Gut microbes and their genes are associated with brain development and cognitive function in healthy children

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Abstract

Both the brain and microbiome of humans develop rapidly in the first years of life, enabling extensive signaling between the gut and central nervous system (dubbed the "microbiome-gut-brain axis"). Emerging evidence implicates gut microorganisms and microbiota composition in cognitive outcomes and neurodevelopmental disorders (e.g., autism), but the influence of gut microbial metabolism on typical neurodevelopment has not been explored in detail. We investigated the relationship of the microbiome with the neuroanatomy and cognitive function of 281 healthy children in a cross-sectional analysis and demonstrated that differences in gut microbial taxa and gene functions are associated with the size of brain regions and with overall cognitive function. Many species, including *Eubacterium eligens* and *Roseburia hominis*, were associated with higher cognitive function, while some species such as *Ruminococcus gnavus* was more commonly found in children with low cognitive scores. Microbial enzymes involved in the metabolism of neuroactive compounds such as glutamate and GABA, were also associated with structure of the brain, including the first brain regions to develop such as the cerebellum, and with overall cognitive function.

Introduction

The first years of life are a unique and dynamic period of neurological and cognitive development. Throughout childhood, a child's brain undergoes remarkable anatomical, microstructural, organizational, and functional change. By age 5, a child's brain has reached over 85% of its adult size, has achieved near-adult levels of myelination, and the pattern of axonal connections has been established (Silbereis et al., 2016). This development is profoundly affected by the child's environment and early life exposures (Fox et al., 2010). The first years of life also witness dramatic changes in the gut microbiome. The gut microbial

community is seeded at birth, and develops over the course of the first year from a low-diversity community dominated by Firmicutes and Proteobacteria, to a more diverse, adult-like microbiome upon the introduction of solid food. This microbial community development is also shaped by the environment, with factors such as mode of delivery and diet (breast milk vs formula) known to have lasting effects on community composition (Bäckhed et al., 2015; Dominguez-Bello et al., 2010).

The gut, the gut microbiome, and the central nervous system are intricately linked through a system known as the gut-microbiome-brain axis (Clarke et al., 2013), and differences in microbial communities are associated with, and in some cases cause, changes in neurocognitive development (Flannery et al., 2020; Gao et al., 2019) and the outcome of neurological disorders such as autism (Sharon et al., 2019). However, the study of the relationships between environmental exposures, neurocognitive development, and the gut microbiome during neurotypical development remains in its infancy (Carlson et al., 2018; Sordillo et al., 2019).

Here, we focused on the relationship of microbial taxa and metabolic potential in the structural development of the brain and in neurocognition. In particular, we show that microbial taxa as well as genes with neuroactive potential, specifically genes encoding enzymes for the metabolism of glutamate and GABA, are associated with the size of important brain regions and with differences in cognitive function. Understanding the relationship of intestinal GABA and glutamate metabolism may be particularly important to understanding the role of the gut microbiome in early childhood cognitive development, as together, GABA and glutamate response neurons make up the main cerebellar output pathways (Carletti & Rossi, 2008; Hoshino, 2006). The cerebellum is one of the earliest brain regions to develop (Leto et al., 2016; Silbereis et al., 2016), making it especially vulnerable for disorder and disease (S.H. Wang et al., 2014). Understanding how the gut microbiome of healthy children interacts with the complex

metabolism of these and other neuroactive molecules will be critical in understanding the etiology of cognitive disorders and how to promote healthy neurological development.

Results and Discussion

To examine the relationships between early childhood gut microbiome and neurocognitive development, we collected stool samples from 281 children enrolled in the RESONANCE study of child development, part of the NIH initiative Environmental influences on Child Health Outcomes (ECHO; Gillman & Blaisdell, 2018), an observational study of healthy and neurotypical brain development that spans the fetal and infant to adolescent life stages, combining neuroimaging (magnetic resonance imaging, MRI), neurocognitive assessments, and rich demographic, socioeconomic, family and medical history information (Table 1). As an initial characterization step, we used shotgun metagenomic sequencing to generate taxonomic and functional profiles for each of our child fecal samples. Participant age was the greatest driver of both taxonomic and functional diversity, as expected (Figure 1b-c; Koenig et al., 2011). Children under one year of age formed a distinct cluster from older children, characterized by high aerobe load and low alpha (within-sample) diversity (Figure 1b, Supplementary Figure 2). Comparing children's profiles with those of unrelated pregnant women (n=251), the microbiomes of children over two years old were similar to those of adults (Supplementary Figure 1a, 2a).

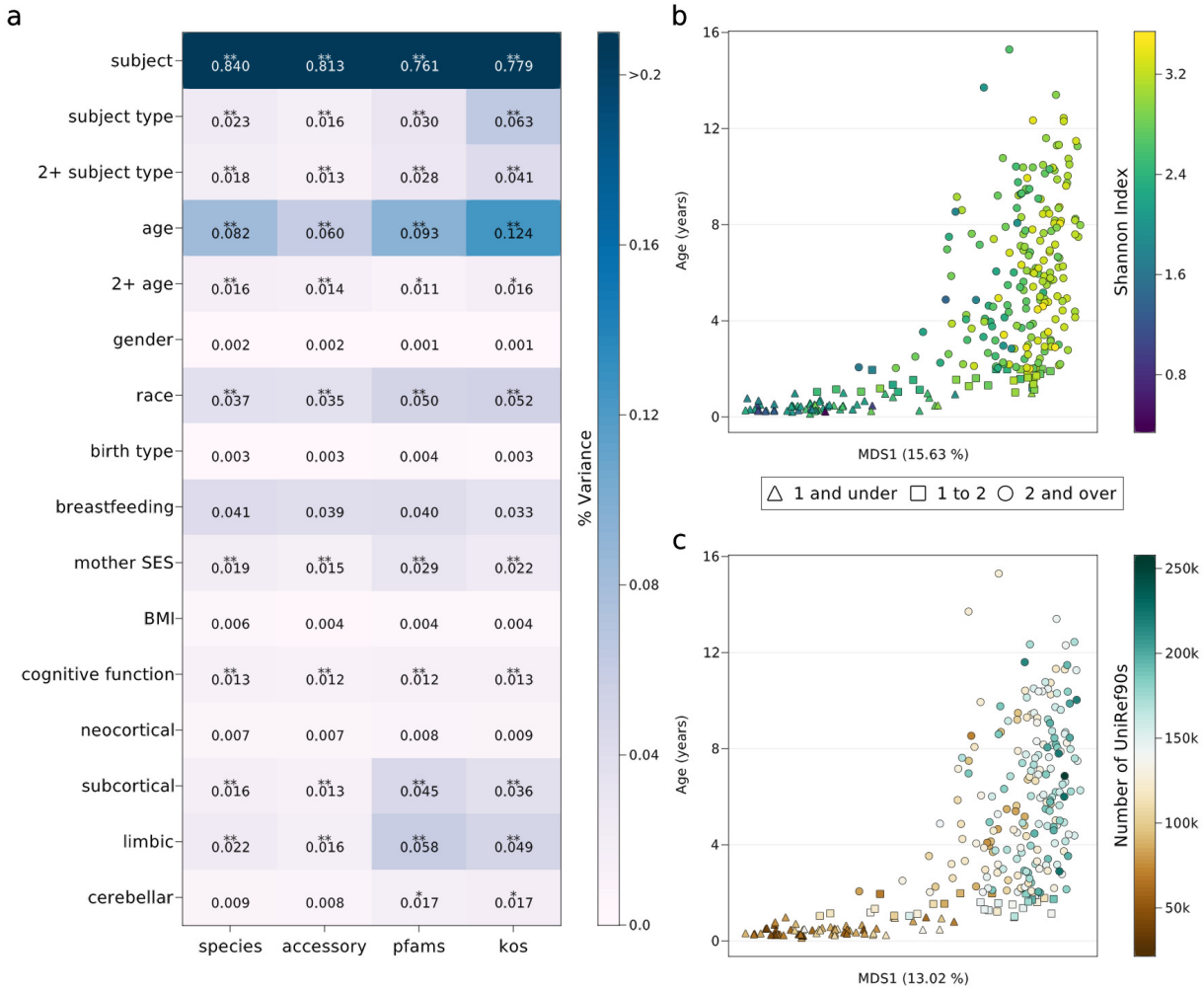
Table 1. Baseline characteristics of ECHO RESONANCE participants

Subjects (n)	281
Under 1yo (n)	60
Over 2yo (n)	192
With high resolution scan (n)	141
With cognitive function score (n)	274

Both scan and cognitive function (n)	134
Non-white (%)	56.77
Mixed race (%)	18.8
Age in years (mean, SD)	4.45, 3.5
BMI (mean, SD)	16.81, 2.61
Maternal SES (mean, SD)	5.93, 1.0

95

96 Figure 1



97

98 **The gut microbiome of healthy children changes dramatically over the first 2 years of life**
99 **and is associated with neurocognitive measures**

100 Stool samples from children aged 0 to 15 years old (N=281, one sample per subject) were
101 analyzed with associated cognitive evaluations (N=274), structural and functional brain imaging
102 (N=141), and rich demographic and environmental exposure information. **b**, PERMANOVA
103 analysis for selected subject metadata vs. pairwise Bray-Curtis dissimilarity for species-level
104 taxonomic or functional profiles. Functional profiles include Kegg-Orthology (KO), Pfams or
105 accessory UniRef90s; subject and subject type include all subjects, 2+ includes only children
106 over 2 years old (N=192), others include all children for which the measure was available
107 (breastfeeding: N=60, maternal socioeconomic status (SES): N=261, BMI: N=226); stars
108 indicate significance after Benjamini-Hochberg FDR correction. (* <0.1, ** < 0.01, *** < 0.001).
109 **c**, First principle coordinate (PCoA) based on Bray-Curtis dissimilarity in taxonomic (species)
110 profiles vs age; younger children cluster away from older children, and are lower in diversity. **d,**
111 same as (c) using the first PCo axis for functional profiles (UniRef90 accessory genes) after

112 removing gene families that were present in >90% of subjects in a given age group; variation is
113 driven by similar effects as for taxonomic profiles.

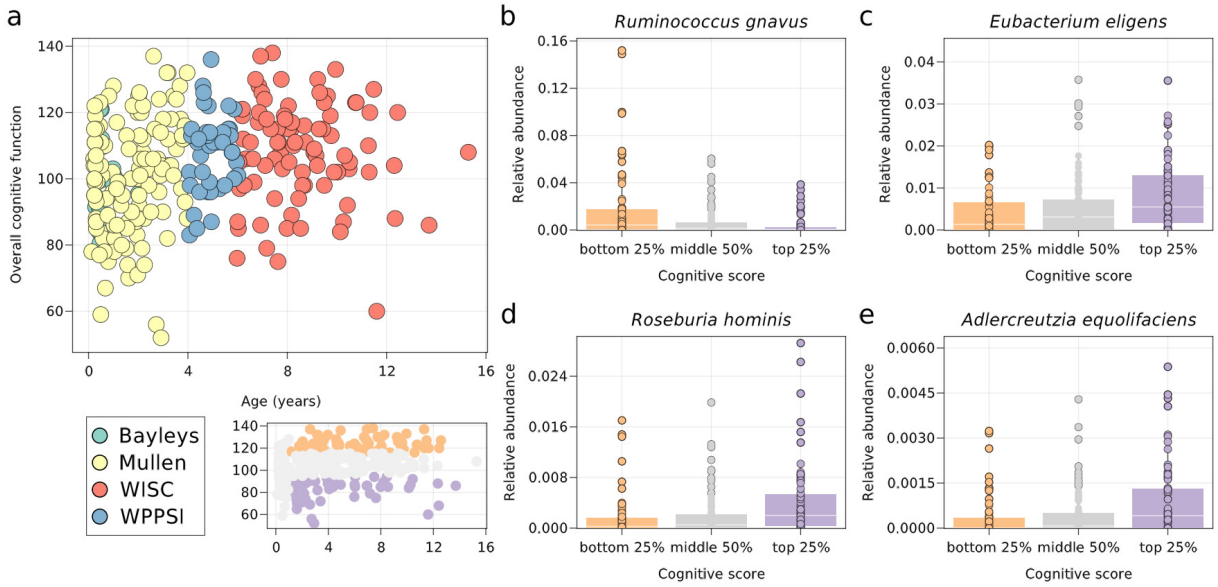
As in previous adult and infant cohorts, functional beta diversity was generally lower than taxonomic diversity (Supplementary Figure 2a), suggesting that healthy guts select for similar gene functions even when different species contribute those functions. However, this interpretation may be complicated by the fact that as many as 50% of sequencing reads in some samples are not mapped to any of the reference genes used, and are thus unclassified (Supplementary Figure 2b). Interestingly, although children under 1 (N=60) tended to have substantially fewer species and, therefore, fewer total genes (Figure 1c, right), those genes tended to be better characterized, likely because the taxa present in this age-range are better represented in experimental studies (Supplementary Figure 2b, Vatanen et al., 2018). Consistent with previous studies of adult cohorts from industrialized countries (Tett et al., 2019), another major driver of variation visible from principal coordinates analysis was the presence of *Prevotella copri* (Supplementary Figure 3). Like samples from very young children, samples from children with *P. copri* had reduced diversity compared with samples from other children and pregnant mothers without *P. copri* (Supplementary Figure 2, 3). Overall, these results are consistent with prior studies of adult and childhood gut microbiomes (Koenig et al., 2011; Lloyd-Price et al., 2017).

To assess the potential role of the microbiome in neuro-structural and -cognitive development, child stool samples were collected alongside MRI (N=141and age-appropriate neurocognitive evaluations (N=274) (Figure 2a). Measures of overall cognitive ability (e.g., intelligence quotient, IQ; Mullen & others, 1995; Wechsler, 1949), MRI measures of cortical volume and morphometry, as well as other potentially relevant clinical metadata, were compared to taxonomic and functional profile dissimilarity by PERMANOVA (Anderson, 2017; McArdle & Anderson, 2001). Consistent with previous studies, inter-individual (subject) variation accounted for the majority of variation in microbial taxonomic and functional profiles ([84%, 78%], $q < 0.01$) (Figure 1b, Supplementary Table 1). Subject type (child or mother) accounted for a moderate amount of variation ([2%, 6%] $q < 0.01$), but this effect dropped when children

under 2 years of age were excluded, suggesting that age, rather than subject type, is responsible for driving much of the taxonomic and functional variation. Among children's samples, age accounted for 8-12% of variation in both taxonomic and functional profiles, but this effect also largely disappeared when children under 2 were excluded (Figure 1b, Supplementary Table 1), suggesting that the age effect is primarily driven by the enormous changes in the microbiome over the first year.

Microbiome taxonomic and functional variation was also associated with small but significant differences in several neurocognitive measures including age-appropriate measures of cognitive ability (1.3%, $q < 0.01$, N=274). We found significant associations between regional brain volumes and microbial taxonomic and functional variation, including the sizes of the cerebellum ([0.9%, 1.8%], q [NS, < 0.01]), the subcortex ([1.7%, 3.7%], $q < 0.01$), and limbic regions ([2.3%, 4.9%], $q < 0.01$) (N = 141 for all high resolution scans) after correcting for the effect of age on brain volume. These results are on par with the magnitude of previously reported drivers of microbial diversity such as antibiotics use and Inflammatory Bowel Disease diagnosis (Lloyd-Price et al., 2019), and suggest that there is a strong relationship between the gut microbiome and neurocognitive development (Figure 1b). Though the direction of causality cannot be determined, experimental models of brain development and neurological disorders have demonstrated that microbes in the intestine may have causal effects on the functioning of the central nervous system through their metabolic action or interactions with the immune system (Blacher et al., 2019; Clarke et al., 2013; Gao et al., 2019; Hsiao et al., 2013).

160 Figure 2



161

162 **Differences in microbial taxa are associated with cognitive function in neurotypical**
163 **children**
164 **a,** Cognitive function measured using age-appropriate IQ-like tests, allowing comparison across
165 multiple developmental stages. Inset is the same as a, but shows top (orange, N=66) and
166 bottom (purple, N=65) quartiles for children older than 1 year used in b-e. **b-e,** relative
167 abundances of taxa that are significantly ($q < 0.1$ after FDR correction) different in the top and
168 bottom quartiles of cognitive score for children over 1 year.
169

170 Table 2. Gut microbial taxa associated with cognitive scores in children
171 older than 1 year.

Species	Association	Lower 25% median	Upper 25% median	N samples	P value	Q value
Ruminococcus gnavus	-	0.004214	0.000492	102	0.000055	0.016523
Coprococcus sp ART55 1	+	< 1E-6	< 1E-6	25	0.000320	0.032148
Eubacterium eligens	+	0.001354	0.005472	101	0.000238	0.032148
Coprobacillus unclassified	-	0.000205	< 1E-6	53	0.000619	0.044157
Roseburia hominis	+	0.000279	0.002013	95	0.000738	0.044157
Adlercreutzia equolifaciens	+	< 1E-6	0.000413	71	0.000935	0.044157
Lachnospiraceae bacterium 2 1 58FAA	-	< 1E-6	< 1E-6	47	0.001027	0.044157
Clostridium symbiosum	-	0.000092	< 1E-6	55	0.001825	0.068654

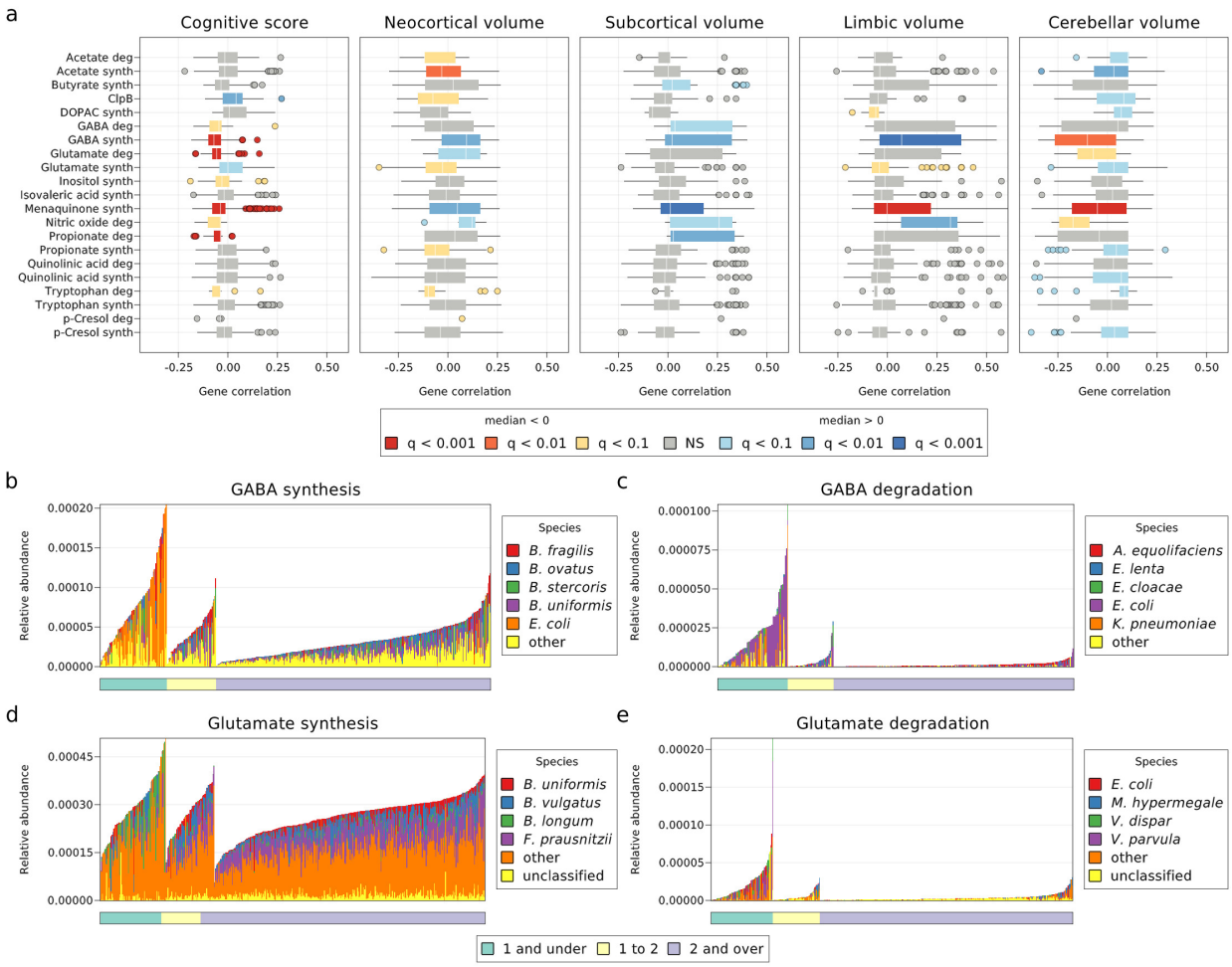
172

To determine if any specific microbial taxa may be associated with these differences, we divided children into quartiles for cognitive function score, and tested for differences in microbe abundance in the top and bottom quartiles (Table 2; Figure 2b-e; Supplementary Table 2). Due to the rapid changes in the microbiomes of very young children, those under 1 year old were excluded from this analysis. Several taxa were significantly different in the upper and lower quartiles (Mann-Whitney U test, $q < 0.1$ after FDR correction). For example, *Ruminococcus gnavus*, which has previously been associated with depression in children (Chahwan et al., 2019) and inflammatory bowel disease (Hall et al., 2017; Schirmer et al., 2019), was more abundant in children that tested in the lowest quartile for cognitive function (Figure 2b). By contrast, *Eubacterium eligens* (Figure 1c) and *Roseburia hominis* (Figure 1d), both of which have been associated with regulating inflammation in the gut (Chung et al., 2016; Patterson et al., 2017), were more abundant in the stool samples of children from the top quartile of cognitive scores. *Adlercreutzia equolifaciens* (Figure 1e) has been linked to autism and multiple sclerosis (Chen et al., 2016; Li et al., 2019) and was also more abundant in children with higher cognitive scores. Multivariate linear models including all children over the age of 1 year showed similar trends in these taxa, but none were significant after FDR correction.

While identifying important taxa in cognitive development is useful to direct further research, the effects of microbes on their hosts are ultimately driven by their metabolism. To investigate potential mechanisms through which the gut microbiome might affect neurostructural and neurocognitive development in infants and young children, we focused on a group of microbial genes that code for enzymes that metabolize neuroactive compounds (Valles-Colomer et al., 2019). We analyzed the association of each of these gene sets with our neurocognitive measures using feature set enrichment analysis (FSEA; de Leeuw et al., 2016; Metwally et al., 2018; Figure 3a, Supplementary Table 3). Briefly, we calculated the Pearson correlation between the relative abundance of all identified UniRef90 gene families with each neurocognitive measure, then calculated the Mann-Whitney U statistic for each potentially

199 neuroactive subset. Using this analysis, we observed that catabolic and anabolic pathways for
200 several molecules known to be important in the developing brain were significantly associated
201 with overall cognitive function scores and the size of brain subregions.

Figure 3



Microbial genes involved in the metabolism of neuroactive compounds are associated with cognition and brain structure

a, FSEA analysis for gene sets with neuroactive potential (see Methods); many gene sets with neuroactive potential are associated with cognitive function and brain structure. **b-e,** Species contributions of GABA and glutamate synthesis and degradation gene sets; the microbiomes of children over 1 year old have substantially lower capacity for degradation of glutamate and GABA. There is a clear shift in glutamate synthesis from *B. longum* and other *Bifidobacterium* spp. to *F. prausnitzii* and *Bacteroides* spp. (common species in adult microbiomes) when comparing children under 1 year old to older children.

In particular, microbial genes for GABA synthesis were positively associated with neocortical ($q < 0.01$, Figure 3a, Supplementary Table 3), subcortical ($q < 0.01$), and limbic ($q < 0.001$) volume, and negatively associated with cerebellar volume ($q < 0.01$) and overall cognitive function ($q < 1e-5$). Interestingly, GABA degradation genes were also positively associated with the size of the subcortex and negatively associated with cognitive function ($q < 0.05$). This may be due to higher GABA synthesis selecting for the ability to catabolize this molecule, making it difficult to assess how actual GABA concentrations in the gut are associated with brain development. GABA synthesis genes that could be assigned to specific taxa were found primarily in *E. coli* in children under 1 year old, and in several different *Bacteroides* species in older children (Figure 3b). GABA degradation in younger children was also seen extensively in *E. coli*, but declines dramatically in abundance in older kids (Figure 3c).

Unlike the metabolism of GABA, glutamate synthesis and degradation genes have an inverse relationship with neurocognitive measures (Figure 3a, Supplementary Table 3). The glutamate degradation gene set was negatively associated with cognitive function ($q < 1e-4$) and cerebellar volume ($q < 0.05$) and positively associated with the size of the neocortex ($q < 0.05$), while glutamate synthesis was marginally negatively associated with overall cognitive function and the size of the neocortex, while positively associated with the size of the cerebellum ($q < 0.05$). However, it remains difficult to predict how gut concentrations of glutamate might be related to microbial metabolism; while it might be intuitive to expect that higher glutamate synthesis and lower glutamate degradation would lead to higher gut concentrations of glutamate, it might also be the case that lower glutamate concentrations select for microbes that can synthesize it and against those that break it down. Glutamate is also far more prevalent in the diet and can be rapidly metabolized by gut epithelial cells, making the relationship between gut concentrations and microbial metabolism even more complex (Reeds et al., 2000). Unsurprisingly, as glutamate is an essential amino acid, Glu synthesis

genes were found in a variety of taxa, including the most common taxa for each age group (eg. *B. longum* for children under 1 year old and *F. prausnitzii* in older children; Figure 3d).

This is the first look at an ongoing study of child neurocognitive and microbiome development. Using cross-sectional data, we have shown that differences in gut microbial taxa and genes are associated with the structural development of the brain and with cognitive development. In addition, we have shown that particular microbial gene sets with neuroactive potential are associated with neurocognitive development, thus perhaps playing a direct role in the gut luminal exposure of children to neuroactive metabolites. Glutamate and GABA metabolism are of particular interest, since these are critical molecules for signaling from the cerebellum during early development and learning, and the cerebellum is one of the first brain structures to develop (Leto et al., 2016; Silbereis et al., 2016). Neurodevelopmental disorders, such as autism spectrum disorder, have been associated with an imbalance of the inhibitory/excitatory system regulated by glutamate and GABA, with recent evidence suggesting an impaired conversion of glutamate to GABA in the disorder (Fatemi et al., 2012), and understanding these pathological outcomes will depend on a deeper understanding of developmental exposures in neurotypically developing children.

This study is ongoing, and we are collecting additional clinical data such as resting state functional brain imaging, participant genetic profiles, lead exposure, air quality data and nutritional information to understand how the environment, microbiome, and biological development interact to shape neurocognition. Future studies assessing gut metabolite pools combined with MR spectroscopic methods to quantify concentrations of neurotransmitters such as GABA and glutamate-glutamine in the brain, as well humanized mouse models and longitudinal human data, will provide further insight into the interactions of microbial metabolism and neurocognitive development. As we continue to follow these subjects, we will be able to identify how early-life microbial exposures, including exposures *in utero*, might affect future neurocognitive outcomes.

Materials and methods

Cohort description

Data used in this study were drawn from the ongoing longitudinal RESONANCE study of healthy and neurotypical brain and cognitive development, based at Brown University in Providence, RI, USA. From the RESONANCE cohort, 281 typically-developing children between the ages of 47 days and 15 years old and 251 healthy unrelated pregnant women were selected for analysis in this study. Only one stool sample per subject was analyzed; either the sample associated with the first time point collected or the first stool sample with an associated neurocognitive measure for the same time point. General participant demographics are provided in **Table 1**. Complete metadata are available in Supplementary Table 4, with children being representative of the RI population. As a broad background, children in the RESONANCE cohort were born full-term (>37 weeks gestation) with height and weight average for gestational age, and from uncomplicated singleton pregnancies. Children with known major risk factors for developmental abnormalities at enrollment were excluded. In addition to screening at the time of enrollment, on-going screening for worrisome behaviors using validated tools was performed to identify at-risk children and remove them from subsequent analysis.

Additional data collection

Demographic and other non-biospecimen data such as race and ethnicity, parental education and occupation, feeding behavior (breast- and formula-feeding), child weight and height, were collected through questionnaires or direct examination as appropriate. All data were collected at every assessment visit, scheduled on the same day of the MRI scan or at least within 2 weeks of the scan date.

Approval for human subject research

All procedures for this study were approved by the local institutional review board at Rhode Island Hospital, and all experiments adhered to the regulation of the review board. Written informed consent was obtained from all parents or legal guardians of enrolled participants.

Stool sample collection and handling

Stool samples (n=532) were collected by parents in OMR-200 tubes (OMNIgene GUT, DNA Genotek, Ottawa, Ontario, Canada), immediately stored on ice, and brought within 24 hrs to the lab in RI where they were immediately frozen at -80˚C. Stool samples were not collected if the infant had taken antibiotics within the last two weeks.

DNA extraction and sequencing of metagenomes

All processing of the samples was done at Wellesley College (Wellesley, MA). Nucleic acids were extracted from stool samples using the RNeasy PowerMicrobiome kit automated on the QIAcube (Qiagen, Germantown, MD), excluding the DNA degradation steps. Extracted DNA was sequenced at the Integrated Microbiome Resource (IMR, Dalhousie University, NS, Canada).

Shotgun metagenomic sequencing was performed on all samples. A pooled library (max 96 samples per run) was prepared using the Illumina Nextera Flex Kit for MiSeq and NextSeq from 1 ng of each sample. Samples were then pooled onto a plate and sequenced on the Illumina NextSeq 550 platform using 150+150 bp paired-end “high output” chemistry, generating ~400 million raw reads and ~120 Gb of sequence.

Computational methods, statistical analyses and data availability

Raw and processed data (excluding PHI) is available through SRA and Zenodo.org (Bonham et al., 2020). All code used for statistical and other analysis is available on github (Kevin Bonham, 2020). Software packages included vegan (R package) for PERMANOVAs, MultivariateStats.jl for MDS analysis, HypothesisTests.jl for Mann-Whitney U tests (used in FSEA analysis), MultipleTesting.jl for false discovery rate correction, and Makie.jl for plotting.

FSEA analyses (de Leeuw et al., 2016)were performed by assessing the Pearson correlation of the relative abundance of each gene with a given measure (brain region volume or cognitive score) across all subjects. The difference between genes within a gene set to all other genes measured was assessed using Mann-Whitney U, a non-parametric test of the null hypothesis that the correlation of a random gene from within the gene set has an equal probability of being higher or lower than a random gene from outside the gene set against the alternative hypothesis that these probabilities are not equal.

Metagenomic data were analyzed using the bioBakery (McIver et al., 2018) family of tools with default parameters. Briefly, KneadData (v0.7.1) was used to trim and filter raw sequence reads and to separate human reads from bacterial sequences. Samples that passed quality control were taxonomically profiled to the species level using MetaPhlAn2 (v2.7.7). Stratified functional profiles were generated by HUMAnN2 (v0.11.1).

MRI Acquisition and data processing

Structural T_1-weighted MRI scans were acquired on a 3T Siemens Trio scanner with a 12-channel head RF array, preprocessed using a multistep registration procedure. Cortical reconstruction and volumetric segmentation were performed with the Freesurfer image analysis suite, which is documented and freely available for download online (http://surfer.nmr.mgh.harvard.edu/). Brain regions were divided into neocortex, cerebellum,

limbic and subcortical regions (for more details on acquisition and processing, see extended methods).

Neurocognitive assessments

Overall cognitive function was defined by the Early Learning Composite as assessed via the Mullen Scales of Early Learning (MSEL; Mullen & others, 1995), a standardized and population normed tool for assessing fine and gross motor, expressive and receptive language, and visual reception functioning in children from birth through 68 months of age.

The third edition of the Bayley Scales of Infant and Toddler Development (Bayley's III) is a standard series of measures used primarily to assess the development of infants and toddlers, ranging from 1 to 42 months of age (Bayley, 2006).

The Wechsler Intelligence Quotient for Children (WISC; Wechsler, 2012) is an individually administered standard intelligence test for children aged 6 to 16 years. It derives a full scale intelligence quotient (IQ) score, which we used to assess overall cognitive functioning.

The fourth edition of the Wechsler Preschool and Primary Scale of Intelligence (WPPSI-IV; Wechsler, 2012) is an individually administered standard intelligence test for children aged 2 years 6 months to 7 years 7 months, trying to meet the increasing need for the assessment of preschoolers. Just as the WISC, it derives a full scale IQ score, which we used to assess overall cognitive functioning.

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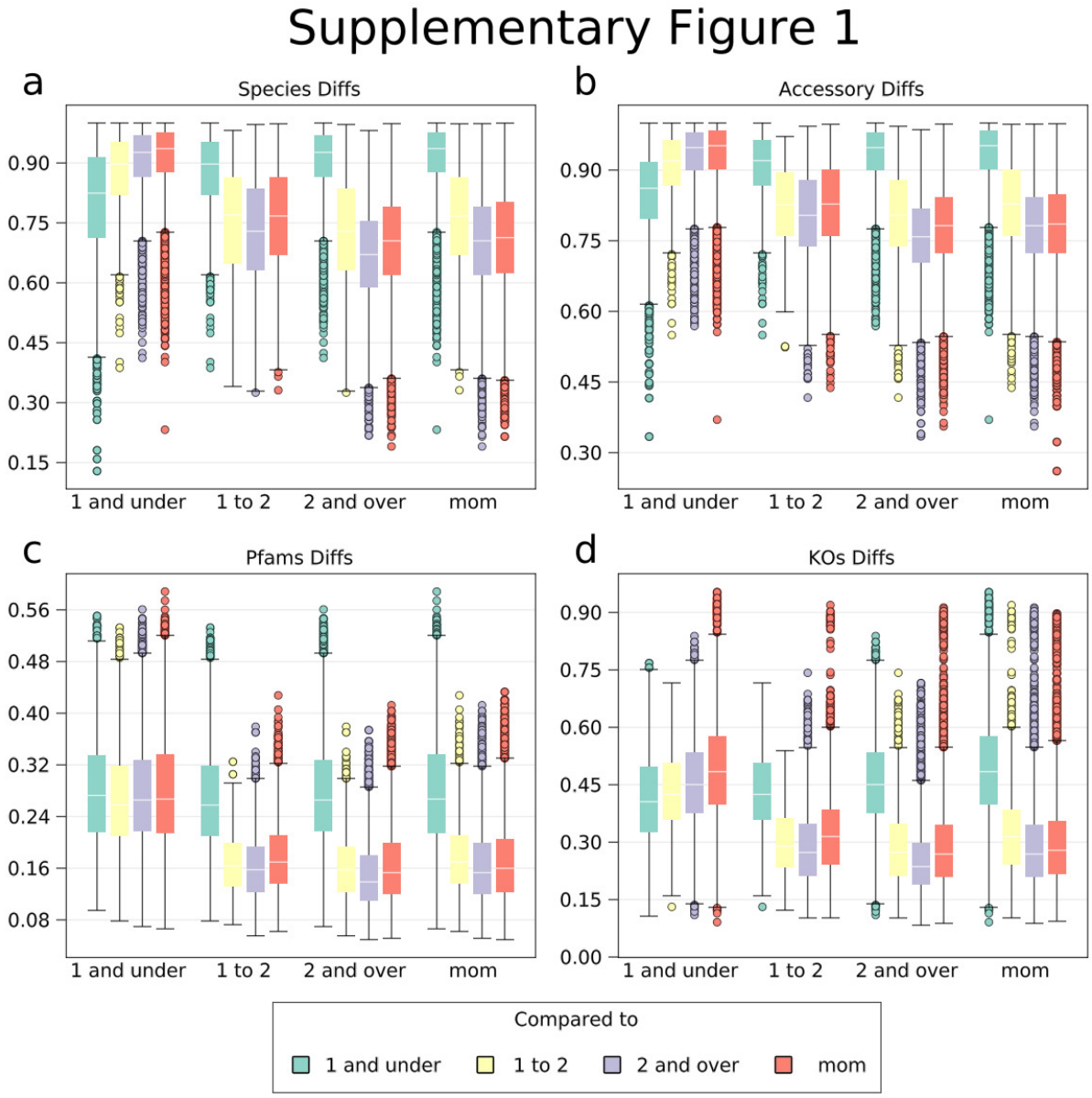
Supplemental Figures

Supplementary Figure 1



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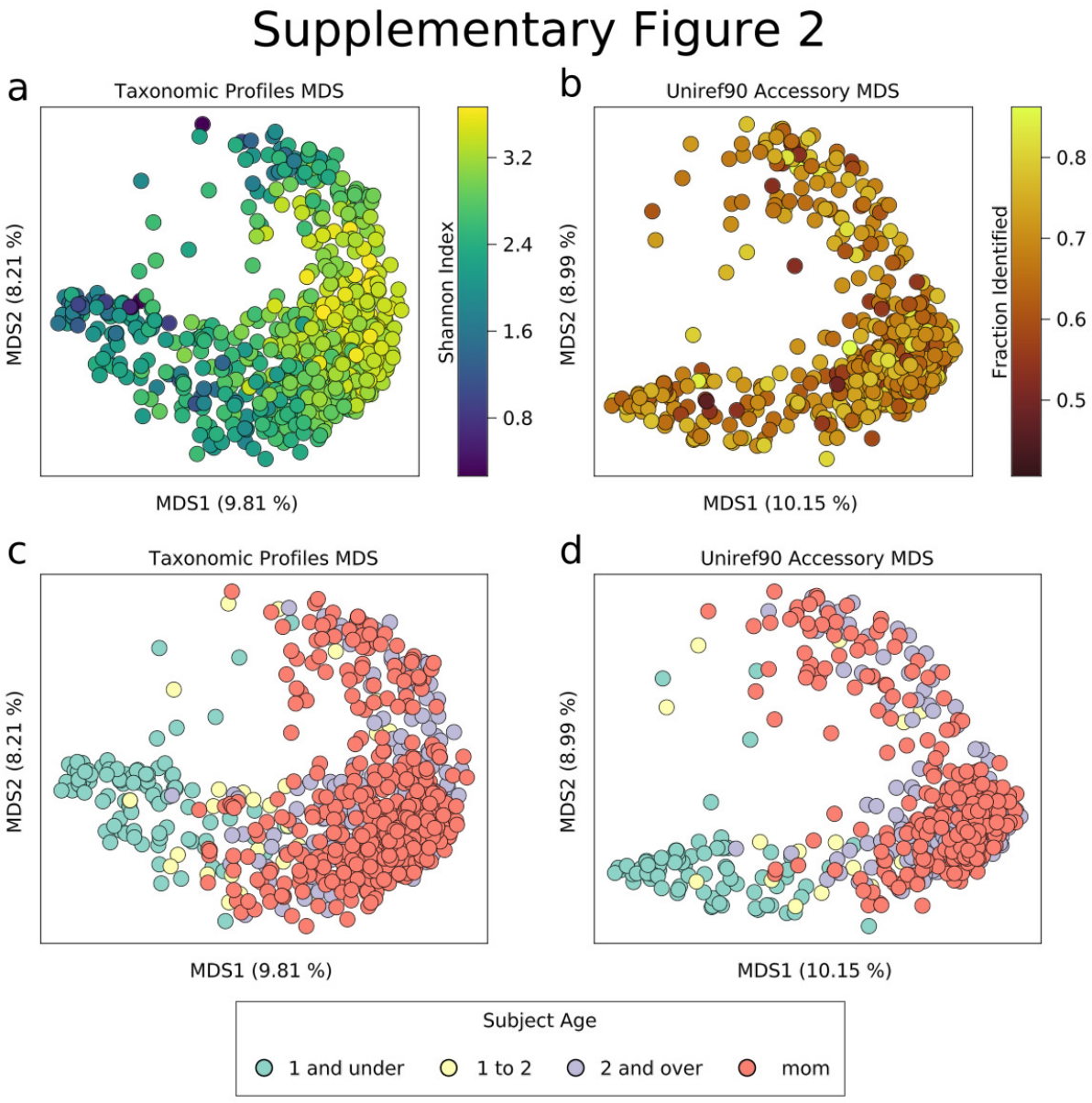

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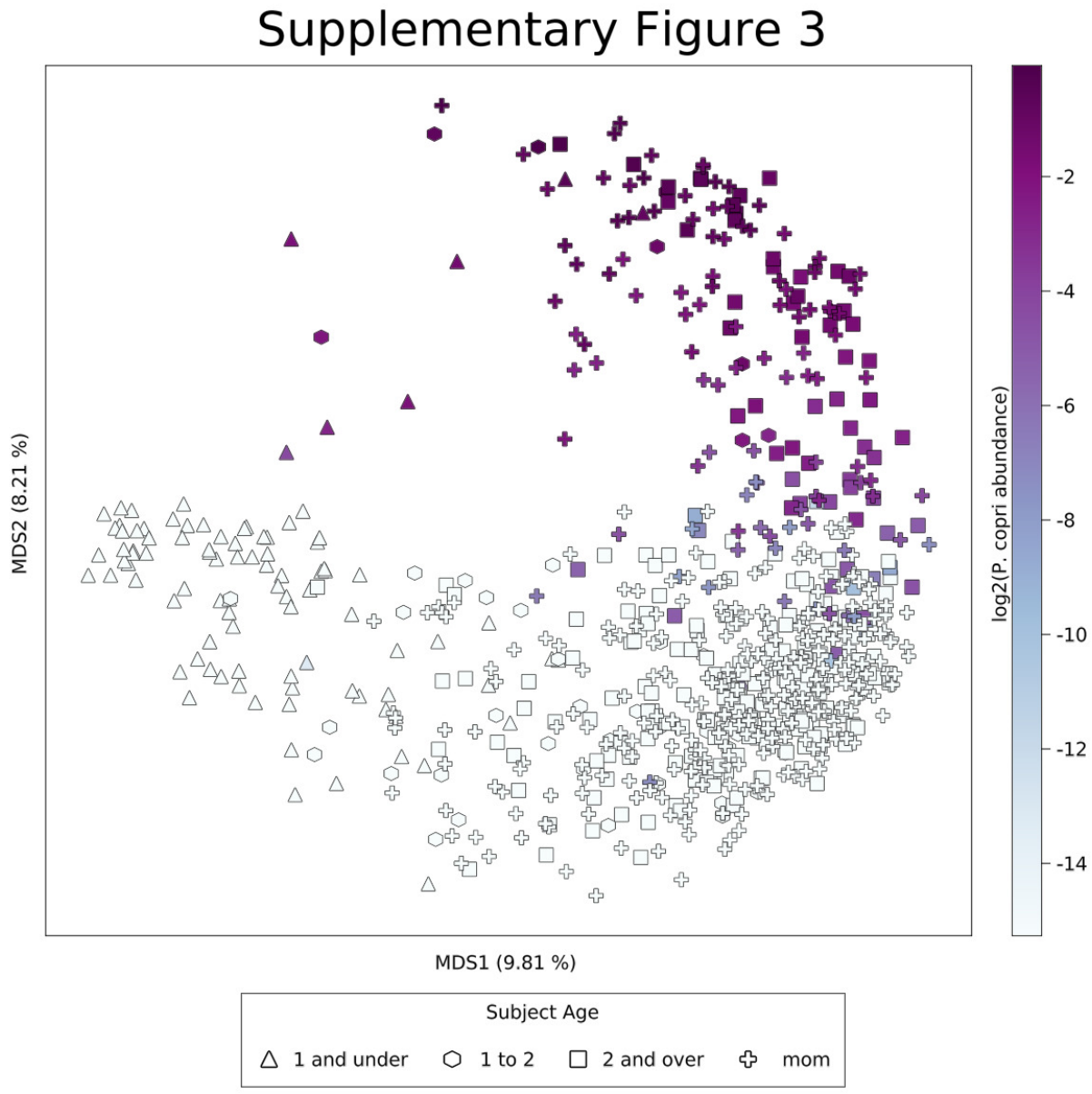

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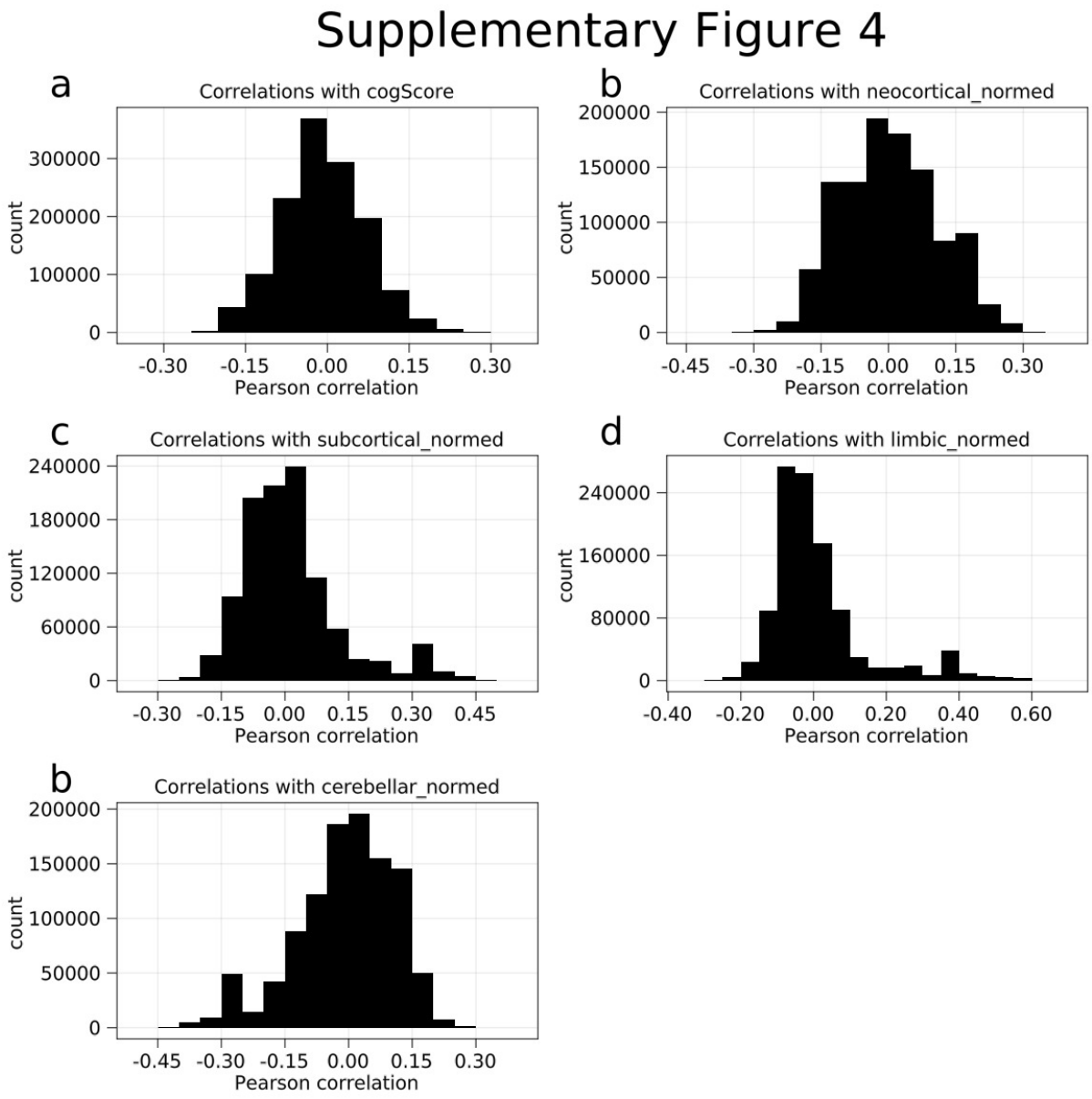

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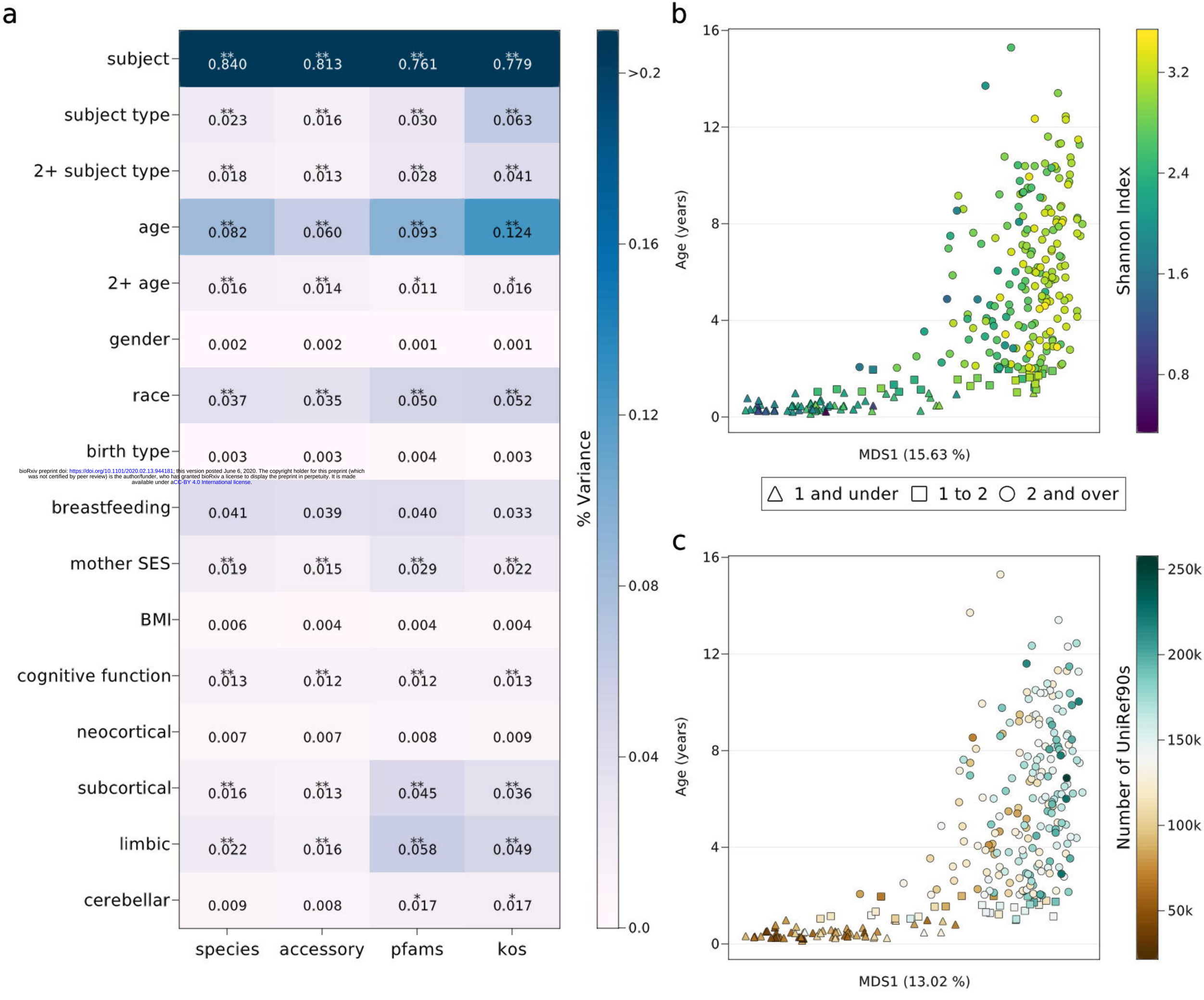

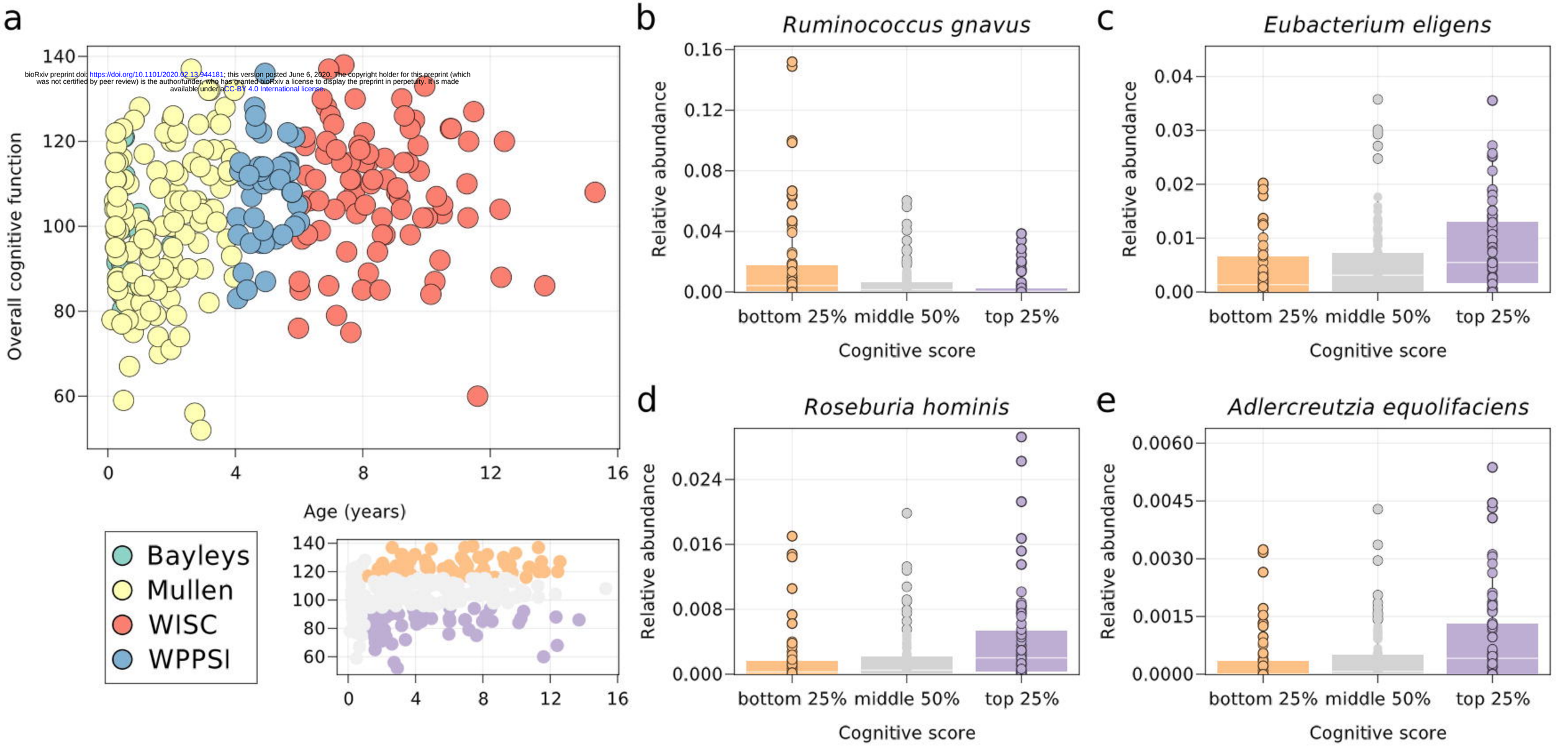

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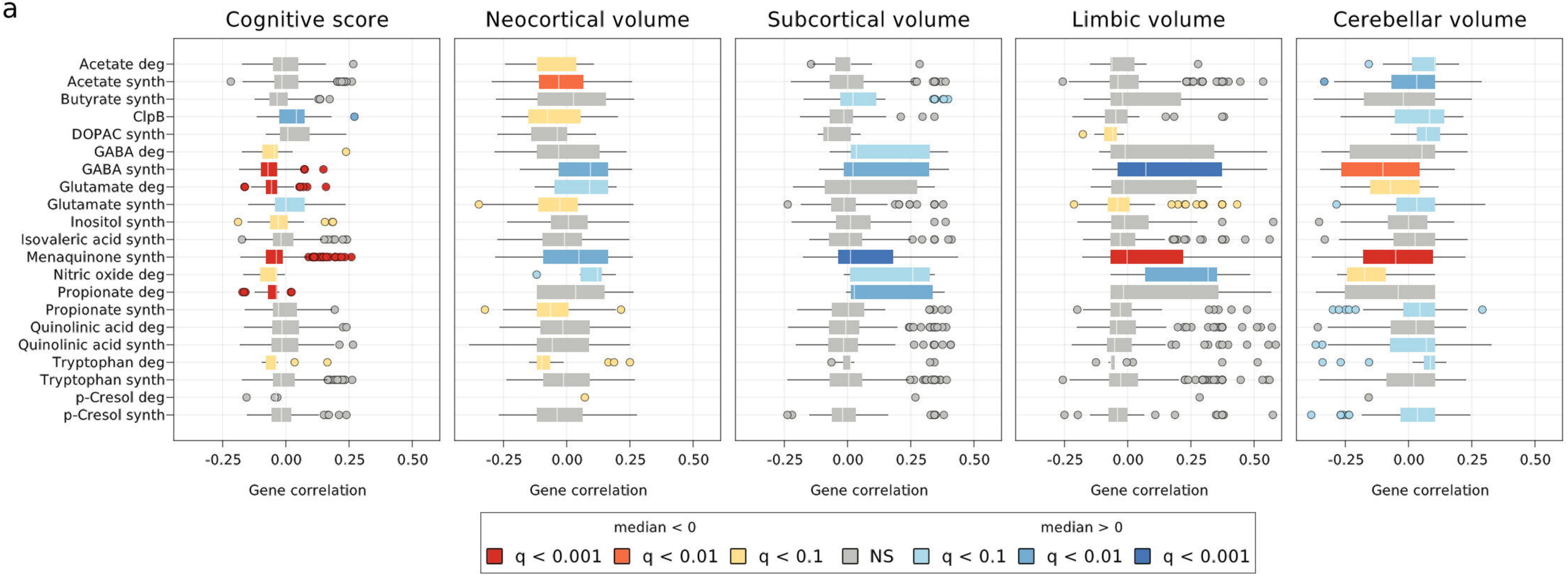

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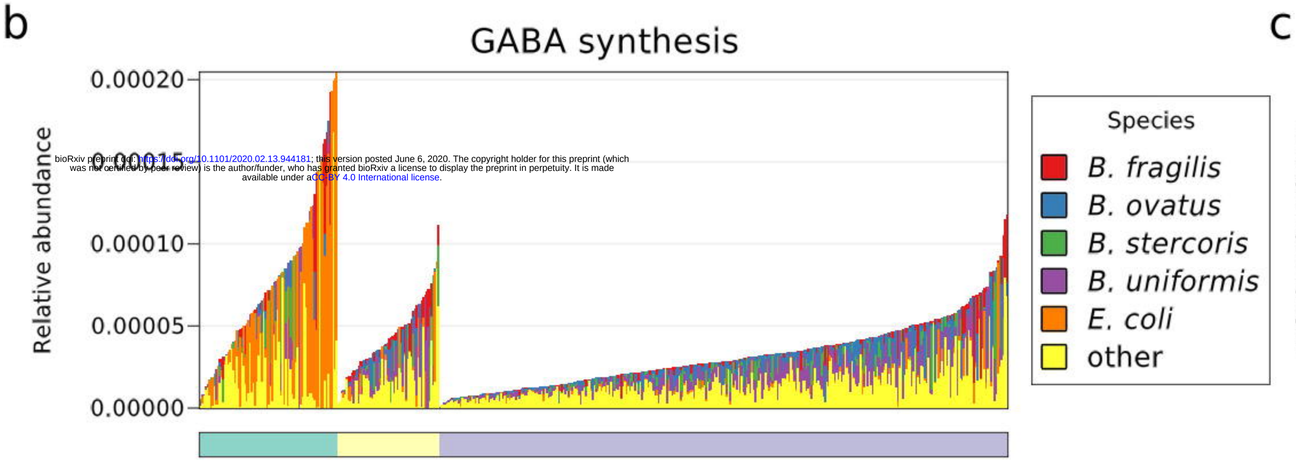

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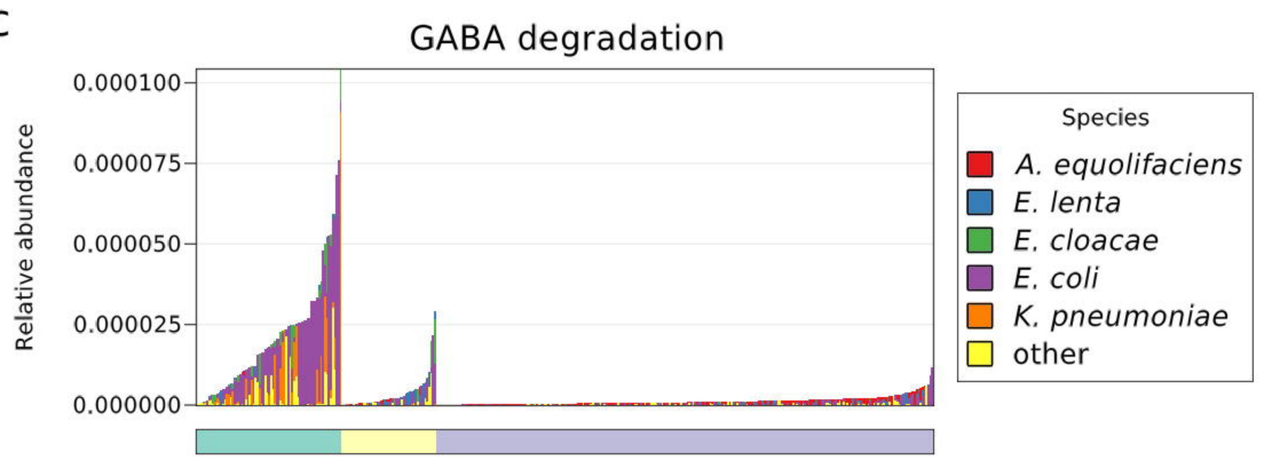

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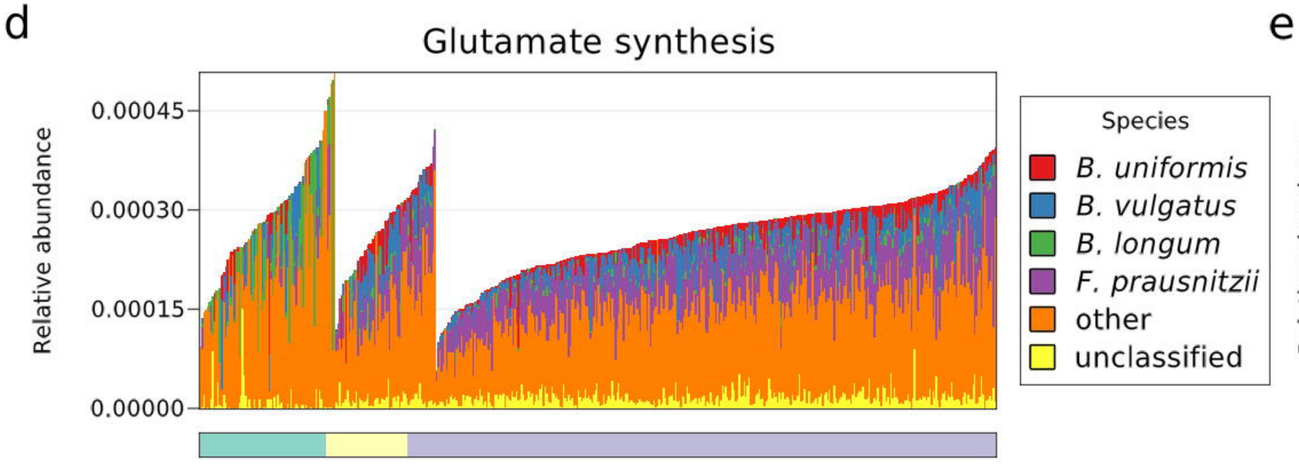

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