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Nitrous oxide abuse in the emergency practice, and Review of toxicity mechanisms and potential markers

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ABSTRACT

Nitrous oxide (N_2O) toxicity is a concern common to several medical fields. Here, retrospective study of four N_2O abuses with neurological signs in the emergency practice provides a preliminary basis for a metabolic Discussion/Review. This latter highlights N_2O abuse as pathology of DNA/RNA/protein methylations, for instance consistent with impairments of protein arginine methyltransferases involved in myelinogenesis and myelopathy in patients. Basically, pathogenesis starts with oxidation by N_2O of coordinated cobalamine cobalt ions at enzyme sites with impairments of vitamin-B12-dependent pathways. Methionine synthase (methylcobalamine) and methymalonyl-CoA mutase (adenosylcobalamine) are inactivated and cofactor-depleted, respectively. The number of impacted pathways (folate cycle, methylation cycle, *S*-adenosylmethionine-dependent methyltransferases, transulfuration pathway, Krebs cycle fueling by methylmalonyl-CoA, glutathione synthesis) explains the variety of potential research/laboratory markers, and may provide new clues and future angles to explore N_2O toxicity. Overall, homocysteine measurements obviously help diagnosis of N_2O abuses. Additional markers may include vitamin-B12, methionine, methylmalonate, dimethylglycine, sarcosine, *S*-adenosylmethionine to *S*-adenosylhomocysteine ratio, various *S*-adenosylamino acids, *S*-adenosylmethionine-dependent cellular methylations, and additional analytes (propionylcarnitine, propionylglycine, cystathionine and derived metabolites, methylated amino acids [eg arginine], betaine).

Keywords

Nitrous oxide abuse; cobalamins; vitamin B12; methionine synthase; methylmalonyl-CoA mutase; methylation cycle; folate cycle; homocysteine; biological markers

1. Introduction

Nitrous oxide (N_2O) was discovered in 1772. This 'laughing gas' has medical indications which include analgesia, anxiolysis and anaesthesia, and date back to 1844 with analgesia in dental surgery, then anaesthesia (Kongara et al., 2021; Oussalah et al., 2019). Recreational use also dates from this period. Facilitated by its easy availability as a propellant gas in aerosol cans for food use, it has recently re-emerged as a cause of hallucinations, excitement and psychological dependence in the young population (van Amsterdam et al., 2015). N_2O poured into “whippits” is inhaled by users to meet euphoria. However, prolonged high consumption of N_2O causes side effects among which vitamin B12 deficiency, severe neurological deterioration such as the degeneration of the spinal cord (Dong et al., 2019; Lan et al., 2019; McArdle and Gaillard, 2020; Seed and Jogia, 2020) and, *via* hyperhomocysteinemia, pulmonary embolisms, venous and arterial thromboses, psychiatric disorders and ischaemic strokes (Bajaj et al., 2018; Chien et al., 2020; den Uil et al., 2018; Pratt et al., 2020).

Though well documented for methylcobalamin at methionine synthase enzyme site, the inactivating effects of N_2O on non-enzyme bound vitamin B12 and adenosylcobalamin encased in methymalonyl-CoA mutase still need to be clarified (see Discussion). Vitamin B12 deficiency is double-edged being consequence and worsening factor of N_2O toxicity. The present retrospective study of data concerns four young (18-23 years old) patients who developed N_2O abuse before the Covid-19 outbreak. The discussion stresses the clinical and biological signs that should alert on N_2O intoxication, and the panoply of biomarkers potentially available to diagnosing this addiction. A brief account of patient 3 has been published previously (Grzych et al., 2020).

2. N_2O abuse in the emergency practice

The present retrospective study of cases of N_2O abuse in the emergency practice serves as a preliminary basis to review underlying toxicity mechanisms and potential markers (Discussion). Plasma amino acids concentrations (µmol/L) were determined as described by Boemer et al., 2015. Briefly, plasma amino acids were amine-modified using the aTRAQ™ labeling reagent, which provides a specific mass tag for tandem mass spectrometry (MS). The tag is identified by MS/MS fragmentation of analytes and standards under the multiple reaction monitoring mode. An internal standard (IS) set of aTRAQ™ labeled amino acids was used for both detection and quantification of analytes by HPLC (Shimadzu C18 column, Kyoto, Japan) separation coupled with MS/MS (Sciex 3200 Qtrap, Framingham, MA, US). Quantification was performed by dividing peak area of the analyte with

peak area of the respective IS, the calculated quotient being mutiplied by the respective IS concentration. Vitamins B1 and B6 were assayed using ChromSystemS protocols (Gräfelfing, Germany) on whole blood and plasma samples, respectively. Sample clean-up was achieved by protein precipitation. Derivatization of supernatants was performed to obtain fluorescent analytes. Isocratic HPLC separation was followed by fluorimetric detection through a procedure adapted from the method of Reynold and Brain (1992). Seric vitamins B9 (folates) and B12 were measured by imunoassays (Access Folates and Access Vitamin B12 cobalamine protocols, respectively, Beckman Coulter, CA).

Patient 1 - A 19-year-old man was admitted for gait disturbance and paresthesia affecting left leg and both hands. These signs had developed gradually over the preceding week. One week ago, patient had also developed balance problems with a fall episode. The history indicated no personal and family history for a neurological disease. Alcohol consumption and smoking (6 packs per year) were occasional. By contrast, cannabis was consumed daily (2 to 3 times per day), and N_2O at 200 to 300 whippits per day. Clinically, the ataxia was major with the need for an assistive device to walk. Generalized areflexia and apallesthesia of the lower limbs were also observed. Vitamin B12 assessed on admission was at the lower limit of reference values (0.2 nmol/L [control range: 0.2-1.0 nmol/L]) (Table 1). The MRI scan showed posterior cervical spinal cord lesion spanning from the C2 to C6 vertebral levels and suggestive of subacute combined degeneration (Fig. 1). This myelopathy associated with vitamin B12 deficiency prompted biological inverstigations to identify any underlying nutritional or metabolic deficiency. Low plasma vitamin B6 (8 nmol/L [control values: 15-73 nmol/L]), and significantly increased total plasma homocysteine (144 µmol/L [control values < 14 µmol/L]) were observed (Table 1). There was no anaemia and the GMV was normal. IV vitamin B12 supplementation was followed by oral relay (1 mg/day for 1 month), as well as completed with vitamin B9 (5 mg/day for 1 month) and vitamin B6 (500 mg/day for 1 month) (Table 1) therapies. Discontinuation of N_2O was also undertaken during upon hospitalization. Signs gradually improved and patient was discharged after a few days of the multi-vitamin therapy.

Patient 2 - A 19-year-old woman was admitted with paresthesia of the lower limbs. The patient followed a meat-free diet. For 1 year and at repeated time intervals, she consumed 200 whippits of N_2O per day (Table 1). Four months ago, paresthesia developed with hypoesthesia extending from the feet to the knees and from the hands to the elbows without motor deficit. The symptoms regressed with the withdrawal of N_2O, and sensory disturbances in the lower limbs reappeared as

soon as patient resumed N_2O consumption for a few days. Unstable walking caused a fall with knee trauma. On examination, patient was conscious without signs of disorientation. MRI of the spine was normal. Vitamin B12 was collapsed below 0.2 nmol/L in parallel with a significant increase in total plasma homocysteine (142 µmol/L [control values < 14 µmol/L]) (Table 1). IM vitamin B12 supplementation followed by oral relay (1 mg/day for 1 month) was combined with oral folic acid (5 mg x2/day for 1 month) (Table 1).

Patient 3 - An 18-year-old man was referred for subacute walking difficulties. For six weeks, he suffered from heavy leg signs. For one year, patient consumed 100 whippits of N_2O every weekend (Table 1); he spontaneously halved consumption when affected with walking difficulties, and stopped intake when convinced of a link between consumption and signs. The sensation of heavy leg was accentuated on exertion, when climbing stairs and walking for long periods. Three weeks before present admission, the patient referred to another hospital and diagnosed with subacute combined spinal cord degeneration caused by N_2O, omitted to take the prescribed vitamin B12 when out of the hospital. Walking difficulties with neuropathic pain were the cause of the present consultation. The electroneuromyogram showed a severe symmetrical sensitivomotor polyneuropathy of axonal origin. Bilateral carpal tunnel syndrome was also diagnosed. Plasma vitamin B12 was at the lower limit of reference values, total serum homocysteine (123 µmol/L [control values < 14 µmol/L]) was increased (Table 1). IM vitamin B12 supplementation was administered at a dose of 1 mg/day. After 7 days of hospitalisation, the patient was transferred to a rehabilitation unit and 10 days later, without medical agreement, escaped from this unit, resulting in a loss of medical follow-up (Table 1).

Patient 4 - A 23-year-old man was admitted for delusion in the context of a paranoid disorientation for 7 days. On arrival he was agitated with hallucinations. He was treated with Tercian and Rivotril. Heteroanamnesis revealed a similar episode a few months ago, and consumption of 100 whippits of N_2O per day for 2 years. Patient had no personal psychiatric history. The cerebral CT scan failed to detect haemorrhagic lesion. Plasma vitamin B12 concentration was below baseline and total serum homocysteine was increased (164 µmol/L [control values < 14 µmol/L]) (Table 1).

3. Discussion, Review of mechanisms and potential markers of N_2O toxicity

3.1. Recapitulative clinics, biology and therapeutic measures in the four reported cases - Alert signs for nitrous oxide abuse

Table 1 provides a brief summary of the clinical history, laboratory data and medical treatments of the four patients. Diagnosing N_2O abuse has been aided with the anamnesis of patients, and,

heteroanamnesis conducted in their immediate social entourage. Recreational uptakes of 100 or more whippits per day caused signs which led patients to consult and be hospitalized. Patients had deficient or low levels of vitamin B12 (Table 1). Gait disturbances were present in all but one patient. In this patient, clinical signs were dominated by a delirium. This change in mental status has been previously reported in cases of N_2O abuse (Sterman and Coyle, 1983). In the other three patients, gait disturbances were associated with paresthesia, heaviness and pain of legs. Hands were also affected as reported spontaneously by patients or diagnosed after medical examination including EMG. Paraesthesia, gait instability and weakness are common in patients with N_2O abuse (Garakani et al., 2016; Xiang et al., 2021). These signs should thus lead to include N_2O abuse in the differential diagnosis (Fernández et al., 2017). One of the two patients with subacute combined degeneration of spinal cord had also vitamin B6 deficiency, raising a possible protection of vitamin B6 against myelopathy caused by vitamin B12 deficiency (see below).

3.2. Effects of nitrous oxide on cobalamin-dependent proteins, and general consequences on metabolism and pathogenesis

Vitamin B12 encompasses a group of molecules usually referred to as cobalamins and which share in common a molecular structure which coordinates a cobalt atom within a corinnoid ring (Froese et al., 2019). Essential forms of cobalamins involved in human metabolism include non-exhaustively methylcobolamin and adenosylcobalamin which are cofactors of cytoplasmic methionine synthase and mitochondrial methylmalonyl-CoA-mutase, respectively (Froese et al., 2019). Vitamin B12 is also involved in erythrogenesis and CNS homeostasis. N_2O impairs cobalamin (vitamin B12)-dependent metabolic pathways through the oxidation by N_2O of cobalt ions from the vitamin corrinoid ring leading to inactivation and deficiency of vitamin B12 (Sharma et al., 2003) along with its biological manifestations including increased homocysteine and methylmalonic acid (Xiang et al., 2021).

Fig.2 illustrates the metabolic pathways dependent on cobalamin cofactors, stressing their targeting by N_2O as well as some reciprocal regulations taking place between methylation and folate cycles. Subcellular sites for the main metabolic reactions are illustrated as well as interactions between pathways including folate cycle, methylation cycle, transulfuration pathway, mitochondrial succinyl-CoA metabolism, cytosolic glutathione synthesis, and cell/tissue S-adenosylmethionine-dependent methyltransferases (for a more complete consideration including the nuclear component of folate metabolism, see Lan et al, 2018; Tibbetts and Appling 2010).

Fig.2 points a potential protective role of vitamin B6 (evoked above for Patient 1) against vitamin B12 deficiency by linking homocysteine to glutathione synthesis *via* cysteine formation. Other mechanisms for neuroprotective properties of vitamin B6 rely on PMK2-NRF2 signaling and up-regulation of glutathione biosynthesis genes (Wei et al., 2020). Reduced availability of vitamin B6

could thus compromise antioxidant defenses and repair mechanisms that might protect against subacute combined spinal cord degeneration. Functional deficiency of S-adenosylmethionine-dependent methyltransferases including protein arginine methyltransferases (PRMT) 1 and 5 which are critical for myelin formation (Hashimoto et al., 2021) might also promote this myelopathy. The inactivation by N_2O of methionine synthase [vitB12-dependent enzyme] might impair PRMT1 and 5 *via* either reduced supply in *S*-adenosylmethionine or increased product inhibition by *S*-adenosylhomocysteine. The latter *vs* former mechanism might prevail based on normal levels of methionine observed in patients.

The analysis of N_2O-impacted pathways suggests two mechanisms contributing to the rise of methymalonic acid : its decreased handling by mitochondrial methylmalonyl-CoA mutase and its enhanced synthesis from the α-ketobutyrate derived, *via* cystathionine, from homocysteine accumulating secondarily to impaired methionine synthase (**Fig.2**).

3.3. Biological markers for N_2O abuse

3.3.1. Distinct inactivation patterns of methionine synthase and methylmalonyl-CoA mutase

Altered levels in blood vitamin B12, homocysteine and methionine, and urinary methylmalonic acid have been largely described in N_2O abuse. Their genesis, and kinetics, may be understood by taking into account the recoveries of methylmalonyl-CoA mutase and methionine synthase activities after exposure to N_2O. Riedel and coworkers (Riedel et al., 1999) provided evidence that cobalamin oxidation by N_2O at methionine synthase enzyme site involves hydroxyl radical and subsequent irreversible oxidative enzyme inactivation. As a result, *de novo* enzyme synthesis is needed to recovering enzyme function. By contrast, the apoenzyme of methylmalonyl-CoA mutase is preserved after N_2O, and recovery of the mutase activity requires only the replenishment of the corrinoid ring with the cobalamin enzyme cofactor (Riedel et al., 1999). As a conclusion, methionine synthase is oxidatively and irreversibly inactivated by N_2O whereas methylmalonyl-CoA mutase is essentially cofactor depleted. In addition, a rapid recovery of the mutase activity, which relies on release of the altered cobalamin cofactor and its replacement by an intact one, is promoted by MMAA (Takahashi-Iñiguez et al., 2017). These two distinct patterns of enzyme alterations by N_2O may explain the persistence of elevated homocysteine but not methylmalonic acid levels when patients both receive vitamin B12 therapy and maintain N_2O consumption (Nunn, 1984). Other considerations for distinct impaction of the two enzymes by N_2O might lie for the mutase (*vs* synthase), in the mitochondrial (vs cystololic) location and cobalamin flanking adenosyl (*vs* methyl) substitution. Whether these enzyme specificities (mitochondrial membrane barriers, steric hindrance in the catalytic site) might protect the mutase enzyme site from N_2O poisoning remains to be clarified.

3.3.2. Mechanisms of vitamin B12 deficiency induced by N_2O oxidation of cobalamin cobalt ions

As mentioned just above, N_2O-driven irreversible oxidation of cobalamin cobalt ions at enzyme sites impacts irreversibly methionine synthase and reversibly methylmalonyl-CoA mutase. The recovery of methionine synthase and methylmalonyl-CoA mutase from N_2O-induced inactive states is inevitably made at the expense of available vitamin B12 stores *via* incorporation of cobalamins in newly synthesized and recycled apoenzymes, respectively. This may contribute to vitamin B12 deficiency which in turn may worsen back the impairment of the two vitamin B12-dependent enzymes.

3.3.3. Potential markers of N_2O abuse

Vitamin B12 is a potential reference biological marker of N_2O abuse. For the reasons just mentioned above, N_2O abuse may induce a drop in blood vitamin B12. The absence of status of vitamin measurements before N_2O abuse does not allow to assess the drop but only the possible dropped value. When vitamin B12 value is in normal range, vitamin B12 deficiency or drop may not be stated in N_2O abuse. In two of the four patients, serum vitamin B12 values were at the lowest normal range values. Such values might be considered to be pathological since reference values are based on a large population inevitably including some deficient patients. Finally, the social food habits of N_2O consumers do not rule out a poor nutritional status responsible for vitamin B12 deficiency. As mentioned above, such a pre-existing deficiency in vitamin B12 would exacerbate the gas toxicity.

Methylmalonic acid accumulation, and inhibition of methylmalonyl-CoA mutase may occur under N_2O abuse. For the reasons mentioned above, methylmalonic acid might be a less reliable marker than homocysteine as more as, in contrast to methionine synthase, inactivation of methylmalonyl-CoA mutase still remains unclear (Frasca et al., 1986; Waclawik et al., 2003). Nevertheless, methymalonic aciduria is regularly observed in patients with N_2O abuse; it should lead to check vitamin B12 status and plasma homocysteine levels when drug abuse is suspected. Increased levels of plasma valine can result from methylmalonyl-CoA mutase deficiency (Riedel et al., 1999). This is consistent with metabolic fuelling of methylmalonyl-CoA by valine (**Fig. 2**).

Increased plasma homocysteine is the direct result of N_2O-targeted methionine synthase. It was observed in the four patients with values 10-fold the reference highest value, suggesting it might be a highly sensitive marker of N_2O abuse. Despite alternative etiologies, differential diagnosis should immediately evoke N_2O abuse in a context of drug intake. Reciprocally, plasma homocysteine might be measured when N_2O abuse is suspected.

Methionine, the product of methionine synthase, is expected to be decreased under N_2O. However, as observed in our patients, plasma methionine concentrations may be in normal range values. So, the drop in plasma methionine should not be considered as a reliable marker of N_2O abuse. Compensatory mechanisms that might maintain normal levels of methionine are diet and body

(proteolysis) supplies, alternative synthesis from homocysteine (BHCMT), and decreased consumption by the methylation cycle.

Dimethylglycine and sarcosine are discussed because of increased plasma sarcosine in one of the four patients. In this light, sarcosine does not appear to be a reliable marker in N_2O abuse. How dimethylglycine levels behave under N_2O needs clarification.

S-adenosylmethionine/S-adenosylhomocysteine balance, and related individual S-adenosyl-amino acid levels are physiologically controlled by GNMT activity. This latter is negatively regulated by 5-methyl THF (**Fig. 2**) (Simile et al., 2018). In return, *S*-adenosylmethionine one of the substrates (the other being glycine) of GNMT inhibits methylenetetrahydrofolate reductase (MTHFR) (Simile et al., 2018) with as consequences (in the absence of N_2O) a rise in 5-methyl THF (THF, tetrahydrofolate) and increased conversion of homocysteine to methionine. It also inhibits the other methionine-synthesizing enzyme BHCMT (Simile et al., 2018). (**Fig.2**). This balance between, from the one hand, methylation cycle inhibition (at the level of GNMT) by a folate cycle intermediate (5-methyl THF) and, on the other hand, folate cycle inhibition (at the MTHFR step) by a methylation cycle intermediate (*S*-adenosylmethionine) represents a fine tuning to adapt the folate cycle activity to the cellular demand for *S*-adenosyl-dependent methylations. N_2O may impact this S-adenosylmethionine/S-adenosylhomocysteine balance and related individual S-adenosyl-aminoacid levels essentially by inducing a huge rise in *S*-adenosylhomocysteine (Molloy et al., 1990), making this S-substituted amino acid a potential interesting biological marker for N_2O abuse.

Cellular S-adenosylmethionine-dependent methylations is expected to be affected by the N_2O-mediated inactivation of methionine synthase. The enzyme inactivation though not leading to decreased levels of methionine (see above) might alter the steady-state concentrations and availability of methionine for S-adenosylmethionine synthesis, and hence the availabitily of S-adenosylmethionine for cellular methylations. The *S*-adenosyladenosine-dependent methylation product to substrate ratios might clarify subsequent impact on the individual *S*-adenosyladenosine methyltransferases (see **Fig. 2** for a rapid non-exhaustive listing), and in turn be exploited as markers of the N_2O abuse. Serious limits in this approach arise when metabolites take place in several metabolic pathways (for instance glycine) or are supplied by the diet. For instance, creatine the product of guanidinoacetate methyltransferase is present in energetic drinks which when taken-up by N_2O users may affect creatine levels and creatine to guanidinoacetate ratio. In the same time, increased guanidinoacetate secondarily to deficient S-adenosyladenosine availability could witness for N_2O abuse. Substrates and products of S-adenosyl-dependent methylations and their ratios warrant future clarifications about their suitability as biological markers of N_2O abuse.

Other metabolites appearing on **Fig.2** might be checked for their suitability to be biological markers of N_2O abuse. Briefly, these might include propionylcarnitine (reduced consumption and increased

production of propionyl-CoA secondarily to inactivated methylmalonyl-CoA mutase and methionine synthase, respectively), propionylglycine (for the same reasons as propionylcarnitine, and hypothetic, increased glycine availability), cystathionine and derived metabolites (secondarily to methionine synthase deficiency) such as 2-oxobutyrate (mentioned above) and intermediates of the homocysteine transulfuration pathway (taurine, for instance). Methylated aminoacids resulting from hydrolysis of proteins which have been subjected to S-adenosylmethionine-dependent methylations are also of interest, for instance symmetric and asymmetric dimethylarginine (SDMA and ADMA). Though, in contrast to homocysteine, ADMA was not impacted by the acute anesthetic use of N_2O (Myles et al., 2008), chronic recreational use of N_2O might affect dimethylarginine levels *via* chronic impairment of the methylation cycle resulting from methionine synthase inactivation. Betaine consumption (and hence reduced levels of betain and its precursor choline) which yields dimethylglycine can be decreased by the stimulated fuelling of BHCMT by high homocysteine levels (Imbard et al., 2015). So, ratios to betain of metabolites which are expected to increase in patients with N_2O abuse obviously also warrant future consideration.

4. Conclusions

Grosso modo, chronic N_2O toxicity exhibits most clinical and metabolic features of vitamin B12 deficiency. Clinically, recreational N_2O users may develop walking difficulties and pain, and more severe neurological signs ranging from sensory-motor polyneuropathy to subacute combined spinal cord degeneration and, delirium. Biologically, patients usually present with decreased serum vitamin B12 (near or below the lower limit of normal) and markers for deficiency of vitamin B12-dependent enzymes (cytosolic methionine synthase and mitochondrial methylmalonyl-CoA mutase) such as increased homocysteine and decreased methionine, and increased methylmalonate.. N_2O abuse should be primarily considered as a severe methionine synthase deficiency associated with partially to severely deficient methylmalonyl-CoA mutase. In this light, the pathogenesis of N_2O abuse should be viewed as a disorder of cellular DNA, RNA and protein methylations, consistent with impairment of S-adenosylmethionine-dependent methyltransferases such as PRMT1 and 5 involved in myelinogenesis and functional deficiencies of which are consitent with myelopathy seen in patients. Treatment relies on cessation of N_2O intake and supplementation of vitamin B12. Treatment with vitamin B6 is also recommended when blood levels of this vitamin are decreased. Moreover, vitamin B12 supplementation should be combined with vitamin B6 treatment in cases of N_2O overdose, even in the absence of vitamin B6 depletion. This dual measure might help the detoxifying of the methylation cycle by removing excess homocysteine *via* methionine [progressive recovery of methionine synthase activity, VitB12-dependent] and cysteine [stimulation of cystathionine synthase,

VitB6-dependent]. Limiting access to N_2O whippits and regular measurements of homocysteine or other potential relevant marker to monitor patient compliance can also be recommended.

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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TABLE

Table 1

Clinics, biology, and medical treatments of four patients with N_2O abuse. Abnormal biological data appear in bold underlined characters. NA, not available. * Reference laboratory range values are given between round brackets.

	Patient 1	**Patient 2**	**Patient 3**	**Patient 4**
Age (years)	19	19	18	23
N_2O consumption	200 to 300 whippits/day	200 whippits/day	100 whippits/week-end	100 whippits/day
Clinicalsigns	gait disorder, paraesthesia of left leg and both hands	gait disorder, paresthesia of lower limbs	gait disorder, walking pain	delirium
Spinal MRI	extensive posterior C2 - C6 cervical cord injury suggestive of subacute combined degeneration	normal	NA	NA
EMG	NA	bilateral L5 root affection more marked on left side	severe symmetrical sensitivomotor axonal polyneuropathy, bilateral carpal tunnel syndrome	NA
Blood/Plasma/Serum Biology*				
Hemoglobin(13-17g/dL)	14.1	**11.4**	16.1	13.1
MGM (82-98 µm3)	95.3	88.6	93.3	89.3
Vit B12 (0.2-1 ng/mL)	**0.2**	**<0.2**	**0.2**	**<0.2**
Vit B9 (3.1-19.9 ng/mL)	7.5	11.9	4.4	21.3
Vit B6 (15-73 nmol/L)	**8**	59	47	NA
Vit B1 (95-180 nmol/L)	167	105	145	109
Homocyteine(<14µmol/L)	**144**	**142 - 153**	**123**	**164**
Methionine (16-29 µmol/L)	22	19.5	19	NA
Sarcosine (0.5-2.7 µmol/L)	**3.5**	2.1	**7.7**	NA
Cystathionine (0.1-1.5µmol/L)	**1.7**	**2.4**	**1.7**	NA
Therapeutic measures				
Cessation of N_2O intakes	Yes	Yes	Yes	Yes
Vit B12	1 mg/day for 1 month	1 mg/day for 1 month	1 mg/day (for 2 weeks before loss of follow-up)	
Vit B9 (folate)	5 mg/day for 1 month	5 mg x2/day for 1 month	None	
Vit B6	500 mg/day for 1 month	None	None	

FIGURES

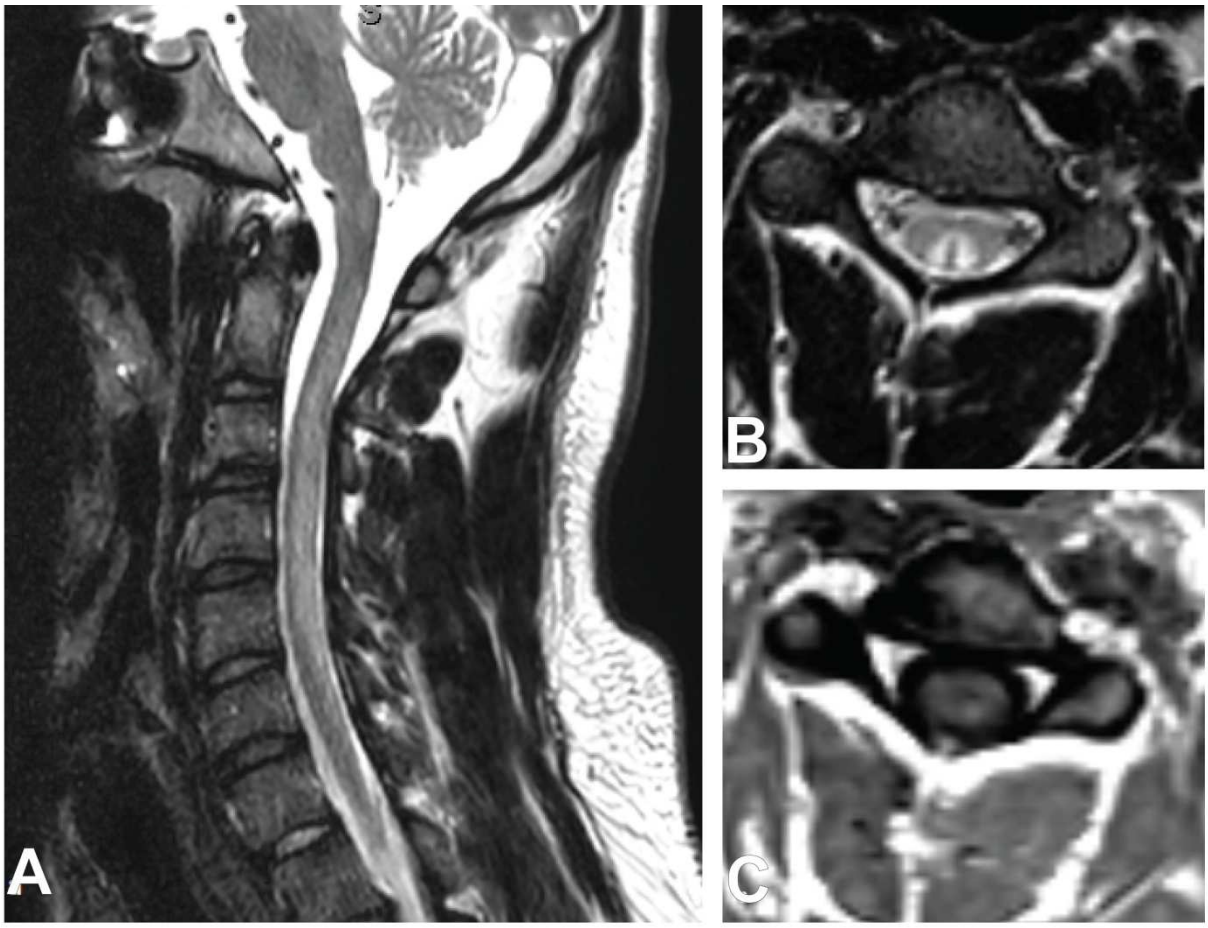

Fig. 1. MRI examination of the cervical spine in patient 1. MRI images show the swelling from C1 to C5 and the strong signal intensity in the dorsal cervical spinal cord on the sagittal (A) and axial (B) T2-weighted images. The contrast-enhanced T1-weighted axial image (C) further highlights the focal enhancement of the left posterior column of the spinal cord. These findings are consistent with subacute combined degeneration of the spinal cord, and possibly with a deficient function of *S*-adenosylmethionine-dependent methylases involved in myelogenesis (see Discussion).

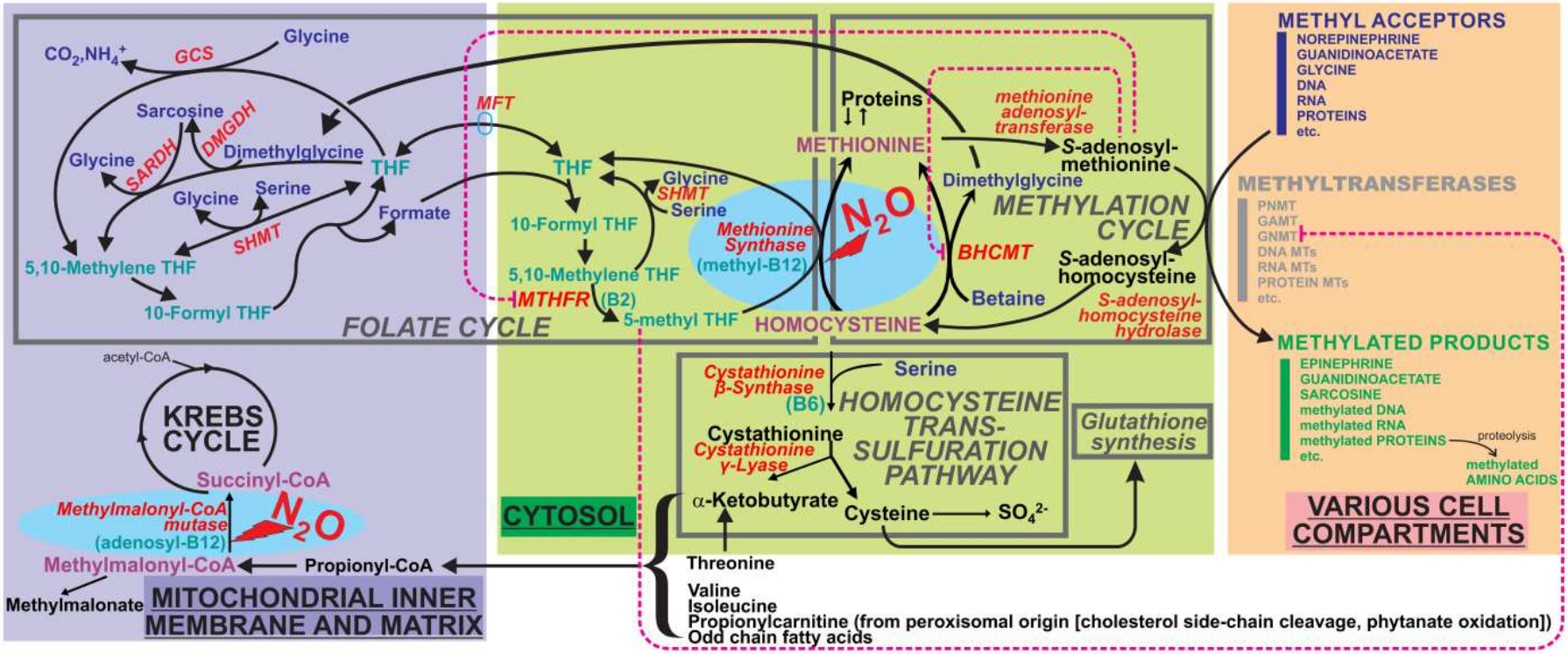

Fig.2. Subcellular location and metabolic role of cellular patways affected by N_2O. The vitamin B12-dependent methionine synthase (cytosol) and methylmalonyl-CoA mutase (mitochondria) are biological metabolic targets of N_2O. As explained in the main article text, methionine synthase is differently and more severely impacted than methylmalonyl-CoA mutase. The figure highlights the cellular roles of these enzymes in major cellular pathways which include folate cycle, methylation cycle, S-adenosylmethionine-dependent methyltransferases, transulfuration pathway, Krebs cycle fueling by methylmalonyl-CoA, and glutathione biosynthesis. A non-exhaustive description of proteins, metabolic steps, and intermediates involved in these pathways is given. Abbreviations are: BHCMT, betaine homocysteine methyltransferase; DMGDH, dimethylglycine dehydrogenase; GAMT, guanidinoacetate methyltransferase; GCS, glycine cleavage system; GNMT, glycine *N*-methyltransferase; MFT, mitochondrial folate transporter; MTHFR, methylenetetrahydrofolate reductase; PNMT, phenylethanolamine*N*-methyltransferase; SARDH, sarcosine dehydrogenase; SHMT, serine hydroxymethyltransferase; THF, tetrahydrofolate (yielded from folic acid, not shown). Note that serine produced by mitochondrial SHMT may fuel serine consumed by cytosolic SHMT, and glycine produced by cytosolic SHMT may fuel that consumed by mitochondrial SHMT. The purple broken lines stress some mutual inhibitions that can take place between the methylation and folate cycles.