

Article

Exosomal miR-92a Concentration in the Serum of Shift Workers

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Abstract: Shift work is associated with alterations in the human biological clock and metabolism. Serum exosomal miR-92a concentration was inversely correlated with brown adipose tissue activity playing a pivotal role in energy balance. In this study, miR-92a was measured in serum exosomes of 30 workers engaged in shift and daytime work. No significant metabolic alterations were shown between daytime and shift workers while a difference in serum exosomal miR-92a levels was found between the two groups. The lower levels of miR-92a in shift workers were suggestive of a higher brown adipose tissue activity compared with daytime workers. However, the possibility that other physiological and pathological processes may influence miR-92a cannot be ruled out. Our results suggest further investigations on brown adipose tissue activity and on miR-92a regulatory mechanisms, such as those related to the estrogen pathway, in shift workers.

Keywords: shift work; night work; circadian disruption; metabolism; metabolic syndrome; brown adipose tissue; BAT activity; miR-92a

1. Introduction

Working on a shift work schedule implies that some daily habits, such as sleeping or eating, are not conducted with regular occurrence [1] resulting in a possible desynchronization between external and biological times [2]. The perturbation of the biological clock leads to alterations of several metabolic processes including reduced glucose tolerance, dislipidemia, weight gain [2–4], and the impairment of some molecules related to fat absorption and metabolism [2,5]. Epidemiological studies associated shift work with an increased incidence of metabolic syndrome [6,7] that, in turn, is an important cardiovascular risk factor [8,9]. Since alterations of metabolic parameters and weight gain in shift workers do not seem to be associated with low physical activity or increased food intake [5,10], it is likely that there are other factors that may influence energy balance.

In the last few decades, brown adipose tissue (BAT) has regained scientific and public interest for its role in metabolism and weight gain [11–16]. BAT is a rich vascularized tissue with a high number of mitochondria. It catabolizes lipids to generate heat playing a major role in thermogenesis and energy expenditure [17–20]. Similar to lipids, BAT also metabolizes carbohydrates, contributing to the homeostasis of these two groups of macromolecules resulting in a beneficial effect on overall metabolism [21–23]. Obese patients have less active BAT [24]. In fact, an inverse relationship between active BAT and body mass index (BMI) has been discovered [25].

The most used method for quantitatively measuring BAT volume and activity is the ¹⁸F-fluor-deoxy-glucose positron emission tomography coupled with computed tomography (FDG-PET/CT) [11]. Since

this method is costly, labor-intensive, and involves exposure to radiation, some parameters were recently studied as biomarkers of BAT activity.

MiRNAs are small, non-coding RNAs which regulate protein expression in several tissues [26–28]. MiRNAs are molecules that complementary bind mRNA and downregulate gene expression by translational repression, mRNA cleavage, and deadenylation [29]. MiRNAs are carried by exosomes, which are small lipid vesicles (40–100 nm in size) that constitute cell-to-cell communication [30,31]. Exosomes allow an exchange of material between cells in an autocrine, paracrine, and even endocrine manner [32,33]. MiRNAs present in human blood are stable and they are resistant to endogenous RNase activity [34]. MiRNAs are involved in the regulation of several pathological processes and represent new diagnostic and prognostic biomarkers for the diagnosis of various diseases [35]. Several miRNAs have proven to play a major role in BAT, such as controlling brown adipocytes differentiation [36]. Among these miRNAs, miR-92a has been involved in several metabolic processes, such as glucose homeostasis [24], regulation of intracellular cholesterol levels [37], and essential hypertension [38]. Furthermore, Chen and collaborators demonstrated that serum levels of exosomal miR-92a were inversely correlated with human BAT activity, confirming the data by FDG-PET/CT [39]. Due to miR-92a in serum exosomes can easily be assessed, it could be considered a non-invasive biomarker for BAT activity [39].

In light of this information, it was decided to study the levels of miR-92a in shift and daytime workers.

2. Materials and Methods

2.1. Subject Enrollment

The concentration of exosomal miR-92a was determined in the serum of 30 workers enrolled among the nursing staff of Area Vasta 2 (Ancona, Italy). All nurses were female, fifteen subjects worked a rotating shift schedule that included night shifts (SW nurses), whereas the other 15 workers were involved exclusively in daytime shifts (DT nurses).

Selection criteria were the following: fertile age; no current use of medications; a negative history of psychiatric disorders, insomnia, cancer or chronic systemic diseases, such as degenerative, cardiovascular or autoimmune disorders; absence of artificial light when sleeping at home; no occupational tasks involving exposure to ionizing radiation or antineoplastic drugs. SW nurses had to be enrolled in the current shift schedule, including more than 60 night shifts/year, for more than 2 years without schedule breaks in the previous 6 months. DT nurses had to go to bed habitually around 23:00 and to wake up around 06:00 without sleepless nights for more than 3 weeks prior to the study.

Workers provided their consent after receiving information about the purpose and procedures of the study, which was conducted according to the Helsinki Statement of Ethical Standards. Being performed as part of required, routine health surveillance, the study required no formal approval by the local ethics committee. The SW nurses worked a fast clockwise shift schedule: day 1, 07:00–14:00; day 2, 14:00–22:00; day 3, 22:00–07:00; rest for 48 h; resumption of the cycle. The DT nurses worked from 07:00 to 14:00 for 6 days/week. To exclude the acute alterations related to night shift, fasting venous blood samples were taken from both SW and DT nurses at the beginning of the morning shift after a regular night's sleep on a day off. Blood samples were processed immediately after collection. An aliquot of serum was stored at -80°C until miR-92a analysis. Blood was analyzed the same day for glycemia, total and HDL cholesterol, and triglycerides.

2.2. Clinical Parameters Collection

Socio-demographic characteristics were collected as part of the medical history. The chronotype was investigated by the "Morningness–Eveningness Questionnaire" (MEQ) [40], a questionnaire with 19 items and a total score ranging from 16 to 86, widely used in adults and workers [41–44]. Sleep quality was investigated using the Pittsburgh Sleep Quality Index (PSQI) [45], while daytime sleepiness was

assessed by the Epworth Sleepiness Scale (ESS) [46,47]. Measurement of anthropometric parameters was performed during the medical examination. The fat mass was calculated using Durnin and Womersley formula [48]. A food diary was kept by workers for 5 consecutive days and daily average values of total energy intake and fat, carbohydrate, and protein intake were calculated [49]. Daily average values of total energy expenditure and energy expenditure due to motor activity were investigated using a metabolic monitor (SenseWear Armband®; BodyMedia Inc., Pittsburgh, PA, USA) [50], secured on each participant's right arm for 5 days. There were no cases of interrupted or invalid recordings.

2.3. Determination of Serum Exosomal miR-92a

Serum exosomal miR-92a was measured after a few months in storage at -80°C . Samples of SW and DT nurses were coded in a mixed order and analyzed by blinded personnel. After all the samples completed the miRNAs extraction, they underwent the reverse transcription and finally the relative qRT-PCR. The determination of serum exosomal miR-92a was completed in about two months.

2.3.1. MiRNAs Extraction

Exosomes were extracted from 1 mL of serum using a "Total Exosome Isolation (from serum)" Kit (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. Exosomal proteins were then quantified through the Bradford method and 20 μg of exosomal protein were diluted in 250 μL of PBS for the following RNA extraction process. We used the same quantity of exosomal protein to prevent differences in miR-92a related to the total amount of exosomes. Before RNA isolation, and to avoid further technical errors during the subsequent quantification of miRNAs by qRT-PCR, 60 fmol of a synthetic spike-in small RNA from *Caenorhabditis elegans* (cel-miR-39) were added to the samples as external normalizer [51]. Total RNA was then extracted by adding 750 μL of Tri-Reagent BD (Sigma-Aldrich, Saint Louis, MO, USA) and 200 μL of chloroform. The Phase Lock Gel (Eppendorf, Hamburg, Germany) was used to enhance the phase separation. Finally, miRNAs were isolated using the Purelink miRNA Isolation Kit (Invitrogen; Thermo Fisher Scientific, Inc.) and eluted in a final volume of 40 μL of RNase-free water, centrifuging twice 20 μL to improve the yield. Samples were stored at -80°C until reverse transcription.

2.3.2. Reverse Transcription

Reverse transcription reactions were performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with miRNA-specific stem-loop primers: small nuclear RNA U6 (Applied Biosystems, #RT: 001973), cel-miR-39 (Applied Biosystems, #RT: 000200), and miR-92a (Applied Biosystems, #RT: 000431). The 7.5 μL volume reaction was as follows: 5.5 μL of master mix (2.58 μL H_2O , 0.75 μL 10 \times reverse-transcription buffer, 0.095 μL RNase-inhibitor (20 U/ μL), 0.075 μL dNTPs (100 mM), 1.5 μL RT primers, and 0.5 μL reverse transcriptase) and 2 μL of input miRNAs. Reverse transcription reactions were carried out on the Biometra thermal cycler (Analytik Jena AG, Jena, Germany) following these conditions: 16 $^{\circ}\text{C}$ for 30 min, 42 $^{\circ}\text{C}$ for 30 min, 85 $^{\circ}\text{C}$ for 5 min, and then held at 4 $^{\circ}\text{C}$. The reverse transcription products were stored at -20°C until relative qRT-PCR.

2.3.3. Relative qRT-PCR

The qRT reactions were performed in duplicate in a final volume of 10 μL : 5 μL of MasterMix FluoCycle II™ Master Mix for Probe (Euroclone S.p.A., Pero, Italy) was mixed with 0.5 μL of miRNA specific primer/probe mix small nuclear RNA U6 (Applied Biosystems, #TM:001973), cel-miR-39 (Applied Biosystems, #TM: 000200), and miR-92a (Applied Biosystems, #TM: 000200) and 1.33 μL of cDNA into RNase-free strip-tubes sealed with their own flat cap (Eppendorf, Hamburg, Germany). The qRT-PCR reactions were run using the Realplex Mastercycler ep gradient S (Eppendorf, Hamburg, Germany) with the following thermal profile: 50 $^{\circ}\text{C}$ for 2 min, 95 $^{\circ}\text{C}$ for 10 min, followed by 40 cycles of 95 $^{\circ}\text{C}$ for 15 s and 60 $^{\circ}\text{C}$ for 1 min. Finally, the temperature was held at 4 $^{\circ}\text{C}$.

Data was normalized for biological variability, using the small nuclear RNA U6, and for possible technical variations during miRNAs extraction, using the cel-miR-39 as the exogenous normalizer. After the reaction was run, Ct values were determined and the relative quantification of miR-92a was calculated using the comparative $2^{-\Delta Ct}$ method [52]. The expression of miR-92a was calculated by double normalization: Both controls (CtU6 and Ctcel-miR-39) were subtracted from the Ct value of the sample of interest [51–53].

2.4. Statistical Analysis

Kolmogorov-Smirnov test was used to assess the normality of distribution. Since miR-92a values were not normally distributed, a non-parametric statistical approach was chosen. Continuous variables were expressed as median (25th–75th percentile) and differences between DT and SW nurses were analyzed by Mann-Whitney U test. Dichotomous or categorical variables were expressed as percentages and differences were analyzed by Chi-square test. All the tests were two-tailed considering a $p < 0.05$ as statistically significant. Data analysis was performed with the Statistical Package for Social Sciences version 19.0 (SPSS Inc., Chicago, IL, USA).

3. Results

All the workers who participated in the study were females. No significant differences were found in age, job seniority, numbers of smokers, or of alcohol drinkers between DT and SW nurses (Table 1). None of the SW nurses had changed the type of schedule from the beginning of the work as evidenced by the same values of job seniority and shift work seniority. SW nurses worked a median of 6.0 nights per month. No significant difference was found in chronotype (MEQ score), sleep quality (PSQI score), and daytime sleepiness (ESS score) between DT and SW nurses.

Table 1. Socio-demographic characteristics of daytime (DT) and shift-working (SW) nurses. MEQ: Morningness–Eveningness Questionnaire; PSQI: Pittsburgh Sleep Quality Index; ESS: Epworth Sleepiness Scale.

	DT Nurses	SW Nurses
Age (years): Median (25th–75th percentile)	39.0 (37.0–41.5)	39.0 (35.5–40.5)
Job seniority (years): Median (25th–75th percentile)	14.0 (11.0–18.3)	13.0 (10.5–16.5)
Shift work seniority (years): Median (25th–75th percentile)	-	13.0 (10.5–16.5)
Night worked per month: Median (25th–75th percentile)	-	6.0 (5.3–6.0)
Smokers (%)	40.0	40.0
Alcohol drinkers (%)	33.3	40.0
Subjects taking physical exercise (%)	46.7	60.0
Coffee consumption (cups/day): Median (25th–75th percentile)	2.0 (1.0–3.0)	2.0 (1.5–3.0)
Chronotype (MEQ score): Median (25th–75th percentile)	57.5 (52.5–64.0)	57.0 (51.3–62.0)
Sleep quality (PSQI score): Median (25th–75th percentile)	6.0 (3.0–8.0)	4.0 (2.5–6.5)
Daytime sleepiness (ESS score): Median (25th–75th percentile)	4.0 (2.3–8.8)	5.0 (4.0–9.3)

There were no significant differences in metabolic parameters between DT and SW nurses (Table 2). DT nurses showed a higher median value of total cholesterol, but the difference was not significant. Other blood parameters, systolic/diastolic pressure, BMI, waist circumference, skin thickness, and body fat were also similar in the two groups.

Table 2. Metabolic characteristics of daytime (DT) and shift-working (SW) nurses. Results are expressed as median (25th–75th percentile).

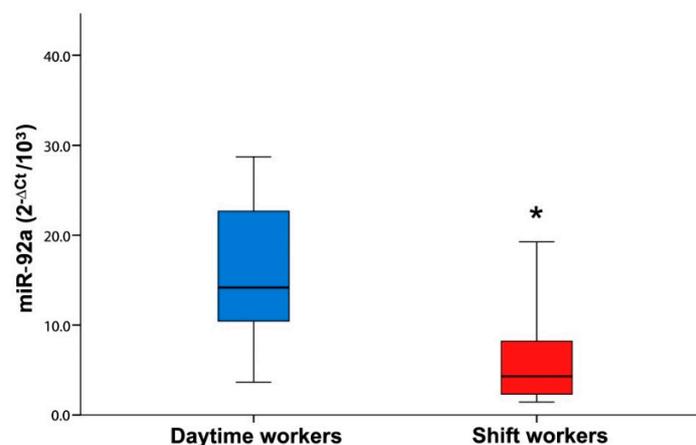
		DT Nurses	SW Nurses
Fasting glycemia (mg/dL)		89.5 (86.3–96.3)	83.0 (78.3–91.0)
Total cholesterol (mg/dL)		206.5 (158.8–225.5)	186.0 (159.5–196.3)
HDL cholesterol (mg/mL)		52.5 (40.8–61.5)	49.0 (41.0–57.5)
Triglycerides (mg/mL)		70.0 (51.5–106.3)	67.5 (55.5–114.5)
Systolic pressure (mmHg)		110.0 (105.0–120.0)	110 (110–117.5)
Diastolic pressure (mmHg)		70.0 (60.0–80.0)	70.0 (65.0–72.5)
BMI (Kg/m ²)		23.6 (21.1–25.7)	22.5 (21.2–29.7)
Waist circumference (cm)		90.0 (83.3–99.0)	87.0 (81.0–103.0)
Skin thickness	Biceps	11.2 (6.7–11.8)	11.2 (8.2–15.2)
	Triceps	21.2 (17.2–28.0)	22.2 (20.0–28.8)
	Subscapular	23.0 (17.5–27.8)	18.8 (13.4–38.4)
	Suprailiac	31.2 (22.4–33.7)	24.0 (18.4–35.8)
Body fat (%)		37.5 (34.1–41.9)	35.9 (32.1–40.1)

Data about total energy intake, diet composition, and total energy expenditure were similar in the two groups (Table 3). The statistical analysis did not show significant differences.

Table 3. Energy intake, diet composition, and total energy expenditure of daytime (DT) and shift-working (SW) nurses. Results are expressed as median (25th–75th percentile).

		DT Nurses	SW Nurses
Total Energy intake (Kcal)		2004 (1712–2272)	1927 (1850–2112)
Diet composition	Carbohydrates (%)	55.0 (53.2–55.5)	55.0 (53.9–55.4)
	Proteins (%)	18.4 (17.8–19.4)	19.0 (18.6–19.6)
	Fats (%)	26.5 (26.1–27.8)	26.1 (25.8–25.8)
Total energy expenditure (Kcal)		2179 (1866–2438)	2225 (1962–2527)
Energy expenditure due to locomotor activity (Kcal)		874 (694–1484)	1050 (923–1294)

Levels of miR-92a in serum exosomes are reported in Figure 1. We found significantly higher levels of miR-92a in DT nurses than in SW nurses [median 14.2 versus 4.3 ($2^{-\Delta Ct}/10^3$), $p = 0.002$]. Since miR-92a in serum exosomes inversely correlate with BAT activity, results were indicative of a lower BAT activity in DT nurses compared with SW nurses.

**Figure 1.** Box plot of levels of miR-92a in serum exosomes of daytime (DT) and shift-working (SW) nurses. * $p < 0.05$, Mann-Whitney U test.

4. Discussion

Shift work may disrupt sleep and meal rhythms. The perturbation of biological rhythms can alter metabolic homeostasis that may lead to weight gain and/or metabolic disturbances [54–57]. Shift work was associated with higher waist circumference [58–60], and a higher risk of metabolic syndrome and cardiovascular events [7,61–63]. In our sample, SW are similar to DT nurses concerning the waist circumference and the other metabolic parameters analyzed. The lack of differences is likely due to the small size effect that shift work has on metabolic parameters. A larger sampling would help this to be more visible. Moreover, our sample constituted of young nurses (median age of 39.0 years) with a median job seniority of 13.0 years. These parameters likely differ from the metabolic alterations shown in older shift workers [64]. Several authors have obtained increased BMI values in SW compared to DT workers [60,65,66], which, however, were not found to be associated to differences in diet style [65,67], nor in physical activity between the two groups of workers [10,68,69]. Other factors, such as a low level of active BAT, may also contribute to weight gain in SW.

This study measured the levels of miR-92a that a study of Chen and colleagues inversely correlated with BAT activity [39]. Contrary to the hypothesis that SW may have a low level of active BAT, we found lower levels of miR-92a in SW nurses compared to DT nurses, indicative of higher BAT activity [39]. Several argumentations may be possible: (1) The increased BAT activity detected in SW nurses may be a physiologic mechanism to counteract metabolic alterations induced by circadian disruption linked to shift work. This counteracting mechanism may be impaired after a long duration of shift work resulting in weight gain and metabolic syndrome. (2) In a study on mice, prolonged daily light exposure (similar to the experiences of shift workers during a night shift) decreases BAT activity [70]. However, mice differ from humans as they usually sleep during the day and are active and eat during the night, resulting in a possible opposite effect of light on BAT. (3) Shift work can induce a sympathodominant state [71]. Since the sympathetic nervous system is a stimulator of BAT [72], the higher BAT activity in SW nurses may be caused by a sympathetic stimulation. (4) Since BAT activity has a circadian rhythm [73,74], our finding may be related to a phase misalignment of a circadian cycle of BAT activity in SW compared to DT nurses. (5) Since the activity of BAT produces heat, its increased activity may be the cause of the higher peripheral skin temperature previously demonstrated in a similar sample of SW nurses [75]. (6) The lower levels of miR-92a found in the serum of shift workers may not only be related to BAT activity, but also to other biological processes [76,77].

This last hypothesis must be carefully considered. Further investigations are necessary due to the inverse correlation between serum exosomal miR-92a and BAT activity, which is based only on the study of Chen and colleagues [39]. Moreover, the miR-92a is involved in the regulation of several genes. MiRTargetLink [78], using information from miRTarBase [79,80], found 1419 genes that are targeted by miR-92a: 30 interactions with strong support, 1301 interactions with weak support, and 88 predicted interactions (Table S1). MiR-92a regulates several processes including cell proliferation and the development of cancer [77,81]. Night shift work has been suggested to be associated with the onset of cancer, specifically breast cancer [82–85]. Interestingly, in breast cancer the miR-92a is deregulated [86–92]. The miR-92a targets and downregulates estrogen receptor beta (ER β), and is controlled by estrogen [93]. Since higher levels of estrogen were found in shift workers [94–97], miR-92a may contribute to the deregulation of estrogen levels induced by shift work. Hence, it would be interesting to perform additional investigations regarding the possible role of miR-92a related to a higher risk of breast cancer in shift workers.

5. Conclusions

The significant lower levels of serum exosomal miR-92a suggest a higher BAT activity in SW nurses compared to DT nurses. Additional studies using gold standard tests for BAT activity in shift workers are needed. The possibility that the lower levels of miR-92a found in the serum of shift workers may not only be related to BAT activity, but also to other factors cannot be ruled out. Considering that

miR-92a takes part in several biological processes, including breast cancer, our results suggest further investigations on miR-92a in shift workers.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2076-3417/10/2/430/s1>, Table S1: miRNA-target interaction network for miR-92a. The central node is the miR-92a, surrounded by validated targets with strong evidence (green), weak evidence (blue), and predicted targets (yellow). Targets are listed below the figure.

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