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ORIGINAL ARTICLE

# Level of alpha amylase activity in human saliva as a non-invasive biochemical marker of sleep deprivation

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## Abstract

To investigate the usefulness of the enzyme salivary alpha amylase as a biochemical marker of sleep deprivation in human subjects. Total 168 healthy school-going adolescents studying in 9th grade were selected randomly from morning shift ($n=84$) and dayshift ($n=84$) schools. The study was undertaken longitudinally for a period of 2 years. Study encompassed administration of questionnaire and collection of saliva samples from the participants. Activity of salivary alpha amylase (sAA) activity was estimated spectrophotometrically and statistical analysis was performed to determine the association between sAA activity and sleep duration. Excessive daytime sleepiness among students was also studied in association with sAA activity. sAA activity of students was found to have a negative correlation with the duration of sleep and a positive correlation with their level of sleepiness. Morning shift students were found to have significantly less sleep and correspondingly higher sAA activity as compared to dayshift students. A significant increase in the sAA activity was noticed in the second year as the students progressed from 9th to 10th grade. Higher amylase activity was also observed in sleep deprived students suffering from excessive daytime sleepiness irrespective of school timings. Salivary alpha amylase activity increases in saliva in response to sleep deprivation. School timings may modulate sleep duration of students. Present finding reveals that sAA could be an appropriate non-invasive biochemical marker for the objective assessment of sleep deprivation among individuals as well as at population level.

**Keywords** Salivary alpha amylase · Sleep deprivation · Adolescents · Excessive daytime sleepiness

## Introduction

Sleep-related problems and susceptibility towards stress, the two most common feature of the modern society, are interrelated. The scientific community has made a considerable effort to establish a valid biomarker for the assessment of the sleepiness and stress level in human beings. To explore useful biomarkers, saliva is considered the most suitable medium due to ease of its collection and causing least discomfort to the subjects. One such biomarker, salivary alpha amylase (sAA) has been studied widely and recommended as a marker of activity of sympathetic nervous system [1–3]. It has been suggested that the level of the enzyme alpha amylase increases in saliva in response to stressful conditions with an increase in the sympathetic nervous system activity. The sympathetic activity is also known to increase due to reduced duration of sleep [4]. Increase in salivary alpha amylase activity has also been reported among individuals suffering from obstructive sleep apnea syndrome (OSAS) [5]. OSAS is a major cause of excessive daytime sleepiness. Though the genetic studies using drosophila as model organism has revealed a strong association between amylase mRNA and sleep deprivation [6, 7], studies on the association between salivary amylase enzyme activity and sleep deprivation in human has not yet been studied extensively on a larger sample size.

Sleep need varies considerably across different age groups and the need for sleep is higher among adolescents as compared to pre-pubertal children, younger children and adults [8, 9]. Adolescence is a stage between childhood and adulthood and the stage is associated with behavioral and psychosocial changes. The sleep requirement of adolescents is more than the adults or child because of their physiological requirement. However, the higher need for sleep in adolescents largely remains unmet due to a number of factors

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such as school timing, increasing academic demand and tendency to stay awake late at night. This sleep deprivation in adolescents is known to interfere with their capacity of learning and ability to sustain attention [10, 11], which results in poor school performance, behavioral problems [12, 13] and even road accidents [14]. Long-term sleep restriction has also been linked to accumulation of sleep debt and activation of both pathways of stress response system, hypothalamic pituitary adrenal (HPA) and sympathoadrenal medullary (SAM) axis [15]. Furthermore, a higher load of sleep debt has been correlated with the suicidal tendency among adolescents [16].

The devastating effects of sleep deprivation has become important to address because not only the adolescents but the adults also experiences excessive daytime sleepiness due to changing living style [17]. Furthermore, there are growing evidences suggesting that neurodegenerative disorders such as Alzheimer's and Parkinson's disease are intrinsically associated with unhealthy sleep [18, 19]. However, a potent quantifiable non-invasive biochemical marker for objective assessment of sleep deprivation has not been established as yet. The present study carried out on a large school-going adolescent population for two consecutive years is an attempt to find out not only the effect of school timings on adolescents sleep but also to validate the candidature of salivary amylase as a non-invasive biochemical marker for assessment of sleepiness level.

## Method

### Subject selection

Five different schools located in the urban area of Raipur (Chhattisgarh, India) were selected out of which two schools had the start timing 7:30 AM (morning shift) and three schools had the start timing 11:00 AM (dayshift). Consent of the school authorities was obtained for conducting the present study. Administration of questionnaire and collection of saliva sample was carried out longitudinally for a period of two consecutive years. The average age of students was $14.19 \pm 0.07$ and $14.25 \pm 0.08$, respectively, in morning and dayshift group. Students were studying in the 9th grade when the present study was initiated and were followed-up till the completion of their 10th grade. The study was initiated in the academic session 2014–2015 and 168 students participated in the study in the first year. 84 students were from morning shift schools (46 girls and 38 boys) and 84 students (28 girls and 56 boys) were from the dayshift schools. Some of the students could not be followed-up in the second year due to migration to other schools or withdrawal from the participation by some students. Hence, for the academic session, 2015–2016, the total number of participants were 150 out of this, 79 from morning shift schools (43 girls and 36 boys) and 71 from dayshift schools (25 girls and 46 boys). 12 students in the first year and 10 students in the second year were eliminated from the study because of the incomplete biographical information obtained from them. All the students enrolled in the present study were healthy, neither suffering from any kind of psychiatric illness and major diseases nor adhering to any kind of medication.

### Administration of questionnaire

Questionnaires were distributed directly to the participants in leisure and completely filled in questionnaires were collected back from them. Questionnaire included biographical information and the questions on their sleep habits (bedtime and wake up time) during weekdays and in the weekends, and their health status. Sleep duration of students was computed using self-reported bedtime and wake time. Sleepiness level among students was measured on Epworth sleepiness scale [20]. ESS score of 10 or more was indicated as excessive daytime sleepiness. The stress questionnaire of Ministry of Social Security, National Solidarity & Reform Institutions (http://www.gov.mu/portal/sites/suicideprevention/file/student) for students [21] was utilized for assessment of stress, which consisted of 20 questions pertaining to student's social, financial and academic problems. Stress score of 0–20 represents a "good control over stress", stress score of 21–40 represents "low level of stress", stress score of 41–60 represents a "medium level of stress" and stress score of 61–80 represents "high level of stress".

### Weekday sleep debt and social jetlag

Weekday sleep debt was determined from the difference between obtained sleep duration during weekend and weekday [22]. The social jetlag was computed from the difference in midpoint of weekend and weekday sleep timing (sum of sleep onset time and half the total sleep duration).

### Saliva collection and study design

Saliva samples were collected by spitting method in an Eppendorf tube and the lid was sealed firmly. Samples were stored in a refrigerator at − 20 °C and assayed for the enzyme alpha amylase within 1 month. Subjects were instructed to refrain from doing any intense physical activity on the day of saliva collection and cleanse the mouth before spitting. However, there was no restriction put on dietary intake, and subjects were allowed to have their morning breakfast as usual before reaching school to maintain their natural setting. Precaution was taken to ensure intake of no other substance by the subjects 2 h before sampling. The wake timings were also not altered to find out the effect of

school timings and to measure sleepiness level of morning and dayshift students in their natural setting. Sampling was done 2–2.5 h before lunch time and the saliva samples for estimation of enzyme activity were collected from the students of either shifts between 11:00 and 11:30 AM to eliminate any time effect. Saliva samples were collected from the students in their natural setting just one month prior to final examination (in the month of January).

### Salivary amylase assay

Activity of the salivary alpha amylase was assessed by utilizing assay kit (Agappe diagnostics ltd., Kerala, India). Samples were thawed and brought up to room temperature before performing the assay. Samples were centrifuged at 3000 rpm for 15 min and diluted (1:400) with normal saline. 25 µl of sample was taken out in the cuvette. To 25 µl of diluted saliva sample, 1 ml of substrate [2-chloro-4-nitrophenyl-α-maltotrioside (CNPG3)] provided in the assay kit was added and the mixture was incubated at 37 °C for 1 min. Change in absorbance per minute was measured at 405 nm for a period of 3 min. Amylase activity was calculated using the formula:

$$\text{Alpha-amylase activity (U/ml)} = \{(\text{OD/min}) \times 3178 \times 400\}/1000$$

### Statistical analysis

SPSS for windows (version 16.0. Chicago, SPSS Inc.) and MS Excel was used for analysis of data. ANOVA and paired *t* test was used for comparison of data in different and related groups, respectively. Pearson correlation was used to study the association between different variables and scatter plot diagram was used to show the significant association. Results with $p$ value of $<0.05$ were considered to be statistically significant in all the analyses.

## Result

Comparative account of sleep habits of school students as a function of school timing in the first and second year have been depicted in Tables 1 and 2, respectively. Table 3 summarizes the sleep behavior of morning as well as dayshift students in the first and second year of the study computed through paired *t* test.

### Comparative account of sleep habits of school students as a function of school timing

ANOVA revealed a significant difference between the sleep duration (in weekdays as well as weekends) and wake up time of morning and dayshift school students for a period of two consecutive years. A significantly less sleep duration and earlier wake up time was observed among morning shift school students.

The weekdays sleep duration of morning shift and dayshift students in the first year was found to be $7.08 \pm 0.10$ and $8.76 \pm 0.16$ h, respectively, whereas the weekend sleep duration of the two groups was found to be $8.67 \pm 0.12$ and $9.11 \pm 0.16$ h, respectively. In the second year, morning shift and dayshift students were found to sleep for an average $6.70 \pm 0.11$ and $8.38 \pm 0.17$ h, respectively, in the weekdays and $8.14 \pm 0.14$ and $8.75 \pm 0.13$ h in weekends. The morning group was also found to have an earlier wake up time of $5{:}22 \pm 00{:}03$ AM and $05{:}18 \pm 00{:}04$ AM, respectively, in the first and second year as compared to the later wake up time of $7{:}01 \pm 00{:}07$ AM and $06{:}56 \pm 00{:}08$ AM observed among the dayshift group in the first and second year, respectively. The results also indicate a higher weekday sleep debt among the morning group in both years of observation ($F_{1,154} = 31.22$, $p < 0.001$ and $F_{1,138} = 29.02$, $p < 0.001$ respectively). Moreover, the morning group was found to have a higher degree

**Table 1** Comparison of sleep variables and salivary alpha amylase activity between morning and dayshift students in 9th grade

| | Morning shift ($n=79$) | Dayshift ($n=77$) | ANOVA summary | | |
|---|---|---|---|---|---|
| | | | *F* | *df* | *p* |
| Sleep duration (weekdays) | $7.08 \pm 0.10$ | $8.76 \pm 0.16$ | 81.71 | 1, 154 | $<0.001$ |
| Sleep duration (weekends) | $8.67 \pm 0.12$ | $9.11 \pm 0.16$ | 4.84 | 1, 154 | 0.029 |
| Bedtime (weekdays) | $10{:}19 \pm 00{:}05$ | $10{:}15 \pm 00{:}07$ | 0.154 | 1, 154 | 0.695 |
| Waketime (weekdays) | $5{:}22 \pm 00{:}03$ | $7{:}01 \pm 00{:}07$ | 139.73 | 1, 154 | $<0.001$ |
| Bedtime (weekends) | $11{:}08 \pm 00{:}13$ | $10{:}06 \pm 00{:}20$ | 6.74 | 1, 154 | 0.010 |
| Waketime (weekends) | $7{:}49 \pm 00{:}06$ | $8{:}26 \pm 00{:}08$ | 11.69 | 1, 154 | 0.001 |
| Sleep debt | $1.58 \pm 0.14$ | $0.35 \pm 0.17$ | 31.22 | 1, 154 | $<0.001$ |
| Social jetlag | $1.66 \pm 0.10$ | $1.48 \pm 0.11$ | 1.44 | 1, 154 | 0.232 |
| ESS score | $7.08 \pm 0.32$ | $5.86 \pm 0.39$ | 5.83 | 1, 154 | 0.017 |
| Stress score | $30.96 \pm 1.16$ | $25.63 \pm 1.32$ | 9.23 | 1, 154 | 0.003 |
| Salivary alpha amylase activity (U/ml) | $27.76 \pm 1.47$ | $25.46 \pm 1.74$ | 1.017 | 1, 166 | 0.315 |

**Table 2** Comparison of sleep variable and salivary alpha amylase activity between morning and dayshift students in 10th grade

| | Morning shift ($n=71$) | Dayshift ($n=69$) | ANOVA summary | | |
|---|---|---|---|---|---|
| | | | *F* | *df* | *p* |
| Sleep duration (weekdays) | 6.70 ± 0.11 | 8.38 ± 0.17 | 70.22 | 1, 138 | < 0.001 |
| Sleep duration (weekends) | 8.14 ± 0.14 | 8.75 ± 0.13 | 9.99 | 1, 138 | < 0.001 |
| Bedtime (weekdays) | 10:15 ± 00:12 | 10:22 ± 00:10 | 0.214 | 1, 138 | 0.644 |
| Waketime (weekdays) | 05:18 ± 00:04 | 06:56 ± 00:08 | 109.62 | 1, 138 | < 0.001 |
| Bedtime (weekends) | 11:02 ± 00:05 | 11:06 ± 00:06 | 0.232 | 1, 138 | 0.631 |
| Waketime (weekends) | 7:11 ± 00:09 | 7:49 ± 00:07 | 10.262 | 1, 138 | 0.002 |
| Sleep debt | 1.45 ± 0.14 | 0.37 ± 0.14 | 29.09 | 1, 138 | < 0.001 |
| Social jetlag | 1.18 ± 0.09 | 0.88 ± 0.09 | 5.50 | 1, 138 | 0.020 |
| ESS score | 7.23 ± 0.31 | 7.23 ± 0.46 | 0.000 | 1, 138 | 0.991 |
| Stress score | 28.21 ± 1.39 | 28.12 ± 1.27 | 0.003 | 1, 138 | 0.96 |
| Salivary alpha amylase activity (U/ml) | 44.62 ± 3.93 | 35.01 ± 3.39 | 4.013 | 1, 143 | 0.047 |

**Table 3** Comparison of sleep variables and salivary alpha amylase activity among students in 9th and 10th grade (irrespective of school timing)

| | First year ($n=156$) | Second year ($n=140$) | Paired *t* test | | |
|---|---|---|---|---|---|
| | | | *t* | *df* | *p* |
| Sleep duration (weekdays) | 7.84 ± 0.13 | 7.53 ± 0.12 | 2.357 | 131 | 0.020 |
| Sleep duration (weekends) | 8.89 ± 0.11 | 8.44 ± 0.10 | 3.03 | 131 | 0.003 |
| Bedtime (weekdays) | 10:13 ± 00:04 | 10:34 ± 00:04 | 3.035 | 131 | 0.003 |
| Waketime (weekdays) | 06:05 ± 00:06 | 06:08 ± 00:06 | 0.503 | 131 | 0.616 |
| Bedtime (weekends) | 10:45 ± 00:12 | 11:04 ± 00:04 | 1.49 | 131 | 0.138 |
| Waketime (weekends) | 8:06 ± 00:05 | 7:30 ± 00:06 | 4.25 | 131 | < 0.001 |
| Sleep debt | 1.07 ± 0.12 | 0.92 ± 0.11 | 0.998 | 131 | 0.320 |
| Social jetlag | 1.55 ± 0.08 | 1.03 ± 0.07 | 5.29 | 131 | < 0.001 |
| ESS score | 6.54 ± 0.31 | 7.67 ± 0.32 | 2.549 | 131 | 0.012 |
| Stress score | 28.25 ± 0.93 | 27.90 ± 0.95 | 0.269 | 131 | 0.789 |
| Salivary alpha amylase activity (U/ml) | 24.07 ± 1.01 | 32.46 ± 1.82 | 4.341 | 131 | < 0.001 |

of social jet lag in the second year of the study ($F_{1,138} = 5.5$, $p < 0.001$).

A significant difference ($p = 0.017$) was also observed in sleepiness level in the two groups in the first year of the study but not in the following year. Morning group was found to have a higher level of sleepiness (ESS score of 7.08 ± 0.32 and 5.86 ± 0.39, respectively, among morning and dayshift students) in the first year. But in the second year, the difference was not found to be significant (7.23 ± 0.31 and 7.23 ± 0.46 respectively among morning and dayshift students). Similarly, stress score was found to differ significantly among morning and dayshift students in the first year (Table 1), but in the second year, there was no significant difference observed (Table 2).

## Analysis of sleep behavior of students in first and second year of the study irrespective of school timings

ANOVA revealed a significant difference in the sleep duration, bedtime and ESS score of students in first and second year of the study. Students were found to sleep for significantly less duration in weekdays ($p = 0.020$) as well as weekends ($p = 0.003$) in the second year as compared to that in the previous year. Results also suggest a significant delay in the bedtime during weekdays ($p = 0.003$) but no significant change in the wake up time of students was observed. In contrast, there was no significant difference observed in the bedtime during weekends but the wake up time was found to

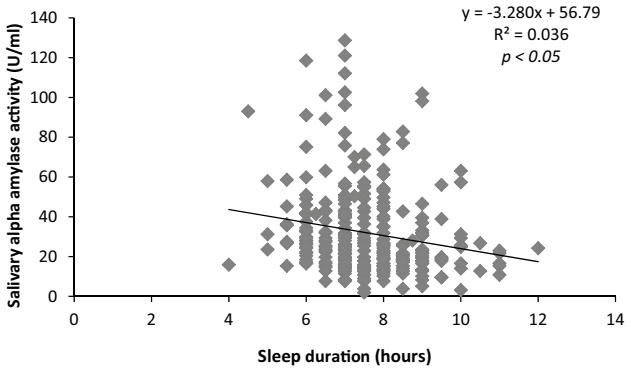


**Fig. 1** Association between sAA and sleep duration

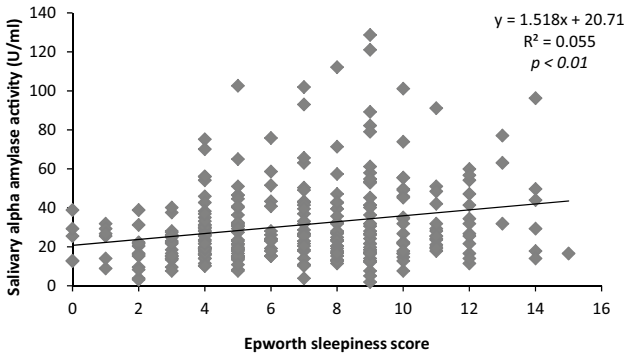


**Fig. 2** Association between sAA and Epworth sleepiness score

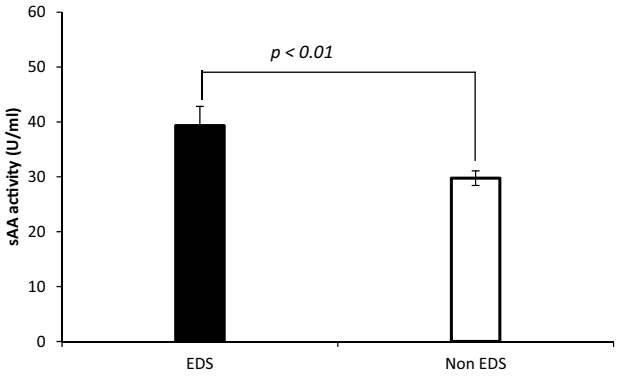


**Fig. 3** Comparison of salivary alpha amylase activity in EDS and non-EDS subjects

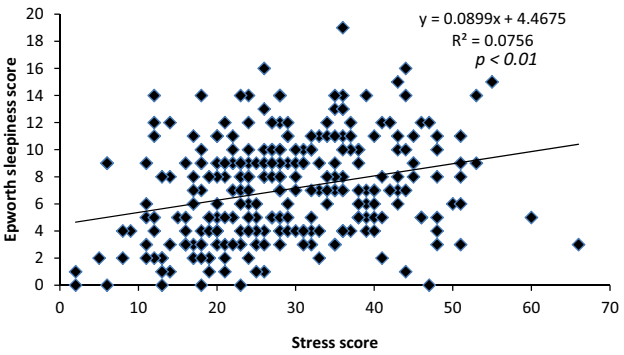


**Fig. 4** Association between Epworth sleepiness score and stress score

significantly earlier in the second year ($p < 0.001$). Students in the second year were also found to have a significantly higher level of sleepiness ($p = 0.012$). However, there was no significant difference in the amount of weekday sleep debt and stress score observed with the progression to higher grade. Significantly lesser social jet lag was observed in the second year ($p < 0.001$).

## Association of salivary alpha amylase activity with sleepiness and stress level

Analysis of data revealed a heightened association of salivary alpha amylase enzyme activity with sleep durations and sleepiness levels of adolescent students (Figs. 1, 2). Amylase activity was found to have a significant negative correlation with sleep duration ($r = -0.129$, $p = 0.026$, $n = 296$), whereas a significant positive correlation of amylase activity was observed with ESS score ($r = 0.262$, $p < 0.001$, $n = 296$). It was also noticed that the level of salivary amylase activity significantly varies with the degree of sleepiness. When level of salivary amylase activity was compared between EDS subjects and non-EDS subjects, amylase activity was found to be significantly higher ($F_{1,294} = 9.13$, $p = 0.003$) among excessive daytime sleepiness (EDS) subjects ($n = 61$) as compared to non-EDS subjects ($n = 235$). The level of amylase activity for EDS and non-EDS subjects was found to be $39.33 \pm 3.51$ and $29.76 \pm 1.33$, respectively (Fig. 3). Using Pearson correlation, amylase activity was shown to have a positive association with stress score ($r = 0.001$, $p = 0.998$) and weekday sleep debt ($r = 0.009$, $p = 0.876$) but it did not reveal a significant association. sAA was also shown to be uncorrelated with social jetlag ($r = -0.031$, $p = 0.591$).

There was a positive association seen between the subjectively reported sleepiness and stress score of students ($r = 0.275$, $p < 0.001$) (Fig. 4) but either stress or sleepiness score was not found to have any significant association with weekday sleep debt.

## Salivary alpha amylase activity as a function of school timing and progression to higher grade

sAA activity for morning shift students in the 9th and 10th grade was found to be $27.76 \pm 1.47$ and $40.75 \pm 3.38$ U/ml, respectively, whereas the level of sAA in dayshift students in 9th and 10th grade was found to be $25.46 \pm 1.74$ and $31.97 \pm 2.71$ U/ml, respectively. As shown in Fig. 5, it was revealed through ANOVA that there is a significant

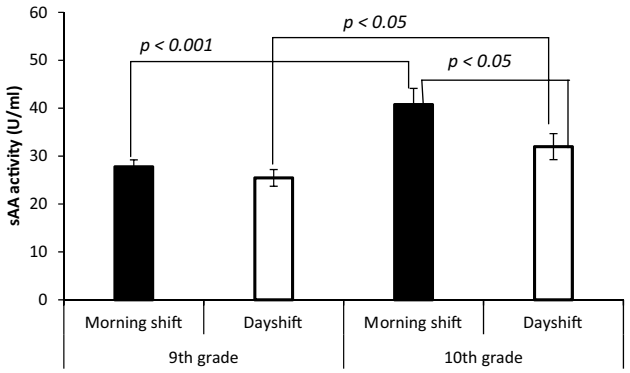


**Fig. 5** Comparative chart of salivary alpha amylase activity in morning and dayshift students in both years

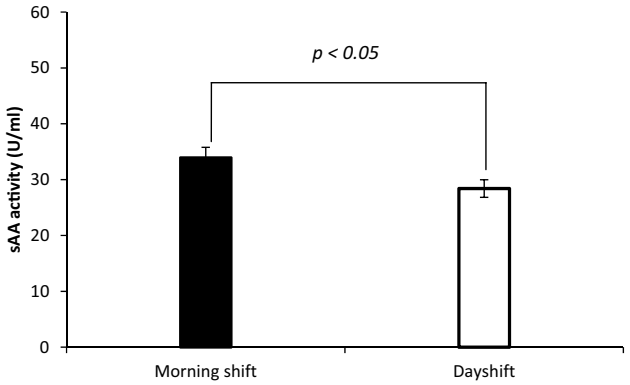


**Fig. 6** Comparison of salivary alpha amylase activity among students as a function of school timing in the pooled data

difference in sAA levels between morning and dayshift students ($F_{1,143} = 4.013$, $p = 0.047$) in the 10th grade but not in the 9th grade ($F_{1,166} = 1.017$, $p = 0.315$).

When the 2 years data was subjected to paired *t* test, it was found that the amylase activity of students has increased with progression to the 10th grade (Table 3). Paired sample *t* test reveals that the difference between amylase activity among morning group students in the 9th and 10th grade is significant ($t_{131} = 4.34$, $p < 0.001$) and that the average amylase activity recorded in second year (10th grade) is higher than that recorded in the 9th grade (95% CI − 12.21, − 4.56). In the pooled data, the salivary alpha amylase activity was found to be $33.93 \pm 1.85$ and $28.40 \pm 1.57$, respectively, among morning and dayshift students and the difference was found to be significant ($F_{1,311} = 5.18$, $p = 0.024$) (Fig. 6).

## Discussion

Prevalence of sleepiness among human population is rampant throughout the world. As a result, it has caught attention of the researchers in the recent past. Besides its widespread prevalence in adults, the school-going adolescents are also not spared of this unwanted social burden. School start timing has been reported to be an important factor in causing sleep deprivation among adolescents leading to deterioration in performance. However, a quantifiable, non-invasive biochemical marker for objective assessment of sleep deprivation is yet to be established. The present study is an attempt to find out utility of enzyme salivary alpha amylase as a biomarker of sleep deprivation among adolescent school-going students in association with the impact of school timings of students. The study also highlights the association of this biomarker with sleep duration of students as they progress towards higher grade.

Studies conducted on Drosophila have revealed that the activity of salivary amylase increases along with a concomitant increase of mRNA for encoding this protein following sleep deprivation [7]. Similar increase in salivary amylase mRNA and level of amylase enzyme activity have also been observed in human after extended waking [6]. Hence both these biochemical variables have been suggested as biomarkers for sleep deprivation in humans. However, this observation was based on a very limited number (< 10) of subjects and barring this lone study, report at population level is completely lacking and the validation at population level is most warranted. The present study was undertaken to examine the use of salivary amylase enzyme as a biomarker of sleep deprivation in large human population followed-up for a period of 2 years. Analysis of data revealed that sAA activity (irrespective of school timings) has a significant negative association with the sleep duration. Amylase activity was also found to have a significant positive correlation with ESS score suggesting that level of sAA activity is a potent indicator of degree of sleepiness. An increased amylase level in saliva among the students suffering from excessive daytime sleepiness (EDS) is a further confirmatory evidence for the association between sAA activity and sleepiness level.

Sleep behavior of students as a function of school timings was found to vary significantly. The findings of the study illustrate a significantly reduced sleep duration in the morning group than that of the day group in the first as well as second year of the study. From the data, it follows that earlier school timings contribute significantly towards the reduction in sleep duration of adolescents. The present results corroborate with our earlier findings where we have reported the sleep deprivation among morning

shift school students due to earlier school timings and have recommended delaying the school timing by 1 or 2 h [23]. It was also revealed that the sleep duration (weekdays as well as weekends) of students irrespective of their school start timings has reduced in the second year. Reduction in sleep of these students in the second year irrespective of the school start timings may be due to their more academic demand in higher grade. When the enzyme activity was compared as a function of school timings, amylase activity was found to be significantly higher among the morning group as compared to the dayshift group in the second year of the study. Higher social jet lag observed among the morning group in the second year, probably due to higher academic demand, also indicates that morning group is deprived of sufficient sleep. A higher weekday sleep debt among morning shift students in both years further substantiates the sleep deprivation among this group. A significantly higher salivary amylase activity among morning school students in the second year may be attributable to their sleep deprivation. Furthermore, this notable reduction in sleep duration can be explained by the significant delay in the bedtime with advanced grade but a constant wake up time of students throughout the study. Thus, it also follows from the findings that school students as they progress towards higher grade, exhibit reduced sleep duration due to higher amount of time required to meet academic demands in the late night hours. The significant association of sleepiness score with stress score suggests that daily stress is an important factor for causing elevation in the sleepiness level of students.

The present report, to the best of our knowledge, highlights the importance of sAA in the assessment of sleepiness level for the first time in a large population studied on longitudinal basis. The data underscore the usefulness of enzyme sAA in the evaluation of sleep deprivation among the adolescent population. The findings of this study clearly indicate that measurement of salivary amylase activity may be a suitable non-invasive biochemical parameter for objective assessment of sleep deprivation among individual as well as at population level. However, studies on larger population comprising different sectors of the society will be helpful for further validation.

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## Compliance with ethical standards

**Conflict of interest** On behalf of all authors, the corresponding author states that there is no conflict of interest.

**Ethical approval** All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee (IEC Ref No. 017/IEC/PRSU/2013 dated April 15, 2013) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

**Informed consent** Informed consent was obtained from all individual participants included in the study.

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