG metagenome functions associated with the ACC volume after 1-year follow-up in the Ironmet cohort (*n*=60). The -$\log_{10}$(pFDR) values are multiplied by the fold change (FC) sign to take into account the direction of the association. Bars are coloured according to the pFDR. Metagenomic functions that were also associated with the ACC volume at baseline are highlighted in bold, and those also associated with the SCWT at baseline at highlighted in italics.

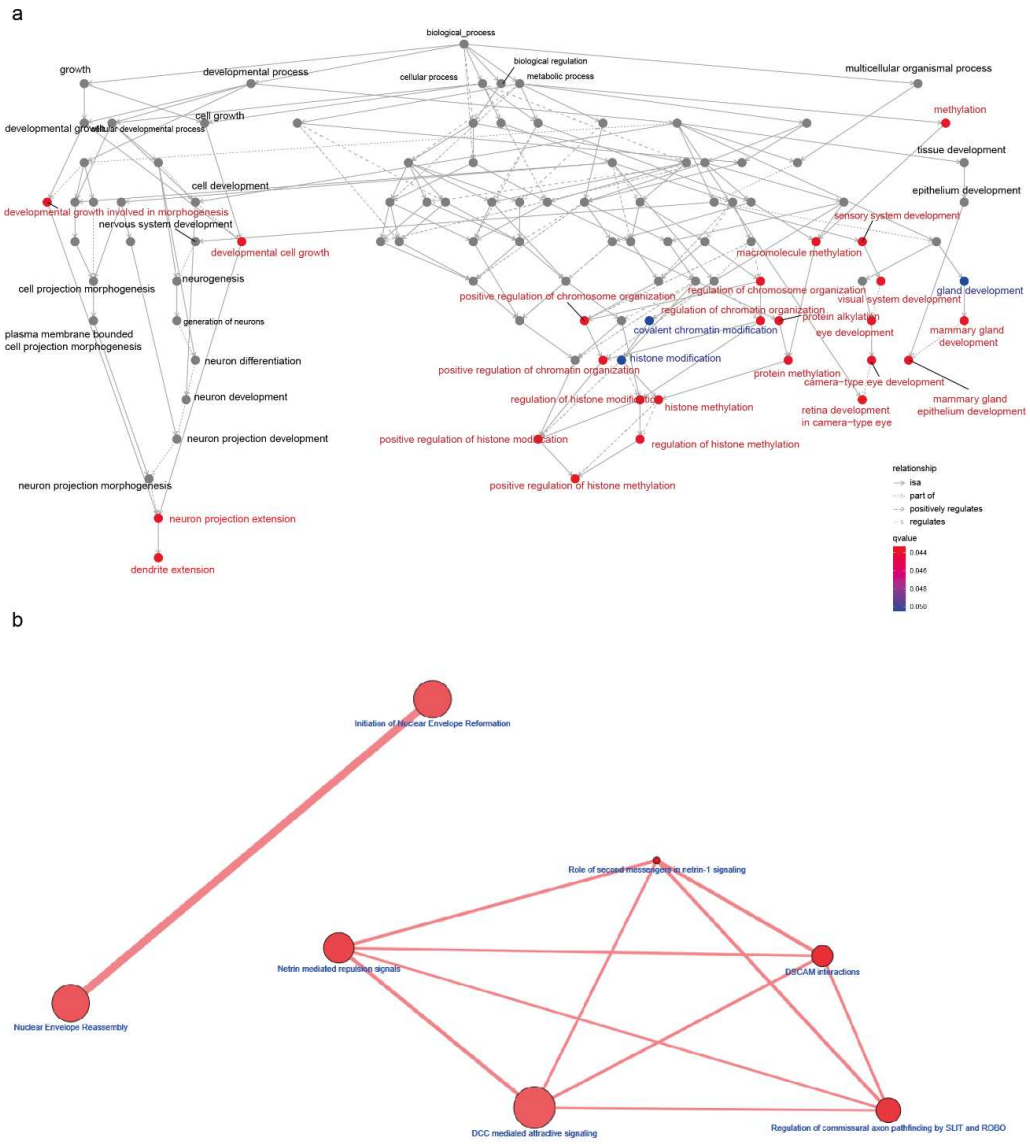


Fig S4. Over-representation analysis from significant recipient mice PFC genes associated with the donors metagenome functions. a) Over-represented biological processes from Gene Ontology based on significant genes linked to the donors dUTP pyrophosphatase (*dut*). Over-represented terms are shown in color, with red indicating higher significance. **b)** Over-represented Reactome pathways from significant genes linked to the donors exodeoxyribonuclease V. Each node is a predefined gene set. The node size reflects the size of the gene set and the node color its *P*-value. An edge denotes shared genes between sets. The edge width reflects the size of this overlap and the color reflects the number of genes from the input list contained in the overlap.

6. GENERAL DISCUSSION

This thesis comprises two original papers which have revealed the substantial role of the gut microbiome in obesity-associated cognitive impairment. The successive sections summarize the principal results; strengths and limitations; practical implications and future recommendations.

6.1. Global discussion of main findings

Obesity and cognitive decline are public health problems nowadays. Unfortunately, there are still gaps in their pathophysiology and therefore, in their treatment. Furthermore, both conditions can appear, coexist and mutually reinforce even since middle age. It implies unfavorable results in terms of obesity management, but also in the prevention; assuming a real challenge to deal with it. The gut microbiome represents one of the most promising breakthroughs in the last decades and increasing evidence supports its role in a myriad of conditions, including obesity and cognition. Delving into the study of the gut microbiome in obesity-associated cognitive impairment may shed some light on the understanding and handling of these conditions.

The present thesis attempts to address these issues studying cognitive function and the gut microbiome in middle-aged subjects with and without obesity. The results point to the existence of a gut bacterial ecosystem simultaneously linked to cognitive function, focused on memory and inhibitory control, and the gray matter volume of the brain areas involved in these domains. Gut bacterial modulation of aromatic amino acid (AAA) and one-carbon (1C) metabolism pathways may underlie these relationships. The findings were also validated in rodent models since the gut microbiota phenocopied cognitive traits from humans to mice.

In the following sections, a comprehensive discussion of the main findings of both papers is presented. It is based on the prior set objectives, the different pathways and mechanism identified and the relevance in the context of previous studies and existent knowledge.

6.1.1. Objective 1: Subjects with obesity exhibited deficits in the cognitive domains of learning and memory and executive function

Impairments in learning, short-term and working memory measured by the scores of the California Verbal Learning Test (CVLT), subtests Immediate Recall (IR); Short Delayed Free Recall (SDFR); and the Total Digit Span (TDS), respectively, were found in obesity (Paper I).

These results are in line with previous studies which evaluated the relationship between obesity and memory function in middle-aged subjects[116,117] and the majority of compiled works in a more recent review.[121] Nonetheless, our findings contradict other studies in that no associations between memory and obesity were observed.[119,120] The existence of fewer studies evaluating learning and memory than executive domain in obesity along with the inconsistent data of some of them add importance to our results. Our findings support the theory that memory function is impaired in middle-aged subjects with obesity.

Impaired inhibitory control assessed by lower scores of the Stroop Color and Word Test (SCWT) was found in subjects with obesity (Paper II). Inhibition is one of the subdomains of executive function[85] and is crucial to overrule automatic intentions to directly respond to stimuli without thought.[292] As expected, out results aligned with previous literature, in which disturbances in executive functions[102,127,129-131] and particularly inhibitory control[131,134-136] have been widely linked to obesity.

Memory[114] and inhibitory control[127] disturbances might lead to poor food choices, uncontrolled eating and finally weight gain; perpetuating a vicious cycle.[115] Conversely, better cognitive function could predict greater weight loss.[139,140] Thus, it is essential to promptly detect and handle cognitive dysfunction to achieve a successful obesity management. Cognitive modulation and training approaches may be promising tools to further investigate[128] in obesity.[293]

6.1.2. Objective 2: A characteristic gut microbiome ecosystem was associated with memory and inhibitory control

6.1.2.1. Metagenomics

A specific community of bacteria was linked to cognitive scores in all the subjects after adjusting for confounding factors. This ecosystem was detected not only at baseline but also one-year follow-up and was replicated in independent cohorts.

At taxonomy level, general associations with memory (Paper I) and inhibitory control (Paper II) were observed, whereas specific bacterial clusters were distinctively found related to immediate and short-term memory and working memory; suggesting both convergent and divergent bacterial-cognitive patterns (Paper I). A brief summary of the most relevant associations between cognitive performance and gut bacterial taxa as well as the latest insights of these species is presented below (Table 7).

Table 7. Summary of the different bacterial taxa associated with cognitive scores (data extracted from Arnoriaga-Rodríguez et al., 2020, Paper I; Arnoriaga-Rodríguez et al., 2021, Paper II).

Bacterial taxa	Tests (Association)	Evidence
p_Firmicutes; f_*Clostridiaceae;* g_*Clostridium*		
s_*Clostridium* sp. 27_14	CVLT-IR, CVLT-SDFR, TDS, NOR24h (+)	Attenuation inflammation (*Clostridium* spp.).[294]
s_*Clostridium* sp. CAG:230	CVLT-SDFR, TDS, SCWT (+)	Attenuation of microglia-mediated neuroinflammation in Alzheimer's disease[295] and adjuvant therapy for hepatic encephalopathy (C. *butyricum*).[296]
s_*Clostridium* sp. CAG:440	TDS (+)	
p_Firmicutes; f_*Eubacteriaceae;* g_*Eubacterium*		
s_*Eubacterium* sp. CAG:603	TDS, SCWT, RL (+)	Inhibition of colorectal cancer and atherosclerosis; modulation of inflammation (*Eubacterium* spp.).[297]
p_Firmicutes; f_na Firmicutes; g_na Firmicutes		
s_*Firmicutes bacterium* CAG:103	TDS (+)	Decrease in atrial fibrillation (*F. bacterium* CAG:103).[298]
s_*Firmicutes bacterium* CAG:238	SCWT, RL (+)	Reduction in AD[299] and depression;[300] increase in sleep efficiency (p_Firmicutes).[301]
p_Firmicutes; f_*Lachnospiraceae;* g_uc *Lachnospiraceae*		
s_*Lachnospiraceae bacterium* 5_1_57FAA	SCWT, RL (-)	Impaired glucose and inflammation; increased in obesity and metabolic syndrome (f_*Lachnospiraceae*).[302]
s_*Lachnospiraceae bacterium* 6_1_37FAA	SCWT, RL (-)	
p_Firmicutes; f_*Lachnospiraceae;* g_*Roseburia*		
s_*Roseburia* sp. CAG:197	CVLT-IR, CVLT-SDFR (+)	Decreased in cognitive deficits in mice (g_*Roseburia*).[303]
p_Firmicutes; f_*Ruminococcaceae;* g_*Ruminococcus*		
s_*Ruminococcus* sp. CAG:353	CVLT-IR, CVLT-SDFR, NOR24h (+)	Associated with healthy eating behavior[304] and positively related to Recognition, Digit Span Backward, Fluency Span and Memory domain in elderly patients with mild cognitive impairment (g_*Ruminococcus*).[305]
s_*Ruminococcus* sp. CAG:177	TDS (+)	
p_Firmicutes; f_*Selenomonadaceae;* g_*Megamonas*		
s_*Megamonas funiformis*	CVLT-IR, CVLT-SDFR, TDS (+)	Decreased in multiple system atrophy (*M. funiformis*).[306]
p_Firmicutes; f_*Veillonellaceae;* g_*Veillonella*		
s_*Veillonella magna*	CVLT-SDFR (+)	Decreased in cognitive decline, T2D (g_*Veillonella*).[307]
p_Bacteroidetes; f_*Bacteroidaceae; g_Bacteroides*		
s_*Bacteroides fragilis* CAG:558	CVLT-IR, CVLT-SDFR, TDS, NOR24h (-)	Lower abundance in cognitive impairment with brain amyloidosis (*B. fragilis*).[308]
s_*Bacteroides* sp. 43_46	CVLT-SDFR, TDS, SCWT, NOR24h (-)	
s_*Bacteroides caccae* CAG:21	CVLT-SDFR, TDS, SCWT, NOR24h (-)	Enriched in subjects with T2D,[309] gout[310] and bloodstream infections (*B. caccae*).[311]
s_*Bacteroides* sp. AR20	CVLT-SDFR, TDS, SCWT (-)	
s_*Bacteroides plebeius*	SCWT, RL, NOR24h (-)	Increased in patients with hypertension (*B. plebeius)*.[312]
s_*Bacteroides gallinarum*	SCWT, RL, NOR24h (-)	Inflammation and T2D in mice (*B. gallinarum*).[313]
s_*Bacteroides mediterraneensis*	SCWT, RL, NOR24h (-)	Increased after post-stroke cognitive impairment (g_*Bacteroides*).[314]
p_Proteobacteria; f_*Enterobacteriaceae;* g_*Citrobacter*		
s_*Citrobacter freundii*	CVLT-IR, CVLT-SDFR, TDS (-)	Bloodstream, abdominal infections (*C. freundii*).[315]
p_Proteobacteria; f_*Enterobacteriaceae;* g_*Enterobacter*		
s_*Enterobacter cloacae*	CVLT-SDFR, TDS (-)	Enriched abundance in lower cognitive functioning in children (f_*Enterobacteriaceae*).[316]
p_Proteobacteria; f_*Enterobacteriaceae;* g_*Klebsiella*		
s_*Klebsiella aerogenes*	CVLT-IR, CVLT-SDFR, TDS (-)	Bloodstream infections (*K. aerogenes*).[317]
p_Proteobacteria; f_*Enterobacteriaceae;* g_*Salmonella*		
s_*Salmonella enterica*	CVLT-IR, CVLT-SDFR, TDS (-)	Amyloid production[318] and impaired cognition in mice (S. *enterica serovar Typhimurium*).[319]

CVLT, California Verbal Learning Test; IR, Immediate Recall; SDFR, Short Delayed Free Recall; f_, family; g_, genus; NOR24h, Novel Object Recognition 24h; p_, phylum; s_, species; SCWT, Stroop Color and Word Test; TDS, Total Digit Span; T2D, type 2 diabetes; RL, Reversal learning.

Furthermore, cognitive-gut microbiota associations depended on obesity status, since bacteria taxa were differentially linked to memory (Paper I) and inhibitory control (Paper II) in subjects with and without obesity (Table 8).

Table 8. Different cognitive-bacterial associations in subjects with and without obesity (data extracted from Arnoriaga-Rodríguez et al., 2020, Paper I; Arnoriaga-Rodríguez et al., 2021, Paper II).

Without obesity		Obesity	
Bacterial taxa	**Tests (Association)**	**Bacterial taxa**	**Tests (Association)**
s_*Alistipes* sp. CAG:157	SCWT (-)	s_*Anaerovibrio lipolyticus*	CVLT-SDFR (+)
s_*Bacteroides ovatus* CAG:22	SCWT (-)	*s_Clostridium* spp.	CVLT-IR, CVLT-SDFR, TDS (+)
s_*Bacteroides* sp. CAG:462	SCWT (-)	s_*Bacteroides nordii*	SCWT (-)
s_*Butyrivibrio* sp. CAG:318	SCWT (+)	s_*Faecalibacterium* sp. CAG:74	TDS (+)
s_*Clostridium* sp. CAG:632	SCWT (+)	s_*Fusobacterium varium*	SCWT (-)
s_*Roseburia* sp. CAG:471	SCWT (+)	s_*Prevotella timonensis*	SCWT (-)
Acidaminococcus spp.	SCWT (-)	*Eubacterium* spp.	CVLT-IR, CVLT-SDFR, TDS (+)
Lactococcus spp.	TDS (+)	*Ruminococcus* spp.	TDS (+)

CVLT, California Verbal Learning Test; IR, Immediate Recall; SDFR, Short Delayed Free Recall; s_Species; SCWT, Stroop Color and Word Test; TDS, Total Digit Span.

Based on the identified relationships, we hypothesized that positive associations of bacterial taxa and cognitive scores may presumably be beneficial, whilst negative associations, detrimental. In this line, our results fit with the current evidence. Concordantly with our negative correlations, patients with mild cognitive impairment had a higher prevalence of *Bacteroides*.[320] Moreover, impaired memory under acute stress conditions was linked to *Citrobacter rodentium* in mice.[321] Conversely, within the positive relationships, *Ruminococcus* was positively linked to Recognition trials, Digit Span Backward, Semantic Fluency Span and Memory Domain in elderly patients with mild cognitive impairment after a Mindful Awareness Program[305] or species from the phylum Firmicutes such as *Roseburia intestinalis* or *Faecalibacterium prausnitzii (f_Ruminoccocaceae)* had been detected in lower abundance in T2D.[309] Dietary quality assessing by Healthy Eating Scores was also linked to some genera in the same direction as our observations; positively with *Roseburia*, *Faecalibacterium* and negatively with *Bacteroides* and *Fusobacterium* (f_*Fusobacteriaceae*, p_Fusobacteria).[322] Other species linked to cognitive scores and aligned with current evidence are shown in Table 7.

At functional level, the analysis of the gut metagenome revealed several bacterial pathways associated with memory and inhibitory control. Bacterial functions related to AAA metabolism were negatively associated with all memory scores (Paper I) whilst bacterial functions related to nucleotide metabolism have the strongest negative associations with inhibitory control (Paper II). Other bacterial functions related to B vitamins and choline and betaine metabolism were also negatively associated with memory (Paper I) and inhibitory control (Paper II). The main bacterial functions associated with memory and inhibition scores are shown below (Table 9).

Table 9. Bacterial functions associated with cognitive scores (data extracted from Arnoriaga-Rodríguez et al., 2020, Paper I; Arnoriaga-Rodríguez et al., 2021, Paper II).

Bacterial functions	Tests
Aromatic amino acids	
AOC3, AOC2*, *tynA primary-amine oxidase [EC:1.4.3.21]	CVLT-IR, CVLT-SDFR
aspC aspartate aminotransferase [EC:2.6.1.1]	CVLT-IR, CVLT-SDFR
dadA D-amino-acid dehydrogenase [EC:1.4.5.1]	CVLT-IR, CVLT-SDFR
enr 2-enoate reductase [EC:1.3.1.31]	TDS
fadB 3-hydroxyacyl-CoA dehydrogenase … [EC:1.1.1.35 4.2.1.17 5.1.2.3 5.3.3.8]	CVLT-IR, CVLT-SDFR
feaB phenylacetaldehyde dehydrogenase [EC:1.2.1.39]	CVLT-IR, CVLT-SDFR
katE, CAT, catB, srpA catalase [EC:1.11.1.6]	CVLT-SDFR
katG catalase-peroxidase [EC:1.11.1.21]	CVLT-IR, CVLT-SDFR
mhpA 3-(3-hydroxy-phenyl) propionate hydroxylase [EC:1.14.13.127]	CVLT-IR, CVLT-SDFR
mhpB 2,3-dihydroxyphenylpropionate 1,2-dioxygenase [EC:1.13.11.16]	CVLT-SDFR
mhpD 2-keto-4-pentenoate hydratase [EC:4.2.1.80]	CVLT-SDFR
mtr tryptophan-specific transport protein	CVLT-IR, CVLT-SDFR
OGDH, sucA 2-oxoglutarate dehydrogenase E1 component [EC:1.2.4.2]	CVLT-SDFR
paaA ring-1,2-phenylacetyl-CoA epoxidase subunit PaaA [EC:1.14.13.149]	CVLT-SDFR
paaC ring-1,2-phenylacetyl-CoA epoxidase subunit PaaC [EC:1.14.13.149]	CVLT-SDFR
paaZ oxepin-CoA hydrolase … [EC:3.3.2.12 1.2.1.91]	TDS
pheP phenylalanine-specific permease	CVLT-IR
quiA quinate dehydrogenase (quinone) [EC:1.1.5.8]	CVLT-IR, CVLT-SDFR
solA N-methyl-L-tryptophan oxidase [EC:1.5.3.-]	CVLT-IR, CVLT-SDFR
tnaB low affinity tryptophan permease	CVLT-SDFR
trpB tryptophan synthase beta chain [EC:4.2.1.20]	CVLT-IR
tyrC cyclohexadieny/prephenate dehydrogenase [EC:1.3.1.43 1.3.1.12]	CVLT-IR, CVLT-SDFR
Nucleotides	
***dut*,DUT** dUTP pyrophosphatase [EC:3.6.1.23]	SCWT
thyX thy1 thymidylate synthase (FAD) [EC:2.1.1.148]	SCWT
purT phosphoribosylglycinamide formyltransferase 2 [EC:2.1.2.2]	SCWT
Signal transduction	
kinB two-component system, NtrC family, sensor histidine kinase KinB [EC:2.7.13.3]	SCWT
Unclassified: metabolism	
E3.1.11.5 exodeoxyribonuclease V [EC:3.1.11.5]	SCWT
Endocannabinoid signaling	
NAPEPLD N-acyl-phosphatidylethanolamine-hydrolyzing phospholipase D [EC:3.1.4.54]	CVLT-SDFR, TDS

Bacterial functions	Tests
Thiamine B1	
ABC.VB1X.A putative thiamine transport system ATP-binding protein	CVLT-IR, CVLT-SDFR
ABC.VB1X.P putative thiamine transport system permease protein	CVLT-IR, CVLT-SDFR
thiB, tbpA thiamine transport system substrate-binding protein	CVLT-IR, CVLT-SDFR
THI4, THI1 cysteine-dependent adenosine diphosphate thiazole synthase [EC:2.4.2.60]	TDS, SCWT
thiK thiamine kinase [EC:2.7.1.89]	TDS
thiP thiamine transport system permease protein	CVLT-IR, CVLT-SDFR
Riboflavin B2	
aphA acid phosphatase (class B) [EC:3.1.3.2]	CVLT-IR, CVLT-SDFR
bluB 5,6-dimethylbenzimidazole synthase [EC:1.13.11.79]	SCWT
fre, ubiB aquacobalamin reductase / NAD(P)H-flavin reductase [EC:1.16.1.3 1.5.1.41]	CVLT-IR, CVLT-SDFR
ribBA 3,4-dihydroxy 2-butanone 4-phosphate synthase … [EC:4.1.99.12 3.5.4.25]	TDS, SCWT
ribE, RIB5 riboflavin synthase [EC:2.5.1.9]	SCWT
Pyridoxine B6	
epd D-erythrose 4-phosphate dehydrogenase [EC:1.2.1.72]	CVLT-SDFR
pdxA 4-hydroxythreonine-4-phosphate dehydrogenase [EC:1.1.1.262]	TDS, SCWT
Folic acid B9	
folK 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine diphosphokinase [EC:2.7.6.3]	SCWT
folKP ´´/dihydropteroate synthase [EC:2.7.6.3 2.5.1.15]	CVLT-IR, CVLT-SDFR
folX D-erythro-7,8-dihydroneopterin triphosphate epimerase [EC:5.1.99.7]	CVLT-IR, CVLT-SDFR
nudB, ntpA dihydroneopterin triphosphate diphosphatase [EC:3.6.1.67]	SCWT
pabB para-aminobenzoate synthetase component I [EC:2.6.1.85]	TDS
pabC 4-amino-4-deoxychorismate lyase [EC:4.1.3.38]	TDS
queE 7-carboxy-7-deazaguanine synthase [EC:4.3.99.3]	TDS
Cobalamin B12	
ABC.VB12.P, btuC vitamin B12 transport system permease protein	CVLT-IR, CVLT-SDFR
btuB vitamin B12 transporter	SCWT
Choline and betaine	
betA, CHDH choline dehydrogenase [EC:1.1.99.1]	CVLT-IR, CVLT-SDFR
betT, betS choline/glycine/proline betaine transport protein	CVLT-IR, CVLT-SDFR
proV glycine betaine/proline transport system ATP-binding protein [EC:3.6.3.32]	CVLT-SDFR, TDS, SCWT
proX glycine betaine/proline transport system substrate-binding protein	CVLT-SDFR, TDS, SCWT

CVLT, California Verbal Learning Test; IR, Immediate Recall; SDFR, Short Delayed Free Recall; SCWT, Stroop Color and Word Test; TDS, Total Digit Span.

In accordance with the bacterial taxa, the associations at the functional level also varied in subjects with and without obesity. For example, the relationship between SCWT scores and *thyX* and *dut* was specifically marked in subjects without obesity, whereas the link with *exodeoxyribonuclease V* was stronger in subjects with obesity (Paper II). Similarly, the associations of tryptophan-related metagenomic functions and memory were mainly detected in subjects with obesity (Paper I).

6.1.2.2. Metabolomics

Untargeted metabolomic approach was able to identify plasma and fecal metabolites linked to memory (CVLT, TDS) and inhibition (SCWT) scores.

Disturbances in AAA metabolism were linked to both cognitive domains. All memory scores were associated with altered plasma levels of tryptophan, tyrosine, phenylalanine and their catabolites (Paper I). Remarkably, positive associations were found between memory function and plasma levels of tryptophan and several indole derivatives metabolized by the gut microbiota (Figure 8). Noteworthy changes in tryptophan and tyrosine metabolism in relation to SCWT scores were also observed. In plasma, tryptophan and 4-hydroxyphenyllatic acid (4-HPLA) had the strongest positive association with SCWT scores, whereas in feces, consistent associations were found with tyrosine and the 5-hydroxyindoleacetic acid (5-HIAA). Tryptophan alterations were also replicated in an independent cohort, in which tryptophan itself and microbial-derived tryptophan metabolites, such as indolepropionamide (IPAM), were positively linked to SCWT (Paper II). Again, cognitive-related tryptophan disturbances, in both memory and inhibitory control, were mainly observed in subjects with obesity.

To the best of our knowledge, the current observations are the first in humans linking tryptophan and its metabolites to cognition.

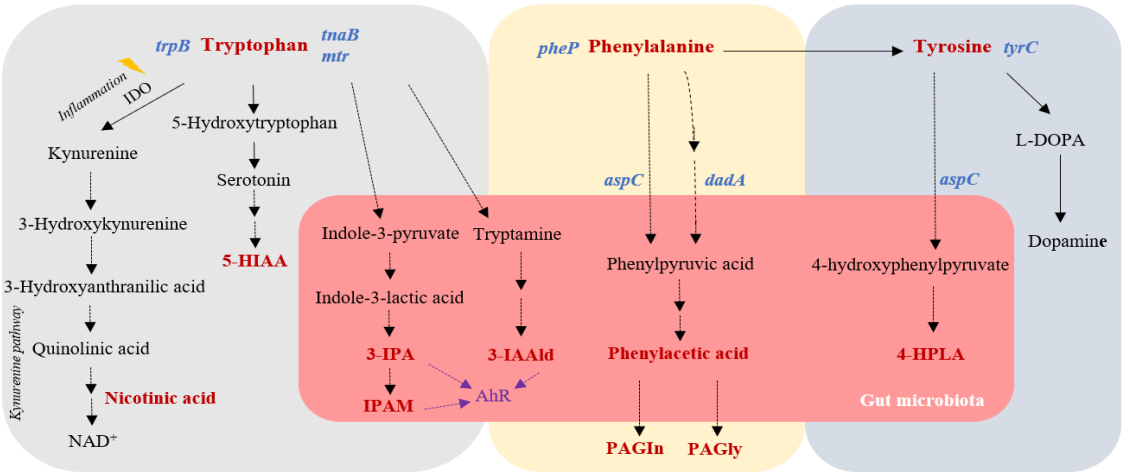


Figure 8. Overview of the main catabolic pathways of aromatic amino acids. Tryptophan and tyrosine are the precursor of the serotonin and dopamine neurotransmitters, respectively. The gut microbiota can metabolize AAA[323,324,325] to indoles and other compounds, some of them ligands of aryl hydrocarbon receptor (AhR). Bacterial pathways and metabolites involved in AAA metabolism and significantly associated with cognitive function are shown in blue and red, respectively. AhR, aryl hydrocarbon receptor; aspC, aspartate aminotransferase; dadA, D-amino-acid dehydrogenase; IDO, indole-amine 2,3-dioxygenase; IPAM, indolepropionamide; mtr, tryptophan-specific transport protein; NAD, nicotinamide adenine dinucleotide; PAGln, phenylacetylglutamine; PAGly, phenylacetylglycine; pheP, phenylalanine-specific permease; trpB, tryptophan synthase beta chain; tnaB, low affinity tryptophan permease; tyrC, cyclohexadieny/prephenate dehydrogenase; 3-IAAld, indole-3-acetaldehyde; 3-IPA, Indole-3-propionic acid; 4-HPLA, 4-hydroxyphenyllatic acid; 5-HIAA, 5-hydroxyindoleacetic acid (mostly adapted from Arnoriaga-Rodríguez et al., 2021, Paper II; some data also extracted from Liu et al., 2020[323] and Nemet et al., 2020).[324]

In addition, other metabolites related to cognitive function were identified. Fecal levels of methionine and microbial-derived methionine catabolite, dimethyl sulfone (DMSO2), showed a negative association with SCWT scores (Paper II) as well as plasma levels of betaine with both memory and inhibition (Figure 9). Similarly, these later relationships were only found in subjects without obesity.

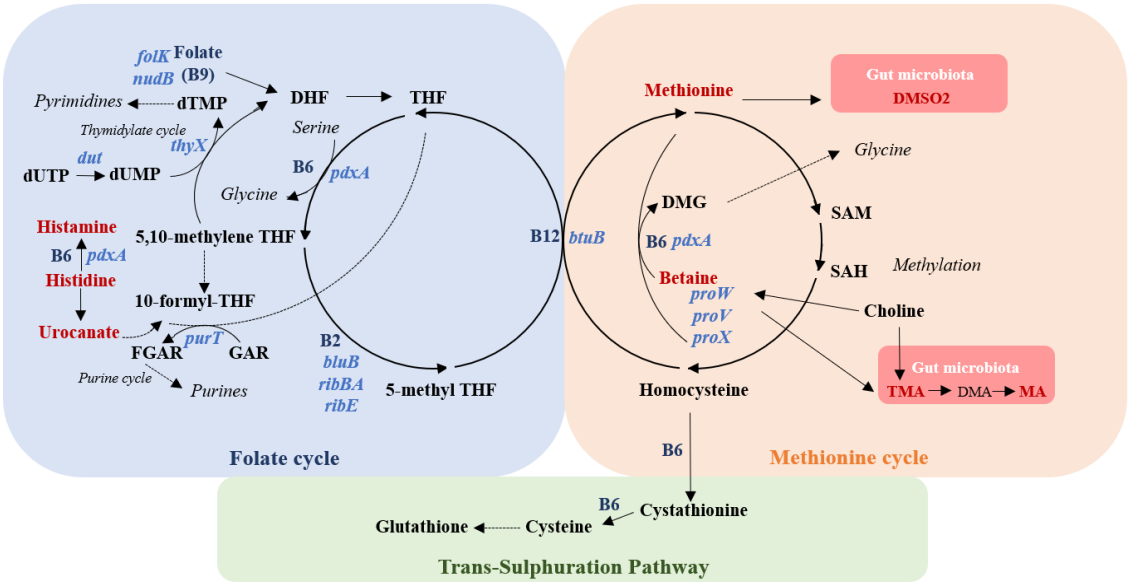


Figure 9. Overview of the one-carbon metabolism. One-carbon metabolism[326,327,328,329] is a metabolic process that transfers 1C units to purine and thymidine synthesis and homocysteine remethylation, among others. Bacterial pathways and metabolites involved in 1C metabolism and significantly associated with cognitive function are shown in blue and red, respectively. bluB, 5,6-dimethylbenzimidazole synthetase; btuB, vitamin B12 transporter; DHF, dihydrofolate; DMA, dimethylamine; DMG, dimethylglycine; DMSO2, dimethyl sulfone; dut, dUTP pyrophosphatase; dUTP, deoxyuridine triphosphate; dUMP, deoxyuridine monophosphate; dTMP, deoxythymidine monophosphate; FGAR, 5'-phosphoribosyl-N-formylglycineamide; folK, 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine diphosphokinase; GAR, 5'-phosphoribosylglycineamide; MA, methylamine; nudB, dihydroneopterin triphosphate diphosphatase; pdxA, 4-hydroxythreonine-4-phosphate dehydrogenase; proV, glycine betaine/proline transport system ATP-binding protein; proW, glycine betaine/proline transport system permease protein; proX, glycine betaine/proline transport system substrate-binding protein; purT, phosphoribosylglycinamide formyltransferase 2; ribBA, 3,4-dihydroxy 2-butanone 4-phosphate synthase/GTP cyclohydrolase II; ribE, riboflavin synthase; SAH, S-adenosylhomocysteine; SAM, 5-adenosylmethionine; THF; tetrahydrofolate; thyX, thymidylate synthase; TMA, trimethylamine (mostly adapted from Arnoriaga-Rodríguez et al., 2021, Paper II; some data also extracted from Lyon et al., 2020[327] and Konno et al., 2017).[329]

Conversely, positive associations between the endocannabinoids oleamide and arachidonoylethanolamide (AEA, anandamide) and memory were observed (Paper I). The endocannabinoids are lipid-derived mediators involved in neurotransmission and learning and memory.[330,331] Oleamide and AEA reversed cognitive impairment[332,333] and hippocampal damage[333] in mice. Interestingly, we found that microbial N-acyl phosphatidylethanolamine-hydrolyzing phospholipase D (NAPEPLD) (Table 9), essential in endocannabinoid biosynthesis,[334] had one of the strongest associations with cognition in both humans and mice.

6.1.2.3. Integrative analysis of metagenomics and metabolomics

The current functional metabolomics and metagenomics analyses highlighted several bacterial functions and circulating metabolites essentially involved in aromatic amino acid (AAA) and one-carbon (1C) metabolism differentially linked to memory and inhibitory control scores, in subjects with and without obesity.

Aromatic amino acid metabolism: Tryptophan and tyrosine are the precursors of serotonin and dopamine, respectively. Disturbances in serotoninergic and dopaminergic neurotransmission have been associated with learning and memory[335] and executive function impairments.[336,337] Projections of both systems in the hippocampus are mainly involved in memory function[338,339] whereas in the prefrontal cortex (PFC) they play a key role in executive function.[340,341] We evidenced plasma tryptophan, tyrosine and some related-catabolites positively associated with memory and inhibitory control (Figure 8). In line with our results, the administration of tryptophan improved memory in rodents through increased serotonergic neurotransmission in the hippocampus[342] and reduced intraneuronal amyloid-beta load in a mouse model of AD.[343] Furthermore, preclinical studies have evidenced an important role of dopamine output in the hippocampus in recognition memory.[344] In case of executive function, our results fit with available evidence in which low levels of tryptophan increased impulsiveness[345] or reaction times[346] in healthy adults; although available data show somewhat inconsistent results.[347,348] These differences may be related to the different cognitive tests assessing inhibitory control, underpinning similar but not identical neurobiological mechanisms.[349] Moreover, our findings related to tyrosine are concordant with previous reports showing that enhancing brain dopamine through tyrosine supplementation[350] or bromocriptine, a dopamine D2 selective agonist,[351] improved inhibitory control in healthy participants.

Gut microbiota can modulate AAA metabolism. Changes in the gut microbiota due to antibiotic treatment in piglets decreased the concentrations of serotonin and dopamine as well as their AAA precursors in the hypothalamus.[352] Gut microbiota has also shown to directly metabolize tryptophan into several indole derivatives which are potent ligands of the aryl hydrocarbon receptor (AhR). In experimental models, these metabolites had an effect on astrocytes to limit central nervous system inflammation[353] and deletion of the AhR altered adult hippocampal neurogenesis and contextual fear memory.[354,355] Consistently, we found several indole derivatives positively associated with memory scores (Figure 8). Furthermore, microbial-derived products including indoles can modulate the tryptophan metabolism through the activation of indole-amine-2,3-dioxygenase (IDO), the rate-limiting enzyme in the kynurenine pathway.[356] The kynurenine pathway is the major catabolic route of tryptophan which is activated under inflammatory conditions.[357]

Notably, cognitive-tryptophan metabolites disturbances aligned with the associations of cognitive-tryptophan-related metagenomic functions mainly observed in subjects with obesity. Low-grade inflammation may underline both cognitive and tryptophan metabolism dysfunction. On the one hand, the role of chronic inflammation in obesity and associated disorders such as cognitive decline is well-established.[358,359] On the other hand, a more recent report has also linked obesity and systemic inflammation to alterations in tryptophan metabolic pathways.[360] Concordantly, we obtained a negative correlation between plasmatic tryptophan and high-sensitive C-reactive protein.

One-carbon metabolism*:* Folate derivatives, tetrahydrofolate (THF) polyglutamates, are coenzymes that donate or accept one-carbon units in a network of reactions known as one-carbon (1C) metabolism.[361] 1C metabolism supports numerous physiological processes, such as purine and thymidine biosynthesis; glycine, serine and methionine homeostasis; epigenetic maintenance and redox defense.[362] Although all the mechanisms are not fully known, folate and other B vitamin deficiencies and/or genetic mutations and polymorphisms can impair 1C metabolism.[363,364] 1C metabolism is compartmentalized in the mitochondria, nucleus and cytoplasm[361] and eukaryotic cells mobilize multiple carbon sources to supply 1C metabolism.[362] 1C sources originate in the diet are metabolized to generate 1C units. The most important are choline, serine and glycine. Glucose, threonine, methionine and histidine can also contribute to the 1C pool.[362] The purine, thymidylate and methionine cycles encompass the 1C metabolism in the cytosol.[326] In this line, we found bacterial metagenomic functions involved in the purine (*purT*), thymidylate (*thyX, dut*) and methionine (*proW, proV, porX*) cycles, showing a negative association with inhibitory control (Figure 9). These alterations were also supported by the metabolomic identification of low fecal levels of methionine and microbial-derived methionine catabolites such as DMSO2 related to SCWT scores.[365]

Remarkably, alterations in those cycles are different in subjects with and without obesity. Thus, disturbances in SCWT and methionine and thymidylate cycles were only detected in subjects without obesity as well as in betaine levels. Similarly, metagenomic betaine-related functions (*proW, proV, proX*) and metabolomics, betaine and gut microbiota-betaine derived, trimethylamine (TMA) and methylamine (MA) were also associated with memory and inhibitory control. Choline is the precursor of the neurotransmitter acetylcholine but it can also be metabolized to betaine, a methyl donor in the 1C metabolism and modulator of homocysteine status (Figure 9). High plasma levels of homocysteine have been associated with cognitive impairment[366] whereas betaine supplementation has shown to prevent these homocysteine-induced deficits.[367] By contrast, plasmatic levels of 1C donor histidine, the precursor of the neurotransmitter histamine[368] were positively associated with SCWT scores in subjects with obesity. Consistently, we also found negative associations between fecal histamine and SCWT.

Bacterial functions related to B vitamin metabolism, including B2, B6, B9, B12 showed negative relationships with memory and inhibitory control (Table 9). Those vitamins are essential for 1C metabolism (Figure 9). Disturbances in 1C metabolism have been associated with cognitive decline[369] and there is compelling evidence for the impact of B vitamins on cognition.[366,370,371] In particular, the role of folate (B9) and thiamine (B1)[372, 373] in memory function has been widely studied. In agreement, we also identified negative associations between thiamine-related bacterial functions (Table 9) and memory scores. We hypothesized that this negative relationship may be explained by preferential consumption or catabolism of B vitamins by internal bacteria to the detriment of the host. Concordantly, we detected low plasma thiamine levels linked to worse memory scores, more marked in subjects with obesity, in turn, particularly susceptible to thiamine deficits.[374]

6.1.3. Objective 3: Brain structure differentially associated with the gut microbiome depending on obesity status

A region of interest (ROI) approach of the brain areas involved in memory and inhibitory control revealed positive associations between the right and left hippocampus with verbal learning and memory (CVLT); the right frontal inferior orbital (FIO) with working memory (TDS) (Paper I); and the anterior cingulate cortex (ACC) with inhibitory control (SCWT) (Paper II) in all the subjects. The relationships remained significant after adjusting for confounding variables, at both cross-sectional and after 1-year follow-up. These findings aligned with previous reports linking verbal memory performance with prefrontal and temporal brain features, such as the hippocampus[261-263] and inhibitory control with ACC.[375] Notably, in a sub-group analysis, the correlations between memory function and brain structure remained significant in subjects without obesity whilst disappearing in obesity.

Interestingly, in line with cognitive-bacterial relationships, different taxa and metagenomic functions were concordantly and longitudinally associated with the volume of the brain areas. A concise compilation of bacterial taxa simultaneously linked to cognitive scores and brain structure is listed below (Table 10). Notably, the strongest negative associations between the bacterial functions and the ACC gray matter volume were with *dut*, *thyX* and *kinB*, that were precisely the bacterial function negatively associated with SCWT scores (Table 9) in subjects without obesity (Paper II).

Again, no associations were found between memory, metagenomics and brain volumes in subjects with obesity, which is in line with the lack of significant associations between memory tests and selected brain volumes in this population (Paper I).

Table 10. Structural and functional brain features correlated with the gut microbiota (data extracted from Arnoriaga-Rodríguez et al., 2020, Paper I; Arnoriaga-Rodríguez et al., 2021, Paper II).

Bacterial taxa	Tests (Association)	Brain volume
Roseburia spp.	CVLT-IR, CVLT-SDFR (+) SCWT (+)	Left hippocampus ACC
Bacteroides spp.	CVLT (-) TDS (-) SCWT (-)	Left hippocampus Right FIO ACC
s_*Firmicutes bacterium* CAG:534	CVLT-IR, CVLT-SDFR (+)	Left hippocampus
s_*Lachnospiraceae bacterium* TF01-11	CVLT-SDFR (-)	Left hippocampus
s_*Anaerovibrio* sp. RM50	SCWT (-)	ACC
s_*Selenomonas* sp. oral taxon 478	SCWT (-)	ACC
s_*Clostridium* sp. CAG:417	SCWT (+)	ACC
s_*Ruminococcus* sp. CAG:724	SCWT (+)	ACC

ACC, anterior cingulate cortex; CVLT, California Verbal Learning Test; IR, Immediate Recall; SDFR, Short Delayed Free Recall; FIO, frontal inferior orbital; SCWT, Stroop Color Word Test; TDS, Total Digit Span.

Our data provides more evidence in support of the theory that gut microbiota is associated with morphological brain features; as it has been described in animal models[376,377] or in humans, at a single point in time.[378,379] For example, previous works have observed associations between the relative abundance of Firmicutes and Bacteroidetes phyla and the gray matter volume of the orbital and triangularis sections of the opercula as well as the temporal cortex in patients with irritable bowel syndrome.[378] In obesity, pilot studies identified changes in gut microbiota at phyla and family level in association with both cognitive function and brain architecture,[241] in particular, focus on brain iron load.[243] Nevertheless, our study has gone one step further illustrating a more in-depth gut-brain ecosystem at two different points in time.

6.1.4. Objective 4. Effects of fecal microbiota transplantation from humans to mice

Finally, in order to validate the results identified in humans and to figure out a potential causal role of the gut microbiota in cognitive impairment, different experiments of fecal microbiota transplantation (FMT) from humans to mice were conducted. Overall, cognitive traits from human donors were aligned with cognition-like behaviors in mice. Mice receiving the microbiota from donors without obesity with high memory scores exhibited better performance in the Novel Object Recognition (NOR) (Paper I). Conversely, mice receiving the microbiota from subjects with obesity with lower inhibitory control had significantly worse reversal learning (RL) performance (Paper II).

Several bacterial species and functions from human donors associated with the SCWT in humans were also linked to the RL task in recipient mice (Table 7) (Paper II). Similarly, common bacterial species associated with CVLT and TDS scores in humans were also linked to NOR performance in transplanted mice (Table 7) (Paper I). Notably, memory-related donor's metagenomic functions linked to vitamin B6 (*pdxJ*, *pdxB*), B12 (*btuB*), tryptophan metabolism (*trpA*, *trpB*) and endocannabinoid signaling (*NAPEPLD*) were also associated with memory-like behavior in mice (Paper I).

Prefrontal cortex (PFC) transcriptomics in recipient mice revealed more than 2,700 genes associated with memory-like performance in mice (Paper I). The most relevant genes identified and available evidence are summarized below (Table 11). To note the associations with SLC6A3, which encodes a dopamine transporter, the 5-HT receptors HTR1A and HTR2A as well as the FOLR1 emphasizing the connection between AAAs, folate metabolism and memory.

Table 11. PFC genes of recipient mice associated with the NOR3h (data extracted from Arnoriaga-Rodríguez et al., 2020, Paper I; Arnoriaga-Rodríguez et al., 2021, Paper II).

PFC genes	Evidence
TTR	Memory deficits in aged animals with altered hippocampal TTR expression.[380] Neuroprotection in AD.[381]
SLC6A3	Alcohol use disorder, attention-deficit/hyperactivity disorder, autism and movement disorders.[382]
HTR1A, HTR2A	HTR1A involved in social behavior;[383] HTR2A in memory and cognition.[384]
FOLR1	Genetic defects affecting folate caused multiple brain manifestations.[385]
NFKB1	Synaptic plasticity, learning, and memory.[386]
DICER 1	The knockout of DICER1 has been reported to enhance memory.[387]
ACSS2	Regulation of histone acetylation in neurons and spatial memory in mammals.[388]

TTR, transthyretin; SLC6A3, solute carrier family 6 member 3; HTR1A, HTR2A, 5-hydroxytryptamine (serotonin) receptor 1A and 2A; FOLR1, folate receptor 1; NFKB1, nuclear factor of kappa light polypeptide gene enhancer in B cells 1; ACSS2, acyl-CoA synthetase short-chain family member 2.

Furthermore, PFC genes of recipient mice were associated with SCWT-related bacterial functions of human donors (Table 12) (Paper II). In particular, *dut* was positively associated with the expression of KCNE2, PRLR, FOLR1, CLDN2, SLC4A5, SOSTDC1 and borderline associations (*p*FDR <0.11) with TMEM72, F5, and TTR. All these genes were found among the top 25 genes changing the hippocampal expression after contextual fear conditioning.[389] Moreover, particularly noticeable is the significant association between the bacterial function of human donors involved in folate-mediated one-carbon metabolism, *dut* and the expression in the PFC of recipient mice of the FOLR1 (Table 11, Table 12) and MECP2 (Table 12), which has a well-established function in neurodevelopment[390] and has been linked to AD.[391] This is in agreement with our previous findings and further highlights the key role of the 1C metabolism and its involvement in DNA methylation.

Enrichment analysis of differentially expressed genes revealed over-representation of biological processes related to neuron development and histone methylation, in case of *dut* and netrin signaling, which has recently shown to play and important role in synaptic plasticity and memory formation,[392] in case of *exodeoxyribonuclase V*.

Table 12. PFC genes of recipient mice associated with bacterial functions of human donors (data extracted from Arnoriaga-Rodríguez et al., 2020, Paper I; Arnoriaga-Rodríguez et al., 2021, Paper II).

PFC genes of recipient mice	Human bacterial functions
KCNE2 (Potassium voltage-gated channel subfamily E regulatory subunit 2)	*dut*
PRLR (Prolactin receptor)	*dut*
FOLR1 (Folate receptor 1)	*dut*
CLDN2 (Claudin 2)	*dut*
AUTS2 (Autism susceptibility candidate 2)	*dut*
MFRP (Membrane frizzled-related protein)	*dut*
HIPK1 (Homeodomain interacting protein kinase 1)	*dut*
SLC4A5 (Solute carrier family 4 member 5)	*dut*
SOSTDC1 (Sclerostin domain containing 1)	*dut*
MECP2 (Methyl CpG binding protein 2)	*dut*
TMEM72 (Transmembrane protein 72)	*dut*
F5 (Coagulation factor V)	*dut*
TTR (Transthyretin)	*dut,exo V*
MTHFD1L (Methylenetetrahydrofolate dehydrogenase 1-like)	*exo V*

dut, dUTP pyrophosphatase; exo V, exodeoxyribonuclease V controlling for age, sex and education years of the donors.

In addition, the examination of the PFC genes could identify the MS4A4A (Membrane-spanning 4-domains) and the SLC16A12 (Solute Carrier Family 16 Member 12) as the main predictors of SCWT scores of the human donors; in turn, associated with SCWT-related bacterial functions *dut* and *thyX*. Supporting our results, both genes have been related to cognition. SLC16A12 is the transporter for creatinine and its alterations have been associated with cognitive impairment.[393] Moreover, it has been described to be linked to folate status.[394] MS4A4A has also been identified as the key modulator of soluble TREM2 (triggering receptor expressed on myeloid cells 2) and AD risk.[395]

6.2. Strengths and limitations

This thesis provides a new insight into the relationships between the gut microbiome and obesity-associated cognitive dysfunction in humans; identifying involved mechanisms and translating prior evidence in animals to humans. In spite of the significant contributions and novel findings, the works compiled in this thesis (Paper I and Paper II) have also some limitations that should be acknowledged. The following sections present a summary of the main strengths and weaknesses of the entire project.

Research topic: While compelling evidence supports the impact of the gut microbiota on cognition in animals' models of obesity,[196,211] there was a lack of consistent evidence in humans. Thus, it makes this project original and innovative. To the best our knowledge, there are no previous descriptions of gut microbiota simultaneously linked to the different memory tests in humans neither evaluating differences in subjects with and without obesity. By contrast, the absence of similar studies does not allow us to directly match our results and we must rely on available data in preclinical models. Only one prospective[243] and two preliminary case-control studies[241,242] in humans have evaluated this topic, assessing other subdomain of executive function, cognitive flexibility. In addition, more recently, in a cohort of patients with obesity, working memory, inhibitory control and cognitive flexibility were measured in order to quantify the effects of a prebiotic administration. Due to the nature of its design, it is not possible to compare between subjects with and without obesity as it can be done in our study.

Sample size: Although the sample size of the discovery cohort seems appropriate and provides subtype-specific associations; large-scale data collection from consortiums related to the microbiome would have had greater power to detect effects. Our study may be considered as an intermediate step between prior underpowered studies and population-based cohorts; since we validated the results in different replication cohorts; one of them, a population study which included nearly 1000 microbiota samples.

Time points: The design of our prospective studies with both cross-sectional and longitudinal data enables us to detect not only associations between cognitive function and the microbiome but also a plausible direction of the relationships. Nevertheless, longer term follow-up would be necessary to better understand the strength of our conclusions.

Selection and characterization of subjects: Extensive phenotype of the subjects was carried out. It implied not only the use of anthropometric, medical and biochemical measurements in the same way as those applied in clinical practice, but also the employment of gold-standard techniques including the clamp or the DXA to assess insulin sensitivity and body composition, respectively. Furthermore, in an attempt to include as much homogenous subsets as possible, we matched subjects with and without obesity by age, sex and menopausal status.

Cognitive assessment: Comprehensible evaluation of cognition was conducted by a trained bilingual neuropsychologist using validated versions of neuropsychological tests and questionnaires. Even though these tests are usually used in clinical practice to assess parameters focusing on the different cognitive domains, it should be taken into account that some of them might rely on multiple brain regions and it is not possible to totally separate their functionality.

MRI examination: Both cross-sectional and longitudinal data related to MRI has been analyzed as a measurement of brain structure in an equipment with a high resolution. MRI has been widely used for non-invasive evaluation of either structural, functional or metabolic changes in the brain. However, it should be acknowledged that the capacity to detect molecular alterations is limited, unlike in the study of postmortem brain samples.

Multi-omics approach: The study of the gut microbiota is complex. To overcome this issue, we applied an integrative multi-omics approach including shotgun metagenomics and metabolomics, which did reveal insight into molecular mechanisms underlying the interactions between the microbiome and the cognitive function. One of the strengths of our study is the application of shotgun metagenomics instead of 16S-RNA sequencing. The latter prevented other reports from performing species-level associations as well as information about microbial functionality. In addition, statistical analysis included not only correlations like previous studies but also compositional univariate and multivariate machine learning strategies, to take into account the compositional structure of the microbiome data and rule out possible spurious associations. Overall, it provided an intricate network of interactions between the gut microbiome and functional and structural cognitive measurements, in general and at the same time, specifically according to the presence or absence of obesity, and allowed us to detect bacterial signatures of cognitive profiles. Nonetheless, experimental models which confirm the potential effects of certain species or microbial-derived compounds may be necessary to close the circle and reach feasible practical applications.

Preclinical models: Fecal microbiota transplantation (FMT) experiments validated our conclusions and enabled causal statements about the role of the gut microbiota in cognition and, particularly, in obesity-related cognitive dysfunction. Despite mouse models being widely used and validated to infer cognition-like phenotypes in real settings, they cannot be exactly comparable with cognitive evaluation and brain morphology in humans. In addition, we only studied male mice to avoid the possible effects of estrogen in female mice. However, rodents as well as humans also exhibit sex differences in cognition-like behavior.[396] Thus, further studies should include female mice. Finally, it would be interesting to assess metabolic alterations in recipients' mice depending on human donor's microbiota to measure whether the cognitive response may be mediated or modulated by some of these changes.

Dealing with confounders: Apart from obesity, several variables are known to influence cognitive function including the age, sex and education,[110] to name just a few. Moreover, the impact of depression in memory function[397] as well as in obesity is also noticed.[398,399] Hence, the associations between the microbiome and the cognitive scores were adjusted for these confounding factors. In addition, other elements such as total intracranial volume and insulin resistance and inflammation parameters were considered in specific or subtype analysis. These adjustments notwithstanding, there may be other determinants that we have not taken into account and that could have meaningful effects in cognitive function. One of the main limitations of our study is that the impact of physical activity and diet on the relationships between the gut microbiome and the cognitive scores was not evaluated. It could be to some extent offset by the fact that we studied individuals from the same environments with expected similar dietary patterns regarding the cultural backgrounds at least within the groups. Nevertheless, further research is warranted to evaluate the role of both factors in this complex interaction.

6.3. Public Health implications

In the light of obesity as a public health concern, due to the increasing prevalence of obesity worldwide and in the framework of the objective 3, Good health and well-being of The Global Goals for Sustainable Development of the United Nations,[400] translational research initiatives are needed to fully understand the underlying mechanisms of the pathophysiology of obesity and related disorders. The interplay obesity-cognitive dysfunction is one of them. Obesity is considered a modifiable risk factor for cognitive decline and, in turn, cognitive impairment predisposes to overeating and weight gain. This idea reinforces the control of modifiable risk factors as well as some non-communicable diseases to prevent cognitive impairment in a bidirectional perspective. As an appreciation, subjects with even only subjective cognitive complaints are referred to the medical specialist, addressing their manifestations from a multidisciplinary view. However, much more remains to be done in the case of obesity, in which a non-depreciable number of cases, hardly appears among the patient's diagnoses. Therefore, it must be imperative to raise awareness not only amongst patients, but also amongst physicians of the importance of recognizing and acting on obesity. It would improve morbidity and mortality rates, reduce caregiver's burnout and health expenditure in the long-term.

The role of cognitive decline in obesity outcomes has also been described. A worse cognitive profile may determine negative results in terms of healthy patterns adherence, food choices and related-behaviors and weight-loss maintenance.

Thus, clinicians should be encouraged to evaluate the presence of cognitive dysfunction in patients with obesity from the initial visits in order to select the best therapeutic option; or almost in bariatric surgery candidates to identify those more prone to respond.

In the future, the analysis of the gut microbiome would be used as an additional tool to detect vulnerable individuals to develop cognitive impairment, even in a preclinical stage, before the start of the symptoms. As it can be seen by our results, in a sample of clinically healthy cognitive subjects, individuals with obesity had lower scores in cognitive tasks that may determine long-term outcomes. Moreover, cognitive traits were phenocopied through gut microbiota, inferring the possible causal role of the microbiota in this interaction. Including a gut microbiota analysis in clinical practice could detect patients at higher-risk to develop cognitive impairment and, consequently, a more aggressive course of disease. Nevertheless, further studies should examine the cost-efficiency of this measure in routine clinical settings.

Overall, albeit the design of this thesis does not allow to have direct clinical applicability, it provides a theoretical basis for formulating and testing novel hypothesis to be addressed in the future. In particular, aromatic amino acid and one-carbon metabolism pathways should be noted.

The development of diets, supplements, pre- and probiotics and like in other conditions, the possibility to design fecal microbiota transplantations towards a personalized medicine based on an individual's microbiome, are some of the possibilities to further consider.

Finally, as a social impact of the present scientific work to reach to general public and in the framework of the European project *ThinkGut*[401], the results of this thesis are treated actively in information campaigns to be disseminated to the general public.

6.4. Perspectives

The findings of this thesis offer a novel perspective of the role of the gut microbiome in obesity and cognition and provide metabolic mechanistic pathways in this host-microbe interplay. Nevertheless, there are new questions and some limitations that need to be addressed in future investigations.

Firstly, one of the drawbacks of the present thesis is not exploring in detail the role of the diet and physical activity in the interaction cognition-microbiota. Therefore, to overcome this limitation our group designed a study (PI18/01022; NCT03889132)[402] which includes the monitorization of physical activity using a wearable smartwatch device in association with an assessment of dietary habits.

Secondly, to tackle the links between tryptophan pathways and cognitive function in subjects with obesity and low-grade inflammation-related disorders, another study is being carried out in our group (PI20/01090). This study combines different approaches to explore molecular mechanisms in order to translate the potential results to a clinical applicability, in a pre- or probiotic or dietary intervention way.

Thirdly, different experiments in rodent models are being conducted. On the one hand, a FMT in a model that includes male and female mice to assess the impact of the sex in this relationship. On the other, a FMT in germ-free mice instead of antibiotic eradication to test whether there may be some differential features to further consider. In both cases, metabolic changes resulting from FMT will be taken into account to identify possible mediators or moderators of this interaction.

Finally, the evaluation of the effects of weight loss in the obesity-associated cognitive dysfunction is an important area of investigation. Hence, long-term studies, in bariatric surgery patients, in particular, could provide interesting information. In addition, projects that involve a greater number of subjects, such as the results of microbiota consortium, may represent the truly global approach to generalize the findings to different populations.

7. CONCLUSIONS

General conclusion:

The studies included in the present thesis identified deficits in memory and inhibitory control in middle-aged subjects with obesity in association with a gut bacterial community of species, functions and metabolites. Alterations in aromatic amino acids and one-carbon metabolism may be involved. Furthermore, obesity-associated cognitive disturbances were phenocopied to recipient mice through fecal microbiota transplantation.

Specific conclusions:

1) Middle-aged subjects with obesity exhibited deficits in memory and inhibitory control compared to their counterparts without obesity.
2) Convergent and divergent patterns of microbial taxa were linked to memory and inhibitory control scores. Functional analysis of the gut microbiome through fecal metagenomics and fecal and plasma metabolomics revealed distinctive alterations in aromatic amino acid and one-carbon metabolism linked to cognitive function in subjects with and without obesity. To note, the changes in tryptophan-related bacterial functions and metabolites that were identified in obesity-cognitive dysfunction.
3) Brain structure also differentially associated with the gut microbiome composition and functionality depending on obesity status.
4) Cognitive scores of human donors and recipient mice became aligned via fecal microbiota transplantation. The microbiota from subjects with obesity led to decreased cognitive performance in recipient mice, which presented shifts in aromatic amino acid- and one-carbon metabolism-related genes in the prefrontal cortex simultaneously associated with different clusters of bacterial species and functions in the same direction as in humans.

Final comments:

- To the best our knowledge, these results are the first to provide broad information of the interactions between the gut microbiome and cognitive function in humans, particularly, in obesity-associated cognitive impairment, integrating different OMICs approaches.
- Finally, the findings emphasize the potential role of the gut microbiome in the prevention and management of obesity-cognitive dysfunction, even from preclinical stages. Further studies are needed to address the practical applications in clinical settings.

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Appendix A

Additional publications related to the thesis topic generated during the PhD program.

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