Increased plasma homocysteine levels in shift working bus drivers

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Background: Previous studies have indicated an association between shift work and cardiovascular disease. There is also considerable epidemiological evidence that hyperhomocysteinemia is an independent risk factor for cardiovascular disorders.

Aims: To analyse plasma homocysteine levels in shift work bus drivers, and to investigate possible relations with sleep parameters and other biochemical factors.

Methods: Blood samples were collected from 30 male shift working long-haul bus drivers in a Brazilian sample and analysed for plasma levels of homocysteine, folic acid, vitamin B₁₂, and serum lipids. A group of 22 daytime workers, matched for age and body mass index served as controls. The incidence of mutations in the gene coding for methylene tetrahydrofolate, an enzyme which is related to hyperhomocysteinemia, was also assessed. Polysomnographic recordings were obtained from the target group.

Results: Bus drivers showed significantly higher levels of plasma homocysteine than the control group (18.57 ± 9.43 µM). Most of the other biochemical, behavioural, and molecular parameters did not differ between groups. Likewise, sleep parameters appeared to be within the normal range.

Conclusions: The significantly increased plasma homocysteine levels in long-haul bus drivers did not appear to be secondary to other biochemical or behavioural problems in this group. These results suggest that hyperhomocysteinemia may be involved in the increased incidence of cardiovascular diseases observed in shift workers.
conventional risk factors. Factors that could potentially lead to hyperhomocysteinemia include some genetic defect in one of the enzymes involved in Hcy metabolism or a nutritional deficiency of one or more of the vitamins that participate in Hcy metabolism (folic acid and vitamins B<sub>6</sub> and B<sub>12</sub>). While Hcy is associated with CVD and the latter is very frequent in shift workers, no studies, to the best of our knowledge, have investigated levels of Hcy in shift workers. Therefore this was the main objective of this study. To this end we studied a sample of Brazilian long-haul bus drivers working shifts. Our analyses also included behavioural factors, sleep, and biochemical and molecular parameters.

**MATERIAL AND METHODS**

**Study subjects**

Data were obtained from 30 male professional long-distance bus drivers ranging in age between 31 and 51 years (mean age 40.6 (SD 5.77) years) and body mass index (BMI) between 18.5 and 37.0. These drivers were shift workers employed by the same interstate transportation company, who followed irregular schedules—that is, whose driving assignments to varying interstate destinations might start at different times of day or night. Subjects included in the study were equated for average duration of driving assignments, which was approximately six hours each trip, with a compulsory rest period of at least 12 hours between trips. The median time at this occupation for this sample was 37 months (range 4–106 months). Mandatory pre-employment medical examinations had been carried out, which screened for pre-existing chronic diseases, including cardiovascular disease, in addition to annual medical examinations mandated by law. They enrolled as volunteers in a sleep study at the Sleep Institute, Department of Psychobiology, Federal University of São Paulo.

Another 22 male workers equated for age (43.5 (5.14) years) and BMI served as controls. These subjects worked regular (non-shift) schedules in several occupations that were not significantly different from bus driving in socioeconomic terms. Each subject answered a yes-no questionnaire which asked whether they often engaged in physical activity, consumed alcohol, and/or smoked. All subjects gave informed written consent to participate in the study and agreed with the procedures, according to the norms of the Medical Ethics Committee of UNIFESP/EPM. The investigation conformed to the procedures, according to the norms of the Medical Ethics Committee of UNIFESP/EPM.

**Blood sampling**

Fasting blood samples for biochemical analyses were collected at 8 am into vacuum tubes (Becton Dickinson). One day before sampling, all subjects underwent the following standardised procedure: they performed a work shift during the day, arrived at the sleep laboratory at 8 pm, and were directed to bed at 10 pm. Before going to the laboratory, they had dinner at the bus company headquarters at 7 pm. After an uninterrupted night of sleep, fasting blood was collected at 8 am. They then had breakfast and answered a questionnaire. Subjects stayed awake until 10 pm when polysomnographic recordings were carried out for eight hours. Blood samples were processed, and serum and plasma aliquots were separated and stored at −80°C until biochemical analyses were carried out.

**Biochemical methods**

Plasma folic acid and vitamin B<sub>12</sub> were determined by the automated chemiluminescence system (ACS:180, Bayer Corporation). Total Hcy values were determined by high performance liquid chromatography (HPLC) with fluorometric detection and isocratic elution. Commercial kits (DIALAB, Diapack Liquid Reagents, Vienna, Austria) were used for spectrophotometric serum determinations of total cholesterol and its fractions (HDL, LDL, and VLDL), triglycerides, and glucose by ADVIA 16/50 (Bayer Diagnostics Corporation).

### Table 1: Characteristics of self reported daytime and bus driver shift workers

<table>
<thead>
<tr>
<th>Variable</th>
<th>Daytime workers (n)</th>
<th>Bus drivers (n)</th>
</tr>
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<tbody>
<tr>
<td>Smoker (%)</td>
<td>35.00 (7)</td>
<td>37.50 (9)</td>
</tr>
<tr>
<td>Alcohol consumer (%)</td>
<td>47.62 (10)</td>
<td>58.33 (14)</td>
</tr>
<tr>
<td>Physically active (%)</td>
<td>33.33 (7)</td>
<td>58.33 (14)</td>
</tr>
<tr>
<td>BMI (mean ± SD)</td>
<td>25.64 ± 3.77 (22)</td>
<td>27.30 ± 3.61 (30)</td>
</tr>
</tbody>
</table>

BMI, body mass index.

*Significantly different from daytime workers (χ<sup>2</sup> test, p<0.05).

#### Sleep recording

All 30 drivers underwent polysomnographic assessment after an initial adaptation night to the sleep laboratory. Sleep recordings were carried out for eight hours (beginning at 22:00 ± 0.30 h) using Medilog Recorders (Oxford Instruments Ltd). The following parameters were obtained, according to the criteria established by Rechtschaffen and Kales<sup>15</sup>: sleep onset latency (SOL; time lag between the beginning of recording and the first 10 minutes of uninterrupted sleep), total sleep time (TST; sum of all sleep periods during the recording), sleep efficiency (SEfF; TST/total recording time), duration of stages 1 and 3, duration of stages 3 and 4 slow wave sleep (SWS), duration of rapid eye movement sleep (REM), REM latency (REML), wakefulness after sleep onset (WASO; sum of all periods of wakefulness after sleep onset), number of arousals/hour, apnoea/hypopnoea index (AHI; number of apnoea and hypopnoea events/hour).

**Gene analysis**

Genomic DNA was obtained from peripheral blood samples using QIAamp DNA Mini Kit (QIAGEN Inc.). To analyse the C677T mutation in the methylene tetrahydrofolate reductase (MTHFR) gene, 50 ng of DNA was used for polymerase chain reaction (PCR), amplification, using upstream and downstream primers flanking the respective sequences. The amplified and digested PCR products were analysed in 8% non-denaturing ethidium bromide stained polyacrylamide gels, and each individual was genotyped.

**Statistical analysis**

Living habits and biochemical parameters were analysed using non-parametric tests for comparison between groups after distribution analysis. Data for biochemical variables are expressed both as means (SD) and medians (range), since not all variables followed a normal distribution. Data for sleep parameters are presented as means (SEM) for ease of comparison to published values. Correlation analyses were performed using Spearman’s rank correlation test (r). The association of occupation types with risk factors for CVD, folic acid, and vitamin B<sub>12</sub>, including C677T polymorphism, was evaluated using unadjusted and adjusted odds ratio (OR) computed with logistic regression analyses. For all analyses, the alpha level was established at p < 0.05 (two tailed) conducted with the SAS statistical system (SAS Institute, Inc., North Carolina, USA).

### RESULTS

#### Sample characteristics

Table 1 shows the living habits of both groups. Statistical analyses revealed no significant differences between groups for most of the behavioural variables, except for a significantly higher percentage of physically active bus drivers compared to controls (χ<sup>2</sup> = 6.28, p < 0.05).
Biochemical measures
Table 2 summarises the results. Both groups had biochemical parameters within normal range, with the exception of Hcy level in bus drivers, which was >15 µM (normal range 5–15 µM). Furthermore, 66.7% of bus drivers versus 9.1% of daytime workers had Hcy levels above 15 µM, which is often used to define increased risk for CVD19 (odds ratio (OR) 20.0, 95% CI 3.88 to 103.1). There were also significant differences between daytime and shift workers in the levels of HDL cholesterol (–20%, p < 0.05), Hcy (+96%, p < 0.05), and folic acid (+40%, p < 0.05). There were no significant correlations among Hcy levels and other biochemical parameters in bus drivers. A stepwise multiple logistic regression analysis including all factors revealed that only homocysteine presented a significant association with shift work drivers. A significant increase in the levels of HDL cholesterol (16.95%, p = 0.03), folic acid (21.11%, p = 0.03), and vitamin B12 (20.0, p = 0.03) was noted in the group of shift workers compared to daytime workers. On the other hand, in the Copenhagen male study there was no difference in the proportion of alcohol consumers between shift and daytime workers. In our study no differences in the proportion of alcohol consumption were found between the groups (table 1).

Sleep parameters
All of the measured sleep parameters in the bus driver sample appeared to be well within the normal range.13 Means and standard errors were: sleep onset latency: 15.75 (3.56) minutes; total sleep time: 401.1 (12.06) minutes; sleep efficiency: 84.17% (2.15%); percentage of sleep spent in stage 1: 15.95% (3.56); stage 2: 49% (1.96%); slow wave sleep: 15.95% (3.56); number of arousals/hour: 5.58 (0.73); apnoea/hypopnoea index: 6.10 (2.14).

Significant correlations were found between Hcy levels and sleep onset latency (r = –0.58), sleep efficiency (r = 0.39), and number of apnoea episodes/hour (r = 0.43) in the group of bus drivers.

DISCUSSION
The present study revealed increased plasma Hcy levels in 67% of the shift workers studied, indicating an increased cardiovascular risk. These data are consistent with the higher incidence of CVD observed in other populations of shift workers.20–22

Among the hypotheses proposed to explain the augmented susceptibility of shift workers to develop CVD, Harma and Ilmarinen23 have suggested that shift work triggers the effect of other lifestyle related risk factors for coronary heart disease. The risk factors that have been studied in this population include disruption of circadian rhythms, disturbed sociotemporal patterns, disrupted social support, stress, behavioural (smoking, diet, alcohol, exercise), and biochemical changes (for example, increased levels of cholesterol and triglycerides).2

In this study the percentage of smokers was similar in the two groups (table 1). It has been suggested that for both daytime and rotating-shift workers the number of cigarettes smoked per day is associated with poor work site social support and/or lower work pace control.20 The lack of differences related to smoking in the present study is consistent with this possibility. It is quite possible therefore that increased smoking is not directly related to shift work; rather, it may be a mediating factor which cannot completely explain the increased risk for CVD among shift workers.24

In the literature there are controversial data concerning alcohol consumption among shift workers. In the Helsinki Heart Study,7 the number of moderate or heavy alcohol consumers was apparently lower among shift workers compared to daytime workers. On the other hand, in the Copenhagen male study there was no difference in the proportion of self reported drinkers between shift and daytime workers; however, among the drinkers, shift workers consumed slightly, but significantly more than day workers.25 In our study no differences in the proportion of alcohol consumption were found between the groups (table 1).

In general, there is no indication of marked differences in physical activity between shift and daytime workers, which is of interest given the general association between physical activity levels and cardiovascular risk.7,24,25 In our sample, 75% of the shift workers reported regularly engaging in physical activity, as defined by Caspersen and colleagues,25 in contrast to 33% of control subjects. However, given that BMI indices did not differ between groups, the amount of physical activity reported by the shift work group may not be sufficient to produce alterations which may be translated in a lower risk for cardiovascular disease.

Bogdil and Knutsson7 observed a slight increase of cholesterol and triglyceride levels in shift workers, which is an important observation considering that these and other lipids are the best markers of atherosclerotic diseases. Peter and

Table 2 Biochemical parameters in shift and daytime workers

<table>
<thead>
<tr>
<th>Variable</th>
<th>Daytime workers (n=22)</th>
<th>Shift workers (n=30)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycemia [mg/dl]</td>
<td>92.62 (13.13)</td>
<td>89.85 (71.97–120.00)</td>
<td></td>
</tr>
<tr>
<td>Total cholesterol [mg/dl]</td>
<td>194.05 (39.41)</td>
<td>190.35 (133.33–298.00)</td>
<td></td>
</tr>
<tr>
<td>Triglycerides [mg/dl]</td>
<td>152.21 (71.34)</td>
<td>148.85 (43.35–312.00)</td>
<td></td>
</tr>
<tr>
<td>HDL cholesterol [mg/dl]</td>
<td>49.11 (13.32)</td>
<td>49.59 (30.99–78.00)</td>
<td></td>
</tr>
<tr>
<td>LDL cholesterol [mg/dl]</td>
<td>113.14 (52.17)</td>
<td>110.82 (57.99–197.00)</td>
<td></td>
</tr>
<tr>
<td>VLDL cholesterol [mg/dl]</td>
<td>30.42 (14.23)</td>
<td>29.71 (8.67–62.00)</td>
<td></td>
</tr>
<tr>
<td>Total cholesterol-HDL cholesterol ratio</td>
<td>4.21 (1.36)</td>
<td>3.97 (2.52–7.64)</td>
<td></td>
</tr>
<tr>
<td>Homocysteine (µM)</td>
<td>9.43 (3.25)</td>
<td>9.22 (4.80–18.23)</td>
<td></td>
</tr>
<tr>
<td>Vitamin B6 (µg/ml)</td>
<td>430.20 (188.79)</td>
<td>385.75 (190.40–1069.00)</td>
<td></td>
</tr>
<tr>
<td>Folic acid (ng/ml)</td>
<td>8.46 (3.91)</td>
<td>7.80 (2.7–20.00)</td>
<td></td>
</tr>
<tr>
<td>Malay to Vitamin B12 ratio</td>
<td>16 19</td>
<td></td>
<td>0.43</td>
</tr>
<tr>
<td>Total cholesterol:HDL cholesterol ratio</td>
<td>2 6–8</td>
<td></td>
<td>1.49</td>
</tr>
</tbody>
</table>

Table 3 Presence of C677T polymorphism in the MTHFR

<table>
<thead>
<tr>
<th>C677T genotype</th>
<th>Daytime workers n (%)</th>
<th>Shift workers n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>11 (50.00)</td>
<td>19 (63.33)</td>
</tr>
<tr>
<td>CT</td>
<td>9 (41.00)</td>
<td>11 (36.67)</td>
</tr>
<tr>
<td>TT</td>
<td>2 (9.00)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

C677T polymorphism: CC, homozygous for the wild type; CT, heterozygous, and TT, homozygous for the mutation.
colleagues' reported an increased total/HDL cholesterol ratio in shift workers. Contrary to what has been previously reported, in folliculocystic polycystic ovary syndrome patients, the total/HDL cholesterol ratio was similar between both groups (table 2).

Homocysteine has been considered an independent risk factor for cardiovascular diseases. 18 19 No previous studies however have analysed plasma levels of this amino acid in shift workers. The shift workers in the present study had homocysteine levels well above the normal range. Elevation of plasma homocysteine levels may provide an explanation for the higher incidence of cardiovascular diseases in shift workers, which has not been satisfactorily accounted for by traditional risk factors for atherosclerosis. Moderate hyperhomocysteinemia has been associated with genetic factors, chiefly polymorphisms in the gene coding for the enzyme MTHFR. 20 The frequency of the particular polymorphism examined in this present study (C677T, table 3) was not different in shift workers, compared to the control group or to values in the overall Brazilian population. 21 Therefore, this finding does not support the possibility that a genetic factor associated with this enzyme explains the increased levels of homocysteine detected in the bus drivers.

Another important factor associated with homocysteine levels is the availability of folic acid and vitamin B 12. Vitamin B 12 status was not different between the groups and was within the normal range. In healthy subjects, however, vitamin B 12 seems to play a minor role as determinant of Hcy concentration. 22 Our sample was composed of subjects with folic acid levels within the normal range, although on average, shift workers did show lower levels than daytime workers (5.08 ± 8.46 nmol/l). Although the relation between folic acid and homocysteine levels is well known, there was no significant correlation between these parameters among the bus drivers. However, when we considered all the subjects (daytime and shift workers) a significant correlation emerged ($r = -0.46$), confirming the existence of a general relation between these two compounds.

The recommended amount of folic acid intake is 300 µg/day, whereas the plasma concentration in healthy subjects is 7–8 nmol/l. 23 The slight reduction in plasma folic acid levels in bus drivers may contribute to the increase in homocysteine levels, although folic acid levels were within expected values. The difference in folic acid levels may be related to the quality of meals taken by the shift workers. Although we have not found studies establishing an association between vitamin status and atherosclerotic diseases in shift workers, other findings have suggested altered quality and quantity of meals taken by these workers, which could affect the risk for CVD. Alternatively, it is possible that shift work does not change the diet's nutritional quality or meal frequency, 24 but may instead lead to a circadian redistribution of relative energy expenditure. 25 26 Redistribution of meals from the afternoon to the evening is associated with increased total and LDL cholesterol; however, when meals are taken in the morning there is a reduction in HDL cholesterol. 27 Recently, it has been observed that protein-rich meals taken in the morning can increase plasma homocysteine levels by 15–20% above fasting levels. 28 Experimental studies with rats show that cholesterol and methionine supplementation diets can produce striking increases in homocysteine levels. 29 It is possible, therefore, that meal redistribution to which shift working bus drivers are constantly subjected, added to the poor quality of these meals (possibly including insufficient folic acid intake), may result in a predisposition towards higher homocysteine levels. This in turn may be responsible for the high incidence of atherosclerotic diseases in shift workers. If this reasoning is correct, folic acid supplementation should be considered as a preventive measure in this population, as in the case of neural tube defects and CVD in general. 30 31 Reduced folate levels combined with increased homocysteine plasma levels has been linked to heavy or chronic smoking and alcohol intake. 32 33 In the present study there were no significant differences in numbers of drinkers and smokers in the two groups. We also failed to find differences in homocysteine and folic acid levels when we separated drinkers from non-drinkers and smokers from non-smokers. Therefore, we have no evidence that lower folic acid and higher homocysteine levels in bus drivers could be attributable to smoking or alcohol intake. In summary, an important finding of this study is that Hcy was the only variable showing a significant association with this type of occupation, as shown by multiple logistic regression analyses.

On the other hand, one of the limitations of the present study was that it did not control adequately for some potentially relevant factors associated with shift work. In particular, information was not available on diet, stress assessments, and, in the case of the control group, sleep parameters. Also unavailable was information on the biochemical status of the participants before they became bus drivers or shift workers. On the other hand, there is no reason to suspect that these drivers, as a group, might already have hyperhomocysteinemia before they started to work irregular schedules. Given the median length of employment (37 months) in their present occupation, it is unlikely that previous status would be a factor in the results obtained in this study.

The most important health complaint of shift workers is related to sleep impairments, with a higher incidence in subjects older than 40 years of age. 34 The circadian resynchronisation to which shift workers are frequently subjected leads to a significant reduction in sleep time. 35 There is limited knowledge on the possible relation between reduced or disrupted sleep and CVD. Sleep apnoea, for example, is a risk factor for CVD, but few studies have looked at the relation between shift work and sleep apnoea syndrome. 36 37 Although we did find a modest correlation between the number of apnoea episodes and homocysteine levels among bus drivers, we also found that only 3.3% (1 subject in 30) presented a pathological apnoea/hypopnoea index (AHI > 10/hour), a low prevalence similar to that described by Mitler and colleagues 38 for long-haul truck drivers. Overall, in the present study there was little indication of disrupted sleep patterns in bus drivers. It must be noted however that this suggestion is based on published normative data, 39 since unfortunately it was not possible to obtain polysomnographic data for the control group in this study. It is also conceivable that sleep abnormalities might have emerged if the subjects had not been allowed one night of adaptation to the laboratory environment before measurements were taken. With these limitations in minds, it seems nonetheless possible to suggest that alterations in plasma Hcy levels were not secondary to sleep deficits.

Conclusions
Long-haul bus drivers working irregular shift hours were found to have increased plasma homocysteine levels, which could not be accounted for by any of the other factors examined in this study. While more investigation is required in order to explain the reason for this increase, the data suggest that hyperhomocysteinemia may be involved in the recently described increased index of cardiovascular diseases in shift workers.

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