



Remission of insomnia in older adults treated with cognitive behavioral therapy for insomnia (CBT-I) reduces p16^{INK4a} gene expression in peripheral blood: secondary outcome analysis from a randomized clinical trial

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Abstract Late life insomnia may increase risk for accelerated biological aging. Intervening to treat insomnia may provide protection from biological aging by reducing the prevalence of senescent cells in the immune system, as indicated by gene expression of a marker of cellular senescence, p16^{INK4a}. In the present study, we determine whether treatment of insomnia in older adults with cognitive behavioral therapy for insomnia (CBT-I) would reduce p16^{INK4a} gene expression in peripheral blood mononuclear cells (PBMC), compared to a sleep education therapy (SET), an active comparator condition. Secondly, we investigate the relationship between sustained insomnia remission and reduced expression of p16^{INK4a}. Participants 60+ years old with insomnia were enrolled in a randomized controlled trial and assigned to CBT-I or SET. Analyses

of 231 older adults (CBT-I=119; SET=112) examine baseline, post (2 months), and 24 months gene expression of p16^{INK4a}. Compared to baseline, expression of p16^{INK4a} increased in the SET group over 24 months ($P=0.03$), but showed no change in the CBT-I group. Those who received CBT-I and experienced sustained remission of insomnia had a significant decline in p16^{INK4a} expression by 24 months compared to baseline ($P=0.02$). Individuals not sustaining remission of insomnia exhibited overall increase expression of p16^{INK4a} by 24 months ($P=0.03$). In older adults with insomnia, p16^{INK4a} increases over 24 months, while CBT-I treatment of insomnia mitigates the increase in p16^{INK4a}. Further, sustained remission of insomnia using CBT-I leads to a decrease in p16^{INK4a}. These results suggest that behavioral interventions that are effective at treating insomnia might reduce the population of senescent cells in circulating blood.

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Introduction

Insomnia is a disorder characterized by an inability to initiate or sustain sleep at night, accompanied by daytime dysfunction. Estimated prevalence of insomnia increases with age with roughly half of adults over age 65 reporting sleep disturbances, and 10–25% experiencing more severe clinical insomnia

[1–4]. Insomnia among older adults raises risk for poorer health, frailty [5, 6], cognitive decline [7, 8], cardiovascular disease [9–11], cancer progression [12–14], and mortality [15, 16].

This effect of insomnia on health in older adults may be particularly detrimental in an aging biological system as aged systems have reduced capacity to respond to new insults. Aging within the immune system has implications for immune defenses, including reduced bacterial clearance, vaccine efficacy, defenses against viral attack, and slowed wound healing [17–21]. However, aging immune system can also have consequences for biological aging across tissues [17, 19, 22, 23], raising risk for chronic disease and death [24–26].

Although disease morbidity and mortality risk are elevated among those with sleep disturbances [1, 27–29], the specific molecular pathways altered by sleep loss, which impact human disease, are poorly defined, and these pathways may be particularly magnified in late life when there is reduced reserve capacity to rebound (i.e., resilience). Potential pathways include accelerating biological aging through an increase in cellular stress and accumulation of damage cells [30, 31] that enter a senescent state [32], which are a major contributor to biological aging [33–38].

Cellular senescence is a state of cell cycle arrest, commonly reached by cell replication (e.g., critically short telomeres) or excess cell stress (e.g., DNA damage) [17, 39–42]. Expression of p16^{INK4a}, a protein that inhibits cells from replicating, has been proposed as a biomarker of human aging, as it is expressed in cells that are senescent [43, 44]. Cellular senescence is thought to not only serve as a biomarker of aging but is also thought to promote the progression of biological aging, predominantly by increasing the release of proinflammatory and degrading secretory factors [41, 45–47]. Removal of senescent cells reverse or delay the aging pathology and improve lifespan in preclinical models [48, 49], resulting in the initiation of human trials to target these cells for elimination or reduction in diseases of aging [50], with the potential to impact cardiovascular disease progression, Alzheimer's disease and related dementias, arthritis, osteoporosis, sarcopenia, and immunosenescence [51]. This burgeoning research that seeks to extend human *healthspan* by reducing and eliminating diseases of biological aging, coined Geroscience, has a need for the identification

of modifiable behaviors that also may be directly targeted to slow the aging process [49, 52].

Insomnia is a modifiable behavioral target with established treatment efficacy that might alter biological aging trajectories. Cognitive behavioral therapy for insomnia (CBT-I) is an effective intervention to treat insomnia among older adults and typically includes cognitive therapy, stimulus control, sleep hygiene, and relaxation training [53, 54]. Insomnia is a behavior amenable to intervention but whether the successful remission of insomnia results in alterations to the expression of a marker of senescence has not been directly tested.

In the present study, we hypothesized that treatment of insomnia with CBT-I would reduce expression of p16^{INK4a} in peripheral blood mononuclear cells (PBMCs) compared to a sleep education therapy (SET), an active comparator condition. We further predicted that insomnia remission would protect from increases in p16^{INK4a}, whereas those with sustained insomnia were expected to have increasing p16^{INK4a} gene expression.

Methods

Trial design and oversight

This trial was investigator initiated, single site, masked (rater), partially blinded (participants), parallel-group, randomized controlled trial with recruitment from July 1, 2012 through April 30, 2015. Full trial design has been reported previously [see Irwin et al., 2022 Protocol Supplement (Appendix)] [55]. The trial and the subsequent R01 to support the current aims was funded by National Institute of Aging, which had no influence on design or conduct of the trial, and was not involved in data analysis, interpretation, or manuscript writing. Approval was obtained from the institutional review board at the University of California, Los Angeles, and conducted in accordance with the provision of the Declaration of Helsinki and Good Clinical Practices guidelines. All participants gave written informed consent.

Participants

Recruitment involved targeted enrollment of adults 60 years and older residing within 15 mi of the

UCLA Westwood campus with current insomnia. Screening eligibility for enrollment assessed presence of general sleep disturbance, determined by self-report (i.e., 15-item Pittsburgh Sleep Quality Index, PSQI, score > 5) [56], and the absence of a current depression (i.e., 10-item Center for Epidemiologic Studies Depression [CESD] score < 4) [57]. Interviews confirmed that participants met criteria for insomnia using diagnostic criteria of the Diagnostic and Statistical Manual for Mental Disorders, fourth edition (DSM-IV), and absence of major depression using Structured Clinical Interview (SCID) for DSM-IV or DSM-V within the last 12 months. Full inclusion and exclusion criteria are provided in a prior published Protocol [55]. In brief, participants with major medical conditions such as cancer, recent stroke or myocardial infarction, neurological disease, autoimmune disorders, regular use of opioids, psychotropic, or steroids were excluded.

Trial procedures

As reported previously [55], participants were randomized using a computer-generated random number sequence, with block sizes from 5 to 10 participants. Treatment allocation was masked so that research assessors were blinded to treatment allocation of the participants, *and participants were partially blinded*, i.e., *modified blind-to-treatment protocol*. CBT-I and SET interventions were delivered over an 8-week (2 months) period in weekly 120-min group sessions as reported previously. Briefly, CBT-I contained five validated components: cognitive therapy, stimulus control, sleep restriction, sleep hygiene, and relaxation. SET contained educational components: sleep hygiene, sleep biology, characteristics of healthy and unhealthy sleep, stress biology, and impact on sleep. SET is an active comparator as previously demonstrated [55], which was matched to CBT-I for time, attention, group interaction, and expectancy of benefit effects.

Peripheral blood samples were collected by venipuncture immediately prior to initiation of treatment (baseline), after completion of treatment (post, 2 months after baseline), and again at 24 months after baseline. PBMCs were isolated from heparinized whole blood by density gradient centrifugation within 2 h of blood draw. Up to 5×10^6 PBMCs were resuspended in RLT Lysis

Buffer (QIAGEN) plus β -2-mercaptoethanol for the preservation of mRNA, and frozen at -80°C until the completion of the study.

Insomnia remission

Remission of insomnia was determined using SCID DSM-IV criterion for insomnia at each post intervention visit as described previously [55]. Percentage of visits with no insomnia was calculated and used to determine two categories: (1) sustained remission (100% of follow up assessments with no insomnia) and (2) no sustained remission of insomnia (any follow up visits with insomnia).

Outcome

The primary outcome was the relative abundance of mRNA transcripts from the *CDKN2A* gene, which encodes p16^{INK4a}, a biomarker for cellular senescence and aging, in peripheral blood mononuclear cells (PBMCs). Assays were conducted as previously described [58], with preserved and frozen PBMC samples thawed and total RNA extracted (QIAGEN RNeasy), tested for suitable mass (PicoGreen RNA) and integrity (Agilent TapeStation), reverse-transcribed to cDNA (Illumina TruSeq stranded), and sequenced on an Illumina HiSeq 4000 instrument using the manufacturer's standard protocols. Assays targeted 10 million reads per sample (achieved average = 14.4 million), each of which was mapped to the GRCh38 reference human transcriptome using the STAR aligner, with *CDKN2A* mRNA abundance quantified as transcripts per million total mapped reads. Transcript abundance data were log₂ transformed for analysis as described below.

Statistical analyses

Analyses are aligned with CONSORT guidelines and were defined in a statistical analysis plan consistent with the primary trial results.⁵⁷ IBM SPSS Version 27 was used for all analyses. Characteristics of participants at baseline enrollment are reported and compared by treatment group (CBT-I vs. SET) using Wilcoxon and chi-square tests for differences. Primary outcome analyses tested *hypothesis 1*: relative to SET, CBT-I will have lower overall expression of p16^{INK4a} over time;

hypothesis 2: relative to unremitting insomnia, remission will be associated with less overall expression of p16^{INK4a} over time, and this will be most pronounced in the CBT-I group. These hypotheses were analyzed using linear mixed models, including all subjects in the intent-to-treat cohort. To test hypothesis 1, a 2 (CBT-I vs. SET) by 3 (baseline, post, 24 months) analysis was performed. Hypothesis 2 was tested using a 4 (CBT-I with remission, CBT-I without remission, SET with remission, SET without remission) by 3 (baseline, post, 24 months) design. Following this, mean differences between groups were evaluated using Fisher's least significant difference contrast. The primary model (model 0) does not control for covariates, providing an unconditional (generalizable) estimate of treatment effects on p16^{INK4a}. It was anticipated that randomization procedures would generate group equivalents on demographic, psychosocial, biobehavioral, and medical factors (i.e., age, BMI, gender, education, comorbidities, etc.); however, potential confounding variables, plus gene expression at baseline, were considered in a subsequent model (model 1) to determine whether the observed effects hold up when these variables are controlled. Given that depression was previously reported to be higher in the SET group compared to CBT-I group [55], we conducted additional secondary analyses controlling for incident depression in the model. While the sample size target was determined by the primary aims of the main trial, the present sample size provides greater than 80% power to detect modest differences ($d \geq 0.35$) within secondary analyses. "Minimally important differences" for health outcomes have been suggested to fall within the range of $d = -0.30$ to $d = 0.50$ [59, 60].

Results

Participants and treatment

A total of 291 participants were enrolled in the primary trial from August 2012 to April 2015, with 156 assigned to CBT-I and 135 to the SET group. Of the enrolled participants, a total of 231 (119 CBT-I; 112 SET) provided blood specimens at baseline plus at least one of two subsequent follow up visits to ascertain our primary outcome, p16^{INK4a} (see Fig. 1, CONSORT). Characteristics of participants enrolled in the trial who did not provide blood did not differ from those providing blood except for a modest difference

in gender (data in supplement Table 1). Among participants in the current analysis, those enrolled in the CBT-I compared to those in the SET group had on average a higher BMI and a higher number of years of education (Table 1). No other demographic or clinical feature differed between groups.

Treatment adherence and acceptability did not differ between treatment arms, as reported previously [55]. For the primary outcome data, p16^{INK4a}, the pattern of missing data was not different across groups ($X^2 = 2.11$; $P = 0.15$).

Primary outcome

Analyses of the effect of treatment condition (CBT-I vs SET) on p16^{INK4a} expression over time (baseline, post, 24 months) was significant ($P = 0.023$), such that participants assigned to the SET condition had increased expression of p16^{INK4a} over 24 months, while those assigned to the CBT-I exhibited no increases in p16^{INK4a} expression over 24 months (Fig. 2; Table 2). The proportion of participants who achieved remission of insomnia disorder immediately following treatment (2 months after baseline) in the current sample was greater in the CBT-I group (61 [51.3%]) compared to the SET group (41 [37.3%], adjusted $\beta = 0.67$; 95%CI, 0.12–0.1.00; $P = 0.02$). For sustained remission of insomnia disorder (i.e., insomnia remission 24 months after baseline), similar results were found in the CBT-I group (33 [27.7%]) compared with the SET group (20 [17.9%]; adjusted $\beta = 0.77$; 95% CI, 0.10–1.00; $P = 0.03$). Linear mixed models testing the interaction of treatment with and without remission (4 groups) on p16^{INK4a} over time (3 timepoints) was significant ($P = 0.016$; Table 2; Fig. 3). Insomnia remission in the CBT-I group was associated with the greatest difference in p16^{INK4a} expression values over time, such that those enrolled in the CBT-I who had full remission of insomnia exhibited a significant decrease in p16^{INK4a} by 24 months, a significant change from baseline in this group ($P = 0.02$). Individuals not sustaining remission of insomnia in the CBT-I or SET group exhibited overall increase expression of p16^{INK4a} by 24 months ($P = 0.025$).

Secondary analyses

Secondary analyses further adjusted for age, BMI, gender, education, and comorbidities, plus the baseline level of p16^{INK4a} expression. The effect of

Fig. 1 CONSORT flow diagram

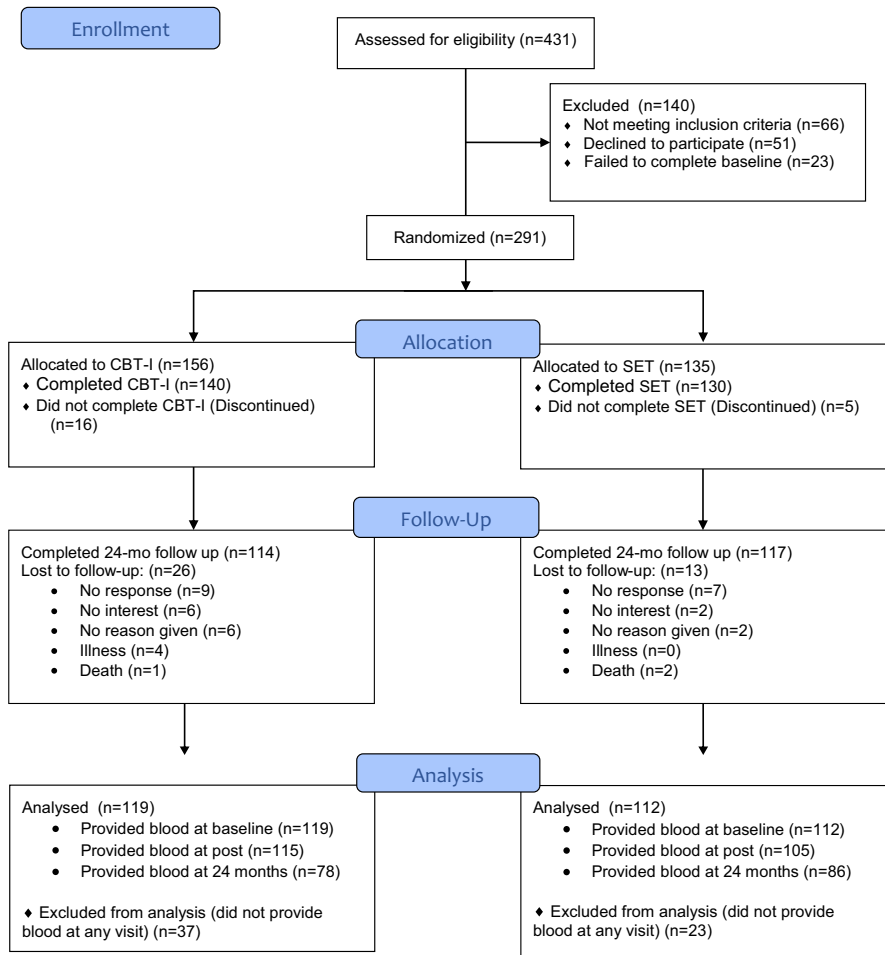


Table 1 Baseline sample characteristics by treatment group

	CBT-I (n = 119)	SET (n = 112)	<i>t</i> (χ^2)	<i>P</i>
	Mean (SD) or %	Mean (SD) or %		
Age (years)	70.2 (7.0)	69.7 (6.0)	0.65	0.52
Race (% White)	84.6%	83.9%	0.02	0.89
Gender (% Female)	51.3%	58.0%	1.07	0.30
Body Mass Index (BMI, kg/m ²)	27.4 (4.2)	26.0 (4.1)	2.51	0.013
Education (Years)	17.1 (2.5)	16.4 (2.4)	2.23	0.027
Smoker (% Yes)	2.5%	6.3%	1.98	0.16
Comorbidity Score	2.8 (1.1)	2.7 (0.8)	0.39	0.70
Baseline log ₂ p16 ^{INK4a} gene expression	0.68 (1.6)	0.65 (1.6)	0.15	0.88

Comorbidity score is derived from the Charlson Comorbidity Index [72]

treatment condition on p16^{INK4a} expression over time remained significant (*P* = 0.048), and analyses testing insomnia remission in the CBT-I group were similar

after adjustment for covariates (*P* = 0.055) (Table 2). Further adjusting for depression incidence had no effect on the results in Table 2 (data not shown).

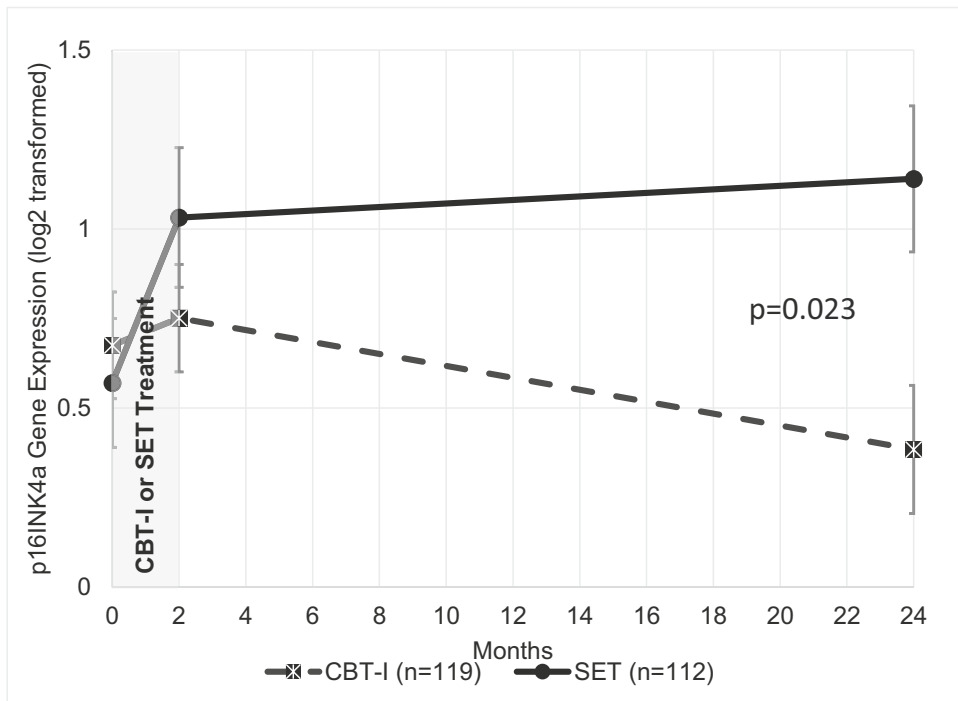


Fig. 2 The effect of treatment on p16^{INK4a} gene expression. The adjusted means and standard errors for log2 transformed p16INK4a are derived from mixed linear models including all subjects in intent-to-treat analyses, and graphically displayed in the figure for the two treatment groups, cognitive behavioral

therapy for insomnia (CBT-I) (dashed line) and sleep education therapy (SET) (solid line) at baseline (prior to treatment), following treatment (2 months), and 24 months after treatment initiation. The *p* value shown is for the time by treatment interaction

Table 2 Mixed model effect of treatment group and remission status on gene expression of p16^{INK4a} (*N*=231 total participants, all available timepoints)

	Model 0		Model 1, Adjusting for age, BMI, education, comorbidity, baseline gene expression	
	<i>F</i> (<i>df</i>)	<i>P</i>	<i>F</i> (<i>df</i>)	<i>P</i>
<i>p16^{INK4a} gene expression</i>				
Time ^a	1.73 (2, 398.0)	0.18	1.20 (2, 407.7)	0.31
Treatment	3.08 (1, 228.7)	0.081	14.16 (1, 197.4)	<0.001
Remission	1.31 (1, 228.7)	0.26	0.84 (1, 201.5)	0.37
Treatment × time	3.82 (2, 398.0)	0.023	3.06 (2, 409.2)	0.048
Time ^a	1.72 (2, 398.0)	0.18	1.20 (2, 407.7)	0.31
Four group ^b	2.42 (3, 228.5)	0.067	6.46 (3, 198.9)	<0.001
Time × four groups	2.64 (6, 396.3)	0.016	2.08 (6, 406.2)	0.055

^aThree timepoints: baseline, post (2 months), 24 months

^bFour groups: cognitive behavioral therapy for insomnia (CBT-I) with remission, CBT-I without remission, sleep education therapy (SET) with remission, SET without remission

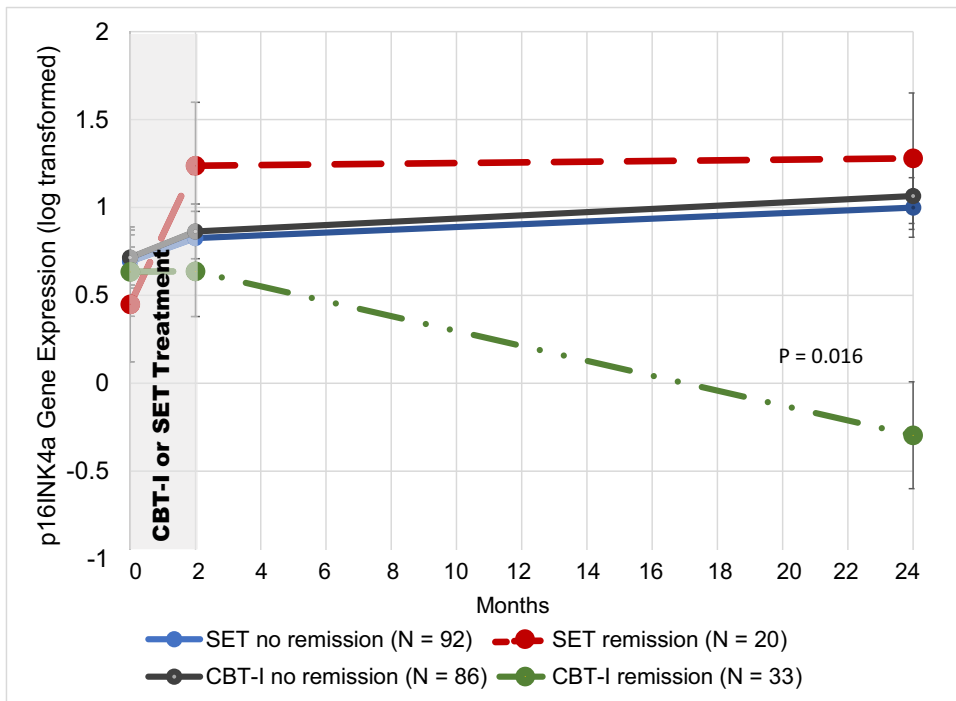


Fig. 3 The effect of treatment and sustained remission status on p16^{INK4a} gene expression. The adjusted means and standard errors for log₂ transformed p16^{INK4a} are derived from mixed linear models including all subjects in intent-to-treat analyses, and graphically displayed in the figure for baseline (prior to treatment), following treatment (2 months), and 24 months

after treatment initiation for four groups, sleep education treatment (SET) without remission (Blue), SET with sustained remission (red), cognitive behavioral therapy for insomnia (CBT-I) without remission (Black), and CBT-I with sustained insomnia remission (green line). The *P* value shown is for the group by time interaction

Discussion

The current study examined whether a randomized clinical trial to treat insomnia among older adults could result in modification of a hallmark of biological aging, namely cellular senescence. Older adults assigned to a cognitive behavioral therapy for insomnia had no increases over 2 years in the expression of the gene for p16^{INK4a}, a marker of cellular senescence, in peripheral blood mononuclear cells. In contrast, those assigned to the control arm of the study had significant increases in p16^{INK4a} gene expression over the same 2-year time interval. In analyses of individuals with sustained insomnia remission 2 years after the initiation of cognitive behavioral therapy treatment, we observed significant decreases in cellular senescence-associated

gene expression over 2 years, while those with chronic unremitting insomnia had increased cell senescence gene expression over 2 years. These results point to a role of insomnia in accelerating biological aging, while successful treatment and remission of insomnia resulted in reduction in this aging marker.

One in four older adults suffer from moderate to severe clinical insomnia [1–4], making this a highly salient modifiable behavioral factor that, when treated successfully, may slow the aging process by reducing cell senescence. As insomnia raises risk for poorer health, frailty [5, 6], cognitive decline [7, 8], cardiovascular disease [9–11], cancer progression [12–14], and mortality [15, 16], there is a high need to address this modifiable target in clinical practice. Our prior work identified lowered inflammation and reduced

risk profiles for disease following successful remission of insomnia in older adults receiving either CBT-I or Tai Chi interventions [61, 62]. This study extends these results, suggesting that successful remission of insomnia alters levels of a marker of cellular senescence in circulating blood. Future research will need to better understand how insomnia drives aging and the mechanisms that are driving declines in p16^{INK4a} expression levels following successful remission. Long-term follow-ups of individuals with successful remission of insomnia compared to those with ongoing chronic insomnia would be needed to understand the lasting effect of the treatment on biological aging and risk for disease.

Another feature of this study that may have strengthened the results is the characteristics of the cohort. As aged systems are thought to be less resilient to challenges such as sleep loss, our intervention may have a stronger effect in an older adult sample than if the intervention was delivered to younger adults with insomnia. Additional research will be necessary to test vulnerability and resilience to the efficacy of the intervention to slow biological aging across the lifespan.

Of note, our marker of cellular senescence captures aging within the circulating immune system, which has implications for immunity [17–21], but may also contribute to aging processes across other tissues in the body [17, 19, 22, 23], and be a link to chronic disease and death [24–26]. Specific molecular pathways altered by insomnia and treatment of insomnia are not entirely clear. Similar to studies testing the biological pathways driving aging in response to chronic stress exposure [63–65], insomnia is associated with elevated anxiety, stress responsivity, and activated catecholaminergic system [66]. Activation of this system is thought to drive inflammation [67] and elevate mitochondrial metabolism, raising cellular stress and accumulation of damage [30, 31, 64]. These pathways are involved in driving aging and cellular senescence [48]. In contrast, a reduction in the overall expression of a senescence-related gene following improvements in sleep may indicate enhanced clearance of senescent cells during sleep intervals, parallel to research investigating the importance of sleep for clearing waste products in the brain [68, 69], or may reflect

reduced development of expression (and therefore, senescence) within the PBMC population with improved sleep, resulting in less overall gene expression over time. Testing the pathways specifically involved in altering the expression of p16^{INK4a} in PBMCs following remission of insomnia by CBT-I will be an important next step.

Despite many strengths of the current study design including a randomized clinical trial, detailed assessments of insomnia status and covariates, and a longitudinal follow up of 2 years, a few limitations of the current study should be noted. First, our sample was comprised of moderately mobile and healthy older adults and may not generalize to those with multimorbidity, frailty, or chronic inflammatory conditions. Second, as our sample was comprised of older adults, the results need to be replicated in other age groups. Last, the sample was representative of the nearby community of patients at our institution, predominantly Caucasian and of upper middle income, and results of the trial may differ when applying it to other socioeconomic or ethnic/racial groups with divergent risk. Further research is needed to understand the role of insomnia remission at slowing biological aging in populations of different racial and ethnic make-up, ages, regions of the US and other countries, and economic backgrounds.

Conclusions

The current randomized clinical trial of Cognitive Behavioral Therapy for Insomnia (CBT-I) compared to a sleep education control group was effective at altering the gene expression for the biological aging biomarker p16^{INK4a} in older adult patients initially diagnosed with insomnia. Those in the CBT-I group who experienced sustained remission of insomnia had significant lowering of p16^{INK4a} gene expression while those in the control group who had sustained insomnia over the 2-year interval exhibited increases in p16^{INK4a}. These results extend prior work linking insomnia in late life with hallmarks of biological aging [70, 71], and point to the utility of an effective intervention to treat insomnia [53, 54] which may also be useful for the field of Geroscience by identifying

a modifiable behavior (i.e., insomnia) that alters one key element of the aging process, cellular senescence.

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Declarations

Competing interests The authors declare no competing interests.

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