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RESEARCH PAPER

Circulating miRNAs are associated with sleep duration in children/adolescents: Results of the I.Family Study

Giuseppe Iacomino[1]* | Fabio Lauria[1]* | Paola Russo[1] | Pasquale Marena[1] | Antonella Venezia[1] | Nunzia Iannaccone[1] | Stefaan De Henauw[2] | Ronja Foraita[3] | Regina Heidinger-Felső[4] | Monica Hunsberger[5] | Yiannis Kourides[6] | Luis A Moreno[7] | Barbara Thumann[3] | Toomas Veidebaum[8] | Alfonso Siani[1] | on behalf of the IDEFICS/I.Family consortium

[1]Institute of Food Sciences, National Research Council, ISA-CNR, via Roma 64, 83100, Avellino, Italy

[2]University of Ghent, C. Heymanslaan 10, 4K3 9000, Gent, Belgium

[3]Leibniz Institute for Prevention Research and Epidemiology, BIPS, Achterstraße 30, 28359, Bremen, Germany

[4]Medical School, University of Pécs, Szigeti út 12, 7624, Pécs, Hungary

[5]Sahlgrenska Academy at the University of Gothenburg, Medicinaregatan 3, 413 90, Göteborg, Sweden

[6]Research and Education Institute of Child Health, 138, Limassol ave, #205 2015, Strovolos, Cyprus

[7]University of Zaragoza, Spain; Domingo Miral, s/n, 50009, Zaragoza, Spain

[8]National Institute for Health Development, Hiiu 42, 11619, Tallinn, Estonia

Correspondence
Fabio Lauria, Institute of Food Sciences, National Research Council, Avellino, Italy, Via Roma, 64, 83100 Avellino, Italy.
Email: flauria@isa.cnr.it

*G. Iacomino and F. Lauria contributed equally to this paper.

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Abstract

It is commonly recognized that sleep is essential for children's health, and that insufficient sleep duration is associated with negative health outcomes. In humans, sleep duration and quality are influenced by genetic, environmental and social factors. Epigenetic mechanisms, likewise, regulate circadian rhythms and sleep patterns. In the present study, we aimed to identify circulating microRNAs associated with sleep duration in a subsample of normal-weight European children/adolescents ($n = 111$) participating in the I.Family Study. Subjects were divided into two groups based upon self-reported sleep duration, according to the recommended amount of sleep for paediatric populations. Sleep needs for children <13 years were at least 9 h per day, and for children >13 were at least 8 h per day. There were group differences (short sleepers *versus* normal sleepers) in circulating levels of miR-26b-3p (mean (95% CI) = 2.0 (1.3–2.7) *versus* 2.3 (1.9–2.7), $P = 0.05$) and miR-485-5p (mean (95% CI) = 0.6 (0.3–0.9) *versus* 0.9 (0.7 – 1.0), $P < 0.001$), adjusting for country of origin, age, sex, pubertal status, screen time and highest educational level of parents. Our findings show for the first time that sleep duration reflects the profile of specific circulating microRNAs in school-aged children and adolescents. It is conceivable that epigenetic modifications, mainly related to circadian rhythm control, may be modulated or interfere with sleep duration.

KEYWORDS
children/adolescents, microRNAs, sleep duration

1 | INTRODUCTION

In humans, sleep duration and pattern are influenced by various environmental factors, including lifestyle, food consumption, temperature, light–dark cycle, stress, diseases, sleep–wake history, occupation and socio-economic status. Several genes with critical roles for sleep quality, sleep duration and sleep timing have been explored (Archer & Oster, 2015). Epigenetic mechanisms may also

regulate circadian rhythms (Wang et al., 2016) as well as sleep patterns (Powell & LaSalle, 2015; Tucci, 2016). Epigenetic modulation of gene expression is primarily accomplished through processes of DNA methylation and histone modification, in addition to the role of non-coding RNAs. microRNAs (miRNAs) represent a class of small RNAs acting as post-transcriptional regulators of gene expression by base-pairing with their target mRNAs (Iwakawa & Tomari, 2015). At present, up to 2595 different miRNAs have been described in humans (miRTarBase, release 7.0).

Each miRNA can target many transcripts, and individual mRNAs may include binding sites for multiple miRNAs (Friedman, Farh, Burge, & Bartel, 2009). The concurrent targeting of several genes leads to a specific fine-tuning through the regulation of specific cellular sub-networks (Lewis, Burge, & Bartel, 2005; Paul et al., 2017; Thomou et al., 2017; Turchinovich, Weiz, Langheinz, & Burwinkel, 2011). It has been reported that changes in circulating miRNA levels might be used as stable and accessible biomarkers for a variety of physiopathological settings (Iacomino & Siani, 2017; Kim, 2015). miRNAs are abundantly expressed also in the brain, where they are actively involved in defining the nervous system development and its physiology (Cao, Li, & Chan, 2016). Remarkably, miRNA expression profiles have been confirmed to be altered in the brain tissue of patients with psychiatric disorders, emphasizing their significant diagnostic roles and offering novel targets for therapeutic development (Issler & Chen, 2015). The involvement of miRNAs in sleep regulation has been addressed also in animal models, confirming that sleep deprivation can affect miRNA levels in both brain and adipose tissue (Davis, Bohnet, Meyerson, & Krueger, 2007; Gharib, Khalyfa, Abdelkarim, Bhushan, & Gozal, 2012). Of note, it was reported that acute sleep deprivation and recovery may differently affect blood miRNA levels in healthy volunteers (Weigend et al., 2018). Moreover, it was recently reported that chronic short sleep is associated with a marked reduction in circulating levels of miR-125a, miR-126 and miR-146a in adults (Hijmans et al., 2019). However, the role of circulating miRNAs in controlling sleep duration has not yet been explored in school-aged children and adolescents.

In the present study, we aimed to identify differential patterns of circulating miRNAs associated with sleep duration in a subsample of normal-weight European children/adolescents of the I.Family Study (www.ifamilystudy.eu) (Ahrens et al., 2017; Iacomino et al., 2016), and to explore their potential functional implications.

New Findings

- **What is the central question of this study?**
  Are differential patterns of circulating miRNAs associated with sleep duration in normal-weight European children and adolescents?
- **What is the main finding and its importance?**
  Differences in the expression level of circulating miR-26b-3p and miR-485-5p are positively associated with total sleep duration in healthy normal-weight children and adolescents.

2 | METHODS

2.1 | Ethical approval

This study was registered on the ISRCTN registry (ISRCTN62310987) and a complete description of the project has been previously published (Ahrens et al., 2017). The study was conducted according to the latest version of the *Declaration of Helsinki*. Approval by the appropriate ethics committees was obtained by each of the eight participating centres carrying out the fieldwork: (1) Belgium: Ethics Committee of the Gent University Hospital, 19/02/2013, No. B670201316342; (2) Cyprus: Cyprus National Bioethics Committee, 21/Feb/2013, No. EEBK/ETI/2012/33; (3) Estonia: Tallinn Medical Research Ethics Committee (TMREC), 17/January 2013, No. 128; (4) Germany: Ethic Commission of the University of Bremen, 11/12/2012; (5) Hungary: Medical Research Council, 18/12/2012, 4536/2013/EKU; (6) Italy: Ethics Committee of the Local Health Authority (ASL) in Avellino, 18/Sep/2012, No. 12/12; (7) Spain: Ethics Committee for Clinical Research of Aragon (CEICA), 13/Feb/2013, No. PI13/0012; (8) Sweden: Regional Ethics Research Board in Gothenburg, 10/Jan/2013, No. 927-12. Participants were not subjected to any study procedure before both the children and their parents gave their oral (children) and written (parents) informed consent for examinations, collection of samples, subsequent analysis, and storage of personal data and collected samples.

2.2 | Study population

The miRNA screening was conducted on plasma samples in a subsample of healthy European children/adolescents belonging to the cohort of the I.Family Study, an European Commission-funded project finalized to investigate the determinants of food choice, lifestyle and related health outcomes in children and adolescents of eight European countries (Ahrens et al., 2017). In the framework of the I.Family survey, we pre-planned a substudy mainly aimed at assessing the association between the expression of circulating miRNAs and patterns of body fat accumulation. A complete description of the subsample and of the selection criteria can be found in Iacomino et al. (2019). Briefly, in each country, we selected 20 children who retained normal weight, i.e. who showed a body mass index (BMI) *z*-score between −1 and +1 at baseline and follow-up and did not change more than ±0.1 in BMI *z*-score per year (defined as normal weight), and 20 children who retained overweight or obese, i.e. who had a BMI *z*-score of more than +1 at baseline and follow-up, respectively, and did not change more than ±0.1 in BMI *z*-score per year (defined as overweight/obese). In the present study we included only the normal weight children/adolescents from eight European countries ($n = 111$: Belgium, $n = 15$; Cyprus, $n = 7$; Estonia, $n = 20$; Germany, $n = 14$; Hungary, $n = 15$; Italy, $n = 11$; Spain, $n = 16$; and Sweden, $n = 13$), with a complete dataset, including the

selected miRNA levels, and self-reported sleep duration and quality data.

2.3 | Anthropometric measurements

Anthropometric data were collected using standardized procedures; a detailed description of methods has been elsewhere published (Stomfai et al., 2011). Body height was measured, without shoes, with a calibrated stadiometer (SECA 225; Seca, Birmingham, UK) to the nearest 0.1 cm. Subjects were weighed in light clothes and without shoes using an electronic scale (Tanita BC 420 SMA, Tanita Europe GmbH, Sindelfingen, Germany) with an approximation of 0.1 kg. BMI was calculated as weight in kg divided by the square of height in metres (kg m^{-2}). Sex- and age-specific BMI *z*-scores were calculated (Cole & Lobstein, 2012). Children/adolescents were classified as normal weight according to the cutoffs released by IOTF (Cole & Lobstein, 2012).

2.4 | Sleep and questionnaires

On the day of the physical examination, information about sleep was collected. Participants reported night sleep duration and napping time (hours and minutes) separately for school/work days and weekend days/vacations. A weighted average of total sleep duration (night sleep + napping time) was calculated for each subject as follows: (sleep duration on school/work days × 5 + sleep duration on weekend days/vacations × 2)/7.

Based on the average sleep onset time of children and adolescents, the population was also split into 'early sleep onset' and 'late sleep onset' subjects to classify different chronotypes. Moreover, to study the increase of sleep time during the weekend (weekend rebound) as a marker of sleep debit, the population was categorized into two groups based on an additional sleep time of 1.5 h during the weekend.

Furthermore, on a subsample of subjects, sleep quality was investigated by asking whether the child/adolescent 'snored' (less than once a week/more than once a week; $n = 76$), had 'trouble getting up in the morning' (yes/no; $n = 91$), had 'difficulties falling asleep' (yes/no; $n = 91$), and what in general was their perceived sleep quality (very good, fairly good, fairly bad, very bad; $n = 91$) (Magee, Robinson, & Keane, 2017; Thumann et al, 2019).

According to the consensus recommendations for the amount of sleep needed to promote optimal health in children and adolescents (Paruthi et al., 2016), children aged 6–12 should sleep at least 9 h, and adolescents aged 13–18 should sleep at least 8 h per 24 h on a regular basis. Consequently, study subjects were divided into two groups: (1) 'short sleepers' including children sleeping less than 9 h per day and adolescents sleeping less than 8 h per day; and (2) 'normal sleepers' including children sleeping at least 9 h per day and adolescents sleeping at least 8 h per day.

Information about the educational level of parents, screen time and pubertal status were collected using a questionnaire filled in at home by parents. The parental education level was assessed by asking parents for their highest educational attainment and categorized according to the International Standard Classification of Education (ISCED) into low (ISCED levels 1 and 2), medium (ISCED levels 3 and 4), and high (ISCED level 5) educational attainment (Unesco Institute for Statistics, 2012). In addition, parents reported how much time their child usually spent watching television or the web (screen time). Pubertal status was evaluated asking parents if menarche has occurred in girls or if voice alterations have started or were completed in boys.

2.5 | miRNA extraction and profiling

Experimental methods for miRNA extraction and screening from plasma have been already published (Iacomino et al., 2016, 2019). Taking advantage of qPCR array technology, we previously identified two circulating miRNAs (hsa-miR-26b-3p, MIMAT0004500, and hsa-miR-485-5p, MIMAT0002175) potentially connected to sleep duration (unpublished data). In the present study, we aimed to confirm their association with sleep duration in a sample of normal-weight children/adolescents, through validation by SYBR Green-based real-time quantitative RT-PCR (RT-qPCR) by using the miScript Primer Assays, according to the manufacturer's recommendations (Qiagen, Hilden, Germany). Individual plasma samples were first screened for haemoglobin levels. miRNAs were extracted and quantified in single assays performed in triplicate by RT-qPCR. Finally, miRNA relative levels were normalized using the spike-in Cel-miR-39 (Iacomino et al., 2019).

2.6 | Bioinformatics

Biological targets of miRNAs were explored *in silico* by using miRPath v3.0 (Vlachos et al., 2015), which achieves an advanced analysis such as hierarchical clustering of miRNAs and pathways based on the levels of their interactions. miRNA targets were predicted by the DIANA-microT-CDS algorithm or experimentally validated miRNA interactions derived from DIANA-TarBase v7.0 (Vlachos et al., 2015). Predicted targets were further evaluated through the use of the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2014). Molecular interactions were likewise predicted by GeneMania (Warde-Farley et al., 2010), a tool for integrated gene analysis. The distribution of the miRNAs in human tissues was assessed using the miRmine Human miRNA Expression Database (Panwar, Omenn, & Guan, 2017).

2.7 | Statistical analysis

Characteristics of the study population are reported as mean (95% confidence interval) or frequency (%) as indicated. The distribution of miRNA levels was assessed by the Shapiro–Wilk test. Kendall's tau non-parametric test was used to study the correlation between variables of interest. Associations between miRNA levels and sleep categories and sleep quality were performed by analysis of variance (general linear model) adjusting for covariates (country of origin, age, sex, pubertal status, screen time – including TV and web consumption – and highest educational level of parents defined according to the ISCED). A *P*-value less than 0.05 was considered statistically

TABLE 1 Characteristics of the sample according to sleep categories

SLEEP	Short sleepers ($n = 25$)	Normal sleepers ($n = 86$)	P
Boys/girls (n)	11/14	39/47	
Age (years)	12.0 (11.5–12.4)	12.1 (11.7–12.5)	0.695
BMI (kg m^{-2})	18.2 (17.3–19.0)	18.3 (17.8–18.7)	0.889
BMI z-score	0.2 (−0.1 to 0.5)	0.2 (0.4–0.3)	0.994
Screen time per week (h)	23.8 (18.4–29.1)	19.4 (16.5–22.3)	0.145
Snoring (% less than once a week)	20.0	24.1	$\chi^2 = 0.452$
Trouble getting up in the morning (% yes)	25.0	21.0	$\chi^2 = 0.398$
Difficulties falling asleep (% yes)	20.7	20.0	$\chi^2 = 0.725$
Perceived sleep quality (% good)	52.0	48.8	$\chi^2 = 0.479$
Pubertal maturation (% yes)	60.0	58.1	$\chi^2 = 0.868$
Parental education (% high)	45.8	39.7	$\chi^2 = 0.596$

Data are expressed as mean (95% confidence interval) or as frequency (%).

significant. As a measure of the effect size, the partial eta-squared value (η_p^2) was also determined.

Statistical analyses were performed using IBM SPSS Statistics v23.0 (IBM Corp., Armonk, NY, USA).

3 | RESULTS

Characteristics of the 111 study subjects are reported in Table 1 in which children/adolescents were divided based on of the recommended sleep needs (short sleepers *versus* normal sleepers) (Paruthi et al., 2016); no significant differences were observed between the two groups.

Based on the Shapiro–Wilk test, miRNA values were not normally distributed ($P < 0.05$), therefore non-parametric Kendall's tau test was used to study the correlation between miRNAs and sleep duration, and between the two miRNAs. Both miRNAs were found to be differentially expressed between the selected groups (Figure 1a,b). In detail, there were group differences in circulating levels of miR-26b-3p ('short sleepers' group: $r = -0.229$, $P = 0.058$; 'normal sleepers' group: $r = 0.148$, $P = 0.024$) and miR-485-5p ('short sleepers' group: $r = -0.075$, $P = 0.303$; 'normal sleepers' group: $r = 0.196$, $P = 0.004$). Specifically, the correlation of both miR-26b-3p and miR-485-5p with sleep duration was statistically significant only in the group of children/adolescent that met the sleep needs recommendation for the specific age range ('normal sleepers'). Dispersion of study subjects according to miRNA levels revealed a strong correlation between miR-26b-3p and miR-485-5p ($r = 0.725$, $P < 0.001$) (Figure 2).

Tables 2 and 3 report the results of the univariate analysis of associations of miRNAs with sleep categories and sleep quality, respectively, adjusted for country of origin, age, sex, pubertal status, screen time and ISCED. In detail, miR-26b-3p showed only a weak association ($P = 0.05$; $\eta_p^2 = 0.140$) with sleep categories, and no association with the sleep quality parameters considered. miR-485-5p showed a statistically significant association with both sleep categories ($P < 0.001$; $\eta_p^2 = 0.510$) and perceived 'sleep quality' ($P = 0.015$; $\eta_p^2 = 0.357$), while no association was found with 'snoring', 'trouble getting up in the morning' and 'difficulties falling asleep'.

Moreover, no association of selected miRNAs was found in (1) 'early sleep onset' and 'late sleep onset' groups, and (2) in subjects that, compared to the weekdays, sleep more or less than 1.5 h during the weekend (data not shown).

To gain a mechanistic understanding of how the miRNAs could be associated with sleep duration, molecular interactions of confirmed miRNAs were predicted by bioinformatics. The functional characterization and enrichment of the biological pathways regulated by the two miRNAs were investigated by miRPath analysis in which the miRNAs were combined. Target genes were classified according to KEGG functional annotations to identify top pathways that were actively regulated by miRNAs. Identified pathways were arranged according to enrichment statistical scores (P-values) in addition to the number and names of miRNA target genes implicated in each KEGG pathway. Predicted pathways were determined for single miRNAs as well as for their association since both donor and target tissues of established miRNAs were unidentified (Tables 4 and 5). Computational predictions indicated the role of these miRNAs in controlling the expression of genes involved in relevant biological processes, with several miRNAs associated with known central clock genes. In detail, miR-26b-3p targets *PRKAA2*, *CREB1*, *CSNK1D* and *RORA*, and miR-485-5p targets *FBXW11*. These results were also confirmed by GeneMANIA (Figure 3), a useful tool to study validated biological networks.

In our analysis, the top predicted target of miR-26b-3p ($P < 0.001$) is the prion protein (PrP^C, KEGG pathway Prion diseases (hsa05020)). Results also showed that miR-485-5p targets hypoxia inducible factor 1 subunit α and participates in the cellular response to hypoxia.

It was noteworthy that exploration of the miRmine Human miRNA Expression Database (a collection of expression profiles from different publicly available miRNA-seq datasets) found that these miRNAs are primarily expressed in both brain and plasma samples (Figure 4).

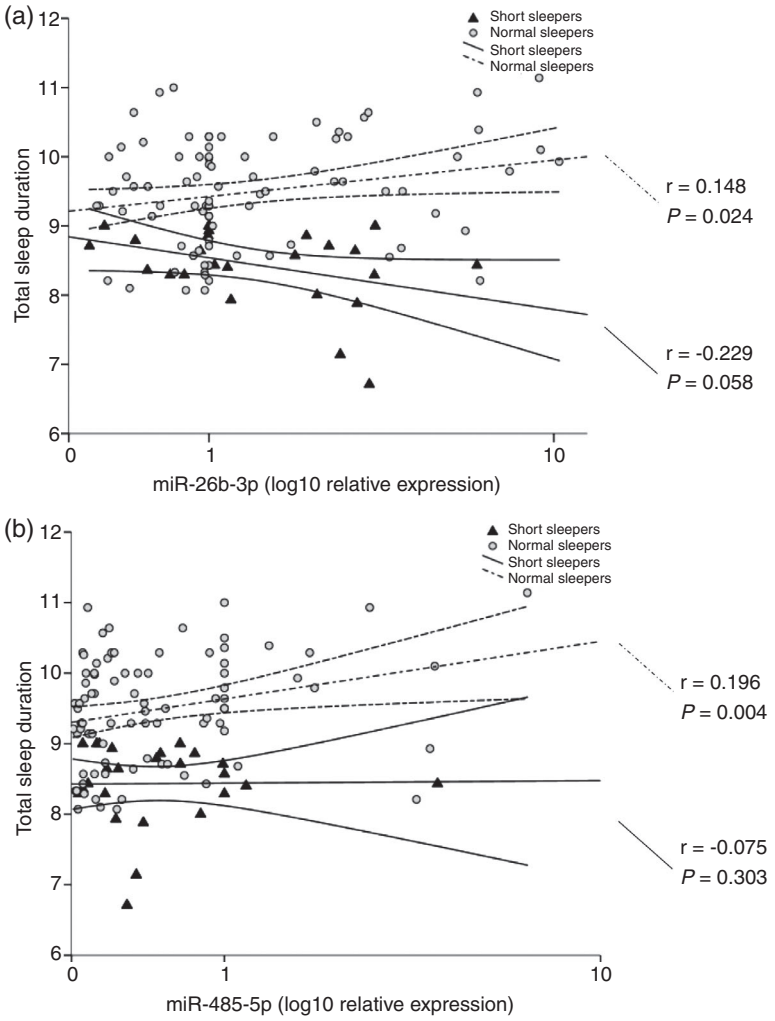

FIGURE 1 Correlation between sleep needs groups and miR-26b-3p (a) and miR-485-5p (b). Triangles and continuous lines refer to subjects belonging to the 'short sleepers' group; circles and dashed lines refer to subjects belonging to the 'normal sleepers' group. Lines show the linear correlation between variables plus confidence intervals (mean)

4 | DISCUSSION

The notion that some circulating miRNAs display diurnal rhythmicity in healthy humans is a relatively novel finding (Heegaard et al., 2016), suggesting that specific circulating miRNAs may have a role in the regulation of circadian rhythms. To the best of our knowledge, the current study shows for the first time that the expression levels of circulating miR-26b-3p and miR-485-5p are different between 'short sleepers' and 'normal sleepers' in a population of healthy normal-weight children and adolescents. The effect size for miR-26b-3p was weaker, as confirmed by confidence intervals and partial eta-squared values.

However, correlation of sleep duration with miR-26b-3p and miR-485-5p was statistically significant only in the group of children/adolescents that meets the sleep needs recommendation for the specific age range. Furthermore, no association was found in subgroup analyses of the selected miRNAs and sleep characteristics

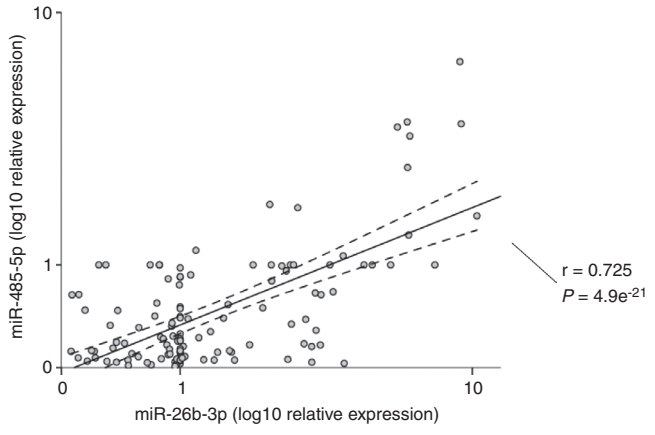

FIGURE 2 Correlation between miR-26b-3p and miR-485-5p. The continuous line shows the linear correlation between the variables. The dashed lines represent the confidence intervals (mean)

TABLE 2 miRNA expression according to sleep categories

SLEEP	Short sleepers ($n = 25$)	Normal sleepers ($n = 86$)	P	η_p^2
miR-26b-3p	2.0 (1.3–2.7)	2.3 (1.9–2.7)	0.050	0.140
miR-485-5p	0.6 (0.3–0.9)	0.9 (0.7–1.0)	<0.001	0.510

Data are expressed as mean (95% confidence interval). Covariates: age, sex, pubertal status, screen time, highest educational level of parents defined according to the International Standard Classification of Education (ISCED).

TABLE 3 Association between miRNAs and sleep quality

Sleep quality parameter		miR-26b-3p	*P*	η_p^2	miR-485-5p	*P*	η_p^2
Snoring ($n = 76$)	Less than once a week	1.8 (1.36–2.23)	0.939	<0.001	0.58 (0.37–0.79)	0.606	<0.05
	More than once a week	1.3 (−1.15 to 2.70)			0.55 (−0.15 to 1.25)		
Trouble getting up in the morning ($n = 91$)	Yes	2.53 (2.00–3.05)	0.052	0.113	0.95 (0.61–1.28)	0.221	0.043
	No	1.99 (1.58–2.41)			0.67 (0.41–0.93)		
Difficulties falling asleep ($n = 91$)	Yes	3.08 (2.34–3.81)	0.076	0.092	1.16 (0.80–1.52)	0.349	0.027
	No	1.78 (1.35–2.22)			0.58 (0.37–0.88)		
Perceived sleep quality ($n = 91$)	Very good	—	0.228	0.144	—	0.015	0.357
	Fairly good	1.97 (1.44–2.50)			0.77 (0.52–1.01)†		
	Fairly bad	1.71 (1.14–2.30)*			0.38 (0.12–0.65)*		
	Very bad	3.46 (2.20–4.70)*			1.79 (1.20–2.37)*,†		

Data are expressed as mean (95% confidence interval). Covariates: age, sex, pubertal status, screen time, highest educational level of parents defined according to the International Standard Classification of Education (ISCED). Statistically significant for multiple comparison: *$P < 0.05$, †$P < 0.001$.

such as snoring, difficulties falling asleep, trouble getting up in the morning and perceived sleep quality. No children and adolescents reported having a 'very good' sleep quality.

Further analysis to address whether the results were due to sleep deprivation or chronotype showed no association of 'early sleep onset' and 'late sleep onset' groups with selected miRNAs. Finally, since children and adolescents tend to increase total sleep time during the weekend in order to compensate sleep debit across the week, we also evaluated whether the sleep time compensation over the weekend would affect miRNA expression. This analysis found no effect.

To mechanistically analyse the biological targets of characterized miRNAs, their putative functions were explored *in silico*. Of course, plasma miRNA levels do not necessarily reflect the effects exerted by miRNAs inside cells and tissues; nevertheless, the general concept that circulating miRNAs can be transferred to other cell types and act through mechanisms of paracrine or endocrine regulation is nowadays supported by experimental evidence.

In our analysis, the top predicted target of differential miRNAs was PrP^C, a cell surface protein mainly known for its notorious role in prion diseases (Wulf, Senatore, & Aguzzi, 2017). Of note, recent evidence suggests a role of PrPC in sleep regulation through noradrenergic and dopaminergic signalling and melatonin synthesis, possibly modulating both healthy circadian activity rhythms and patterns, and sleep dysfunction during neurodegenerative disorders (Roguski & Gill, 2017).

Experimental results have also shown that miR-485-5p potentially participates in the cellular response to hypoxia and the HIF-1α signalling pathway. miR-485-5p is down-regulated in the serum of subjects with obstructive sleep apnoea, a sleep-related breathing disease (Li, Wei, Qin, & Wei, 2017). Moreover, dysregulation of miR-26a has been detected in plasma from patients affected by narcolepsy and idiopathic hypersomnia (Holm et al., 2014).

New epigenetic mechanisms regulating sleep are emerging, with several investigations showing that some miRNAs are related to circadian control (Goodwin et al., 2018). In our study, computational predictions suggest that selected miRNAs are potentially associated with the circadian rhythm in humans. Among miR-26b-3p predicted targets, 5′-AMP-activated protein kinase catalytic subunit α-2 (PRKAA2) is a crucial cellular energy sensor that also acts as a regulator of circadian rhythm by mediating phosphorylation of CRY1.

Among the miR-26b-3p predicted targets, *CSNK1D* is involved in the regulation of apoptosis, circadian rhythm, microtubule dynamics and p53-mediated effects on growth. Intriguingly, *CSNK1D* has been implicated in advanced sleep phase syndrome-2. Moreover, it has been predicted that miR-26b-3p targets *RORA*; the protein encoded by this gene is a member of the NR1 subfamily of nuclear hormone receptors involved in circadian rhythm control. Our analysis also revealed that miR-485-5p putatively targets FBXW11, the substrate recognition component of SKP1–CUL1–F-box protein (SCF) E3 ubiquitin-protein ligase complex. SCF/FBXW11 mediates the ubiquitination of phosphorylated CTNNB1 and participates in the positive regulation of the circadian rhythm.

Finally, the intersection analysis of the targeted genes established that 2-phosphoglycerate enolase (*ENO1*) is potentially the common target of both miR-26b-3p and miR-485-5p. *ENO1* consists of a relevant hub in which several energy-source pathways overlaps (glycolysis/gluconeogenesis, hsa00010; carbon metabolism, hsa01200; biosynthesis of amino acids, hsa01230; RNA degradation, hsa03018; HIF-1 signalling pathway, hsa04066).

There are several limitations concerning this study that deserve mention. The first, and most important, is that the correlation between miRNA expression and sleep duration observed in 'normal' sleepers, although statistically significant, is very weak and should be cautiously interpreted. Moreover, lifestyle factors might possibly affect the results, but the addition of covariates to the statistical models did not influence the association with sleep duration. Total sleep duration was assessed by self-report questionnaires, which may introduce measurement errors and inaccuracies. However, a fair agreement has been found in earlier studies between self-reported and objectively measured sleep duration (actigraphic observations) (Lauderdale, Knutson, Yan, Liu, & Rathouz, 2008). Accordingly, although self-reported data are not as precise as actigraphy, they are often used in children, due to their ease of use and minimal time and costs involved in data collection (Matricciani, 2013; Thumann et al, 2019).

TABLE 4 miRPath analysis – gene union

KEGG pathway	*P*	miRNAs	Number of targets	Target gene	Ensemble gene ID
Prion diseases	1.36×10^{-35}	1	1	*PRNP*	ESG00000171867
Viral carcinogenesis	0.0022	2	13	*RASA2*	ENSG00000155903
				HDAC7	ENSG00000061273
				CREB5	ENSG00000146592
				DLG1	ENSG00000075711
				TRAF5	ENSG00000082512
				CDK6	ENSG00000105810
				DDX3X	ENSG00000215301
				CREB1	ENSG00000118260
				EIF2AK2	ENSG00000055332
				HIST1H2BG	ENSG00000273802
				PIK3R1	ENSG00000145675
				USP7	ENSG00000187555
				CDKN1A	ENSG00000124762
Lysine degradation	0.0082	2	4	*ASH1L*	ENSG00000116539
				KMT2D	ENSG00000167548
				WHSC1	ENSG00000109685
				KMT2A	ENSG00000118058
Circadian rhythm	0.0408	2	5	*PRKAA2*	ENSG00000162409
				CREB1	ENSG00000118260
				CSNK1D	ENSG00000141551
				RORA	ENSG00000069667
				FBXW11	ENSG00000072803
Oestrogen signalling pathway	0.0408	2	7	*GNAS*	ENSG00000087460
				CREB5	ENSG00000146592
				CREB1	ENSG00000118260
				HSP90AB1	ENSG00000096384
				ITPR1	ENSG00000150995
				PIK3R1	ENSG00000145675
				HSPA8	ENSG00000109971
Vasopressin-regulated water reabsorption	0.0408	2	7	*GNAS*	ENSG00000087460
				CREB5	ENSG00000146592
				DYNC1H1	ENSG00000197102
				ARHGDIB	ENSG00000111348
				DCTN4	ENSG00000132912
				CREB1	ENSG00000118260
				DYNC2H1	ENSG00000187240

Data analysis for multiple miRNAs was performed by gene union selection. A *P*-value threshold of 0.05 and false discovery rate correction were applied.

Since data about physical activity were available only for about half of the study population, we did not include this variable in our analysis in order to investigate the largest available sample. However, when running our model in the subsample for which self-reported physical activity data were available, this covariate did not change the results (data not shown). Finally, the molecular characterization of the detected miRNAs, although relevant, is far beyond the aims of the present study, which is exploratory in essence.

The hypothesis-generating study presented here suggested that specific miRNAs are potentially associated with sleep duration during evolutive age. It is conceivable that epigenetic changes in human genes may be interrelated with sleep duration differences. Further studies

TABLE 5 miRPath analysis – gene intersection

KEGG pathway	*P*	miRNAs	Number of targets	Target gene	Ensemble gene ID
RNA degradation	0.0017	2	1	*ENO1*	ENSG00000074800
Glycolysis/gluconeogenesis	0.0475	2	1	*ENO1*	ENSG00000074800
Carbon metabolism	0.0475	2	1	*ENO1*	ENSG00000074800
Biosynthesis of amino acids	0.0475	2	1	*ENO1*	ENSG00000074800
NF-κB signalling pathway	0.0475	2	1	*ENO1*	ENSG00000074800
HIF-1 signalling pathway	0.0475	2	1	*ENO1*	ENSG00000074800

Data analysis for multiple miRNAs was performed by gene intersection selection. A *P*-value threshold of 0.05 and false discovery rate correction were applied. HIF-1, hypoxia-inducible factor 1; NF-κB, nuclear factor-κB.

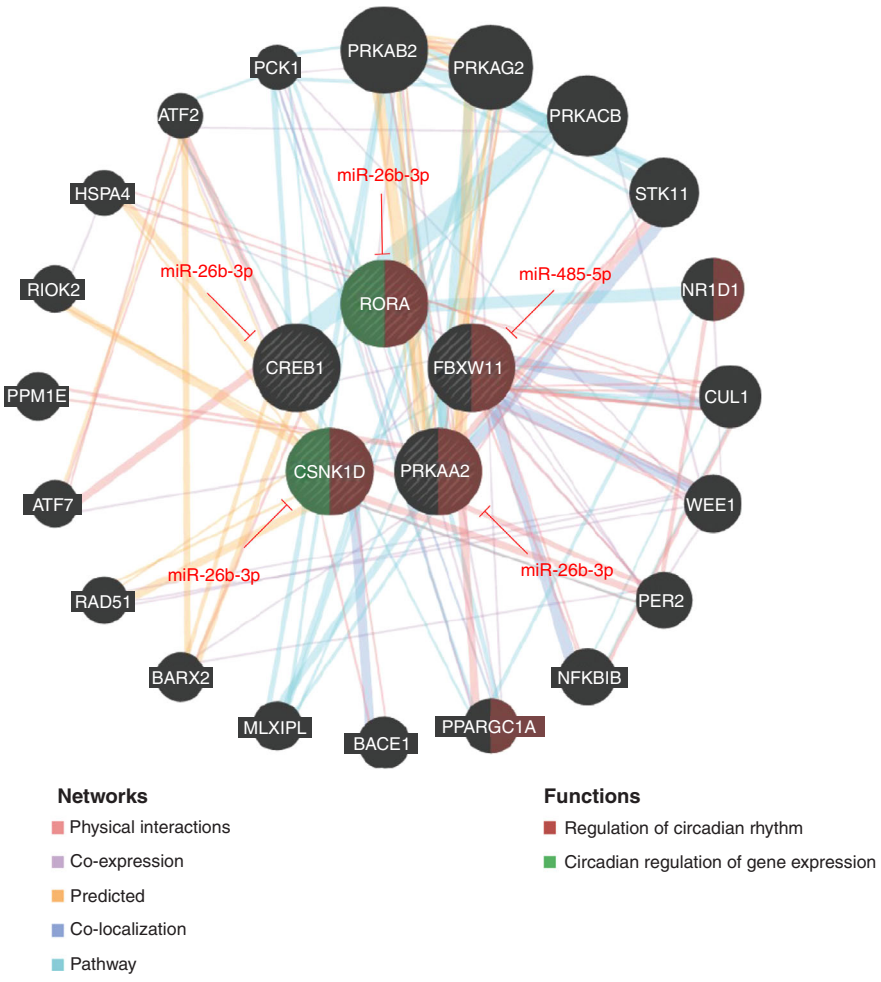

FIGURE 3 Molecular wiring diagram of the core circadian clock interactions obtained by GeneMANIA analysis. Computational prediction shows that both characterized miRNAs are associated with circadian rhythm control (miR-26b-3p targets *PRKAA2*, *CREB1*, *CSNK1D* and *RORA*; miR-485-5p targets *FBXW11*)

are required to explore the functional relevance of the miRNA species identified.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

G.I., F.L., P.R. and A.S. conceived, designed, oversaw the analyses, and drafted the manuscript. P.M., A.V. and N.I. conducted the analyses at the Institute of Food Sciences, National Research Council, Avellino-Italy. R.F., B.T. and S.D.H., contributed to the interpretation of data with critical revision of the manuscript. R.H.F., M.H., Y.K., L.A.M. and T.V. contributed to the critical revision of the manuscript. All authors have read and approved the final version of this manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work

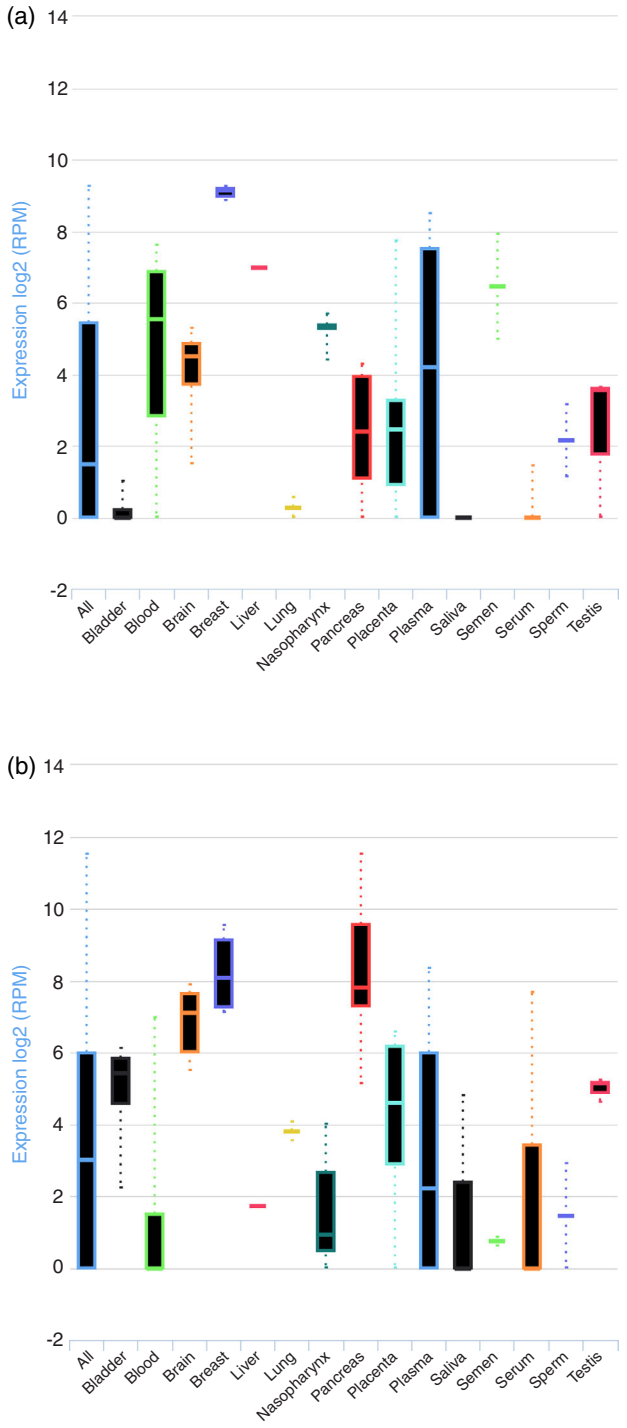

FIGURE 4 Boxplots of expression values of miRNAs in different human tissues. Expression values are scaled to $\log_2$ transformed reads per million (RPM). (a) miR-26b-3p; (b) miR-485-5p

are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

ORCID

Fabio Lauria https://orcid.org/0000-0002-1663-1582

REFERENCES

Ahrens, W., Siani, A., Adan, R., De Henauw, S., Eiben, G., Gwozdz, W., … Pigeot, I. (2017). Cohort Profile: The transition from childhood to adolescence in European children – how I.Family extends the IDEFICS cohort. *International Journal of Epidemiology*, *46*(5), 1394–1395j.

Archer, S. N., & Oster, H. (2015). How sleep and wakefulness influence circadian rhythmicity: Effects of insufficient and mistimed sleep on the animal and human transcriptome. *Journal of Sleep Research*, *24*(5), 476–493.

Cao, D. D., Li, L., & Chan, W. Y. (2016). MicroRNAs: Key regulators in the central nervous system and their implication in neurological diseases. *International Journal of Molecular Sciences*, *17*(6), E842.

Cole, T. J., & Lobstein, T. (2012). Extended international (IOTF) body mass index cut-offs for thinness, overweight and obesity. *Pediatric Obesity*, *7*(4), 284–294.

Davis, C. J., Bohnet, S. G., Meyerson, J. M., & Krueger, J. M. (2007). Sleep loss changes microRNA levels in the brain: A possible mechanism for state-dependent translational regulation. *Neuroscience Letters*, *422*(1), 68–73.

Friedman, R. C., Farh, K. K., Burge, C. B., & Bartel, D. P. (2009). Most mammalian mRNAs are conserved targets of microRNAs. *Genome Research*, *19*(1), 92–105.

Gharib, S. A., Khalyfa, A., Abdelkarim, A., Bhushan, B., & Gozal, D. (2012). Integrative miRNA-mRNA profiling of adipose tissue unravels transcriptional circuits induced by sleep fragmentation. *PloS One*, *7*(5), e37669.

Goodwin, P. R., Meng, A., Moore, J., Hobin, M., Fulga, T. A., Van Vactor, D., & Griffith, L. C. (2018). MicroRNAs regulate sleep and sleep homeostasis in *Drosophila*. *Cell Reports*, *23*(13), 3776–3786.

Heegaard, N. H., Carlsen, A. L., Lilje, B., Ng, K. L., Ronne, M. E., Jorgensen, H. L., … Fahrenkrug, J. (2016). Diurnal variations of human circulating cell-free micro-RNA. *PloS One*, *11*(8), e0160577.

Hijmans, J. G., Levy, M., Garcia, V., Lincenberg, G. M., Diehl, K. J., Greiner, J. J., … DeSouza, C. A. (2019). Insufficient sleep is associated with a pro-atherogenic circulating microRNA signature. *Experimental Physiology*, *104*(6), 975–982.

Holm, A., Bang-Berthelsen, C. H., Knudsen, S., Kornum, B. R., Modvig, S., Jennum, P., & Gammeltoft, S. (2014). miRNA profiles in plasma from patients with sleep disorders reveal dysregulation of miRNAs in narcolepsy and other central hypersomnias. *Sleep*, *37*(9), 1525–1533.

Iacomino, G., Russo, P., Stillitano, I., Lauria, F., Marena, P., Ahrens, W., … Siani, A. (2016). Circulating microRNAs are deregulated in overweight/obese children: Preliminary results of the I.Family study. *Genes & Nutrition*, *11*, 7.

Iacomino, G., Russo, P., Marena, P., Lauria, F., Venezia, A., Ahrens, W., … Siani, A. (2019). Circulating microRNAs are associated with early childhood obesity: Results of the I.Family Study. *Genes & Nutrition*, *14*, 2.

Iacomino, G., & Siani, A. (2017). Role of microRNAs in obesity and obesity-related diseases. *Genes & Nutrition*, *12*, 23.

Issler, O., & Chen, A. (2015). Determining the role of microRNAs in psychiatric disorders. *Nature Reviews Neuroscience*, *16*(4), 201–212.

Iwakawa, H. O., & Tomari, Y. (2015). The functions of MicroRNAs: MRNA decay and translational repression. *Trends in Cell Biology*, *25*(11), 651–665.

Kanehisa, M., Goto, S., Sato, Y., Kawashima, M., Furumichi, M., & Tanabe, M. (2014). Data, information, knowledge and principle: Back to metabolism in KEGG. *Nucleic Acids Research*, *42*(Database issue), D199–D205.

Kim, Y. K. (2015). Extracellular microRNAs as biomarkers in human disease. *Chonnam Medical Journal*, *51*(2), 51–57.

Lauderdale, D. S., Knutson, K. L., Yan, L. L., Liu, K., & Rathouz, P. J. (2008). Self-reported and measured sleep duration: How similar are they? *Epidemiology*, *19*(6), 838--845

Lewis, B. P., Burge, C. B., & Bartel, D. P. (2005). Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell*, *120*(1), 15–20.

Li, K., Wei, P., Qin, Y., & Wei, Y. (2017). MicroRNA expression profiling and bioinformatics analysis of dysregulated microRNAs in obstructive sleep apnea patients. *Medicine*, *96*(34), e7917.

Magee, C. A., Robinson, L., & Keane, C. (2017). Sleep quality subtypes predict health-related quality of life in children. *Sleep Medicine*, *35*, 67–73.

Matricciani, L. (2013). Subjective reports of children's sleep duration: Does the question matter? A literature review. *Sleep Medicine*, *14*(4), 303–311.

Panwar, B., Omenn, G. S., & Guan, Y. (2017). miRmine: A database of human miRNA expression profiles. *Bioinformatics*, *33*(10), 1554–1560.

Paruthi, S., Brooks, L. J., D'Ambrosio, C., Hall, W. A., Kotagal, S., Lloyd, R. M., … Wise, M. S. (2016). Consensus Statement of the American Academy of Sleep Medicine on the Recommended Amount of Sleep for Healthy Children: Methodology and Discussion. *Journal of Clinical Sleep Medicine*, *12*(11), 1549–1561.

Paul, P., Chakraborty, A., Sarkar, D., Langthasa, M., Rahman, M., Bari, M., … Chakraborty, S. (2017). Interplay between miRNAs and human diseases: a review. *Journal of Cellular Physiology*, *233*, 2007–2018.

Powell, W. T., & LaSalle, J. M. (2015). Epigenetic mechanisms in diurnal cycles of metabolism and neurodevelopment. *Human Molecular Genetics*, *24*(R1), R1–R9.

Roguski, A., & Gill, A. C. (2017). The role of the mammalian prion protein in the control of sleep. *Pathogens*, *6*(4), E58.

Stomfai, S., Ahrens, W., Bammann, K., Kovacs, E., Marild, S., Michels, N., … Molnar, D. (2011). Intra- and inter-observer reliability in anthropometric measurements in children. *International Journal of Obesity*, *35*(Suppl 1), S45–S51.

Thomou, T., Mori, M. A., Dreyfuss, J. M., Konishi, M., Sakaguchi, M., Wolfrum, C., … Kahn, C. R. (2017). Adipose-derived circulating miRNAs regulate gene expression in other tissues. *Nature*, *542*(7642), 450–455.

Thumann, B. F., Börnhorst, C., Michels, N., Veidebaum, T., Solea, A., Reisch, L., … Ahrens, W.; IDEFICS and I.Family consortia. (2019). Cross-sectional and longitudinal associations between psychosocial well-being and sleep in European children and adolescents. *Journal of Sleep Research*, *28*(2), e12783.

Tucci, V. (2016). Genomic imprinting: A new epigenetic perspective of sleep regulation. *PLoS Genetics*, *12*(5), e1006004.

Turchinovich, A., Weiz, L., Langheinz, A., & Burwinkel, B. (2011). Characterization of extracellular circulating microRNA. *Nucleic Acids Research*, *39*(16), 7223–7233.

Unesco Institute for Statistics. (2012). *International Standard Classification of Education ISCED 2011*. Montreal, Canada: UNESCO Institute for Statistics.

Vlachos, I. S., Zagganas, K., Paraskevopoulou, M. D., Georgakilas, G., Karagkouni, D., Vergoulis, T., … Hatzigeorgiou, A. G. (2015). DIANA-miRPath v3.0: Deciphering microRNA function with experimental support. *Nucleic Acids Research*, *43*(W1), W460–W466.

Wang, H., Fan, Z., Zhao, M., Li, J., Lu, M., Liu, W., … Yan, J. (2016). Oscillating primary transcripts harbor miRNAs with circadian functions. *Scientific Reports*, *6*, 21598.

Warde-Farley, D., Donaldson, S. L., Comes, O., Zuberi, K., Badrawi, R., Chao, P., … Morris, Q. (2010). The GeneMANIA prediction server: Biological network integration for gene prioritization and predicting gene function. *Nucleic Acids Research*, *38*(Web Server issue), W214–W220.

Weigend, S., Holst, S. C., Meier, J., Brock, M., Kohler, M., & Landolt, H.-P. (2018). Prolonged waking and recovery sleep affect the serum microRNA expression profile in humans. *Clocks & Sleep*, *1*(1), 8.

Wulf, M. A., Senatore, A., & Aguzzi, A. (2017). The biological function of the cellular prion protein: An update. *BMC Biology*, *15*(1), 34.

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