Manganese Provision in Parenteral Nutrition: An Update

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Abstract

Manganese (Mn) is an essential micronutrient required for the activity of metalloenzymes. It is an essential component of parenteral nutrition (PN), but requirements are low. Mn status is difficult to assess, with the commonest method being measurement of its concentration in whole blood. This method has limitations, including artificially high concentrations resulting from contamination of specimen tubes. Mn toxicity is a well-recognized complication of PN, the risk of which increases if there is cholestasis or if the patient has received high doses. It usually presents with parkinsonian-like symptoms but may be detected presymptomatically as hypermanganesemia or as increased signal intensity of the basal ganglia upon T1-weighted magnetic resonance imaging. Caution is necessary when providing Mn for patients on long-term PN (>1 month). It is advisable to withhold supplementation if hypermanganesemia or cholestasis develops. Deficiency of Mn is rare in patients treated with PN. PN regimens are contaminated with Mn in amounts likely to meet requirements. Consequently, it is debated whether PN should be routinely supplemented with Mn. The currently recommended dose of Mn in adults treated with PN is 55 μg/d, but the doses provided by most currently available multi–trace element products exceed this. In response to calls for new products to be developed, 2 new multi–trace element products are currently available in Europe that provide Mn doses of 55 μg/d. Once these products are in general use, it is likely that the incidence of Mn toxicity will decrease. (Nutr Clin Pract. XXXX;xx:xx-xx)

Keywords

managanese; manganese toxicity; manganese deficiency; parenteral nutrition; selenium; iron

The trace element (TE) manganese (Mn) is an essential dietary component because it is required for the activity of numerous metalloenzymes. These participate in physiologic processes, including energy metabolism, antioxidant defense, tissue maintenance, wound healing, and functioning of the central nervous and immune systems.1 In health, homeostatic mechanisms prevent Mn deficiency or accumulation. However, in patients treated with parenteral nutrition (PN), the intravenous (IV) delivery of Mn increases its bioavailability to 100%. If provision of Mn exceeds the capacity for excretion, positive balance occurs, eventually leading to toxicity.

It is appropriate to review this subject at the present time because recent developments have important implications for clinical practice. First, there has been considerable progress in the understanding of Mn toxicity. Second, studies have suggested that underprovision of Mn has adverse effects. Finally, new multi-TE (MTE) products have been developed that contain Mn doses in line with the 2012 American Society for Parenteral and Enteral Nutrition (ASPEN) recommendations on TE provision in PN. This article begins by covering the physiology of Mn and then provides an overview of Mn toxicity and deficiency, focusing on the findings of recent studies. The implications of these findings for patients treated with PN are considered. Finally, the article considers practical aspects of the provision of Mn in PN.

Physiology

Mn is the 12th-most abundant element in the earth’s crust, ubiquitously present in food and water supply.2 It exists in 11 oxidation states, of which Mn2+ and Mn3+ are the commonest in biological systems. The total Mn content of the human body is about 15 mg, the highest concentrations being in liver, kidney, and bone.3 Most of the Mn in tissues is located in mitochondria.4 Mn metalloenzymes participate in the metabolism of amino acids, carbohydrates, and lipids. They include arginase, glutamine synthase, mitochondrial Mn superoxide dismutase (MnSOD), and 3-hydroxy-3-methylglutaryl-coenzyme A (HMG CoA) reductase, the rate-limiting enzyme in cholesterol biosynthesis. Mn is required for the activity of glycosyltransferases needed for formation of connective tissue. It is further required for synthesis and secretion of insulin,5 and it upregulates insulin-like growth factor I.6 As such, Mn is necessary for anabolism. It is essential for normal development and maintenance of bone.7

Under normal circumstances, exposure to Mn is mainly from food. Mn is naturally abundant in grains, nuts, fruits, and...
vegetables. Nuts, for example, contain up to 46 mg/kg.8 Tea is a rich source of Mn, containing 0.4–1.3 mg in an average cup. The Mn content of milk varies widely, ranging from 3.5–15 μg/L in human milk9 to approximately 300 μg/L in commercial infant formulas.9 Mn intake from water is usually considerably lower than that from food. The Mn concentration of drinking water varies widely among sources, but at the median Mn concentration of 10 μg/L, daily intake is likely to be about 20 μg. The U.S. Environmental Protection Agency has recommended that Mn concentrations in drinking water not exceed 50 μg/L and has stated a lifetime health advisory value of 300 μg/L.10 Small amounts of Mn are inhaled, with the ambient concentration in urban air being about 40 ng/m³.10 Mn reaches air as a result of industrial activities, including steel production and its use in the gasoline additive methylcyclopentadienyl Mn tricarbonyl.

Dietary Mn is absorbed in the proximal small bowel by the divalent metal transporter 1, also known as SLC11A2. The proportion of dietary Mn absorbed is small, reflecting low requirements. Only about 3% is absorbed from a typical diet containing 3–4 mg/d.11 After reaching the liver in portal blood, it is exported to tissues. About 60%–80% of Mn in blood is located inside red blood cells (RBCs).12 In blood plasma, Mn is bound to albumin (84%), hydrated (6.4%), or complexed to bicarbonate and citrate ions.13,14 Before it can enter tissues, it is oxidized to Mn³⁺ by ceruloplasmin, enabling it to bind to transferrin. Transferrin binds to the transferrin receptor, which is endocytosed. Transport of Mn into cells occurs via divalent metal transporter 1 and ZIP8, an electroneutral influx symporter. Mn efflux from cells is mediated by 3 transporters—namely, SLC30A10, ferroportin, and the P-type ATPase secretory pathway Ca-ATPase 1 (SPCA1).14,15 SLC30A10 is a solute carrier abundant in liver, and it appears to protect cells against excessive accumulation of Mn. The roles of the other 2 transporters in maintaining Mn balance are less clear. Excretion of Mn from the body occurs largely in bile (90%),16 with the remainder being excreted in urine. Homeostasis of Mn is achieved mainly by regulation of biliary excretion. Normal hepatic function is therefore vital for Mn homeostasis. Intestinal absorption of Mn is subject to regulation, the percentage absorbed being inversely associated with the amount ingested. Mn absorption is influenced by iron (Fe) status, increasing when Fe stores are low and decreasing when dietary Fe is high.17,18 Fe shares the same transport mechanisms as Mn and conversion back to the neurotransmitter glutamate.19 Uptake of glutamate by astrocytes depends on glutamine synthase activity. This activity prevents extracellular glutamate concentrations from climbing to toxic levels. In experimental systems lacking glutamine synthase, the ability of astrocytes to protect neurons from excitotoxicity is impaired.20 MnSOD clears superoxide radicals generated by aerobic metabolism in mitochondria.21 It catalyzes the dismutation of superoxide anions to hydrogen peroxide and oxygen, thereby protecting neurons against oxidative stress.

Mn is transported into the brain mainly across the blood-brain barrier and choroid plexuses, but transport may occur via the olfactory nerve.13,14 Mn leaves the brain more slowly than it leaves other tissues.15 Kinetic studies suggest that this occurs by diffusion rather than being carrier mediated.22 Regulation of Mn influx and efflux is poorly understood, but studies of animals and cultured cells have investigated possible mechanisms. Briefly, Park9, also called ATP13A2, is believed to shuttle cations across lysosomal membranes and to protect against Mn accumulation when its expression is increased.23 SPCA1 is believed to be an important regulator of Mn in brain. It is thought that, when cytosolic Mn concentrations are excessive, Mn is pumped into the Golgi lumen through SPCA1 and subsequently excreted from cells. Expression of SPCA1 increases in brain mitochondria of animals chronically exposed to Mn, and loss of function of SPCA1 increases sensitivity to Mn toxicity.24 The metal-binding protein metallothionein may have a role in detoxification of Mn. Mn exposure decreases mRNA for metallothionein in cultured astrocytes and induces metallothionein in mouse liver in the presence of interleukin 6.25,26 This subject has been discussed in detail elsewhere.19

Assessment of Mn Status

The method most commonly used to assess Mn status in patients is measurement of the whole blood Mn concentration. As a test of Mn status in patients treated with PN, it is considered superior to serum or plasma Mn.29–32 Whole blood Mn concentrations are affected by age, sex, and pregnancy. In neonates, concentrations are threefold higher than in adults.33 Concentrations are higher in women after puberty, possibly reflecting increased Mn absorption in response to lower Fe status.34 Concentrations increase in late pregnancy.35 For clinical purposes, Mn is usually measured either by atomic absorption spectroscopy or by inductively coupled plasma-atomic emission spectrometry.36

As a test of Mn status, whole blood Mn suffers from 2 notable limitations—namely, that biological variation is wide and measured concentrations may be artifactually high.37 Artificial results may occur because Mn, owing to its ubiquitous nature, tends to contaminate glassware, solutions, and specimen tubes. Mn concentrations cannot be reliably interpreted unless glassware has been acid-washed to minimize contamination.16 Researchers have considered whether the confounding effect of
Mn Toxicity

Mn-Induced Neurotoxicity

The earliest description of neurotoxic effects resulting from Mn toxicity was by Couper in 1837 among patients who had inhaled Mn oxide dust during the manufacture of bleach. Since 1967 it has been recognized that welders and miners are at risk of Mn toxicity. Workers in these occupations may be exposed to excessively high airborne Mn. In welding workshops, for example, air Mn concentrations of 0.2 mg/m³ have been reported, considerably higher than in ambient urban air. While the amount of Mn inhaled by these workers is low versus oral intake, inhaled Mn predisposes to neurotoxicity because it reaches the brain directly via the olfactory nerve.

When Mn is present in excess, it accumulates in brain regions responsible for control of motor and nonmotor functions—namely, the globus pallidus, subthalamic nucleus, and substantia nigra. The globus pallidus appears to be particularly susceptible because there are abundant dopamine receptors that bind Mn. Mn accumulation initially occurs asymptomatically. The patient later develops a parkinsonian-like syndrome consisting of tremor, hypertonia, bradykinesia, and gait disturbance, but nonspecific symptoms have been reported, including visual disturbance, headache, anxiety, memory loss, and seizures. The features are described as “parkinsonian-like” because they mimic, but are not identical to, those of Parkinson’s disease (PD). Tremor is less frequent and hypertonia more frequent than in PD. Furthermore, PD tends to affect the striatum, and Lewy bodies (the histologic hallmark of PD) are absent in Mn toxicity. Mn accumulation in brain can be assessed through T1-weighted magnetic resonance imaging (MRI) of the globus pallidus. A pallidal index is calculated by dividing the signal from the globus pallidus by the signal from frontal cortex white matter and multiplying by 100. In the context of occupational Mn exposure, the pallidal index is specific for Mn exposure during and even before clinical neurotoxicity develops. The pallidal signal disappears some months after cessation of exposure, irrespective of whether the Mn exposure is airborne or IV.

The mechanism whereby Mn causes neurotoxicity is unknown, but the hypothesis best supported by evidence is that it results in oxidative stress–induced damage to the dopaminergic system. Reactive oxygen species may be generated as a result of the pro-oxidant effect of Mn when present at high concentrations and by direct inhibition of cellular respiration, leading to progressive neuronal degeneration. There is further support for this hypothesis from observations that the antioxidant N-acetylcysteine protects cells against toxicity and from evidence for oxidative damage in welders exposed to Mn occupationally. In studies of animals, Mn intoxication paradoxically decreases glutamine synthase activity in the basal ganglia. Mn exposure decreases glutamate uptake by astrocytes. These effects would be expected to impair the ability of astrocytes to protect neurons from the toxic effects of excessive extracellular glutamate.

Mn-induced neurotoxicity and PN. Mn toxicity is a well-recognized complication of PN. It was first reported in 1990 in a 32-year-old woman with cholestatic jaundice who presented with parkinsonian-like symptoms and hypermanganesemia after receiving Mn doses of 300 μg/d for 4 months. The symptoms and hypermanganesemia resolved after supplemental Mn was withheld. Patients were later described in whom cerebral Mn accumulation was detected by MRI of the basal ganglia. Mn accumulates in liver, but it remains unclear whether this is directly hepatotoxic.

Mn is a ubiquitous contaminant of PN. This raises safety concerns, especially for patients on long-term PN (>1 month) in whom there is impaired biliary excretion. The Mn that contaminates PN originates from component solutions. Contamination is highest in solutions of calcium gluconate, magnesium sulfate, sodium chloride, and potassium chloride, but it is present in dextrose and amino acid solutions. In one study, for example, Mn concentrations were 280 μg/L in potassium phosphate and up to 225 μg/L in magnesium sulfate solutions, whereas amino acid solutions contained 5.2–17.0 μg/L and 50% dextrose contained 0.64–2.5 μg/L. Data on the total Mn contamination of a PN regimen are more useful to the clinician than data on individual components. In the aforementioned study, the total Mn dose delivered by contamination was 8.07–21.75 μg/d. Less than 3.3 μg of this originated from potassium phosphate and magnesium sulfate because of the
small volumes of these solutions used. Elsewhere, estimates of total Mn doses delivered by contamination of PN regimens range from 3–38 μg/d.16,66,67

Hypermanganesemia is common in patients treated with long-term PN. At least 50% of these patients, receiving Mn provision in accordance with the 1979 recommendations,58 develop hypermanganesemia, but neurotoxicity is relatively uncommon.31,32,47 In 1 study, 11 patients treated with long-term PN all had whole blood Mn concentrations, twice those of controls, as well as positive MRI results, but only 1 patient had parkinsonian-like symptoms.69 In a study that investigated 16 patients on long-term PN (receiving Mn doses of 400 μg/d), 8 had hypermanganesemia, and 13 had positive MRI results, of whom only 2 had parkinsonian-like symptoms.70 Whole blood Mn correlates with the dose of Mn and with cerebral accumulation as measured by MRI of the basal ganglia,66,71 but it does not reliably predict the development of toxicity in individuals. Serum Mn correlates poorly with accumulation, tending to remain within the reference range despite increased intracerebral Mn concentrations.7 It reflects IV delivery rather than Mn accumulation. It has recently been suggested that RBC Mn is preferable to whole blood Mn as a test of Mn accumulation.37 This is to be expected because whole blood is an inhomogeneous mixture of RBCs and plasma, the Mn content of the latter reflecting IV delivery of Mn, whereas RBCs are homogeneous. One study in which supplemental Mn was withheld from patients on long-term PN reported that RBC Mn correlated well with disappearance of cerebral Mn accumulation.39 While RBC Mn has potential as a test of Mn accumulation, there is currently limited information available on its utility, and its measurement is not widely available in clinical laboratories.

The features of PN-associated Mn neurotoxicity closely resemble those of neurotoxicity resulting from occupational Mn exposure.14 The symptoms and brain regions affected are the same, and the route whereby Mn enters the bloodstream bypasses the liver.7 Another factor common to both conditions is that it is important to detect Mn accumulation at an early stage, if symptoms are to be avoided. There is more information available on occupational Mn toxicity given that it was not widely available in clinical laboratories.

Risk factors for Mn neurotoxicity. It is difficult to predict whether patients will develop toxicity when exposed to Mn because susceptibility varies widely among individuals. However, numerous risk factors have been identified. While not all of these have been demonstrated in studies of PN, all are worthy of mention because they potentially apply to patients treated with PN.

Excessive provision. Toxicity may result from the use of MTE products containing too much Mn. Most reports of toxicity have been in adults receiving Mn doses >500 μg/d or in children receiving doses >40 μg/kg/d.16 However, critically ill patients may be susceptible to neurotoxicity at lower doses. A recent report described a 62 year old man, chronically critically ill with acute pancreatitis, who developed parkinsonian-like symptoms after 10 weeks of PN containing Mn doses of 270 μg/d.74 Most case reports of Mn toxicity in PN have been for patients treated for >3 months, suggesting that the cumulative dose is important.47 Neurotoxicity has been reported in the context of short-term PN. A 22-year-old woman with acute pancreatitis developed Mn encephalopathy after only 2 weeks of PN.48 The patient had hypermanganesemia and Mn accumulation in brain. She recovered, albeit with some residual visual impairment, after supplemental Mn was withheld. Mn toxicity presenting with parkinsonian-like symptoms has recently been reported among individuals abusing IVephedrine.72 This may contain Mn concentrations up to 0.6 g/L, originating from potassium permanganate. The doses of Mn delivered may be 60–180 mg/d, which greatly exceed those in PN. Given that Mn efflux from the brain is slow, the risk of toxicity would be expected to increase as a result of recent exposure to Mn, irrespective of the source.

Liver disease. When biliary excretion is impaired, Mn tends to accumulate in both liver and brain. Patients with cholestatic liver disease, including PN-associated liver disease (PNALD), should therefore be considered at risk of developing neurotoxicity.69 Mn toxicity can occur in patients with chronic liver disease who have not received PN.75 The presence of collateral vessels, such as esophageal varices, enables Mn absorbed in the gastrointestinal tract to reach the systemic circulation directly, resulting in hypermanganesemia.76 Cerebral accumulation of Mn in patients with liver disease can be reversed by liver transplant.5,77,78 Mn that accumulates in the liver may be directly hepatotoxic. This is considered in a later section.

Age. Infants are more susceptible than adults to developing hypermanganesemia and accumulation of Mn because immaturity of regulatory mechanisms results in greater absorption and retention of Mn. Mn uptake into the brain is greater because neuronal barriers are more permeable.47 This can lead to permanent brain damage.80 In the infant population, hypermanganesemia is associated with poorer neurodevelopment.81

Fe deficiency. Fe deficiency in children is associated with hypermanganesemia,82,83 accumulation of Mn in brain,84 and neurotoxicity.85 These observations are of concern given the high prevalence of Fe deficiency worldwide. The mechanism whereby Fe deficiency predisposes to Mn toxicity has been studied in animals. There is increased intestinal absorption and cerebral uptake of Mn caused by increased activity of transport proteins divalent metal transporter 1, transferrin receptor, and
ZIP8, expressed in the gastrointestinal tract and brain.\textsuperscript{86-89} It is unknown whether Fe deficiency influences the risk of Mn toxicity in patients treated with PN.

\textit{Selenium deficiency.} Patients who are deficient in selenium (Se) may be more susceptible to Mn toxicity. Conversely, normal Se status may have a protective effect. A recent study of TE concentrations in cord blood suggested that Se protects infants against neurotoxicity resulting from prenatal Mn exposure.\textsuperscript{90} A cord blood Se concentration $\geq 63.1 $ μg/L appeared to block toxic effects of Mn on neurodevelopment. The mechanism of this effect is unclear, but Se could counteract the oxidative stress believed to contribute to Mn neurotoxicity. These findings suggest that it is advisable to maintain optimal Se status for infants at risk of Mn neurotoxicity. The authors recommended that Se supplementation should be considered for pregnant women living in regions of low natural Se. It is unknown whether Se deficiency influences the risk of Mn toxicity in patients treated with PN, but it should be noted that Se doses delivered in PN are commonly below the recommended amounts.\textsuperscript{91}

\textit{Genetic factors.} At least 3 lines of evidence suggest that genetic variation may in part explain the large interindividual differences in susceptibility to Mn neurotoxicity. First, single-nucleotide polymorphisms of genes participating in detoxification of Mn are associated with increased susceptibility to Mn neurotoxicity.\textsuperscript{92,93} These variants were considered likely to predispose to Mn toxicity irrespective of the route of exposure. Second, mutations affecting the parkin gene, which are implicated in juvenile-onset PD, predispose to Mn toxicity.\textsuperscript{94} Third, recessively inherited mutations of SLC30A10 cause familial Mn-induced parkinsonism.\textsuperscript{95} Patients with this disorder have severe inherited hypermanganesemia and develop parkinsonian-like symptoms. Milder defects of these genes potentially render individuals more susceptible to hypermanganesemia in the presence of increased intake. Conversely, genetic factors have been identified that may decrease susceptibility to Mn neurotoxicity. For example, a variant of the gene encoding the Fe-regulatory protein hepcidin is associated with blood Mn concentrations that are 10% lower than in the general population.\textsuperscript{96} Overexpression of parkin may protect against Mn-induced dopaminergic cell death.\textsuperscript{97} In addition, findings of studies in cellular systems have suggested that Mn has an epigenetic role in neurotoxicity.\textsuperscript{98} Mn exposure resulted in overexpression of α-synuclein, a protein leading to apoptosis, which is known to participate in the development of PD.\textsuperscript{99,100}

\textit{Management of Mn neurotoxicity.} The key therapeutic intervention for Mn intoxication is to remove the patient from exposure. Thereafter, symptoms usually resolve, and brain Mn concentrations decrease. In a review of cases of Mn toxicity in PN, Dickerson reported that resolution of symptoms may take from 3 days to many months.\textsuperscript{47} Symptoms may fail to resolve completely in some patients. Blood Mn concentrations decrease but, as observed in 1 study of children receiving PN, may take up to 9 months to return to normal.\textsuperscript{71} Signal intensity of the basal ganglia on MRI has been reported to take up to 5 months to return to normal after Mn was withheld from adults treated with PN.\textsuperscript{53} In this study, whole blood Mn concentrations decreased in parallel with MRI signal intensity.

If symptoms persist, other causes should be excluded. Other treatments may be considered, but there is relatively little information available on treatments other than cessation of exposure. Levodopa is unlikely to be effective.\textsuperscript{46} Similarly, the chelating agent ethylenediaminetetraacetic acid fails to improve symptoms despite increasing urinary Mn, probably because, being water soluble, it has limited access across the blood-brain barrier.\textsuperscript{13,14} However, another chelating agent, para-aminosalicylic acid, shows promise in the treatment of Mn neurotoxicity because its metabolite N-acetyl para-aminosalicylic acid reaches the brain.\textsuperscript{100} Patients may benefit from Fe supplementation. In a recent report of a patient with occupational Mn toxicity treated with chelation therapy, the symptoms improved only after supplementation of Fe.\textsuperscript{102} Fe may decrease Mn uptake into the brain by competing with it for transport.

\textbf{Mn-Induced Hepatotoxicity}

Mn may accumulate in the liver of patients treated with long-term PN. An autopsy study of patients on long-term PN, who had received Mn doses of 700 μg/d, found increased concentrations of Mn in liver, with the highest concentrations being in patients with liver disease.\textsuperscript{63} The consequences of this accumulation are unclear, but it is implicated in the development of PNALD. A large study compared infants treated with PN containing either high (50 μg/kg/d) or low (0.91 μg/kg/d) Mn doses.\textsuperscript{103} It found that serum direct bilirubin and whole blood Mn concentrations were significantly higher in the high Mn group. In addition, severe hyperbilirubinemia was more common in this group. Further evidence implicating Mn in PNALD is the finding that, in children with cholestasis and hypermanganesemia, serum bilirubin decreased significantly after Mn was withheld.\textsuperscript{49}

Studies of animals suggest that hepatic Mn accumulation is hepatotoxic. There is evidence, dating back to the 1970s, that infusion of Mn contributes to the development of cholestasis.\textsuperscript{104-107} More recently, administration of Mn followed by bilirubin led to a decrease in bile flow, thought to be caused by changes in permeability at the canalicular membrane.\textsuperscript{107} Mn increases the activity of HMG CoA reductase and decreases conversion of cholesterol to bile acids,\textsuperscript{106} both of which could lead to an increase in hepatic cholesterol concentrations. Excessive hepatic cholesterol could predispose to cholestasis, either by accumulating in the canalicular membrane or by increasing the formation of biliary sludge.\textsuperscript{109} In a study of animals exposed to excess Mn, there was inhibition of the hepatic enzymes MnSOD and glutathione peroxidase and a decrease in hepatic
concentrations of glutathione. There were also histopathologic changes in liver indicative of hepatocellular damage. Taken together, the findings suggested that excess hepatic Mn caused oxidative damage. The effect of hepatic Mn accumulation in humans merits further investigation. Such studies would be facilitated by the development of techniques capable of measuring hepatic Mn concentrations noninvasively.

New Biomarkers of Mn Exposure

Presymptomatic neurochemical changes that occur following Mn exposure have been quantitated by magnetic resonance spectroscopy. For example, GABA (γ-aminobutyric acid) concentrations doubled in the basal ganglia and thalamus of workers exposed to airborne Mn. Increased thalamic GABA is associated with decreased fine motor performance as assessed by the Purdue pegboard test. The Mn:Fe ratio in RBCs and plasma has been used as a marker of occupational Mn exposure. It correlates with airborne Mn concentrations and with neurobehavioral changes. The rationale for using this ratio is that, by combining the 2 measurements, the sensitivity of the test is increased. In vivo neutron activation analysis is a sensitive, noninvasive, and portable method, recently developed to quantify Mn in bone, as a test of past exposure. It can measure concentrations as low as 0.5 ppm. Bone Mn concentrations were significantly higher in occupationally exposed subjects than in nonexposed subjects. These biomarkers have not yet been studied in patients treated with PN.

Mn Deficiency

Relatively little is known about Mn deficiency in the context of PN, but information is available from other areas of investigation that is potentially applicable to PN. For the purpose of discussion, Mn deficiency can be considered as either severe, in which there are overt clinical features, or mild, in which the features are nonspecific or observed in epidemiologic studies. Mild deficiency of any micronutrient is more difficult to study because it may not be associated with measurable changes in the blood.

Severe Mn Deficiency

Severe deficiency of Mn is rare, probably because requirements are readily met by the amounts normally present in the diet, but it has occurred in individuals consuming diets artificially low in Mn. Subjects consuming a diet containing only 110 μg/d of Mn for 39 days developed a skin rash and hypocholesterolemia, which responded to Mn supplementation. Other symptoms of Mn deficiency described in humans are weight loss, decreased growth of nails and hair, and reddening of black hair. Skeletal abnormalities and permanent ataxia have been reported in experimental Mn deficiency in animals. Its metabolic effects include impaired antioxidant defense and decreased lipoprotein metabolism. In studies of rats, decreased serum insulin and insulin-like growth factor 1 concentrations have been reported.

Nondiary Mn deficiency has severe effects that overlap with those of dietary deficiency. A defect affecting ZIP8 was recently reported that caused urinary Mn wasting, hypomanganesemia, and impaired galactosyltransferase activity. The clinical features were short stature, intellectual impairment, developmental delay, and hypotonia. Recessively inherited defects of Mn metalloenzymes result in features comparable to those of dietary deficiency. For example, arginase deficiency results in impaired growth, intellectual impairment, and ataxia, and glutamine synthase deficiency causes seizures, developmental delay, and hypotonia. The same systems are potentially adversely affected by partial impairment of Mn metalloenzymes, resulting from insufficient Mn supply.

Mild Mn Deficiency

Mild or marginal micronutrient deficiency is a state in which overt features of deficiency are absent but which may nevertheless worsen the clinical outcome—for example, by predisposing to infection or by delaying wound healing. These clinical problems result from suboptimal metabolism, caused by impaired activity of enzymes. Mn metalloenzymes participate in a range of physiologic processes, all of which are potentially compromised by Mn deficiency. Evidence suggesting that Mn deficiency is associated with adverse outcomes is discussed here.

Epidemiologic studies have suggested that dietary deficiency of Mn, sufficient to cause hypomanganesemia, may adversely affect cognitive function and growth. A study of 12-month-old infants found an inverted U-shaped association between whole blood Mn concentration and mental development score, with the lowest scores being in the lowest and highest Mn quintiles. This finding was consistent with Mn acting as both an essential nutrient and a toxicant. The mechanism whereby Mn deficiency affects neurodevelopment is unknown, but oxidative injury could result from decreased activity of MnSOD. In another study, decreased maternal whole blood Mn was associated with intrauterine growth retardation and, and, more recently, studies have shown an inverted U-shaped association between maternal whole blood Mn and birth weight. In adults, low and high blood Mn are both associated with decreased cognitive function. In these study populations, the whole blood Mn concentration appears to be sufficiently sensitive to detect clinically significant Mn deficiency.

Various observations have suggested that deficiency of Mn contributes to diseases affecting growth and cerebral function. Mn deficiency at birth is implicated in Perthe’s disease, a condition associated with avascular necrosis of the head of the femur and disordered growth. Children with this condition have lower blood Mn concentrations. Mn deficiency is implicated in increased susceptibility to seizures in patients with epilepsy. Whole blood Mn was significantly
lower in hospitalized patients with epilepsy than in control subjects.\textsuperscript{135} This association could reflect the requirement of the nervous system for Mn metalloenzymes. Another study observed that whole blood Mn concentrations were significantly lower in patients with type 2 diabetes than in control subjects.\textsuperscript{136} The consequences of this are unknown, but it could impair insulin secretion. In addition, by increasing oxidative stress and endothelial dysfunction, it is implicated in the development of atherosclerosis.\textsuperscript{137} Dietary Mn deficiency in animal models has been observed to decrease arginase activity in liver and aorta, thereby increasing synthesis of nitric oxide.\textsuperscript{138,139} A similar effect occurring in the brain would be expected to compromise neuroprotection.

**Mn Deficiency During PN**

The only reported case of severe Mn deficiency in a patient treated with PN is of a child with short bowel syndrome who developed osteoporosis and weight loss after receiving PN unsupplemented with Mn.\textsuperscript{140} The osteoporosis was probably caused by lack of bone matrix resulting from deficient glycosyltransferase activity. Severe Mn deficiency has not been reported in adults treated with PN. Mn deficiency, unlike Cu deficiency, does not appear to occur when supplementation is withheld from PN, possibly because requirements are usually met by the amount of Mn present as a contaminant of PN.

In practice, however, patients from whom supplemental Mn is withheld usually have cholestasis that, by decreasing Mn excretion, might be expected to protect against deficiency. The risk of Mn deficiency could be higher in noncholestatic patients treated with PN from which Mn is withheld. One study investigated such patients who received Mn doses of only 3–6 μg/d from contamination of the PN.\textsuperscript{66} While whole blood Mn concentrations were significantly lower in these patients than in patients receiving doses of 55 μg/d, none of the unsupplemented patients developed overt features of Mn deficiency during the 13–16 months of the study. However, this small study was not specifically designed to investigate hypomanganeseemia. In addition, neurophysiologic tests and tests of cognitive function were not carried out. Another study of patients on long-term PN reported blood Mn concentrations significantly below the reference range 1 year after supplemental Mn had been withheld.\textsuperscript{90} Significantly decreased blood Mn concentrations were observed in a study of patients undergoing cisplatin chemotherapy for esophageal cancer who received 28 days of PN unsupplemented with Mn.\textsuperscript{141} Although no adverse effects of hypomanganeseemia were reported in these studies, this does not necessarily mean that it is an unimportant finding. For example, it is unknown whether hypomanganeseemia would be tolerated well over longer periods than in these studies or among patients receiving lower background doses of Mn from contamination of PN. While the significance of hypomanganeseemia during PN is uncertain, it appears that it could impair neurodevelopment and weight gain in infants and impair cognitive function in adults. Until more information is available on its effects, hypomanganeseemia should be considered potentially harmful.

**Practical Considerations**

**Mn Contamination of PN**

Per a study by Pluhator-Murton et al, the amount of Mn contaminating PN varies significantly among manufacturers and among lots from the same manufacturer.\textsuperscript{67} The former is likely accounted for by differences in methods among manufacturers. Another study by the same authors reported that the concentration of Mn delivered to the patient depends on the temperature and duration of storage of the bag.\textsuperscript{142} Mn concentrations in bags stored at 20°C decreased to a trough, 88% of the starting concentration, at 36 hours, whereas concentrations decreased to the same trough over a period of 30 days in bags stored at 4°C. The decrease in concentration could have been caused either by precipitation of Mn or by its adsorption to the bag. These findings suggest that PN regimens with short storage and short infusion times deliver slightly more Mn to the patient.

In 1984, it was recommended that levels of contamination be known before supplementing Mn.\textsuperscript{68} Although Mn contamination of PN has been extensively measured since then, there is a need for studies on currently available regimens, especially pediatric ones. ASPEN has recommended that such research be carried out and that total Mn contamination not exceed 40 μg/d in a typical adult PN regimen.\textsuperscript{91} It has been suggested that manufacturers of PN products state the maximum possible Mn contamination on product labels.\textsuperscript{16} It will be difficult for manufacturers to comply with these recommendations because it will require them to consider the Mn content of numerous component solutions.

**Mn Provision in PN**

Between 1979–2014, the standard doses of Mn recommended in adults treated with PN decreased 80-fold, from 800 μg/d\textsuperscript{68} to 10–50 μg/d,\textsuperscript{141} following reports of Mn toxicity and research findings suggesting that requirements were lower than previously thought (Table 1). However, MTE products available in the United States still contain Mn doses in line with the 1979 American Medical Association recommendations.\textsuperscript{68} The current recommendation for adults is based on the observation that, in patients receiving 55 μg/d, the whole blood Mn concentration remained stable and within the reference range.\textsuperscript{66} MRI signal intensity of the globus pallidus remained stable but increased in patients receiving doses of 110 μg/d, suggesting that doses >55 μg/d can lead to accumulation. It is likely that the average parenteral Mn requirement in adults is <55 μg/d and that the standard recommendation will be decreased following further research. However, doses of 55 μg/d appear to be safe, as long as there are no risk factors for toxicity or
significant intake of Mn from other sources. Authors have questioned whether patients treated with long-term PN should be routinely supplemented with Mn. A recent systematic review concluded that there was limited evidence to support the practice of withholding supplemental Mn for all patients receiving long-term PN. The authors suggested that further studies were required to assess the safety of this approach.

**Individualization of Mn provision.** Mn doses, in common with those of other TEs, should ideally be tailored to patients’ requirements. While this may not always be feasible, prescribers of PN should certainly be alert to situations in which patients’ individual requirements may be below the standard recommendations.

**Additional intake.** Before deciding on supplementation of PN, clinicians should consider other sources of Mn intake. These include IV infusions, such as electrolytes or blood products, given separately from PN. It is difficult to account for the amounts delivered in this way because there is limited information available on the Mn content of these infusions. Nevertheless, the amounts may be considerable when large volumes are infused. For example, calcium gluconate is given in large volumes to PN-treated preterm infants to prevent osteopenia of prematurity. There may be additional intake via the oral or enteral routes. Clinicians should be aware that oral Mn supplements are available that deliver doses of 2–4 mg/d.

The value of such supplements is questionable given that Mn is readily available in most diets and that excessive intake can be harmful. Mn concentrations in drinking water in the United States commonly exceed 300 μg/L. Excessive consumption of tea resulted in hypermanganesemia in an enterally fed patient. Previous IV ephedrine abuse should be considered as a possible source of intake.

**Cholestasis.** Mn provision should be decreased or withheld in patients with significant cholestasis, irrespective of the duration of PN. For neonates, it has been recommended that Mn be withheld if the serum bilirubin concentration exceeds 2 mg/dL. For adults, Mn should be withheld if aminotransferases and alkaline phosphatase are more than twice normal levels. However, caution is required when assessing the severity of cholestasis because liver function tests have limited sensitivity. The finding that liver function tests are within normal limits does not rule out the possibility of liver disease sufficiently severe to cause Mn accumulation. If there is doubt about the severity of cholestasis, withholding supplemental Mn is unlikely to be harmful. The MTE product should be withheld, and the other TEs, normally provided by the product (Cu, Se, and Zn), should be given as individual TE products, either in the PN or as a separate infusion.

**Critical illness.** Limited data are available to guide Mn provision for the critically ill, but requirements are likely to vary. The TEs most likely to be deficient in this patient group are Zn, Cu, and Se.

### Table 1. Recommendations on Parenteral Mn Supplementation.

<table>
<thead>
<tr>
<th>Specific Group</th>
<th>Source</th>
<th>Year</th>
<th>Mn Dose</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adults</td>
<td>NAG-AMA</td>
<td>1979</td>
<td>150-800</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>AMA</td>
<td>1984</td>
<td>400-800</td>
<td>144</td>
</tr>
<tr>
<td></td>
<td>AMA</td>
<td>1995</td>
<td>10-50</td>
<td>143</td>
</tr>
<tr>
<td></td>
<td>ASPEN</td>
<td>2004</td>
<td>60-100</td>
<td>145</td>
</tr>
<tr>
<td></td>
<td>ESPEN</td>
<td>2009</td>
<td>200-300</td>
<td>146</td>
</tr>
<tr>
<td></td>
<td>ASPEN</td>
<td>2012</td>
<td>55</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>AuSPEN</td>
<td>2014</td>
<td>55</td>
<td>147</td>
</tr>
<tr>
<td>Children</td>
<td>NAG-AMA</td>
<td>1979</td>
<td>2-10</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>ASCN</td>
<td>1988</td>
<td>1</td>
<td>148</td>
</tr>
<tr>
<td></td>
<td>ASCN</td>
<td>1988</td>
<td>1</td>
<td>148</td>
</tr>
<tr>
<td>Children</td>
<td>ASCN</td>
<td>1988</td>
<td>1</td>
<td>148</td>
</tr>
<tr>
<td>Preterm neonates (&lt;3 kg)</td>
<td>ASPEN</td>
<td>2004</td>
<td>1</td>
<td>145</td>
</tr>
<tr>
<td>Term neonates (3–10 kg)</td>
<td>ASPEN</td>
<td>2004</td>
<td>1</td>
<td>145</td>
</tr>
<tr>
<td>Children (10–40 kg)</td>
<td>ASPEN</td>
<td>2004</td>
<td>1</td>
<td>145</td>
</tr>
<tr>
<td>Adolescents (&gt;40 kg)</td>
<td>ASPEN</td>
<td>2004</td>
<td>40–100c</td>
<td>145</td>
</tr>
</tbody>
</table>

AMA, American Medical Association; ASCN, American Society for Clinical Nutrition; ASPEN, American Society for Parenteral and Enteral Nutrition; AuSPEN, Australian Society for Parenteral and Enteral Nutrition; ESPEN, European Society for Clinical Nutrition and Metabolism; Mn, manganese; NAG-AMA, Nutrition Advisory Group of the American Medical Association.

*a*If applicable.

*b*For adults, μg/d; for children, μg/kg/d (unless noted otherwise).

*c*Value presented as μg/d.
decrease. In 2009 the European Society for Clinical Nutrition and Metabolism recognized that Mn requirements of critically ill patients were below the amount provided by standard MTE products available at the time (200–500 μg/d).154 In a study of trauma patients receiving hemofiltration, the amount of Mn delivered in PN (300 μg/d) exceeded Mn losses, suggesting that there was accumulation.155 This study reported that Mn was present as a contaminant of dialysate solutions. The authors concluded that Mn doses of 60–100 μg/d may exceed the requirements of patients on hemofiltration. Another reason to decrease Mn supplementation in critically ill patients is the additional intake from electrolyte infusions and blood products. In summary, it is advisable to decrease Mn provision in critically ill patients, especially those treated with hemofiltration. Mn supplementation should not exceed 55 μg/d.

Hypermanganesemia. Patients with hypermanganesemia should be considered at risk of developing toxicity and their Mn provision reviewed. ASPEN recommends decreasing or withholding Mn provision in patients with hypermanganesemia.91 If it is uncertain whether the hypermanganesemia is genuine or artifactual, the safest approach is to withhold supplementation. Withholding Mn in the absence of accumulation is unlikely to be harmful, whereas continuing provision in the presence of genuine hypermanganesemia risks toxicity.

Hypomanganesemia. Hypomanganesemia is an unusual metabolic complication in patients treated with PN. It is unlikely to occur in patients whose PN is supplemented with the MTE products currently available, but, as discussed, it can occur when supplemental Mn is withheld.66 Because there is limited information available, it is uncertain what action should be taken, but clinicians should be alert to possible features of Mn deficiency. It is unknown whether these features would resemble those observed in subjects with artificially induced Mn deficiency.48 If hypomanganesemia is persistent or accompanied by features consistent with deficiency, the clinician may consider restarting Mn provision. This should be done cautiously, either by restarting the MTE product—adjusting the doses of any single TE product (Cu, Se, Zn) as necessary—or by delivering Mn as an individual TE product. The Mn dose should not exceed 55 μg/d, and whole blood Mn should be measured 1 month after restarting supplementation.53 Overt features of Mn deficiency might be expected respond promptly to supplementation, given that the skin rash observed in studies of artificially induced Mn deficiency settled within a few days.137 Again, however, it is unknown whether this observation is applicable to patients treated with PN.

MTE products. In practice, decisions on Mn supplementation are constrained, first by the formulation of the MTE product available and second by the availability or otherwise of individual TE products. In 2012 ASPEN reviewed the MTE products available in the United States and Europe.91 At the time, the products for use in adults delivered Mn doses of 100–800 μg (United States) and 200–550 μg/d (Europe). Pediatric products delivered doses of 2–10 μg/kg/d. It was concluded that adult and pediatric products both provided excessive amounts of Mn. Observational studies of patients treated with long-term PN have found evidence of potentially toxic accumulation of Mn resulting from these doses.70,156 The ASPEN review recommended that Mn doses in MTE products for use in adults should be decreased to 55 μg/d to match the recommendation on provision and that pediatric and neonatal products should be reformulated to deliver doses of 1 μg/kg/d. These recommendations were reinforced by a recent call to action to bring safer MTE products to the U.S. market.157 The publication listed the evidence for actual or potential Mn toxicity resulting from the currently available products. It was hoped that the ASPEN recommendations would encourage commercial companies to develop new MTE products.

In addition to delivering excessive doses of Mn, the currently available MTE products do not facilitate individualization of dosing in patients whose requirements are below the standard recommendation. In this situation, the MTE product can be withheld, and the other TEs can be provided via individual TE products at doses that meet patients’ individual requirements for these TEs. However, this approach is not without problems. In countries where individual TE products are unavailable—for example, because of product shortages—the options available to the prescriber are either to provide the MTE product, thereby risking Mn toxicity, or to decrease or withhold the product, risking deficiency of Cu, Se, or Zn. The approach is costly, increases the risk of errors, and islogistically more complex when PN is compounded off-site. There may be a case for developing an Mn-free MTE product to facilitate provision of TEs other than Mn for patients in whom Mn is withheld. Ideally, a range of MTE products would be available, containing TE doses appropriate for different, commonly occurring situations.

Since 2012, 2 new MTE products have been developed for use in adults.157 Both provide Mn doses of 55 μg/d, in line with the ASPEN recommendations. These are a welcome development for patients and prescribers in Europe, but they are not yet available for use in the United States, because they contain Fe, fluoride, iodide, and molybdenum, not contained in the standard products available in the United States. However, it is likely that the currently available U.S. products will be reformulated to bring their Mn content into line with the recommendations. Clinicians intending to prescribe a new MTE product should ensure that it is compatible with the PN regimen of choice.

Monitoring of Mn Status During PN

Given that Mn accumulation is detectable presymptomatically, it should be possible to prevent toxicity developing. When Mn is withheld from patients with accumulation, the outcome appears to be good in that both hypermanganesemia and
cerebral Mn accumulation improve and the patient remains asymptomatic.\textsuperscript{71} Mn status should therefore be monitored in all patients treated with long-term PN. Clinical monitoring should include regular neurologic examination, with the clinician being alert to both parkinsonian-like and nonspecific symptoms of toxicity.\textsuperscript{47,49} Mn provision should be withheld if toxicity is suspected.\textsuperscript{91} Neurophysiologic tests, such as tests of fine motor coordination, are not routinely carried out but could be used as an adjunct to clinical examination.\textsuperscript{37,114} Biochemical monitoring consists of measuring whole blood Mn and liver function tests. Recommendations on the frequency of Mn measurement are based on expert opinion (Table 2). Essentially, measurements should be more frequent for patients at increased risk of Mn accumulation. In an effort to avoid artifactual results, specimen tubes should be approved by the local TE laboratory. If artifactual hypermanganesemia is suspected, clinicians should consider rechecking the whole blood Mn concentration both on a specimen collected into a standard tube and on a second specimen collected simultaneously into a TE-free tube. It is advisable to monitor Fe status and, if necessary, correct deficiency.

Currently, there is no consensus on the utility of cerebral MRI in monitoring patients treated with PN. Authors have recommended MRI for monitoring Mn accumulation in patients who have liver dysfunction or are receiving long-term PN.\textsuperscript{16,162} Others have stated that MRI may be appropriate if toxicity is suspected.\textsuperscript{149,160} In my opinion, MRI is appropriate in patients who have neurologic symptoms, the cause of which is uncertain or which fail to resolve after Mn is withheld. In this situation, MRI may have utility in distinguishing Mn toxicity from other causes. Whether MRI has a place in routine monitoring of asymptomatic patients is more doubtful. It is too costly and impractical. Moreover, the results would be unlikely to influence clinical management because, in patients with hypermanganesemia, Mn is likely to be withheld irrespective of the MRI findings. While increased signal intensity in a normomanganesemic patient could be used as a basis for withholding Mn provision, this situation would be unusual because signal intensity and whole blood Mn correlate strongly.\textsuperscript{53,66} Whole blood Mn is the preferred test and should be considered the mainstay of monitoring. A practical point to note is that use of MRI is precluded when ferrous equipment is being used and for patients who are bedbound.\textsuperscript{74}

### Table 2. Recommendations on Frequency of Monitoring of Whole Blood Mn.

<table>
<thead>
<tr>
<th>Patient Category</th>
<th>Frequency, mo</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stable adults receiving 55 µg/d of Mn</td>
<td>12</td>
<td>147</td>
</tr>
<tr>
<td>Patients in whom Mn was withheld from PN</td>
<td>6–12</td>
<td>63</td>
</tr>
<tr>
<td>Patients receiving high doses of Mn</td>
<td>3–6</td>
<td>147, 158, 159</td>
</tr>
<tr>
<td>Infants receiving prolonged PN</td>
<td>3</td>
<td>149, 160</td>
</tr>
<tr>
<td>Patients with cholestasis (total bilirubin &gt;3.5 mg/dL)</td>
<td>1</td>
<td>158, 161</td>
</tr>
<tr>
<td>Chronically critically ill patients receiving &gt;55 µg/d of Mn</td>
<td>1</td>
<td>48, 74</td>
</tr>
</tbody>
</table>

Mn, manganese; PN, parenteral nutrition.

### Mn and Long-Term PN

It is worth emphasizing some points about the management of patients who are receiving long-term PN because these patients, whether at home or critically ill in the hospital, are at increased risk of developing Mn toxicity. Clinicians should be aware of factors predisposing to Mn toxicity, especially high doses of Mn. Mn intake in fluids, drugs, nutritional supplements, and PN should be assessed. At present, there is limited information available on Mn doses delivered in fluids and drugs, but it is likely that, in the future, pharmaceutical companies will make more information of this sort routinely available. It is important to be alert to the possibility of cholestasis and PNALD and to remember that when standard liver function tests are within reference limits, this does not rule out susceptibility to toxicity. Whole blood Mn should be monitored at least annually among stable patients and more frequently if there are risk factors for toxicity. If a patient has symptoms consistent with Mn toxicity, blood Mn should be measured promptly, MRI considered, and supplemental Mn withheld. In this event, I recommend monitoring blood Mn monthly thereafter until it returns to normal. Other risk factors for Mn toxicity, including Se and Fe deficiency, should be treated. A specialist neurologic opinion should be sought and other causes of parkinsonian-like symptoms, such as PD or drugs, ruled out.

Mn deficiency could occur if supplemental Mn has been withheld long-term, although the risk thereof would appear to be low. While hypomanganesemia has been observed when supplemental Mn has been withheld, it is uncertain whether this indicates that the patient is at increased risk of developing features of deficiency. However, clinicians should monitor patients for overt deficiency, keeping in mind that this would not necessarily present with features, such as hair discoloration, as described in the studies of artificially induced Mn deficiency, but could conceivably present with nonspecific features, such as cognitive dysfunction, poor wound healing, or susceptibility to infection. In addition, whole blood Mn should be monitored at least annually among patients with hypomanganesemia. The finding of normomanganesemia in a patient with features consistent with Mn deficiency should suggest the possibility of an artifactual increase resulting from specimen contamination. In this situation, it would be appropriate to repeat the measurement on a blood specimen collected into a
TE-free tube. Current evidence does not support routinely withholding Mn long-term for patients who do not have risk factors for toxicity.

**Future Directions**

There is a need for new tests, such as genetic tests, to be developed to help identify patients at risk of Mn toxicity. It is likely that advances will be made in imaging techniques, enabling cerebral and hepatic Mn accumulation to be detected presymptomatically. Neurophysiologic tests show promise as a means of detecting neurotoxic features at an early stage. RBC Mn merits further investigation as a test of Mn accumulation.

Pharmaceutical companies should consider developing Mn-free MTE products to simplify TE prescribing in patients with cholestasis or hypermanganesemia. Prescribers would welcome information on Mn contamination of PN regimens and IV fluids given in large volumes to critically ill patients. In time, purification methods may be developed, which will decrease or even eliminate Mn contamination. It is hoped that new MTE products, which provide Mn in line with the 2012 ASPEN recommendations, will soon be widely used in clinical practice. This would be expected to decrease the incidence of Mn toxicity. In this event, it would be appropriate for recommendations on monitoring of Mn to be reviewed. Future research into Mn requirements may lead to a further decrease in recommended Mn provision, and as standard provision moves closer to average requirements, Mn deficiency may increase in incidence, ultimately becoming a commoner concern than toxicity. Future research should study hypomanganesemia and its consequences in patients treated with PN.

**Statement of Authorship**

C. Livingstone contributed to the conception/design of the research; contributed to the acquisition, analysis, or interpretation of the data; drafted the manuscript and critically revised it; agrees to be fully accountable for ensuring the integrity and accuracy of the work; and read and approved the final version.

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