ORIGINAL ARTICLE

Association of plasma nitrite levels with obesity and metabolic syndrome in the Old Order Amish

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Summary

Objectives

Plasma nitrite is a metabolite of nitric oxide and reflects endogenous nitric oxide synthase (NOS) activity. Although plasma nitrites were previously linked with obesity and metabolic syndrome (MetS), the direction of association remains inconsistent, possibly due to sample heterogeneity. In a relatively homogeneous population, we hypothesized that nitrite levels will be positively associated with overweight/obesity and MetS.

Methods

Fasting nitrite levels were measured in 116 Old Order Amish (78% women). We performed age-and-sex-adjusted ANCOVAs to compare nitrite levels between three groups (a) overweight/obese(−)MetS(−), (b) overweight/obese(+)MetS(−) and (c) overweight/obese(+)MetS(+). Multivariate linear regressions were conducted on nitrite associations with continuous metabolic variables, with successive adjustments for demographics, body mass index, C-reactive protein and neopterin.

Results

Nitrite levels were higher in the obese/overweight(+)MetS(+) group than in the other two groups ($p < 0.001$). Nitrites were positively associated with levels of triglycerides ($p < 0.0001$), total cholesterol ($p = 0.048$), high-density lipoprotein/cholesterol ratio ($p < 0.0001$) and fasting glucose ($p < 0.0001$), and negatively correlated with high-density lipoprotein-cholesterol ($p < 0.0001$). These associations were robust to adjustments for body mass index and inflammatory markers.

Conclusion

Further investigation of the connection between obesity/MetS and plasma nitrite levels may lead to novel dietary and pharmacological approaches that ultimately may contribute to reducing the increasing burden of obesity, MetS and cardiovascular morbidity and mortality.

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Introduction

Over the past two decades, metabolic syndrome (MetS) has increasingly featured as a central factor in the pathogenesis of atherosclerotic cardiovascular disease (1). The whole constellation of cardiovascular risk factors in MetS including dyslipidaemia, hypertension, diabetes mellitus Type 2 and its primary clinical outcome, i.e. atherosclerotic cardiovascular disease are major contributors to global disease burden, morbidity and mortality (2,3). As a result, there has been considerable interest in identifying risk factors, early clinical markers and pathogenic mechanisms related to MetS.

In recent years, dysfunctions in nitric oxide (NO˙) metabolic pathways have been associated with MetS (4,5). NO˙ plays an important role in endothelial functioning. It serves as a potent vasodilator in cardiovascular homeostasis, relaxation of gastric pyloric sphincter, fluid-electrolyte balance and penile erection. Endogenous NO synthesis through the L-arginine-NO pathway is mainly regulated by three isoforms of nitric oxide synthase (NOS): endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). Under physiological conditions, eNOS and nNOS are the primary regulators. Using murine models, Sansbury and colleagues showed that a high fat diet increased eNOS expression, which limited diet-induced obesity (6). However, other studies have shown that, as obesity sets in, eNOS expression and function decreases (7), which probably reflects ensuing endothelial dysfunction with increasing body mass index (BMI). Furthermore, obesity and MetS have also been linked to inflammatory cytokines and increased iNOS expression (8,9).

As NO˙ has a very short half-life of 1–2 ms (10,11), several studies have investigated associations of plasma NO˙ levels with MetS and individual cardiovascular risk factors using the oxidized NO˙ metabolites (NOx) nitrate (NO_3-) and nitrite (NO_2-) (12–14). Plasma NOx, in particular nitrite (15), is an indirect marker of endothelial NO synthase activity. Some studies have reported negative associations between nitrite/nitrate levels and waist circumference (WC) (16), obesity (17) and blood pressure (13), with Kleinbongard et al. concluding that decreased plasma nitrite levels are associated with increased cardiovascular risk (13). In contrast, other studies have found positive associations between plasma nitrite levels and BMI (18), fasting blood glucose, systolic blood pressure (19), total cholesterol (14) and triglyceride levels (20), with Ueyama et al. concluding that increased nitrite levels are associated with a clustering of MetS components (12).

Plasma NOx also have an exogenous source in the form of dietary nitrite/nitrate consumption. Eighty per cent of dietary nitrate originates in vegetables and fruits. Dietary nitrite, on the other hand, is primarily consumed from cured meats (~39%) along with cereal and baked cereals (~34%). Seventy-five per cent of dietary nitrate is excreted by the kidneys (21), while ~25% enters systemic circulation to be secreted into the salivary gland. Once in the salivary gland, the nitrate gets secreted back into the oral cavity where it is reduced to nitrite by bacteria. Nitrite then enters the acidic environment of the stomach where protons reduce nitrite to NO˙ (21). This NO˙ serves important antibacterial and mucosal vasodilatory actions in the stomach and, later on, is oxidized to nitrite and re-enters circulation. Once in circulation, many enzymes can reduce nitrite to NO˙. These reducing agents include deoxyhaemoglobin, xanthine oxidoreductase, carbonic anhydrase, aldehyde oxidase and vitamin C (11). Dietary nitrite/nitrate supplementation has been shown to decrease blood pressure, triglyceride levels and oxidative stress in some human randomized control studies (11). It also improves insulin signalling, glucose uptake and vascular function, both in animal models and humans (22,23).

The aim of this study was to estimate the associations of fasting plasma nitrites with obesity, MetS and its components. We hypothesized that nitrite levels would differ between subjects with and without MetS, independent of the presence of obesity. To reduce exogenous sources of variation in nitrite metabolism, we performed our study in an Old Order Amish population because of their homogenous and agrarian lifestyle (24,25).

Methods

Study population

This report is based on 116 Amish individuals from Lancaster, Pennsylvania. The Amish immigrated to the USA in mid-17th century to escape religious persecution (26).

The Amish population from Lancaster County descended from about 275 founders. Ninety-five per cent of the average founder contribution can be traced back to 76 members (45 women and 31 men) (27). This accounts for less genetic heterogeneity in Amish population as compared with general population. Moreover, they have relatively uniform socio-economic status and lifestyle, and substance abuse is minimal. The Amish diet shows relatively less inter-individual variation and contains a significant proportion of food products that are rich in nitrate and nitrite content, such as fresh vegetables, cured meat and baked cereals (37). These factors reduce the potentially confounding influences of variation in environmental exposures on complex traits such as obesity and MetS (28).

Inclusion and exclusion criteria

We performed a secondary analysis on clinical and biological data collected during the 'Old Order Amish Gut Microbiota Study' that had as a primary aim the exploration of dysbiosis of gut microbiota in obesity and its metabolic complications in Old Order Amish (24). Recruitment was conducted from April 2008 to September 2010. Eligibility criteria consisted of (a) Amish descent and (b) age between 20 and 80 years. Exclusion criteria were antibiotic treatment within the last 6 months; currently taking a medication (e.g. anti-inflammatory agents, glucocorticoids, antibiotics or immune modulating medications); having been pregnant in the last 6 months or currently pregnant; unwilling to discontinue supplements, vitamins or probiotics for at least 14 d; uncontrolled thyroid disease (thyroid stimulating hormone >5.5 or <0.4 IU mL^{-1}); history of intestinal surgery (except cholecystectomy or appendectomy); co-existing malignancy; renal insufficiency (serum creatinine >2 mg dL^{-1}); haematocrit <32%; and history of chronic pancreatitis, inflammatory bowel disease, celiac disease, lactose intolerance or other malabsorption disorders.

A total of 310 adults were enrolled in the Gut Microbiota Study (24); however, we included only those individuals ($N = 116$, 78.4% women) in our analyses who had sufficient plasma volume in their samples to have their fasting plasma nitrite levels measured. Recruitment was performed by a field team consisting of an Amish liaison and a nurse during an initial home visit. For inclusion purposes, a screening questionnaire regarding medical conditions and treatment history and a blood test were obtained. The blood test consisted of a complete blood count, comprehensive metabolic panel, celiac screen and thyroid stimulating hormone measurement (Quest Diagnostics, Inc., Horsham, PA, USA). The blood test was obtained at initial visit. A follow-up home visit was conducted, and the following were obtained after an overnight (>8 h) fast: blood pressure measurements, a second blood sample and questionnaires (including personal medical history and family medical history).

All procedures were performed by trained personnel following the Amish Research Clinic and University of Maryland guidelines (29). Trained nurses measured height and weight using a stadiometer and calibrated scale in subjects wearing light clothing and without shoes. Weight in kilograms divided by height in meters squared was used to calculate BMI. An inelastic tape was used to measure WC to the nearest 0.1 cm. During the follow-up visit, blood pressure was obtained in each subject after he or she had been sitting quietly for 5 min; an average of three measurements were taken manually for analysis. After a >8 h fast, blood was drawn, and the following were assayed by Quest Diagnostics: total cholesterol, triglycerides, high-density lipoprotein (HDL)-cholesterol, C-reactive protein (CRP), neopterin and serum glucose. Low density lipoprotein (LDL)-cholesterol was calculated using Friedewald's formula.

Fasting plasma nitrite concentrations were measured at the Division of Biological Chemistry, Biocenter, Medical University of Innsbruck, Austria, using the same protocol as described in Giustarini *et al*. (30). A modified Griess–Ilosvay diazotization reaction assay (Merck KGaA, Darmstadt, Germany) was used to measure nitrite concentrations. Briefly, this protocol consisted of the reaction of sulfanilamide with nitrite present in samples under acidic conditions (phosphoric acid), thereby forming a diazonium salt. This diazonium salt was then coupled with N-(1-naphthyl) ethylenediamine dihydrochloride forming an azo dye which was then analysed at 562 nm in a spectrophotometer (KC4 reader, Bio-Tek Instruments Inc., Winooski, VT, USA).

The protocol was approved at the University of Maryland, School of Medicine by the Institutional Review Board. Informed consent was obtained from all subjects.

Definition of variables

Metabolic syndrome was defined using NCEP ATP III criteria (1) as having three or more of the following risk factors: WC >102 cm (men) or >88 cm (women); systolic blood pressure >130 mmHg and diastolic blood pressure >85 mmHg; triglycerides >150 mg dL^{-1}; HDL <40 mg dL^{-1} (men) and <50 mg dL^{-1} (women) and fasting glucose level >110 mg dL^{-1}. Normal weight, overweight and obesity were defined using the AHA criteria: normal weight (BMI <25 kg m^{-2}); overweight (BMI 25–29.9 kg m^{-2}) and obese (BMI ≥30 kg m^{-2}).

We hypothesized that nitrites are associated with obesity and, independently of obesity, with MetS. In order to examine the difference between obesity and MetS in terms

of nitrite levels, we categorized subjects into three groups (a) overweight/obese(−)MetS(−), (b) overweight/obese(+)MetS(−) and (c) overweight/obese(+)MetS)(+).

Statistical analyses

Plasma nitrite values were not normally distributed and were log transformed prior to analyses. Age-adjusted and sex-adjusted three-way ANCOVAS were performed to compare the three groups, as defined by MetS and BMI group status. Tukey's honestly significant difference post hoc group vs. group analyses with Bonferroni adjustments were carried out if the overall model reached statistical significance. Linear multivariate regression analyses were also performed to test associations between log-transformed nitrite concentrations and continuous measures of cardiovascular risk factors. The linear models were adjusted for age, sex, BMI and, in a second analysis, for inflammatory markers, CRP and neopterin. Data analysis was performed using SAS/STAT 14.2 (Cary, NC, USA), and statistical significance was set at a two-tailed p-value of 0.05.

Results

This study population had a mean age of 54.0 ($R = 25$–79) years and had a skewed distribution in terms of gender (predominantly women) and obesity status ($n = 94$). Twenty-seven (23.3%) participants met the criteria for MetS (24 women and 3 men). Nineteen individuals (16.3%) had normal weight (BMI <25 kg m^{-2}), while only three individuals (2.5%) were included in the overweight group (BMI 25–29.9 kg m^{-2}). Ninety-four individuals (81%) met the criteria for obesity (BMI $\geq$30 kg m^{-2}) (80 women and 14 men). When we categorized individuals based on BMI and MetS status, 19 were included in obese/overweight(−)MetS(−) category, 70 in obese/overweight(+)MetS(−) and 27 individuals fell into obese/overweight(+)MetS(+) category (see Table 1 for subject characteristics). Mean fasting plasma nitrite was 9.52 μmol L^{-1} (SD = 7.53).

Nitrite levels were significantly associated with BMI ± MetS category following adjustment for sex and age ($p < 0.0001$). Tukey's honestly significant difference post hoc analysis with Bonferroni adjustments revealed higher nitrite levels in the overweight/obese(+)MetS(+) group than in both the overweight/obese(+)MetS(−) (mean difference = 0.57, CI: 0.33–0.81, $p = 0.001$) and the overweight/obese(−)MetS(−) (mean difference = 0.91, CI: 0.60–1.20, $p = <0.0001$) groups. The overweight/obese(+)MetS(−) group, in turn, had higher nitrite levels than the normal weight group (mean difference = 0.34, CI: 0.08–0.610, $p = 0.016$) (Figure 1).

We also categorized subjects into (a) obesity(−)MetS(−), (b) obesity(+)MetS(−) and (c) obesity(+)MetS(+) to explore if there was a significant difference of nitrite levels between individuals with obesity/MetS and individuals with normal weight/overweight. Nitrite levels were significantly associated with BMI category/MetS category in this analysis (age-adjusted and sex-adjusted $p < 0.0001$). Post hoc analysis showed that nitrite levels were higher in the obesity(+)MetS(+) group than in the

Table 1 Characteristics of subjects based on BMI ± MetS category

	BMI ± MetS category			
	Overweight/obese(−)MetS(−)	Overweight/obese(+)MetS(−)	Overweight/obese(+)MetS(+)	Total
Women N (%)	19 (42)	70 (84)	27 (89)	116 (78.4)
Age (years)	47.0 (13.98)	53.8 (11.37)	59.5 (8.90)	54.0 (11.88)
Total cholesterol (mg dL^{-1})	208.3 (36.37)	223.7 (53.20)	225.2 (30.50)	221.5 (46.37)
HDL (mg dL^{-1})	63.2 (11.61)	58.9 (13.23)	47.7 (12.86)	57.0 (13.87)
LDL (mg dL^{-1})	133.0 (34.13)	146.8 (47.47)	143.5 (28.20)	143.8 (41.71)
HDL/cholesterol ratio	3.4 (.83)	3.9 (.95)	4.9 (1.20)	4.0 (1.12)
Triglycerides (mg dL^{-1})	60.3 (21.24)	89.7 (36.92)	169.9 (63.27)	103.3 (57.09)
Glucose (mg dL^{-1})	84.3 (6.95)	90.4 (10.11)	103.1 (21.92)	92.3 (14.66)
Waist circumference (cm)	82.5 (7.56)	97.6 (9.60)	103.4 (7.51)	96.5 (11.00)
Hip circumference (cm)	95.6 (5.72)	116.6 (9.78)	120.7 (10.35)	114.1 (12.55)
BMI (kg m^{-2})	22.7 (1.62)	33.8 (3.42)	35.7 (4.44)	32.4 (5.59)
SBP (mmHg)	109.8 (13.26)	117.4 (12.49)	132.7 (17.31)	119.8 (15.75)
DBP (mmHg)	67.3 (7.09)	71.4 (7.70)	77.1 (10.41)	72.1 (8.82)
MAP (mmHg)	81.5 (8.28)	87.9 (8.49)	95.7 (11.23)	88.0 (10.21)
Nitrite (μmol L^{-1})	5.53 (1.13)	7.70 (3.15)	17.06 (12.02)	9.52 (7.53)

BMI, body mass index; DBP, diastolic blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MAP, mean arterial pressure; MetS, metabolic syndrome; SBP, systolic blood pressure; SD, standard deviation.
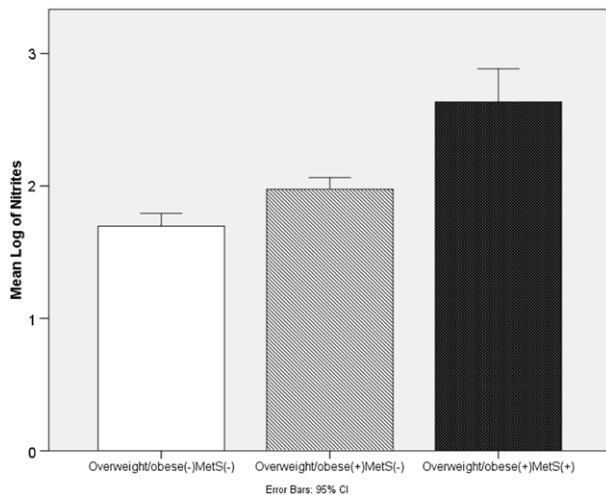
Figure 1 Mean nitrite levels (log-transformed) according to overweight/obese ± MetS category.

obesity(+)MetS(−) group (mean difference = 0.57, CI: 0.33–0.81, p = 0.001). The obesity(+)MetS(−) group, in turn, had higher nitrite levels than the non-obese group (mean difference = 0.29, CI: 0.03–0.54, p = 0.044).

We conducted a secondary data analysis to explore if the association between nitrite and BMI ± MetS groups was confounded by inflammation markers previously associated with obesity and MetS (31,32). Neopterin and CRP, markers for inflammation, were separately added in two ANCOVA models. The association of nitrite levels with BMI ± MetS category remained significant after neopterin and CRP adjustments.

After finding an association between nitrite levels and MetS, we performed linear regression analyses to test associations of MetS components with nitrite levels. After adjusting for age and sex, plasma nitrite levels were significantly associated with BMI ($p < 0.0001$); HDL ($p < 0.0001$); triglycerides ($p < 0.0001$); H/C ratio ($p < 0.0001$); WC ($p < 0.0001$); hip circumference ($p = 0.0001$) and glucose ($p < 0.0001$). When additionally controlled for BMI, the following remained significant: HDL ($p < 0.0001$); total cholesterol ($p = 0.048$); triglycerides ($p < 0.0001$); H/C ratio ($p < 0.0001$) and glucose ($p < 0.0001$).

To explore whether the observed associations were mediated by inflammation, we conducted linear regression while adjusting for two inflammatory markers, i.e. CRP and neopterin. After adjusting for gender, BMI and CRP, nitrite levels were predicted by HDL ($p < 0.0001$); triglycerides ($p < 0.0001$); H/C ratio ($p < 0.0001$); total cholesterol ($p = 0.027$) and glucose ($p < 0.0001$). After additionally controlling for neopterin, the following remained significant: HDL ($p < 0.0001$); total cholesterol ($p = 0.033$); triglycerides ($p < 0.0001$) and H/C ratio ($p < 0.0001$).

Glucose was no longer significant ($p = 0.052$). See Table 2 for all p-values.

Discussion

The main finding of this study was that individuals with obesity and MetS have higher nitrite levels than individuals with normal weight or obesity alone. Individuals with obesity but without MetS also have higher nitrite levels than individuals with normal weight. When we examined individual continuous cardiovascular risk factors, we found positive associations between plasma nitrite and BMI, total cholesterol, triglycerides and fasting glucose, as well as a negative association with HDL. Although there has been considerable variability across previous studies, our results are consistent with several of the previously reported association between nitrite levels and MetS. Amish subjects had an overall elevated fasting nitrite level of 9.52 μmol L^{-1} (7.53), which was higher than the levels previously reported in other non-Amish studies (13,15,33–37) (Table 3).

One speculative explanation of our findings could be that, analogous to hyperinsulinaemia, there might be increased endogenous NO production leading to a hypernitritemic state in MetS. These effects have been linked to increased production of tumour necrosis factor (TNF) and other pro-inflammatory cytokines in obesity (38). Obesity-induced inflammatory cytokines decrease eNOS expression but also increase the expression of iNOS (8,9). iNOS is expressed in macrophages after stimulation by pro-inflammatory cytokines and produces NO in far greater amounts than eNOS. iNOS derived NO, in higher amounts, serves important immune function (39), but also damages normal tissues (9). Therefore, high levels of nitrite in MetS may be attributed to iNOS overexpression. In such cases, plasma nitrite has the potential to serve as a marker for inflammatory activation in MetS and its components. Indeed, nitrites increased from overweight/obese without MetS to overweight/obese with MetS phenotype in our study.

While the conjecture of iNOS and inflammatory mechanisms inducing high amounts of NO is consistent with our findings, our study showed that the associations of plasma nitrite with obesity, MetS and individual cardiovascular risk factors remained significant when controlling for levels of the inflammatory markers CRP and neopterin. This discrepancy may be due to selective immune activation rather than general activation. Adipocytes have been shown to secrete Th2 cytokines, including IL-4 and IL-13, which maintain adipose tissue macrophages in anti-inflammatory M2 phenotype (40). However, diet-induced obesity induces adipose tissue macrophages to switch from anti-inflammatory (alternatively activated) M2 phenotype to

Table 2 Regression models testing associations between log-transformed nitrite and individual cardiovascular risk factors

| | Model 1 | | | Model 2 | | | Model 3 | | | Model 4 | | |
| | Adjusted for age and gender | | | Adjusted for age, gender and BMI | | | Adjusted for gender, BMI and CRP | | | Adjusted for gender, BMI, CRP and neopterin | | |
	β	Model p-value	*p*-value	β	Model p-value	*p*-value	β	Model p-value	*p*-value	β	Model p-value	*p*-value
N (116)												
Age												
Sex												
BMI	0.474	<.0001	<.0001*									
SBP	0.041	0.224	0.713	−0.069	<.0001	0.498	0.006	<.0001	0.942	0.006	<.0001	0.949
DBP	0.091	0.159	0.333	−0.022	<.0001	0.801	−0.015	<.0001	0.867	−0.005	<.0001	0.961
MAP	0.075	0.186	0.449	−0.043	<.0001	0.642	−0.005	<.0001	0.956	0.001	<.0001	0.993
HDL	−0.519	<.0001	<.0001*	−0.412	<.0001	<.0001*	−0.413	<.0001	<.0001*	−0.407	<.0001	<.0001*
LDL	0.109	0.132	0.243	0.313	<.0001	0.149	0.137	<.0001	0.104	0.158	<.0001	0.077
Triglycerides	0.107	<.0001	<.0001*	0.803	<.0001	<.0001*	0.793	<.0001	<.0001*	0.768	<.0001	<.0001*
Total cholesterol	0.142	0.089	0.132	0.167	<.0001	0.048*	0.187	<.0001	0.027*	0.191	<.0001	0.033*
HDL/chol ratio	0.629	<.0001	<.0001*	0.544	<.0001	<.0001*	0.563	<.0001	<.0001*	0.573	<.0001	<.0001*
Waist circumference	0.427	<.0001	<.0001*	0.130	<.0001	0.449	0.176	<.0001	0.299	0.154	<.0001	0.363
Hip circumference	0.434	<.0001	<.0001*	0.178	<.0001	0.908	0.065	<.0001	0.743	0.076	<.0001	0.704
Glucose	0.375	<.0001	<.0001*	0.353	<.0001	<.0001*	0.358	<.0001	<.0001*	0.171	<.0001	0.052

BMI, body mass index; CRP, C-reactive protein; DBP, diastolic blood pressure; HDL, high density lipoprotein; HDL/chol, HDL/total cholesterol ratio; LDL, low-density lipoprotein; MAP, mean arterial pressure; SBP, systolic blood pressure; SD, standard deviation. Results of regression models showing the relationship of log-transformed fasting plasma nitrite variable and the listed variables: non-adjusted, adjusted for age, sex and BMI adjusted for sex, BMI and CRP, and adjusted for sex, BMI, CRP and neopterin.

pro-inflammatory (classically activated) M1 phenotype (41). In fact, Weisberg *et al.* showed that obesity was associated with the infiltration of macrophages into adipose tissue, which, in turn, was a major contributor to TNF and iNOS expression (42). Adipose tissue macrophages selectively secrete the pro-inflammatory mediators TNF, interleukin-6 (IL-6), interleukin (IL-1), monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1a (MIP-1a), and their secretion is even higher than classical M1 macrophages (8). These studies suggest that plasma nitrite

may be a better marker for obesity-related inflammation than other inflammatory mediators. Further studies are needed in order to advance the understanding of the link between MetS, iNOS and other inflammatory markers.

This study also found a strong association between the individual components of MetS and nitrite. Amish participants had fasting nitrites that were positively associated with total cholesterol, triglycerides, HDL/cholesterol ratio and negatively associated with HDL. Similar, although not identical, findings have been reported elsewhere. For example, Ueyama *et al.* found an association between high

Table 3 Human plasma nitrite levels found in the literature

First author	*N*	Plasma nitrite (µmol L^{-1})	Blood sample condition
Kapil *et al.* (36)	21	0.12–0.75	Fasting
Kelm *et al.* (37)	33	0.2 (SD: 0.03)	Fasting
Zand *et al.* (33)	30	0.25–0.5	Fasting
Geisler *et al.* ((34))	100	44.9 (SD: 32)	Non-fasting
Kleinbongard *et al.* (13)	351	0.27 (SD: 0.01)	Not known
Sastry *et al.* (35)	10	3.68 (SD: 0.56)	Not Known
Kleinbongard *et al.* (15)	24	0.31 (SD: 0.023)	Not known

SD, standard deviation; SEM, standard error of mean.

NOx and low HDL-cholesterol (12). Choi *et al*. (2004) reported that men with high total cholesterol and triglycerides had higher nitrite levels (14), whereas Caimi *et al*. reported a positive correlation of nitrites with triglycerides and low density lipoprotein-cholesterol and a negative correlation with HDL-cholesterol (43).

One consistent finding in most of these studies has been that raised blood pressure was not significantly associated with nitrite levels. This study also found no association between nitrites and blood pressure. Although it may seem unlikely considering the relation of NO to vasodilation and blood pressure, this discrepancy may be explained by the hypothesis that nitrite in MetS predominantly derives from iNOS expressed in macrophages, and the endothelium still remains relatively NO deprived because of endothelial dysfunction. Interestingly, Sindler *et al*. reported that dietary nitrite improved vascular function and decreased arterial stiffening associate with ageing (22). Low dose sodium nitrite infusion also increases blood flow in ischaemic myocardium of patients (44). In such circumstances, dietary nitrite may serve as both a precursor of NO and an NO-like molecule itself.

In contrast to Bahadoran *et al*. who observed increased serum NOx levels in relation to WC (20), in our study, the association with WC became non-significant after adjusting for age and sex. Overall, studies aimed at finding associations between nitrite levels and cardiovascular risk factors have shown variable yet overlapping results. The discrepancy in results across various studies may have resulted from non-representative population samples, methodological issues and study designs. For example, most studies have measured combined nitrate and nitrite (NOx) rather than of nitrite alone, which correlates better with endogenous NO˙ production (15). In addition, the Griess method employed by most studies to measure NO˙ metabolites is less reliable than other methods (30). Variations in techniques used within the Griess method are associated with different sensitivities and specificities, making it even more challenging to compare nitrite levels across studies (35,45). Tsikas has already pointed out the urgent need of quality control systems to monitor the overall reliability of the Griess method (46).

Whether or not high plasma nitrite has any role in the causation or worsening of individual components of MetS is a matter of interest because, theoretically, dietary nitrites can cause even higher levels of plasma NOx. Although most studies have found, as in our study, higher nitrite levels in association with MetS, evidence suggests that dietary nitrite supplementation may have beneficial effects in patients with MetS (11). For example, Ohtake *et al*. showed that dietary nitrate supplementation could reverse some features of MetS in murine model of postmenopausal MetS induced by high fat diet and ovariectomy (47). Similarly, ingestion of sodium nitrite improved insulin signalling and decreased insulin resistance in Type 2 diabetic mice (23). In humans, dietary nitrite and nitrate supplementation have been shown to increase NO˙ and decrease triglyceride levels (33). These beneficial effects of dietary nitrates in the presence of hypernitritemia may appear analogous to the effects of exogenous insulin in hyperinsulinaemic state of MetS. It remains to be explored whether, along with insulin resistance, there is concurrent NO resistance in MetS.

One of the limitations of our study was that we did not collect individual dietary data. As many Amish live an agrarian lifestyle, they may be exposed to foods with higher concentrations of nitrate and nitrite as compared with non-Amish populations. One study showed that 30% of Amish individuals in Ohio cured their own meat and 38% made their own sausages, compared with 8% and 15% of non-Amish controls (25). In addition, nitrate was found as a contaminant in drinking water in agricultural areas inhabited by the Amish recruited in our study (48). Considering the lifestyle of Old Order Amish, dietary habits, including consumption of well water, may be one explanation of higher nitrite levels found in our study. However, our subjects had their blood drawn after a >8 h fast. Because ~90% of circulating nitrite in fasted patients is derived from the L-arginine-NO pathway, it is likely that the high nitrite levels seen in our subjects are due to endogenous sources (49). In addition, the effect of dietary habits on the associations reported in our study may have been minimized because of homogeneous dietary habits across the Amish population. Nevertheless, even if the short-term effects of dietary nitrate were minimized by fasting, it remains to be determined whether long-term environmental exposure, such as diet, resulted in higher nitrite levels.

Another limitation of our study is that its cross-sectional design does not allow us to make inferences on the direction of causality in our dataset. To be sure, nitrite levels only provide an indirect link between endothelial function and obesity/MetS. Direct assessments of endothelial function need to be explored in relation to nitrite and obesity/MetS. In addition, study population had a skewed distribution in terms of gender (predominantly women) and obesity status (94 obese and 19 healthy). Out of 310 individuals recruited, we included only those individuals (*N* = 116) in our analyses who had sufficient plasma volume in their samples to have their fasting plasma nitrite levels measured. Remaining amount of plasma might be one of the factors that skewed the distribution. Indeed, the average BMI of 310 adults was 29.2 as compared with 31.05 in 116 individuals. We also did not measure complete panel of inflammation molecules and

secondary causes of obesity, such as Cushing's disease or polycystic ovarian syndrome were neither explored clinically, nor considered in the exclusion criteria.

In conclusion, the strong positive associations found between fasting blood nitrite levels and overweight/obese status, MetS and some of the components of MetS support the potential role of nitrite in MetS and associated cardiovascular risk factors, either as a marker or a mediator. It remains to be further investigated whether high nitrite levels in obesity and MetS are mediators of inflammatory mechanisms, a marker of a biochemical predisposition or a detrimental dietary regimen or, alternatively, a compensatory response to increased vasodilatory demands. Regardless, they have a potential to serve both as a clinical marker and as a target for interventions in MetS.

Conflict of interest statement

F. A., D. F., M. D., G. N., A. R., M. E. B., O. O., E. B., L. A. B., C. A. L., K. A. R., M. P., B. D. M. and T. T. P. declared no conflict of interest.

S. S. became an employee of Novo Nordisk A/S, after the analysis of data was completed, and he affirms that his work on the manuscript is fully unrelated to his employment at Novo Nordisk A/S.

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