# Simultaneous measurement of $CO$, $CH_4$ and $H_2$ gases in exhaled breath taken during a medical examination of students and evaluation of their stress levels

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## Introduction

There has been much talk recently of the influence of different stressors in daily life on the heart and on the body, and it is thought that excessive anxiety or tension caused by stress risks inducing abnormal secretion of hormones and autonomic disorders which provoke various mental illnesses such as depression or the illnesses known as "lifestyle diseases" such as high blood pressure.[1] In order to study these problems, the recently created Japanese Society of Fatigue Science held its first congress in June 2005. A number of university hospitals have begun to set up Clinical Fatigue Centres in order to study and treat social fatigue and stress, these being subjects that have been neglected until recently.[2]

Taking advantage of the opportunity presented by the medical examination of students at the Kawasaki University of Medical Welfare, where we specialize in the field of training of experts in medical care, we measured the density in the breath of CO produced in the cells by haemo-oxygenase-1 (HO-1),[3] which is induced by stress as well as the level of stress using a Stressometer device.[4] We also measured the density in the breath of methane ($CH_4$) produced by fermentation in the intestines caused by constipation, abnormal fermentation or intestinal ailments, etc.,[5] as well as the density of hydrogen ($H_2$) in the breath caused by intolerance to lactose, to the length of time required for food to pass through the intestines, or to dietary fibre, etc.[6] We thought that these various measures would make it possible for us to evaluate the level of stress experienced by students and their way of life, as manifest in their smoking habits (considered as exogenous CO),[7] consumption of alcohol, eating habits and sport, and that this evaluation would be useful for orienting students towards the medical career on which they would embark in the future.

In effect, the analysis of gas in the breath is non-aggressive and adequate for repeated analysis.[8] In addition, a new gas measurement device makes it possible to analyse CO, $CH_4$ and $H_2$ simultaneously.[9]

For all these reasons, we planned this experiment at the time of the students' medical examination in order to acquire knowledge of their levels of stress and way of life.

This paper presents a summary of the results of this trial. The second trial is currently under way. We will present the results of this second trial on a future occasion.

## Objects and methodology

**Medical examination of students**

The medical examination of the students at our university (three faculties consisting of eleven departments and with 4,000 students) took place between 28 March and 13 April 2005. During this period and depending on our availability, we took samples of the exhaled breath of 993 students over four days, namely 5, 7, 12 and 13 April, from 8.30 a.m. to 12.00 noon and from 1.30 to 4.00 in the afternoon, taking advantage of the time that the students were waiting to receive their X-ray examination. At the same time, we also measured the stress levels of 445 students using a Stressometer for four days, but avoiding times of overcrowding.

496 students were examined in the morning and 497 in the afternoon for exhaled breath, and 359 were examined in the morning and 86 in the afternoon for measurement of stress levels.

Before measurement of exhalation and stress levels, the students filled out a questionnaire (see Table 1: "Survey on measurement of gas density in breath"), which was used for self-evaluation at the time of the analysis.

The present research has been approved by the Ethics Committee of our university under the Approval Number 018.

**Exhalation sampling**

Each student breathed in lightly and then waited for 15 seconds before exhaling. Breath was taken at the last moment of exhalation, with each subject exhaling between 100 and 200 ml into a breath sampling bag manufactured by Otsuka Seiyaku.[10]

**Measurement of exhaled gas**

1 ml of exhaled gas was taken with a syringe and introduced into a gas analysis device, TRIlyser mBA-3000, manufactured by Taiyo, which makes it possible to analyse the three gases simultaneously for 4 minutes 20 seconds per sample. All the analyses were performed two to five days after the exhalation was sampled.

The analysis device used is a gas chromatograph equipped with a high-sensitivity semiconductor gas detector. The lower limit for detection of the three gases is 0.1ppm, and its reproductivity is +2%. Its linearity reaches a level of 100 ppm.[9] As the carrier gas we used pure synthetic air (impurity of less than 0.1 ppm). We carried out calibration on every 100 samples using mixed gas of high density (50 ppm for the three gases) and low density (5 ppm). The density of CO, $CH_4$ and $H_2$ in the air where the analysis was performed was 0.6 ppm, 1.5 ppm and 1.0 ppm respectively.

**Stress level**

The stress level was measured using a Stressometer developed by TNR TECHNOLOGY in France. While the measurement was being made the students maintained the standard position for measurement (standing but relaxing). They lightly grasped the sensor handle with the right hand (or the left hand in the case of left-handed subjects) and waited 10 to

20 seconds for the TNR (Tremor of the Nervous System at Rest) to stabilize at a level below 50 in the “Continuous” mode. We then measured stress for 20 seconds in the “Measurement” mode.[4] If the value obtained was in excess of 100 TNR, we carried out the measurement again after asking the student to assume a relaxed posture.

The Stressometer makes it possible to detect tremor of the living body (tremor of the hand with an amplitude of between 3 and 20 Hz) by means of a piezoelectric sensor. The electronic circuit processes the signal and displays the measured TNR value on the screen. In most cases the average TNR value is situated between 35 and 40 in the case of a healthy male. As a criterion for judgment, a TNR value higher than 25 is considered to be “stressed”. A level of 50 or above indicates that stress should be considered “very important”, while a TNR higher than 100 indicates tremor ascribable to illness.

As shown in Table 2, we reckon that this device detects primarily physiological tremor in addition to a part of essential tremor which may vary depending on levels of fatigue, stress, anxiety and tension.

Several students showed major tremor which should be treated as essential tremor or hyperthyroidism. (A second series of tests is being conducted on these students.)

**<u>Statistics</u>**

The results obtained are expressed in terms of the average value + the average absolute deviation, and the significant difference is verified by means of Student t test and (X2) test.

We estimated the significant difference when “p” is less than <0.05.

## Results

1. <u>CO in the breath: CO considered as a Stress Marker</u>

The density of CO in the breath is classified into the following three categories:
- Category 1: Under 3 ppm
- Category 2: 3 to 5 ppm
- Category 3: Over 5 ppm

Table 3 shows the results. Among the 993 students who took part in the test, 11.3% came within Category 3 (over 5 ppm). The figure for male students only (n = 382) indicated that 20.2% came within Category 3, while the figure for female students (n = 611) shows that only 5.7% came within this category. This difference is significant ($p<0.001$) (see Table 3 and Figure 1).

Figure 2 shows the number of smokers and non-smokers as well as the risk of passive smoking by non-smokers, with a classification of the risk into the following three categories:
- Risk of passive smoking
- Very little risk
- No risk

CO density is markedly higher in the case of smokers, and the density increases in proportion to the number of cigarettes smoked.[7]

In the case of non-smokers, CO density is higher in the case of those who are subjected to the risk of passive smoking in comparison with non-smokers who are not exposed to this risk ($p<0.01$) (see Figure 2).

These results show that the difference due to gender on the density of CO in the breath is likely to be due to the difference in the number of smokers, because of the greater proportion of smokers among male students (79.3%).

Among non-smokers who are not exposed to any risk of passive smoking, we also carried out a comparison of the density of CO involving division into the two following groups:
- Those who engage in sporting activities (two or three times a week and four or five times a week).
- Those who do not engage in sporting activities.

As shown in Figure 3, the density of CO is highest in the group engaging in sporting activities (two or three times a week and four or five times a week) than for the group not engaging in sporting activities. The density of CO increases in accordance with the frequency with which sporting activities are engaged in, and the difference in this respect is significant ($p<0.01$). (The number of students engaging in sporting activities once a week or six or seven times a week is very small and negligible.)

We also conducted a comparison of CO density in the breath by dividing the non-smoking students with no exposure to passive smoking into the following two groups:
- Those who engaged in energetic sporting activity the preceding day (n=11).
- Those who did not engage in energetic sporting activity the preceding day (n=24).

We did not find any significant differences through this comparison.

We also performed a comparison of the density of CO in the breath in accordance with the replies "Yes" or "No" given by students in the questionnaire (alcoholic beverages, regular meals (three times a day), constipation, duration of sleep, taking of medicines or dietary supplements, unstable health), but we failed to find any significant differences.

We finally carried out a comparison of the density of CO in the breath in accordance with the replies given by students to questions concerning stress (n=167) based on division into the following groups:
- Group with heavy stress.
- Group with moderate stress.
- Group with almost no stress.

However, we found no significant differences here either.

2. Level of stress according to level of tremor

We measured the level of stress of students using a Stressometer and classified the results according to gender into the following five categories:
- Under TNR 25.
- TNR 25 to TNR 50.

- TNR 51 to TNR 70.
- TNR 71 to TNR 100.
- Over TNR 100.

Table 3 shows the results of measurement of students' stress levels.

The TNR for most students (68.8%) was less than 50. But 13.0% of students had a TNR higher than 70. We will re-check their state of stress during the second tests which are currently under way.

Between men and women, a TNR higher than 70 is more important for a man than it is for a woman ($p<0.01$) (see Table 3 and Figure 1).

If we divide students with a TNR higher than 70 according to year, second-year students are significantly more important (16.8%) than third-year (12.8%) or fourth-year (7.9%) students (($p<0.01$).

(By chance, there were no first-year students among the students we measured for stress levels.)

As regards measurements carried out in the morning and the afternoon, the number of students with a TNR higher than 70 was higher in the afternoon measurement (18.6%) than the morning measurement (11.7%) ($p<0.01$).

And if we divide students with a TNR higher than 70 according the departments of faculties, section "J" (subjects: 19 male and 9 female students) shows the most important results (22.4%) in relation to the average result of 13.0% (174 male and 271 female students) ($p<0.01$). The number of male students is much higher than the number of female students in this department. But we did not observe any significant difference in other departments where male students outnumber female students. This means that it is possible that there may be factors other than gender difference which contribute to raising levels of stress of students in this department.

We also studied the correlation between stress levels and the density of CO in the breath (considered as a Stress Marker) for the case including smokers and passive smokers as well as for the case excluding smokers and passive smokers, but we did not manage to find any clear correlation between these two cases.

On the other hand, there was a significant correlation for non-smoking female students ($n=36$, excluding passive smokers) ($y = 0.0281X + 2.07$, $R^2 = 0.1706$, $p<0.01$) (see Figure 4).

There was no clear correlation with replies to the questionnaire (smoking habits, daily sporting activities, energetic sporting activities, regular meals (three times a day), constipation, length of sleep), nor did we find any correlation as regards replies concerning fatigue or stress.

During the second tests currently under way, we are classifying students with a high TNR according to Table 2 and we will examine the correlation with the results of the GHQ28 survey as well as variations in stress level before and after stress charge on the basis of the Uchida-Kraepelin test.

3. <u>Density of $CH_4$ and $H_2$ in the breath: Analysis of lifestyle on the basis of fermentation produced by intestinal flora</u>

Certain studies show that the density of $H_2$ in the breath is influenced by the intake of food, but this influence is not important on the density of $CH_4$.[11] In the questionnaire filled out by the students before measurements were taken, either in the morning or in the afternoon, there were no questions related to the intake of food products resistant to absorption (such as starch or oligo-saccharide) which might raise the density of $H_2$ for breakfast or lunch. We therefore studied mainly that density of $CH_4$ in the breath based on division into the following four categories:
- Category 1: Under 1 ppm
- Category 2: 1 to 3 ppm
- Category 3: 3 to 5 ppm
- Category 4: Over 5 ppm

The results of measurement according to gender are shown in Table 3.

Students in Category 4 ($CH_4$ higher than 5 ppm) constituted 3.6%. According to gender, the number of female students in Category 4 (4.4%) was larger than that of male students (2.4%) in this same category. There was thus a significant different ($p<0.01$) (see Table 3 and Figure 1).

As regards correlation with defecation, the density of $CH_4$ was lower for students who had defecated in the morning than for those who had not defecated ($p<0.01$) (see Table 5). In addition, the density of $CH_4$ was generally lower for students who did not have any problems as regards constipation (student who had defecated in the morning or on the previous day) than for those who were constipated ($p<0.02$) (see Table 5). The difference in the density of $CH_4$ according to gender might well be due to the fact that the problem of constipation is more important for female students.

Between smokers and non-smokers, the density of $CH_4$ is lower for smokers than for non-smokers.

As regards alcoholic beverages, the density of $CH_4$ is significantly lower for students who drink alcoholic beverages every day than for those who drink rarely or never ($p<0.05$). This result may be connected with the fact that there are very high rates of smokers and drinkers among male students (80% and 100% respectively).

As to the correlation between $CH_4$ and $H_2$ in the case of all the students (n=993), students with a high $CH_4$ density generally have a low density of $H_2$. In contrast, students with a low density of $CH_4$ generally have a high density of $H_2$ ($y = -0.2681X + 17{,}888$, $R^2 = 0.0094$, $p<0.01$).

We also verified the correlation between $CH_4$ and $H_2$ on the majority of students with a density of between 1.5 and 5 ppm for the two gases while eliminating students with a density of less than 1.5 ppm and more than 5 ppm (n=892 corresponding to 89.9% of the total number of students). We found a clear correlation ($y = -4.14245X + 24.388$, $R2 = 0.0195$, $p<0.01$) (see Figure 6). In the case of comparison between average values shown graphically in columns, there is a significant difference between students with a $CH_4$ density of less than 1 ppm and other students with a $CH_4$ density of between 1 and 3 ppm, 3 and 5 ppm, and over 5 ppm. The $H_2$ density of students with a $CH_4$ density of less than 1 ppm is significantly higher ($p<0.01$).

## Discussion

The medical term “stress” was coined more than half a century ago, since when the world has experienced a succession of wars, conflicts and disasters. Not only in developed countries but also in the developing countries, the inhabitants of these regions experience anxiety and excessive stress that are destroying their health all over the world.

There are people resistant to attack by different stressors (stress factors) of all kinds, but there are also people without such resistance due to personal stress resistance factors. Stress reactions are more important for people without resistance, but if such people have friends and family members who understand their anxiety and accept their feelings, the stress reaction will be alleviated thanks to their support.

Stress reactions are experienced through many different and very complicated mechanisms, such that it is extremely difficult to quantify the level of stress.

If there were a trustworthy biomarker that made it possible to evaluate stress, this would prove useful for a “healing science” based on scientific studies of rest which would make it possible to regulate the problem of stress and to provide recuperation from fatigue.

Despite numerous studies conducted up to the present time,[12] [13] we have still not found a good and simple method for evaluating stress.

But since CO is produced by an enzymatic reaction of HO-1 induced by stress, one can consider CO to be a powerful antioxidant stress element. Accordingly, a series of reaction induced by HO-1 should provide a biological defence.[14]

A number of non-smokers also show a high level of CO in their breath. It seems likely that this phenomenon is due to passive smoking or to the inhalation of exhaust gas from road vehicles.[15] But, among non-smoking students with no risk of passive smoking (n=168), we noticed a CO density in excess of 5 ppm for five out of 168 students, and this might be connected, for example, to sporting activities.

It should be observed too that there is a correlation between the level of stress and the density of CO for non-smoking female students with no risk of passive smoking, and also that male students in this department had a particularly high density of CO.

In order to clarify the influence of stress on illness, we need to re-check the variation of CO density during the day as well as before and after the stress charge.

As regards the stress level analysed by means of a Stressometer, 13.0% of students showed a TNR higher than 70. A high TNR is more important for male students than for female students. We reckon that this tremor can be classified as either a physiological tremor or an essential tremor. In order to find out more about the influence of stress and fatigue on students, we will perform a GHQ28 survey whose aim will be to evaluate their state of mental health and an Uchida-Kraepelin test so as to evaluate variation in the level of stress before and after the stress charge.

We also studied the way of life of the students while simultaneously measuring the density of $CH_4$ and $H_2$ in the breath while CO is being measured. It should be noted that $CH_4$ density is higher in the case of female students and that this is connected with defecation or constipation. He need to conduct further studies to see if the simultaneous performance of measurement of $CH_4$ and $H_2$ in addition to measurement of CO is a good method for evaluating the state of stress, smoking habits, energetic sports, eating habits, intake of fibre in the food, constipation, the amount of time it takes for food to pass through the intestines, intolerance to lactose, etc.

As regards $CH_4$, 3.6% of students showed a high density in their breath.[16] And we observed also an inverse correlation between the density of $CH_4$ and that of $H_2$. We reckon that, as far as students are concerned, $CH_4$ is produced by *Methanobrevibacter smithi* (bacteria produced by $CH_4$ from $H_2$ and $CO_2$) and less numerous *Methanosphaera stadtmaniae* (bacteria produced by CH4 from methanol and $H_2$).[17] But we do not yet know how and when these bacteria penetrated students' bodies or the factors which bring about a variation in the production of $CH_4$. We will check these matters during the second test.

Due to the fact that the production of $CH_4$ increases through the addition of arginine during the cultivation of faecal matter in vitro,[18] we intend to study variations in production of $CH_4$ in the case of assimilation of buckwheat protein (a protein resistant to absorption) through the mouth.[19] There is thus still much left for us to study.

A future topic of interest for us will be to construct a theoretical system of nutritious food that is capable of lowering stress and to devise a concrete strategy. We hope to study whether certain anti-stress oxidizing foods are capable of allaying stress and whether certain foods are able to improve the "support level" for lowering stress levels. With this goal in mind we intend to clarify the roles played by magnesium and calcium in food aimed at conquering or allaying stress from the standpoint of Evidence-Based Nutrition (EBN). On the other hand, we will also be studying the cultural aspect of food in terms of food and the dinner table seen as a place for communication.

**Explanation of the tables:**

**Table 1**: Questionnaire on measurement of the density of gas in exhaled breath. We prepared two questionnaire forms, one for use in the morning and the other for use in the afternoon.

**Table 2**: Measurement of stress levels by Stressometer and its significance

**Table 3**: Results of measurement of the density of GO and $GH_4$ gases in exhaled breath and of measurement of stress levels among students (according to gender, in %)

**Explanation of the figures:**

**Figure 1**: Comparison of high values for the density of CO in exhaled breath, the density of $CH_4$ in exhaled breath and stress levels and differences therein according to gender.
* $p<0.01$

**Figure 2**: Comparison of the density of CO in exhaled breath between smokers and non-smokers (classified in accordance with the risk of passive smoking)
* $p<0.01$

**Figure 3**: Correlation between sporting activities and the density of CO in exhaled breath
$p<0.02$
The total number of those engaging in sporting activities once a week and six or seven times a week is less than n=5.

**Figure 4**: Correlation between stress levels and the density of CO in exhaled breath for non-smoking female students with no risk of passive smoking
$y = 0.0281X + 2.07$, $R^2 = 0.1706$, ($p<0.01$)

**Figure 5**: Correlation between defecation, constipation and density of $CH_4$ in exhaled breath
* $p<0.02$
The figures in brackets show the percentages of female students. We observed a gender-based difference in connection with defecation ($p<0.01$) and constipation ($p<0.02$). Students who defecated in the morning or on the previous day are included in the group not experiencing problems as regards constipation.

**Figure 6**: Correlation between the density of $CH_4$ and $H_2$ gases in exhaled breath.
We verified this correlation on 892 students, corresponding to 89.9% of all volunteers, while excluding students with a density of less than 1.5 ppm and more than 5 ppm.
$y = -4.1425X + 24.388$, $R^2 = 0.0195$ ($p<0.01$)

**Table 1**: **Questionnaire on measurement of gas density in exhaled breath**

After reading the attached document, which will explain to you the object of this study, please sign below to confirm that you agree to take part in this study:
Department:
Year:
Family name and first name(s):
----------------------------------------------------------------------------------------------------
**Circle the appropriate reply**: Date: 2005

**Your result (basic values)**
- CO: ______ppm (less than 2)
- $H_2$: ______ppm (less than 10)
- $H_2$: ______ppm (less than 1)
- Stress: ____TNR (less than 50)

**<u>* Do you smoke?</u>**
No
Yes (number of cigarettes a day)
Less than 10 / 10 to 20 / 20 to 30 / More than 30

*** (Question for non-smokers only**)
**Do you run the risk of passive smoking?**
No risk / Maybe no / Perhaps / Yes (i.e. there are smokers in my room or family)

*** Do you drink alcoholic beverages?**
No / Occasionally /
Almost every day (Type of drink: Volume (ml) )
What was the quantity of alcoholic beverages that you drank yesterday?
(Type of drink: Volume (ml) )
Have you got a hangover today? Yes No

*** Do you eat three meals regularly every day?**
Yes No (once a day, twice a day)
Did you have breakfast this morning? Yes No

*** Are you defecating regularly?**
Yes, I defecated this morning
Yes, I defecated ( ) days ago
I am constipated

*** Do you engage in sporting activities?**
No
Yes (more than 30 minutes at a time, more than twice a week)
Type of sport:
Frequency: times per week
Did you engage in an energetic sporting activity yesterday?

*** Duration of sleep**
Normal duration of sleep: hours
Duration of sleep yesterday: hours

*** Do you feel stressed at the moment?**
Yes: Strong fatigue (strong stress) A little Almost not
Reason for stress:

*** Are you taking take medicine or dietary supplements?**
No
Yes: Name of medicine:
Name of dietary supplement:

*** Describe as far as you can your state of health, if you are in imperfect health (cold, illness being treated by a doctor, etc.)**

Thank you for your assistance.

**Table 2: Measurement of stress levels (tremor) by Stressometer and the importance thereof**

**Tremor which is not considered to be an illness**
1. Physiological tremor*

The tremor is varied according to the stress, the anxiety, the tension and the excitation.

**Tremor which is considered to be an illness**
2. Essential tremor (tremor due to benign heredity)*
From young people to the elderly, hereditary in the family.
Treatment is needed for people in certain professions requiring manual dexterity such as designers

3. Alcoholism*
Tremor due to alcoholic mania

4. Hyperthyroidism (Basedow's disease)
Particularly important for young women, defect in internal secretion

5. Other neurosis
Parkinson's disease. Tremor due to cerebellar disorder (intentional tremor)

* One might cite tremor at reset, attitudinal tremor, tremor in motion and intentional tremor, but tremor is not included among the diagnostic criteria for chronic fatigue syndrome.

**Table 3: Results of measurement of the density of CO and $CH_4$ gases in exhaled breath and measurement of stress levels in students (according to gender, expressed as %)**

| Designation | Category | Total number (%) | Male (%) | Female (%) |
|---|---|---|---|---|
| **Density of CO in exhaled breath (ppm)** | Under 3 | 494 (49.7) | 148 (38.7) | 346 (56.6) |
| | 3 to 5 | 387 (39.0) | 157 (41.1) | 230 (37.7) |
| | Over 5 | 112 (11.3) | 77 (20.2) | 35 (5.7) |
| | **Total (%)** | **993 (100)** | **382 (100)** | **611 (100)** |
| **Stress level (TNR)** | Under 25 | 61 (13.7) | 23 (13.2) | 38 (14.0) |
| | 25 to 49 | 245 (55.1) | 82 (47.1) | 163 (60.2) |
| | 50 to 69 | 81 (18.2) | 36 (20.7) | 45 (16.6) |
| | 70 to 99 | 45 (10.1) | 23 (13.2) | 22 (8.1) |
| | Over 100 | 13 (2.9) | 10 (5.8) | 3 (1.1) |
| | **Total (%)** | **445 (100)** | **174 (100)** | **271 (100)** |
| **Density of $CH_4$ in exhaled breath (ppm)** | Under 1 | 65 (6.6) | 24 (6.3) | 41 (6.7) |
| | 1 to 3 | 839 (84.5) | 321 (84.0) | 518 (84.8) |
| | 3 to 5 | 53 (5.3) | 28 (7.3) | 25 (4.1) |
| | Over 5 | 36 (3.6) | 9 (2.4) | 27 (4.4) |
| | **Total (%)** | **993 (100)** | **382 (100)** | **611 (100)** |

**Figure 1**: Comparison of high values for the density of CO in exhaled breath, the density of $CH_4$ in exhaled breath and stress levels and differences therein according to gender

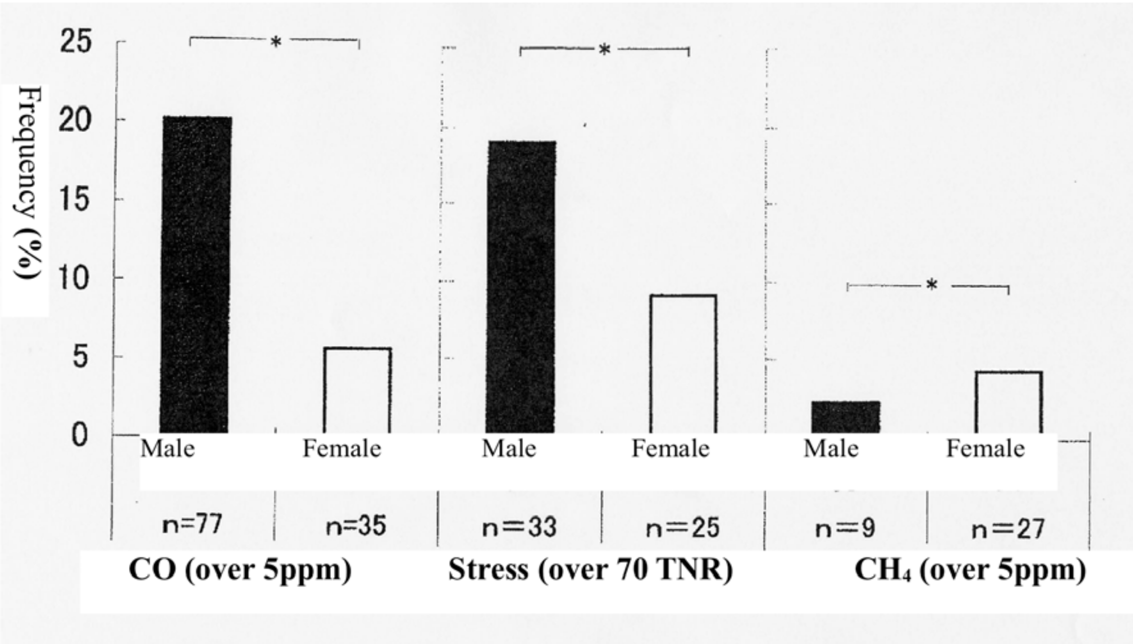


**Figure 2**: Comparison of the density of CO in exhaled breath between smokers and non-smokers (classified in accordance with the risk of passive smoking)

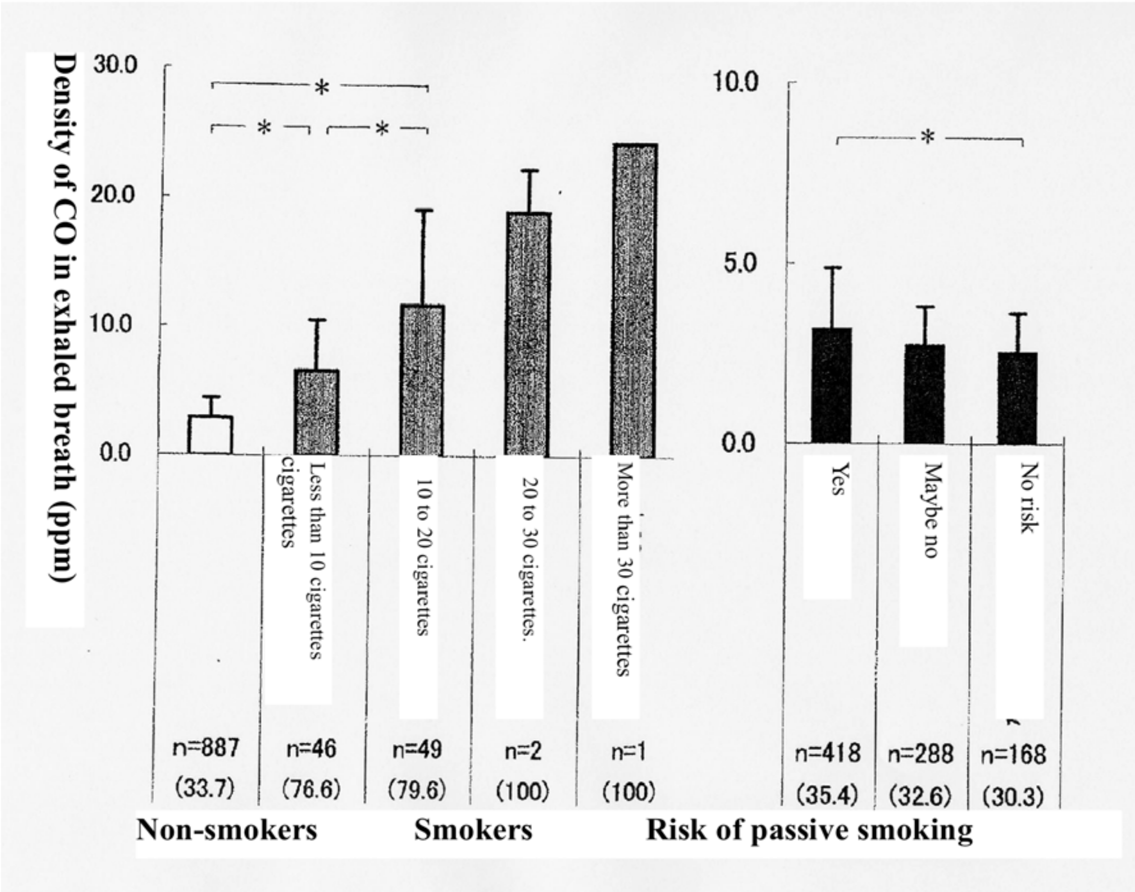


**Figure 3**: Correlation between sporting activities and the density of CO in exhaled breath

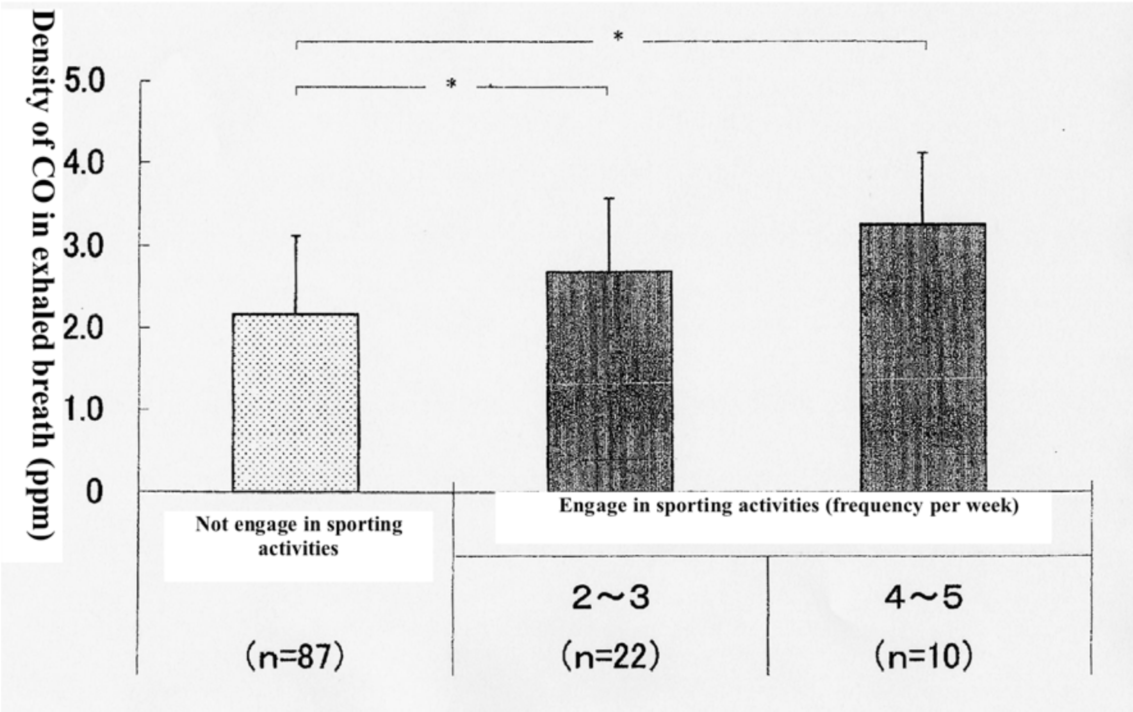


Figure 4: Correlation between stress levels and the density of CO in exhaled breath for non-smoking female students with no risk of passive smoking

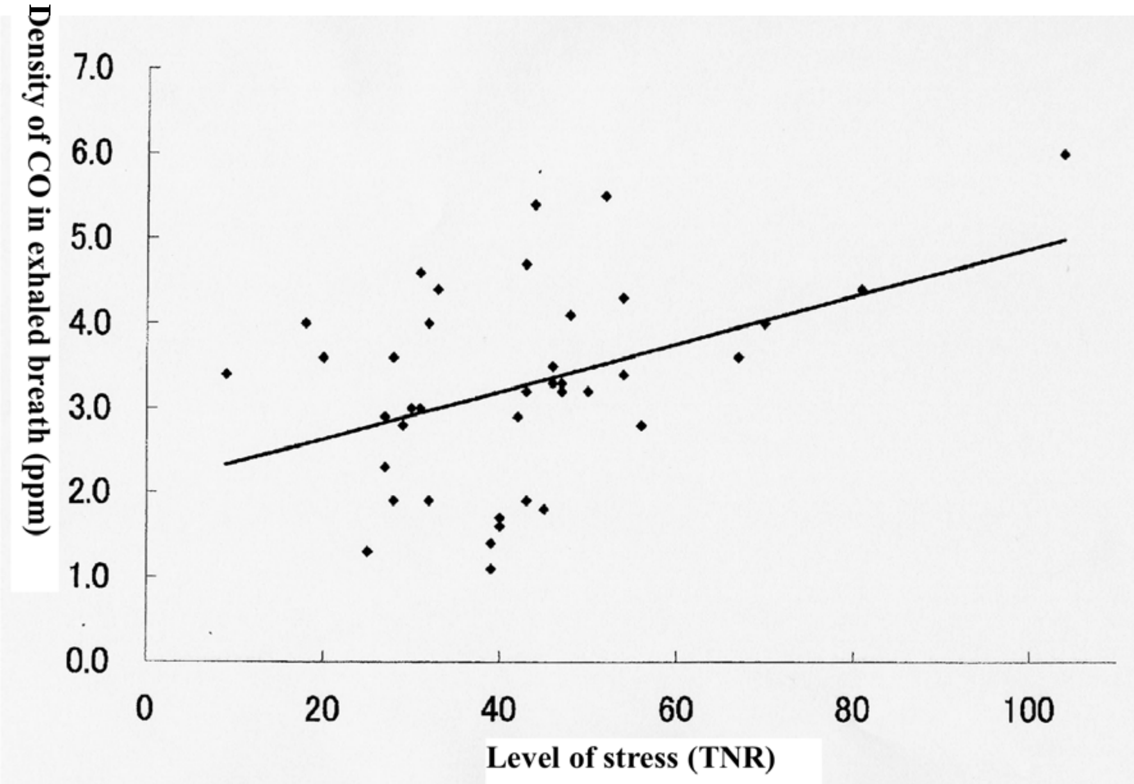
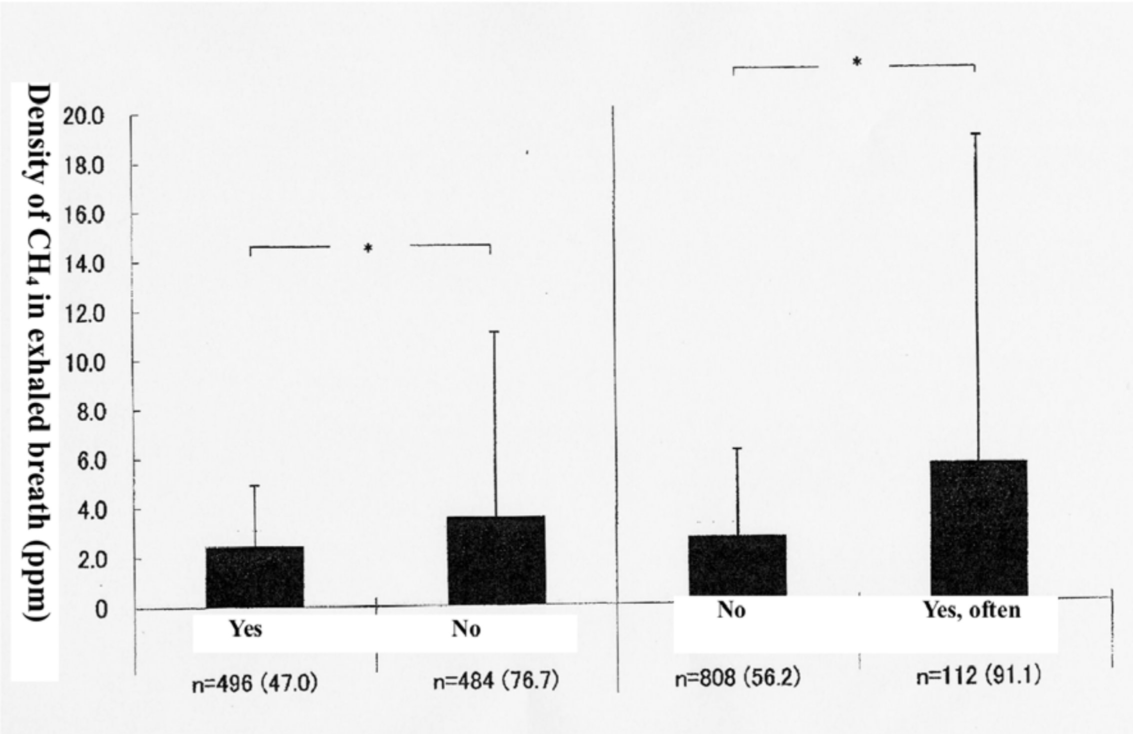


**Figure 5**: Correlation between defecation, constipation and the density of $CH_4$ in exhaled breath

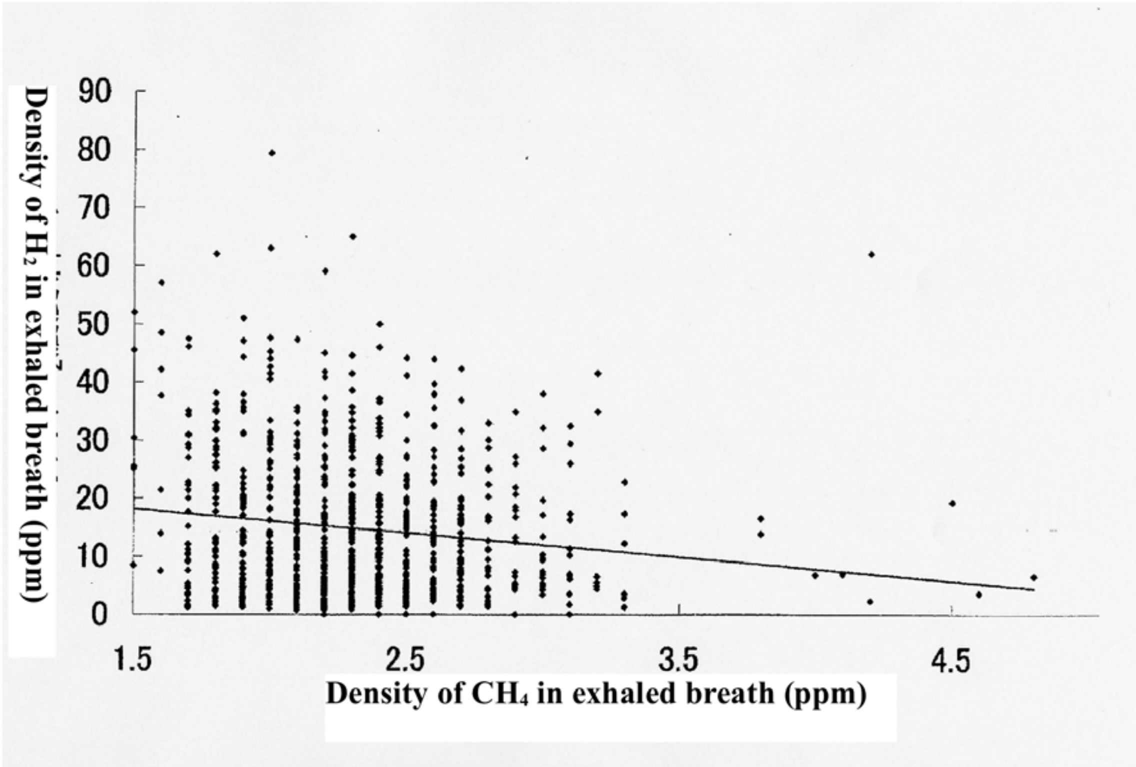
Figures in brackets indicate the percentage of female students.




Did you have a bowel movement this morning?

Are you bothered with constipation?

**Figure 6**: Correlation between the density of $CH_4$ and $H_2$ gases in exhaled breath



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