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RESEARCH

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Uncovering longitudinal changes in the brain functional connectome along the migraine cycle: a multilevel clinical connectome fingerprinting framework

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Abstract

Background Changes in large-scale brain networks have been reported in migraine patients, but it remains unclear how these manifest in the various phases of the migraine cycle. Case-control fMRI studies spanning the entire migraine cycle are lacking, precluding a complete assessment of brain functional connectivity in migraine. Such studies are essential for understanding the inherent changes in the brain of migraine patients as well as transient changes along the cycle. Here, we leverage the concept of functional connectome (FC) fingerprinting, whereby individual subjects may be identified based on their FC, to investigate changes in FC and its stability across different phases of the migraine cycle.

Methods We employ a case-control longitudinal design to study a group of 10 patients with episodic menstrual or menstrual-related migraine without aura, in the 4 phases of their spontaneous migraine cycle (preictal, ictal, postictal, interictal), and a group of 14 healthy controls in corresponding phases of the menstrual cycle, using resting-state functional magnetic resonance imaging (fMRI). We propose a novel multilevel clinical connectome fingerprinting approach to analyse the FC identifiability not only within-subject, but also within-session and within-group.

Results This approach allowed us to obtain individual FC fingerprints by reconstructing the data using the first 19 principal components to maximize identifiability at all levels. We found decreased FC identifiability for patients in the preictal phase relative to controls, which increased with the progression of the attack and became comparable to controls in the interictal phase. Using Network-Based Statistic analysis, we found increased FC strength across several brain networks for patients in the ictal and postictal phases relative to controls.

Conclusion Our novel multilevel clinical connectome fingerprinting approach captured FC variations along the migraine cycle in a case-control longitudinal study, bringing new insights into the cyclic nature of the disorder.

Keywords Clinical connectome fingerprinting, fMRI, Functional connectome, Longitudinal, Migraine, Menstrual cycle, Resting state

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Introduction

Migraine is a cyclic condition characterized by recurrent headache attacks, accompanied by sensory and cognitive disruptions, alternated with headache-free periods. It is defined by four distinct phases: preictal (before the headache begins), ictal (the headache phase lasting 4–72 hours), postictal (following headache cessation) and interictal (in-between the attacks) (Peng & May, 2020). Migraine is considered the second cause of disability worldwide and the first cause for women between 15 and 49 years old (Steiner et al., 2020), with its prevalence being two to three times higher in women than in men. Furthermore, menstrual migraine and menstrual-related migraine are among the most common subtypes for female patients (20–25%) (Vetvik & MacGregor, 2021), with menstrual-related attacks being considered the most debilitating (Pavlović et al., 2015).

Over the past decades, magnetic resonance imaging (MRI) has provided insights beyond clinical assessment, revealing structural and functional brain alterations in migraine (Colombo et al., 2019; Messina et al., 2022, 2023). Changes in functional connectivity across large-scale brain networks, collectively referred to as functional connectome (FC), have been observed in migraine patients using resting-state functional magnetic resonance imaging (fMRI); however, findings are highly variable across studies (Chou et al., 2023; Schramm et al., 2023; Skorobogatykh et al., 2019). Notably, most reports pertain to the interictal phase and studies examining the migraine cycle are limited, with a remarkable scarcity of investigations during the peri-ictal phases (preictal, ictal and postictal). In fact, studying the phases surrounding the attack is more challenging due to practical hurdles, such as the unpredictability of the attack and patient discomfort (Tolner et al., 2019). Consequently, longitudinal studies of the migraine cycle are scarce although they are essential to investigate the brain changes associated with the different migraine phases and in this way gain a better understanding of the mechanisms specifically linked to the initiation and termination of migraine attacks. In a comprehensive review spanning fMRI studies from 2014 to 2021, only a small fraction (9 out of 114) included data partially collected during the ictal phase, and often the cycle phases were poorly defined (Schramm et al., 2023). Moreover, only 14 FC studies sampled more than one phase, and of these only 9 were longitudinal (Amin et al., 2018; Araújo et al., 2023; Filippi et al., 2022; Marciszewski et al., 2018; Meylakh et al., 2018; Schulte, Menz, et al., 2020; Schulte & May, 2016; Stankewitz et al., 2021; Stankewitz & Schulz, 2022), with some of them relying on the same sample and including 2 case studies. Furthermore, only 3 of the longitudinal studies sampled all migraine cycle phases; none of these 3 studies included a group of healthy controls. Results varied significantly across studies, including reports of both increases and decreases as well as no changes in FC across different networks. Importantly, most studies included a majority of (or even only) female participants. However, only a few of these studies indicate how the menstrual cycle was controlled for, although it has been shown to influence FC (Dubol et al., 2021).

In an impactful work, FC has demonstrated the ability to represent unique profiles of individual subjects, exhibiting stability over time, irrespective of the task a subject is engaged in, resembling a functional fingerprint (Finn et al., 2015). Dimensionality reduction approaches can further improve subject identifiability, resulting in greater brain-behaviour relationships (Amico & Goñi, 2018; Svaldi et al., 2021), and opening the door to examine the link to cognitive and clinical variables in the context of diseases. Building on the framework proposed in Amico & Goñi (2018), Sorrentino et al. (2021) introduced the concept of clinical connectome fingerprinting, which has been applied to magnetoencephalography (MEG) data in different pathologies (Cipriano et al., 2023; Romano et al., 2022; Sorrentino et al., 2021; Troisi Lopez et al., 2023). A slightly different approach was applied to the study of psychosis patients using fMRI (Tepper et al., 2023). In general, these studies found reduced identifiability in patients compared with healthy controls, and that differences relative to controls can predict individual clinical features. To our knowledge, no study has yet employed a connectome fingerprinting approach to study patients longitudinally over more than 2 sessions, while differentiating them from controls.

In summary, the literature clearly points to changes in connectivity across several networks, suggesting a widespread impact of migraine on the whole-brain FC. However, the literature presents several inconsistencies with no systematic direction in the differences found. This may be likely due to the fact that most reports studied heterogeneous samples including both chronic and episodic migraine, as well as patients with and without aura, and did not specifically control for the migraine phase or the menstrual cycle. In this study, we hypothesize that a whole-brain FC fingerprinting approach along the four phases of the migraine cycle, applied to a homogeneous cohort of low-frequency episodic migraine female patients without aura compared with menstrual controls, may help clarify this literature. Here, we leverage the identifiability of individual FC to investigate changes along the migraine cycle, controlling for the menstrual cycle. For this purpose, we employ a case-control longitudinal design to study a group of patients with episodic menstrual or menstrual-related migraine without aura, in the four phases of their spontaneous migraine

cycle (preictal, ictal, postictal, interictal), and a group of healthy controls in corresponding phases of the menstrual cycle. We develop a novel multilevel clinical connectome fingerprinting approach to investigate whether the migraine cycle influences individual FC fingerprints, relative to menstrual controls. We hypothesize that: 1) the identifiability of patients differs from healthy controls and is dependent on the migraine cycle phase; 2) the peri-ictal FC fingerprints of patients differ from interictal ones and from the matching ones for healthy controls; and 3) differences in identifiability metrics and the FC fingerprints between the groups are associated with migraine clinical features.

Materials and Methods

This study is part of a larger research project on brain imaging in migraine (MigN2Treat). It employed a prospective, longitudinal, within-subject design, and consisted of a comprehensive neuroimaging protocol including arterial spin labelling MRI, diffusion MRI and task-fMRI as well as resting-state fMRI. In this report we focus specifically on the resting-state fMRI data. The methods and results corresponding to the other MRI protocols are described elsewhere (Domingos et al., 2023; Fouto, Henriques, et al., 2024; Fouto, Nunes, et al., 2024; Matoso et al., 2024; Ruiz-Tagle et al., 2024). The research protocol and statistical analysis were not preregistered. The study was approved by the Hospital da Luz Ethics Committee and all participants provided written informed consent according to the Declaration of Helsinki 7th revision.

Participants

The flowchart of the recruitment and participant selection/exclusion is presented in Fig. 1.

The recruitment of patients with migraine was performed by a neurologist during routine medical consultations at the headache outpatient clinics of Hospital da Luz. We did not perform a statistical power analysis before initiating the study. Nevertheless, we targeted a sample size of 15 migraine patients, consistent with prior MRI cohort studies that identified group differences during spontaneous migraine attacks with sample sizes of $N=13$ (Coppola et al., 2016, 2018) or $N=17$ (Amin et al., 2018). Healthy control participants, comprising adult females, were recruited through social media advertisements targeting the general population and university campus, ensuring they matched the clinical sample in

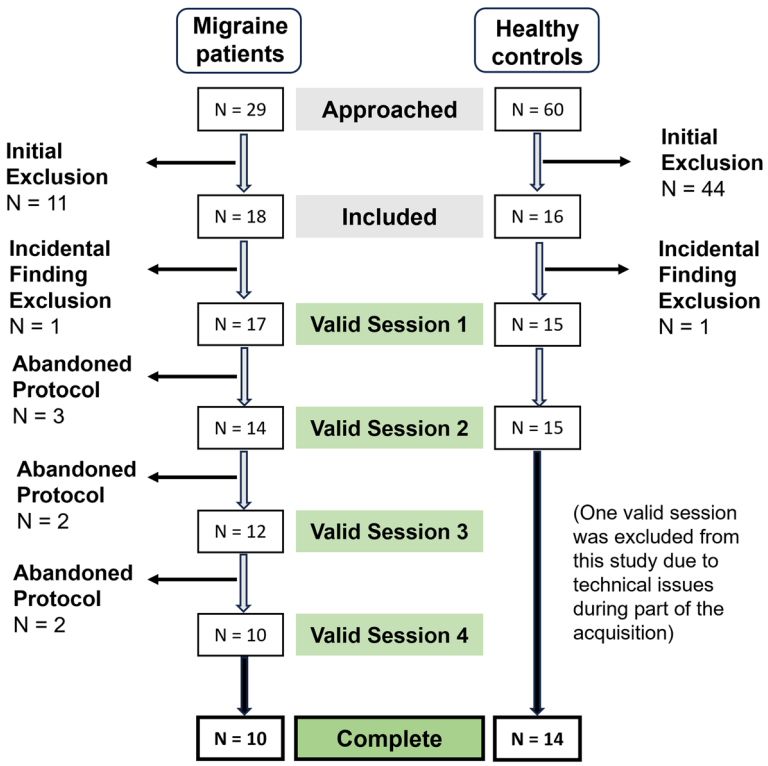

Fig. 1 Flow chart of the study showing the number of participants approached, included, and completing valid sessions. Invalid sessions were those scheduled as preictal but not followed by an attack within 72 hours, as well as sessions from participants excluded after an incidental finding was detected during the MRI exam

gender, age, contraceptive use, and menstrual phase at the time of scanning. The inclusion criteria for the participants were the following: having an age between 18 and 55 years; having at least 9 years of education; Portuguese being their first language; being healthy (excluding migraine, for the patients), without any diagnosed condition significantly hindering active and productive life or causing life expectancy to be below 5 years; suffering from low-frequency episodic migraine without aura, diagnosed according to the criteria of the 3rd edition of the International Classification of Headache Disorders (ICHD-3) (International Headache Society, 2018) with menstrual-related migraine attacks (for patients only). The exclusion criteria were: history or presence of a neurologic condition (excluding migraine, for the patients); history or presence of a psychiatric disorder and/or severe anxiety and/or depressive symptoms as indicated by questionnaires; history or presence of vascular disease; receiving treatment with psychoactive drugs, including anxiolytics, antidepressants, anti-epileptics, and any migraine prophylactics; being pregnant or trying to get pregnant, breastfeeding, being post-menopausal, or using contraception precluding cyclic menses; contraindications for MRI. An initial pool of 29 migraine patients and 60 controls were approached out of which 18 and 16 were included in the study, respectively.

We aimed to study the migraine cycle (between peri-ictal and interictal phases) longitudinally, in a specific stage of evolution of the disease in each patient. Data was acquired from 18 female patients that we planned to assess in 4 sessions: preictal (M-pre), ictal (M-ict) and postictal (M-post) phases (around menses), and interictal phase (M-inter) (post-ovulation). The preictal session was acquired within a window of 72 hours preceding the onset of a spontaneous migraine attack (Giffin et al., 2003; Stankewitz A et al., 2011). For each patient, we scheduled the preictal session based on the usual migraine attack onset in relation to menstruation and their prediction of menses start. A preictal session was considered valid upon confirmation with the patient after 72 hours that a spontaneous migraine attack had occurred during the period following our assessment. Considering this, 3 sessions were deemed invalid and discarded because during the 72 hours follow-up to confirm the occurrence of a spontaneous migraine attack it was found that the attack had not taken place. The postictal session was acquired within 48 hours after pain relief from the spontaneous migraine attack (International Headache Society, 2018). Given that some patients struggle to recognize their symptoms (Gago-Veiga et al., 2018; Schulte et al., 2015) and functional changes may occur even before symptoms become apparent, we relied solely on the temporal criteria, which were consistently applied across all patients. For the interictal session, participants had to be free from pain for at least 48 hours before, with confirmation of the absence of a migraine attack obtained 72 hours post-scan. Of the 18 patients, one was excluded due to an incidental finding. Only 10 migraine patients (M group) completed the 4 sessions and were included in the analysis. The demographic and clinical data are presented in Table 1. The attack features concern a typical migraine attack, not being specific to the examined ictal session.

Furthermore, we collected data from 16 healthy controls matched for gender, age, contraceptive use, and menstrual phase at the time of scanning. One subject was excluded due to an incidental finding and another one due to technical issues during the recording. Therefore, the final sample included 14 healthy controls (HC group). They were assessed in two phases of the menstrual cycle to match the peri-ictal and interictal phases of the patients, yielding respectively the perimenstrual phase (HC-pm) (approximately 5 days before or after the menses) and the post-ovulation phase (HC-postov) (approximately on day 19 of the menstrual cycle). The demographic data for this group is also presented in Table 1.

The experimental protocol is schematically represented in Fig. 2. Due to logistic constraints, for each participant the sessions were, in general, performed during different migraine/menstrual cycles. The data acquisition from both patients and controls took place between June 2019 and December 2022. All scanning sessions were performed in the evening (7:00–9:00 p.m.).

MRI data acquisition

The MRI data was acquired using a 3T Siemens Vida system, using a 64-channel radio frequency (RF) coil. Functional images were obtained using a T2*-weighted gradient-echo Echo Planar Imaging (EPI) with the

Table 1 Demographic characteristics and migraine clinical features. The median values are presented along with the interquartile range (IQR)

	M group (N = 10)	HC group (N = 14)
Age (years)	38.0 (15)	30.5 (13)
Sex (F)	100%	100%
Years of Education	16.5 (1)	17 (1)
Migraine Onset (years)	18.0 (11)	
Disease Duration (years)	12.0 (17)	
Attack Frequency (/month)	1.8 (2.9)	
Attack Duration (hours)	42.0 (32)	
Attack Pain Intensity (1–10)	7 (1.9)	

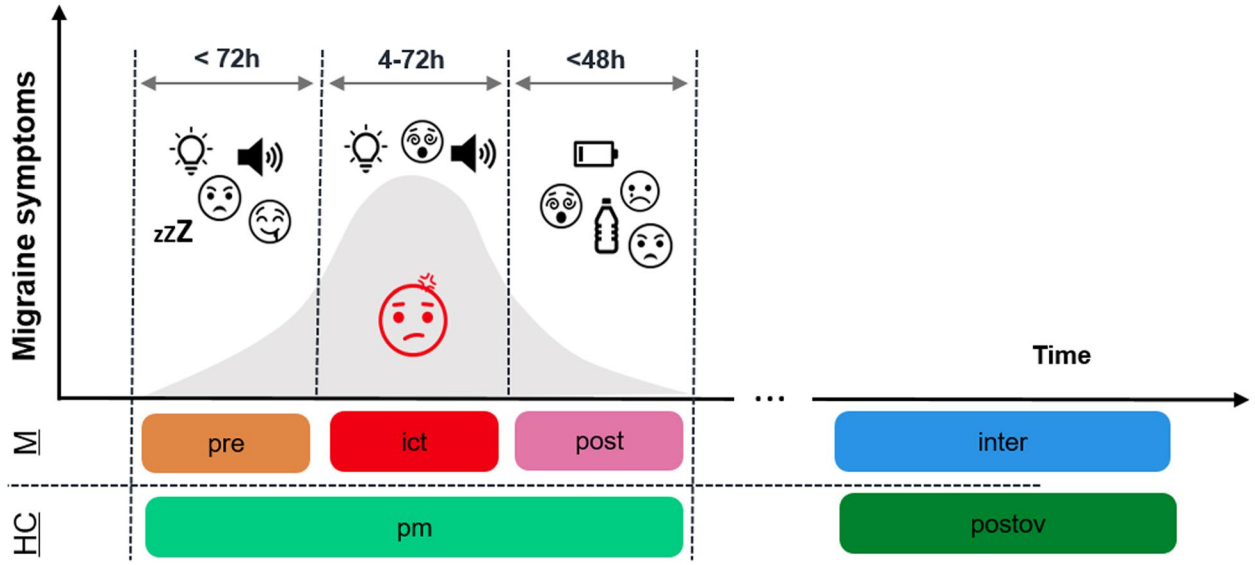

Fig. 2 Definition of preictal (M-pre), ictal (M-ict), postictal (M-post) and interictal (M-inter) phases for the migraine patients (M) and the corresponding phases for the healthy controls (HC), matched for the menstrual cycle, perimenstrual (HC-pm) and post-ovulation (HC-postov). The symptoms illustrated across the migraine cycle are only present for the M group and include sleepiness, food cravings, increased sensitivity to light/sound, mood changes, cognitive dysfunction, fatigue, and thirst

following parameters: repetition time (TR)/echo time (TE) = 1260/30ms, flip angle = 70°, 60 slices, 2.2 mm isotropic voxel resolution. We used techniques to speed up the data acquisition, namely, in-plane generalized autocalibrating partially parallel acquisition (GRAPPA) acceleration factor = 2 and simultaneous multi-slice (SMS) factor = 3. High resolution structural images were acquired with a T1-weighted magnetization-prepared rapid gradient echo (MPRAGE) sequence, with TR = 2300 ms, TE = 2.98 ms, inversion time (TI) = 900 ms, and 1 mm isotropic voxel resolution. To improve image quality, we also captured fieldmap magnitude and phase images using a double-echo gradient-echo sequence (TR = 400.0 ms, TE = 4.92/7.38 ms, voxel size: $3.4 \times 3.4 \times 3.0$ mm^3, flip angle = 60°), which helps correct for distortions caused by magnetic field variations. Participants were instructed to keep their eyes open, looking at a black screen, and to not think about anything in particular for 7 min (333 fMRI volumes). To mitigate acoustic noise exposure and minimize head motion, earplugs were used and small cushions were placed beneath and on the sides of the head of the subject.

MRI data preprocessing

The MRI data was preprocessed using FMRIB Software Library (FSL)'s tools (Jenkinson et al., 2012).

Structural images were preprocessed to remove non-brain tissue using Brain Extraction Tool (BET) and corrected for bias field inhomogeneities using FMRIB's Automated Segmentation Tool (FAST). Each structural image was then aligned to the subject's functional space (reference volume) as well as the standard Montreal Neurosciences Institute (MNI) space using FMRIB's Linear Image Registration Tool (FLIRT) and FMRIB's Non-Linear Image Registration Tool (FNIRT). White matter (WM) and cerebrospinal fluid (CSF) masks were derived by tissue segmentation using FAST (a threshold equal to 1 was applied to the obtained tissue probability maps), and subsequently registered to the functional space. The CSF mask was further refined by intersecting it with the lateral ventricles (originally in the MNI space and transformed to the subject's functional space) was performed.

fMRI preprocessing consisted of: distortion correction based on the acquired fieldmap; volume realignment; high-pass temporal filtering with a cut-off frequency of 0.01 Hz to remove slow drift fluctuations; regression of motion parameters, WM, CSF and motion outliers in a single step; spatial smoothing was employing a Gaussian kernel with a full-width half maximum of 3.3 mm. The code used for fMRI preprocessing is available here: https://github.com/martaxavier/fMRI-Preprocessing.

MRI data analysis

The framework used for data analysis is inspired by the work of Amico & Goñi (2018), in which principal component analysis (PCA) is applied in the connectivity domain and the individual FC are then reconstructed using the number of principal components (PCs) that maximizes

identifiability, i.e. how similar a subject is to himself/herself and how different he/she is from other subjects. We extend this to a novel multilevel clinical connectome fingerprinting framework in which identifiability is maximized over three levels: within-subject, within-session and within-group. Therefore, in this study, the concept of identifiability is expanded to encompass the ability to distinguish unique FC patterns at various levels, including individual subjects, specific sessions, or distinct groups. This approach offers a novel perspective on brain connectivity by focusing on level-specific patterns of functional networks and their distinguishability, rather than solely examining shared or common patterns as usually done in FC analysis. The resulting FC fingerprints are then used for two separate analyses: computing the correlation among them within-subject, within-session and between-group, and performing Network-Based Statistic (NBS) analysis for within/between-group comparisons. By adding network-based FC analysis, we can uncover alterations in specific parts of the connectome and more directly relate our findings with previous FC studies, offering a complementary perspective to the FC fingerprinting analysis. Finally, the association between the results of both analyses and clinical features for the M group were investigated. The schematic pipeline representing the workflow employed to perform these steps is depicted in Fig. 3. The code was implemented in MATLAB software version R2016b and is available in https://github.com/isesteves/multilevel-fingerprinting_migraine.

Functional connectomes (FC)

fMRI data was parcellated by computing region average time courses using Schaefer atlas (Schaefer et al., 2018) with 100 parcels, directly corresponding to the 7 intrinsic networks defined by Yeo et al. (2011): fronto-parietal network (FPN), default mode network (DMN), dorsal attention network (DAN), limbic network (LN), ventral attention network (VAN), somatomotor network (SMN) and visual network (VN). Furthermore, given the importance of subcortical regions for migraine, as well as the cerebellum, two additional networks were retrieved from the Automated Anatomical Labelling 116 (AAL116) atlas (Tzourio-Mazoyer et al., 2002): one comprising the hippocampus, amygdala, caudate, putamen, pallidum and thalamus (SUB) and another one comprising 9 bilateral cerebellar regions and the vermis (CRB). Care was taken to ensure that regions overlapping less than 50% with the fMRI data coverage were excluded from further analysis. This procedure led to the bilateral exclusion of 4 cerebellar regions: Crus II, Cerebellum7b, Cerebellum8 and Cerebellum9, for all subjects. Overall, the 9 networks encompassed a total of 130 regions (Fig. 3.A). The time series of each region were demeaned and bandpass-filtered from 0.01 to 0.1 Hz using a 2nd order Butterworth filter. Symmetric FC matrices (130 regions×130 regions, 16900 edges) were generated by computing the Pearson correlation coefficient for each pair of regions.

Principal Component Analysis (PCA)

For each subject and session, the lower triangular part of the FC matrix (excluding the diagonal) was vectorized (8385 edges x 1). The individual FC vectors (10 M subjects × 4 M sessions = 40 samples; 14 HC subjects × 2 HC sessions = 28 samples; total = 68 samples) were concatenated across all subjects and sessions into a single FC matrix (8385 edges×68 samples) (Fig. 3.B). Subsequently, PCA was applied to obtain a number of PCs equal to the number of samples (i.e., 68 PCs), which were ordered by decreasing explained variance. We expect that higher variance PCs will contain global FC information (session- and group-level), intermediate variance PCs will contain subject-level information, and the lowest variance PCs will capture confounding effects, noise and artifacts. Consequently, the FC matrix was reconstructed by using an increasingly larger number of PCs (starting with the largest explained variance), from 2 to 68 for our multilevel clinical connectome fingerprinting approach.

Multilevel clinical connectome fingerprinting framework

Multilevel identifiability matrix Previous fingerprinting studies assessed subjects in at most two sessions (test and retest), either by evaluating them on two different days or by splitting single-day data into two halves. In contrast, the present study assesses the M group in 4 time points and the HC group in 2 time points. To account for the larger number of sessions, we built a modified identifiability matrix by computing the Pearson correlation between all possible pairs of subjects/sessions (see Fig. 3.C). This multilevel identifiability matrix is organized by subject: the first 40 rows/columns correspond to M, with each group of 4 being M-pre, M-ict, M-post and M-inter for each of the 10 M; the last 28 rows/columns correspond to HC, with each group of 2 being HC-pm and HC-postov for each of the 14 HC (68 samples x 68 samples). Hence, unlike the originally proposed identifiability matrix, this modified version is symmetric. For ease of interpretation and to differentiate it from the original, we display our multilevel identifiability matrix by using only the corresponding lower triangular matrix.

Multilevel differential identifiability The FC fingerprinting framework is based on the premise (already substantiated by evidence) that a subject's FC profile is more similar to their own FC profile assessed on a different

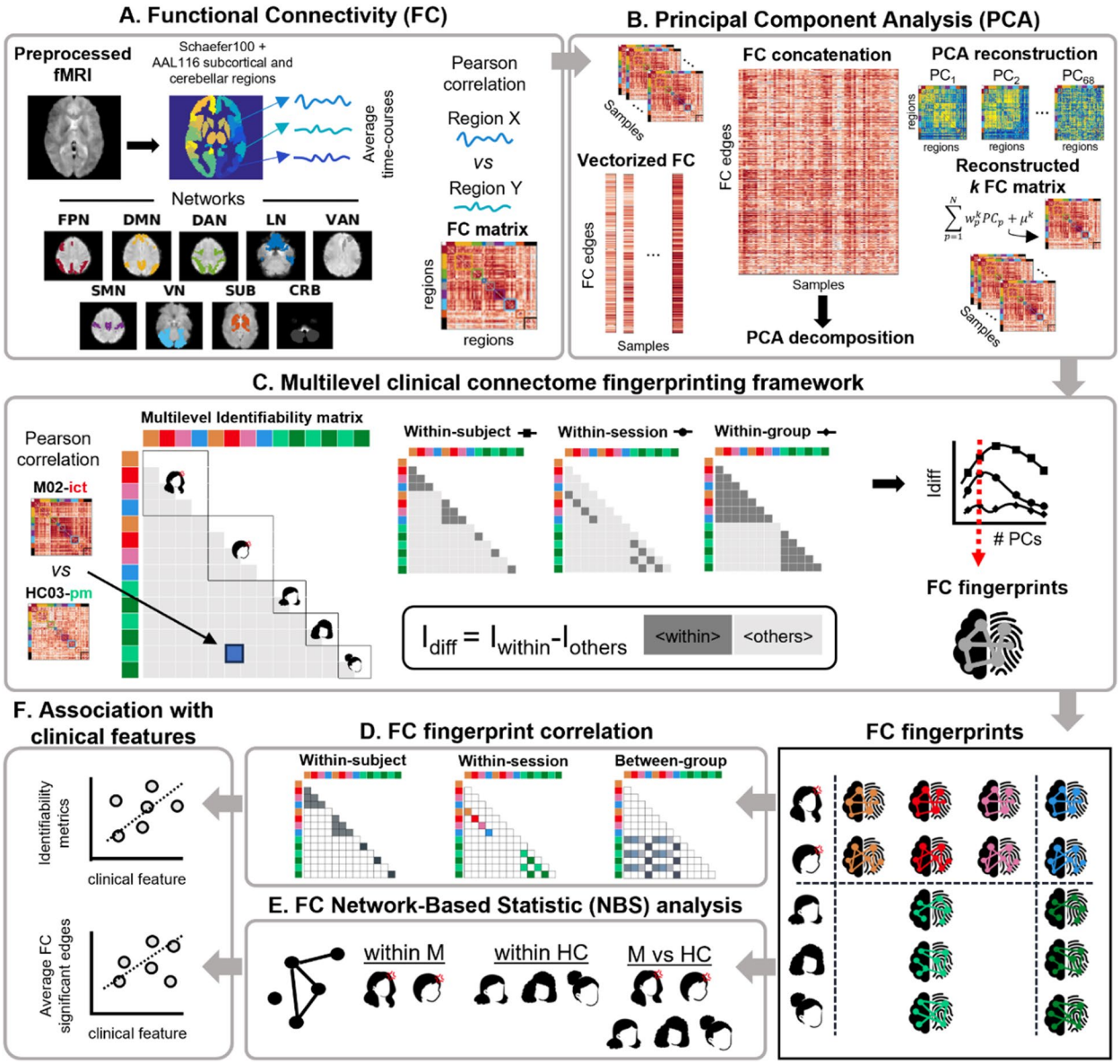

Fig. 3 Pipeline scheme: **A**. Parcellation into cortical regions of intrinsic networks, based on the Schaefer atlas (100 regions, representing 7 networks) and subcortical and cerebellar regions from the AAL116 atlas: fronto-parietal (FPN), default mode (DMN), dorsal attention (DAN), limbic (LN), ventral attention (VAN), somatomotor (SMN), visual (VN), subcortical (SUB) and cerebellar (CRB). The parcellated data is used for FC computation, by calculating the Pearson correlation between the average time-courses of each pair of regions; **B**. The lower triangular matrix (excluding the diagonal) of each FC matrix was vectorized and they were all concatenated for PCA decomposition and reconstruction varying the number of PCs N, for each sample k, using the corresponding weights w and mean μ; **C**. Multilevel clinical connectome fingerprinting framework with PCs selection using a multilevel identifiability matrix corresponding to the Pearson correlation between vectorized FC matrices for all possible combinations of subjects/sessions (samples). In this matrix, for each level, I_{within} corresponds to the average of the elements in dark gray, I_{others} corresponds to the average of the elements in light gray and I_{diff} (%) corresponds to $100\times(I_{within}-I_{others})$. The FC fingerprints are obtained by reconstructing the data using only the selected number of PCs; **D**. FC fingerprint correlation was computed within-subject, within-session and between-group by averaging the correlation values per subject for each of these cases; **E**. Computation of Network-Based Statistic (NBS) for within- and between-group FC analysis. **F**. Association of identifiability metrics and average FC significant edges (found with NBS) with clinical features, using Spearman correlation.

day or task than to those of other subjects (Finn et al., 2015, 2017). The original framework introduced self-identifiability (I_{self}) as the Pearson correlation between two sessions of the same subject (Amico & Goñi, 2018). Besides within-subject similarity, we extend this differential identifiability concept to consider within-session

and within-group similarity. To accommodate the different levels of similarity, we propose the use of within-identifiability (I_{within}), a broader concept which corresponds to the average Pearson correlation between FC matrices belonging to the same subject, the same session, or the same group. Correspondingly, for each of the levels, the elements that are not included in I_{within} are considered I_{others}, *i.e.*, average Pearson correlation between different subjects, different sessions, or different groups. The differential identifiability for each level is defined as the difference between these two terms: $I_{diff}(\%) = 100 \times (I_{within} - I_{others})$ (Fig. 3.C). The higher the I_{diff}, the more pronounced the fingerprint along that specific level. In this case, instead of maximizing only the within-subject I_{diff}, we aim for a balance that simultaneously achieves higher values for within-subject, within-session and within-group I_{diff}. In other words, we manually selected the number of PCs used for the PCA reconstruction that provided the best trade-off across all I_{diff} levels. Having selected the number of PCs, we consider that the final reconstructed individual FC matrices correspond to the individual FC fingerprints.

FC fingerprint correlation

Upon obtaining the individual FC fingerprints, our goal was to interpret the results in the context of the longitudinal assessment of the M group relative to HC. We extracted Pearson correlation values from the multilevel identifiability matrix computed on individual FC fingerprints, in order to average them for different comparisons and examine their distribution (see Fig. 3.D). These individual FC fingerprints correspond to the matrices of Pearson correlation coefficients between all pairs of brain regions (i.e., involving all connections within and between all networks), after the reconstruction with 19 PCs only. First, within-subject, by separately analysing M subjects (averaging 6 values for each subject, corresponding to combinations of 4 sessions, two by two) and HC subjects. Second, within-session, considering all the points that correspond to either M-pre, M-ict, M-post or M-inter for the M group, and HC-pm and HC-postov for the HC group. For each subject and session, we extracted the correlation values with the rest of the subjects in the same session and averaged them. Third, between-group, considering all the correlation values between each M session and the corresponding HC session: M-pre vs HC-pm, M-ict vs HC-pm, M-post vs HC-pm and M-inter vs HC-postov. For each subject in the M group and session, we extracted and averaged the correlation values with all the other subjects in the corresponding session of the HC group.

Due to the limited sample size, non-parametric statistical tests were used. Specifically, for comparisons within the M or HC group, we applied the signed rank Wilcoxon test. For comparisons between the M and HC groups, conducted specifically for matching sessions (M-pre, M-ict, M-post compared to HC-pm; M-inter compared to HC-postov), we employed the Wilcoxon rank sum test. The significance level was set to $\alpha = 0.05$ and multiple comparisons were corrected for the false-discovery rate (FDR) with the Benjamini-Hochberg method (Benjamini & Hochberg, 1995) using the Multiple Testing toolbox (Martínez-Cagigal, 2021). For the analysis of FC fingerprints average correlation within-session we corrected for the performance of 11 tests, and for the between-group analysis we corrected for 6 tests.

FC Network-Based Statistic (NBS) analysis

Using the previously obtained individual FC fingerprints for each subject and session, we employed NBS to test for differences in the FC strength (Fig. 3.E). This is a cluster-level tool for human connectome statistical analysis that models FC as a graph and controls for family-wise error rate (FWER) when performing mass univariate testing, which is available as a MATLAB toolbox (Zalesky et al., 2010). The NBS analysis detects network components that consist of connections surpassing a certain threshold. Subsequently, permutation testing is carried out to establish a p-value adjusted for FWER for each network, considering its size. The advantage of performing NBS lies in its ability to identify statistically significant patterns of connectivity across entire networks, rather than isolated connections, providing a more robust statistical approach than performing multiple univariate analysis. The initial threshold for the test statistic was established at a t-value of 4.0, and 5,000 permutations were executed to identify a network component with a p-value < 0.05 after correction for FWER.

Association with clinical features

We investigated the association of FC identifiability and FC strength with relevant clinical features (Migraine Onset, Disease Duration, Attack Frequency, Attack Duration and Attack Pain Intensity), using Spearman correlation (Fig. 3.F), with a significance level of $\alpha = 0.05$ and multiple comparisons FDR correction with the Benjamini-Hochberg method (Benjamini & Hochberg, 1995; Martínez-Cagigal, 2021).

Regarding identifiability, we evaluated the association with: 1) within-subject average correlation, 2) between-group average correlation for each M session of each patient; 3) between-group average correlation computed across all sessions for each patient. For FC strength, we tested the association with the average FC across all

edges that showed significant differences when comparing M and HC, for each patient. In the association with clinical variables, for each case we corrected for the number of clinical variables (5 tests). Additionally, for point 2) we also corrected for the number of sessions (4 tests).

Results

Functional connectomes

The FC matrices obtained for all subjects and sessions are presented in Fig. 4. The distributions display a shift towards positive correlation values and some distinct patterns are discernible, both within networks (e.g., strong FC within the SMN for patients 005, 008, 034 and 041 during the M-pre session) and between networks (e.g., strong FC among DAN, VAN, SMN and VN for patients 008, 009, 034 and 041 during the M-pre session). Furthermore, several patients showed stronger FC for the interictal phase compared to the peri-ictal ones, whereas controls also displayed stronger FC for post-ovulation compared to the perimenstrual phase. However, a clear pattern is not evident across subjects within the same session.

Multilevel clinical connectome fingerprinting framework

The I_{diff} values computed for each level (within-subject, -session and -group) as a function of the number of PCs are presented in Fig. 5.A, showing that the number of PCs that maximizes I_{diff} is not the same for all levels (within-subject: 24 PCs; within-session: 6 PCs; within-group: 36 PCs), as expected. Moreover, across all the numbers of PCs, I_{diff} values were larger within-subject than within-session and within-group. For our multilevel-informed selection of the number of PCs, we used the within-subject level as a starting point following previous studies (Amico & Goñi, 2018; Svaldi et al., 2021) and focused on its plateau region. In this region, we opted for a local maximum of the within-session and within-group levels, selecting a cutting-off point of 19 PCs. With this reconstruction, 80% of the variance of the original data was preserved. In Fig. 5.B, we show the multilevel identifiability matrix after reconstruction with 68 PCs (100% of variance explained) and the selected 19 PCs (80% of variance explained), where especially the within-subject pattern is evidenced.

FC fingerprint correlation

The results of the FC fingerprint correlations are presented in Fig. 6. At the within-subject level (Fig. 6, left), no significant differences were observed between the M and HC groups. Although the M group exhibited a generally smaller dispersion, it is noteworthy that there were two outliers within this group. At the within-session level (Fig. 6, center), significant differences were observed between HC-pm and M-pre. For the HC group, no significant differences were found. In contrast, for the M group, M-pre was significantly different from M-post and M-inter, and M-ict was significantly different from M-inter. Therefore, during the preictal phase, the FC of the M group not only deviates from that of the HC group, but also stands out as the most distinct among other migraine phases of the M group. At the between-group analysis (Fig. 6, right), no significant differences were detected. Although there is a trend towards growing similarity between the HC and M groups throughout the migraine cycle, particularly peaking during the M-inter relative to HC-postov, the substantial variability prevents us from drawing conclusions.

FC Network-Based Statistic analysis

The results for the NBS analysis between-group (M and HC in matching phases, i.e., M-pre, M-ict and M-post compared to HC-pm; M-inter compared to HC-postov) and within the M group (migraine phases within the same menstrual phase, i.e. M-pre, M-ict and M-post) are shown in Fig. 7. Comparing the M and HC groups (Fig. 7, left), in the corresponding phases, both M-ict and M-post showed a significantly stronger FC compared to HC-pm. While M-ict had a stronger FC primarily in connections involving nodes of the SMN, DAN, VN and VAN, M-post had a stronger FC in fewer connections mostly involving nodes of the VN with the DAN and VAN networks. In contrast, no significant differences were found between M-inter and HC-postov. Regarding the within-group comparisons (Fig. 7, right), for M in the same menstrual phase, M-ict showed higher FC compared to M-pre, mainly involving connections between nodes of the CRB and other networks (specially SMN, DAN, VAN, VN and SUB). It should be noted that, when considering the full data (i.e., all 68 PCs) instead of the reconstructed data using only the selected 19 PCs, the differences between M-post and HC-pm did not survive the cluster threshold (Supplementary Figure S1). This indicates that the PCA reconstruction of the multilevel clinical connectome fingerprinting framework was advantageous for refining this analysis.

Additionally, the results obtained by comparing the different phases within each group across different menstrual phases (i.e., M-pre, M-ict and M-post compared to M-inter for M; HC-pm compared to HC-postov for HC) are presented in Supplementary Figures S2 and S3 for the reconstruction with 19 PCs and 68 PCs, respectively. Regarding the reconstruction with 19 PCs, for HC, FC was weaker for HC-pm relative to HC-postov. For M, FC was also weaker in the peri-ictal phases (M-pre, M-ict, M-post) compared to the interictal phase (M-inter). It is noteworthy that, with 19 PCs, all comparisons indicated

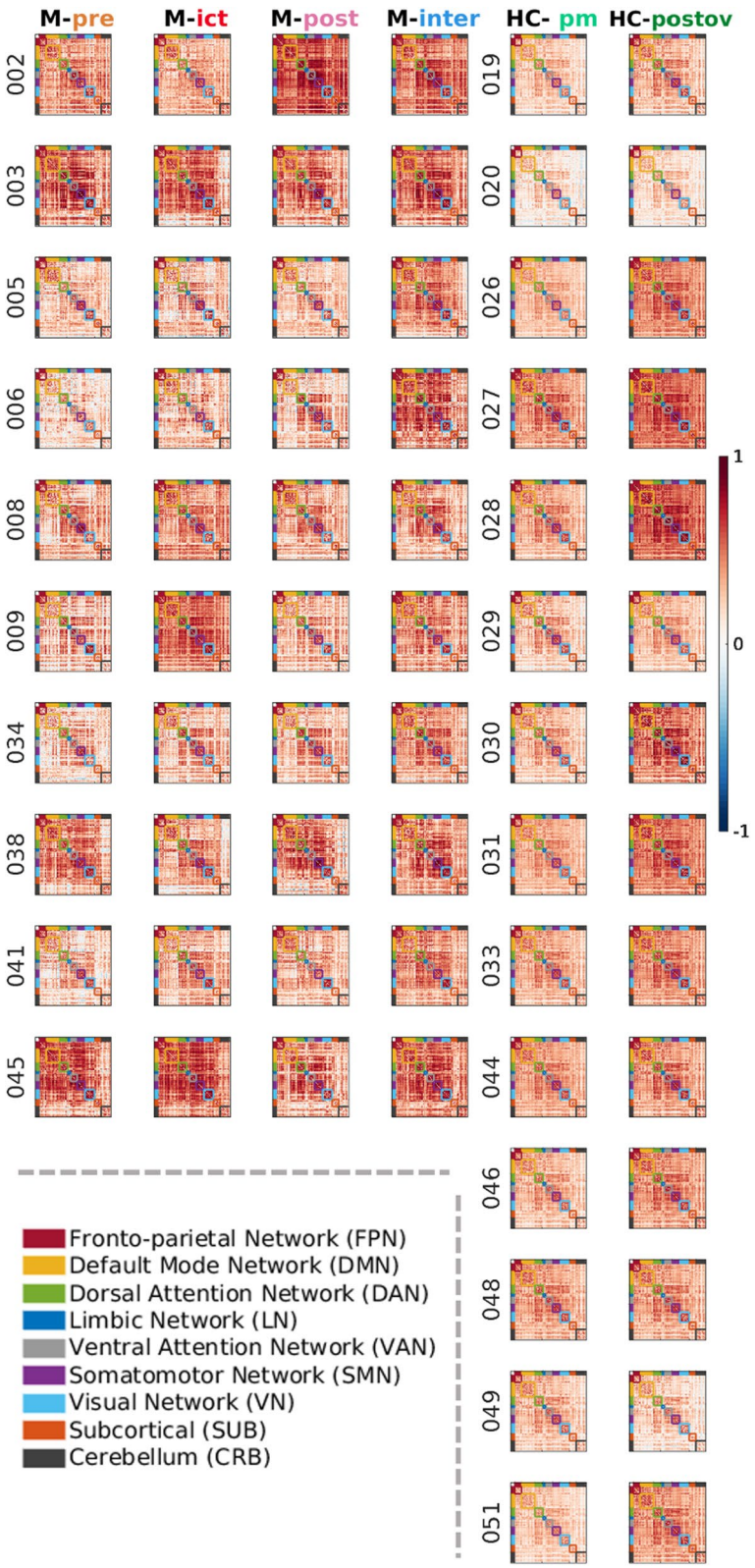

Fig. 4 Functional connectivity matrices computed using Pearson correlation for each participant in the M group and HC group, in each session. In each FC matrix, the brain regions are ordered by network. The networks are represented by colors, as indicated in the legend

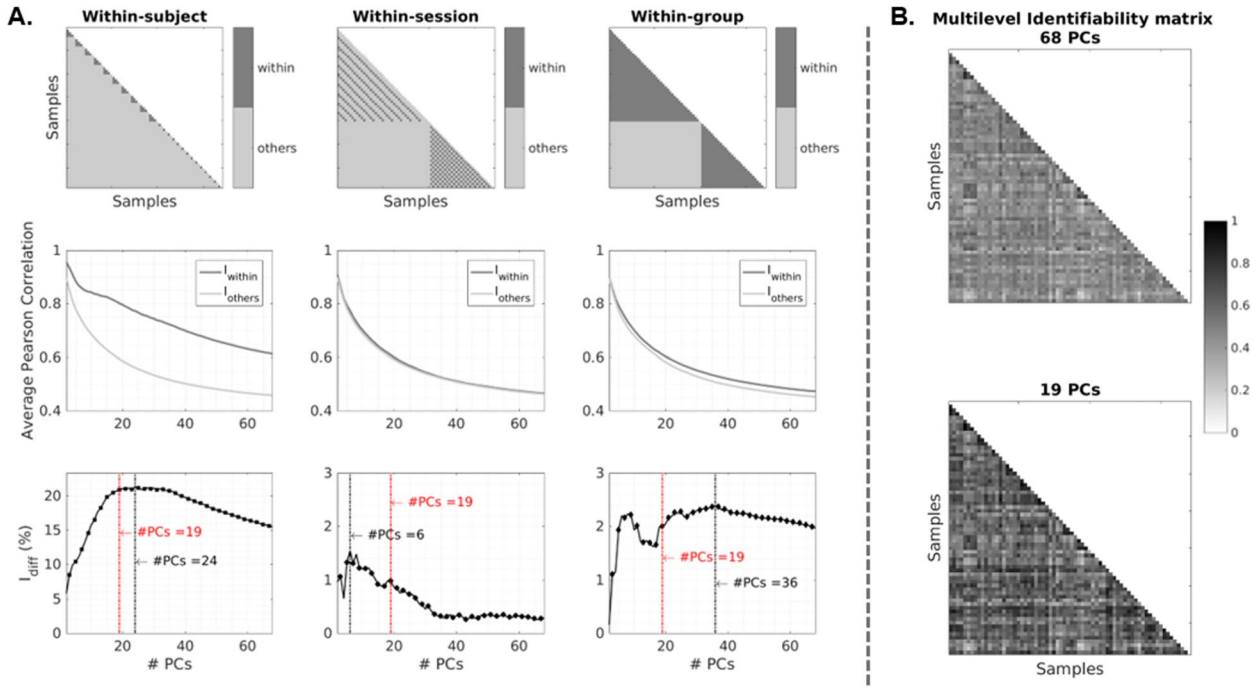

Fig. 5 Multilevel Identifiability analysis: **A**. Multilevel templates for I_{within} (average correlation among equivalent samples) and I_{others} (average correlation among non-equivalent samples)(Top); I_{within} and I_{others} as a function of PCs (Center); $I_{diff}(\%) = 100x(I_{within}\text{-}I_{others})$ as a function of PCs: 19 PCs (indicated in red) were chosen for reconstruction as a compromise between the values of all the levels (Bottom). **B**. Multilevel identifiability matrices computed using all PCs (68 PCs), and using only the selected ones (19 PCs), which shows localized increases in correlation values.

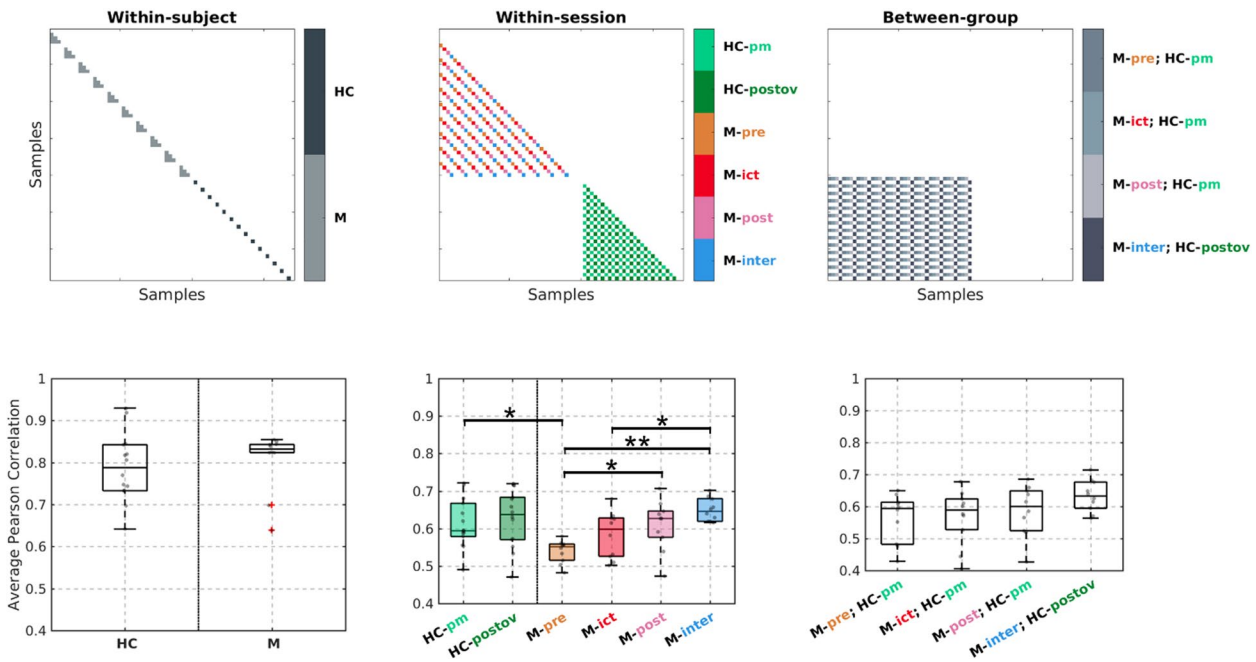

Fig. 6 FC fingerprints average correlations: templates for computation (Top) and distributions across subjects (Bottom), for: within-subject (all pairwise session combinations for M; HC-pm vs HC-postov), within session (M/HC: all other M/HC during the same session), and between-group (only for M: all HC in the corresponding session) correlations. Significant differences are indicated; (Wilcoxon signed-rank test (1-sample) and Wilcoxon rank-sum tests (2-sample); Benjamini-Hochberg FDR correction; $*p < 0.05$;$**p < 0.01$)

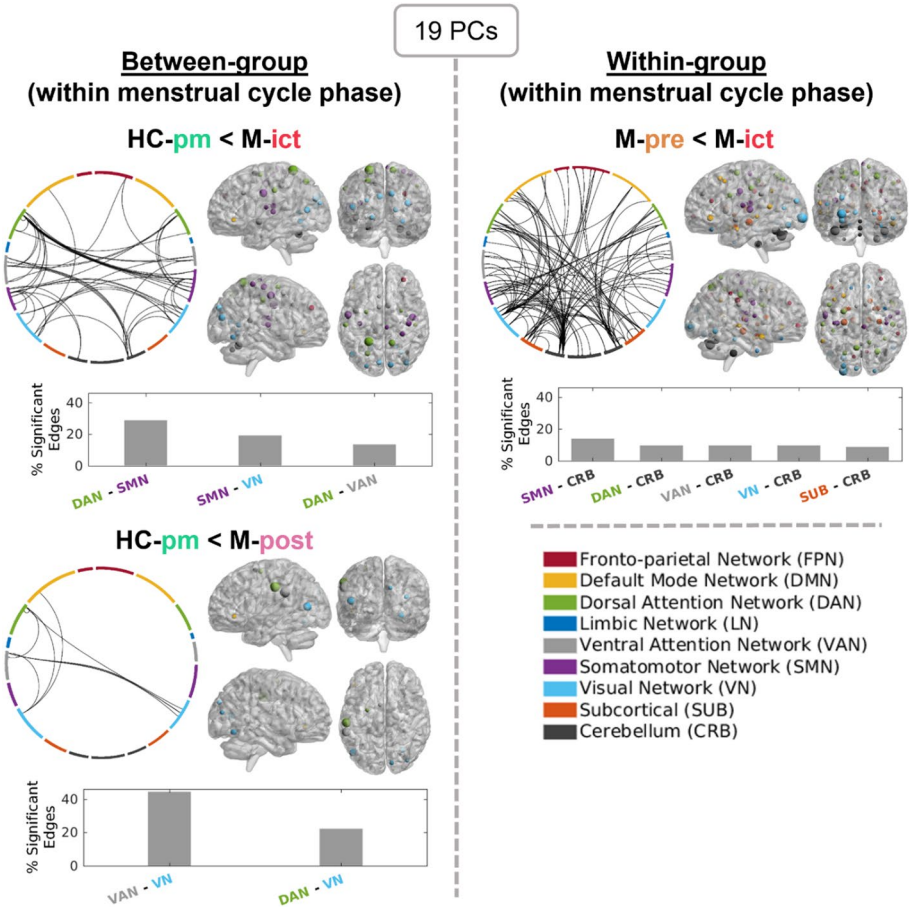

Fig. 7 FC analysis between-group and within menstrual cycle phase (Left) and within-group (Right), using NBS after FC fingerprints reconstruction with 19 PCs (based on extent, cluster threshold = 4; α = 0.05; 5000 permutations). For each comparison, two representations are shown: a chord diagram with the edges that significantly differ (generated with NiChord Python toolbox (Bogdan et al., 2023)); four brain views showing the nodes that contribute to significantly different edges (generated with BrainNet Viewer MATLAB toolbox (Xia et al., 2013)); a bar plot in which each bar indicates the percentage of the total significant edges belonging to a network or inter-network connection (the bars are displayed in descending order of contribution, with only those contributing up to a cumulative 50% being included in the figure). For each comparison, the node size is scaled according to the node degree, which corresponds to the sum of the number of significantly different edges linked to that node. The networks are represented by colours, as indicated in the legend

differences in connections between nodes of the VN and DMN, as well as between nodes of the VN and the DAN. This pattern was also observed with 68 PCs, except for the M-ict vs HC-pm comparison. These findings highlight the significant impact of the menstrual cycle on FC, both in strength and configurations, and hence the importance of controlling for it when studying the migraine cycle. For this reason, within-patient comparisons were considered only within the same menstrual cycle phase in Fig. 7.

Association with clinical features

The average FC in edges significantly different between M-post and HC-pm was negatively correlated with the Attack Frequency ($r_s = -0.83$, $p < 0.05$). On the contrary, it was positively correlated with the Migraine Onset at the uncorrected significance level ($r_s = 0.66$, $p < 0.05$), but failed to surpass the multiple comparisons correction threshold. There were no significant correlations of differential FC identifiability measures or FC strength with other clinical features.

Discussion

We report the first case-control longitudinal study of brain FC across all phases of the migraine cycle, where we employ a novel multilevel clinical connectome fingerprinting approach to retrieve individual FC fingerprints of patients and matched healthy controls. We found decreased FC identifiability of patients in the preictal phase compared with controls, which increased after the

attack and reached normal values in the interictal phase. Moreover, we found that patients' FC fingerprints exhibited increased strength in the ictal and postictal phases but were otherwise comparable to those of healthy controls in the corresponding menstrual phases.

Multilevel clinical connectome fingerprinting framework

The proposed multilevel clinical connectome fingerprinting framework is based on previous clinical connectome fingerprinting methods (Sorrentino et al., 2021; Svaldi et al., 2021; Tepper et al., 2023), but presents some important unique features. Our between-group average correlation is similar to the $I_{clinical}$ metric proposed in Sorrentino et al. (2021). However, in their case and in related work (Cipriano et al., 2023; Romano et al., 2022; Troisi Lopez et al., 2023), data were acquired in only two sessions and these were used as test-retest, i.e., no longitudinal changes were considered to occur. In our case, the different sessions corresponded to different phases of the migraine/menstrual cycles and we wished to assess each of them individually. By employing our multilevel clinical connectome fingerprinting framework we were able to capture representative FC fingerprints, not only of individual subjects and patient/control groups but also of different phases of the migraine/menstrual cycle.

We observed significantly increased FC heterogeneity for patients in the preictal phase compared with healthy controls, which decreased with the progression of the attack. When not experiencing symptoms, patients' FCs were as homogeneous as controls. This is partially in line with previous FC fingerprinting studies of disease, which also found higher patient heterogeneity in amnesic mild cognitive impairment (Sorrentino et al., 2021), amyotrophic lateral sclerosis (Romano et al., 2022), Parkinson's disease (Troisi Lopez et al., 2023) and multiple sclerosis (Cipriano et al., 2023). Nevertheless, these studies also observed larger intra-subject variability for patients, which was not the case in our migraine patients. It is noteworthy, though, that they all employed MEG instead of fMRI, and focused on a very different time scale. In fact, test and re-rest sessions were measured on the same day, with only 1 min break between them, while in our study the different sessions were separated by several days.

Concerning the menstrual cycle, to our knowledge, there is only one study evaluating fingerprints across three phases: peri-ovulatory, mid-luteal and early follicular (Cipriano et al., 2024). This study observed that FC identifiability remained consistent across phases. Nonetheless, a more detailed edgewise analysis revealed that the connectomes during the peri-ovulatory and mid-luteal phases showed less stability (Cipriano et al., 2024). Although the peri-ovulatory and early follicular phases in that study partially coincide with the post-ovulation and perimenstrual phases in the present study, the methodological differences hinder us from establishing strong connections between the two studies.

The value of FC fingerprint measures (reflecting the patterns of connectivity across the whole-brain) is based on previous reports of changes across several functional networks, suggesting that alterations in specific pathways due to the presence of migraine impact the whole connectome. The specific FC fingerprint approach proposed to address the longitudinal case-control design of our study allows accounting for potential changes along the migraine cycle. Therefore, this may be informative of some aspects of the pathophysiology of migraine, namely how whole-brain FC patterns converge/diverge as the cycle progresses. Nevertheless, we go beyond the FC fingerprinting approach and employ the NBS approach to identify more spatially-specific patterns of connectivity changes.

FC differences between patients and healthy controls

When comparing patients with controls in matching phases of the menstrual cycle, there were no differences in the interictal phase (relative to post-ovulation controls). In agreement with our findings, a few recent reviews reveal no evidence in the literature for FC changes in migraine patients in the interictal phase. A systematic review shows that, although FC studies had found differences in more than 20 networks, these differences were not reproducible and there was no common pattern that could be associated to migraine (Skorobogatykh et al., 2019). A more recent review by Schramm et al. (2023), which mostly included studies performed during the interictal phase, examined reported differences in FC and further emphasized the lack of reproducibility across studies. Consistently, a recent study examined a large sample of patients suffering from migraine with aura and did not find any resting-state FC differences compared to controls (Hougaard et al., 2023) and the meta-analysis performed by Chen et al. (2022) did not observe consistent FC differences. In contrast, another recent meta-analysis focusing only on the DMN found significant interictal differences compared to healthy controls (Hu et al., 2023). Given the prevalence of menstrual-related migraine and the significant proportion of female participants in most studies, disregarding the hormonal influence on brain functional connectivity related to the menstrual cycle phase may lead to results unrelated to migraine, potentially explaining some of the documented disparities.

When analysing patients in the peri-ictal phases, we did find significant changes compared to peri-menstruation controls. In the ictal and post-ictal phases, FC strength

was increased mainly in connections involving the DAN, VAN and VN, as well as the SMN only for the ictal phase. The few previous studies that analysed peri-ictal phases report divergent results. While Coppola et al. (2018) observed increased FC during the ictal phase, Coppola et al. (2016) also reported decreased FC. Other studies found no differences comparing patients during the postictal phase (among other phases) with healthy controls (Meylakh et al., 2018, 2021).

FC changes across the migraine cycle

When comparing the peri-ictal phases directly with one another, we found significant increases in FC strength from the preictal to the ictal phase. These increases affected all brain networks, bilaterally, and most of them involved the cerebellum. These findings suggest that the occurrence of migraine may lead to an abnormal increase in FC during peri-ictal phases after the migraine attack has initiated (following the preictal phase), when compared to the FC expected for a normal perimenstrual phase. Therefore, experiencing a migraine attack may lead to a quicker reset of FC perimenstrually that would not occur as fast considering only normal hormonal fluctuations across the menstrual cycle. Stankewitz et al. (2021) and Stankewitz & Schulz (2022) applied a linear mixed-effects model to explain the trajectory of FC strength across several points in the migraine cycle. A negative FC peak was detected during the ictal phase between limbic structures and the hypothalamus (Stankewitz et al., 2021) as well as within the limbic network (Stankewitz & Schulz, 2022). Stankewitz & Schulz (2022) detected a descending trajectory with a negative FC peak during the preictal phase in visual and cerebellar regions. However, in contrast to our findings, they also observed an increase in FC peaking during the preictal phase in 22 networks. These networks predominantly included regions from visual, cerebellar, somatomotor, ventral attention, limbic and default mode networks. In addition to methodological differences, contradictory results could be attributed to the fact that they included patients both with and without aura, whereas our sample is restricted to migraine without aura (usually occurring during the preictal phase). Moreover, it is challenging to pinpoint the start of the pre-ictal phase in general, since definitions rely on convention rather than biological markers.

FC changes across the menstrual cycle

In healthy controls, we found widespread increases in FC strength during post-ovulation compared to perimenstrual phases. A comprehensive review performed by Dubol et al. (2021) shows FC changes across the menstrual cycle in regions such as the hippocampus, amygdala, anterior cingulate cortex, insula, inferior parietal lobule, and prefrontal cortex. Nevertheless, they also report several methodological challenges as well as a large variety of scanning protocols and timings. Furthermore, they did not include studies comparing the early luteal phase (post-ovulation) and late luteal phase or early follicular phase (perimenstrual), which hinders a direct comparison with our study.

The observed FC differences across the menstrual cycle in healthy controls underline the need to control for the menstrual phase when comparing different phases of the migraine cycle in female participants, as was done in our study, and direct comparisons between the peri-ictal phases and the interictal phase should be carefully interpreted. Nevertheless, given the literature reporting such comparisons, we discuss here our own findings in relation to previous reports. Compared to the interictal phase, patients in the peri-ictal phases showed a widespread decrease in FC throughout the brain. As we saw, this can be at least partly explained by the menstrual cycle, as it aligns with the differences between menstrual phases in healthy controls. To our knowledge, only two studies comparing all migraine phases attempted to control for the menstrual cycle by stating that the sampling was distributed equally across the cycle for female patients (Stankewitz et al., 2021; Stankewitz & Schulz, 2022). Other studies reporting differences between the interictal and peri-ictal phases did not control for, or report, the menstrual cycle phase (Amin et al., 2018; Araújo et al., 2023; Schulte, Menz, et al., 2020). Consistently with our results, Araújo et al. (2023) and Amin et al. (2018) reported FC decreases during the ictal phase compared to the interictal phase, though the latter also found FC increases. Contrary to our results, (Schulte, Menz, et al., 2020) observed increased FC during the preictal phase compared to the interictal phase.

Limitations

Despite the uniqueness of our dataset, we must acknowledge several limitations in our study. The main limitation is the relatively small sample size, in terms of the number of subjects studied, which may not be representative of the diversity of migraine symptom manifestations and FC patterns. This makes the results more vulnerable to extreme values, possibly affecting the extrapolation of the results. However, we believe that this is at least partly compensated for by the longitudinal design of the study, including multiple samples from each individual. To mitigate variability, we evaluated a homogeneous sample (only female patients with low-frequency episodic migraine without aura, with menstrual-related migraine attacks). Furthermore, the low attack frequency ensured that there was a clear

separation between the migraine phases of consecutive attacks. However, this choice limits the generalizability of the findings to other types of migraine. If indeed our findings would also be verified in other types of migraine, then a larger albeit more heterogeneous cohort could have produced more statistically significant results. Furthermore, since several studies analyse datasets with a mixture of episodic/chronic and/or migraine with/without aura, some findings cannot be replicated in our study.

Although each patient was studied in different phases of the migraine cycle, the data were not always recorded within the same migraine cycle in each patient, leading to potential additional variability due to variations between attacks in the same patient, including pain intensity. Preictal/postictal symptoms also differ in type and duration across cycles and among patients. Due to this variability, future studies may also rely on clinical symptoms or other objective indicators to define these phases, in addition to temporal criteria, which was what we employed. In our study, we chose a 72-hour interval for the preictal phase because, at the time the protocol was developed, the recommendations by Peng & May (2020), which suggest a 48-hour interval, had not yet been published. Therefore, we followed the review on migraine pathophysiology by Goadsby et al. (2017), in *Physiological Reviews*, which refers to a preictal phase of 72h citing Giffin et al. (2003). Additionally, Stankewitz A et al. (2011) also defined an interval of 72h. Even though they recommend a 48-hour interval, in Peng & May (2020) also mention that this definition may not apply to all patients/attacks and that the preictal phase might be longer, up to 72 hours, in a subset of patients. In our final sample, the median interval was 39 hours, with only 2 out of 10 patients with intervals longer than 48 hours (62 and 70 hours). In light of these, we believe that our 72-hour interval definition of the preictal phase is still appropriate and should not limit the validity of our results.

Another limitation is that some participants used hormonal contraception, which introduces a potentially confounding variable, since hormones play a key role in the FC and the pathogenesis of migraine. Unfortunately, restricting the use of hormonal contraception would reduce the sample size even further. Moreover, we did not measure hormonal levels, which renders uncertainty in the definition of the menstrual cycle in women with natural menstrual cycles. This introduces an additional confounding factor, making it challenging to distinguish changes associated solely with migraines from those influenced by hormonal fluctuations. Furthermore, we did not establish inclusion/exclusion criteria focused on the regularity of the menstrual cycle or presence of hormone-related disorders such as premenstrual syndrome of premenstrual dysphoric disorder, which may have an impact on brain functional networks (Peng & May, 2025).

Finally, although our extension of the already established connectome fingerprinting framework was useful for this longitudinal study concerning the migraine cycle, it lacks further validation using a distinct and larger dataset.

Novelty and impact

Limited research has delved into FC changes across the migraine cycle, as most studies focus on the interictal phase only. Challenges in studying the phases around attacks, attributed to logistical hurdles and patient discomfort, have hindered thorough investigation, despite their crucial role in understanding the complete migraine cycle. Even when multiple phases of the migraine cycle are compared, the designs are most often employed using different patient cohorts for each phase. Longitudinal assessments are scarce, with a few focusing only on the ictal and interictal phases, and a couple measuring multiple time points within the same cycle phase. Notably, longitudinal studies covering all cycle phases did not include a control group. Furthermore, most studies include mostly or only female patients, but did not control for the menstrual cycle phase.

Migraine is characterized by cyclic phases, each potentially associated with distinct changes in brain activity and network connectivity. FC fingerprinting enables the detection of nuanced, phase-specific connectivity variations that are often overlooked by traditional analysis methods. Reduced FC identifiability (the ability to distinguish an FC pattern) may reflect disruptions in the brain's ability to maintain stable and distinct network configurations, which could underlie the cognitive, sensory, and emotional symptoms experienced by migraine patients. This reduction could serve as a biomarker for network instability in migraine. Although in this study we were not able to find a link between identifiability metrics and clinical features, probably due to the small sample size, we believe that this path may be further explored in future, larger studies. Indeed, other studies investigating the use of FC fingerprinting to characterize pathologies have found relationships with relevant clinical features, which showcases the potential of this approach for disease characterization and prediction of treatment response (Cipriano et al., 2023; Romano et al., 2022; Tepper et al., 2023; Troisi Lopez et al., 2023).

Conclusions

We determined the influence of the migraine cycle on individual FC fingerprints in a longitudinal study of patients with episodic menstrual or menstrual-related migraine without aura across the four phases of the

migraine cycle and healthy controls in corresponding phases of their menstrual cycle. Our novel multilevel clinical connectome fingerprinting approach enabled the detection of increased variability among patients, beyond menstrual effects, especially around migraine attacks when patients also differ more from controls. However, this approach should be further validated using a larger dataset of migraine or other pathology. To our knowledge, this work represents the first case-control longitudinal fMRI study across the whole migraine cycle.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s10194-025-01969-6.

Supplementary Material 1.

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Authors' contributions
IE: Methodology, Software, Formal analysis, Investigation, Data Curation, Writing – original draft, Writing – review and editing, Visualization; ARF: Investigation, Resources, Data Curation; ART: Investigation, Resources, Data Curation; GC: Investigation, Resources, Writing – review and editing; RGN: Conceptualization, Project administration; NAS: Resources; PV: Conceptualization; IPM: Conceptualization, Writing – review and editing, Supervision, Project administration; RGG: Conceptualization, Resources, Writing – review and editing, Supervision, Project administration; CCG: Methodology, Conceptualization, Writing – review and editing, Supervision; PF: Methodology, Conceptualization, Resources, Writing – review and editing, Supervision, Project administration, Funding acquisition. All authors have approved the final manuscript.

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Data Availability
Data will be made available by the corresponding author upon reasonable request. Code can be found in the following repository: https://github.com/isesteves/multilevel-fingerprinting_migraine

Declarations

Ethics approval and consent to participate
All participants provided written informed consent and the study was carried out following the Declaration of Helsinki upon approval by the local Ethics Committee of Hospital da Luz.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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