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Microbiota–gut–brain axis and treatment resistance in epilepsy: a multicentre prospective study protocol (CARE)

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STRENGTHS AND LIMITATIONS OF THIS STUDY

⇒ The multicentre prospective design enables standardised longitudinal collection of clinical, neurophysiological and biological data across heterogeneous epilepsy populations.
⇒ Repeated sampling of stool and blood allows within-subject analysis of temporal changes in microbiota composition, metaproteomics and host DNA methylation.
⇒ Integration of multi-omics data with clinical metadata is supported by predefined bioinformatic and statistical pipelines, including mixed-effects and multivariate models.
⇒ Smaller sample sizes in selected intervention cohorts (vagus nerve stimulation and ketogenic diet) restrict analyses in these groups to exploratory and hypothesis-generating objectives.
⇒ Residual confounding related to age, epilepsy aetiology, diet and concomitant medications may influence microbiota and epigenetic measures despite planned adjustments.

ABSTRACT

Introduction Approximately one-third of people with epilepsy (PWE) experience resistance to treatment, including pharmacological therapies, epilepsy surgery, vagus nerve stimulation (VNS) and dietary interventions such as the ketogenic diet (KD). Emerging evidence suggests that the gut microbiota may influence seizure susceptibility and treatment response through the microbiota-gut-brain axis, potentially contributing to treatment resistance. The MiCrobiota-gut-brain Axis in Resistant Epilepsy project investigates how gut microbial features and associated host epigenetic signatures affect clinical outcomes in PWE undergoing diverse treatment strategies.

Methods and analysis This is a multicentre, prospective, longitudinal study involving four clinical centres in Italy and one self-financing partner. Participants aged 3–50 years will be enrolled and stratified into four intervention cohorts: newly diagnosed drug-naïve epilepsy scheduled to start anti-seizure medications, focal drug-resistant epilepsy (DRE) undergoing epilepsy surgery, DRE receiving VNS, and DRE initiating KD. Clinical assessments (including body mass index calculation, self-reported monthly seizure count, dietary evaluation, quality of life scale and gastrointestinal symptoms scale), electroencephalography, MRI and biological sample collection (stool and blood) will be obtained at baseline and longitudinally at two or three timepoints over a 12-month observation period. Gut microbiota changes over time will be assessed via metagenomics (using 16S ribosomal RNA sequencing) and metaproteomics; the associated host DNA methylation profiles will be obtained from blood using Illumina EPIC arrays. Primary endpoints include identification of microbial or host methylation changes predictive of therapeutic response (ie, reduction from baseline in monthly seizure count) to the intervention. Data will be analysed using multivariate models and mixed-effect regression. Further, omics data and corresponding metadata will be integrated using multi-omics approaches to identify molecular signatures biomarkers predictive of treatment response and prognosis in PWE.

Ethics and dissemination The study received ethical approval from the Research Ethic Board (Comitato Etico Territoriale Lombardia 3, ID 4896 – parere numero 4896_17.07.2024_N_bis). All participants or their legal guardians will provide written informed consent. Results will be disseminated through peer-reviewed publications, conference presentations or lay summaries targeting patient organisations.

Trial registration number ClinicalTrials.gov Identifier NCT07010445, registered on 2 May 2025.

INTRODUCTION

Epilepsy is a prevalent neurological disorder affecting over 50 million people worldwide.[1 2] While anti-seizure medications (ASMs) remain the first-line treatment, approximately one-third of individuals still develop drug-resistant epilepsy (DRE),[3–5] defined as the failure to achieve sustained

seizure freedom after adequate trials of two appropriately chosen and tolerated ASMs.[6] DRE severely impacts the quality of life (QoL) of patients and is associated with an increased risk of premature death.[7] Treatment options for DRE include epilepsy surgery, vagus nerve stimulation (VNS) and the ketogenic diet (KD).[8 9] Selected patients with DRE may benefit from epilepsy surgery, with seizure freedom rates ranging from 58% to 77%, depending on the underlying pathology.[10–12] For patients who are not surgical candidates, VNS provides an alternative, yielding an average seizure reduction of 24.5% and a responder rate—defined as a ≥50% reduction in seizure frequency—of approximately 31%.[13 14] The KD offers another viable therapeutic option, with a reported responder rate of about 53%.[9] However, a substantial proportion of individuals continue to experience uncontrolled seizures despite these interventions.

Interindividual differences in treatment response remain poorly understood. Notably, all major interventions for DRE—including ASMs, VNS and the KD—have been shown to modulate the gut microbiota, a key player in the microbiota–gut–brain axis (MGBA).[15] The MGBA refers to the complex, bidirectional communication between the central nervous system (CNS) and the gut microbiota, involving neuroimmune, neuroendocrine and metabolic pathways.[15 16] The intestinal microbiome is believed to influence CNS homeostasis, neurodevelopment and neuroinflammation and is increasingly implicated in the pathophysiology of neurological disorders.[17–19] Alterations in gut microbial composition (dysbiosis) have been observed in people with epilepsy (PWE), and emerging data show that gut microbiota profiles differ not only between PWE and healthy individuals, but also between drug-responsive and drug-resistant PWE.[19–21] In particular, preliminary findings from the MiCrobiota-gut-brain Axis in Resistant Epilepsy (CARE) group support the hypothesis that selected gut microbial features may influence seizure burden and shape therapeutic outcomes.[20] Moreover, recent preclinical and clinical studies suggest that the MGBA may modulate seizure susceptibility and treatment responsiveness, particularly in the context of dietary therapies such as the KD.[22–26] However, the existing literature remains focused on dietary modulation of seizure susceptibility via the gut microbiome, and no study to date has specifically investigated the role of the MGBA in modulating outcomes following non-pharmacological interventions such as epilepsy surgery or neuromodulation (eg, VNS). Current evidence remains limited by a lack of longitudinal studies examining microbiota changes across different treatment stages, especially from the pre-ASM stage onward.

Within this framework, the MGBA is increasingly recognised as both a potential therapeutic target and a source of biomarkers for disease progression and treatment response. The CARE project was designed to address this critical knowledge gap by integrating longitudinal clinical and neurophysiological data with multi-omics analyses in patients undergoing diverse therapeutic interventions. Through detailed characterisation of gut microbiota composition and function, alongside host DNA methylation profiles, the study will enable high-resolution association analyses to identify biomarkers of treatment response in different stages of epilepsy. The ultimate goals of this study are to identify robust, clinically relevant microbial and host-derived molecular biomarkers that can predict treatment response and disease trajectories in epilepsy. By integrating clinical, neurophysiological and multi-omics data over time, the CARE project aims to generate predictive models that support early patient stratification, inform personalised therapeutic strategies (eg, selecting candidates for epilepsy surgery, KD or VNS) and improve monitoring of treatment efficacy—thus advancing precision medicine approaches for PWE.

METHODS AND ANALYSES

Study design and setting

CARE project is an ongoing 24-month, multicentre, prospective, non-pharmacological, interventional study involving four Italian tertiary epilepsy centres and one self-financing partner. Eligible participants are between 3 and 50 years of age, meet the diagnostic criteria for epilepsy or DRE,[6] and are either initiating ASM if drug-naïve or undergoing one of three non-pharmacological interventions if DRE: epilepsy surgery, VNS or KD. Exclusion criteria include the presence of severe systemic illness or significant gastrointestinal disorders (eg, inflammatory bowel disease); the current use of antibiotics, corticosteroids or probiotics (within 30 days prior to enrolment); and current or recent adherence to special dietary regimens. Written informed consent—and assent, in the case of minors—will be obtained from all participants and/or their legal guardians prior to enrolment in the study.

Patient and public involvement

Patients were not formally involved in the design or conduct of this study. However, feedback from patients and caregivers in routine clinical practice informed the selection of patient-reported outcome measures. Study findings will be shared with participants through lay summaries and institutional communication channels.

Subject withdrawal

Participants, or their parents or legal guardians in the case of minors, may choose to discontinue their involvement in the study at any time. Additionally, the principal investigator, as the study guarantor, may decide to withdraw a participant in the event of adverse events, non-compliance with study procedures or withdrawal of informed consent.

Intervention and data collection

Participants are stratified into four study cohorts: (1) n=50 newly diagnosed drug-naïve patients (naïve), (2) n=50 DRE patients undergoing epilepsy surgery

(DRE-surgery), (3) n=10 DRE patients receiving VNS (DRE-VNS), and (4) n=10 DRE patients commencing a medically supervised KD (DRE-KD). These interventions will be administered as part of standard clinical care, and no experimental treatment is involved. Biological samples and clinical data are collected at four predefined time points: baseline (T0), 1 month (T1), 6 months (T2) and 12 months (T3).

Stool samples are obtained at all four time points, while blood samples and anthropometric measurements, including weight, height and body mass index (BMI), as well as a standardised neurological examination, are obtained at time points at T0, T2 and T3.

Gut microbiota taxonomic profiles will be derived from 16S ribosomal RNA sequencing of the V3–V4 region, followed by bioinformatic processing using QIIME2 (microbiome analysis platform and pipeline, QIIME 2 Development Team (Northern Arizona University), Flagstaff AZ, USA) and Phyloseq. Gut metaproteomic data will be obtained from tryptic peptides analysed by LC–MS/MS (Liquid Chromatography–Tandem Mass Spectrometry) and identified in Proteome Discoverer V.2.5 (proteomics data analysis software, Thermo Fisher Scientific, Waltham MA, USA using Sequest-HT, with peptide validation by Percolator (semi-supervised machine learning software for proteomics post-processing, Lukas Käll et al. (KTH Royal Institute of Technology), Stockholm, Sweden) at 1% false discovery rate (FDR).

Blood samples will be used to perform genome-wide DNA methylation measurement with the Illumina EPIC array. Bisulphite converted DNA will be subjected to methylome profiling using the Illumina Infinium Methylation EPIC Beadchip array, consisting of around 850 000 CpG (Cytosine–phosphate–Guanine) sites over the whole genome.

Epilepsy-specific collected data include seizure type, frequency and severity (documented via participant-reported seizure diaries); age at seizure onset; history of neonatal, febrile or acute symptomatic seizures; the presence of neurological, psychiatric or cognitive comorbidities. Epilepsy aetiology is classified based on available clinical and diagnostic information. In accordance with ILAE (International League Against Epilepsy) recommendations,[6] aetiologies consistent with clinical presentation are categorised as symptomatic; when a specific cause cannot be identified, the epilepsy is classified as of unknown aetiology. Family history of epilepsy is recorded, and seizure semiology is documented both at onset and throughout follow-up.

Treatment-specific information is collected at every study timepoint for all participants including documentation of the intervention received (epilepsy surgery, VNS or KD) along with any associated complications or adverse events. Current ASM treatment and all previously administered ASMs are recorded, as well as the use of any non-ASM medications during the study.

Electroencephalography (EEG) data (background, interictal and ictal findings) are collected for all participants. Clinical brain MRI is acquired and reviewed at baseline for all patients and subsequently as clinically indicated, except for the participants in epilepsy surgery where longitudinal MRI data is acquired at the T0 and T2 timepoints.

Dietary and nutritional assessments are conducted at all timepoints through a 3-day food diary completed by participants or caregivers, covering 2 weekdays and 1 weekend day. Instructions are provided to ensure consistency. Nutritional intake, including micronutrient and macronutrient composition, will be obtained using Metadieta software (METEDA S.r.l., Rome, Italy, https://www.meteda.it/en/product/metadieta/). Gastrointestinal function is assessed at each time point using the Gastrointestinal Symptom Inventory. (GISI)[27] and the Bristol Stool Form Scale.[28]

Health-related QoL is evaluated using age-appropriate, validated instruments. For adults, the Quality of Life in Epilepsy Inventory (QOLIE-31 V.1.0)[29] is administered at T0 and T3. This 31-item questionnaire produces a total T-score ranging from 0 to 100, with interpretive thresholds indicating poor (<30), moderate (31–50), or good (>50) QoL.[29] For children and adolescents aged 2–25 years, the Italian-validated Paediatric Quality of Life Inventory 4.0 Generic Core Scales (PedsQL)[30] is used. This 23-item instrument covers four domains—physical, emotional, social and school functioning—and yields three composite scores: total, physical health and psychosocial health. Scores are scaled from 0 to 100, with higher scores reflecting better QoL.[30] A summary of assessments and sampling activities across all study visits is provided in table 1.

Sample size justification

This study is designed as a biomarker discovery protocol integrating clinical, microbiota and host epigenetic data in patients with epilepsy undergoing different therapeutic interventions. Based on previously published literature in the fields of gut microbiota and DNA methylation in neurological and metabolic disorders, effect sizes for taxa-level microbial abundance or CpG methylation differences typically fall within the moderate range (Cohen's d=0.4–0.6). For example, Gomez-Eguilaz *et al*[31] and Peng *et al*[32] reported moderate-to-large shifts in microbial profiles among individuals with epilepsy and DRE, while studies in peripheral methylation profiling, such as those by Uddin *et al*[33] and Kundakovic *et al*,[34] demonstrate that sample sizes of approximately 40–50 participants per group are sufficient to detect biologically relevant methylation differences exceeding 10%, with 80% power and α=0.05. Additionally, general guidance from large-scale microbiome studies has suggested that sample sizes of 30–50 individuals per group are typically sufficient to detect moderate changes in microbial diversity and taxa abundance, accounting for the high interpersonal variability observed in gut microbial composition.[35 36] Taking into account an anticipated dropout rate of 30% over the 12-month follow-up period, we plan to enrol

Table 1 Schedule of assessments and study procedures

Study procedures					
Timepoint*	**t0**	**i†**	**t1**	**t2**	**t3**
Eligibility screen	✓				
Informed consent/assent acquisition	✓				
Medical history	✓				
Pharmacological history	✓		✓	✓	✓
Physical and neurological examination	✓			✓	✓
Anthropometric measurements (weight, height, BMI)	✓			✓	✓
MRI	✓‡			✓‡	
Self-administered 3-day food diary	✓		✓	✓	✓
Self-administered daily seizure diary	✓		✓	✓	✓
GISI scale	✓		✓	✓	✓
BSFS	✓		✓	✓	✓
QoL (PedsQL/QOLIE-31) questionnaires	✓				✓
Stool collection	✓		✓	✓	✓
Blood collection	✓			✓	✓

Timing of clinical examinations, patient-reported outcomes, and biological sample collection at enrolment (T0), intervention (i), and follow-up visits (T1: 1 month, T2: 6 months, T3: 12 months). EEG and MRI are performed at baseline and follow-up (longitudinal MRI in the surgery cohort only)
*t0, enrolment; i, intervention; t1: 1 month after intervention; t2: 6 months after intervention; t3: 12 months after intervention
†Either ASM initiation, epilepsy surgery, VNS implantation or KD initiation
‡Epilepsy surgery participants only
ASM, anti-seizure medications; BMI, body mass index; BSFS, Bristol Stool Form Scale; EEG, electroencephalography; GISI, Gastrointestinal Symptom Inventory; KD, ketogenic diet; PedsQL, Paediatric Quality of Life Inventory; QoL, Quality of Life; QOLIE 31, Quality of Life in Epilepsy Inventory; VNS, vagus nerve stimulation.

50 participants each in the drug-naïve and surgery groups. Smaller exploratory cohorts (n=10 each) undergoing VNS or KD will be included for descriptive and hypothesis-generating analyses. The results of this study will inform effect size estimates for future confirmatory trials with formal power calculations. Figure 1 illustrates the flow of participants from enrolment to allocation into study groups, including patients at epilepsy onset and those with DRE, who are further subdivided according to treatment (resective surgery, VNS or KD).

Data management

All study data will be collected and managed using REDCap (Research Electronic Data Capture, Vanderbilt University Medical Center (VUMC), Vanderbilt Institute for clinical and translational Research (VICTR), Nashville, TN, USA), a secure, web-based platform hosted by the coordinating centre.[37] REDCap provides a validated and auditable infrastructure for electronic data entry, storage and monitoring. Each participating centre will be granted access credentials for authorised study personnel, ensuring traceability and data integrity through time-stamped logs and role-based permissions. Data entered into electronic case report forms have been reviewed in the Interventions and data collection section. Built-in data validation rules and range checks will minimise entry errors. Biological samples (stool and blood) will be pseudonymised using unique alphanumeric codes before laboratory processing. Sample tracking, processing status and analytical outputs (eg, sequencing files, methylation arrays and metaproteomic results) will be recorded in linked metadata spreadsheets stored on encrypted institutional servers. Sample aliquots will be stored at either −20°C or −80°C in designated biobanks with controlled access. All personal data will be managed in full compliance with European Union General Data Protection Regulation (GDPR; Regulation EU 2016/679). Data will be anonymised before analysis, and identifiable information will be accessible only to authorised personnel. After publication of primary results, de-identified datasets will be made available on reasonable request, in line

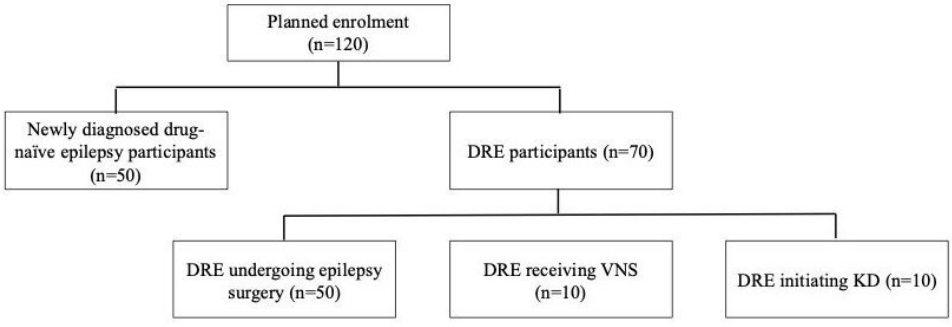

Figure 1 Schematic representation of the planned recruitment and cohort allocation in the CARE study protocol. Eligible participants will be enrolled and stratified into drug-naïve epilepsy and DRE cohorts. Participants with DRE will be further allocated to surgery, VNS or KD according to standard clinical indications and predefined study criteria. CARE, MiCrobiota-gut-brain Axis in Resistant Epilepsy; DRE, drug-resistant epilepsy; KD, ketogenic diet; VNS, vagus nerve stimulation.

with FAIR (Findable, Accessible, Interoperable, Reusable) data principles and with approval by the data access committee.[38]

Study endpoints

The main objective of the current proposal is to investigate whether baseline gut microbiota characteristics and associated host DNA methylation profiles can predict seizure control in PWE undergoing different therapeutic regimens. The primary clinical outcomes are the changes in seizure frequency and EEG features from baseline, assessed at defined timepoints after intervention. Seizure frequency will be measured using participant-reported seizure diaries at all time points. EEG features will be evaluated independently at 6 and 12 months from intervention by two trained neurologists through blinded qualitative visual assessment, focusing on background activity, the frequency and duration of interictal epileptiform discharges, as well as the frequency and duration of EEG-registered seizures. Inter-rater reliability will be quantified using Cohen's kappa for categorical variables and intraclass correlation coefficients (ICCs) for continuous measures. In cases of minor disagreement, the mean of the two ratings will be used, whereas substantial discrepancies (eg,κ<0.6 or ICC <0.75) will be resolved through joint re-evaluation. These outcomes will be assessed within each treatment cohort. Secondary outcomes include improvement in health-related QoL—defined as an increase of at least 10 points in PedsQL (for children) or QOLIE-31 (for adults)—and a reduction of at least 2 points in gastrointestinal symptoms measured by the GISI (maximum score=17), evaluated at the same time points.

Statistical analysis

Descriptive statistics will summarise baseline demographic and clinical characteristics. The effect of baseline characteristics on longitudinal changes in seizure burden or electrographic outcomes will be analysed using generalised mixed-effects models, with the intervention group included as a factor, and individual patients included as random effects to account for repeated measures. Statistical analyses will be adjusted for relevant confounders such as age, sex, BMI, baseline diet or ASM use. Statistical analyses will be performed using R (R Foundation for Statistical Computing, Vienna, Austria; https://www.r-project.org) or custom code written in Python (https://www.python.org).

Gut microbial taxa significantly associated with specific groups will be identified through ANCOM (National Institute of Environmental Health Sciences/National Institute of Health statistical (NIEHS/NIH) method for microbiome differential abundance analysis, primary developer: Lin Huang and Shyamal Das Peddada, Research Triangle Park, North Carolina, USA. Bioconductor: administrative/fiscal headquarters company name NumFOCUS, Inc, Austin, Texas, USA. Technical/Scientific headquarters organization name: fred Hutchinson cancer center, Seattle, USA) analysis (R/Bioconductor), and relative log2 fold changes will be calculated to evaluate shifts in microbial profiles over time. The Principal Component Partial R-Squared method will be applied to calculate the proportion of variability in the whole omic datasets explained by the variable 'therapies' or 'before' versus 'ongoing' therapy. The set of peptides most discriminating between groups will be identified through discriminant analyses (DA) on log-transformed peptide intensities using sparse Partial Least Squares regression (sPLS) implemented in the Bioconductor package mixOmics.

Blood DNA methylation data will be pre-processed with standard routines for quality control (low quality samples will be removed), batch correction, removal of sex chromosomes and normalisation.

For the main statistical analysis, the association between the methylation level at each CpG and the metadata of the patients (including response to therapy) will be quantified by fitting logistic regression models, including the pseudocontinuous M-value of methylation at each CpG as predictor the predictor .

Enrichment analysis

Differential and discriminating features (alternative splicing variants, peptides and proteins) will be tested for over-representation of specific taxa, functions and pathways. Significant enrichments will be obtained via the Fisher test comparing observed versus expected proportions of features in a specific category. A permutation procedure will be applied to control for multiple testing and the hierarchical nature of annotations.

Correlation analysis

Correlation analysis will be performed to evaluate the association between the different features measured in the study (taxa, functions, protein families etc) and between these features and the clinical variables of patients. Statistical analyses will be performed using R, applying correction for multiple testing when relevant and considering an FDR <0.05 as significant.

High-dimensional data will be analysed using machine learning approaches including sPLS-DA (mixOmics package, R Foundation for Statistical Computing; https://www.r-project.org) to identify molecular features (microbial taxa, methylation markers and DNA methylation signatures) associated with clinical outcomes.

ETHICS AND DISSEMINATION

The study protocol has been approved by the Ethics Committee of Comitato Etico Territoriale Lombardia 3 (protocol number 44896_17.07.2024_N_bis). Data will be handled according to the GDPR (Regulation EU 2016/679) and national guidelines. Dissemination will address both the scientific community and the wider public, through academic outputs and lay channels developed in collaboration with patient associations.

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Competing interests None declared.

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