Impaired vitamin B12 metabolic status in healthcare workers occupationally exposed to nitrous oxide

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Background. Previous studies demonstrated inactivation of vitamin B12 by nitrous oxide (N2O). The intraoperative exposure to N2O was shown to induce megaloblastic anaemia and myelopathy in subjects with subclinical vitamin B12 deficiency. In contrast, no data concerning the influence of occupational exposure to N2O on vitamin B12 metabolic status are available to date. In the present study, the vitamin B12 status in operating theatre personnel was assessed in relation to the extent of exposure.

Methods. Ninety-five operating theatre nurses with the history of exposure to N2O and 90 unexposed counterparts were examined. Vitamin B12 and folic acid were measured by immunoassay. Total homocysteine (tHcy), an indicator of impaired vitamin B12 metabolism, was determined by high performance liquid chromatography. N2O concentration was monitored by adsorption gas chromatography and mass spectrometry.

Results. No significant differences were found between both groups with respect to haematological parameters and folic acid. However, subjects exposed to N2O presented with lower vitamin B12 [372.8 (12.1) vs 436.8 (13.2) pmol litre−1, P<0.001] and higher tHcy [11.2 (0.5) vs 8.9 (0.5) μmol litre−1, P=0.006]. The changes in vitamin B12 status were aggravated in subjects exposed to N2O in concentrations substantially exceeding occupational exposure limit (180 mg m−3) [vitamin B12: 341.9 (17.7) vs 436.8 (13.2) pmol litre−1, P=0.006; tHcy: 12.9 (0.7) vs 8.9 (0.5) μmol litre−1, P=0.047].

Conclusions. Exposure to N2O in healthcare workers is associated with alterations of vitamin B12 metabolic status, the extent of which depends on the level of exposure.

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Nitrous oxide (N2O) is an inhaled anaesthetic commonly used in surgery. Whereas N2O is generally regarded as a safe agent, there is considerable evidence that the intraoperative exposure to N2O and the habitual N2O abuse cause adverse effects on haematopoietic and nervous systems. Complications ranging from subtle haematological changes to agranulocytosis, spinal cord degeneration, and polyneuropathy were reported in subjects anesthetized with N2O1−6 or inhaling N2O for recreational purposes.7−9 Clinical symptoms after the exposure to N2O are highly reminiscent of those encountered in vitamin B12 (cobalamin) deficit. Accordingly, adverse effects accompanying N2O anaesthesia were aggravated in subjects with subclinical cobalamin deficiency,10−13 either due to low dietary consumption or due to genetic predisposition.

N2O acts by oxidizing vitamin B12 from the active cob(I)alamin to the inactive cob(III)alamin.14 This renders methylcobalamin, a metabolite of cobalamin, inactive as a cofactor of methionine synthase and impairs the conversion of homocysteine to methionine, which is essential for
myelin synthesis. Increased level of total homocysteine (tHcy) in serum is, therefore, an indicator of cobalamin deficiency and a biomarker of N₂O exposure.

Healthcare workers active in operating theatres are repeatedly exposed to N₂O in the ambient air. Whereas deleterious effects of N₂O in patients undergoing anaesthesia and in habitual N₂O abuse were reported, little effort has been directed towards evaluation of the influence of N₂O on vitamin B12 metabolism in operating personnel. This prompted us to investigate biochemical indices of vitamin B12 metabolic status among surgical nurses working under various levels of exposure to N₂O. Our results for the first time document alteration of vitamin B12 metabolism in subjects occupationally exposed to N₂O and relate them to the degree of exposure.

Methods

Study subjects and design

This study included 185 female nurses aged between 25 and 56 yr and with 5–26 yr employment history in 10 hospitals in the city area of Lodz. The exposed group was made up of 95 operating theatre nurses (surgical nurses), who routinely provide full-time assistance during operations on a day-to-day basis. They usually stayed in the polluted area of the operating room not less than 5 h and worked for the whole occupational activity in the environment polluted with N₂O and volatile anaesthetics such as isoflurane, sevoflurane, or halothane. Previous studies revealed that this group is the most exposed to N₂O among operating theatre staff. The control group consisted of 90 nurses from other departments of the same hospitals, who have never been occupationally exposed to N₂O or volatile anaesthetics in the course of their professional career. Excluded were subjects periodically active in polluted areas. All examined subjects received information on the purpose of the study and signed the participation consent. The protocol was approved by the local ethical committee.

Each subject underwent general medical examination. Information concerning alcohol and coffee consumption and medication within the past 12 months was gathered using a questionnaire. To avoid inclusion of additional confounding factors, subjects with overt haematological diseases (three people), serious symptoms of neurological deterioration (one person), or heart failure (one person) were excluded. Except for minor illnesses nurses in both examined groups were in good health and presented with no clinical signs of vitamin B12 deficiency. No apparent symptoms of acute N₂O intoxication (headache, drowsiness, nausea and vomiting, paraesthesia, and reduced tendon reflexes) were registered in an exposed group. All subjects denied receiving vitamin B12 or folic acid therapy during 3 yr preceding the study.

Blood collecting and analytical procedures

Nurses in both groups were examined once in the course of the study. Blood and air samples were collected on the same day. Blood was obtained simultaneously in both groups at the end of the daily shift. The serum was separated by centrifugation immediately after blood collection and stored at −20°C. For determination of haematological parameters [red blood cell count (RBC), haemoglobin (Hb), haematocrit (Hct), mean cell haemoglobin (MCH), mean cell volume (MCV) and mean cell haemoglobin concentration (MCHC)], EDTA-blood was collected and blood counts were tested within 2 h. Vitamin B12 and folic acid were determined in the Central Laboratory of the University Hospital in Münster using electrochemiluminescence immunoassay (ECLIA) on a routine laboratory analyzer (Elecsys 2010, Roche, Mannheim, Germany). No changes in vitamin B12 concentrations were observed in serum samples saturated with N₂O excluding the direct interference of N₂O with the immunoassay. Serum vitamin B12 concentrations (normal range 156–672 pmol litre⁻¹) were arbitrarily categorized as low (150–250 pmol litre⁻¹), border low (250–300 pmol litre⁻¹), medium (250–350 pmol litre⁻¹), or high (>350 pmol litre⁻¹). Values in the first interval are encountered at increased frequency in subjects with disturbed vitamin B12 metabolism. tHcy levels in plasma were determined as described elsewhere. Because of inter-assay variability leading to method-dependent normal ranges, tHcy concentration of 12.8 μmol litre⁻¹ reflecting 95th percentile value in the control group was taken in this study as a cut-off value discriminating between elevated and normal tHcy levels. Nevertheless, this value is close to the tHcy normal range upper limit recommended in Germany. Haematological parameters were analysed by a Sysmex XE-2100 Blood Analyzer.

Air sampling and quantitative analysis of N₂O and volatile anaesthetics

N₂O and volatile anaesthetics in the ambient air were evaluated in 26 operating theatres of 10 hospitals, in each of which one out of the three following ventilation systems was installed: natural with supplementary pressure ventilation (6–10 air changes h⁻¹), or pressure/exhaust ventilation (12–15 air changes h⁻¹), or complete laminar flow air-condition system preventing the recirculation of exhaust air into operating room (>15 air changes h⁻¹). Pressure/exhaust ventilation and air conditioning were installed at least 18 and 11 yr, respectively, before the start of this study. Fifteen of 26 operating theatres had provision of anaesthetic gas scavenging devices.

Anaesthetic concentrations were measured continuously during consecutive surgeries carried out with anaesthesia maintained with N₂O and sevoflurane, isoflurane, or halothane. Each monitoring session was carried out for the duration the anaesthetic was being used but not shorter.
than for 75% of the working shift, which allows expressing the N2O air concentration in terms of occupational exposure limit (OEL). Static monitoring was carried out for N2O measurement as previously described and individual dosimeters were applied for volatile anaesthetics. Quantification of N2O and halogenated anaesthetics was done using, respectively, adsorption gas chromatography and partition gas chromatography followed by mass spectrometry.

Statistical analysis
An exploratory statistics was performed using the Statistical Package for the Social Sciences (SPSS-X). The results were expressed as mean (SEM) or as frequencies. Analysis of covariance (ANCOVA) with age and smoking as covariances were used for comparison of means. Pearson’s χ² or Fisher’s exact test were used to compare frequency distributions. Spearman’s rank correlation test was used to examine serial correlations. P-values <0.05 were considered significant.

Results

Characteristics of examined populations
The characteristics of the exposed group and the control group are shown in Table 1. Both groups did not differ significantly with respect to age, smoking, and alcohol and coffee consumption. The employment period was comparable in both groups.

Long-term effects of exposure to anaesthetics on haematological parameters and indices of vitamin B12 metabolic status
The values of haematological parameters and biochemical indices reflecting vitamin B12 status in exposed subjects and controls are shown in Table 2. No differences were observed in erythrocyte count, haemoglobin concentration, haematocrit, MCH, MCV, and MCHC. In contrast, serum concentrations of vitamin B12 were significantly lower in exposed subjects. As levels of vitamin B12 may not adequately reflect disturbances in cobalamin metabolism, plasma concentrations of tHcy were additionally determined. As shown in Table 2, the exposed group presented with increased mean baseline levels of tHcy. Figure 1 demonstrates the distribution (%) of subjects with tHcy concentrations exceeding the cut-off value (>12.8 μmol litre⁻¹) in groups with low, borderline low, medium, and high vitamin B12 concentrations. Owing to an inverse relationship between vitamin B12 and tHcy, the tendency towards over- and under-representation of subjects with increased plasma tHcy levels in groups with low and high vitamin B12 concentrations, respectively, was evident in the control population (χ²=10.5, df=3, P=0.015). This tendency was, however, attenuated in the exposed population (χ²=1.9, df=3, P=0.52).

Levels of vitamin B12, folic acid, and tHcy in relation to the extent of exposure to N2O
Monitoring of anaesthetics in operating theatres revealed that N2O most severely contributed to ambient air pollution.

Table 1 Demographic characteristics of the study subjects. Age and employment duration are given as years. Values are means (SEM). For other parameters values represent % of examined population

<table>
<thead>
<tr>
<th></th>
<th>Controls (n=90)</th>
<th>N₂O-exposed subjects (n=95)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td>0.29</td>
</tr>
<tr>
<td>Employment duration</td>
<td>35.7 (1.2)</td>
<td>39.4 (1.3)</td>
<td>0.29</td>
</tr>
<tr>
<td>Smokers (&gt;5 cigarettes day⁻¹)</td>
<td>14.6 (0.6)</td>
<td>15.8 (0.5)</td>
<td>0.17</td>
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<tr>
<td>Alcohol consumption</td>
<td>58 (0.6)</td>
<td>53 (0.5)</td>
<td>0.97</td>
</tr>
<tr>
<td>Coffee intake (&gt;3 cups day⁻¹)</td>
<td>5</td>
<td>4</td>
<td>0.67</td>
</tr>
</tbody>
</table>

Table 2 Values of haematological parameters and biochemical indices of cobalamin metabolic status in study subjects. Values are means (SEM). Hb, haemoglobin concentration; RBC, red blood count; Htc, haematocrit; MCH, mean cell haemoglobin; MCV, mean cell volume; MCHC, mean cell haemoglobin concentration. Significance by ANCOVA with age and smoking as covariances

<table>
<thead>
<tr>
<th></th>
<th>Controls (n=90)</th>
<th>Exposed subjects (n=95)</th>
<th>P-value</th>
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<tbody>
<tr>
<td>Hb (g dl⁻¹)</td>
<td>12.5 (0.17)</td>
<td>12.2 (0.11)</td>
<td>0.13</td>
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<tr>
<td>RBC (T litre⁻¹)</td>
<td>4.9 (0.2)</td>
<td>5.2 (0.3)</td>
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</tr>
<tr>
<td>Htc (%)</td>
<td>30.8 (0.1)</td>
<td>30.3 (0.1)</td>
<td>0.45</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>18.1 (0.17)</td>
<td>29.4 (0.17)</td>
<td>0.09</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>91.0 (0.51)</td>
<td>90.3 (0.48)</td>
<td>0.31</td>
</tr>
<tr>
<td>MCHC (g dl⁻¹)</td>
<td>34.2 (0.13)</td>
<td>32.7 (0.17)</td>
<td>0.11</td>
</tr>
<tr>
<td>Vitamin B12 (pmol litre⁻¹)</td>
<td>436.8 (13.2)</td>
<td>372.8 (12.1)</td>
<td>0.001</td>
</tr>
<tr>
<td>Folic acid (nmol litre⁻¹)</td>
<td>8.1 (0.3)</td>
<td>8.9 (0.4)</td>
<td>0.11</td>
</tr>
<tr>
<td>Homocysteine (µmol litre⁻¹)</td>
<td>8.9 (0.5)</td>
<td>11.2 (0.5)</td>
<td>0.006</td>
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</table>

Fig 1 Distribution of study subjects with elevated tHcy levels among groups categorized by various plasma vitamin B12 concentrations. The proportion of subjects with tHcy exceeding cut-off value in the subsets of exposed and control groups with low (150–250 pmol litre⁻¹), border low (250–300 pmol litre⁻¹), medium (300–350 pmol litre⁻¹), and high (>350 pmol litre⁻¹) is shown. Plasma tHcy>12.8 µmol litre⁻¹ reflecting 95th percentile in the control group was adopted as a cut-off value discriminating subjects with normal and elevated tHcy levels. Note markedly attenuated relationship between tHcy and vitamin B12 concentration in operating theatre nurses exposed to anaesthetics.
Nitrous oxide (N\textsubscript{2}O) levels measured in the respiratory area of nurses varied over three orders of magnitude and ranged from 35.8 to 1502 mg m\textsuperscript{-3} depending on the ventilation system (Tables 3 and 4). However, in operating theatres equipped with one ventilation system, N\textsubscript{2}O levels remained fairly stable with variation coefficients not exceeding 34\%, 54\%, and 52\% for natural, pressure/exhaust, and air-condition systems, respectively. In 19 out of 26 operating theatres examined in this study, concentration of N\textsubscript{2}O during operations exceeded the German OEL value of 180 mg m\textsuperscript{-3}. Moreover, in five and three operating theatres, respectively, N\textsubscript{2}O levels conformed to threshold values recommended by the American Conference of Governmental Industrial Hygienists (ACGIH) and the National Institute of Occupational Safety and Health (NIOSH). In contrast, none of the operating theatres concentration of volatile anaesthetics, including isoflurane and halothane, exceeded values recommended in Germany or by ACGIH (Table 3). The analysis of the distribution of N\textsubscript{2}O levels in polluted areas revealed that in operating theatres equipped with complete air condition or pressure/exhaust ventilation system N\textsubscript{2}O concentrations were below, equal, or exceeded the (German) OEL value less than twice (mean 185.2 mg m\textsuperscript{-3}, range 35.8–320.4 mg m\textsuperscript{-3}, Table 4). In contrast, N\textsubscript{2}O concentrations substantially higher than OEL (mean 752.5 mg m\textsuperscript{-3}; range 447.4–1502 mg m\textsuperscript{-3}) were registered in operating rooms equipped only with the natural ventilation system. Therefore, to assess the impact of the N\textsubscript{2}O exposure on the magnitude of changes in the vitamin B12 metabolic status in relation to OEL value, the levels of vitamin B12, folic acid, and tHcy were evaluated in two arbitrary subsets of study subjects: individuals working in operating theatres with N\textsubscript{2}O exposure below 2×OEL (360 mg m\textsuperscript{-3}) or above 2×OEL were categorized as ‘low exposure’ or as ‘high exposure’ group, respectively.

The effect of the low- and high-exposure levels on biochemical indices reflecting vitamin B12 status is shown in Table 5. Serum concentrations of vitamin B12 were significantly decreased, whereas tHcy concentrations in plasma were considerably increased in the group of nurses exposed to N\textsubscript{2}O in concentrations substantially exceeding (German) OEL value (‘high exposure’ group). In contrast, the levels of vitamin B12 and tHcy were not significantly different in ‘low exposure’ group. Moreover, there was a significant negative correlation between N\textsubscript{2}O exposure level and vitamin B12 concentration (r = −0.22, P = 0.038) and a significant positive correlation between N\textsubscript{2}O exposure level and tHcy concentration (r = 0.51; P < 0.001) (Fig. 2a and n). No significant correlations were observed between N\textsubscript{2}O exposure levels and haemoglobin (r = 0.078, P = 0.62), haematocrit (r = 0.09, P = 0.29), MCV (r = 0.061, P = 0.45), and folic acid (r = 0.16, P = 0.09). Moreover, no significant correlations were observed between duration of exposure to N\textsubscript{2}O and vitamin B12 (r = 0.18, P = 0.3) and tHcy (r = 0.18, P = 0.09). The distribution of subjects exposed against high and low N\textsubscript{2}O levels across vitamin B12 and tHcy quartiles in unexposed population is shown in Figure 2c and d. Subjects from high exposure group were significantly over- and under-represented in lower and higher vitamin B12 quartiles, respectively, and in higher and lower tHcy quartiles, respectively.

**Discussion**

Current knowledge regarding adverse effects of the occupational exposure to N\textsubscript{2}O on vitamin B12 metabolism is incomplete. In one study, Salo and colleagues\textsuperscript{30} examined...
haematological changes among anaesthetists and surgical nurses exposed to N₂O and failed to find any signs of vitamin B₁₂−N₂O interaction. The present study recapitulates these results, as no changes in blood count and other haematological parameters were noted in subjects occupationally exposed to N₂O. However, frank haematopoietic changes such as macrocytic anaemia are known to occur late in course of vitamin B₁₂ deficiency and their absence is not sufficient to exclude the impairment of vitamin B₁₂ metabolism. Hence, we here directly determined vitamin B₁₂ in sera of subjects exposed to N₂O. We found significantly reduced vitamin B₁₂ levels in surgical nurses when compared with hospital staff working outside operating theatres. This observation was the first indication that repeated occupational exposure to N₂O may disturb vitamin B₁₂ metabolic status.

Decreased levels of vitamin B₁₂ were previously reported in habitual N₂O abuse and sporadically during N₂O anaesthesia.⁸ ¹¹ ¹³ As N₂O preferentially targets metabolically active cob(I)alamin, the suppressing effect of this anaesthetic on plasma vitamin B₁₂ levels is surprising. In this study, we excluded the possibility that N₂O interferes with the assay used for vitamin B₁₂ determination. In our view, it is more likely that N₂O perturbs tissue distribution or degradation of cobalamin and thereby affects its concentration in plasma. Actually, gradual development of vitamin B₁₂ deficiency due to depletion of cobalamin in the liver and its conversion to inactive cobalamin analogues was observed in rats exposed to N₂O.³¹ Moreover, xenobiotics, similarly to N₂O inactivate cob(I)alamin, were shown to decrease vitamin B₁₂ in plasma.³²

A vast body of evidence suggests that serum levels of vitamin B₁₂ are not the most sensitive indicators of cobalamin deficiency. For instance, neurological abnormalities related to vitamin B₁₂ deficiency were repeatedly seen in subjects with normal serum cobalamin concentrations.³³ ³⁴ Therefore, in addition to determination of vitamin B₁₂, the levels of tHcy were measured in the present study. tHcy excessively accumulates in various forms of vitamin B₁₂ deficiency and its increased concentrations precede the overt vitamin B₁₂ deficiency.¹⁷ Several studies demonstrated that intraoperative acute exposure to N₂O is invariably associated with postoperative increases in plasma tHcy.¹⁸ ⁻²⁰ In the present study, we extend these observations to show that increased plasma tHcy levels are found in operating theatre personnel under repeated occupational exposure to N₂O. Moreover, abnormal tHcy concentrations were found to be roughly equally distributed among N₂O-exposed subjects with various vitamin B₁₂ concentrations (Fig. 1), suggesting that disturbances of cobalamin metabolism may occur in this group in the absence of measurable changes in vitamin B₁₂ levels in plasma. Cumulatively, our data for the first time provide the evidence that occupational exposure to N₂O leads to alterations of vitamin B₁₂ metabolic status.

Previous studies demonstrated that the air pollution with N₂O during surgical procedures is critically dependent on

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**Table 5** Values of selected biochemical indices of cobalamin metabolic status in study subjects exposed to low and high N₂O concentrations. Values are means (SEM). Low and high exposure indicate exposure at level of 35.8–320.4 mg m⁻³ (below 2×OEL=360 mg m⁻³) and 447.4–1502 mg m⁻³ (above 2×OEL=360 mg m⁻³), respectively. Statistically significant differences are shown between high exposure and control groups. No significant differences were found between low exposure and control groups (ANOVA with age and smoking as covariances).

<table>
<thead>
<tr>
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<th>Control (n=90)</th>
<th>N₂O-exposed subjects</th>
<th>P-value</th>
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<tbody>
<tr>
<td></td>
<td>Low exposure</td>
<td>High exposure</td>
<td></td>
</tr>
<tr>
<td>Vitamin B₁₂ (pmol litre⁻¹)</td>
<td>436.8 (13.2)</td>
<td>402.4 (21.3)</td>
<td>0.76</td>
</tr>
<tr>
<td>Folic acid (nmol litre⁻¹)</td>
<td>8.1 (0.3)</td>
<td>8.4 (0.6)</td>
<td>0.38</td>
</tr>
<tr>
<td>Homocysteine (µmol litre⁻¹)</td>
<td>8.9 (0.5)</td>
<td>9.6 (0.7)</td>
<td>0.83</td>
</tr>
</tbody>
</table>

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**Fig 2** Relationship between vitamin B₁₂ and tHcy levels and the magnitude of N₂O exposure. Correlation between plasma vitamin B₁₂ (A) and tHcy (B) levels and N₂O concentrations determined in the ambient air in operating theatres. Spearman’s rank correlation test: (A) ρ=−0.22; P=0.038; (n)=52; P<0.001. Distribution of study subjects exposed to N₂O between vitamin B₁₂ and tHcy quartiles in control (unexposed) group. The proportion of subjects exposed to low and high N₂O concentration in the ambient air in each quartile of vitamin B₁₂ and tHcy calculated for the control group is shown. The proportion of control subjects in each interval is by definition 25%. (C) Distribution between vitamin B₁₂ quartiles. (n)= Distribution between tHcy quartiles. Double OEL (2×OEL=360 mg m⁻³) was adopted to discriminate between ‘low’ and ‘high’ exposure groups. *P<0.01, **P<0.001 high-exposed vs unexposed group (Fisher’s exact test). No significant difference was observed between low-exposed and unexposed groups.

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Krajewski et al.
N2O recommended by US agencies (ACGIH, NIOSH) in spite of substantial overstepping of threshold values for OEL, but at the same time shed new light on the validity of metabolic alterations appeared only in operating personnel exposed to the highest N2O levels.

The clinical relevance of the present results remains unclear. Theoretically, healthcare workers active under excessive occupational exposure to N2O, by whom disturbances of vitamin B12 metabolism were evident, might be more susceptible to development of symptomatic vitamin B12 deficiency under certain permissive conditions such as dietary vitamin B12 restriction. Moreover, they would be likely to develop hyperhomocysteinaemia, which is a well-recognized independent risk factor for arterial and venous thrombosis and coronary heart disease. tHcy levels similar to those observed here in subjects exposed to high N2O concentrations were previously demonstrated to substantially increase cardiovascular risk. Unfortunately, no epidemiological data concerning the frequency of coronary or thrombotic events among subjects occupationally exposed to N2O are available to date. Clearly, further prospective studies will be necessary to fully assess the clinical relevance of the long-term excessive exposure to N2O.

The cross-sectional design of the present study is a source of further limitations. Plasma levels of vitamin B12 and tHcy were determined once in the course of the study. Whereas symptoms of acute N2O intoxications were absent in the examined cohort, it cannot be entirely excluded that some short-term influence additionally compounded the effects of chronic N2O exposure. This contention is supported by the absence of a clear-cut relationship between the duration of employment and the degree of abnormalities, albeit the tendency ($P=0.09$) towards higher tHcy levels has been noted among nurses with longest occupational exposure. A longitudinal assessment of vitamin B12 metabolic status will be necessary to fully discriminate between chronic and temporary effects of excessive N2O exposure in operating theatre personnel.

In conclusion, the present study demonstrates that maintaining N2O concentrations in operating theatres under OEL is sufficient for preventing disturbances of vitamin B12 metabolism. Conversely, excessive repeated occupational exposure to N2O is associated with alterations of vitamin B12 metabolic status, the extent of which is critically dependent on the level of exposure.

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