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Gut-derived metabolites mediating cognitive development in 5-year-old children: Early-life transplant in mice has lasting effects throughout adulthood

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ABSTRACT

The gut microbiota has been causally linked to cognitive development. We aimed to identify metabolites mediating its effect on cognitive development, and foods or nutrients related to most promising metabolites. Faeces from 5-year-old children (DORIAN-PISAC cohort, including 90 general population families with infants, 42/48 females/males, born in 2011–2014) were transplanted (FMT) into C57BL/6 germ-free mice. Children and recipient mice were stratified by cognitive phenotype, or based on protective metabolites. Food frequency questionnaires were obtained in children. Cognitive measurements in mice included five Y-maze tests until 23 weeks post-FMT, and (at 23 weeks) PET-CT for brain metabolism and radiodensity, and ultrasound-based carotid vascular indices. Children (faeces, urine) and mice (faeces, plasma) metabolome was measured by 1H NMR spectroscopy, and the faecal microbiota was profiled in mice by 16S rRNA amplicon sequencing.

Cognitive scores of children and recipient mice were correlated. FMT-dependent modifications of brain metabolism were observed. Mice receiving FMT from high-cognitive or protective metabolite-enriched children developed superior cognitive-behavioural performance. A panel of metabolites, namely xanthine, hypoxanthine, formate, mannose, tyrosine, phenylalanine, glutamine, was found to mediate the gut-cognitive axis in donor children and recipient mice. Vascular indices partially explained the metabolite-to-phenotype relationships. Children's consumption of legumes, whole-milk yogurt and eggs, and intake of iron, zinc and vitamin D appeared to support protective gut metabolites.

Overall, metabolites involved in inflammation, purine metabolism and neurotransmitter synthesis mediate the gut-cognitive axis, and holds promise for screening. The related dietary and nutritional findings offer leads to microbiota-targeted interventions for cognitive protection, with long-lasting effects.

1. Introduction

Delays in neurocognitive development during early childhood are considered predictors of mental disorders in adult life (Gow et al., 2008; Nees et al., 2021), underscoring the need of effective prevention strategies. Several human studies highlight communication pathways linking neurological function and gut bacteria. For instance, the diversity of the gut bacterial community, or the abundance of selected bacterial taxa

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have been related to specific components of cognitive function in middle age adults (Meyer et al., 2022), and in preschool children (Guzzardi et al., 2022; Carlson et al., 2018). Germ-free animal models support a causal connection, e.g., anxiety and/or depression have been related to the absence of gut microbiota (Sun et al., 2019; Gheorghe et al., 2021). In addition, modifications of behavioural and cognitive phenotypes reflecting donors' characteristics have been demonstrated in mice undergoing faecal material transplantation (FMT) (Sun et al., 2019; Sharon et al., 2019). Underlying mechanisms have not been fully established, but modulation of inflammation and neurotransmitter production are supported (Koopman et al., 2017). However, it is important to recognize that studying gut microbiota composition alone does not provide mechanistic understanding because different bacteria combinations may be functionally similar. A multi-omics approach combining e.g., microbiota and metabolome data, is more likely to provide insights into the effectors of gut-brain cross-talk and underlying mechanisms (Parker et al., 2020). Moreover, since the diet is the main driver of gut microbiota and metabolome variation, relationships between foods and nutrients intake and promising gut molecules may offer candidate therapeutic leads.

The present study was conducted in healthy preschool 5-year-old children from the DORIAN-PISAC, a birth-cohort of 90 infants born in 2011–2014, 42/48 females/males, and parents, representing the general population (Guzzardi et al., 2022; Guzzardi et al., 2020; Granziera et al., 2021). Children were stratified based on higher/lower cognition (phenotype) as objectively measured by the Griffiths Mental Developmental Scales (GMDS), or based on protective gut metabolites to address mechanistic aspects. Children faeces were used for FMT in germ-free mice, for cause-effect validation. The Y maze was applied to quantify the cognitive and behavioural phenotype of recipient mice longitudinally, and positron emission tomography with ^{18}FDG (^{18}FDG-PET) and computed tomography (CT) were used to quantify brain glucose uptake and brain radiodensity. Carotid vascular resistance (resistivity index, RI) and pressure (pulsatility index, PI) were measured by ultrasound as potential effector of metabolites, affecting the brain, based on their relationship with cognition (inversely) and Alzheimer's disease (directly) in humans (Mitchell et al., 20112011; Yew and Nation, 2017). Metabolome was measured in children (faeces, urine) and mice (faeces, plasma) by 1H NMR spectroscopy, and faecal microbiota composition of mice was assessed at baseline and 15 weeks post-FMT. Lastly, we analysed children's diets and identified food categories and nutrients related to promising microbiota mediators, providing nutritional therapeutic leads.

2. Materials and methods

2.1. Children study

The study was conducted in children of the DORIAN-PISAC birth cohort (Guzzardi et al., 2022; Guzzardi et al., 2020; Granziera et al., 2021). Follow-up visits were carried out from birth to the age of 5 years, including anthropometric, blood pressure, echocardiography, cognitive measurements, and collection of nutritional information and biological samples. At the age of 5 years, the assessment included cognition (n = 56), and faeces (n = 39) and urine (n = 62) collection for metabolome-microbiota analyses and for FMT. Parents' socioeconomic status was assessed from job data according to the European Socio-economic Classification (EseC), 10-class model, as previously described (Granziera et al., 2021). The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Massa and Carrara, and latest amendments by the Ethical Committee of the Area Vasta Nord-Ovest (CEAVNO), Pisa, Italy (study ID 394, decrees n. 75 and 71512). Parents gave their written informed consent before inclusion.

2.2. Measurement of children's cognitive score and parents' intelligence

Cognitive development was objectively measured by GMDS and the Extended revised version (GMDS-ER), as described (Guzzardi et al., 2020). The GDMS measures the rate of development of infants and young children in six domains, *i.e.*, locomotor, personal-social, hearing and language, eye and hand coordination, performance and practical reasoning (the latter from age of 36 months) (Ivens and Martin, 2002; Luiz et al., 2004; Luiz et al., 2006). Mothers' and fathers' intelligence quotients (IQ) were measured by the Raven's progressive matrices. All assessments were conducted by a trained psychologist in a dedicated hospital room.

2.3. Nutritional assessment

At the age of 5 year, the nutritional assessment was conducted by a self-administered, semiquantitative food frequency questionnaire (FFQ), as described (Granziera et al., 2021), consisting of 53 commonly used food items, and frequency the following response categories: never, less than once a month, 1–3 times a month, once a week, 2–4 and 5–6 times a week, once a day and 2–3 and 4–5 times a day. The level of consumption of dietary components linked to the traditional Mediterranean diet (MD) was estimated according to Sofi et al. (Sofi et al., 2008).

2.4. Children's faecal material preparation for transfer into mice

Faecal samples were collected in sterile stool collection tubes at home by mothers, stored at −20 °C and brought in ice to the Institute of Clinical Physiology within 24 h from collection. All faecal samples were stored at −80 °C until use for FMT, occurring after 10–20 months.

Faecal material for FMT was prepared according to published protocols (Turnbaugh et al., 2009; Ridaura et al., 2013), with few modifications to reduce the administration volume to 0.15 ml, given the small stomach size of the 4/5-week-old recipient mice. Briefly, a small piece of faecal material (around 100 mg) was rapidly cut, weighted and allowed to thaw in a closed tube. Then, sterile PBS was added to the tube in 1:7.7 ratio, the lid was immediately closed, the tube vortexed for 5 min and the content passed through a 100 μm filter to remove large particulate. The filtrate obtained from each faecal sample was administered to 3 germ-free mice by single oral gavage.

2.5. Mice protocol

The mice study was conducted in 72 C57BL/6 germ-free, 4/5-week-old mice, 50% males (Charles River Laboratories, Calco (LC), Italy), as a consolidated model in FMT experiments (Turnbaugh et al., 2009; Ridaura et al., 2013). One week after arrival, mice were transplanted by oral gavage with faecal samples (3 recipient mice for each donor), with gender coherence between donor and recipient, while a sham group (n = 12, 50% male/female) received only sterile PBS (150 μL) via single oral gavage. Animals were housed in individually ventilated cages, in a controlled environment, under 12-h light/12-h dark cycles and controlled room temperature (22 °C). The 3 mice receiving the same faecal material (same donor) were housed in the same cage, considering that cross-contamination from faecal ingestion would reinforce gut microbiome/metabolome engraftment. Mice received standard diet low in fat (53.5% carbohydrate, 3% fat, 18.5% protein, autoclavable 4RF21, Mucedola, San Donato Milanese, MI, Italy), and had *ad libitum* access to food and water. Cage, bedding material, food and water were sterilized and changed weekly. Body weight and food intake were monitored bi-weekly. Cognitive function was assessed by the Y maze test at 1, 4, 15, 22 and 23 weeks after FMT, in 59, 59, 58, 56 and 54 mice, respectively. At the end, mice underwent UHFUS and ^{18}FDG-PET-CT, and were then killed by anaesthetic overdose for brain and blood collection. One male mouse was dead at arrival, and 5 male mice

developed urinary tract infection. The study protocol was approved by the Italian Ministry of Health (approval decree n 464/2018-PR).

2.6. Y-maze test

The test was performed using a standard 3-arm Y-maze (Panlab, Harvard Apparatus, Barcelona, Spain) to assess working term memory and other aspects of neurodevelopment including locomotor activity and stress/anxiety in mice. Two different protocols were used, *i.e.*, the spontaneous alternation test (at 1 and 23 weeks after FMT), and the forced alternation test (at 4, 15 and 22 weeks after FMT). In the former, each animal was allowed to move freely through the 3-arms for 8 min. The forced alternation test is a two-trial memory task (Dellu et al., 2000). During the first 5-minute trial, the animal was allowed to visit two arms of the Y-maze, the third (arm B) being blocked by a door. After an inter-trial interval of 1.5 h, the door was opened, and a second 5-minute trial was run with the animal free to access all three arms. All trials were carried out between 9:00 and 12:00 AM. A visual automatic tracking system allowed to quantify the number of spontaneous alternation triplets and the percentage of alternation triplets in the spontaneous alternation test (spatial working memory), or the number of entries, time, distance and speed in the novel arm in the forced alternation test (spatial reference memory). Latency time until first entrance to novel arm can be inversely related to memory, whereas latency time until first arm choice and resting time are indicators of stress/anxiety, and zone transition number, total arm entries, travelled distance and speed in the total maze are indices of locomotor activity. A colourless and odourless chlorhexidine gluconate (CHG)/alcohol-based solution (Alcoholic Neoxidina, Nuova Farmec srl, Varese, Italy) was used to disinfect the Y-maze surface before and between trials to remove odours and avoid microbial cross-contamination between animals.

2.7. PET-CT scanning and image processing

PET-CT images were acquired in fasted (7 h) animals, under anaesthesia (1–2% (v/v) isofluorane in 1.0 l/min pure oxygen, IsoFlo®, Abbott Laboratories, Chicago, IL, U.S.), in a dedicated tomograph (IRIS PET/CT, Inviscan SAS, Strasbourg, France). After low-dose CT scan (20 s total scan time at 80 kVp, with a total time–current product of 18 mAs), ^{18}FDG was injected i.p. and a 100-min whole-body dynamic PET scan was performed, followed by high-resolution CT (112 s total scan time, 65 kVp, 112 mAs). Glucose levels were measured in tail blood by glucometer (OneTouch, Johnson&Johnson Medical SpA, Italy) at 30, 60 and 90 min from injection. CT images were reconstructed by cone-beam filtered backprojection (FBP) with correction of geometric misalignments, beam hardening and ring artifacts and exported to DICOM format. High-resolution CTs have a matrix of 600x600x1458 voxels, with an isotropic pitch of 76.95 μm. PET data were corrected for dead time, random coincidences, photon attenuation and radioactive decay, reconstructed by three-dimensional iterative ordered-subset expectation–maximization algorithm (OSEM), and co-registered to CT images (AMIDE Medical Image Data Examiner v.1.0.5). Volumes of interest (VOIs) were manually drawn on fused PET/TC brain images of the neocortex, right and left entorhinal cortex, right and left hippocampus, hypothalamus, thalamus and whole brain, verifying VOIs positioning in the Allen Mouse Brain Atlas (https://mouse.brain-map.org/). A VOIs were also drawn in left ventricular cavity images to derive the blood activity. Tissue activity levels in the last 20 min were divided by the integrated blood activity over 100 min (input function) to obtain the fractional uptake rate constant, which was multiplied by blood glucose levels and divided by brain lumped constant (0.6) to compute tissue glucose uptake rates (Guzzardi et al., 2022; Sanguinetti et al., 2019). Whole brain mean radiodensity (HU) was measured on CT high-resolution images.

2.8. Ultra-high frequency ultrasound (UHFUS)

Pulsed wave Doppler images of the right common carotid were acquired in anesthetized mice (1.5% isoflurane in 1.0 l/min pure oxygen) with a high-resolution imaging system (Vevo 3100, FUJIFILM VisualSonics Inc., Toronto, Canada) equipped with a 55 MHz ultrasound probe (MX550D, FUJIFILM VisualSonics Inc., Toronto, Canada). Peak systolic velocity (PSV) and end-diastolic velocity (EDV) were manually measured; mean velocity (MV) was obtained from tracing the envelope of the flow signal corresponding to a single cardiac cycle. Measurements were averaged over 3–5 cardiac cycles. Carotid resistivity index (RI) was calculated as (PSV-EDV)/PSV, while carotid pulsatility index (PI) was assessed as (PSV-EDV)/MV.

2.9. Faecal, urinary and serum metabolome by 1H NMR spectroscopy

In children, faecal samples were collected as described above, and urine samples were collected in sterile tubes during the visits (5:00–7:00 PM), and immediately brought in ice to the Institute of Clinical Physiology to be stored at −80 °C until analysis. In mice, faecal samples were collected and kept on ice during Y-maze sessions, to be stored at −80 °C immediately thereafter. Blood was centrifuged at 3000 rpm for 10 min, and plasma stored at −80 °C.

Metabolome was analysed at 1, 4 and 23 weeks post-FMT, as described (Sanguinetti et al., 2018). Briefly, 1H-NMR spectra of faecal or urine extracts, or serum (20 μL) supplemented with D2O (2 μL) were recorded in a Bruker Avance DRX 600 spectrometer (Valencia, Spain). Samples were measured at 310 K, and a single pulse presaturation experiment was acquired in all samples. Spectra were processed using MestReNova 8.1 (Mestrelab Research S.l., Santiago de Compostela, Spain). MATLAB (MathWorks, 2012) with in-house scripts was used for data analysis. Metabolite spin systems and resonances were identified by literature data and Chenomx resonances database (Chenomx NMR 7.6). Spectra were normalized to the total aliphatic spectral area to eliminate differences in metabolite total concentration, binned into 0.01 ppm buckets, and then subjected to mean-centering. Signals belonging to selected metabolites were integrated and quantified using semi-automated in-house MATLAB peak-fitting routines to obtain metabolite relative abundances.

2.10. Faecal microbiome

Faecal microbiota was profiled in mice before FMT, *i.e.*, upon arrival (F0, n = 4) and after 1 week spent in the animal facility (PRE, n = 51), as well as at 1 (F1, n = 39), and 15 weeks after FMT (F4, n = 64). Microbial DNA was extracted from stool samples using the QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions. For each sample, the hypervariable V3–V4 regions of the 16S rRNA gene were PCR-amplified using the S-D-Bact-0341-b-S-17/S-D-Bact-0785-a-A-21 primers (Klindworth et al., 20132013) with Illumina overhang adapter sequences. Amplicons were purified using a magnetic bead-based clean-up system (Agencourt AMPure XP; Beckman Coulter, Brea, CA, USA) and indexed by limited-cycle PCR using Nextera technology. Library sequencing was performed on an Illumina MiSeq platform using a 2 × 250 bp paired-end protocol as per manufacturer's instructions (Illumina, San Diego, CA, USA). Sequencing reads were processed using a pipeline combining PANDAseq (Masella et al., 2012) and QIIME 2 (Bolyen et al., 2019). After length and quality filtering, reads were binned into amplicon sequence variants (ASVs) using DADA2 (Callahan et al., 2016). Taxonomy was assigned via the VSEARCH algorithm (Rognes et al., 2016), using the Greengenes database as reference. Genus-level community composition was generated for all samples. Alpha diversity was measured using the Shannon index, the number of observed ASVs and Faith's phylogenetic diversity. Beta diversity was computed based on weighted and unweighted UniFrac distances and visualized on a Principal Coordinates Analysis (PCoA) plot.

PCoA and bar plots were built using the R packages made4 (Culhane et al., 2005) and vegan (https://www.cran.r-project.org/package = vegan).

2.11. Statistical analysis

IBM® SPSS® Statistics for Mac OS X (version 24.0, Chicago, IL, USA) and the free online software MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/MetaboAnalyst/) were used. Pearson's correlation coefficient was used to explore univariate associations between metabolome data and cognitive, imaging, or nutritional parameters in children and/or mice. Partial correlations were used to explore the role of vascular parameters in the metabolite-to-cognition relationship. Correlation coefficients ≥ 0.4 identified strong associations (Toubiana and Maruenda, 2021) in metabolome analyses. Group differences in metabolome composition were assessed by both multivariate orthogonal partial least-squares (OPLS) model, with multivariate variable importance in projection (VIP) analysis to identify specific relevant metabolites (Thevenot et al., 2015), and univariate Student's *t*-test for independent samples, with false discovery rate (FDR) applied to correct for multiple testing. Metabolite-based children clusters were obtained by K-means clustering algorithm. Chi-square test was used to analyse the distribution of gender, breastfeeding, delivery type and maternal gestational diabetes mellitus between groups of donors. For mice microbiota, statistical analysis was performed using R Studio 1.0.44 on R software v3.3.2 (https://www.r-project.org). Group differences in alpha diversity and relative taxon abundances were assessed by Wilcoxon test. The significance of separation between study groups in PCoA was tested using PERMANOVA (adonis function in vegan package). P-values ≤ 0.05 were regarded as statistically significant.

3. Results

3.1. Pipeline

The analysis and presentation of results were intended to 1) prove the causal role of the gut microbiota/metabolome on cognitive-behavioural performance in a durable fashion (via FMT), and 2) to identify a gut metabolite-based biomarker able to predict cognitive development to the benefit of screening and/or primary prevention by e.g., nutritional stimulation or supplementation in children. Out of the DORIAN-PISAC children with available faeces and cognitive data, we originally selected the 10 children with the highest and 10 children with the lowest global function (total GMDS score, 50% male/female), as distant as possible, intending to have two clearly stratified groups. We found that the cognitive phenotype was transferred by the transfer of gut microbiome/metabolome, that the total GDMS score (in children) was the parameter most correlated with cognitive-behaviour parameters in corresponding recipient mice, and that metabolite-to-phenotype links were shared among children and recipient mice. Then, key metabolites were used to identify a simple gut-derived biomarker able to screen or predict cognitive development. Since the full spectrum of above-identified metabolites was insufficiently effective, the top 3 metabolites, showing strongest positive associations with children's cognition were tested as stratification criterion in children and mice (metabolite-enriched vs -depleted donors and recipients). Comparing cognitive function and faecal and blood metabolome in recipients of metabolite-enriched or -depleted FMTs supported causality and candidate metabolites. Then, we performed an explorative analysis to capture protective nutritional modulators in children.

3.2. Cause-effect role of FMT on cognitive development

Children in the high- and low-GMDS groups were similar for anthropometrics, parents' age, parents' intelligence quotients and socio-economic status, but significantly different for most of neurocognitive GMDS-based scales (Table 1). The Y-maze test in corresponding FMT recipient germ-free mice (high-GMDS-R and low-GMDS-R; R = recipients) showed that 1 week after FMT, high-GMDS-R ran longer distance, reached higher speed, and spent less time resting compared to low-GMDS-R and sham mice (Fig. 1A). Four weeks after FMT, high-GMDS-R showed higher short-term working memory and locomotor activity (greater distance, fast time in the novel arm B), compared to the other groups, with a decreasing trend from high- to low-GMDS-R, and to sham mice (Fig. 1B). At 22 and 23 weeks, high-GMDS-R showed lower alternation triplet % (which might indicate habituation), and lower latency to enter the novel arm (Fig. 1C) or do their first choice (Fig. 1D) compared to low-GMDS-R or sham mice, with low-GMDS-R showing very high latency to enter the novel arm (>2-fold greater than the other groups).

Table 1
Description of donors stratified based on GMDS total score.

Parameter	High-GDMS	Low-GDMS
Boys/girls, N	5/5	5/5
Weight at birth (kg), mean ± SEM	3.3 ± 0.1	3.1 ± 0.1
Delivery mode (vaginal/C-section), N	8/2	7/3
Breastfeeding (exclusively/non-exclusively), N	3/7	3/7
Age (months) at weaning, mean ± SEM	5.5 ± 0.2	5.7 ± 0.2
Weight at age 5 (kg), mean ± SEM	19.6 ± 0.7	20.2 ± 0.6
Height at age 5 (cm), mean ± SEM	112.2 ± 1.7	109.6 ± 1.0
BMI at age 5 (kg/m^2), mean ± SEM	16.0 ± 0.5	16.8 ± 0.4
Locomotor score, mean ± SEM	108.3 ± 1.4*	96.8 ± 2.2
Personal-social score, mean ± SEM	106.5 ± 1.4*	97.6 ± 3.4
Hearing and speech score, mean ± SEM	103.7 ± 2.0#	96.4 ± 3.5
Eye-and-hand coordination score, mean ± SEM	102.3 ± 1.8	99.1 ± 3.7
Performance score, mean ± SEM	116.1 ± 0.8*	110.2 ± 2.4
Practical reasoning score, mean ± SEM	107.4 ± 3.3**	88.7 ± 1.8
Total score, mean ± SEM	107.9 ± 1.1**	98.7 ± 1.7
Mothers' IQ estimate, mean ± SEM (N = 17)	118.5 ± 2.8 (10)	113.1 ± 3.1 (7)
Father's IQ estimate, mean ± SEM (N = 9)	116.2 ± 7.5 (4)	115.7 ± 3.5 (5)
Mother's age (years), mean ± SEM	41.8 ± 1.4	39.4 ± 1.4
Father's age (years), mean ± SEM	44.3 ± 1.6	43.4 ± 1.4
Mother' ESeC, mean ± SEM	6.9 ± 0.9	6.2 ± 1.0
Fathers' ESeC, mean ± SEM (N = 17)	6.3 ± 0.9 (9)	6.0 ± 0.8 (8)

Table 1 Groups characteristics are given as mean ± standard error (SEM) and/or number (N), as appropriate. Abbreviations: BMI = body mass index; C-section = caesarean section; IQ = intelligence quotient; ESeC = European Socio-economic Classification 10-class model [ref]. Statistical test: *t*-test, *p-values = ≤ 0.05, *p-values = ≤ 0.01, #p-values = ≤ 0.1.

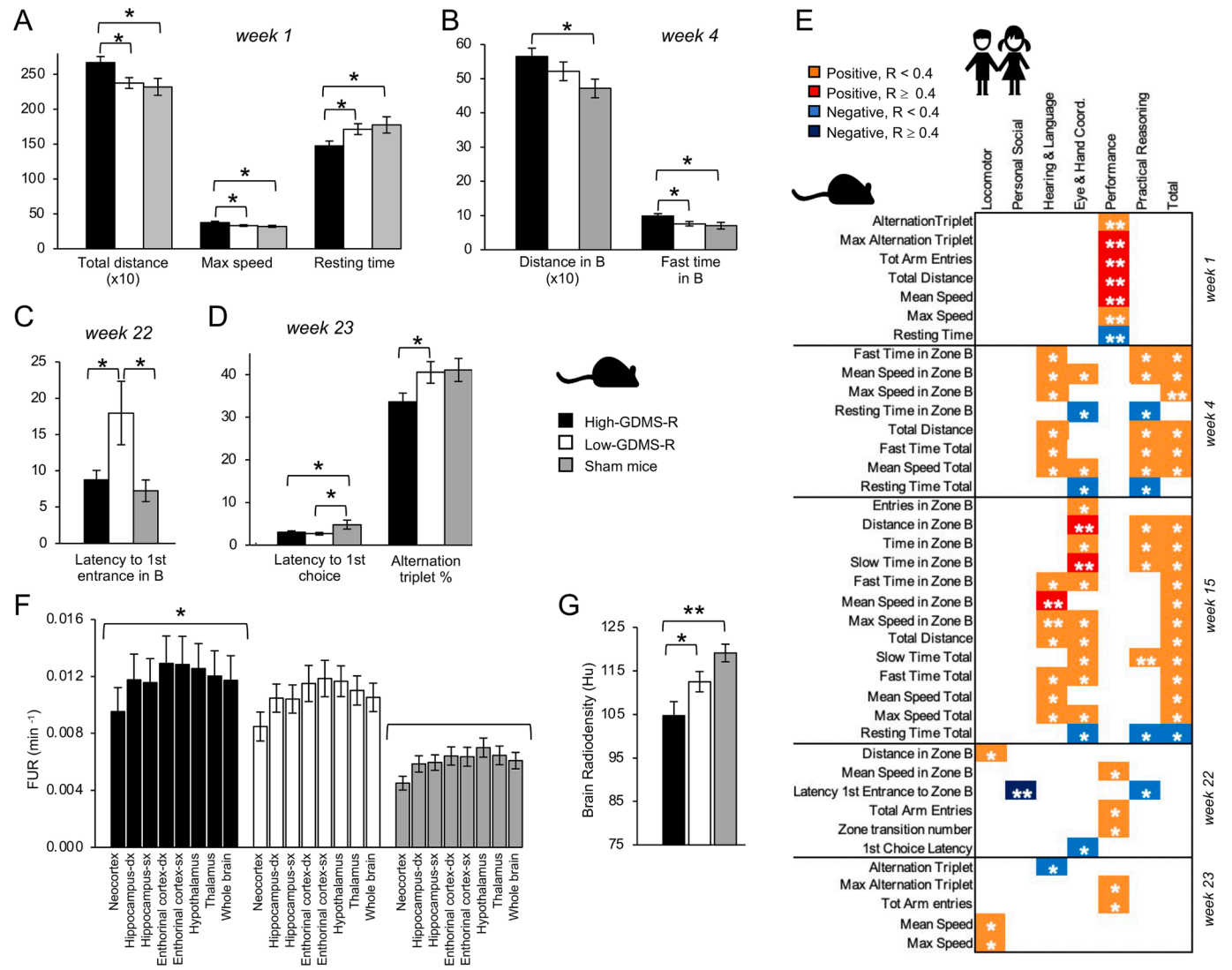

Fig. 1. Donor-to-recipient phenotype transfer. Cognitive and behavioural parameters were significantly different between mice receiving faecal metabolome from high-GMDS or low-GMDS donors or sham mice, as assessed by the spontaneous alternation test at 1 and 23 weeks after FMT (A, D), or by the forced alternation task at 4 (B) and 22 (C) weeks after FMT. Moreover, several donor-to-recipient correlations in cognitive-behavioural parameters were found, as shown in E. Higher brain glucose fractional uptake (F) and lower radiodensity (G) were measured in high-GMDS-R compared to others. x10 = the reported number should be multiplied by 10. * $p \leq 0.05$, ** $p \leq 0.01$.

Correlative findings (Fig. 1E) provided even stronger evidence of donor-to-recipient phenotype transfer. Positive associations were observed between donors' cognitive scores and recipients' variables related to the cognitive-memory domain (alternation triplets, total arm entries, zone transition number, and distance, speed, time in the novel arm or in the whole maze). Negative correlations were sporadic, but notably, children's practical reasoning predicted lower latency to enter the new arm (*i.e.*, better function) in adult recipients. Total GMDS, performance, practical reasoning, hearing and language, and eye-hand coordination scores in children were the parameters most consistently associated with mice variables over time.

Glucose uptake was measured in several brain areas of recipients by *in vivo* PET imaging at the end of the protocol. In all areas, glucose fractional uptake rate was higher in high-GMDS-R compared to sham mice, with a decreasing trend from high- to low-GMDS-R to sham mice (R −0.281 to −0.253, p trend 0.023 to 0.042) (Fig. 1F). Brain radiodensity measured *in vivo* by CT was lower in high-GMDS-R compared to low-GMDS-R and sham mice, the latter showing highest values (Fig. 1G). No group difference was found in brain weight (ex vivo) or brain-to-body mass ratio.

Overall, these results strongly support that a phenotype transfer can be achieved through FMT, with some effects lasting until full adult age.

3.3. Metabolites linked to the translatable phenotype

In children, multivariate *ortho*-PLSDA analysis of faecal metabolome (available in 18/20 cases) identified 15 metabolites with VIP score > 1, six of which, *i.e.*, fumarate, formate, mannose, hypoxantine plus adenine, uracil plus unknown, and 3-hydroxybutyrate, showed increasing trends in high-GMDS compared to low-GMDS children (Fig. 2A). Moreover, univariate correlation revealed that 15 faecal metabolites were associated with cognitive development in at least one of the GMDS domains (Fig. 2B). Specifically, mannose, xanthine and formate, and the amino acids valine, tyrosine, and phenylalanine were positively related to eye-hand coordination and/or total GMDS scores, whereas caprylate was directly associated with performance. Conversely, butyrate, leucine plus unknown, glutarate plus unknown, acetate, propionate plus glutarate, creatines plus lysine and creatinine were negatively related with one or more of the GMDS domains.

In FMT mice, the multivariate *ortho*-PLSDA analysis identified 19

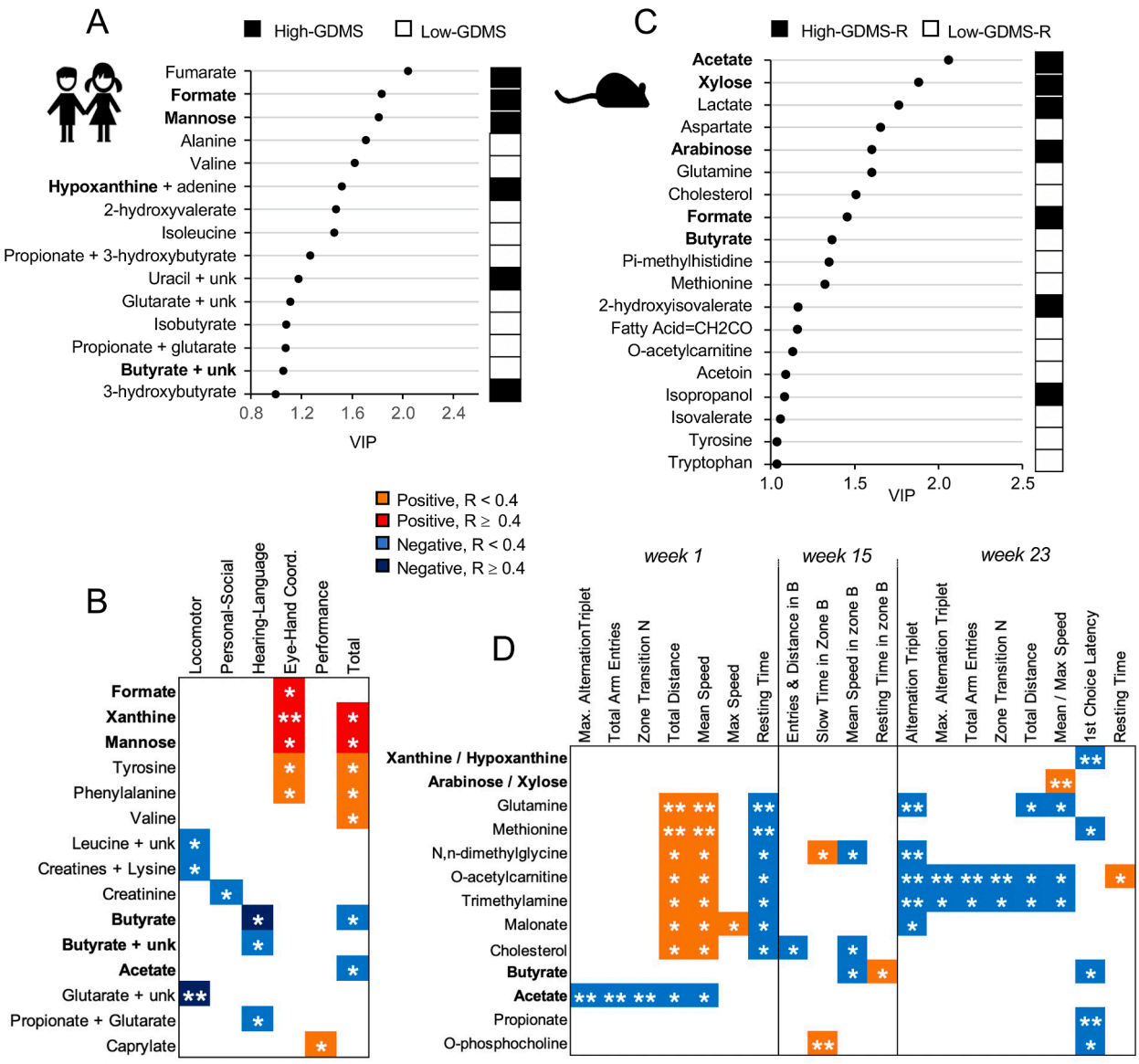

Fig. 2. Metabolites linked to the translatable phenotype in donor children and in recipient mice. Multivariate orthogonal partial least-squares (OPLS) model with variable importance in projection (VIP) analysis (the black and white colours indicate the group in which the specific metabolite in enriched) in high-GMDS and low-GMDS donors (A) and recipients (C), and univariate correlative analysis between faecal metabolites and cognitive scores in donors (B) and recipients (D). Metabolite-to-phenotype links shared among children and recipient are in bold and include formate, xanthine/hypoxanthine, and sugars, namely mannose in children and xylose/arabinose in mice, with positive associations, and acetate and butyrate with negative associations.

metabolites with VIP score > 1 (Fig. 2C), 7 of which, *i.e.*, acetate, xylose, lattate, arabinose, formate, 2-hydroxisovalerate, isopropanol, showing an increasing trend in high-GMDS-R compared to low-GMDS-R. In addition, in the univariate correlation analysis a small panel of metabolites including those identified in children was correlated with cognitive-behavioural parameters (Fig. 2D). In fact, the faecal levels of xanthine/hypoxanthine, and of xylose/arabinose (dietary sugars) were positively related to mean speed or negatively related to first choice latency at 23 weeks, respectively, whereas butyrate and acetate were negatively correlated with cognitive and locomotor variables at 1 and 15 weeks, respectively, and directly to resting time in B (butyrate) at 15 weeks.

Overall, these results show that there are shared metabolites predicting the cognitive phenotype in a consensual manner in children and mice, including formate, xanthine/hypoxanthine, and sugars, namely mannose in children and xylose/arabinose in mice, with positive associations, and acetate and butyrate with negative associations.

3.4. *Identification of a gut-derived biomarker of cognitive development*

We consider that an effective biomarker should be able to significantly separate individuals with high or low cognitive development scores, and include the minimum number of molecules to save costs and time for biological assays.

We first tested a combination of the above-mentioned metabolites. By using the K-means clustering algorithm, we clustered children in two groups based on the faecal levels of formate, xanthine/hypoxanthine, mannose, acetate, butyrate. However, this metabolite-based clustering approach was insufficiently effective in separating phenotypes in both children and mice groups. In fact, a regulatory metabolite is not necessarily a good biomarker.

Thus, we combined only gut-derived metabolite showing the strongest positive associations with cognitive scores, namely formate, mannose and xanthine (Fig. 2B). The 3 metabolites were summed, and children were ranked by the sum (cumulative) value to be stratified into

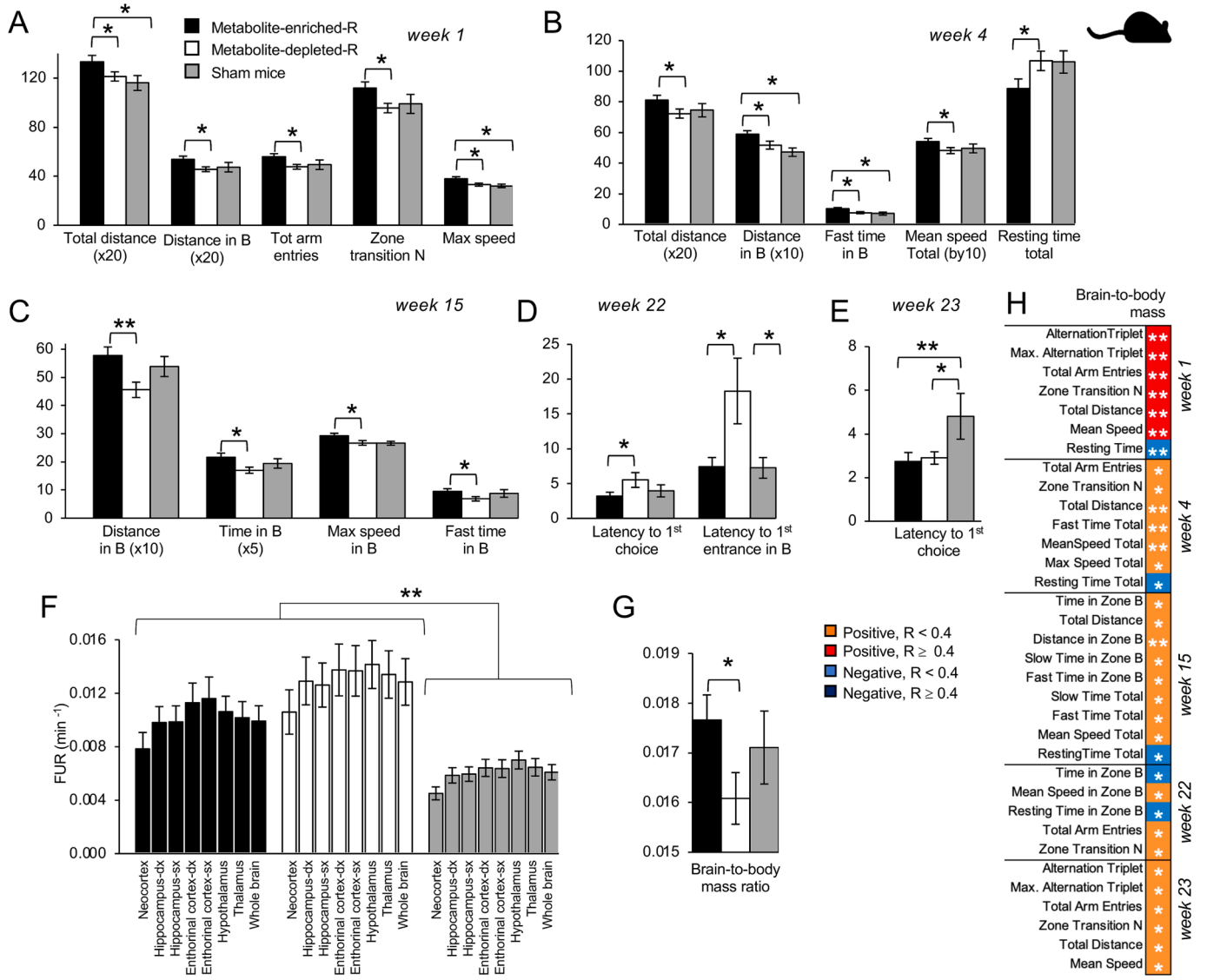

Fig. 3. Validation of the gut-derived biomarker. Cognitive-behavioural scores were significantly different between mice receiving faecal metabolome from metabolite-enriched or metabolite-depleted donors or sham mice, as assessed by the spontaneous alternation test at 1 and 23 weeks after FMT (A, E), or by the forced alternation task at 4 (B), 15 (C) and 22 (D) weeks after FMT. The metabolite-based stratification highlighted FMT-dependent modifications of brain metabolism (F), and group differences in brain-to-body mass ratio (G), which correlated to cognitive-behavioural scores are shown (H). x5, x10, x20 = the reported number should be multiplied by 5, 10, 20, respectively. * $p \leq 0.05$, ** $p \leq 0.01$.

metabolite-enriched or metabolite-depleted. This combined biomarker was able to stratify two groups of children with significantly different scores in several neurocognitive GMDS-based scales (Supplementary Table S1), in spite of no significant differences in anthropometrics, parents' age, parents' intelligence quotients and socioeconomic status, and sex distribution.

3.5. *Validation of the gut-derived metabolomic biomarker: Cognitive-behavioural scores and in vivo imaging in recipients*

In order to validate the identified metabolomic biomarker, FMT recipient germ-free mice were stratified into metabolite-enriched-R and metabolite-depleted-R, in coherence with respective donors' groups, and phenotypic parameters were reanalysed. One week after FMT, metabolite-enriched-R scored better in several cognitive-behavioural parameters (total distance, maximum alternation triplet, total arm entries, zone transition number and maximum speed) (Fig. 3A), as assessed by the spontaneous alternation test, compared to metabolite-depleted-R, which were comparable to sham mice. Four weeks after FMT, metabolite-enriched-R showed higher short-term working memory and locomotor activity, as assessed by greater distance run in the whole maze and in the novel arm (B), higher fast time in the novel arm, higher mean speed, and lower resting time compared to metabolite-depleted-R (Fig. 3B). Again, similar results were obtained 15 weeks after FMT, when the metabolite-enriched-R spent more time, ran longer distance and reached higher speed in the novel arm compared to mice in the metabolite-depleted group (Fig. 3C). Sham mice were similar to metabolite-depleted mice or intermediate between FMT groups. At 22 and 23 weeks after FMT, the metabolite-enriched-R showed significantly lower latency to first choice and to enter the novel arm (Fig. 3D-E) compared to metabolite-depleted or sham mice. Again, metabolite-depleted-R (similar to low-GMDS-R) showed very high latency to enter the novel arm at this adult age.

In vivo PET data showed higher fractional uptake rate in FMT than sham mice, with no difference between metabolite-enriched-R and metabolite-depleted-R. Brain radiodensity decreased from sham to metabolite-depleted-R and metabolite-enriched-R ($R = -0.284$, $p = 0.028$), with lower values in metabolite-enriched than sham mice ($p =$

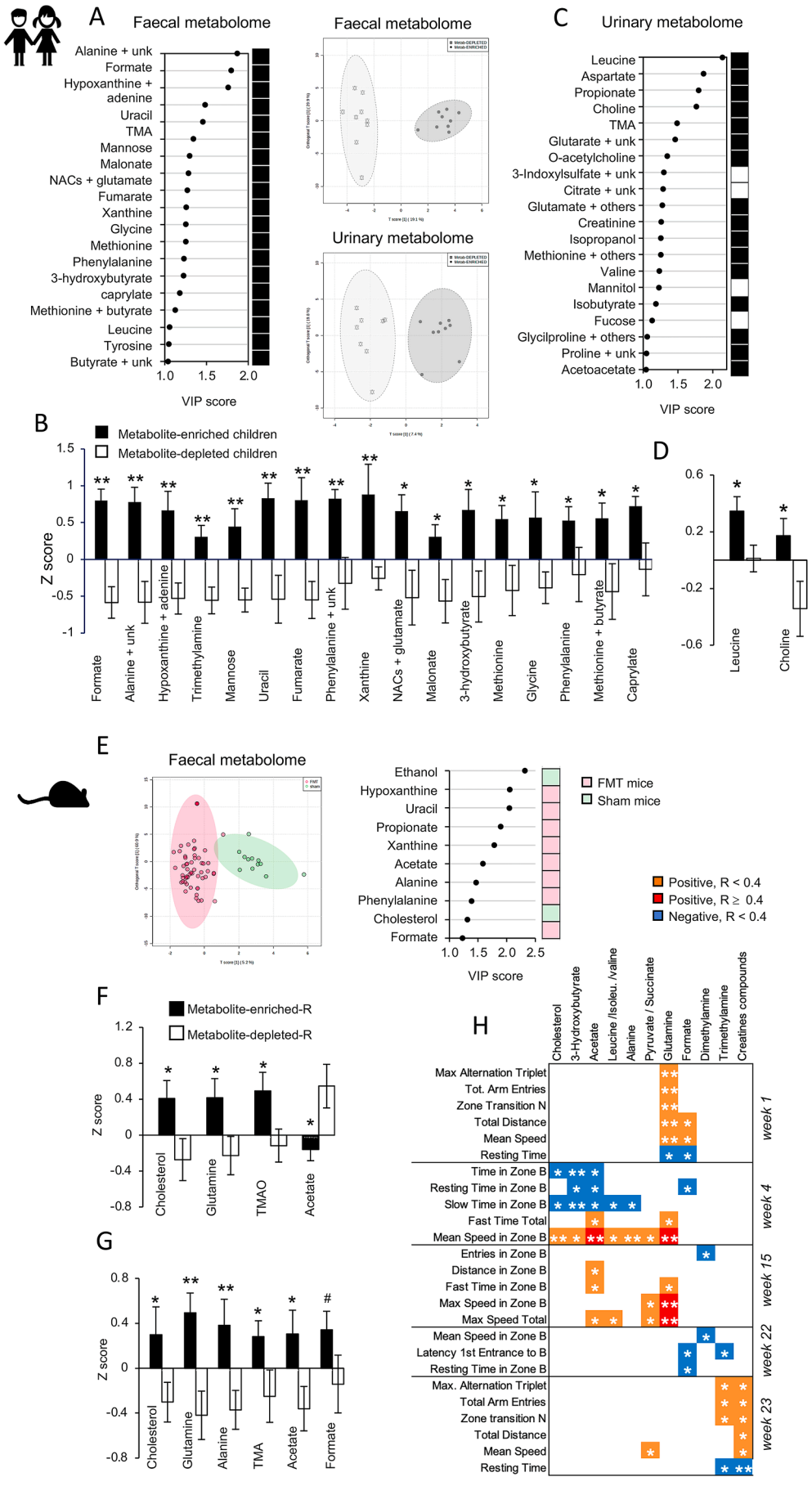

Fig. 4. Metabolite signatures of cognitive development in biomarker-based analysis In donor children, multivariate orthogonal partial least-squares (OPLS) model with variable importance in projection (VIP) analysis (the black and white colours indicate the group in which the specific metabolite in enriched) showed that both faecal (A) and urinary (D) metabolomes were significantly different between metabolite-enriched and metabolite-depleted children. Bottom panels show the levels of faecal (B) and urinary (D) metabolites achieving significant group differences according to univariate analysis. In recipient mice, OPLS model with VIP analysis (the pink and green colours indicate the group in which the specific metabolite in enriched) showed that faecal metabolome was significantly different between FMT and sham mice at the end of the protocol (E). Faecal levels of cholesterol, glutamine, TMAO, and acetate were significantly different between recipients' groups at 1 weeks after FMT (F). Significant group differences were not detected in the blood metabolome as a whole, but were found in specific metabolites (G), which correlated with cognitive-behavioural parameters (H). * $p \leq 0.05$, ** $p \leq 0.01$ and p-FDR ≤ 0.05 (in group comparisons), # $p = 0.06$.

0.016). Conversely, metabolite-enriched compared to -depleted mice had higher brain-to-body mass ratio (Fig. 3G), which was also correlated with several cognitive-behavioural parameters (Fig. 3H).

3.6. Metabolite signatures of cognitive development in biomarker-based analysis

Any stratification based on selected metabolites would carry-over other metabolites with potential synergic effects. Therefore, we re-analysed the metabolic profiles of both donors and recipients. In donor children faecal metabolome was significantly different between metabolite-enriched and metabolite-depleted group (Fig. 4A), with 19 top metabolites contributing to the separation (VIP score > 1). In univariate analysis, 17 of them were significantly different (relative abundance, standardized values) between groups, and 9 remained significantly different after FDR correction (Fig. 4B). Specifically, higher faecal levels of alanine, phenylalanine, TMA, fumarate, hypoxanthine plus adenine, and uracil, in addition to mannose, xanthine and formate, were observed in metabolite-enriched than metabolite-depleted children. These 9 metabolites surviving FDR correction were among the top 10 metabolites contributing to group separation in the *ortho*-PLSDA analysis, demonstrating good agreement between multivariate and univariate analyses.

Urinary metabolome was also analysed as potentially contributing to the gut-brain cross-talk. The urinary metabolome was different between metabolite-enriched and metabolite-depleted children in multivariate *ortho*-PLSDA analysis (Fig. 4C), with 20 top metabolites contributing to the separation (VIP score > 1). Three out of them, namely O-acetylcholine, choline, and TMA, were related to faecal biomarkers and/or positively associated with cognitive scores (Supplementary Fig. S1A and B). In univariate analysis, only choline and leucine were significantly different between groups (relative abundance, standardized values) (Fig. 4D).

Overall, the panel of potential protective gut-derived biomarkers for cognitive development in 5-year-old children includes faecal xanthine/hypoxanthine, formate and mannose, and co-segregating faecal alanine, tyrosine, phenylalanine, valine, uracil and TMA. The co-segregation between faecal mannose, xanthine and formate and urinary O-acetylcholine, choline and TMA may indicate a mechanistic synergy.

In mice, the faecal metabolome of FMT recipients was significantly different from that of sham individuals (Fig. 4E), and metabolites mostly contributing to this separation (VIP score > 1) included the biomarkers related to cognitive scores in children, namely xanthine, hypoxanthine, alanine, phenylalanine, acetate, formate.

The *ortho*-PLSD analysis did not reveal significant difference between FMT groups (data not shown), but the relative abundance of specific metabolites was increased, *i.e.*, cholesterol, glutamine, and TMA-N-oxide (TMAO), or reduced, *i.e.*, acetate, in metabolites-enriched-R metabolite-depleted-R at 1 week after FMT (Fig. 4F), with no differences at 15 and 23 weeks post-FMT. Notably, at the end of the protocol, donors' faecal metabolites appeared to be more closely reflected in the circulation of recipients. In fact, higher plasma levels of glutamine, alanine, TMA, acetate, formate and cholesterol were found in metabolite-enriched-R than metabolite-depleted-R (Fig. 4G). In particular, plasma levels of glutamine in recipients were associated positively with cognitive and memory-related parameters (e.g., maximum alternation triplets, distance and speed) and negatively with the anxiety parameter resting time (Fig. 4H). Glutamine was not detected in donors' faeces, but the NAC plus glutamate peak was elevated in metabolite-enriched compared to metabolite-depleted children (Fig. 4B) and showed a positive trend with the plasma glutamine level of mice ($R = 0.256$, $p = 0.082$), suggesting an augmented glutamate-glutamine cycle in metabolite-enriched children and mice. These findings, together with the direct correlations between donors' faecal TMA and recipients' faecal TMAO ($R = 0.332$, $p = 0.020$), and between donors' faecal alanine and recipients' plasma alanine ($R = 0.331$, $p = 0.023$), suggest the transfer of metabolites from donor to recipient.

3.7. Faecal microbiome of recipients

As expected, 16S rRNA amplicon sequencing of faecal samples from germ-free mice upon arrival and after 1 week, prior to FMT, yielded no or few high-quality reads. In contrast, sequencing of recipients' faecal samples at 1 week (F1, $n = 39$) and 15 weeks post-FMT (F4, $n = 64$) yielded a total of 3,352,420 high-quality reads, with an average of $32{,}547 \pm 10{,}898$ sequences per sample. Regardless of the time point, the faecal microbiota of FMT recipients was significantly different from sham mice in alpha and beta diversity (Fig. 5). Particularly, alpha diversity was significantly higher in FMT than sham mice with all metrics used ($p < 0.001$, Wilcoxon test) (Fig. 5A). As for beta diversity, PCoA of inter-sample variation based on weighted and unweighted UniFrac distances showed significant segregation between FMT recipients and sham mice at both time points ($p < 0.001$, PERMANOVA) (Fig. 5B).

Next, we investigated the taxonomic composition of metabolite-enriched and metabolite-depleted recipients over time (Fig. 5C). One week after FMT, metabolite-enriched-R showed overabundance of [*Eubacterium*] compared with metabolite-depleted-R ($p = 0.02$) (Fig. 5D). The two groups also differed at 15 weeks in relative abundances of *Ruminococcus* and *Turicibacter*, both being higher in metabolite-enriched mice ($p = 0.006$, $p = 0.03$). Intra-group differences over time showed a significant increase in relative abundance of *Turicibacter*, *Blautia*, *Coprobacillus* and *Adlercreutzia* ($p \leq 0.03$), and depletion of *Bifidobacterium* ($p = 0.02$) in metabolite-enriched-R. However, some of these differences, namely those in [*Eubacterium*], *Blautia* and *Coprobacillus* were also shared by sham mice ($p \leq 0.04$). In contrast, in the metabolite-depleted group, fewer compositional variations were observed over time, namely a significant increase in *Alistipes* ($p = 0.02$).

We observed that the two stratification criteria resulted in similar compositional variations, especially in intra-group comparisons. One week after FMT, high-GMDS-R were found to be significantly enriched in *Clostridium* (from the *Erysipelotrichaceae* family) and *Ruminococcus* ($p = 0.02$, $p = 0.04$; Wilcoxon test), and depleted in *Parabacteroides* and *Enterococcus* ($p = 0.02$, $p = 0.04$) compared to low-GMDS-R (Supplementary Fig. S2). Changes in *Ruminococcus* and *Parabacteroides* persisted until 15 weeks post-FMT ($p = 0.001$, $p = 0.05$). In addition, at this time point, a significant overrepresentation of *Akkermansia* was observed in low-GMDS-R compared to high-GMDS-R ($p = 0.009$). Intra-group differences over time showed a significant increase in relative abundance of *Turicibacter*, *Blautia*, *Alistipes*, *Adlercreutzia* and *Coprobacillus* ($p \leq 0.04$), and depletion of *Bifidobacterium* ($p = 0.03$) in high-GMDS-R. Like in the metabolite-based stratification, fewer compositional variations were observed over time in low-GMDS-R, including a significant increase in *Blautia* and *Turicibacter* ($p = 0.005$, $p = 0.02$), vs high-GMDS-R. As mentioned above, changes in *Blautia* and *Coprobacillus* were shared by sham mice ($p = 0.02$, $p = 0.04$), which also showed a significant increase in the relative abundances of the genera [*Eubacterium*], *Clostridium* (from the *Lachnospiraceae* family), and *Oscillospira* ($p \leq 0.04$).

3.8. Vascular resistance mediates correlations between metabolites and cognition

Vascular carotid resistivity index (RI) and pulsatility index (PI) were tested as potential mediators of the gut-brain relationship, based on their relationship with cognition (inversely) and Alzheimer's disease (directly) in humans (Mitchell et al., 20112011; Yew and Nation, 2017). RI and PI decreased from sham mice to metabolite-depleted-R to metabolite-enriched-R (Fig. 6A), and RI was inversely correlated with brain-to-body mass ratio (Fig. 6B). In addition, RI and PI correlated with some cognitive-behavioural parameters and (Fig. 6C) with faecal and blood metabolites relating to cognitive variables (Fig. 2D). By using

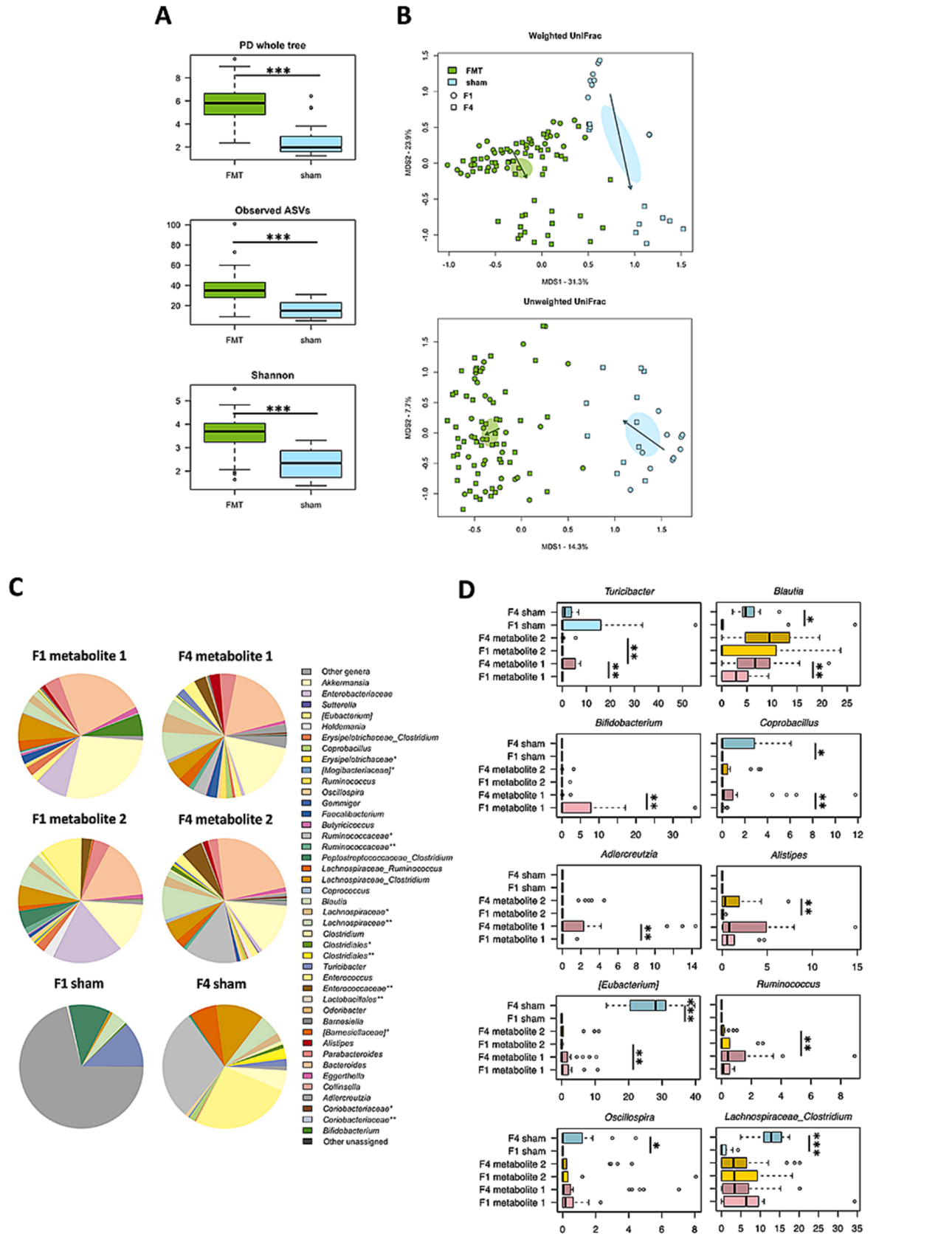

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Fig. 5. The faecal microbiota of FMT mice segregates from that of sham mice, and metabolites-enriched-R differ from metabolites-depleted-R in genus-level microbiota composition. Boxplots showing the distribution of alpha diversity, measured according to Faith's Phylogenetic Diversity (PD whole tree), the number of observed ASVs and Shannon index, in faecal samples from FMT recipients vs sham mice, collected at 1 week (F1) and 15 weeks (F4) post-FMT (A). Alpha diversity was significantly higher in FMT than sham mice with all metrics used (*** $p < 0.001$, Wilcoxon test). Principal Coordinates Analysis (PCoA) based on weighted (top) and unweighted (bottom) UniFrac distances between the microbiota profiles of FMT recipients and sham mice over time (B). A significant separation between groups was found, regardless of time point ($p < 0.001$, PERMANOVA). The arrows indicate the direction of temporal variations, from week 1 (F1) to week 15 post-FMT (F4). Pie charts summarizing the mean relative abundance of genera in FMT recipients stratified using metabolite-based stratification (metabolites-enriched – metabolite 1 vs metabolites-depleted – metabolite 2), and sham mice at 1 and 15 weeks post-FMT (F1 and F4, respectively) (C). Only genera present in at least 20% of samples with relative abundance $\geq 0.5\%$ are shown. Boxplot showing the relative abundance distribution of differentially represented genera between study groups and over time (* $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$, Wilcoxon test) (D).

multivariate analysis, we found that correlations between faecal levels of cholesterol and O-acetylcarnitine and total distance or speed in the maze at 1 week after FMT were abolished when controlling for RI, and correlations between butyrate or N,n-dimethylglycine and mean speed in the novel arm at 15 weeks after FMT were abolished when controlling for PI, indicating that vascular parameters mediate these metabolite-to-function relationships. Conversely, the relationship between faecal glutamine and methionine and cognitive function was not mediated by vascular indices (not abolished by partial correlation). Similarly, correlations between plasma glutamine and total distance and speed at 1 week, or speed parameters at 15 weeks after FMT were independent of RI. Overall, these results indicate that vascular resistance and pressure in the carotid artery may represent the anatomical link between some metabolites and cognitive development, and reinforce the independent role of glutamine, participating in different pathways, e.g., glutamate-glutamine cycle.

3.9. Nutritional factors related to metabolomic biomarkers

Children's dietary information were analysed to identify foods and nutrients potentially able to improve cognitive development by targeting the gut-brain axis. Notably, the majority of children who had been exclusively breastfed in the first 6 months of life were in the metabolite-enriched group, as opposed to children receiving mixed or formula feeding (Fig. 7A). In the DORIAN-PISAC, exclusive breastfeeding was related to higher levels of faecal TMA and malonate at 5 years (Supplementary Fig. S3), in line with higher levels of these metabolites in metabolite-enriched compared to metabolite-depleted children (Fig. 2E).

At the age of 5 years, metabolite-enriched children consumed more animal-derived foods, in particular milk and other dairy products, more specifically whole-milk yogurt (Fig. 7B). In terms of macro- and micronutrient composition, a higher intake of simple carbohydrates and animal proteins was observed in metabolite-enriched children (Fig. 7C).

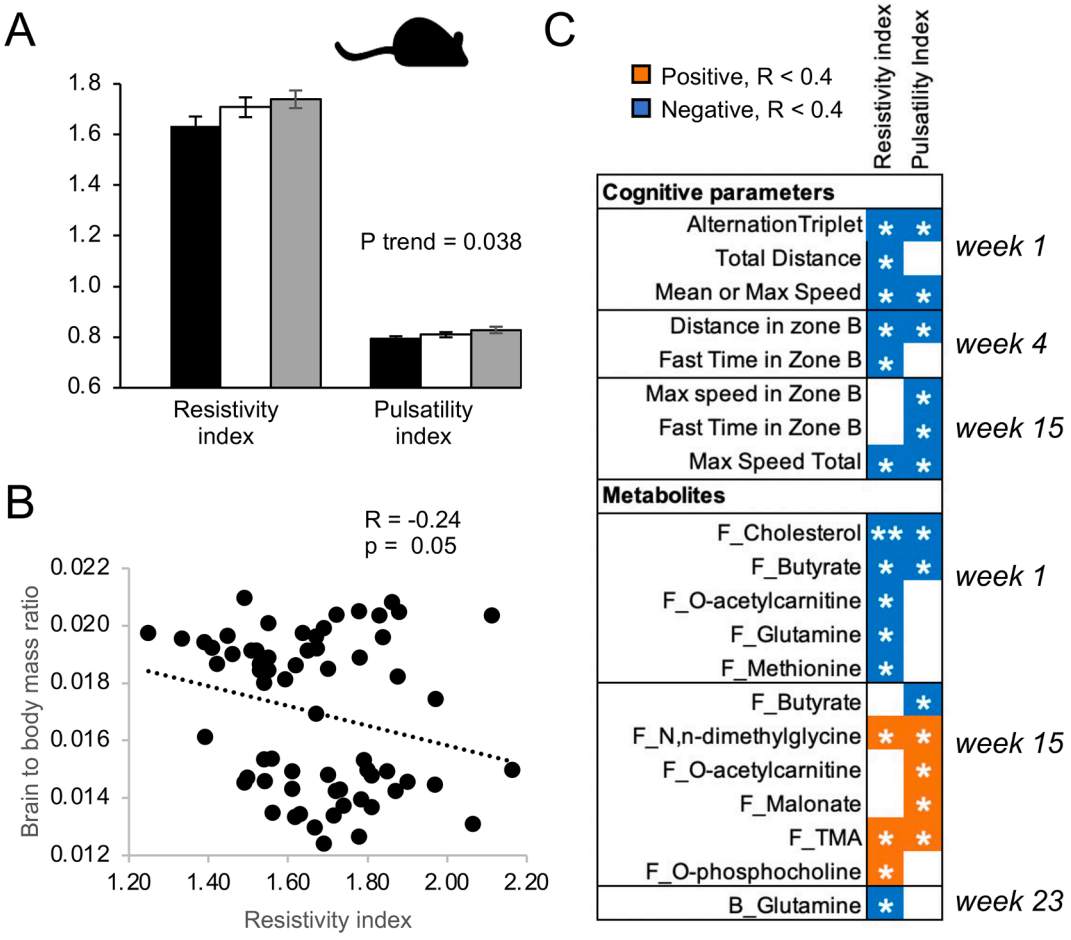

Fig. 6. Vascular resistivity and pulsatility indices. Carotid artery resistivity index (RI) and pulsatility index (PI) tended to lower values in metabolite-enriched-R (A) compared to others, and RI correlated inversely with brain-to-body mass ratio (B). Both RI and PI were related to cognitive-behavioural parameters and faecal (identified as F_) and blood (identified as B_) key metabolites (C).

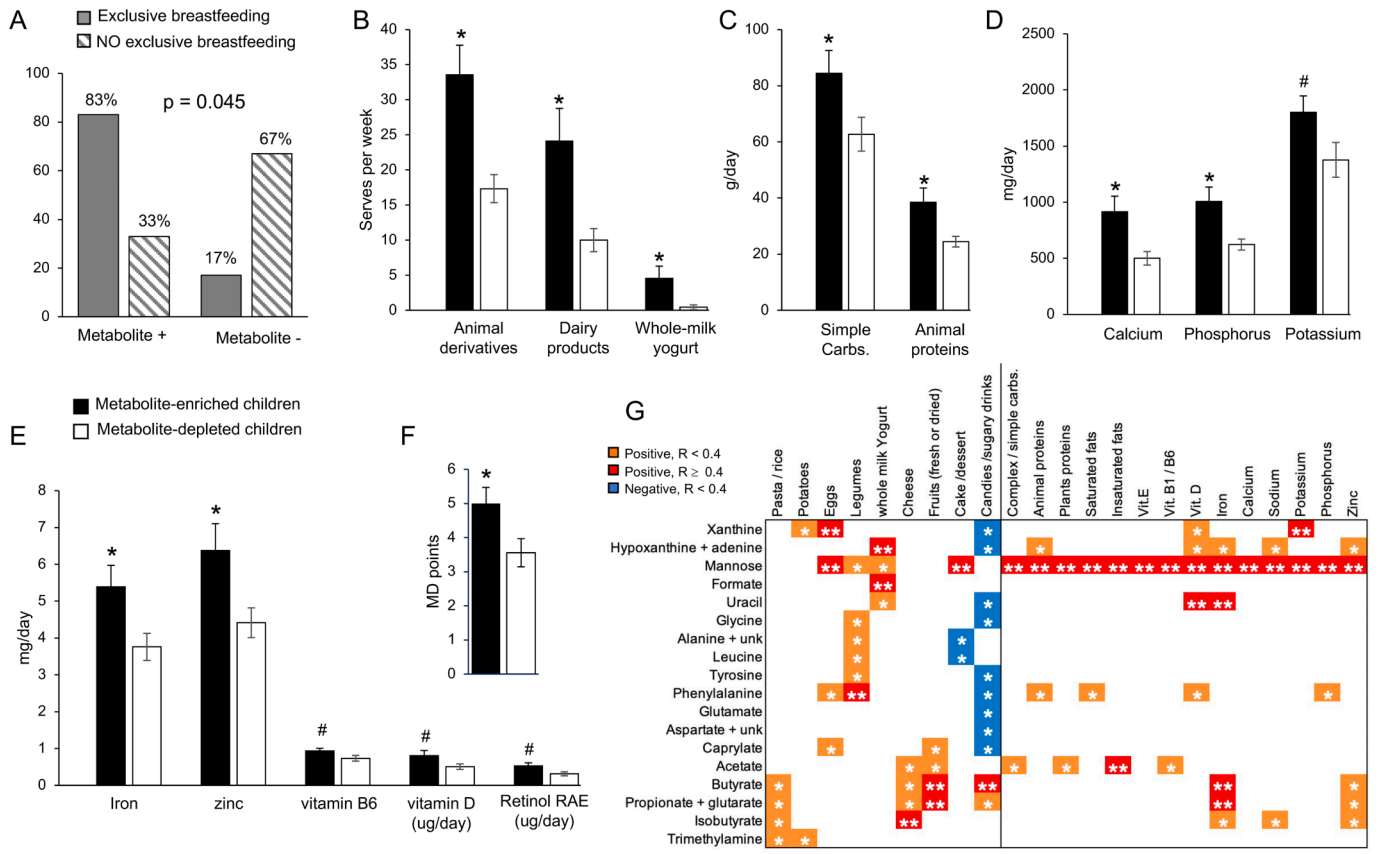

Fig. 7. Relationships between nutrition and faecal metabolome in children. Differences between metabolite-enriched and metabolite-depleted children were found in early feeding practice (A), in the consumption of food categories (B) and in the intake of macro- (C) and micro-nutrients (D-E) at the age of 5 years. Overall, metabolite-enriched children showed higher consumption of dietary components associated to the traditional Mediterranean diet (F). Specific foods and nutrients were related to the level of promising gut-derived metabolites (G). ** $p \leq 0.01$, * $p \leq 0.05$, # $p \leq 0.07$.

Also, the diet of metabolite-enriched children was characterized by a greater intake of iron, calcium, phosphorus, zinc, retinol, vitamin B6, and vitamin D compared to the metabolite-depleted group (Fig. 7D-E). Vitamin supplementation was generally not, or only sporadically used (only 2 children among donors), and therefore not considered in the analysis. In addition, a higher consumption of dietary component associated to the traditional Mediterranean diet was found in metabolite-enriched children (Fig. 7F). No group differences were detected in total calorie intake (1530 ± 151 vs 1219 ± 115 kcal/day), consistent with similar BMI (15.6 ± 0.5 vs 16.8 ± 0.5 kg/m^2), in metabolite-enriched vs metabolite-depleted children.

Significant relationships were found between consumption of specific food or nutrient categories and relevant gut metabolites. Specifically, higher consumption of eggs and whole-milk yogurt was positively related to faecal xanthine, uracil, hypoxanthine, formate, mannose and phenylalanine faecal contents, whereas legumes consumption was related to higher faecal levels of several amino acids, including glycine, alanine, tyrosine, phenylalanine, and valine, all being predictors of higher cognitive scores in children and in recipient mice (Fig. 7G). Cereals, cheese and fruit consumption positively predicted higher faecal levels of SCFAs acetate, butyrate, propionate plus glutarate and isobutyrate, which were negative markers of children's GMDS and of mice explorative scores in the present study. Candies and sugary drinks were negatively associated with most of the faecal metabolites playing a positive role for cognitive development, including xanthine and amino acids. Faecal mannose was positively related to the consumption of eggs, legumes and whole milk yogurt, but also of cakes and desserts. Consistently, positive correlations were found between faecal mannose and intake of total calories ($R = 0.605$, $p < 0.005$), and all macronutrients (R range 0.435 – 0.651, p range 0.005–0.002), and micronutrients (R range 0.400 – 0.557, p range 0.005–0.014). At the level of micronutrients, higher intakes of vitamin D, iron, potassium and zinc were positively correlated with faecal xanthine, hypoxanthine and uracil faecal content. Iron and zinc intakes were also associated with faecal butyrate, propionate plus glutarate and isobutyrate (Fig. 7G).

4. Discussion

Altered gut microbiota has been associated with neurocognitive function in children (Guzzardi et al., 2022; Carlson et al., 2018), and in adults (Meyer et al., 2022). We have previously demonstrated in a cohort of healthy children that the gut microbiota at birth predicts behavioural-cognitive development 3 to 5 years later (Guzzardi et al., 2022). However, the evaluation of microbiota composition alone does not provide mechanistic understanding or unequivocal therapeutic leads, considering that different bacterial combinations may be functionally similar. Metabolites produced by gut bacteria and their urinary catabolites are the potential effectors of the gut-brain cross-talk and can be more directly translated into nutritional products or strategies. They can also serve as more direct and unequivocal biomarkers. Therefore, we first demonstrated that the cognitive phenotype can be transferred by the transfer of stools from high and low cognitive score children into recipient mice, and identified shared metabolite-cognitive links. Then, we identified an effective gut-metabolite-based biomarker able to predict cognitive development, offering potential for (time–cost efficient) primary prevention. This was achieved by combining faecal metabolites showing the strongest positive associations with cognitive scores, namely formate, mannose and xanthine. Finally, we demonstrated

strong and durable effects in mice receiving such metabolite-stratified faeces transplant. Faecal metabolites may hold the possibility to be modifiable by nutritional intervention, affecting the cognitive phenotype. In this line, we explored children's diets and identified food categories and nutrients related to promising gut-derived metabolic candidates, providing nutritional therapeutic leads.

Xanthine/hypoxantine, formate and a sugar molecule (mannose in children or xylose and arabinose in mice), were the key gut-derived molecules showing positive correlations with cognitive scores in both donors and recipients, and were combined as stratifying biomarker.

Xanthine, hypoxanthine and formate are related to purine metabolism. Xanthine is a purine base deriving from the breakdown of hypoxanthine, and alterations in its hippocampal metabolites are related to fear memory processing and consolidation (Koyanagi et al., 2021); in fact, treatment with the xanthine catabolite allantoin increased proliferation of hippocampal immature neurons with memory-enhancing effects (Ahn et al., 2014). In humans, lower levels of xanthine, hypoxanthine and adenosine have been detected in the frontal cortex of patients with early Alzheimer's disease (Alonso-Andres et al., 2018). Formate is a by-product of anaerobic fermentation of gut bacteria, feeding the synthesis of nucleotide and methyl groups (Pietzke et al., 2020). In line with the current understanding, our results provide evidence that the purine metabolic pathway links gut metabolome and cognitive development, extending to children, outside of pathological adult models. Sugars like mannose arabinose and xylose are related to anti-obesity and anti-inflammatory function. Mannose is an epimer of glucose, affecting energy generation and storage (Mardinoglu et al., 2017); boosting anti-inflammatory pathways in rodents with neuroinflammation (Wang et al., 2021). Arabinose and xylose are pentoses monosaccharides, can be derived by dietary fibers and metabolized by gut bacteria (Desai et al., 2016). They are considered functional nutrients for the beneficial effects on postprandial glycemic and insulin responses (Pol and Mars, 2021).

The stratification based on the above metabolites carries-over other metabolites with potential synergic effects, such as amino-acids (tyrosine, phenylalanine, glutamine), and TMA. These amino-acids are related to neurotransmitters metabolism. Phenylalanine and tyrosine are dopamine precursors. The dopamine system is involved in reinforcement, motor control and cognition (Linssen et al., 2011). In healthy volunteers, acute phenylalanine and tyrosine depletion impaired stimulus processing during working memory performance (Linssen et al., 2011). In mice lacking conversion of phenylalanine into tyrosine, tyrosine supplementation improved motor function and stress behaviour (Kwak et al., 2013), independent of brain levels of dopamine, not substantially increased. Thus, these amino acids express dopamine-dependent and -independent targeting of brain health. In addition, glutamine feeds the glutamate/glutamine cycle (Waagepetersen et al., 2000), which is essential for synaptic activity and recognition memory (Cheung et al., 2022). Alterations of the glutamatergic transmission are strongly implicated in causing cognitive deficits (Takado et al., 2019). Notably, blood glutamine levels, which reflect brain levels (Takado et al., 2019), were increased in metabolite-enriched-R.

Methylamines TMA and TMAO were also relevant in our analyses, since higher TMA levels were found in metabolite-enriched children (faeces and urine) and respective recipients (faeces and blood) and related to a protective role. TMA is produced by microbial metabolism of dietary choline and L-carnitine, and converted to TMAO in the gut or liver (Janeiro et al., 2018). The TMA/TMAO ratio shows a dual relationship with cognitive disorders. TMAO predicts progression of Alzheimer's disease (Vogt et al., 2018), and TMA/TMAO suppression improved cognitive function in APP/PS1 mice (Gao et al., 2019), but TMAO has also been recently shown to enhance blood–brain barrier integrity and prevent inflammation (Hoyles et al., 2021).

Cholesterol may have an additional role, since its faecal and plasma levels were both increased in metabolite-enriched-R compared to metabolite-depleted-R. Together with sphingolipids, cholesterol is a major structural brain component, with key roles in neurodevelopment and function, glial cell proliferation, neurite outgrowth, microtubules stability, synaptogenesis and myelination (Goritz et al., 2005); consistently, higher brain lipid content measured by *in vivo* CT imaging metabolite-enriched-R compared to others.

It is important to point out that not all metabolites that can be detected in faeces are microbiota products. Specific classes of metabolites may be substantially ascribed to the microbiota, such as short-chain fatty acids, branched-chain amino acids (BCAAs), trimethylamine N-oxide, and tryptophan, whereas other metabolites are derived from foods, such as sugars and several amino-acids. However, the level of each metabolite, including the ones derived from the diet, might be directly or indirectly remodulated by gut microbiota (and vice versa), e.g., their use to synthesize other products, or modification of their intestinal absorption. This field of research is still in its infancy and for several metabolites it remains difficult to determine whether they are fully microbiota-derived or if other sources are involved, including diet or the host itself, as recently reported (Agus et al., 2021).

Besides metabolites detected in faeces, we identified a panel of few urinary candidates predictive of cognitive development in children and/or relating with faecal xanthine and formate. By reflecting circulating markers (Kirsch et al., 2010), urinary catabolites provide further information on gut-derived effectors on brain in a non-invasive (children-suited) manner. These included choline, o-acetylcholine, TMA, and BCAAs valine, isoleucine and leucine. Choline-derived phosphatidylcholine is implicated in structural integrity of cell membranes. Acetylcholine is fundamental in neurogenesis, spine and synapse formation. Cholinergic neurons innervate the hippocampus and parahippocampal regions, where acetylcholine modulates memory functions. Impairment of the cholinergic system has been shown in patients with Alzheimer's disease (Haam and Yakel, 2017). BCAAs are precursors in the synthesis of neurotransmitters involved in memory and learning, such as acetylcholine, glutamate and GABA (Tournissac et al., 2018), and their plasma levels were negatively associated with the risk or progression of dementia (Sanguinetti et al., 2018; Toledo et al., 2017).

A further novelty of the present study is the combination of FMT with multi-modal *in vivo* imaging, *i.e.*, ^{18}FDG-PET-CT and UHFUS, demonstrating that gut microbial colonization *per se* modifies brain metabolism and structure. Increased brain glucose extraction rate was measured in FMT compared to sham mice, suggestive of increased neural function. However, brain metabolism and cognition were not significantly correlated, suggesting that different gut-mediated pathways underlie the two brain functions. To identify a potential physical link between metabolites and cognition, *in vivo* UHFUS was applied to measure carotid RI and PI, which are inversely related to cognition (Mitchell et al., 20112011; Yew and Nation, 2017). Here, RI and PI were inversely related to memory and locomotor scores, mediating the gut-to-brain relationship involving the anti-inflammatory and antioxidant molecule O-acetylcarnitine (Wang et al., 2020). Conversely, faecal glutamine was correlated with cognition independent of vascular indices.

We are aware that faecal metabolome is the product of multiple factors, the main being microbiota and diet, and can in turn influence microbiota composition. Therefore, to identify potentially discriminating bacterial genera, faecal microbiota was analysed in mice. We observed that *Ruminococcus, Turicibacter* and [*Eubacterium]* genera were overexpressed in high-GMDS-R and metabolite-enriched-R mice. *Ruminococcus* is a major intestinal SCFA producer, which has been positively correlated with several subscales of neurodevelopment in 3-year-old children (Zhang et al., 2021). Both *Ruminococcus* and *Turicibacter* are considered potential gut-brain negative mediators in Alzheimer's disease (Liu et al., 2019; Zhang et al., 2017; Dunham et al., 2022) for their role in the production of SCFA (Zhang et al., 2021) and serotonin (Fung et al., 2019), respectively. Serotonin was not detected in our faecal metabolome, but tryptophan, its primary precursor, was not correlated with cognitive scores. >90% of the body's serotonin is made in the gut, but does not cross the blood–brain barrier, therefore other mechanisms,

like inflammation, might underlie the role of *Turicibacter* in the gut-brain axis, as suggested by the reported inverse correlations with IL-1beta and IL-6 (Liu et al., 2021). Finally, not many information is available on [*Eubacterium]* genus, and further studies are warranted to confirm and clarify its role on cognitive development.

Our findings extend the above pathophysiological concepts to healthy children, but our main intent was to identify a restricted panel of non-invasively measurable and modifiable metabolites for prevention. Advancements provided by this study to the understanding and perspective use of such metabolic mediators are three-fold. First, faecal and urinary findings converged towards the same pathways of neurotransmission, anti-inflammatory function, and purine metabolism. Via those pathways, we were able to demonstrate that children's faeces can affect neurodevelopment in young recipients until adult age, proving that gut metabolite manipulation in early life holds promise for long-lasting effects, which may be directed towards prevention. Second, our data also validate a panel of main and synergistic faecal and urinary metabolites, circumscribing laboratory demands in future risk-screening and nutritional supplementation. Third, the longitudinal design allowed to appreciate that donors' metabolites were found in recipients' faeces initially, migrating into blood subsequently, in proportion to groups stratification. Our data suggest that after gut colonization, faecal metabolites affect circulating metabolites both directly (entering the bloodstream) and indirectly (by modifying related pathways).

In order to translate our findings in practical perspectives and identify modifiable nutritional elements to the benefit of cognitive development, we sought for food categories and macro- and micronutrients associated with the relevant faecal metabolites. We acknowledge that the concurrent diet is only one of the factors influencing gut microbiome/metabolome development during early life. Here, we also accounted for early feeding (breastfeeding, with relevant results discussed below), and did not find any difference in delivery mode, anthropometrics, sex, parental factors between stratified groups. Consumption of legumes, whole-milk yogurt, and eggs seemed important to augment the levels of protective metabolites. Legumes are plant-based source of proteins, iron and zinc, with positive roles on brain development, consistent with their elevated intake in metabolite-enriched children. Iron is a cofactor in the production of myelin and neurotransmitters, including dopamine, and is essential for brain development and function (Todorich et al., 2009; Youdim and Yehuda, 2000). Zinc is also an essential trace mineral present in the brain that contributes to cerebral structure and function (Black, 1998). Iron or zinc deficiency in preschool children has been related to impairment in cognitive development, short-term memory, and motor delays (Monk et al., 2013). Iron and zinc supplementation in early infancy are recommended by WHO for children whose diet does not include fortified foods. We have previously shown that consumption of dairy products was related to performance scores in this same population (Granziera et al., 2021), and the present results point especially towards whole-milk yogurt, as well as breast-feeding observed in a majority of metabolite-enriched and a minority of metabolite-depleted children. Bioactive peptides, α-lactalbumin, vitamin B12, and calcium, as well as phospholipids in the milk fat globule membrane (Lee et al., 2018) and prebiotics in breastmilk may explain the biological link. Consumption of whole-milk yogurt produces beneficial changes in the gut microbiota (Le Roy et al., 2022). Another food emerging from our analysis was eggs, as source of brain-boosting vitamins A, D and B12, along with choline. Besides the above discussed role of choline, the pleiotropic actions of vitamin D include neuroprotection, by modulation of oxidative stress, calcium homeostasis, and inflammation (Bivona et al., 2019). The contribution of the current findings, beyond the above knowledge was to demonstrate strong links between these nutrients and faecal metabolites. Importantly, we reported very pronounced differences in diet composition between metabolite-enriched and depleted children, as the latter consumed one-tenth to one-half of the foods and 30–50% less macro- and micronutrients in the above categories. Likewise, the consumption of dietary components associated to the traditional Mediterranean diet was 45% higher in metabolite-enriched children; this diet promotes healthy gut metabolome, and predicted higher cognitive scores in these children (Granziera et al., 2021).

Strengths of the present study include deep phenotyping of both donor children and recipient mice, demonstrating that FMT influences cognitive development and allowing to test biomarker-based stratifications. The longitudinal design is an additional strength, demonstrating a lasting role of a single early-life FMT. We are aware that the 3 metabolites selected for stratification will carry-over other metabolites that differ between groups, including some that might correlate with cognitive development in spite of no group difference. However, if only 3 are sufficient to effectively stratify a larger and incisive panel, this would simplify the screening procedure and its translational value. The study combines in an original manner multimodal imaging (PET, CT, US) with FMT to track organ crosstalk leading to a specific cognitive phenotype *in vivo*.

Critical experimental aspects need further discussion. The age of recipient mice was chosen to be matched, as much as possible, in terms of development to the one of donor children. However, to avoid the stress of an early separation from mothers, their phase of development is slightly more advanced than that of 5-year-old children. The mice age of 4–5 weeks represents the period between childhood and adolescence, during which brain and cognitive development are extremely active until adulthood, which is compatible with the plasticity and translational value we wished to target in this study. In fact, one of the most important findings is that early seeding of healthy gut microbiota/metabolome can programme in durable fashion, until adulthood, which is the critical period of life in which cognition starts declining. The use of germ-free animals is also a critical aspect since they are abnormal in term of brain function and behaviour. However, the alternative pseudo germ-free mice obtained by heavy antibiotic cocktail administration would have introduced other confounders related to intestinal and global health. To cope with this limitation, a group of sham animals was studied. This does not completely abolish germ-free limitations in FMT mice, but provides robustness to the identification of FMT specific results compared to the no-FMT group. Moreover, since baseline cognitive characterization is missing to avoid germ-free contamination before FMT, sham mice were also important as no-FMT longitudinal controls. To cover more than a single aspect of neurodevelopment by using a single test (to the sake of sterility), the Y maze was chosen to fulfil a spectrum cognitive-behavioural aspects, including locomotor activity or anxiety, in addition to memory.

From a statistical standpoint, FDR estimation is not yet widely adopted in the field of metabolomics, as evident from the lack of automated FDR approaches. Moreover, a low level of correction is accepted in new biomarkers discovery (compared to clinical biomarkers validation), as these annotations would require further experimental validation. Therefore, FDR correction was reported only in children, and raw p-values in preclinical analyses, though we acknowledge that this may increase the risk of false positives. Preparation of faecal material for transplantation was very rapid to limit oxygen bacterial exposure, however we recognize that aerobic conditions might have affected viability of anaerobic bacteria.

The administration of each metabolite or a combination, outside of the faecal material, remains to be pursued, since the study was meant to interrogate metabolites occurring in their natural microbial environment. Brain-tissue molecular alterations underpinning metabolite-behaviour relationships remain to be investigated. Moreover, it is important to underline that the observed faecal metabolites may have microbiota or diet or other origin and further studies with multi-omics approaches will be needed to dissect the contribution of the gut microbiota.

In conclusion, as summarized in Fig. 8, we have delineated and validated a panel of gut-derived metabolites underlying cognitive development, with a predictive role lasting through adulthood

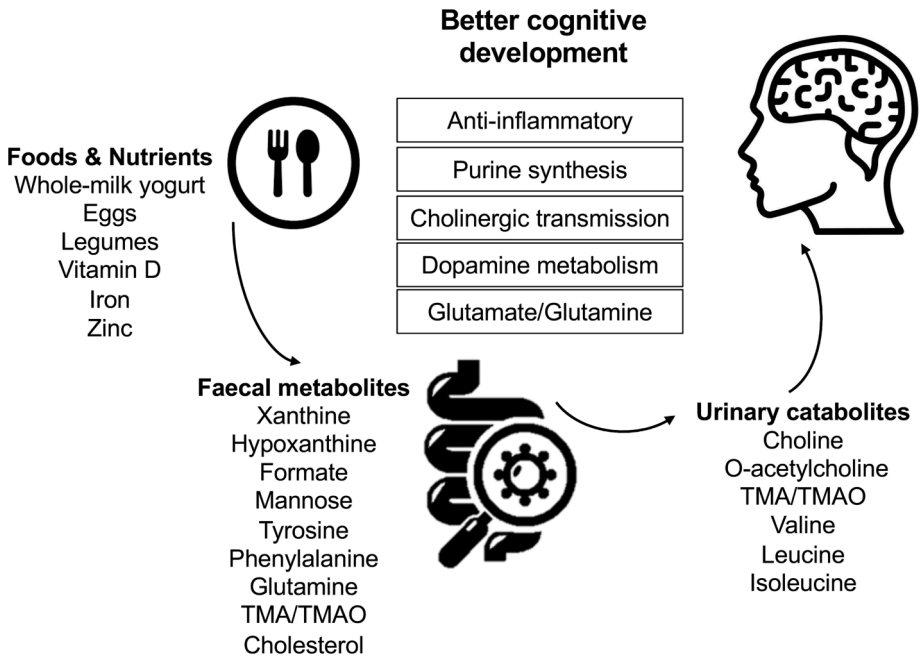

Fig. 8. Graphical summary. Schematic diagram summarizing faecal and urinary predictors (gut-derived candidate molecules) of high cognitive development, and the biochemical pathways involved. Food and nutrients categories associated with the identified biomarkers represent potential primary intervention targets. Correlations between faecal metabolites and urinary catabolites suggest the potential occurrence of synergistic mechanisms between underlying biochemical pathways.

outcomes. Consensus between findings in faeces, blood and urine points towards inflammation, purine and neurotransmitters (dopamine, glutamine, acetylcholine) pathways. Our panel of gut-derived biomarkers holds promise for screening. The related dietary and nutritional findings offer leads to microbiota-targeted intervention for cognitive protection, with short time-to-implementation and long-lasting effects, supporting that the enrichment of legumes, whole-milk yogurt, eggs, and iron, zinc and vitamin D (micronutrients) in our diet may optimize gut mediators to the benefits of neurodevelopment.

Authors' contributions

MAG: data acquisition, analysis and interpretation, drafting of the manuscript; FLR: conduct of all animal experiments, technical coordination; FG: data acquisition and analysis of PET-CT images; MPT and DM: human and mice metabolome analysis; MB and ST: mice microbiota analysis and interpretation; PB: manuscript critical revision and editing; DP, FF and CK: acquisition of images, processing, interpretation; PI: conceptualization and funding acquisition, manuscript critical revision and editing. All authors have revised the manuscript and approved the submitted version.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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We wish to acknowledge the families participating in the study.

Informed consent statement

Informed consent was obtained from all subjects involved in all studies.

Data availability statement

The data presented in this study (except for faecal microbiota) are available on request from the corresponding author, as they have not yet

been uploaded in a public database. Microbiota sequencing reads were deposited in the National Center for Biotechnology Information Sequence Read Archive (Bioproject ID: PRJNA956738)

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2023.08.009.

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