

CLINICAL PRACTICE



## Effect of nitrous oxide on plasma homocysteine and folate in patients undergoing major surgery

P. S. Myles<sup>1\*</sup>, M. T. V. Chan<sup>2</sup>, K. Leslie<sup>3</sup>, P. Peyton<sup>4</sup>, M. Paech<sup>5 6</sup> and A. Forbes<sup>7</sup>

<sup>1</sup>Department of Anaesthesia and Perioperative Medicine, Alfred Hospital, Monash University, Commercial Road, Melbourne, Vic. 3004, Australia. <sup>2</sup>Department of Anaesthesia and Intensive Care, The Chinese University of Hong Kong, Prince of Wales Hospital, Hong Kong. <sup>3</sup>Department of Anaesthesia and Pain Management and Department of Pharmacology, Royal Melbourne Hospital and University of Melbourne, Melbourne, Australia. <sup>4</sup>Department of Anaesthesia, Austin Hospital, Melbourne, Australia. <sup>5</sup>School of Medicine and Pharmacology, University of Western Australia, Perth, Australia. <sup>6</sup>Department of Anaesthesia and Pain Medicine, King Edward Memorial Hospital for Women, Perth, Australia. <sup>7</sup>Department of Epidemiology and Preventive Medicine, Monash University, Melbourne, Australia

\*Corresponding author. E-mail: p.myles@alfred.org.au

**Background.** Nitrous oxide (N<sub>2</sub>O) inhibits methionine synthetase resulting in elevated plasma homocysteine (Hcy) concentration after surgery. In epidemiological studies, hyperhomocysteinaemia is associated with increased risk of cardiovascular disease and dementia.

**Methods.** Blood samples were obtained to measure plasma folate and Hcy concentrations from two centres participating in a multicentre randomized trial investigating the effects of N<sub>2</sub>O on the outcome after major surgery. The effect of N<sub>2</sub>O and duration of anaesthesia on plasma Hcy, and the relationship between hyperhomocysteinaemia and outcomes were assessed.

**Results.** We enrolled 394 patients. The N<sub>2</sub>O Group had an increase in plasma Hcy concentration after surgery when compared with the N<sub>2</sub>O-free Group: 11.1 (3.8) vs 8.5 (4.0)  $\mu\text{mol litre}^{-1}$ ,  $P < 0.0005$ . Postoperative hyperhomocysteinaemia was associated with an increased risk of major complications: risk ratio (RR) 2.8 (95% CI: 1.4–5.4),  $P = 0.002$  and cardiovascular events, RR 5.1 (95% CI: 3.1–8.5),  $P < 0.0005$ . There was a significant association between duration of anaesthesia and the relative change in plasma Hcy concentration, particularly in the N<sub>2</sub>O Group:  $r = 0.42$ ,  $P < 0.001$ .

**Conclusions.** N<sub>2</sub>O increases plasma Hcy concentration; this effect is greater with a longer duration of anaesthesia. Hyperhomocysteinaemia is a risk factor for major postoperative complications. N<sub>2</sub>O-induced increases in plasma Hcy concentration may be a cause of postoperative cardiovascular morbidity.

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Nitrous oxide (N<sub>2</sub>O) oxidizes the cobalt atom on vitamin B<sub>12</sub>, leading to inhibition of the enzyme methionine synthetase.<sup>1–5</sup> Because methionine synthetase is required for folate and DNA synthesis, prolonged or repeated exposure to N<sub>2</sub>O can lead to megaloblastic anaemia and subacute degeneration of the spinal cord.<sup>6–11</sup> These adverse effects of N<sub>2</sub>O exposure are not limited to

intensive care patients and those undergoing exceedingly prolonged surgery. A 2-h exposure to N<sub>2</sub>O is associated with a 50% reduction in methionine synthetase activity.<sup>3</sup>

Methionine synthetase catalyses the transfer of a methyl group from N<sup>5</sup>-methyltetrahydrofolate to homocysteine (Hcy), producing tetrahydrofolate and methionine.<sup>1–5</sup> Hcy has gained importance in cardiovascular medicine because

it induces endothelial dysfunction and has atherogenic properties,<sup>12–17</sup> both of which are associated with increased risk of myocardial infarction (MI), stroke, and dementia in the general population and those with other risk factors for cardiovascular disease.<sup>12–18</sup>

A few small studies have found that N<sub>2</sub>O exposure during surgery leads to increased plasma Hcy concentrations after operation,<sup>4 19–21</sup> but the extent and clinical implications of this are unclear. In a recently completed study,<sup>22</sup> we took the opportunity to collect blood samples in patients from two centres to measure the effect of N<sub>2</sub>O on postoperative plasma folate and Hcy concentrations. This enabled us to identify factors associated with elevated Hcy concentration and its relationship with postoperative complications.

## Methods

The ENIGMA Trial evaluated outcomes associated with N<sub>2</sub>O exposure in 2050 adult patients undergoing major non-cardiac surgery.<sup>22</sup> In brief, adult patients undergoing general anaesthesia and in whom surgery was anticipated to exceed 2 h duration were recruited in the study. Patients were randomly assigned to receive either N<sub>2</sub>O-free or N<sub>2</sub>O-containing general anaesthesia. The N<sub>2</sub>O-free Group could receive higher inspired concentration of oxygen, such that most of them received 80% oxygen with air.<sup>22</sup> The patient and all surgical and research staff were blinded to group identity. Postoperative complications occurring in the first 30 days after surgery were recorded. Outcomes included wound infection, pneumonia, severe nausea and vomiting, MI, and death.<sup>22</sup> We defined major postoperative complication as one or more of: wound infection, MI, stroke, pneumonia, thromboembolism, awareness, and death; adverse cardiovascular events included thromboembolism, MI, and stroke; respiratory complications included atelectasis, pneumonia, pneumothorax, and pulmonary embolism.

In the present study, a sequential cohort of patients enrolled in the ENIGMA trial from two of the participating centres (Alfred Hospital, Melbourne; Prince of Wales Hospital, Hong Kong) were included in a sub-study of folate and Hcy flux after surgery. The study protocol was approved by the ethics committee of both the participating institutions. Patients were approached before surgery and provided written, informed consent.

Patients' dietary folate and vitamin B<sub>12</sub> status were assessed by a brief screening questionnaire, with yes or no responses to the following items:<sup>23</sup> (i) vegan or vegetarian status; (ii) regular breakfast cereal intake; (iii) daily fresh fruit and/or vegetables intake; (iv) regular vitamin B or folate tablets; (v) vitamin B<sub>12</sub> injection in the previous 3 months.

On the day of surgery and in the morning of the first postoperative day, a 10 ml fasting venous blood sample was obtained from each of the study participants. The blood samples were stored at 4°C, and then centrifuged

within 30 min at 3500 rpm for 10 min. Plasma was separated and stored at –70°C until analysis. Plasma folate and Hcy concentrations were measured by immunoassay, as previously described.<sup>23</sup>

## Statistical analysis

We initially planned our sample size based on previous data,<sup>21</sup> using a mean increase in plasma Hcy concentration from 11 to 15 µmol litre<sup>–1</sup>, with a standard deviation (SD) of 6 µmol litre<sup>–1</sup>, and this required 47 patients per group (type I error of 0.05 and a type II error of 0.2). We subsequently increased the sample size to 394 patients in this study to allow meaningful exploratory analyses of a number of potential variables that may affect plasma Hcy concentration. The analyses were based on intention-to-treat.

We used the 90th centile of the preoperative Hcy concentration to define postoperative hyperhomocysteinaemia.<sup>15</sup> Group patient characteristics and perioperative data were tabulated using mean (SD), median [interquartile range (IQR)], or number (%). Numerical data were first tested for normality and then analysed using *t*-tests. Categorical data were analysed using  $\chi^2$ ; risk ratio (RR) and 95% confidence intervals (CI) were used as an estimate of risk. Associations between numerical variables were measured using Pearson correlation coefficients (*r*). We used logistic regression analyses to identify the risk of postoperative complications, adjusting for age, ASA physical status, and duration of anaesthesia, and then explored the effect of plasma Hcy and folate flux. We also did exploratory stepwise linear regression analysis to identify significant factors associated with plasma Hcy and folate flux. The effect of N<sub>2</sub>O exposure and duration of anaesthesia on the postoperative change in plasma Hcy was analysed with generalized linear models. All analyses were done using SPSS for Windows version 15.0 (SPSS Inc., Chicago, IL, USA). All reported *P*-values were two-sided and not adjusted for multiple comparisons.

## Results

We enrolled 394 patients undergoing a broad range of surgical procedures (Table 1). There were no dropouts or exclusions. The median (IQR) duration of anaesthesia was 4.5 (3.1–6.0) h, and after operation 80 patients (20%) were admitted to the intensive care unit directly from the operation theatre. A total of 67 patients (17%) had at least one major postoperative complication. There were only six patients with confirmed MI (N<sub>2</sub>O Group, *n*=2; N<sub>2</sub>O-free Group, *n*=4), and three deaths (all in the N<sub>2</sub>O Group).

The N<sub>2</sub>O-free Group received a higher inspired oxygen concentration and slightly more inhalation agent during maintenance of anaesthesia, both *P*<0.001 (Table 2). The N<sub>2</sub>O Group had a significantly increased plasma Hcy concentration after surgery compared with the N<sub>2</sub>O-free Group (Table 3).

The 90th centile of the preoperative plasma Hcy concentration was  $13.5 \mu\text{mol litre}^{-1}$ . N<sub>2</sub>O increased the risk of postoperative hyperhomocysteinaemia, N<sub>2</sub>O Group 13 (7.7%) vs N<sub>2</sub>O-free Group 38 (19%), RR 1.9 (95% CI: 1.2–3.1),  $P=0.002$ . This adverse effect was apparent across all sub-groups (Fig. 1). There was an increased risk of major complications in those with hyperhomocysteinaemia, RR 2.8 (95% CI: 1.4–5.4),  $P=0.002$ ; but this was modified by N<sub>2</sub>O exposure (interaction term  $P=0.06$ ), and for those in the N<sub>2</sub>O Group, the risk of major complications in those with hyperhomocysteinaemia was less, RR 1.44 (95% CI: 0.76–2.71),  $P=0.31$ ; N<sub>2</sub>O-free Group 6.02 (95% CI: 2.24–16.1),  $P<0.0005$ . There was also an increased risk of adverse cardiovascular events in those with hyperhomocysteinaemia, RR 5.1 (95% CI: 3.1–8.5),  $P<0.0005$ . There was no evidence that preoperative folate

or vitamin B supplementation protected against postoperative hyperhomocysteinaemia (OR 1.4,  $P=0.32$ ). N<sub>2</sub>O was associated with more complications, but most were not affected by postoperative plasma folate concentration or hyperhomocysteinaemia (Table 4). The exception was major complications, for which hyperhomocysteinaemia had an independent additional effect on the risk of major complications (Table 4). This suggests that N<sub>2</sub>O-associated complications cannot be entirely attributed to elevated plasma Hcy. Regular ingestion of cereals ( $P=0.17$ ) or preoperative folate or vitamin B supplementation ( $P=0.97$ ) had no significant effect on the risk of major complications.

There was a negative correlation between plasma folate and Hcy concentrations before operation,  $r=-0.27$  ( $P<0.001$ ), but not after operation,  $r=0.02$  ( $P=0.65$ ). However, this lack of association in the postoperative period was confounded by N<sub>2</sub>O exposure: for patients in the N<sub>2</sub>O-free Group there was a negative correlation between postoperative folate and postoperative Hcy,  $r=-0.24$  ( $P=0.002$ ); for patients in the N<sub>2</sub>O Group there was no correlation between postoperative folate and postoperative Hcy,  $r=0.04$  ( $P=0.57$ ).

There was a significant effect of duration of anaesthesia on the relative change in plasma Hcy concentration, and this effect differed between the groups ( $P<0.001$ ): N<sub>2</sub>O Group  $r=0.42$  ( $P<0.001$ ); N<sub>2</sub>O-free Group  $r=0.26$  ( $P=0.001$ ) (Fig. 2). An exploratory logistic regression analysis identified several significant predictors of hyperhomocysteinaemia (Table 5). Preoperative folate or vitamin B supplementation was protective in this regard.

An exploratory stepwise linear regression analysis identified N<sub>2</sub>O exposure (increase), smoking (increase), female gender (increase), regular breakfast cereal (decrease),

**Table 1** Patient and surgical characteristics expressed as number (%), unless otherwise stated. ASA, American Society of Anesthesiologists; ENT, ear, nose and, throat

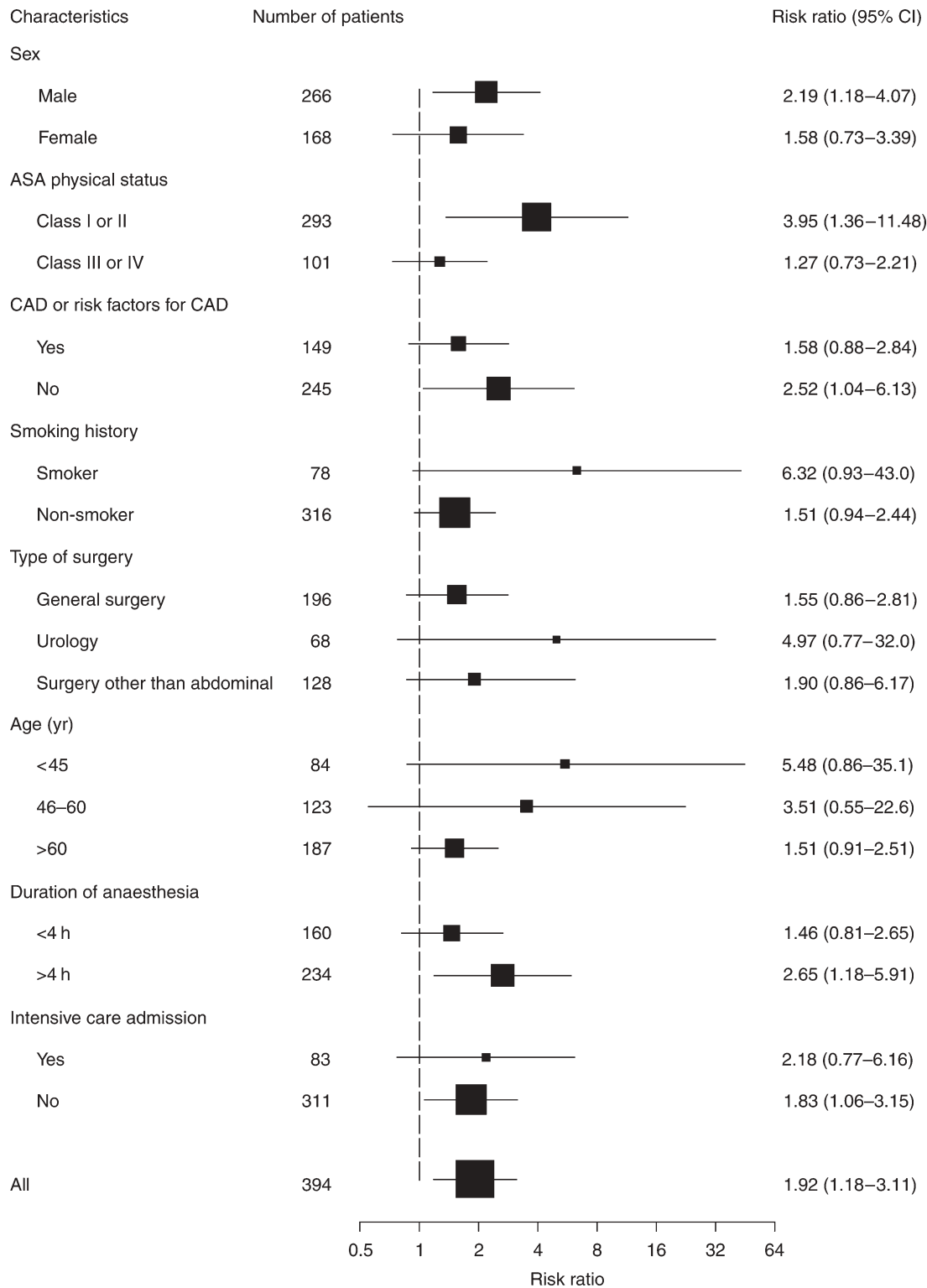
Factors	N <sub>2</sub> O Group <i>n</i> =215	N <sub>2</sub> O-free Group <i>n</i> =179
Males	118 (55)	108 (60)
Age (yr), mean (SD)	59 (15)	58 (16)
Weight (kg), mean (SD)	63 (16)	63 (17)
ASA physical status		
I	29 (14)	30 (17)
II	129 (60)	105 (59)
III or IV	57 (27)	44 (25)
Dietary factors		
Vegan	2 (0.9)	3 (1.7)
Regular breakfast cereals	66 (31)	63 (35)
Regular fruit/vegetables	124 (58)	98 (55)
Folate/vitamin B supplementation	61 (28)	43 (24)
Medical conditions		
Smoker	51 (24)	27 (15)
Diabetes	29 (14)	33 (18)
Hypertension	90 (42)	66 (37)
Coronary artery disease	28 (13)	16 (8.9)
Heart failure	8 (3.7)	2 (1.1)
Previous thromboembolism	5 (2.3)	5 (2.8)
Previous stroke	16 (7.4)	8 (4.5)
Type of surgery		
General	104 (48)	92 (51)
Urology	35 (16)	33 (18)
Neurosurgery	19 (8.8)	17 (9.5)
Orthopaedic	13 (6.0)	11 (6.1)
Vascular	17 (7.9)	7 (3.9)
Gynaecology	14 (6.5)	7 (3.9)
ENT or faciomaxillary	10 (4.7)	9 (5.0)
Plastics	3 (1.4)	3 (1.7)
Duration of anaesthesia (h), mean (SD)	4.9 (2.4)	4.9 (2.5)

**Table 3** Changes in plasma folate and homocysteine (Hcy) concentration according to nitrous oxide (N<sub>2</sub>O) exposure during surgery, expressed as mean (SD). \*Adjusted for preoperative value

Factors	N <sub>2</sub> O Group <i>n</i> =215	N <sub>2</sub> O-free Group <i>n</i> =179	<i>P</i> -value*
Preoperative			
Hcy ( $\mu\text{mol litre}^{-1}$ )	9.2 (3.0)	9.7 (5.3)	–
Folate ( $\text{mmol litre}^{-1}$ )	23.5 (5.1)	23.9 (5.4)	–
Postoperative			
Hcy ( $\mu\text{mol litre}^{-1}$ )	11.1 (3.8)	8.5 (4.0)	<0.0005
Folate ( $\text{mmol litre}^{-1}$ )	26.5 (5.7)	22.4 (6.0)	<0.0005

**Table 2** Anaesthetic factors expressed as mean (SD). MAC, minimum alveolar concentration. MAC values used for sevoflurane, isoflurane, and desflurane as 1.80, 1.15, and 6.0, respectively

Factors	N <sub>2</sub> O Group <i>n</i> =215	N <sub>2</sub> O-free Group <i>n</i> =179	<i>P</i> -value
Inspired oxygen concentration (%)	32 (5.8)	73 (19.3)	<0.001
End-tidal volatile concentration (MAC equivalents)	<i>n</i> =162 0.63 (0.02)	<i>n</i> =130 0.76 (0.02)	<0.001
Target propofol plasma concentration ( $\mu\text{g ml}^{-1}$ )	<i>n</i> =48 3.28 (0.50)	<i>n</i> =46 3.30 (0.58)	0.85
Intraoperative fentanyl ( $\mu\text{g}$ )	<i>n</i> =154 106 (50)	<i>n</i> =114 117 (72)	0.12
Intraoperative morphine (mg)	<i>n</i> =169 11.0 (5.2)	<i>n</i> =142 11.0 (7.2)	0.99



**Fig 1** The risk of hyperhomocysteinaemia with nitrous oxide exposure in selected sub-groups, expressed as risk ratio (95% confidence interval). The vertical interrupted line represents a risk ratio of 1.0 (no effect). Sub-group differences existed for ASA ( $P<0.0005$ ) and CAD ( $P<0.0005$ ), but not for others (all  $P>0.05$ ). ASA, American Society of Anesthesiologists physical status; CAD, coronary artery disease.

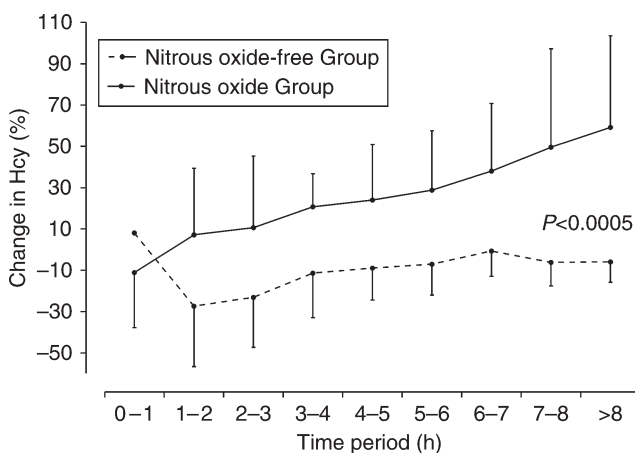
folate or vitamin B supplementation (decrease), and duration of anaesthesia (decrease) as the only significant predictors of a relative change in plasma folate concentration after surgery (all  $P<0.001$ ).

## Discussion

We found that  $N_2O$  exposure increased plasma Hcy concentration and this was dose-related, in which a longer duration of anaesthesia (and surgery) had a greater impact

**Table 4** The risk of postoperative complications in patients receiving nitrous oxide (N<sub>2</sub>O) adjusted for age, ASAs' physical status, and duration of anaesthesia. Hyperhomocysteinaemia and plasma folate variables were then added to logistic regression models to determine whether they had an independent effect. \*Respiratory complications included pneumonia, atelectasis, pneumothorax, or pulmonary embolism within 30 days of surgery. †Major complications included pneumonia, pulmonary embolism, wound infection, myocardial infarction, venous thromboembolism, stroke, awareness, or death within 30 days of surgery. ‡Hyperhomocysteinaemia had an independent effect on the risk of major complications, adjusted odds ratio (OR) 2.54 (95% CI: 1.28–5.01), modifying the N<sub>2</sub>O effect, adjusted OR 1.56 (95% CI: 0.87–2.80)

Complication	Incidence (%)		Total N <sub>2</sub> O effect, OR (95% CI)	Total N <sub>2</sub> O effect, P-value	Independent Hcy effect, P-value	Independent folate effect, P-value
	N <sub>2</sub> O Group (n=215)	N <sub>2</sub> O-free Group (n=179)				
Severe nausea or vomiting	27	12	2.64 (1.54–4.55)	<0.0005	0.54	0.5
Wound infection	16	7.8	2.31 (1.18–4.53)	<0.0005	0.91	0.46
Any respiratory complication*	24	13	2.17 (1.26–3.74)	0.005	0.72	0.94
All major complications†	21	12	1.93 (1.10–3.41)	0.023	0.007‡	0.57



**Fig 2** The effect of duration of anaesthesia on the postoperative change in plasma homocysteine (Hcy) according to nitrous oxide exposure, expressed as % change from the preoperative value. Values are mean (SD). There was a group–time interaction ( $P<0.0005$ ).

on postoperative Hcy concentration. Furthermore, this effect was apparent in a variety of patient categories, both with and without comorbidity, and undergoing a diverse range of surgical procedures. We identified hyperhomocysteinaemia as a risk factor for major postoperative complications in this cohort.

N<sub>2</sub>O is a potent inhibitor of methionine synthetase, with even low concentrations (10–50%) decreasing enzyme activity to undetectable concentrations within hours.<sup>1 2</sup> Hyperhomocysteinaemia may then ensue.<sup>4 19–21</sup> Hyperhomocysteinaemia is a recognized risk factor for MI, dementia, and stroke.<sup>12–18</sup> A meta-analysis of randomized trials found that reducing plasma Hcy concentration by 3  $\mu\text{mol litre}^{-1}$  could reduce the risk of MI by 16% (95% CI: 11–20%), stroke by 24% (95% CI: 15–33%), and venous thromboembolism by 25% (95% CI: 8–38%).<sup>17</sup> A randomized trial of 90 patients undergoing carotid endarterectomy under general anaesthesia<sup>21</sup> found that N<sub>2</sub>O exposure led to a significant increase in postoperative Hcy concentration and myocardial ischaemia. Acute increases in Hcy concentration lead to endothelial dysfunction<sup>24</sup> and

**Table 5** Patient and anaesthetic factors associated with hyperhomocysteinaemia after surgery. ASA, American Society of Anesthesiologists; ICU, intensive care unit; OR, odds ratio

Factors	Univariate OR (95% CI)	P-value	Adjusted OR (95% CI)	P-value
Male sex	1.47 (0.79–2.72)	0.22	–	–
Age >65 yr	2.16 (1.19–3.92)	0.01	0.97 (0.39–2.40)	0.94
ASA III or IV	4.15 (2.25–7.66)	<0.0005	6.48 (2.95–14.2)	<0.0005
Coronary artery disease	2.99 (1.63–5.48)	<0.0005	1.86 (0.74–4.72)	0.19
Smoker	1.92 (0.98–3.73)	0.053	1.53 (0.71–4.72)	0.27
Folate/vitamin B supplementation	0.26 (0.10–0.67)	0.003	0.09 (0.03–0.28)	<0.0005
Abdominal surgery	0.68 (0.37–1.25)	0.21	–	–
Nitrous oxide exposure	2.80 (1.44–5.46)	0.002	3.91 (1.82–8.40)	<0.0005
Duration of anaesthesia >4 h	1.42 (0.78–2.56)	0.25	–	–
Admission to ICU	1.59 (0.80–3.08)	0.19	–	–

thrombogenesis,<sup>12 13 15 17</sup> both of which could increase the risk of perioperative MI.<sup>14 15</sup>

As found in our clinical trial from which this cohort was selected,<sup>22</sup> avoidance of N<sub>2</sub>O and replacement with additional inspired oxygen is associated with a reduction in severe nausea or vomiting, wound infection, respiratory complications, and major complications. The study design leaves open the possibility that supplemental oxygen could have had an independent effect on some of the results, but there are no pre-existing data suggesting an oxygen effect on methionine synthetase or plasma Hcy. In the present study, we found no apparent modification of the risk of any of these complications by preoperative vitamin supplementation or postoperative plasma folate concentrations. Similarly, postoperative hyperhomocysteinaemia had no effect on nausea or vomiting, wound infection, or respiratory complications. There was however an independent effect of hyperhomocysteinaemia on the risk of major complications, and this can be attributed to increased rates of cardiovascular complications.

Low plasma folate concentrations are a common problem in the elderly,<sup>25</sup> and there is an association between folate



status and total Hcy concentrations.<sup>15 23</sup> Preoperative vitamin B supplementation for 1 week before surgery can prevent N<sub>2</sub>O-induced hyperhomocysteinaemia,<sup>26</sup> but this is dose-dependent. Folinic acid 30 mg given immediately before and 12 h after anaesthesia prevents the N<sub>2</sub>O-induced increase in plasma Hcy concentration in most patients, whereas smaller amounts are ineffective.<sup>27</sup> We found that regular vitamin supplementation reduced the risk of hyperhomocysteinaemia but did not protect against complications associated with N<sub>2</sub>O administration. However, we did not record the vitamin dosage or compliance.

As shown by other studies,<sup>1 2 4</sup> we found that N<sub>2</sub>O exposure was associated with increased plasma folate concentrations after operation. This may be explained by a probable failure of the intracellular transport of folate,<sup>3 5</sup> and enhanced excretion,<sup>1</sup> such that a folate deficiency state may exist in the setting of normal plasma (total) folate concentrations. This is known as the 'methyl trap hypothesis',<sup>1 5</sup> whereby decreased amounts of 10-formyl- and 5,10-methylenetetrahydrofolate are available for purine and thymidine synthesis. In rats, N<sub>2</sub>O exposure is followed by increased plasma folate concentrations but reduced tissue folate stores, this loss being most marked in the liver.<sup>1</sup> In humans, N<sub>2</sub>O also results in diminished intracellular folate concentrations and a redistribution of folate derivatives.<sup>5 28</sup> Our study findings of no association between plasma folate and Hcy concentrations after operation in those exposed to N<sub>2</sub>O, in contrast to those in the N<sub>2</sub>O-free Group, supports a mechanism of N<sub>2</sub>O-induced impairment of intracellular transport of folate in the early postoperative period.

Our results have implications for patients undergoing surgery and general anaesthesia. Elderly, vegan, and chronically ill patients may be deficient in dietary folate, methionine, or vitamin B<sub>12</sub>.<sup>8 10 13 25</sup> N<sub>2</sub>O can interfere with folate and Hcy metabolism and may increase the risk of postoperative cardiovascular and other adverse outcomes.<sup>21 22</sup> Such data provide some reasons to question the routine use of N<sub>2</sub>O in contemporary practice.<sup>22 29</sup>

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