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Distinct gut microbiota composition in pediatric patients with central nervous system (CNS) tumors: A comparative study

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Abstract

Background. Central nervous system (CNS) tumors are the leading cause of cancer-related deaths in children aged 0–14 years. Despite significant efforts, targeted therapies based on identified pathways have not improved survival rates. Research has shown that the gut microbiota (GM) can influence brain tumor cell proliferation, suggesting that the microbiota–gut–brain axis plays a role in CNS cancer. Our study aims to assess whether the GM composition in pediatric CNS tumors exhibits specific characteristics.

Methods. The study included 18 pediatric patients, 9 diagnosed with CNS tumors (CNS tumors group) and 9 with other tumor types (extra-CNS tumors group). Microbial DNA was extracted from stool samples, and 16S DNA libraries were generated and sequenced. GM composition was analyzed using amplicon sequence variant (ASV) tables.

Results. Alpha-diversity analysis, represented by the number of observed features, was lower in the CNS tumors group ($P = .0054$), while Pielou's evenness index was similar between groups. LEfSe analysis revealed a significantly reduced abundance of the Firmicutes phylum in CNS tumors group, along with other taxa within this phylum, such as the Clostridia class, Clostridiales order, and Lachnospiraceae family, compared to extra-CNS tumors group. Further analysis using sPLS-DA showed a distinct pattern in GM composition in the CNS tumors group, with lower levels of several taxa, particularly the Firmicutes phylum, Lachnospiraceae family, Clostridiales order, Clostridia class, Ruminococcaceae and Coriobacteriaceae families, and *Blautia* genus.

Conclusions. Pediatric patients with CNS tumors have a distinct GM composition. The reduction of specific beneficial microbial taxa may contribute to tumor growth through the microbiota–gut–brain axis.

Key Points

1. Pediatric CNS tumors show lower gut microbiota diversity than other tumors.
2. Reduced beneficial gut bacteria in CNS tumors may promote tumor growth.
3. The brain–gut axis exerts selective pressure on the gut microbiota.

Central nervous system (CNS) tumors are the primary cause of cancer-related mortality in both adults and children aged 0–14 years. These tumors are notably prevalent among children diagnosed before the age of 6, representing the highest incidence rate in this age group.[1] Although research has uncovered numerous genetic alterations that contribute to the

Importance of the Study

Our study addresses a key gap in understanding pediatric CNS tumors, the primary cause of cancer-related deaths in children. This research is the first to show how GM might influence CNS cancers via the gut–brain–microbiota axis, a pathway linking microbial activity to brain health. By comparing GM compositions in children with CNS tumors and other tumor types, we observed distinct microbial patterns in CNS tumor patients, especially a reduction in beneficial microbial taxa. These differences suggest that GM dysbiosis could support tumor growth through the microbiota–gut–brain axis. This insight underscores the need for further research to explore whether targeting GM may improve outcomes for children with CNS tumors.

pathogenesis of CNS tumors, the development of targeted therapies addressing these specific pathways has largely been unsuccessful. These treatments have either failed to produce significant results or have had minimal impact on improving patient survival rates, highlighting the urgent need for more effective therapeutic strategies.[2]

It is becoming increasingly clear that the gut microbiota (GM) and their collective genomes, which are referred to as the microbiome, play an important role in human biology; it contributes to the body's homeostasis and health by preventing colonization by pathogens and modulating local and systemic immune-inflammatory responses.[3] The gastrointestinal tract and the CNS exhibit a significant interrelationship, wherein the CNS is integral in regulating the function and homeostasis of the GI tract. Conversely, GM can influence the regulation of nervous system functions and may affect the development and progression of neurological disorders.[4] This bidirectional communication and mutual influence are referred to as the grain–gut–microbiota axis.[4] The main mechanisms underlying the brain–gut axis function include: (i) immunological modulation; (ii) microbial metabolites-mediated modulation. The GM has been reported to act as a paramount player in the initiation, instruction, training, and regulation of the immune system, influencing the immune system development.[4] A deregulation in GM impairing the immune environment and resulting in chronic inflammation has been associated with oncogenesis and tumor progression.[5] The metabolites and products generated by GM are significant contributors to the brain/gut axis and exert their functions predominantly via receptor-mediated interactions in the tissues and cells of the host. Among them, short-chain fatty acids (SCFAs) and endogenous tryptophan are the metabolites that have received the greatest attention from researchers. The host metabolic status affects the microbial composition, and microbial metabolites can induce epigenetic modifications. The isocitrate dehydrogenase 1 or 2 (IDH1 or IDH2) mutations in glioma inhibit multiple α-ketoglutarate-dependent enzymes, leading to aberrant DNA methylation.[6] Thus, the GM and its derived metabolites as SCFAs might modify the epigenetic status, contributing to glioma progression.[6] Over the last decade, mutations in epigenetic modulator IDH1 or IDH2, and in the histone genes H3F3A or HIST1H3 B have been identified as key biomarkers for tumor classification. This emphasizes the central role of epigenetic alterations in the evolution and biology of gliomas.[7] The DNA methylation status of genes is an epigenetic modulation of the gene expression, whose variation is often a result of environmental factors, including metabolism.[8]

Following radiation therapy, a temporary response to treatment is frequently observed in most high-grade gliomas; however, predicting the timeline of tumor progression remains currently very difficult. It is essential to understand the biological mechanisms behind the response to radiation, to stop the growth of the tumor for as long as it is feasible, and possibly to identify biomarkers that might direct therapy. It has been demonstrated that GM plays a key role in pathophysiological processes[9]; it can mediate the proliferation of brain tumor cells and metastasis through different mechanisms, such as the aryl hydrocarbon receptor modulation, the regulation of SCFAs release, arginine and tryptophan metabolism, inflammatory cytokine production, microglia maturation, and T cell proliferation.[10] For these reasons, the involvement of the microbiota–gut–brain axis—a complex network of interactions between the gastrointestinal tract, microbiota, enteric nervous system, and brain—must be studied in the context of CNS cancer development and treatment response. In the present study, we evaluated the composition of GM in a cohort of oncologic pediatric patients and investigated its peculiarities in patients with CNS tumors, compared to the one of patients affected by extra-CNS tumors (soft tissues sarcomas, osteosarcoma, lymphomas, and neuroblastoma) through gut-microbiome taxonomic analysis.

The importance of this study lies in the demonstration of the huge influence of brain–gut–microbiota axis also in pediatric neoplastic diseases and it represents a step forward: treat GM to improve CNS cancer management.

Methods

Clinical Dataset

In 2020, the clinical study named "Cross-Domain interactions among SARS-CoV-2, microbiome and host in pediatric cancer patients" was started at the Pediatric Oncology Unit of our institute (Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy (INT)). It aimed at exploring the microbiota in COVID-19-positive/negative pediatric cancer patients. Details about the enrolment criteria are reported in Supplementary Materials. The study was conducted in accordance with ethical guidelines and regulations and it was approved by the local ethical committee (INT77/20). In the context of the clinical study, from June 2020 to July 2022, 138 patients were enrolled, after the informed

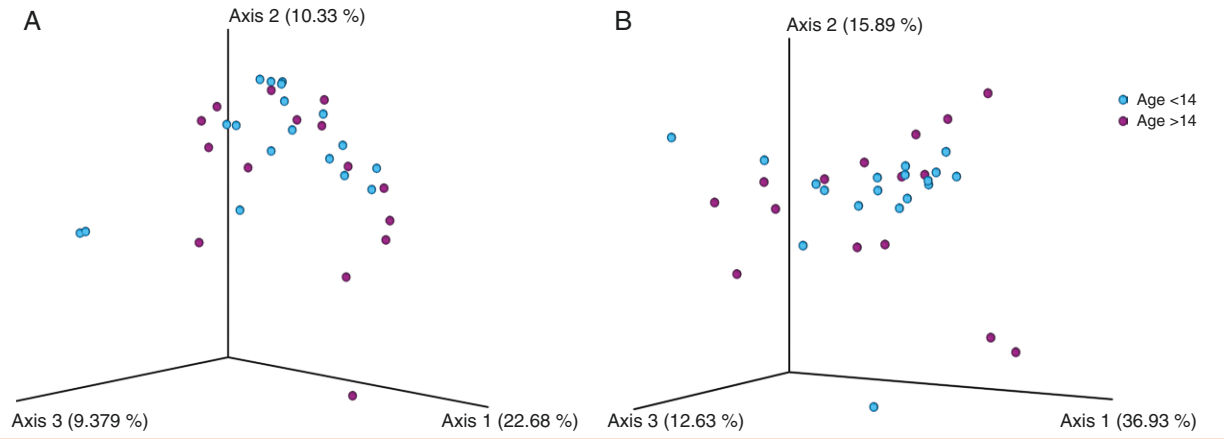

Figure 1. The unweighted (A) and weighted Unifrac (B) index is shown for inter-sample diversity. Statistics in alpha-diversity measures is according to the Mann–Whitney test.

consent was signed by themselves/their caregivers. The present study is based on a subset of 33/90 pediatric patients (Supplementary Figure S1) with full clinical data and stool collection available (see Supplementary Materials for details).

This clinical study allowed us to explore the role of the brain–gut–microbiota axis in a pediatric population. Out of the 33 patients selected, 11 were affected by CNS tumors (CNS group), while 22 were affected by other extra-CNS solid tumors (extra-CNS group) (consort diagram in **Supplementary Figure S1**). A beta-diversity analysis on these subgroups demonstrated that age did not influence the GM composition, both with unweighted and weighted UniFrac metrics (*P*-value = .398 and *P*-value = .389; **Figure 1A**, **1B**). However, the 2 groups show a significant difference in age distribution, with CNS patients being younger than those with other tumors (*P*-value = .031). To match the 2 subcohorts for age, we choose to exclude patients older than 14 years old (y.o.). Therefore, we refined the 2 groups, and we depicted the microbiota landscape in the following subgroups: (i) CNS tumor patients ($n = 9$, CNS tumors group) consisting of 6 females and 3 males; (ii) with extra-CNS solid tumors patients ($n = 9$, extra-CNS tumors group) comprising 5 females and 4 males. The 2 groups were balanced for age (Fisher test *P*-value = .59), sex (Fisher test *P*-value = 1), BMI (Wilcoxon–Mann–Whitney [WMW] test *P*-value = .41), and treatment (Fisher test *P*-value = .58) (**Table 1**). Clinical characteristics, such as sex and body mass index (BMI), did not influence the GM composition (**Supplementary Figure S3**).

Stool Sample Collection and Microbiota DNA Extraction

Stool samples were collected with the OMNIgene•GUT kit (DNA Genotek), containing a microbic DNA preservation buffer, following the manufacturer's instructions. The microbic DNA was extracted with the QIAsymphony DSP/Virus/pathogen Midi (QIAGEN) used on the QIAsymphony SP instrument (QIAGEN) that provides fully automated and simultaneous purification of viral nucleic acids and bacterial DNA. In particular, it is based on magnetic-particle technology and 4 steps of purification: sample lyses, bind of DNA to magnetic beads, wash and elution of microbic DNA. DNA concentration was quantified by the fluorimetric instrument Qubit 4.0 with dsDNA high sensitivity kit (Thermo Fisher) and the DNA integrity was evaluated with 4150 TapeStation system and genomic DNA screen tape (Agilent Technologies).

Sequencing of Microbic DNA

For each sample, starting from 100 ng of DNA, 16S rRNA gene amplicons libraries were generated with ION 16S Metagenomics kit (Thermo Fisher Scientific) which includes 2 primers sets for the selective amplification of the hypervariable regions of the bacterial gene encoding for 16S rRNA. Libraries were equimolarly pooled and sequenced by a sequencing service onto an Ion 550 barcoded chip for sequencing with an S5Primesequencer system (Thermo Fisher Scientific). The sequencing data were demultiplexed, and each FASTQ file containing sequences from multiple hypervariable regions was deconvoluted into separate files for each V region using the MetagenomicsPP[11] and Deconvolution Tool.[12]

Bioinformatics Analysis

The fastQ resulting from sequencing was imported into the QIIME 2 environment (ver 2023.7) where quality control was carried out and the amplicon sequencing variants (ASVs) tables were created using the DADA2 algorithm[13] and the GreenGenes database (ver 2022.10) was then used to infer the correct taxonomy associated with each sequence.

To assess the richness of GM, we computed 2 alpha-diversity metrics. The first is the observed characteristics, which are calculated by counting the number of different taxa in an ecological environment, and the second one is

Table 1. Summary of the Clinical Characteristics of the Sample

Clinical characteristics		CNS tumors (*n* = 9)	Extra-CNS tumors (*n* = 9)	*P*-value
Pathology	Ependymoma	1		
	Low-grade glioma	3		
	High-grade glioma	1		
	Brainstem/midline glioma	2		
	Medulloblastoma/PNET	1		
	Pineal tumor	1		
	Hodgkin's lymphoma		1	
	NRSTS		2	
	Osteosarcoma		2	
	Neuroblastoma		4	
Age	median	6.83	9.25	0.59 (WMW)
	range	[2.64; 13.65]	[2.36; 12.13]	
Sex	male	3	4	1.00* (F)
	female	6	5	
BMI	median	16.84	15.74	0.41 (WMW)
	range	[14.62; 30.84]	[12.89; 19.233]	
Oncologic treatment before stool collection	RT + CT	5	3	0.29 (CS)
	CT	3	6	
	none	1		
Oncologic treatment in progress at time of stool collection	CT	7	6	0.85 (F)
	none	2	3	

PNET = primitive neuro-ectodermal tumors; NRSTS = non rhabdomyosarcoma soft tissue sarcomas; CS = chi-square; WMW = Wilcoxon–Mann–Whitney test; F = Fisher test. For the F, the statistical value is the *q*-value.

the Pielou's evenness index,[14] which characterizes the distribution of abundance across the species in a community, with values ranging from 0 to 1.

The beta-diversity between the groups was then calculated using the unweighted Unifrac and weighted Unifrac metrics,[15] which both calculate the distance between groups by considering how close or distant the taxonomies are in the phylogenetic tree between the different groups. The first metric considers only qualitative taxonomic differences (presence or absence of a given species), while the second also considers the relative abundance of each taxonomic group.

After these first analyses, the ASVs tables were imported into the R environment (ver 4.3.1). The LEfSe analysis was performed employing the microbiomeMarker package (ver 1.6.0); in particular, the parameters used were 0.05 of non-Kruskal–Wallis cutoff and Wlicoxon cutoff and a lda score > 4 and the mixOmics package (ver 6.24.0) was used for the sparse partial least-squares discriminant analysis.[16]

Lymphocyte Count

Peripheral venous blood samples were collected from 16 of the 18 cases eligible for the CNS vs. extra-CNS comparison (8 CNS cases and 8 extra-CNS cases). Venous blood collection was performed using polypropylene tubes containing EDTA as an anticoagulant, following standard clinical practice. Absolute lymphocyte counts were measured in the same institutional laboratory and reported as cells 10^3/μl. The differences in absolute lymphocyte counts across CNS and extra-CNS cases were visualized as box plots and significance was assessed using the Wilcoxon signed-rank test. Discrimination capability was visualized by receiver operating characteristic (ROC) curve and inferred by the area under the curve (AUC), summarizing sensitivity and specificity along with the 95% confidence interval (CI). ROC and AUC were calculated using the pROC R package.

Data Availability

Raw and processed sequencing data are available through GEO repository[17] at the following ID: GSE278463.

Results

The research titled "Cross-Domain Interactions among SARS-CoV-2, Microbiome, and Host in Pediatric Cancer

Patients" was a single-center study aimed at exploring how the gut microbiome in pediatric cancer patients might impact the progression of COVID-19, either beneficially or adversely. The findings related to the clinical study are described in the Supplementary Material.

GM Composition of the Two Patients' Groups

The GM of the 2 groups is different in terms of the abundance of each microbial taxon. At the phylum level, the CNS tumors group is composed by 66.1% of Bacteroidetes, 20.9% of Firmicutes, 6.6% of Proteobacteria, 4.9% of Actinobacteria, 1.6% of Lentisphaerae, and 0.03% Fusobacteria. The overall GM of the extra-CNS tumors group is composed by 56.16% of Bacteroidetes, 35.42% of Firmicutes, 5.65% of Proteobacteria, 2.71% of Actinobacteria, and 0.01% of Fusobacteria (**Supplementary Figure S2**).

Upon Actinobacteria phylum, in the CNS tumors group it has been individuated only 2 genera: *Bifidobacterium* (4.16%) and *Collinsella* (0.71%). In the extra-CNS tumors group, there are more different genera: 1.60% *Bifidobacterium*, 0.79% *Collinsella*, and 0.03% of other genera (*Corynebacterium*, *Actinomyces*, *Adlercreutzia*, and other 2 genera from Coriobacteriaceaae family). Firmicutes is the most variegated phylum, with many different genera. The most abundant in both groups is a genus from the Lachnospiraceae family (4.01% in the CNS tumors group and 4.81% in the extra-CNS tumors group). It follows *Dialister* (3.80% in the CNS tumors group and 2.97% in the extra-CNS tumors group), *Oscillospira* (1.83% in the CNS tumors group and 2.29% in the extra-CNS tumors group), unknown genus from Ruminococcaeae (1.67% in the CNS tumors group and 4.13% in the extra-CNS tumors group) and Lachnospiraceae (1.25% in the CNS tumors group and 1.88% in the extra-CNS tumors group) families, *Ruminococcus* (1.76% in the CNS tumors group and 1.12% in the extra-CNS tumors group), *Blautia* (0.83% in the CNS tumors group and 2.63% in the extra-CNS tumors group), *Roseburia* (0.41% in the CNS tumors group and 2.93% in the extra-CNS tumors group), an unknown genus from Clostridiales family (0.35% in the CNS tumors group and 3.71% in the extra-CNS tumors group) and *Lachnospira* (0.96 in the CNS tumors group and 2.15% in the extra-CNS tumors group). Other genera were totally not identified in CNS tumors group, like *Peptoniphilus*, *Anaerofustis*, *Enterococcus*, *Leuconostoc*, *Lachnobacterium*, *Clostridium*, and *Anaerostipes*. Some genera were not identified also in the extra-CNS tumors group, as *Acidaminococcus*, *Megamonas*, *Pseudoramibacter Eubacterium*, and *WAL_1855D*. Among Proteobacteria phylum, *Sutterella* and *Citrobacter* genera are the most abundant (respectively, 4.23% and 0% in the CNS tumors group; 1.60% and 2.1% in the extra-CNS tumors group). Other genera were not identified in the CNS tumors group, but only in the extra-CNS tumors group: *Campylobacter, Bilophila, Desulfovibrio*, and 2 unknown genera from Oxalobacteraceae and Pasturellaceae families. In our clinical dataset, the prevalent phylum is Bacteroidetes, in particular, the genus *Bacteroides* represents the 48.9% in the CNS tumors group and 42.1% in the extra-CNS tumors group. Other consistent genera present were *Prevotella* (3.92% in the CNS tumors group and 0.3% in the extra-CNS tumors group), an unknown genus from Rikenellaceae family (4.6% in the CNS tumors group and 7.42% in the extra-CNS tumors group), and *Parabacteroides* (6.2% in the CNS tumors group and 3.6% in the extra-CNS tumors group). *Porphyromonas* and other genera from Porphyromondaceae family were found only in the CNS tumors group. Other less abundant genera from Bacteroidetes phylum were present in both groups, like *Odoribacter*, *Butyricimonas*, and *Paraprevotella*. Finally, *Fusobacterium* was found only in the CNS tumors group.

In **Supplementary Figure S2**, the GM composition of the 2 groups at class, order, and family level is plotted.

Alpha-Diversity Analysis Suggests a Lower GM Biodiversity of CNS Tumors Group Compared to Extra-CNS Tumors Group, But Not Beta-Diversity

Alpha-diversity was assessed by 2 different metrics: (i) observed features; (ii) Pielou's evenness. Alpha-diversity expressed by the number of observed features (ASV) is statistically significant lower in the CNS tumors group than in the extra-CNS tumors group (P-value = .0054) (**Figure 2A**). However, Pielou's evenness index is not significantly different between the 2 groups (P-value = .200) (**Figure 2B**). These results indicate clearly a decreased biodiversity of GM composition in pediatric patients affected by CNS tumors, without an alteration of the homogeneity of the different species present.

Beta-diversity analysis measured with unweighted UniFrac metrics revealed that the taxonomic and phylogenetic composition of GM is not different between CNS tumors group and 2 (P-value = .145) (**Figure 2C**).

Linear Discriminant Analysis Effect Size (LEfSe) Reveals a Significant Difference between GM of CNS Tumors Group Compared to Extra-CNS Tumors Group

From the linear discriminant analysis effect size (LEfSe) resulted that the phylum Firmicutes, Clostridia class, Clostridiales order, and Lachnospiraceae family are decreased in the CNS tumors group compared to the extra-CNS tumors group, in terms of relative abundance (**Figure 3B**). These different taxonomic groups represent the features that better differentiate the 2 groups. The LEfSe combines standard statistical test and additional tests explaining biological consistency and relevance.[18]

In particular, the abundance plot highlights clearly that Firmicutes phylum, Clostridiales order, Clostridia class, and Lachnospiraceae family are less abundant in CNS tumors group than in 2 (**Figure 3A**).

Moreover, in the cladogram, it is also visible that the taxa identified in GM as decreased in the CNS tumors group have all the same localization on the phylogenetic tree, as they belong to the Firmicutes phylum (**Figure 3C**).

Abundance of Firmicutes Phylum is Lower in Pediatric Pwith CNS Tumors

As one of the most important taxa present in the health GM, Firmicutes phylum abundance was taken in consideration.

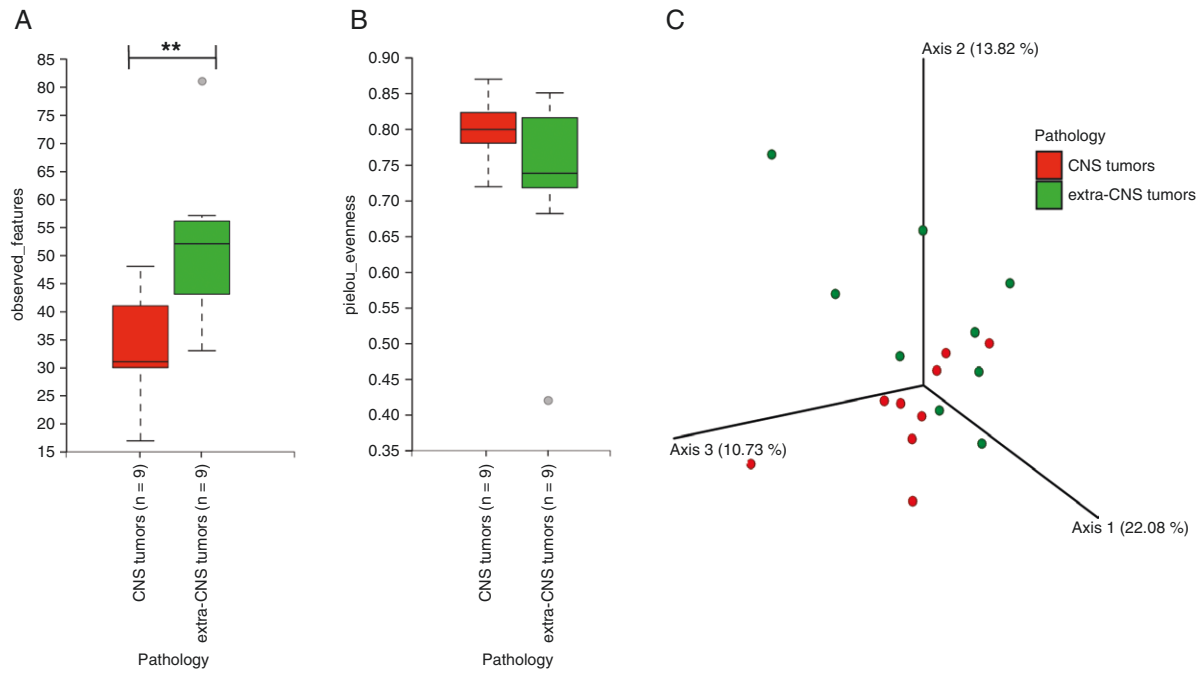

Figure 2. Comparison of the intra-sample biodiversity of the fecal bacterial communities between CNS tumors group and extra-CNS tumors group, according to the alpha-diversity indexes "observed features" (A) and "Pielou evenness" (B). The "weighted Unifrac" index (C) is shown for inter-sample diversity. Statistics in alpha diversity measures is according to the Mann–Whitney test. $^{**}P < .01$.

The median relative abundance of Firmicutes was 35% in the CNS tumors group, and 42% in the extra-CNS tumors group. With the WMW test, we observed a statistically significant difference between the 2 groups (*P*-value = .014) (**Figure 4A**). Then, we have evaluated the Firmicutes/Bacteroidetes ratio (F/B), that it is considered important for describing health microbiota. The median F/B is 0.35 in CNS tumors group and 0.42 in the extra-CNS tumors group, and there is not a significant difference between them (*P*-value = .136) (**Figure 4B**).

Components of the GM Explain the Difference between the Two Groups

To unveil the presence of consistent patterns in GM composition that explain the differences between the CNS tumors group and 2, a sPLS-DA, a multivariate dimensionality-reduction tool, was performed. First of all, it emerged that samples belonging to the CNS tumors group cluster together, meaning they have more similar GM profiles to each other and they belong to the same group (**Figure 5A**). In particular, the component 2 retains most of the variability in metagenomics composition explaining the differences among the CNS tumors group and the extra-CNS tumors group. In the sPLS-DA with background prediction graphic, it is visible that the model fails to classify in the right group 2 patients of the extra-CNS tumors group, while the patients of the CNS tumors group are predicted to have the same background (**Figure 5B**). The separation of a cluster corresponding to the CNS tumors group is driven by different bacteria, in particular, by microorganisms belonging to Firmicutes phylum, Coriobacteriaceae, Lachnospiraceae, and Ruminococcaceae families, and *Blautia* genus. The model assigns to each taxa a weight that represents the contribution of that taxa to the discrimination between the 2 groups. The most discriminating components are represented by Coriobacteriaceae family and *Blautia* genus, with sPLS-DA loads of –0.645 and –0.680, respectively. The other taxa have a lower discriminative power in the model, with lower sPLS-DA loads: –0.183 for Lachnospiraceae family, –0.179 for Ruminococcaceae family, –0.143 for Clostridia class and Clostridiales order, –0.091 for Ruminococcus genus, and –0.083 for Firmicutes phylum (**Figure 5C**). The performance of the clusterization is described by the ROC curve, with an area under the curve (AUC) of 0.975 (**Figure 5D**).

Lymphocyte Count in CNS and Extra-CNS Cases

The number of circulating lymphocytes in peripheral blood was retrieved from blood tests conducted at the same time as stool collection. The relationship between the absolute lymphocyte count in CNS and extra-CNS cases was investigated. **Supplementary Figure 4A** presents the distribution of absolute lymphocyte counts across the 2 groups, revealing significantly higher levels in CNS cases compared to extra-CNS cases (*P*-value = .0421). To evaluate the discriminative performance of the absolute lymphocyte count in distinguishing between the 2 groups, an ROC curve was generated (**Supplementary Figure 4B**). The ROC curve indicates a moderate discriminative capability, with an AUC of 0.781 (95% CI: 0.534–1.000).

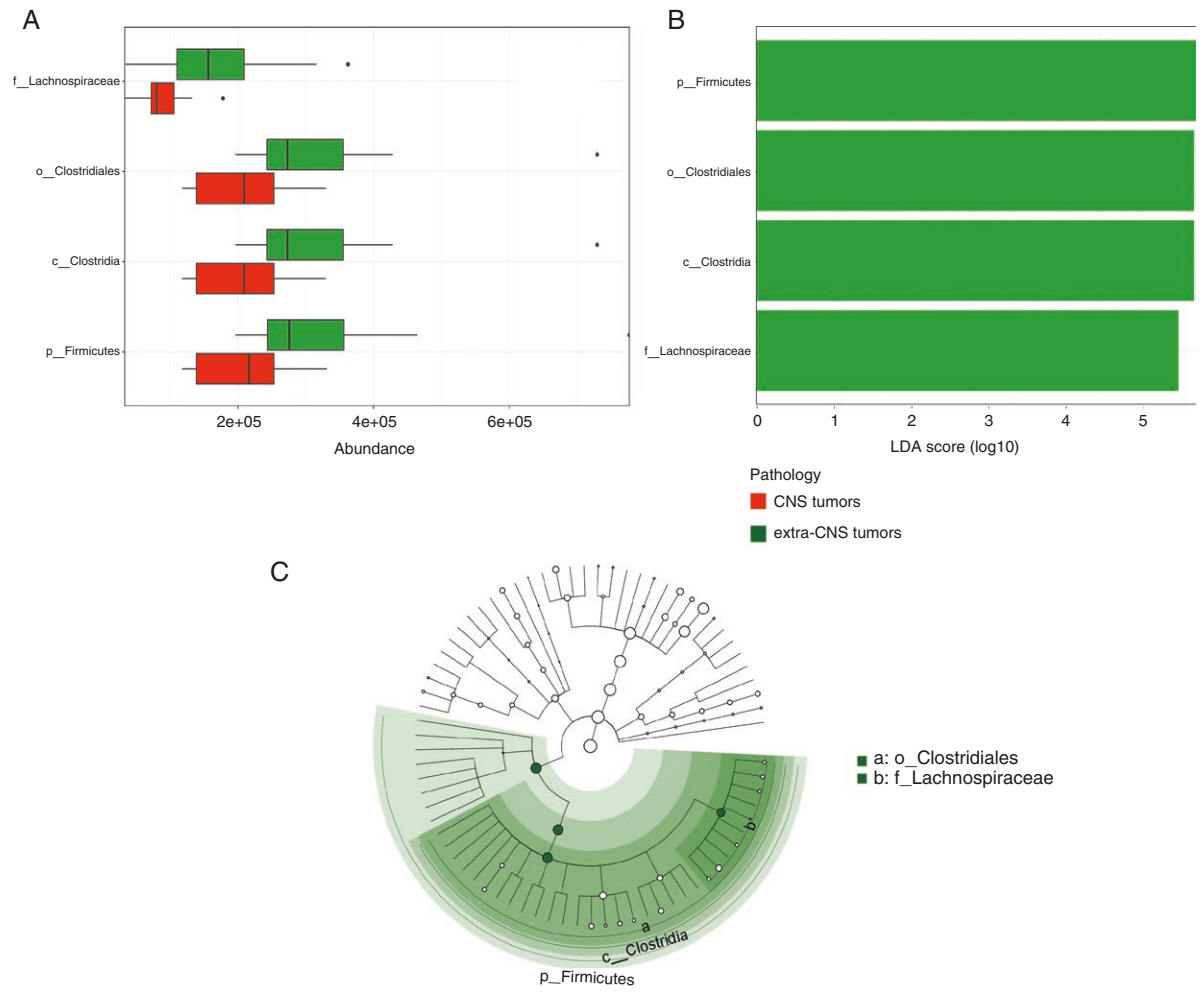

Figure 3. (A) Abundance box-plots for all the taxonomies of interest found by performing the discriminant analysis effect size (LEfSe) analysis. (B) LEfSe and linear discriminant analysis (LDA) based on operational taxonomic units that were used to characterize differences between microbiomes of CNS tumors group and extra-CNS tumors group. (C) Cladogram generated using the LEfSe method indicating the phylogenetic distribution of fecal microbes associated with CNS tumors group and extra-CNS tumors group. The taxonomic levels represented by rings with phyla in the outermost the ring and genera in the innermost ring are shown. Each circle is a member within that level.

Discussion

In recent years, extensive research has explored the interconnection between the gut–brain–microbiota axis, shedding light on its potential causal relationship with brain tumor development. These studies have identified specific microbial taxa associated with an increased risk of brain tumors, offering valuable insights into the role of GM in the etiology of these malignancies, and showing divergent host–microbe interactions compared to healthy subjects.[19] The findings underscore the importance of gut–brain axis interactions, particularly their influence on immune modulation and neuroinflammation, as key mechanisms contributing to tumor initiation, progression, and the broader pathophysiological processes underlying brain tumor development. This growing body of evidence highlights the potential of targeting the gut–brain–microbiota axis as a novel therapeutic approach for brain tumor treatment.[20] Through the exploration of GM composition, we have unveiled a difference between pediatric patients affected by CNS tumors and those affected by other solid tumors. The reduction of observed ASVs coupled with a slightly increasing of evenness, among pediatric patients with CNS tumors in comparison to other patients, suggests that the growth of few specific microbial taxa is promoted by CNS tumor, probably because microbial metabolites sustained their growth through the gut–microbiota–brain axis. Our findings align with recent research conducted in pediatric patients diagnosed with brain tumors undergoing CT, which demonstrated a significant reduction in the diversity and abundance of gut microbial genera. Notably, this disrupted GM composition has been shown to correlate with alterations in key metabolic pathways, including the carnitine shuttle, fatty acid metabolism and activation, as well as tryptophan metabolism. These pathways have

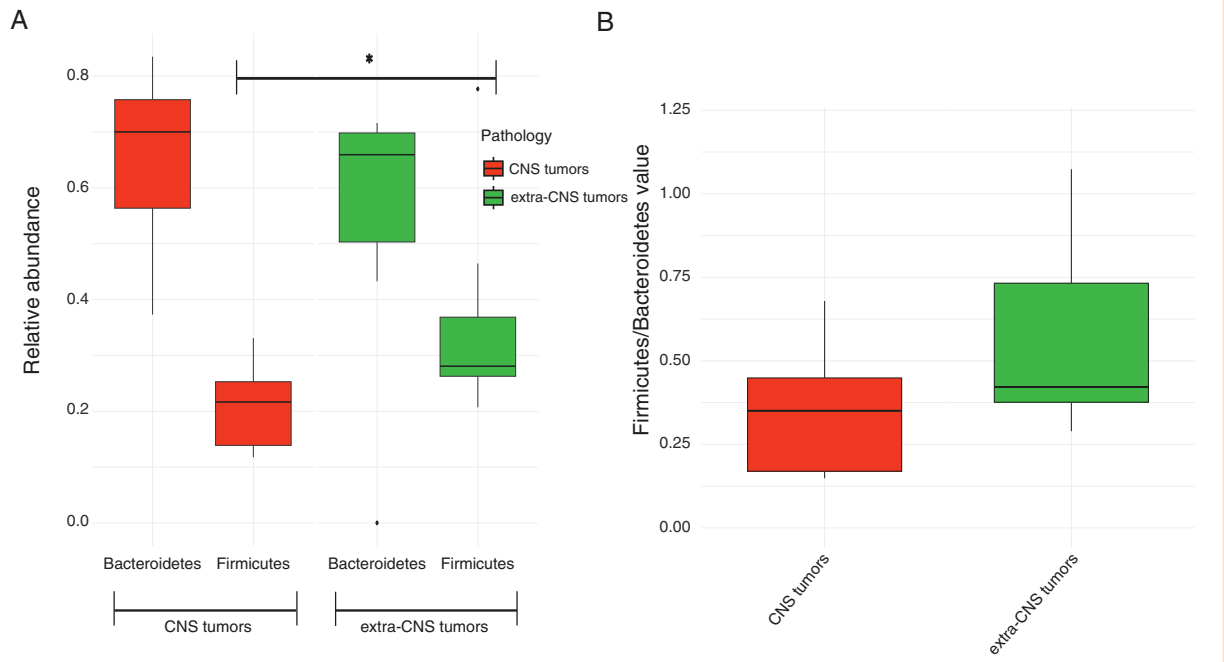

Figure 4. (A) Comparison of the relative abundance of Firmicutes and Bacteroidetes phyla between CNS tumors group and extra-CNS tumors group with WMW test (*$P < .05$). (B) Comparison of the F/B ratio between the 2 groups.

been consistently implicated in various studies examining the systemic effects of chemotherapy across different types of cancer, highlighting potential mechanistic links between microbial dysbiosis and metabolic reprogramming induced by anticancer treatments.[21] The same trend has been found in adult patients with brain tumors by Yang et al., in which the results showed a lower gut microbial richness and evenness.[20]

As a matter of fact, it is already known that brain development and neurological functions can be influenced by GM through microbial metabolites, like SCFAs and amino acids (tryptophan, glutamate, and glutamine).[8,22] In particular, SCFAs are of utmost importance for brain–blood barrier (BBB) formation and maintenance[23] and there are evidences that demonstrated that GM metabolites are fundamental in CNS homeostasis.[24–27] Overall, GM dysbiosis induces CNS homeostasis deregulation, which has a key role in neurological diseases development, like Parkinson's disease (PD)[28] and Alzheimer's disease (AD),[29] but also in brain tumors.[30] GM dysbiosis has been studied in different types of adult brain tumors[31,32] but our study takes into account pediatric patients for the first time.

In particular, a significant decrease in **Firmicutes** phylum in CNS tumors group compared to extra-CNS tumors group was found, like Jiang et al. have demonstrated in the GM of patients with non-small-cell lung cancer (NSCLC) with brain metastasis and Biddle et al. in AD patients[33] (Supplementary Table S2). Firmicutes not only have been correlated to the regulation of anti-inflammatory and apoptotic functions but also regulate intestinal and BBB permeability, through the production of SCFAs.[32,34] In particular, **Clostridia** class and **Clostridiales** order are the main Firmicutes depleted in GM of CNS tumors group patients. The Clostridiales order has been found to decrease also in patients affected by schizophrenia,[35] while Clostridiaceae1 were associated with slight increase in the brain tumor risk.[20] This result could be in contradiction with ours, but it could be explained by the high heterogeneity of the clinical dataset on which the study has been done. The majority of ***Clostridium*** species are commensal bacteria and it has been demonstrated to have anti-inflammatory activities.[36] Among them, a genus within the **Lachnospiraceae** family was decreased in patients affected by Schizophrenia,[37] in PD patients[38] and in a mouse model of depression,[39] and a reduction of Lachnospira has been demonstrated also in adult patients with CNS tumors.[20] The genus ***Blautia*** has been found to decrease in patients affected by PD,[40] cognitive impairment due to AD,[41] and in children with ASD.[42] ***Ruminococcus*** genus, described in the literature as one of Firmicutes phylum to have anti-inflammatory metabolites production, has been found to increase in patients affected by amyotrophic lateral sclerosis (ALS).[43] In a mouse model of AD (APP/PS1)[44] and in AD patients a significant decrease of *Ruminococcus* was found, compared to controls.[45] Interestingly, the Ruminococcus genus was individuated as one of the most involved gut microorganisms in the development of psychoneurological symptoms associated with CT and alteration of metabolic pathways of fatty acids and tryptophan in pediatric patients with CNS tumors.[21]

A reduction in the population of SCFA-producing microorganisms may potentially contribute to a deficiency in SCFAs. This deficiency has been linked to dysregulated immune responses. In our study, we analyzed the circulating immune cell populations and observed a significant increase in the absolute levels of lymphocytes in CNS tumors compared to tumors located outside

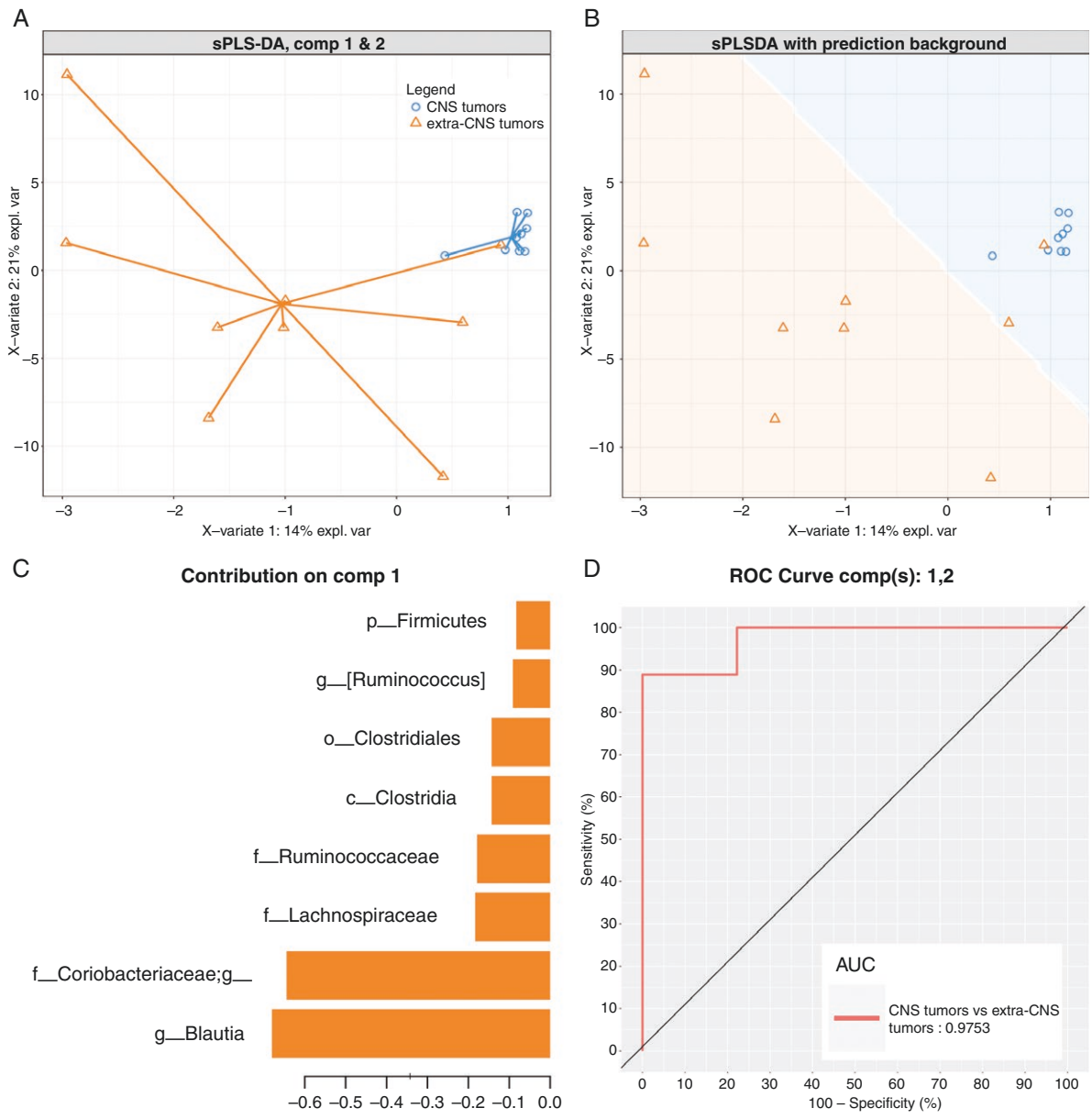

Figure 5. (A) sPLS-DA plot depicting sample distribution, with CNS tumors group samples (circles) and extra-CNS tumors group samples (triangles). (B) sPLS-DA with background prediction plot. (C) The weight of the most important taxonomy for the first component in generating the sPLS-DA model. For all the variables considered the classification value is in favor of extra-CNS tumors group, which is why all of them are negative. (D) ROC curve showing the performance of the sPLS-DA classification model.

the CNS. In addition, reduced SCFAs levels were detected in patients with chronic inflammation associated with long-term multiple sclerosis. These findings highlight the bidirectional regulatory roles of SCFAs and their G-protein-coupled receptors in the context of autoimmune neuroinflammation.[46] In fact, SCFAs induce antigen-presenting cells (APCs) resident in CNS and lymphocyte T to produce interleukin-10 (IL-10), a potent anti-inflammatory cytokine, which limits host immune response to prevent tissue damage.[47] In particular, SCFAs regulate histone deacetylases (HDACs) and mTOR activity, stimulating IL-10 expression in lymphocyte T.[48]

In the context of GM dysbiosis, local inflammation occurs first in the gut, then becomes peripheral when inflammatory molecules, also produced by GM, enter the systemic circulation.[49] These signals induce lymphocytes to differentiate into proinflammatory subtypes (Th1 and Th17), which produce proinflammatory cytokines, such as interleukin-2 (IL-2), interleukin-12 (IL-12), TNFα, and IFNγ. Therefore, the BBB integrity is disrupted by this inflammation, causing the infiltration of inflammatory mediators, immune cells, and bacterial toxins in the CNS,[50,51] which could activate resident microglia's immune response.[52] CNS tumor itself produces damage-associated molecular patterns (DAMPs),

that activate resident microglia to release inflammatory molecules chemoattractants.[53] They recruit peripheral immune cells, such as neutrophils, monocytes, and CD4 +T cells, which also produce other proinflammatory cytokines, that together with DAMPs could enter the circulation and reach distal tissues.[54] Proinflammatory signals could induce gut inflammation that leads to a reduction of regulatory T cells and their production of IL-10 and TGFβ. The loss of anti-inflammatory signals, with the induction of proinflammatory pathways, augments the activation of the immune cells and exacerbates the inflammation of the CNS.[55] Also, the intestinal barrier permeability is promoted, causing a leak of microorganism into circulation and exacerbating systemic inflammation.[56] Last of all, **Coriobacteriaceae** family has been found to decrease in ASD,[57] chronic stress disease,[58] AD patients,[59] in a mouse model of PD[60] while was increased in patients affected by daily epileptic seizures[61] and PD patients.[38]

Overall, evidences in literature published in the last few years support our hypothesis that GM is involved in CNS disease, particularly in cancer and that brain tumors are associated with a reduction of GM richness, in particular of beneficial microbial taxa. Our results corroborate the existence of a bidirectional communication and mutual influence known as the gut–microbiota–brain axis and for the first time, this was observed in a population of pediatric cancer patients. Further studies are warranted to validate our findings and to disclose the clinical role of microbiota in CNS tumors.

Conclusions

In conclusion, our results suggest that pediatric patients affected by CNS tumors have a peculiar composition of GM, significantly different from pediatric patients with other solid tumors and that it could be involved in the process of tumor growth, through the specific interactions of the brain–gut–microbiota axis.

Supplementary Material

Supplementary material is available online at *Neuro-Oncology Advances* (https://academic.oup.com/noa).

Keywords

brain–gut axis | microbiota | pediatric brain tumors

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Author Contributions

Data analysis: A.G.L., F.R., and C.D.; data collection: L.B., V.B., E.S., O.N., and S.C.; experimental implementation: A.G.L., L.V.V., M.O., and Man.M.; interpretation of the data: C.D.; provision of the study materials: P.G., L.B., and L.V.V.; study conception: L.D.C., Mau.M.; study design: L.D.C., L.B., Mau.M.; study supervision: L.D.C., L.B.; visualization: F.R.; writing—original draft: C.D., F.R., and L.D.C.; and revising manuscript: all authors.

Data Availability

The datasets generated and/or analyzed during the current study are available in the GEO repository, [https://www.ncbi.nlm.nih.gov/geo/] ID: GSE278463.

Ethics Approval and Consent to Participate

The study was conducted in accordance with ethical guidelines and regulations and approved by the local ethical committee (INT77/20). The subjects were enrolled after the informed consent was signed by them (if they were older than 18 y.o.) or by their parents/legal guardians (for people younger than 18 y.o.).

Consent for Publication

All authors have read and approved the manuscript and agree with submission to Biomarker Research.

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