ORIGINAL ARTICLE

PER2 C111G polymorphism, cognitive reserve and cognition in subjective cognitive decline and mild cognitive impairment: a 10-year follow-up study

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Background and purpose: *CLOCK* and *PER2* genes have been implicated in sleep–wake cycle alterations and neurodegenerative diseases. Our aim was to evaluate the effect of *CLOCK* T3111C and *PER2* C111G on cognitive functioning in subjective cognitive decline (SCD) patients and mild cognitive impairment (MCI) patients at the baseline of a longitudinal study, and the effect of these two polymorphisms on the progression to Alzheimer's disease (AD) of the two groups.

Methods: Sixty-eight subjects (41 SCD and 27 MCI) who underwent clinical evaluation, neuropsychological assessment, *CLOCK* and *PER2* genotyping at baseline and neuropsychological follow-up every 2 years for a mean time of 10 years were included. Subjects who developed AD (SCD-c and MCI-c) and non-converters (SCD-nc, MCI-nc) were considered.

Results: *CLOCK* T3111C was detected in 47% of cases (21 SCD, 11 MCI) and *PER2* C111G in 19% of cases (eight SCD and five MCI). *PER2* G carriers presented lower premorbid intelligence score ($P = 0.049$), fewer years of education ($P = 0.007$) and a lower frequency of family history of AD ($P = 0.04$) than G non-carriers. MCI *PER2* G carriers had worse performance in tests assessing memory, executive function, language and visuospatial abilities at baseline. During follow-up, two SCD and 15 MCI subjects progressed to AD: both of the SCD-c subjects presented the *PER2* G allele, while none of the SCD *PER2* G non-carriers converted to AD ($P = 0.003$).

Conclusion: *PER2* seems to have a role in cognitive reserve and cognition in SCD and MCI patients. Nevertheless, further studies are needed to assess the role of *PER2* C111G on the risk of progression to AD.

Introduction

Alzheimer's disease (AD) presents a presymptomatic period lasting from several years to decades [1]. Early stages of AD have been identified [2–4]: mild cognitive impairment (MCI) describes subjects with objective cognitive impairment without impact on instrumental activities of daily living [5] and it is considered transitional between a normal cognitive state and dementia; subjective cognitive decline (SCD) is defined as a self-experienced persistent decline in cognitive capacity in comparison with the subject's previously normal status, during which the subject has normal age-, sex- and education-adjusted performance on standardized cognitive tests [6]. Studies of patients with SCD have described the evidence of amyloid load by biomarker positivity similar to that seen in AD patients [7,8]. Recent meta-analyses suggested that older people with SCD are twice as likely to develop dementia as individuals without it [9,10], leading to the hypothesis that self-perceptions of change in cognition may be considered as a very early stage

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of AD before the detection threshold of current neuropsychological instruments is reached [11].

Amongst the symptoms of AD, sleep and circadian rhythm alterations are very common and several studies have suggested that sleep–wake cycle disturbances are associated with an increased risk of dementia and cognitive decline [12,13]. Sleep-related abnormalities have also been described in community-dwelling elderly people with subjective cognitive complaints [14] and in MCI patients [15]. Moreover, in preclinical AD, amyloid deposition seems to be associated with lower sleep quality [16]. It has been proposed that sleep–wake cycle alterations probably contribute to disease pathogenesis [17]; however, this role has to be confirmed.

The circadian system is hierarchically organized, with the central 'master' clock in the suprachiasmatic nucleus (SCN) of the hypothalamus, which synchronizes the peripheral clocks distributed in different brain regions and throughout the body, in order to coordinate physiology and behavior rhythm within a 24-h period. Both in central and in peripheral clocks, cells use a complex 'molecular machinery' composed of transcriptional–translational feedback loops to drive clock-controlled gene expression. The positive transcriptional limb of this loop is composed of the transcription factors BMAL1 (Brain Muscle ARNT-Like 1) and CLOCK, which heterodimerize and bind E-box motifs to drive the circadian transcription of clock-controlled genes, including *PER1*, *PER2*, *PER3* and *CRY* (cryptochrome), which are part of the negative regulatory limb of the molecular machinery [18].

Current research is focusing on polymorphisms of *CLOCK* and *PER* genes to better understand their role in sleep–wake cycle alterations, psychiatric disturbances and neurodegeneration. *CLOCK* T3111C (rs1801260) has recently been related to the quality of aging [19] and has been reportedly associated with the circadian phenotype of eveningness (i.e. individual preference for a later sleep schedule and presenting later peaks of alertness and performance) [20]. Also *PER* variants have been associated with alterations of circadian phenotypes: a correlation between *PER2* C111G (rs2304672) and morningness (i.e. individual preference for an earlier sleep schedule and presenting earlier diurnal peaks of alertness and performance) have been highlighted [21]; however, a recent study in a healthy Italian population did not confirm this result and did not support a role of this polymorphism in chronotypes [22]. The role of *CLOCK* T3111C and *PER2* C111G on cognitive function has not yet been studied, in particular in early stages of cognitive decline, nor has the role of these polymorphisms on the risk of development of AD been explored so far. The aim of our study was to evaluate the effect of *CLOCK* T3111C and *PER2* C111G polymorphisms on cognitive function and progression to AD in a population of SCD and MCI patients.

Materials and methods

Participants and clinical assessment

As part of a longitudinal, clinical–neuropsychological–genetic survey on SCD and MCI, 74 consecutive spontaneous subjects who self-referred to the Center for Alzheimer's Disease and Adult Cognitive Disorders of Careggi Hospital in Florence between April 1996 and May 2014 were included. All participants underwent a comprehensive family and clinical history, general and neurological examination, extensive neuropsychological investigation, estimation of premorbid intelligence and assessment of depression. A positive family history was defined as one or more first-degree relatives with documented cognitive decline. Sleep quality was assessed according to anamnestic data: those subjects who had difficulty falling asleep or woke up early or experienced frequent sleep interruptions were considered 'poor sleepers'; patients who did not report sleep disturbances were classified as 'good sleepers'. Inclusion criteria were (i) complaining of cognitive decline with a duration of ≥6 months; (ii) normal functioning on the activities of daily living and the instrumental activities of daily living scales [23]; (iii) unsatisfied criteria for dementia at baseline [24,25]; (iv) attainment of the clinical endpoint, i.e. conversion to AD, according to the National Institute on Aging and Alzheimer's Association (NIA-AA) criteria [25] during follow-up, regardless of follow-up duration; (v) a follow-up time of more than 2 years from the baseline visit for those patients who did not develop AD. Exclusion criteria were (i) history of head injury, current neurological and/or systemic disease, symptoms of psychosis, major depression, alcoholism or other substance abuse; (ii) complete data loss of patients' follow-up; (iii) progression to dementia other than AD.

From the initial sample, six subjects were excluded: two patients had a follow-up shorter than 2 years; two were diagnosed with psychiatric disturbances and one with frontotemporal dementia [26]; one patient received a diagnosis of vascular dementia [27]. Ultimately 68 subjects were included. All of them underwent apolipoprotein E (ApoE), *CLOCK* and *PER2* genotyping. This sample was divided into two groups: 41 subjects classified as SCD, according to the terminology proposed by the Subjective Cognitive Decline Initiative Working Group [6] (i.e. the presence of a

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self-experienced persistent decline in cognitive capacities with normal performance on standardized cognitive tests); 27 subjects classified as MCI according to NIA-AA criteria for the diagnosis of MCI [3] (i.e. evidence of lower performance in one or more cognitive domains with preserved independence of function in daily life). All patients underwent clinical and neuropsychological follow-up every 12 or 24 months.

On the basis of progression from SCD to AD during the follow-up, SCD subjects were classified respectively into SCD-converters (SCD-c) and SCD-non-converters (SCD-nc). In the same way, MCI subjects were classified as MCI-converters (MCI-c) and MCI-non-converters (MCI-nc).

The local ethics committee approved the protocol of the study. All participants gave written informed consent to participate.

Neuropsychological assessment

All subjects were evaluated by means of an extensive neuropsychological battery standardized and described in further detail elsewhere [28]. The battery consisted of global measurements (Mini-Mental State Examination, MMSE), tasks exploring verbal and spatial short-term memory (digit span; Corsi tapping test), verbal long-term memory (five words and paired words acquisition; recall after 10 min; recall after 24 h; Babcock Short Story immediate and delayed recall) and language (token test; category fluency task) [28]. Visuospatial abilities were also evaluated by the Rey–Osterrieth Complex Figure copy and visuospatial long-term memory was assessed by means of recall of the Rey–Osterrieth Complex Figure test [29]; attention/executive function was explored by means of dual task [30], phonemic fluency test [31] and trail making test [32]. Everyday memory was assessed by means of the Rivermead Behavioral Memory Test [33]. All raw test scores were adjusted for age, education and gender according to the correction factor reported in validation studies for the Italian population [28–33]. Premorbid intelligence was estimated by the Test di Intelligenza Breve (TIB) [34], an Italian version of the National Adult Reading Test [35]. The presence and severity of depressive symptoms were evaluated by means of the 22-item Hamilton Depression Rating Scale (HDRS) [36].

Apolipoprotein E ε4, *CLOCK* T3111C and *PER2* C111G genotyping

A standard automated method (QIAcube; QIAGEN, Hilden, Germany) was used to isolate DNA from peripheral blood samples. ApoE genotypes were investigated by high resolution melting analysis [37]. Two sets of polymerase chain reaction primers were designed to amplify the regions encompassing rs7412 (NC_000019.9: g.45412079C>T) and rs429358 (NC_000019.9: g.45411941T>C). The samples with known ApoE genotypes, which had been validated by DNA sequencing, were used as standard references. The ApoE genotype was coded as ApoE ε4– (no ApoE ε4 alleles) and ApoE ε4+ (presence of one or two ApoE ε4 alleles).

The analyses of *CLOCK* and *PER2* were performed using high resolution melting analysis in order to detect the 3111T/C *CLOCK* polymorphism using primers as reported [38] and the *PER2* C111G polymorphism with the following primers: forward 5′-ACAGAAAGAGTCAAATGGGTGC-3′, reverse 5′-TGTCCACATCTTCCTGCAGT-3′ with annealing temperature 60°C.

Statistical analysis

Patient groups were characterized using mean and standard deviation (SD). Scores on cognitive tests were reported as *z*-scores (*z*-scores were calculated as the raw score of the patient minus the mean score of the Italian general population divided by the SD of the Italian general population). The normal distribution of the data was tested using the Kolmogorov–Smirnov test. Depending on the distribution of the data, the *t* test or non-parametric Mann–Whitney *U* test was used for between group comparisons and Pearson's correlation coefficient or the non-parametric Spearman's ρ to evaluate correlations of between group numerical measures. The two-sided chi-squared test was used to compare categorical data and the effect size was calculated using Cohen's *d* for numerical measures and Cramer's *V* for categorical data. All statistical analyses were performed with SPSS software v.25 (SPSS Inc., Chicago, IL, USA). The significance level was set at $P < 0.05$.

Results

Demographic, clinical features and distribution of *CLOCK* and *PER2* genotypes

In the whole cohort, 32 of 68 subjects (47%) were *CLOCK* C carriers (29 TC, 3 CC), whilst 13 of 68 (19%) were *PER2* G carriers (13 CG, 0 GG). The genotypic distribution of the *CLOCK* and *PER2* genes in this sample was in Hardy–Weinberg equilibrium (*CLOCK* T3111C $\chi^2 = 0.91$, $P > 0.05$; *PER2* C111G $\chi^2 = 0.77$, $P > 0.05$). There were no differences in the prevalence of *CLOCK* T3111C and *PER2* C111G polymorphisms either in SCD subjects (21/41, 51.21% *CLOCK* T3111C carriers; 8/41, 19% *PER2* C111G

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carriers) or in MCI subjects (11/27, 41% *CLOCK* T3111C carriers; 5/27, 19% *PER2* C111G carriers) (Fig. 1 and Table 1).

Concerning *CLOCK* T3111C, at baseline there were no statistically significant differences between C carriers and non-carriers with respect to age at onset of symptoms, age at baseline evaluation, disease duration (time from onset of symptoms and baseline evaluation), follow-up time, familiality, sex, education, TIB, MMSE, HDRS score, sleep quality and ApoE ε4 allele status, not in the whole assay (Table 2) nor in SCD and MCI groups.

On the other hand, *PER2* G carriers had lower premorbid intelligence scores on TIB (104.29 ± 10.74 vs. 109.98 ± 8.06, $P = 0.049$), fewer years of education (7 ± 3.05 vs. 10.73 ± 4.51, $P = 0.007$) and lower frequency of family history of AD (15.38% vs. 60%, χ^2 8.37, $P = 0.004$) (Table 2). When the same analysis was performed on the SCD and MCI groups apart, a statistically significant difference was found between subjects carrying the *PER2* G allele and non-carriers in years of education only in the SCD group (7.50 ± 3.29 vs. 12.42 ± 4.13, $P = 0.004$); in the MCI sample, a family history of AD was more frequent in *PER2* G non-carriers as none of the *PER2* G carriers presented AD familiality (0% vs. 59.09%, χ^2 5.69, $P = 0.017$), while in the SCD group this difference was not confirmed (Table 3).

Cognitive functions and neuropsychological assessment

In the SCD group, no significant differences were found at baseline for any neuropsychological tests between *CLOCK* C carriers and non-carriers; similarly, scores on neuropsychological tests did not differ between SCD *PER2* G carriers and G non-carriers. In the MCI sample, *CLOCK* T3111C did not show any statistically significant influence on neuropsychological tests. On the other hand, MCI carrying *PER2* G allele had worse performances at baseline in tests assessing language (token test, $P = 0.030$), executive function (phonemic fluency test, $P = 0.033$), memory (five words acquisition, $P = 0.042$) and visuospatial abilities (Rey–Osterrieth Complex Figure copy, $P = 0.049$) (Fig. 2). In order to exclude that sleep quality could influence neuropsychological performance, the MCI sample was divided into two groups: good sleepers and poor sleepers. When scores at neuropsychological tests between the two groups were compared between these two groups, no significant differences were found (data not shown).

Table 1 *CLOCK* and *PER2* genotypes in SCD and MCI patients

		SCD (41)	MCI (27)	Total (68)
CLOCK	TT	20	16	36
	TC	20	9	29
	CC	1	2	3
PER2	CC	33	22	55
	CG	8	5	13
	GG	0	0	0

MCI, mild cognitive impairment; SCD, subjective cognitive decline.

Effect of *CLOCK* T3111C and *PER2* C111G on progression to AD

During the follow-up, 17 patients converted to AD, 2 of 41 SCD (7.14%) and 15 of 27 MCI (55.55%). Mean conversion time to AD was 12.39 (±2.28) years for SCD and 3.60 (±2.55) years for MCI. A total of 39 SCD subjects did not convert to AD (SCD-nc) and their mean follow-up time was 11.74 (±3.91) years (range 4.33–19.37 years, IQR 5.44 years); 12 MCI patients did not convert to AD (MCI-nc), with a

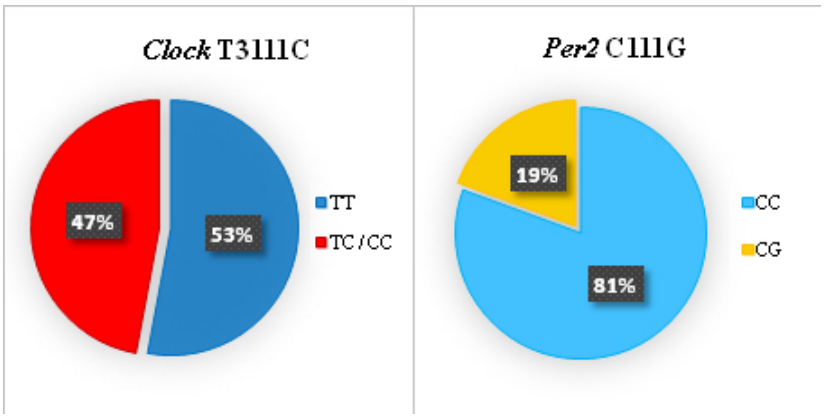

Figure 1 F Prevalence of *PER2* C111G and *CLOCK* T3111C polymorphisms. [Colour figure can be viewed at wileyonlinelibrary.com]

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Table 2 Demographic data of total samples in relation to *PER2* and *CLOCK* polymorphisms

	PER2				*CLOCK*			
Features	G non-carriers (55)	G carriers (13)	*P*	SE	C non-carriers (36)	C carriers (32)	*P*	SE
Age at baseline (±SD) in years	63.72 ± 9.32	65.22 ± 7.26	0.668	−0.180	64.29 ± 9.33	63.69 ± 8.60	0.777	0.067
Age at onset (±SD) in years	59.36 ± 10.21	62.38 ± 7.96	0.502	−0.330	60.56 ± 10.87	59.25 ± 8.64	0.694	0.133
Follow-up time (±SD) in years	10.79 ± 4.24	12.60 ± 4.68	0.204	−0.405	11.34 ± 4.31	10.90 ± 4.45	0.815	0.100
Disease duration (±SD) in years	4.36 ± 3.60	2.83 ± 2.33	0.082	0.505	3.73 ± 3.65	4.44 ± 3.18	0.195	−0.207
Sex (no. females, no. males)	38, 17	10, 3	0.577	0.068	26, 10	22, 10	0.754	0.038
Familiality (%)	60	15.38	**0.004**	0.351	41.66	62.5	0.086	0.086
Education in years (±SD)	10.73 ± 4.51	7 ± 3.05	**0.007**	0.969	9.86 ± 4.80	10.19 ± 4.21	0.737	−0.073
TIB (±SD)	109.98 ± 8.06	104.29 ± 10.74	**0.049**	0.599	110.30 ± 7.84	107.80 ± 9.45	0.427	0.288
MMSE (±SD)	28.47 ± 1.64	28.31 ± 1.49	0.583	0.102	28.09 ± 1.78	28.81 ± 1.33	0.064	−0.458
HDRS (±SD)	26.47 ± 4.01	27.77 ± 3.03	0.118	−0.366	26.44 ± 3.88	27.03 ± 3.86	0.506	−0.152
Poor sleepers (%)	45.09	63.63	0.264	0.142	48.48	48.27	0.987	0.002
ApoE ε4 (%)	36.36	23.07	0.362	0.110	30.55	37.5	0.546	0.073

ApoE ε4, apolipoprotein E; HDRS, Hamilton Depression Rating Scale; MCI, mild cognitive impairment; MMSE, Mini-Mental State Examination; SCD, subjective cognitive decline; SE, size effect; TIB, Test di Intelligenza Breve. Values indicated in the table are mean (±SD) or %. *P* indicates the level of significance for comparison between groups (statistical significance at $P < 0.05$, in bold characters). Age at baseline, age at onset, disease duration, follow-up and education are expressed in years. Age at baseline indicates age at the baseline evaluation; age at onset indicates age at the onset of symptoms of SCD or MCI; disease duration indicates time from onset of symptoms for MCI patients and time from the onset of subjective disturbances for SCD subjects at baseline evaluation.

Table 3 Demographic and clinical data of SCD and MCI patients: *PER2* G carriers versus non-G carriers

	SCD (41)				MCI (27)			
Features	*PER2* G non-carriers (33)	*PER2* G carriers (8)	*P*	SE	*PER2* G non-carriers (22)	*PER2* G carriers (5)	*P*	SE
Age at baseline (±SD) in years	61.05 ± 9.46	63.24 ± 6.30	0.409	−0.048	67.73 ± 7.68	68.37 ± 8.27	0.928	−0.080
Age at onset (±SD) in years	56.24 ± 10.38	60 ± 6.48	0.373	−0.088	64.05 ± 8.08	66.20 ± 9.33	0.832	−0.246
Follow-up time (±SD) in years	11.23 ± 3.58	14.68 ± 4.22	0.058	−0.319	10.12 ± 5.09	9.27 ± 3.45	1	0.121
Disease duration (±SD) in years	4.80 ± 3.79	3.24 ± 2.82	0.176	0.514	3.68 ± 3.25	2.17 ± 1.23	0.377	0.856
Sex (no. females, no. males)	23, 10	7, 1	0.308	0.159	15, 7	3, 2	0.726	0.067
Familiality (%)	60.60	25	0.07	0.283	59.09	0	**0.017**	0.459
Education in years (±SD)	12.42 ± 4.13	7.50 ± 3.29	**0.004**	0.85	8.18 ± 3.87	6.20 ± 2.77	0.344	0.412
TIB (±SD)	111.09 ± 7.34	107.82 ± 8.74	0.213	0.043	107.88 ± 9.13	99.87 ± 12.60	0.203	0.112
MMSE (±SD)	28.88 ± 1.43	28.50 ± 1.77	0.640	0.019	27.80 ± 1.79	28.00 ± 1.00	1	−0.010
HDRS (±SD)	26.61 ± 3.77	27.13 ± 3.27	0.528	−0.027	26.25 ± 4.48	28.80 ± 2.58	0.097	−0.124
Poor sleepers (%)	56.66	83.33	0.221	0.204	28.57	40	0.619	0.098
ApoE ε4 (%)	33.33	25	0.650	0.071	40.9	20	0.382	0.168

ApoE ε4, apolipoprotein E; HDRS, Hamilton Depression Rating Scale; MCI, mild cognitive impairment; MMSE, Mini-Mental State Examination; SCD, subjective cognitive decline; SE, size effect; TIB, Test di Intelligenza Breve. Values indicated in the table are mean (±SD) or %. *P* indicates level of significance for comparison between groups (statistical significance at $P < 0.05$, in bold characters). Age at baseline, age at onset, disease duration, follow-up and education are expressed in years. Age at baseline indicates age at the baseline evaluation; age at onset indicates age at the onset of symptoms of SCD or MCI; disease duration indicates time from onset of symptoms for MCI patients and time from the onset of subjective disturbances for SCD subjects at baseline evaluation.

mean follow-up time of 12.51 (±4.81) years (range 4.06–20.55 years; IQR 6.51 years). In the SCD group, no significant differences were found between SCD-c and SCD-nc in sex, familiality, disease duration, schooling, MMSE, HDRS and TIB. On the other hand, the MCI-c group was older than the MCI-nc at onset of symptoms (68.20 ± 7.48 vs. 59.75 ± 6.59, $P = 0.004$) and at baseline visit (71.39 ± 6.30, $P = 0.004$). ApoE ε4 was statistically significantly more frequent in MCI-c than MCI-nc (60% vs. 8.33%, $P = 0.006$) subjects (Table 4).

CLOCK T3111C polymorphism prevalence did not significantly differ between converters and non-converters, both SCD and MCI. Both the SCD-c subjects presented the *PER2* G allele, while none of the SCD *PER2* G non-carriers converted to AD ($\chi^2 = 8.67$,

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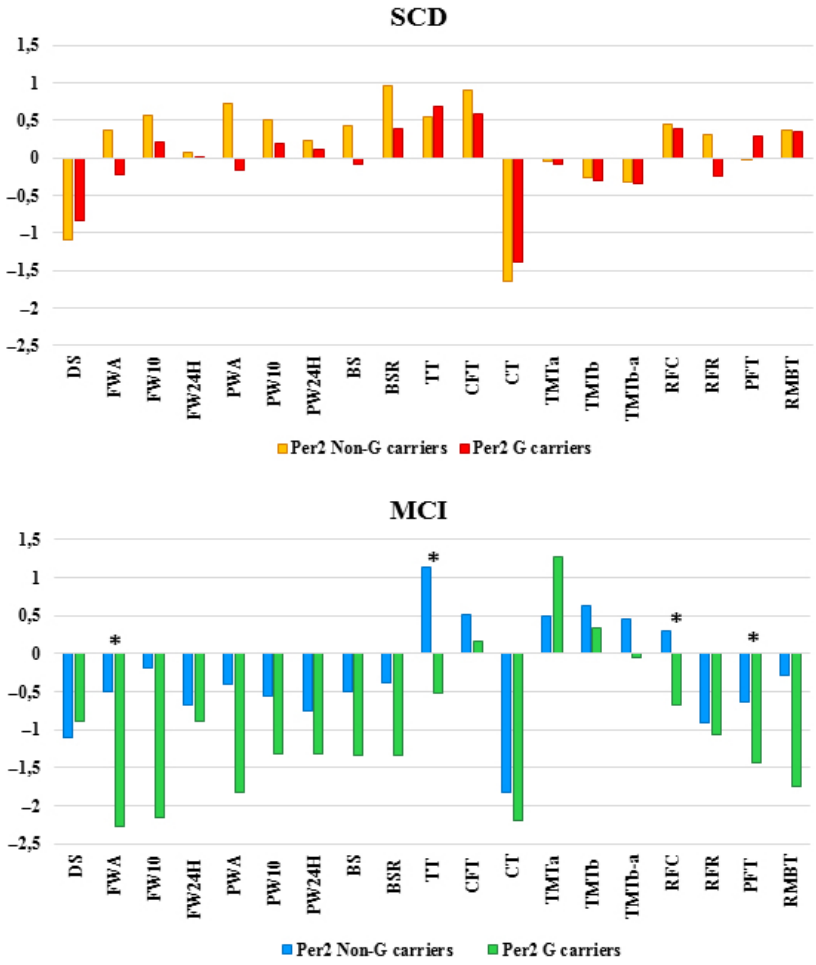

Figure 2 Neuropsychological assessment in SCD and MCI patients. Neuropsychological test scores are expressed as *z*-scores based on normative values. Negative values indicate worse performances than the age-matched normal population. For TMT-a, TMT-b, TMT-b-a, the higher the score, the worse the performance. DS, digit span; FWA, five words acquisition; PWA, paired words acquisition; BS and BSR, Babcock Short Story immediate and delayed recall; TT, token test; CFT, category fluency task; CT, Corsi tapping test; TMT, trail making test; RFC, Rey–Osterrieth Complex Figure copy; PFT, phonemic fluency test; RBMT, Rivermead Behavioral Memory Test. *Significant differences at $P < 0.05$. [Colour figure can be viewed at wileyonlinelibrary.com]

$P = 0.003$). There were no significant differences in the prevalence of *PER2* polymorphism between MCI-c and MCI-nc.

Discussion

This is the first study to investigate the role of *CLOCK* T3111C and *PER2* C111G polymorphisms on cognition and on the progression to AD in SCD and MCI patients.

No association between *CLOCK* T3111C and performances on neuropsychological tests and with the progression to AD was found, in either the SCD or the MCI group.

Differences between MCI *PER2* G carriers and G non-carriers were detected in tests assessing language, memory, executive functions and visuospatial abilities. The challenging question is how *PER2* C111G influences cognitive functions, since the underlying mechanisms are largely unknown. Circadian rhythm and sleep–wake cycle are strictly connected with cognitive functions, which have been shown to be impaired by sleep disorders and circadian alterations [39], and this link might be found at molecular level. Clock genes are widely expressed throughout the central nervous system, in particular in the hippocampus [40] and amygdala [41] (involved in learned behaviors) and in the prefrontal and orbitofrontal cortex, in particular in Brodmann areas 11 and 47 (involved in focused attention and executive functions) [42]. During a 24-h period, the expression of clock genes exhibits oscillations which seem to be region-specific and important

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Table 4 Demographic, genetic and clinical data: SCD-c versus SCD-nc and MCI-c versus MCI-nc

	SCD (41)				MCI (27)			
Features	SCD-c (2)	SCD-nc (39)	*P*	SE	MCI-c (15)	MCI-nc (12)	*P*	SE
Age at baseline (±SD) in years	64.33 ± 3.54	61.33 ± 9.09	0.624	0.435	71.39 ± 6.30	63.42 ± 6.96	**0.004**	1.201
Age at onset (±SD) in years	61 ± 4.24	56.77 ± 9.98	0.585	0.552	68.20 ± 7.48	59.75 ± 6.59	**0.004**	1.199
Follow-up time (±SD) in years	12.39 ± 2.28[a]	11.74 ± 3.91	0.512	0.203	3.60 ± 2.55[a]	12.51 ± 4.81	**0.000**	−2.315
Disease duration (±SD) in years	3.33 ± 0.69	4.56 ± 3.73	0.746	−0.459	3.19 ± 3.79	3.67 ± 1.75	0.083	−0.163
Sex (females, males)	2, 0	28, 11	0.380	0.137	10, 5	8, 4	1	0.000
Familiality (%)	50%	53.84%	0.915	0.017	53.33%	41.66%	0.547	0.116
Education in years (±SD)	6.50 ± 2.12	11.72 ± 4.36	0.102	−1.523	8.47 ± 4.10	7 ± 3.19	0.373	0.400
TIB (±SD)	102.92 ± 14.24	111.09 ± 7.06	0.270	−0.727	107.67 ± 9.68	104.21 ± 10.91	0.5	0.335
MMSE (±SD)	29 ± 1.41	28.79 ± 1.50	1	0.144	28 ± 1.03	27.64 ± 2.24	1	0.206
HDRS (±SD)	26.50 ± 0.70	26.72 ± 3.74	0.790	−0.082	27 ± 5.24	26.45 ± 2.73	0.727	0.132
ApoE ε4 (%)	50%	30.76%	0.569	0.089	60.00%	8.33%	**0.006**	0.532
Poor sleepers (%)	100%	57.14%	0.230	0.197	28.57%	33.33%	0.793	0.051
PER2 C111G carriers (%)	100% (2/2)	15.38 % (6/39)	**0.003**	0.459	13.33% (2/15)	25% (3/12)	0.438	0.149
CLOCK T3111C carriers (%)	50% (1/2)	51.28% (20/39)	0.972	0.006	33.33% (5/15)	50% (6/12)	0.381	0.169

ApoE ε4, apolipoprotein E; HDRS, Hamilton Depression Rating Scale; MCI, mild cognitive impairment; MCI-c, MCI-converters; MCI-nc, MCI-non-converters; MMSE, Mini-Mental State Examination; SCD, subjective cognitive decline; SCD-c, SCD-converters; SCD-nc, SCD-non-converters; TIB, Test di Intelligenza Breve. Values indicated in the table are mean (±SD) or %. *P* indicates level of significance for comparison between groups (statistical significance at $P < 0.05$, in bold characters). Age at baseline, age at onset, disease duration, follow-up and education are expressed in years. Age at baseline indicates age at the baseline evaluation; age at onset indicates age at the onset of symptoms of SCD or MCI; disease duration indicates time from onset of symptoms for MCI patients and time from the onset of subjective disturbances for SCD subjects at baseline evaluation; follow-up time indicates the time from baseline visit to the last evaluation. SE indicates size effect for comparison between SCD-c and SCD-nc and between MCI-c and MCI-nc. [a]In the SCD-c and MCI-c groups, follow-up time indicates conversion time to Alzheimer's disease.

for cognitive performance [43]: clock gene proteins and other molecules involved in long-term potentiation (LTP) present circadian oscillation in the hippocampus [44]. Studies conducted on mouse models have shown that *PER2*-mutant mice present reduced LTP and a decreased CREB phosphorylation, a critical step in the signaling pathway leading to strong LTP and certain long-term memories [45,46]. The reduced levels of p-CREB but not of total CREB due to *PER2* mutations seem to be regionally specific, since they were detected only in the hippocampus and not in the SCN, leading to the hypothesis that *PER2* has a specific role in the intracellular signaling pathways of LTP and implications for synaptic plasticity and learned behaviors [47].

Considering that clock genes encode for transcriptional factors and epigenetic modulators [48] with a role in memory and learned behaviors, it can be hypothesized that *PER2* may have implications for other cognitive functions using specific pathways not yet explored in other brain regions. In addition, it has been demonstrated that *PER2* expression oscillates in the orbitofrontal cortex, in particular in Brodmann areas 11 and 47, commonly associated with executive functions and focused attention [42].

It was also found that *PER2* G carriers presented lower premorbid intelligence scores and fewer years of education, which represent cognitive reserve proxies. Cognitive reserve is defined as the capacity to better cope with greater amounts of cerebral damage in brighter individuals [49]. The role of cognitive reserve in the progression from SCD and MCI to AD has recently been studied [50,51]. Many investigations have suggested that both genetic and environmental factors contribute to the amount of cognitive reserve [52]. Nevertheless, no conclusive data are available about the effect of genetic factors on cognitive reserve and most studies on this topic are centered on the influence of genetic factors on synaptic density [53]. In particular, a possible influence of *PER2* on cognitive reserve has not been explored yet and should be thoroughly investigated in future investigations. Furthermore, the issue of whether the effect of *PER2* on cognitive reserve might be a consequence of the detrimental effect of *PER2* polymorphism on cognitive functions or whether cognitive reserve might modulate the effect of *PER2* polymorphism on cognitive functions would be an interesting point to explore in future studies.

Concerning sleep disturbances, no differences were detected between good and poor sleepers in the prevalence of *CLOCK* and *PER2* polymorphisms, both in the whole cohort and in the SCD and MCI groups. The role of these two polymorphisms is controversial: Katzenberg *et al.* [20] showed that both homozygous and heterozygous individuals for *CLOCK* T3111C

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allele had greater evening preference in comparison to TT carriers in a Caucasian cohort. Similar results were found in a Japanese population [38], while other studies did not find any association between the C allele and morning or evening preferences [23,54,55]. The role of *CLOCK* T3111C on circadian rhythm phenotype preference is not clear. Some authors speculated that the same polymorphism could act differently depending on the latitude in which populations live [23]. Similarly, in one report *PER2* C111G was associated with alterations of circadian phenotypes, particularly with morningness [22], but another study did not confirm this result and did not support a role of this polymorphism in chronotypes [23].

Considering the conversion to AD, although the two SCD-c subjects were *PER2* G carriers and none of the SCD *PER2* G non-carriers converted to AD, no differences in the prevalence of *PER2* polymorphism were detected in the more likely to convert MCI group. Moreover, MCI patients carrying the *PER2* G allele presented a family history of AD less frequently.

PER2 gene and AD seem to be strictly connected since several studies on mouse models have shown that the expression of *PER2* is abnormal both in the SCN and hippocampus: circadian oscillation of *PER2* mRNA and protein in mouse SCN was disrupted by intrahippocampal injection of Aβ31–35 [56,57], leading to speculation that the alterations of *PER2* induced by Aβ may be relevant to the circadian rhythm disruption which characterizes AD [56]. Moreover, changes in the phase oscillations of clock genes like *PER2* have also been observed in the brain tissue of AD patients, and these alterations could lead to a reduced synchronization of clock gene activity across brain regions in AD patients [58].

Despite these interesting earlier results, no clear correlation was found between this polymorphism and conversion to AD. In fact, the absence of a clear prevalence of *PER2* G carriers in the MCI-c group and the limited number of SCD-c subjects did not allow to estimate the real contribution of both *PER2* gene and *PER2* C111G on the risk of progression to AD.

Indeed, the relatively small size of our cohort of patients is the first limitation of our study. For future work, the aim is to expand our sample in order to clarify our current findings and perform multivariant analysis to correct for possible confounding factors. Another limitation is the lack of AD biomarker data. Future studies including cerebrospinal fluid or neuroimaging data could provide interesting and additional information. Finally, as it is a single-center study, there may be biases with regard to assessment and diagnosis procedures.

On the other hand, this study has some remarkable strengths. To the best of our knowledge, this is the first study investigating the role of these specific clock gene polymorphisms on cognitive functions and on the risk of progression to AD in a cohort of well-defined SCD and MCI subjects. The very long mean follow-up time is an additional strength. In fact, follow-up time in the SCD-nc group is comparable to time of conversion in SCD-c, and MCI-nc is even much longer than the conversion time in MCI-c. This information allows the possible underestimation of conversion to AD and the risk of classifying subjects as stable who carry an Alzheimer pathology and will convert later in the follow-up to be minimized.

In conclusion, our preliminary study suggests a role of *PER2* C111G in the development of cognitive reserve and cognition, while *CLOCK* T3111C seems not to exert any influence on this construct. Even if a higher prevalence of *PER2* C111G was found in SCD subjects who converted to AD compared to patients who did not progress, the detrimental role of this polymorphism regarding the risk of progression to AD is still unclear. Further studies are needed to better explore and understand the role of genetic factors in cognition and in the development of AD from the earliest phases of the disease, in particular the influence of those genes which drive circadian rhythm and are implicated in cognitive reserve and cognitive functions.

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Disclosure of conflicts of interest

The authors declare no financial or other conflicts of interest.

Data availability statement

Data that support the findings of this study will be shared upon request from any qualified investigator.

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