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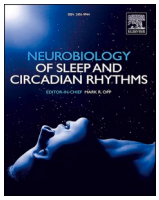

Circadian profile, daytime activity, and the Parkinson's phenotype: A motion sensor pilot study with neurobiological underpinnings

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ABSTRACT

Circadian rhythm impairment may play a role in Parkinson's disease (PD) pathophysiology. Recent literature associated circadian rhythm features to the risk of developing Parkinson and to its progression through stages. The association between the chronotype and the phenotype should be verified on a clinical and biological point of view. Herein we investigate the chronotype of a sample of 50 PD patients with the Morningness Eveningness Questionnaire and monitor their daily activity with a motion sensor embedded in a smartphone. Fibroblasts were collected from PD patients (n = 5) and from sex/age matched controls (n = 3) and tested for the circadian expression of clock genes (CLOCK, BMAL1, PER1, CRY1), and for cell morphology, proliferation, and death. Our results show an association between the chronotype and the PD phenotype. The most representative clinical chronotypes were "moderate morning" (56%), the "intermediate" (24%) and, in a minor part, the "definite morning" (16%). They differed for axial motor impairment, presence of motor fluctuations and quality of life ($p < 0.05$). Patients with visuospatial dysfunction and patients with a higher PIGD score had a blunted motor daily activity ($p = 0.006$ and $p = 0.001$, respectively), independently by the influence of age and other motor scores. Fibroblasts obtained by PD patients (n = 5) had an impaired BMAL1 cycle compared to controls (n = 3, $p = 0.01$). Moreover, a PD flat BMAL1 profile was associated with the lowest cell proliferation and the largest cell morphology. This study contributes to the growing literature on CR abnormalities in the pathophysiology of Parkinson's disease providing a link between the clinical and biological patient chronotype and the disease phenomenology.

1. Introduction

Circadian rhythms (CR) are biological cycles that are regulated by an endogenous process. They have a periodicity of about 24 h, which can persist even in absence of environmental cues. At a molecular level, the circadian clock consists of an autonomous regulatory genetic network involving interconnected negative and positive transcription-translation feedback loops (i.e., the CLOCK/BMAL1 system). The latter works in a cycle lasting approximately 24 h to complete (Dibner et al., 2010). CR disorders are disturbances of the sleep-wake rhythm due to alterations of the circadian timing system or to a misalignment between the timing of the endogenous CR and the sleep-wake times required by social schedules (Gros and Videnovic, 2020).

Parkinson's disease is a frequent degenerative disease, which is second only to Alzheimer's disease in prevalence, but has a growing incidence worldwide that will reach ~14 million cases by 2040. Despite its core phenomenology being featured by motor symptoms and signs such as bradykinesia, rigidity, tremor and postural instability, non-motor symptoms have a leading role in leading the pre-diagnostic phase and drive the prognosis towards the disease progression. Sleep

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and wake behaviors are disrupted in PD from the premotor stage on a qualitative and quantitative point of view (Hunt et al., 2022). More than 80% of the PD population experiences sleep issues such as difficulties in falling asleep and in maintaining sleep for a consistent period (insomnia) or the REM sleep Behavior Disorder (RBD) (Gros and Videnovic, 2020). On the other hand, PD patients' wakefulness might be seriously impaired by the presence of diurnal somnolence or Excessive Daytime sleepiness (EDS) (Gros and Videnovic, 2020). Sleep and wake disorders are interconnected between each other through an interrelationship which is influenced by various endogenous (e.g., hormonal and genetics) and environmental (e.g., dopaminergic therapies) factors (Breen et al., 2014). Therefore, the pathophysiological mechanism of the sleep-wake modifications in PD subjects is still poorly understood.

Despite their relevance, the relationship between PD sleep and wake symptoms with CR has not been yet fully clarified. Indeed, the CR has been poorly investigated in the PD population and the amount of the CR research in PD is not comparable with the high load of specific sleep studies. However, epidemiological studies suggested that an altered CR might be an additional risk factor of developing PD (Lauretti et al., 2017; Leng et al., 2019). Moreover, there is growing evidence of the direct involvement of CR in PD pathophysiology with insights coming from molecular research, animal models and preclinical evidence (Hunt et al., 2022). The suprachiasmatic nucleus (SCN), the director of the CR orchestra, shows early dysfunctions in PD. This, together with peripheral (e.g., retinal/ganglionic) alterations, could partially justifying the compromission of the response to the light that is observed in animal models and patients (Dibner et al., 2010). Nevertheless, manipulations of the environment and of light exposure are currently under investigation for their putative role in alleviating the PD pathology in animals and symptoms in patients (Gros and Videnovic, 2020).

The complex interplay between environmental, hormonal, and genetic factors makes the feasibility of a comprehensive study on CR in PD hard to attain and few research groups worldwide have dedicated enough resources to the topic. Nevertheless, the research on the role of CR abnormalities in neurodegenerative diseases and PD pathophysiology is constantly growing (Hunt et al., 2022; Lauretti et al., 2017; Leng et al., 2019). Indeed, the CR disruption may play a significant role in the PD pathophysiology. Herein, we aimed at investigating the relationship between the patient's circadian behavior (i.e., chronotype obtained through questionnaires), their daytime motor activity (i.e., through a motion sensor analysis) and disease phenotype in a prospective uncontrolled pilot study. Furthermore, since human fibroblasts follow a rhythmicity of ~24 h in vitro, we also performed a small pilot on the oscillating expression of core circadian genes and the possible effect that CR could exert on fibroblast features shown to be altered in PD cells (i.e., morphology, proliferation, and death).

2. Materials and methods

Patients with idiopathic PD have been consecutively enrolled at Fondazione Policlinico Universitario Campus Bio-Medico, Rome, Italy, from January to March 2021. All of them were initially evaluated in the morning during their usual ON time for a global clinical assessment including Unified Parkinson's Disease Rating Scale (UPDRS) part I-IV subscores and total score, modified Hoehn and Yahr (H&Y), Montreal Cognitive Assessment (MoCA), quality of life (PDQ39) and non-motor symptoms questionnaire (NMSQ). The MoCA was recorded as total score and sub-scores (i.e., visuospatial, naming, attention, language, abstraction, delayed memory). The UPDRS includes 4 subscales. The part I is a 4 items scale for "mentation, behavior and mood", the part II is a 13-item scale for activity of daily living; part III is a 27-item scale on the motor examination of PD; the part IV is a 11-item scale on the complication of pharmacotherapy. Each item of the I-III has a 0 to 4 score. The scale ranges from 0 to 176 but is sensible to the effect of therapies. The modified H&Y is a well acknowledge clinical scale aimed at staging the motor syndrome from 1 to 5 (1 - Unilateral; 1.5 - Unilateral and axial; 2 - Bilateral without impairment of balance; 2.5 - Mild bilateral with recovery on pull test; 3 - Mild to moderate bilateral with some postural instability; 4 – Severe but still able to walk or stand unassisted; 5 - Wheelchair bound or bedridden). The postural instability and gait disorder (PIGD) score was also calculated (Stebbins et al., 2013). The PIGD score is derived by the UPDRS motor part (III) and motor related activity of daily living (II) and relates to the prevalence of postural and gait symptoms among others and is representative of a more severe disease phenotype overall and associated with a poorer prognosis (Stebbins et al., 2013). Data on sleep quality were collected through the PD sleep scale (PDSS) and through the RBD questionnaire (RBDQ) (Kurtis et al., 2018). The excessive daytime sleepiness (EDS) was evaluated through the Epworth Sleepiness Scale (ESS). Data on pharmacological therapies and comorbidities were recorded and the levodopa equivalent daily dose (LEDD, a method to globally estimate the dopaminergic therapy burden) has been derived.

Patients were interviewed through the Morningness Eveningness Questionnaire Self-Assessment (MEQ-SA) score to characterize their clinical chronotype (Roveda et al., 2017) (Supplementary Table 1). Subjects with a "definite morning" (MEQ-SA score 70–86) and with a "moderate morning" (MEQ-SA score 59–69) chronotype will be collectively grouped as "morning" patients, while subjects with a "intermediate" (MEQ-SA score 42–58), a "moderate evening" (MEQ-SA score 31–41) and a "definite evening" (MEQ-SA score 16–30) chronotype will be collectively grouped as "evening" and compared; a subgroup analysis across specific chronotypes will be conducted, afterwards.

We also stratified the sample according to (i) the presence of any deficit at MoCA sub-score (i.e., the loss of at least 1 point for item) and to (ii) the median PIGD index value of 0.6, to allow the evaluation of the effect of cognitive and motor signs on the chronotype. The presence of motor complications was assessed through the UPDRS part 4 "OFF duration" item >0 or UPDRS part 4 "Dyskinesias duration" item >0. The presence of EDS was assessed through an ESS >9 and the presence of RBD through a RBDQ>6, as described elsewhere (Marano et al., 2020).

All subjects were equipped with a smartphone (Samsung S series, fully charged) with an embedded application (Mon4t ToGo, www.mon4t.com) to monitor the quantity of motion (activity index, AI) during a 24-h period. The latter have been previously successfully tested by our group for the "on demand" remote patient monitoring in PD and validated versus standard technologies and clinical evaluations (Tchelet et al., 2019; Marano et al., 2021; Motolese et al., 2020). Patients wore the smartphone in a chest band and the application recorded continuously for as long as there is power in the device. The smartphone integral 3D linear accelerometers were used for motion sensing. Each is sampled at 50 Hz for as long as the app is running. The average of all three sensors is calculated for each 10-min period for ~48 h (~11:00 of day 1 to ~11:00 of day 3), the score is the sum of these averages factored by a scaling normalization number (set according for each subject). We decided to analyze only 18 h of data (from 6:00 to 24:00) of a single day (day 2) because (i) all subjects shown a full rest activity (<0.55) during the 24:00–6:00 timespan and (ii) to avoid further registration biases. Therefore, it was calculated a single "total AI" value (mean AI of patient motion from 6:00 to 24:00) and 6 "partial AI" values. Each partial AI was obtained by the mean AI of a 3-h time frame in the 6:00 to 24:00 timeframe (i.e., 6:00–9:00 or T1, 9:00–12:00 or T2, 12:00–15:00 or T3, 15:00–18:00 or T4, 18:00–21:00 or T5, 21:00–24:00 or T6).

Exclusion criteria were: 1) inability to manage a smartphone or to sign the informed consent; 2) known clinical signs of dementia; 3) a modified Hoehn and Yahr (H&Y) > 4 (advanced patients, unable to walk unaided were excluded) and any other neurological or physical condition other than PD capable of altered mobility; 4) non-corrigible visual or sensory disturbances that could interfere with a proper neuropsychological testing through the MoCA.

Dermal skin biopsies of 5 PD patients and 3 controls (a summary of subject information is provided in Supplementary Table 2) were collected using a 4 mm biopsy punch and handled according to the

protocol described in supplementary methods. All primary skin fibroblasts were cultured in DMEM High Glucose supplemented with 20% FBS (Sigma-Aldrich), 2 mM L-Glutamine (Sigma-Aldrich), 100U/mL Penicillin-Streptomycin (Sigma-Aldrich) and 1x Non-essential Amino Acids (Sigma-Aldrich). Fibroblasts were maintained in an incubator at 37 °C and 5% CO2. Cell growth and cell death were determined using Trypan Blue dye (Sigma-Aldrich) by direct counting the dead and live cells after 7 (T1) and 14 (T2) days of culture. The mean cell count/well was recorded for each cell line and growth curves were plotted against culture time. Growth rates of cells were calculated by dividing the number of cells for each time point by the number of cells seeded at time zero (T0, 100.000 cells). Death curves were estimated as a percentage of death cells at each time point (T1 and T2). All experiments were performed at least in triplicate.

The circadian cycling of the expression of core circadian genes CLOCK, BMAL1, PER1 and CRY1 was analyzed through qPCR by a timed RNA sampling from the cultured fibroblasts. All cells were synchronized by 1 μM of dexamethasone 22 h before measures were taken. Successively, fibroblasts were collected every 6 h for a total of 6 timepoints following synchronization (22, 28, 34, 40, 46 and 52 h after synchronization – T1 to T6, respectively) and the total RNAs extraction and qPCR analysis were conducted according to standardized methodologies described in supplementary methods. SYBR Green gene primers are listed in Supplementary Table 3.

Fibroblast morphology was analyzed to investigate for their distinctive tracts of PD versus controls (Teves et al., 2018; Cartelli et al., 2012). To allow clear demarcations of cell size and shape, fibroblasts were stained with Anti-acetylated α-tubulin antibody (Sigma-Aldrich) and Hoechst 33,342 (Life Technologies) according to the methodology described in supplementary methods. Morphological analysis was performed using ImageJ program on 5 to 10 random images for each subject. In each image, 10 cells were analyzed for a total of 50 cells for each fibroblast line. Area and Axes ratio were calculated with ImageJ software as pixels for the selected area and Max/Min axes length, respectively.

Continuous variables are presented as mean ± standard deviation and nominal/ordinal data are presented as frequencies (%), according to the distribution. Continuous data has been checked for normality through the Shapiro-Wilks test. Inferential statistics have been carried out through Wilcoxon's or the Chi-squared test for paired and unpaired data, accordingly, and corrected for multiple testing. Data correlations have been tested through the Spearman's test and reported as correlation coefficient (p-value). Time series have been investigated through univariate and multivariate MANOVA models with intra- and intergroup comparison and time*variable interaction studies. Differences in cell proliferation, death, morphology and CR gene expression were evaluated through Mann-Whitney *U* test and two-way ANOVA followed by Sidak's test for multiple comparisons, when required. A p-value <0.05 was considered statistically significant. Statistics and figures have been generated through the JMP software (SAS Institute Inc., v 16.0) for patients and GraphPad Prism version 8.0 for fibroblast experiments.

The study protocol was approved by the local medical ethics committee. All participants received verbal and written information about the study protocol and signed a consent form prior to participation, in line with the Declaration of Helsinki. Raw data will be made available to any identifiable researcher, upon formal request.

3. Results

This study included fifty PD subjects (17 females, 34%) with a mean age of 67 ± 10 years and a disease duration of 7.3 ± 5.8 years. The H&Y and the UPDRS part III were 2.5 ± 0.5 and 21 ± 9.25, respectively. The PIGD score was equal to 0.82 ± 0.5. The mean total MoCA score was 23.7 ± 3.5. Moreover, among the MoCA subscales, patients obtained a visuospatial score of 3.75 ± 1.25, a naming score of 2.75 ± 0.6, an attention score of 5.25 ± 1, a language score of 2.45 ± 0.8, an abstraction score of 1.5 ± 0.6, a delayed memory score of 2 ± 1.45 and an orientation score of 5.75 ± 0.6 points (Supplementary Table 4). All subjects were on chronic levodopa therapy, 15 (30%) were on dopamine agonist (DA) and the mean LEDD of the sample was 725 ± 326 mgs. Twelve patients (24%) were on low dose melatonin (1–2 mgs daily). According to the MEQ-SA score, 36 out of 50 (72%) were classified as "morning" patients ("definite morning", n = 8, 16%, mean MEQ-SA score 72.1 ± 1.8; "moderate morning", n = 28, 56%, mean MEQ-SA score 63.7 ± 3.1) and 14 out of 50 (28%) were "evening" patients ("intermediate", n = 12, 24%;, mean MEQ-SA score 52.9 ± 4; "moderate evening", n = 2, 4%, mean MEQ-SA score 34.5 ± 3.1). No one reported a "definite evening" chronotype. Main demographic and clinical description of the sample are reported in Table 1.

"Morning" versus "Evening" subjects presented a lower PIGD score (0.72 ± 0.43 vs 1.07 ± 0.62, Z-score 2, p = 0.045) and a consensually lower incidence of patients with a PIGD phenotype (66.6% vs 92.8%, p = 0.038). There were no other differences across these two main groups according to the other demographic or disease-related features. A subgroup analysis across "definite morning", "moderate morning", "intermediate", "moderate evening" chronotypes was then performed.

Age was similar across groups, but there was a significant prevalence of male sex in the "moderate morning" group. Chronotypes were significantly different in their UPDRS and PIGD scores, quality of life (PDQ39) and disease duration. In particular, the "intermediate" versus the "moderate morning" chronotype group had higher scores (i.e., worse performances) at UPDRS total (Z-score 2.2, p = 0.025), UPDRS IV (Z-score 2.7, p = 0.006) and PDQ39 (Z-score 2.11, p = 0.034), PIGD (Z-score 2.08, p = 0.036). They also presented with a higher incidence of motor fluctuations (p = 0.011) and a longer disease duration (Z-score 2.2, P = 0.028). Also, the "definite morning" versus the "moderate morning" showed a higher UPDRS III (Z-score 2.2, p = 0.023) and higher disease duration (Z-score 2.5, p = 0.018). No linear relationship was found between MEQ-SA score, UPDRS or its subscales and MoCA. Noteworthy, there were no differences across groups in ESS and EDS prevalence, RBDQ and RBD prevalence. In line with this, the MEQ-SA did not relate to the PDSS score, to the RBDQ score or to the presence of RBD. Results are detailed in Table 2.

A complete 6:00 to 24:00 AI curve was obtained for 47 out of 50 subjects. Three subjects (2 moderate morning subjects and 1 definite morning subject discontinued the recording due to lack of compliance or technical reasons) did not complete the motion sensor monitoring due to the lack of compliance/technical issues.

Table 1
Main demographic and clinical features of 50 patients with PD.

	N (%)	Mean ± SD
Sex (F)	17 (34%)	
Age (years)		67 ± 10
Disease duration (years)		7.3 ± 5.8
Modified Hoehn and Yahr		2.5 ± 0.5
UPDRS III		21 ± 9.25
UPDRS IV		4.1 ± 3.2
PIGD score		0.82 ± 0.5
MoCA score		23.7 ± 3.5
LEDD (mgs)		725 ± 326
PDQ39		25.6 ± 18.8
NMSQ		10.2 ± 4.9
ESS score		8.5 ± 4.7
EDS	13 (26%)	
RBDQ score		6.5 ± 2.7
RBD	30 (60%)	
PDSS score		91.6 ± 25.9
MEQ-SA score		61.3 ± 8.7
Chronotypes (MEQ-SA score)		
Definite morning	8 (16%)	72.1 ± 1.8
Moderate morning	28 (56%)	63.7 ± 3.1
Intermediate	12 (24%)	52.9 ± 4
Moderate evening	2 (4%)	34.5 ± 3.1
Definite evening	0	

Table 2
Comparison of demographical and clinical features across chronotypes.

	Definite morning (n = 8)	Moderate morning (n = 28)	Intermediate (n = 12)	Moderate evening (n = 2)	p-value
Age (years)	69.3 ± 4.1	66.4 ± 10.4	67.3 ± 13.2	66.5 ± 6.3	0.951
Sex (M)	2 (25%)*	23 (82%)	6 (50%)*	2 (100%)	***0.008***
Disease duration (years)	9.6 ± 4.1*	5.7 ± 5.3	10.2 ± 6.8*	3 ± 1.4	***0.015***
H&Y	2.3 ± 0.4	2 ± 0.6	2.4 ± 0.7	2.25 ± 0.35	0.115
LEDD (mgs)	855,9 ± 241.8	675.8 ± 362.5	815.8 ± 431.3	400 ± 0	0.129
UPDRS total	47 ± 12.9*	24.8 ± 16.8	44.8 ± 13*	29.5 ± 2	***0.027***
UPDRS I	2.2 ± 1.5	1.9 ± 2	2.7 ± 1.7	1 ± 0	0.331
UPDRS II	13.6 ± 4.5	10.7 ± 5.3	12.3 ± 4.8	10.5 ± 6.4	0.405
UPDRS III	25.6 ± 7*	19.1 ± 9.8	23.5 ± 8.5	16 ± 4	***0.042***
UPDRS IV	5.5 ± 3.6	2.9 ± 2.5	6.1 ± 3.4 **	2 ± 0	***0.019***
Motor fluctuations	5 (62%)	9 (32%)	9 (75%)*	0	0.032
MoCA	20.7 ± 4.9	24 ± 4	25.5 ± 2.5	25 ± 2.8	0.058
NMSQ	13.5 ± 4.5	8.7 ± 4.7	11.5 ± 5.1	9 ± 1.5	0.076
ESS score	9.9 ± 6.6	8 ± 4.5	7.5 ± 3	15 ± 7	0.375
EDS	4 (50%)	6 (21.5%)	2 (16.7%)	1 (50%)	0.279
RBDQ score	7.1 ± 2	6.4 ± 3	6.6 ± 2.5	6 ± 1.4	0.874
RBD	5 (62.5%)	17 (60.7%)	7 (58.3%)	1 (50%)	0.988
PDSS score	95 ± 10.7	88.5 ± 24.7	94.4 ± 29.8	104 ± 28.3	0.795
PDQ 39	33.6 ± 16	21.2 ± 18	33.5 ± 18*	7.2 ± 2.2	***0.026***

*p < 0.05 vs "moderate morning" chronotype, **p < 0.01 vs "moderate morning" chronotype. Motor fluctuations are estimated through the presence of UPDRS IV OFF duration item >0.

The mean AI was 1.1 ± 0.30 units. The index significantly varied during time points and the AI*time curve significantly related to the MEQ-SA score ($F_{5,40} = 2.501$, $p = 0.045$).

The two main groups differed in the early motor activity, which was higher in "Morning" than in "evening" subjects (T1, AI 6:00–9:00, which was higher in the former group as expected (0.97 ± 0.50 vs 0.57 ± 0.34, $p = 0.001$). As also performed for the demographic and clinical analysis, the motion sensor study was compared across chronotype subgroups. The distribution of AI across chronotypes is reported in Table 3. The "definite morning", the "moderate morning" and "intermediate" chronotypes showed significant changes in their early morning activity with the "intermediate" group exhibiting a significantly lower index (T1, AI 6:00–9:00) and, together with the "definite morning" chronotype, they showed a dampened and less significant fluctuation in their activity profile (Fig. 1 and Table 3). The presence of motor fluctuations or dyskinesias did not relate to the total AI value (i.e., there was no correlation between the UPDRS part IV adopted criteria, and the total AI as happened with the clinical chronotype). While the analysis of partial AI values showed that patients with UPDRS part IV OFF duration >0 had a higher AI at 21:00–24:00 (0.66 ± 0.22 vs 0.49 ± 0.15; $p < 0.001$). Only a statistical trend was found between the total AI and the UPDRS III ($p = 0.0501$).

The AI curves varied significantly according to the presence of a visuospatial impairment at MoCA (i.e., visuospatial score <5; 31 subjects, 62%) and according to the mean PIGD score with a time*group significant interaction at MANOVA (repeated measures) (Fig. 2A $F_{5,40} = 3.77$, $p = 0.006$ and Fig. 2B $F_{5,40} = 4.73$, $p = 0.001$, respectively). The AI curve of patients with vs without visuospatial impairment was significantly flat and featured by lower AIs. Noteworthy, according to the respective multivariate models, the relationship between the AI and the visuospatial impairment was independent by the age, the UPDRS part III, the PIGD score and the disease duration.

AI values did not relate to the total LEDD, but higher AIs (i.e., 6:00–9:00) were significantly associated with the use of DAs (1.15 ± 0.60 vs 0.71 ± 0.36, $p = 0.031$). Noteworthy, the use of melatonin did not influence either the MEQ-SA chronotype or the AI distributions.

In the analysis of CR gene expression, the fibroblasts of PD patients (n = 5) differed from those of healthy controls (n = 3) in the BMAL1 curve ((Time factor $p = 0.001$, Group factor $p = 0.049$; Fig. 3A). All the gene expression values are shown in Supplementary Table 5). The other circadian master gene CLOCK (Supplementary Table 5 and Supplementary Figure 1A), while the circadian transcriptional repressors CRY1 and PER1 showed to be differently expressed between the two groups (CRY1: Time factor $p = 0.025$, Group factor $p = 0.0018$; PER1: Group factor $p = 0.048$; Supplementary Table 5 and Supplementary Fig. 1B and C, respectively). We focused our attention only on BMAL1 gene expression because it is, together with CLOCK gene, the principal player of the core circadian clock. The BMAL1 cycle was featured by two main expression peaks, T2 (28 h after synchronization) and T5 (46 h after synchronization). To confirm the validity of this subdivision, we compared the BMAL1 cycle between the two cell groups and we found that they differed at T1 (22 h after synchronization) and T5 (Fig. 3D). The higher expression of BMAL1 at T1 in type A cells indicated an advance of the ascending phase of the oscillation, therefore explaining the greater expression at T2 in these cells compared to that observed at T5. The same analysis was conducted on CLOCK (Supplementary Figure 2A) and on the transcriptional repressors CRY1 and PER1 (CRY1: Group factor $p < 0.0001$; PER1: Group factor $p = 0.0026$;

Table 3
Mean activity Index curve values (SEM) across timepoints in patients according to their chronotype.

	Definite morning (n = 7)	Moderate morning (n = 26)	Intermediate (n = 12)	Moderate evening (n = 2)	p-value
T1 (6:00–9:00)	1.18 ± 0.40	0.92 ± 0.51	0.60 ± 0.35$^{\bullet\bullet +}$	0.39 ± 0.26	0.007
T2 (9:00–12:00)	1.70 ± 0.75	1.66 ± 0.79**	1.63 ± 1.06**	0.94 ± 0.69	0.500
T3 (12:00–15:00)	1.37 ± 0.49*	1.22 ± 0.49*$^{\circ}$	1.42 ± 0.45**	1.22 ± 0.50	0.585
T4 (15:00–18:00)	1.38 ± 0.59*	1.35 ± 0.91*$^{\circ}$	1.12 ± 0.53*	0.95 ± 0.34	0.657
T5 (18:00–21:00)	1.08 ± 0.41°	0.94 ± 0.37$^{\circ\&\#}$	1.04 ± 0.94$^{*\&\&}$	1.55 ± 0.73	0.462
T6 (21:00–24:00)	0.60 ± 0.15$^{*\circ\&\&\#\#\hat{}\hat{}}$	0.53 ± 0.15$^{**\circ\&\&\#\#\hat{}\hat{}}$	0.67 ± 0.32$^{\circ\&\&\#\hat{}\hat{}}$	0.59 ± 0.20	0.548

• p < 0.05 vs "definite morning"; •• p < 0.01 vs "definite morning"; $^{+}$p<0.05 vs "moderate morning"; $^{++}$p<0.01 vs "moderate morning"; *p < 0.05 vs T1 or 6:00–9:00, **p < 0.01 vs T1 or 6:00–9:00; $^{\circ}$ p < 0.05 vs T2 or 9:00–12:00, p < 0.01 vs T2 or 9:00–12:00; $^{\&}$ p < 0.05 vs or 12:00–15:00, $^{\&\&}$ p < 0.01 vs T3 or 12:00–15:00; $^{\#}$p < 0.05 vs T4 or 15:00–18:00, $^{\#\#}$p < 0.01 vs T4 or 15:00–18:00; $^{\hat{}}$ p < 0.05 vs T5 or 18:00–21:00, $^{\hat{}\hat{}}$ p < 0.01 vs T5 or 18:00–21:00. Missing cases: 1 "definite morning" subject, 2 "intermediate morning" subjects.

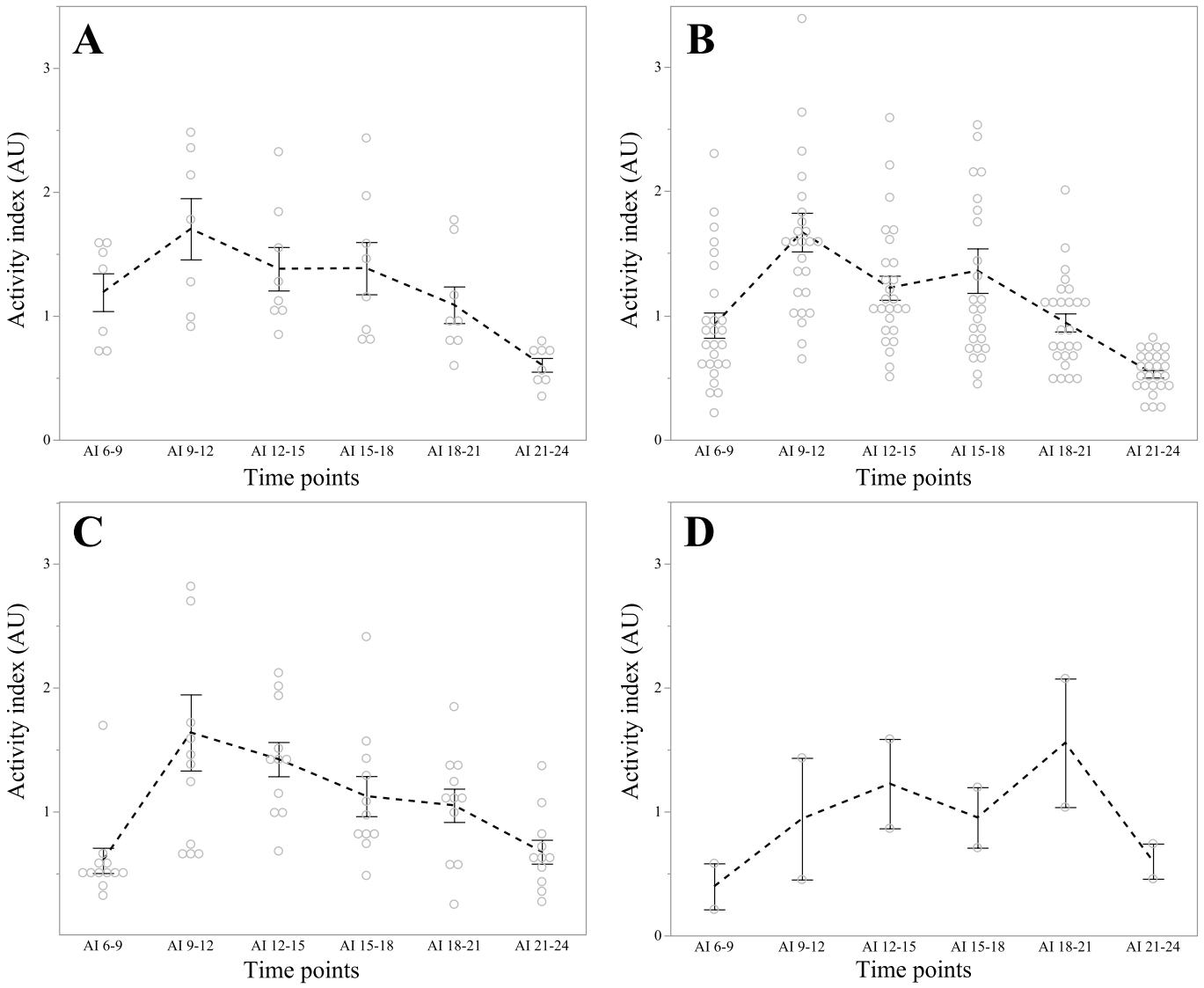

Fig. 1. Mean activity Index curve values (SEM) across timepoints in patients with definite morning (A, n = 7, 1 missing case), moderate morning (B, n = 26, 2 missing cases), intermediate (C, n = 12) and moderate evening (D, n = 2). Definite evening counted no cases. Statistical analysis reported in Table 1.

Supplementary Fig. 2B and C, respectively). Among the three genes analyzed, CRY1 was the only one to manifest a significant difference between the two cell types (at T1) when Sidak's test for multiple comparison was performed. When we went into detail on the cellular characteristics of the two circadian types, we found that while Type B PD cells did not manifest any significant difference in either cell proliferation or cell death compared to Type B CTRs (Fig. 3F), Type A PD cells showed instead a significant reduction of proliferation rate compared to their respective Type A CTR (Fig. 3E, left graph). Moreover, since within this circadian type the % of dead cells between healthy and PD patients did not vary in any of the timepoints analyzed (Fig. 3E, right graph), the lower number of live cells was supposed to be related to a reduced proliferation, rather than to an imbalance between life and death of cells. The cell count and % of dead cells in all patient-derived fibroblasts are reported in Supplementary Table 6. Considering the controversial relationship between cell size and proliferation, our morphology data indicated that PD cells with a lower proliferation rate also had a larger cell size. Type A PD cells had in fact a significantly larger area and a trend to be more elongated than Type A CTR (Fig. 4A, Supplementary Figure 3). Interestingly, within Type B fibroblasts the opposite happened: Type B PD cells were smaller and even manifested a slight tendency to be more rounded than Type B CTR cells (Fig. 4B, Supplementary Figure 3).

4. Discussion

The presented results showed that "morning" versus "evening" people with Parkinson could differ in a main disease phenotype index, with the latter being featured by a higher prevalence of postural instability and gait disturbances. Given the pilot nature of our analysis and despite numbers being too low to allow firm conclusions, we performed a further stratification of the sample based on the five original morningness-eveningness questionnaire chronotypes. Specific chronotypes (i.e., the "intermediate" and possibly the "definite morning") and a blunted and less variable daytime motion activity profile were associated with motor and non-motor signs of a worse disease phenotype with visuospatial cognitive dysfunctions and a more likely PIGD subtype. This is a preliminary observation, which is partially corroborated by the analysis of a small sample of fibroblast showing a dampened BMAL1 cyclicity, that in turn is linked to an altered cell life and fitness. Despite a low detrimental effect on motor function and PD scoring overall was found also in the "definite morning" group, the "intermediate" group showed a more widespread impairment of several part of the disease core such as motor, motor complications and quality of life.

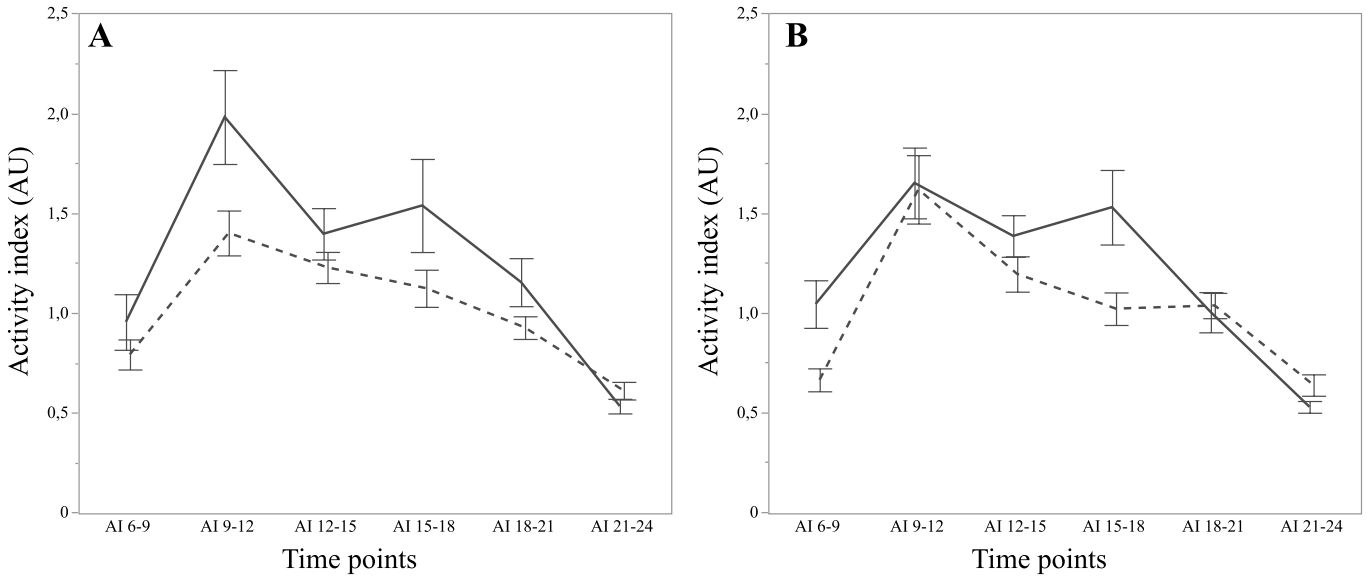

Fig. 2. Comparison between the mean activity index curve values (SEM) in patients without (A, n = 19) versus with (B, n = 31) visuospatial impairment; comparison between the mean activity index curve values (SEM) in patients with lower (C, n = 26) versus higher (D, n = 24) PIGD score.

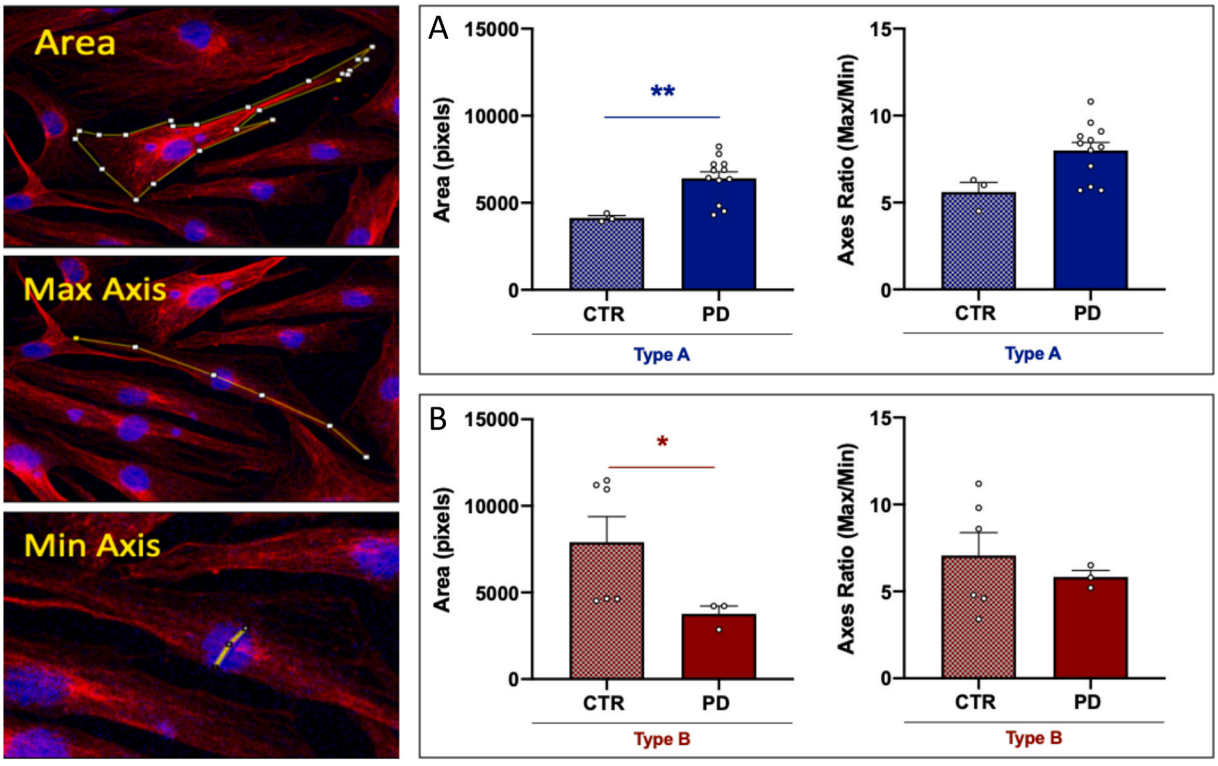

Fig. 3. Gene expression curve of BMAL1 in all PD vs CTRL patients (A) and in single subjects split in Type A (B) and Type B (C) based on T2 vs T5 BMAL1 mRNA expression. BMAL1 cycle differences between Type A and B cells are also shown (D). Cell growth curves and % of dead cells in Type A PD and CTR (E) and Type B PD and CTR (F) fibroblasts with multiple comparisons within types are displayed in the graphs below. Data are expressed as mean ± Standard Error of Mean (SEM). All the Statistical analyses were performed on three independent experiments, using GraphPad Prism 8, through a Two-Way ANOVA test followed by Sidak's test for multiple comparisons, when required. White dots indicate biological replicate (n = 3) for each patient (Type A: CTR N = 1, PD N = 4; Type B: CTR N = 2, PD N = 1). *p < 0.05, **p < 0.01, ***p < 0.0001.

Alterations of sleep and wakefulness are strictly related to the PD pathophysiology at any stage (Gros and Videnovic, 2020; Hunt et al., 2022). For instance, the deposition of Lewy bodies in lower brain stem nuclei might justify at least in part the presence of RBD during the prodromal phase, while the widespread degeneration of wake promoting neural groups have a putative leading role in diurnal hypersomnia of later stages. Our data, obtained by a sample of intermediate stage (mean H&Y 2.5) PD patients, confirms the high incidence of RBD (>50%) and EDS (~25%) in the PD population. Noteworthy, 72% of subject were allocated among the "definite morning" and the "moderate morning" chronotypes, due to a MEQ-SA score ≥59. PD is a disease of aging, and age is related with circadian rhythm patterns. Studies adopting the

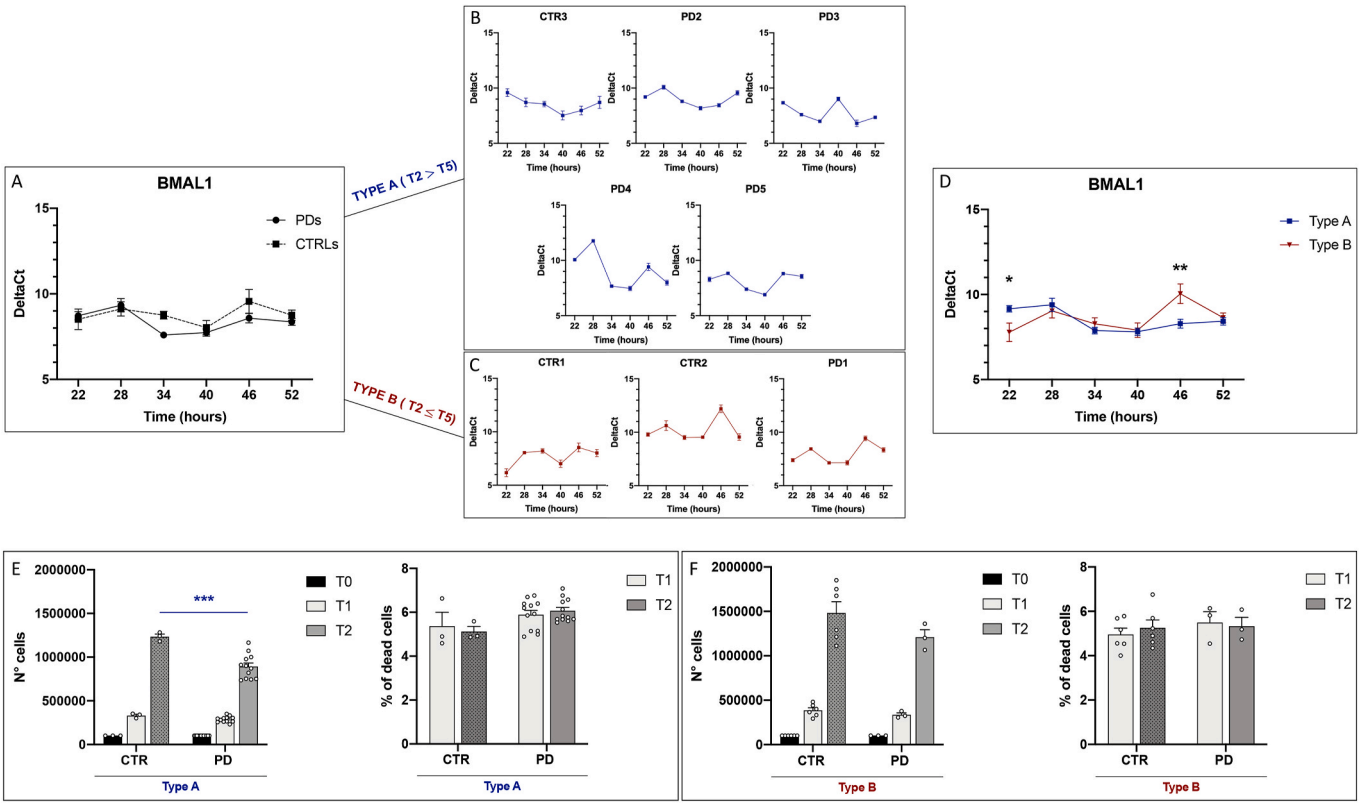

Fig. 4. Fibroblast area and Max/Min axes ratio of Type A (A) and Type B (B) PD and CTRL cells. On the left, representative photos of the morphometric ImageJ analysis are shown. Data are expressed as mean ± Standard Error of Mean (SEM). All the Statistical analyses were performed on three independent experiments, using GraphPad Prism 8, through a Mann-Whitney *U* test. White dots indicate biological replicate (n = 3) for each patient (Type A: CTR N = 1, PD N = 4; Type B: CTR N = 2, PD N = 1). *p < 0.05, *p < 0.01.

MEQ-SA suggested that the incidence of morning chronotypes grow with aging with a large variability (Roepke and Duffy, 2010). Interestingly, despite our sample included a wide age range (40–88), such a relationship did not reveal. On the other hand, our sample showed a relationship between the morning chronotypes and the male sex, in line with what is known by the literature in subjects over the age of 40 (Fischer et al., 2017). However, such affirmations need to be furtherly verified in larger samples to draw any firm conclusions.

Animal models of PD presented changes in the circadian amplitude of the firing neuronal pattern of suprachiasmatic nucleus (SCN) neurons, suggesting that the weakened alerting circadian signaling could contribute to the EDS and to the pathophysiology of early PD (Gros and Videnovic, 2020; Fifel, 2017). In line with this, alterations of the rhythm of the core body temperature - a circadian activity regulated by SCN - is reduced in patients with RBD and synucleopathies (Raupach et al., 2020; Zhong et al., 2013). Despite our expectations, patients' sleep and wake features as estimated through questionnaires (PDSS and ESS) did not relate with a specific chronotype on a clinical (MEQ-SA) and an instrumental (AI) point of view. Possibly, the patient circadian activity generators act through mechanisms that run in parallel to those producing insomnia or EDS. Nevertheless, as also suggested by the existing literature, an uncoupling of circadian and sleep regulation in PD could exist (Bolitho et al., 2014).

The CR evaluated through motion sensors was able to select a subgroup of patients with prominent visuospatial impairment and PIGD features. The relationship between such clinical features and the motion index occurred without any influence of age and disease duration at multivariate analysis. Hence, it is conceivable to hypothesize that both motor and non-motor manifestations of PD may depend, at least in part, by the CR. This is of relevance, given that both the presence of subclinical visuospatial impairment and of a PIGD phenotype are known predictors of a worse disease profile in trajectory studies (Aleksovski et al., 2018; Weil et al., 2016).

Motor complications are of particular importance in the history of patients with PD, given their relation to an impaired quality of life and loss of independence. Our data suggests that motor fluctuations could present more frequently in some chronotypes ("intermediate") than in other ("moderate morning") – adding to the literature that support an effect of disease progression, dopaminergic load, and the presence of levodopa related motor complication in influencing melatonin secretion patterns (Bordet et al., 2003). Despite, the dopaminergic medication load (i.e., LEDD) was not related either to the chronotype or to the motion sensor output, the use of DAs was associated with a higher AI. Indeed, dopamine and the circadian system have bidirectional modulatory relationships and the therapeutic effect of dopamine agonists - which act on a wide dopamine receptor landscape - might justify such an effect (Korshunov et al., 2017).

Controlled preclinical experiments suggested that CR might have a direct role on PD manifestations (Hunt et al., 2022). For instance, an experiment performed on a murine model of dopaminergic denervation suggested that there is a correlation between the locomotor activity and the light/dark exposure (Fifel and Cooper, 2014). Moreover, mice exposed to MPTP manifested a worse motor profile and brain tissue abnormalities (i.e., inflammatory glial changes) if exposed to light/dark circadian disruption when compared with those that maintained a regular CR (Liu et al., 2020). Light response of the circadian system may be compromised in PD due to a degeneration with consequent impairment of the retinal melanopsin system (Ortuno-Lizaran et al., 2018), Such evidence suggests that PD might be associated with a loss of CR endogenous control and as already acknowledged, there might be a strong link between circadian dysfunction and neurodegeneration (Leng et al., 2019). Supporting such pre-clinical observations, hormonal (i.e.,

Melatonin) and molecular (i.e., CLOCK system genes) biomarkers documented a general blunting in their pulsating activity even in patients with PD (Breen et al., 2014; Videnovic et al., 2014; Delgado-Lara et al., 2020).

Various neural, hormonal, and environmental factors contribute to the determination of the human CR, but the CLOCK/BMAL1 system is the main genetic director of the orchestra. It consists in a positive feedback loop, where the transcriptional activator CLOCK interacts with BMAL1 during the day to activate and sustain the transcription of Per and Cry genes, resulting in high levels of their transcripts. The resulting PER and CRY proteins heterodimerize and translocate to the nucleus to interact with the CLOCK-BMAL1 complex and to inhibit its own transcription (i.e., negative feedback loop). During the night, the PER-CRY complex is degraded, and CLOCK-BMAL1 can then start a new cycle of transcription (Fifel, 2017).

Previous experiments suggested that BMAL1 was reduced and lacked time dependent variability in patients with PD and in PD patient derived peripheral blood mononuclear cells (Breen et al., 2014; Cai et al., 2010), while other experiments found a reduced peripheral expression of the entire Clock gene system and lower BMAL1 levels being associated with a worse sleep/wake profile (Li et al., 2021).

These data were confirmed in PD patients-derived fibroblasts that, as a primary cell type, retain the specific environmental and aging history, and polygenic risk factors of the patient. We demonstrated that BMAL1 was the best fitting CR master gene for the PD diagnosis, because no differences in CLOCK were observed. In addition, we observed that the flat BMAL1 profile in PD cells was associated with a significant deregulation of cell proliferation, in line also with morphological data. Indeed, Type A PD cells had a deregulated shape (i.e., "larger and more elongated") than their respective controls (Fig. 4A). This led us to hypothesize that a less marked oscillation of the gene under examination could explain the reduction in PD cell proliferation. Moreover, literature data suggest that the decrease of proliferation rate could be responsible for an increase in cell shape. That is because the cell duplicates its chromosomes and accumulates proteins to prepare itself for a mitosis, which in this case will be delayed compared to a healthy condition. This may contribute to the growing literature suggesting a possible role of genetic Clock alterations in PD pathophysiology and clinical manifestations (Lou et al., 2017; Shkodina et al., 2022). Moreover, these results are of particular importance since they overcome one of the main limits of the CR research: the impossibility to isolate the patient or the animal model from the environment (Fifel, 2017). Indeed, for this preliminary experiment we successfully adopted fibroblasts - a trusted and isolable disease model - to further demonstrate the presence of a dysfunctional CR activity in PD, confirming some known results and opening new research paths.

Hence, our study added to the growing preclinical and clinical experiences that indicate the importance of CR investigations in PD. Given the strong interrelationship between CR and PD pathophysiology, the CR investigation should have a defined and systematic role in PD phenotyping and risk prediction. To such regard, our experiment observed that more serotine subjects have a more significant axial phenomenology, and that such difference in phenotype might be influenced in part by the disease duration. All the above-mentioned results are partially in line with recent studies, that found an interrelation between the disease progression and axial symptoms (i.e., postural control) with the distribution of the daily locomotor activity (i.e., later acrophase), the activity amplitude (i.e., lower amplitude) (Stewart et al., 2018; Obayashi et al., 2021). Furthermore, Leng and colleagues, adopting a similar sensor-based methodology, found a 3% incidence of PD in a retrospective longitudinal cohort of almost 3000 elderlies followed up for 11-year. Authors observed that a reduced CR (lower amplitude, mean activity and robustness as analyzed by actigraphy) was associated with three-fold increased risk of developing PD (Leng et al., 2020).

The presented study has several limitations. The major one is the lack of a sample of matched controls. Hence, data on the distribution of chronotypes and on the effect of potential confounders such as age are inferred by the available literature. Indeed, subjects were not equally distributed across clinical chronotypes, and the absence of a control population made difficult to address an association between PD, a specific chronotype and age. Several influencing factors exist, and former clinical observations need to be further verified in larger and controlled trials. Moreover, most patients appertained to the "moderate morning" (n = 28) or to the "intermediate" (n = 12) group, with a minor part being grouped as "definite morning" (n = 8) and a comparison with the other 2 classes (n = 2 "moderate evening" and n = 0 "definite evening") was not possible. Hence, a statistical comparison among these two classes and the other was flawed by the specific sample size. However, whether such distribution was an effect of age or of the disease itself will be further investigated in larger trials with more structured methodology. Nevertheless, the patient subdivision in chronotypes was verified and significantly depicted through motion sensor data (Fig. 5). Such information came from an ecologic monitoring provided through smartphone sensors. The latter are trustable highly accurate since have been already validated for various neurological tests, compared against ratings provided by movement disorder specialists, wearable sensors, pressure sensitive walkaways, and 3D video motion laboratories (Tchelet et al., 2019; Karlinsky et al., 2022). To such regards, diurnal activity patterns have been already described in PD, with a more favorable trend observed in the motion quality of the early part of the day as also described by our cohort (van Wamelen et al., 2021). In our case, the early morning activity (6:00–9:00) index differentiated the clinical chronotypes, such as the average diurnal pattern that was in line with the clinical characterization despite no statistical significance across groups was shown overall. However, the early morning activity is an acknowledged marker of the human circadian behavior (Roveda et al., 2017) and was an inside validator of our methodology – requiring larger study. Further motion sensor investigations, regarding the sleep pattern and fragmentation and a reduction in precision of the activity onset, are warranted.

Herein, various factors that might potentially influence the CR have not been addressed and deserve further investigations. Circadian oscillations are ubiquitous throughout the body and there may be as many clocks as there are cells. The SCN is a master synchronizer orchestrating a multitude of peripheral subnetworks in a coherent timing system integrating signals like photic information, temperature, hormonal metabolites, and feeding-fasting cycle and its role in the pathophysiology of PD is well documented in both preclinical mouse models and in human post-mortem studies (Dibner et al., 2010; Kudo et al., 2011; De Pablo-Fernandez et al., 2018).

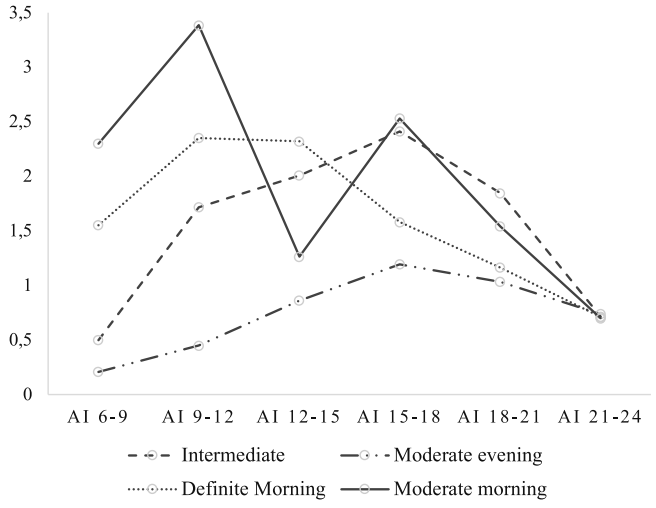

Fig. 5. Individual activity profiles over time as obtained from the smartphone sensor monitoring of 4 patients with different clinical chronotypes.

Studies on larger samples will also help in better understanding the magnitude and significance of the relationship between the MEQ-SA and the motion analysis in PD, as also investigated by previous investigations on healthy subjects (Kurtis et al., 2018).

Despite the use of an arbitrary methodology (i.e., classification of the fibroblasts in type A or B based on the BMAL1 expression profile), our biological results were in line with our clinical observations and with previous literature (Pacelli et al., 2019) and should go through a replication study of larger numbers and on more robust cellular models (i.e., dopaminergic neurons). Indeed, the very low sample size of our biological experiment does not allow to draw any firm conclusion. Nevertheless, the relationship between circadian rhythm and neurodegenerative disorders is a promising and challenging research field and the present study may contribute to pave the way for future translational studies aimed at opening new pathophysiological and therapeutic paths for patients with Parkinson's disease.

Credit author statement

Massimo Marano, Jessica Rosati, Ziv Yekutieli, Alessia Casamassa: Conceptualization, Methodology, Software; Massimo Marano, Alessia Casamassa, Alessandro Magliozzi: Data curation, Writing Original draft preparation. Gabriele Sergi, Miriam Iannizzotto, Alessia Rappa: Investigation. Angelo Vescovi, Vincenzo Di Lazzaro: Supervision. Massimo Marano, Jessica Rosati, Alessia Casamassa: Writing- Reviewing and Editing.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Ziv Yekutieli is CEO of Mon4t.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbscr.2023.100094.

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