DFG Senate Commission
on Food Safety

SKLM

Nitrate and Nitrite in the Diet: An approach to assess Benefit and Risk for Human Health

Adopted on: April 15th 2014

Deutsche Forschungsgemeinschaft
Kennedyallee 40 · 53175 Bonn
www.dfg.de/sklm

DFG

Members and guests of the DFG Senate Commission on Food Safety 2011-2013

Members:

Gerhard Eisenbrand (Chairman), Patrick Diel, Karl-Heinz Engel, Johanna Fink-Gremmels, Jan G. Hengstler, Hans-Ulrich Humpf, Hans-Georg Joost, Dietrich Knorr, Doris Marko, Ivonne Rietjens, Pablo Steinberg

Permanent guests:

Christian Hertel, Sabine Kulling, Alfonso Lampen, Gerhard Rechkemmer, Richard H. Stadler, Stefan Vieths

Members and guests of the DFG Senate Commission on Food Safety 2014-2016

Members:

Pablo Steinberg (Chairman), Patrick Diel, Gerhard Eisenbrand, Karl-Heinz Engel, Bernd Epe, Volker Heinz, Hans-Ulrich Humpf, Hans-Georg Joost, Dietrich Knorr, Theo de Kok, Doris Marko, Ivonne Rietjens, Rudi Vogel

Permanent guests:

Peter Fürst, Sabine Kulling, Alfonso Lampen, Gerhard Rechkemmer, Richard H. Stadler, Stefan Vieths

The Senate Commission gratefully acknowledges the contribution of experts invited to the round table meeting “Nitrate and Nitrite in the Diet, Benefit / Risk for Human Health” held in Bonn on November 27th 2012:

Amrita Ahluwalia (Queen Mary University, London, UK), Stephen Hecht (University of Minnesota, Minneapolis, USA), Theo De Kok (University of Maastricht, Maastricht, NL), Gunter Kuhnle (University of Reading, Reading, UK), Jon Lundberg (Karolinska Institute, Stockholm, SE), David Shuker (The Open University, Milton Keynes, UK), Rashmi Sinha (National Cancer Institute, NIH, Rockville, USA), Marco Zeilmaker (RIVM, Bilthoven, NL)

Thanks go to the scientists of the SKLM office Sabine Guth, Michael Habermeyer and Angelika Roth for the preparation of this opinion.

SKLM Commission Secretariat

Institut für Lebensmitteltoxikologie und Chemische Analytik, Stiftung Tierärztliche Hochschule Hannover, Bischofsholer Damm 15, 30173 Hannover, Germany

E-Mail: SKLM@tiho-hannover.de • Tel.: +49 511 8567227 • Fax: +49 511 8567409

The Senate Commission on Food Safety (SKLM) of the Deutsche Forschungsgemeinschaft (German Research Foundation, DFG) organized a round table meeting on "Nitrate and Nitrite in the Diet, Benefit / Risk for Human Health" in Bonn on 27th November 2012. Experts from the Netherlands, Sweden, UK and USA were invited to discuss benefit / risk aspects of dietary nitrate and nitrite. Extended abstracts of the presentations given at the meeting are available as supplementary material to this opinion via the DFG homepage of the SKLM (www.dfg.de/sklm). Following its mandate for evaluation and advice concerning the effects of food and food constituents on human health the SKLM has prepared conclusions, identified gaps in knowledge and highlighted areas deserving further research. The following opinion was adopted on April 15th 2014.

„Nitrate and Nitrite in the Diet: How to assess Benefit and Risk for Human Health"

Content

1 Summary

Nitrate is a naturally occurring or chemically synthesized compound that forms part of the nitrogen cycle and is also present in the human diet as a natural constituent as well as an approved food additive. In the mammalian organism, nitrate, nitrite and nitrogen oxides are metabolically interconvertible. In addition, nitrogen monoxide (NO), a multifaceted physiological signaling agent, is consistently generated in the organism from arginine by NO synthases. There is increasing evidence for beneficial health effects of dietary nitrate, based on the fact that it represents an alternative source for NO. The nitrate-nitrite-NO pathway has been proposed to serve as a backup system to ensure a certain NO level required for maintenance of cellular and physiological functions in situations characterized by inappropriate/dysfunctional NO synthase activity. NO is known as endothelium derived relaxation factor, inducing vasodilation and thereby decreasing blood pressure. This effect is associated with a reduced risk regarding cardiovascular disease, myocardial infarction, and stroke. As a consequence of its partial conversion to NO in the organism, dietary nitrate has also been claimed to be associated with beneficial effects in patients with gastric ulcer, renal failure or metabolic syndrome. Experimental evidence and first results from human intervention studies indicate that such beneficial health effects due to dietary nitrate may be achievable at intake levels resulting from the daily consumption of nitrate-rich vegetables. In addition to the partial conversion of ingested nitrate to nitrite by oral commensal bacteria, bacterial infections and/or inflammatory processes may also contribute to nitrite formation in the organism.

On the risk side, there is compelling evidence for endogenous formation of *N*-nitroso compounds in humans. Many *N*-nitroso compounds exhibit potent mutagenic and carcinogenic effects. However, the relevance of the endogenous formation of *N*-nitroso compounds for human health has not been adequately explored up to now. Nitrate and nitrite are not considered to be carcinogens *per se*. However, under conditions that result in endogenous nitrosation (i.e. in combination with certain amines or amides) ingested nitrate and nitrite may probably be carcinogenic to humans. At present, the available evidence does not allow to perform a reliable benefit/risk assessment in terms of long-term human health consequences. There is a need for further research addressing potentially negative and/or positive long-term health effects associated with dietary nitrate and nitrite ingestion. Biomarkers

indicative of adverse and/or beneficial health effects need to be developed and applied in appropriately designed human intervention studies.

2 Introduction

The SKLM has repeatedly addressed the potential health risks due to nitrosamines, nitrate and nitrite in connection with endogenous nitrosation reactions. Gaps in knowledge and research needs were lastly summarized by the SKLM in 1994 [1].

In view of the controversial discussion concerning potential detrimental versus beneficial health effects related to dietary nitrate and nitrite intake, the SKLM reviewed the pertinent evidence at a round table meeting on “Nitrate and Nitrite in the Diet, Benefit/Risk for Human Health” in Bonn on 27th November 2012. Experts from The Netherlands, Sweden, UK and USA were invited to discuss benefit/risk aspects of dietary nitrate and nitrite. The aim was to attempt a benefit/risk analysis on the premise that the available data were sufficient to achieve this aim. If this were not the case, missing data and research needs ought to be identified. State-of-the art information on potential nitrate- and nitrite-related beneficial and detrimental health effects was presented by the invited experts and discussed during the round-table meeting.

An increased consumption of vegetables is widely recommended because of their generally recognized beneficial health effects. In 2007 the World Cancer Research Fund/American Institute for Cancer Research (WCRF/AICR) rated the evidence as “convincing to probable” that diets high in vegetables and/or fruits protect against cancers of the mouth and pharynx, oesophagus, lung, stomach, colon and rectum, larynx, pancreas, breast and bladder [2]. It is not clear at present whether such beneficial health effects associated with high vegetable intake become evident because or in spite of a concomitantly high exposure to dietary nitrate. Recent evidence from experimental and human intervention studies as well as epidemiological observations suggest beneficial health effects including, for instance, effects on blood pressure, myocardial infarction or stroke associated with enhanced dietary nitrate intake [3-7]. However, nitrate from exogenous sources has also given rise to health concerns because of the potential endogenous formation of *N*-nitroso compounds

(NOC). In view of the controversial discussion concerning potential detrimental versus beneficial health effects related to dietary nitrate/nitrite intake, the SKLM has reviewed the pertinent evidence to identify gaps in knowledge and research needs.

It is to be mentioned in this context that exposure to nitrate or nitrite may also be associated with the adverse health effect of methemoglobin formation, especially in infants considered to be at elevated risk for methemoglobinemia [8]. However, methemoglobinemia is not in consideration within the current opinion of the SKLM.

2.1 Exposure and former risk assessment of nitrate and nitrite

Nitrate is a naturally occurring or chemically synthesized compound that forms part of the nitrogen cycle. It is a natural constituent as well as an approved food additive. In general, uptake of nitrate from exogenous sources in humans predominantly results from the consumption of nitrate-rich foods. In addition to dietary uptake, endogenous nitrogen monoxide (NO) formation from arginine is considered to be another major contributor to the overall internal exposure of humans to NO, nitrite, nitrate and several nitrosyl intermediates. A major part of dietary nitrate exposure is due to the consumption of vegetables and is the consequence of their often rather high nitrate contents. In view of the large variation in the median concentrations of nitrate in different vegetables (e.g. from 1 mg/kg in peas and Brussels sprouts to 4,800 mg/kg in rocket (*Eruca sativa* and *Diplotaxis tenuifolia*), the European Food Safety Authority (EFSA) considered different scenarios for nitrate exposure estimations, assessing for adults a mean dietary nitrate uptake of 157 mg/day, equivalent to 2.6 mg/kg body weight (b.w.)/day based on a b.w. of 60 kg [9]. An acceptable daily intake (ADI) for nitrate was deduced by the Scientific Committee on Food (SCF) in 1990 [10] and retained in 1995 [11]. The No Observed Effect Level (NOEL) of 370 mg nitrate/kg b.w./day was derived from long-term studies in rats and a subchronic toxicity study in dogs. Applying an uncertainty factor of 100 an ADI of 222 mg/day (0 - 3.7 mg/kg b.w./day) for nitrate was calculated. The ADI was confirmed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 2002 [12]. The CONTAM Panel of EFSA concluded that in the absence of significant new toxicological and toxicokinetic data, there was no need to re-consider this ADI [9].

In its nitrate uptake assessment, the CONTAM panel of EFSA also noted that:

1) individual consumption habits appear to be of great importance;

2) the nitrate uptake of people consuming high amounts of nitrate-rich vegetables such as lettuce, spinach, rocket, beets and radish may be considerably higher than the mean value and may thereby exceed the current ADI.

Nitrite is formed naturally at rather low steady-state concentrations during the nitrogen cycle by nitrogen fixation and is subsequently converted to nitrate, a major nutrient assimilated by plants. Consumer exposure to nitrite results as a consequence of its use as a food preservative and, to a lesser extent, from its presence in certain vegetables [9]. Assuming a mean nitrite concentration of 0.5 mg/kg for all vegetables, as reported in the United Kingdom's 1997 total diet study, a recommended consumption of 400 g vegetables per day would result in a dietary exposure of 0.2 - 0.8 mg nitrite/day, equivalent to 3 - 13 µg/kg b.w./day based on a b.w. of 60 kg [9]. A further publication by EFSA on nitrite in meat products reported mean dietary exposure levels of adults to nitrite in the range of 0.04 to 0.23 mg/kg b.w./day (by combining nationwide data on food consumption with the maximum permitted usage levels for the additive). Assuming reported average nitrite levels, a consumer exposure of 5 - 30 µg/kg b.w./day was assessed [13]. In 2002 JECFA set an ADI of 0 - 0.07 mg/kg b.w. for nitrite, based on heart and lung toxicity in a long-term NTP study in rats and a safety factor of 100 [14].

3 Metabolism of nitrate and nitrite

In the organism, nitrate and nitrite may function as an alternative source for nitrogen monoxide (NO), an important and multifaceted physiological signaling molecule. Although NO is rather short-lived, it may react under oxidative conditions, i.e. in the presence of oxygen and/or reactive oxygen species (ROS) to give rise to nitrite (NO_2^-), nitrate (NO_3^-) as well as nitrosyl peroxide (NOOH) and/or corresponding radical/ionic intermediates contributing to oxidative/nitrosative damage. These NO-derived species may lead to an array of reaction products under cellular or *in vivo* conditions, including *N*-, *S*-, and *O*-nitroso compounds as well as nitro derivatives of amino acids, peptides, proteins and DNA bases [15-18]. The biological activities of such NO-related secondary products have not been fully explored up to now. They may include pharmacological effects, e.g. on blood vessels and blood pressure, the induction of oxidative stress/inflammation and ultimately the endogenous formation of NOCs.

Nitrate biosynthesis in humans was first described more than 35 years ago [19, 20]. Early on, NO_2^- and NO_3^-, whether endogenously synthesized or taken up from exogenous sources, were taken into consideration as an alternative source for endogenous NO [21-26]. NO can endogenously be oxidized to nitrate and nitrite [26-28] and the latter can in part undergo reduction and cycling back to bioactive NO in blood and tissues before terminal excretion, e.g. as urinary nitrate [29]. On the other hand, NO is a biological messenger that is commonly generated by oxygen-dependent NO synthases (NOS) from L-arginine [26, 30]. The NOS family comprises inducible NOS (iNOS) as well as constitutive NOS, like endothelial (eNOS) and neuronal NOS (nNOS) [31].

The Griess nitrite test, which is used to determine nitrite in urine, has a long history as diagnostic tool for the recognition of urinary tract infections [32]. Infections induced by bacteria, parasites or viruses as well as inflammatory disease, such as gastritis, hepatitis and colitis have been recognized as risk factors for human cancers of the stomach, liver and colorectum. Such inflammatory conditions have been shown to favour the enhanced biosynthesis of NO, nitrite and nitrate [33, 34]. An increase in blood nitrate was also shown to occur in lipopolysaccharide-treated mice or mice infected with *Mycobacterium bovis* and was reported to result from enhanced NO/nitrate biosynthesis via T-lymphocyte-mediated activation of macrophages [35]. It has been shown that inflammation in many tissues is accompanied by an up-regulation of iNOS that is capable of producing NO in excess for a prolonged period of time [36, 37].

An increased endogenous formation of NOC has been shown to occur in humans infected with liver fluke (*Opisthorchis viverrini*), later confirmed experimentally in a study with hamsters [33, 38]. Nitrite concentrations in the gastric fluid have been shown to be (directly) correlated with intragastric pH. Under conditions of insufficient production of gastric acid (hypo- or achlorhydria) bacterial contamination of gastric juice is common and nitrate-reducing microorganisms are regularly found. Thus, formation of NOC under microbial catalysis in human gastric juice or urine has been shown [39-44]. Evidence has been accumulating that NO-derived reactive nitrogen species possess pathogenic potential, and it has been proposed that DNA and tissue

damage induced by these reactive species may contribute to increased mutation rates, genome instability, apoptosis and associated tissue regeneration, encompassing a proliferative response of cells [45-47]. Enhanced formation of 8-nitroguanine and 3-nitrotyrosine has also been observed during microbial infection. These compounds may be used as potential biomarkers for “nitrative stress” [45, 47].

Dietary nitrate is rapidly absorbed in the upper gastrointestinal tract and distributed via the blood circulation. It reaches the salivary glands and is actively transported from blood into saliva. The salivary nitrate level may be up to 20 times higher than the plasma level [48, 49]. The protein sialin has been proposed to play a role in nitrate transport [50]. In the oral cavity salivary nitrate can be reduced to nitrite, predominantly by commensal bacteria [51]. The secreted nitrate as well as the nitrite generated in the oral cavity are swallowed, thereby reentering the gastrointestinal tract. Approximately 25% of nitrate originally ingested is secreted through the salivary glands, 6 – 7% of the total nitrate being converted to nitrite in the oral cavity during enterosalivary circulation [48, 52]. To some extent, saliva-derived nitrite may also contribute to NO formation under acidic conditions in the stomach. Exhalation of NO in the breathing air has been taken as an indication for such an intragastric NO formation from NO_2^- [53, 54]. Some nitrite may survive gastric passage and enter the systemic circulation. Nitrite levels in plasma appear to be directly correlated to the intake of nitrate [23, 55, 56].

The nitrate-nitrite-NO pathway is believed to affect NO homeostasis. This may be relevant under circumstances when oxygen-dependent NOS become dysfunctional [56-59]. In blood and tissues, NO can be generated independently from NOS by a variety of enzymes and proteins acting as nitrite reductases, including flavoproteins and cytochrome P-450, deoxygenated hemoglobin, myoglobin, xanthine oxidase and mitochondrial respiratory chain enzymes, among others [22, 24, 57, 60-65].

4 Potential beneficial health effects of nitric oxide, nitrate and nitrite

4.1 Physiological effects of NO

Since the discovery of endogenous NO formation, it became clear that NO is a pleiotropic signaling molecule relevant for a number of NO-mediated physiological effects. The radical NO easily diffuses across cell membranes and is capable of interacting with various receptor proteins. A key NO-related event is the coupling of a

nitroso moiety from NO-derived metabolites to a reactive cysteine leading to the formation of *S*-nitrosothiols. Such NO-induced *S*-nitrosation processes take part in a multitude of signaling events in the cell, influencing many physiological functions. These comprise, amongst others, the regulation of blood pressure [66, 67] and blood flow by mediating vasodilation [68], the maintenance of blood vessel tonus [69], the inhibition of platelet adhesion and aggregation [70, 71], and certain neurotransmitter functions [72-74]. Likewise, modulation of mitochondrial function and energetics by reactive nitrogen oxides [75], modulation of the immune and the endocrine system [76-79], and of retina function [80] have been reported. In addition, the role of NO-related effects in liver regeneration [81], heart development [82], and diseases like cochlear function and hearing diseases [83], cluster headache [84], and cystic fibrosis [85] are under investigation.

NO also has been shown to induce apoptosis in macrophages and endothelial cells [86, 87]. A potential role of NO in carcinogenesis and tumor progression is largely unexplored. Depending on the concentration of NO and the tumor microenvironment, divergent effects may be expected [88, 89]. High cellular activity of NOS appears to be associated with cytostatic or cytotoxic effects on tumor cells [90]. *In vitro* studies with human cancer cells point to an inhibitory effect of nitrite on cancer cell replication mediated by NO [91].

Based on the observation that nitrate and/or nitrite can contribute to endogenous NO formation, dietary uptake of these compounds has been associated with NO-like physiological effects in humans and other mammals. For instance, this applies to blood pressure, vascular control and vasodilation [25, 92-98]. Furthermore, protection against ischemia reperfusion injury in brain, heart, liver and kidney [99-102], improvement of revascularization in chronic ischemia [103], decreasing leukocyte recruitment in microvascular inflammation [104], mobilization of angiogenic cells [105] as well as anti-platelet and anti-aggregation effects in blood [106-108] have been reported. Moreover, attenuation of oxidative stress [109] and stimulation of mucosal blood flow and mucus formation in the gastrointestinal tract [110, 111] have been observed. This may contribute to the reported gastroprotective role of salivary nitrite, exemplified by studies indicating increased gastric mucosal blood flow and mucus thickness in rats.

In addition, dietary inorganic nitrate has been shown to alleviate features of the metabolic syndrome in endothelial NOS-deficient mice [112]. The metabolic syndrome is a combination of physiological disorders that increase the risk of developing cardiovascular diseases and type-2 diabetes. Decreased synthesis of bioavailable NO by endothelial NOS has been proposed to represent a central event in the development of the metabolic syndrome [113, 114].

4.2 Influence of nitrate and nitrite on the cardiovascular system

Potential protective mechanisms related to cardiovascular diseases include vasodilation, inhibition of endothelial dysfunction and inhibition of platelet aggregation. Endothelium-derived NO is an important signaling agent in the regulation of blood pressure [67]. NO-mediated regulation of vascular tone involves increased cyclic guanosine monophosphate (cGMP) and subsequent relaxation of vascular smooth muscle [66, 115].

It has been shown that relaxation of the rat aorta correlated with NO generation from nitrite *in vitro*. This relaxation could be prevented by an inhibitor of NO-sensitive guanylyl cyclase, confirming the involvement of the NO-mediated signaling pathway [116]. Local forearm blood flow at rest und during exercise [95] was found to depend on nitrite reduction to NO by deoxyhemoglobin [61]. Nitrite in the vascular tissue has been proposed to function as a NO reservoir, enabling the release of physiologically relevant quantities of NO in cases of compromised NOS activity and independently of NOS [117]. Accordingly, hypertension in eNOS knock-out mice was prevented by supplementation with dietary nitrate [112]. Nitrite is considered to be a major NO source under hypoxemic conditions, favoring development of acidosis and supporting eNOS inactivation. Under such conditions, deoxyhemoglobin and xanthine oxidoreductase (XOR) are supposed to play a substantial role in NO formation [118]. In a clinical study with a randomized double-blind crossover design, blood pressure decreased in healthy volunteers after supplementation with 0.1 mmol nitrate/kg b.w. for three days [94]. According to the authors, this would correspond to the consumption of about 150-250 g nitrate-rich vegetables such as spinach, lettuce or beetroot.

The enterosalivary circulation of nitrate and its reduction to nitrite by the microbiota of the oral cavity influence plasma concentrations of nitrate and nitrite. Antiseptic mouthwash treatment was found to reduce circulating nitrite concentration [49] and to correlate with an increase of systolic and diastolic blood pressure in rats and humans [119, 120].

Nitrite may also become an important alternative source for NO during ischemia and contribute to reducing myocardial ischemia-reperfusion damage. Enzyme-independent nitrate and/or nitrite reduction to NO in the ischemic heart influenced blood flow regulation and metabolic activity during hypoxia/ischemia and was proposed to mediate post ischemic injury, depending on the duration of NO production [121, 122].

Homogenized human and rat myocardium was found to generate NO from nitrite under ischemic conditions. Moreover, reduction of infarct size in rat hearts *in vitro* via nitrite reduction to NO depended on xanthine oxidoreductase activity [100]. Cytoprotective effects of sodium nitrite during *in vivo* ischemia-reperfusion were confirmed in heart and liver of mice [123] and in the brain of rats [99]. The protective effects against ischemia/reperfusion injury in the kidney were shown to depend on xanthine oxidoreductase activity in rats [102]. In human studies, orally ingested nitrate was found to protect against endothelial ischemia-reperfusion injury [124, 125].

The role of nitric oxide and cGMP in the inhibition of platelet adhesion to vascular endothelium [70] was shown almost simultaneously with the discovery of NO as the endothelium-derived relaxation factor [69, 126, 127]. Ingestion of potassium nitrate (KNO_3) was discovered to cause an increase in the formation of gastric *S*-nitrosothiols and an inhibition of platelet aggregation in humans [108]. The attenuation of platelet reactivity was found to be dependent on erythrocytes and deoxygenation; nitrite alone at physiological concentrations had no effect on platelet aggregation in plasma [128].

4.3 Beneficial health effects of dietary nitrate from foods

Consumption of fruits and vegetables, particularly of green leafy vegetables, appears to protect against coronary heart disease and ischemic stroke risk [129, 130]. In a study with two prospective cohorts, increased fruit and vegetable consumption was associated with modest benefits regarding the progression of cardiovascular diseases

[131]. The high nitrate content of some vegetables may explain some of the beneficial health effects of this food group, including the protection against cardiovascular disease and type-2 diabetes [132].

Beetroot juice, known to often contain elevated nitrate levels [9], was found to exhibit blood pressure-lowering and vasoprotective effects [93]. The consumption of 250 to 500 ml beetroot juice per day led to a decrease in systolic blood pressure of 5.4 to 12 mm Hg and to a decrease in diastolic blood pressure of up to 10 mm Hg. Mean nitrate concentrations in the consumed beetroot juice were 22 to 45 mmol/L; corresponding to an intake of 341 - 1395 mg nitrate per person per day or 5.7 to 23.3 mg/kg b.w./day [133, 134]. Dietary nitrate ingestion with beetroot juice was also reported to prevent endothelial dysfunction subsequent to an acute ischemic insult in the human forearm and to attenuate *ex vivo* platelet aggregation. Disruption of the enterosalivary circulation with partial conversion of nitrate to nitrite (by spitting out all the saliva) blocked the decrease in blood pressure and abolished the inhibitory effects on platelet aggregation, thereby confirming the pivotal role of nitrite generated by the oral microbiota. In a meta-analysis of sixteen studies (7 - 30 participants/study, duration 2 h to 14 days), beetroot juice supplementation as well as nitrate ingestion were associated with a significant reduction in systolic blood pressure. The daily amount of inorganic nitrate (sodium or potassium nitrate) consumed in these studies ranged from 2.5 to 24 mmol/dose (155 – 1488 mg), and the amounts of inorganic nitrate in the consumed beetroot juice varied between 5.1 and 45 mmol/dose (316 – 2790 mg) [135]. In a randomized cross-over trial, the effect of a 10-day period of consumption of Japanese traditional diet on blood pressure in 25 healthy volunteers was investigated. The authors reported that nitrate in the traditional Japanese diet (18.8 mg/kg b.w./day) lowered diastolic blood pressure in healthy volunteers on average about 4.5 mm Hg when compared to the control group, and this effect was suggested to explain in part the beneficial health effects of such foods [136]. Besides lowering the diastolic blood pressure at rest and during cardiopulmonary exercise, the consumption of 250-500 ml beetroot juice containing 5.1 - 11.2 mmol nitrate, i.e. 316 - 694 mg nitrate/ person/day or 5.3 - 11.6 mg nitrate/kg b.w./day was reported to increase exercise tolerance in healthy humans [137-140]. Moreover, in humans with peripheral arterial disease characterized by intermittent disruption of blood and oxygen supply, 500 ml beetroot juice containing 9.1 mmol nitrate, i.e. 564 mg nitrate/person/day or 9.4 mg nitrate/kg b.w./day significantly increased exercise

performance, most probably due to an NO-mediated increase in tissue perfusion and oxygenation [141].

A reduction in blood pressure was noted in healthy volunteers after dietary supplementation with nitrate, an effect consistent with the formation of vasodilating NO. In addition to vasodilation, mitochondrial function, leukocyte adhesion and platelet aggregation also seem to be positively affected.

Of note, randomized clinical intervention trials have indicated a reduction in blood pressure of about 5 mm Hg to be significantly associated with decreased cardiovascular morbidity and mortality [142, 143], whereas a comparable increase was associated with the opposite effect [144]. Thus, it is proposed that intervention studies should be undertaken to achieve a target dietary nitrate intake, e.g. through vegetable consumption, leading to a consistent reduction in blood pressure in a slightly hypertonic population.

5 Potentially detrimental health effects of *N*-nitroso compounds

It has been shown that oral application of nitrosatable amino compounds together with nitrite can lead to the formation of malignant tumours indistinguishable from those induced by the corresponding *N*-nitroso carcinogen [145]. Furthermore, enhanced exposure to nitrate results in enhanced urinary excretion of *N*-nitrosated amino acids in humans. The latter are highly water-soluble non-carcinogenic NOCs that are routinely used as (surrogate) biomarkers to monitor endogenous *N*-nitrosation under various conditions [146, 147]. However, despite many years of research, the potential health risk resulting from the endogenous formation of carcinogenic NOCs has still not been assessed in detail up to the present time. This is in part due to the fact that validated biomarkers reflecting carcinogenic NOC formation are largely missing. Furthermore, the multitude of physiological and disease-related processes such as infections and/or inflammation, which may give rise to nitrosating agents, in addition to the potential NOC formation from dietary precursors during passage through the gastrointestinal tract complicate any approach to study long-term health effects.

In addition, NOCs may already be formed in foods, e.g. as a consequence of processing them with nitrite or nitrate (meat curing). Thus, humans are also exposed to exogenously formed NOCs from various sources such as foods and cosmetics [148]. The WCRF/AICR reported “convincing” evidence that a high consumption of red and processed meat is associated with an enhanced risk of developing colorectal cancer, thereby suggesting a potential contribution of NOCs to the formation of such tumors [149].

5.1 Formation of N-nitroso compounds (exogenous, endogenous)

NOCs comprise *N*-nitrosamines and *N*-nitrosamides (see Figure 1). Whenever nitrosating agents encounter *N*-nitrosatable amino compounds, NOCs may be formed. The classical situation is reflected by the reaction of an amine with nitrous acid in aqueous solution [150, 151]. The nitrosating species, e.g. dinitrogen trioxide (N_2O_3), dinitrogen tetroxide (N_2O_4) or nitrous acidium ion, may be generated from nitrite or nitrous acid via proton catalysis, the acidity of the medium determining the relative prevalence of the nitrosating species. In addition, the basicity of the nitrosatable amine precursor greatly influences nitrosation in aqueous solution. Only non-protonated nitrogen atoms are available for nitrosation. Therefore, strongly basic dialkyl amines ($pk_a > 9.5$) are not considered to exhibit nitrosation rates that favour substantial NOC formation in aqueous media. Weakly basic amines ($pK_a < 9.5$) are much more rapidly nitrosated [52]. Moreover, under neutral to alkaline conditions formaldehyde has been shown to strongly catalyze the conversion of various secondary amines to nitrosamines, probably as a result of forming intermediate hydroxymethylamine adducts [152]. Because acid-catalyzed nitrosation in the absence of formaldehyde and potentially other foodborne aldehydes is inappreciable at pH > 5, NOCs in food and certain consumer products or under specific working place conditions most likely arise from exposure to gaseous NO_x. The *N*-nitrosation reaction can also be inhibited, e.g. in the presence of ascorbic acid, primary amines, tannins or other phenolic compounds [153].

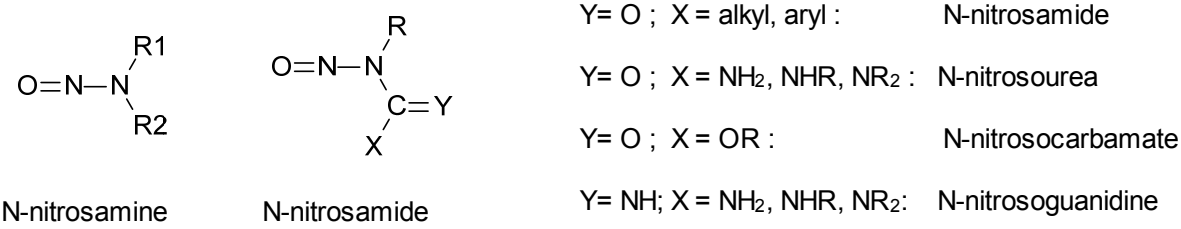


Figure 1: Structures of NOCs.

Nitrosamines most frequently found in food are *N*-nitrosodimethylamine (NDMA), *N*-nitrosopyrrolidine (NPYR), *N*-nitrosopiperidine (NPIP) and *N*-nitrosothiazolidine (NTHZ). Nonvolatile NOCs mainly consist of *N*-nitrosated amino acids, including the *N*-nitroso products of sarcosine (NSAR), 3-hydroxyproline and proline (NPRO), thiazolidine-4-carboxylic acid (NTCA), oxazolidine-4-carboxylic acid (NOCA) and *N*-nitroso-2-methyl-nitroso thiazolidine-4-carboxylic acid (NMTCA) as well as the oxazolidine analog (NMOCA) [154, 155]. The formation and occurrence of NOCs in cosmetics and consumer products has recently been summarized in two opinions by the EU Scientific Committee on Consumer Safety (SCCS) [156, 157].

There is compelling evidence for endogenous formation of NOCs in humans. Thus, formation of NOCs is to be expected not only under environmental, technical or household conditions, thereby favouring the reaction of nitrosatable amino compounds in food with nitrosating agents, but also after food ingestion, e. g. during the stomach passage due to the reaction of precursor amines or amides with nitrite. It has been shown that dietary nitrate and nitrite can lead to NO formation in the stomach and the large intestine [158-160]. NO is not a *N*-nitrosating agent *per se*, but may be rapidly converted into nitrosating and oxidizing reactants such as NOx and peroxynitrite. In addition, certain infections, which lead to inflammatory processes, may also contribute to endogenous nitrosation reactions [34], as shown, for example, by an increased urinary elimination of nitrosated amino acids. An early study by Sander and Bürkle in 1969 proved that endogenous NOC formation can occur *in vivo*. When weakly basic amines like *N*-methylbenzylamine and morpholine were given together with nitrite to rats, the same tumours were induced as observed after application of the corresponding NOC [145]. Further indications came from the observation that malignant liver and lung tumours developed in rats fed the drug amidopyrine (AP) together with nitrite. This was attributed to the extremely high reactivity of AP towards

nitrosating agents, releasing NDMA by endogenous nitrosation [161]. The *in vivo* nitrosation of AP in humans was proven after ingestion of AP together with a nitrate-rich vegetable (radish). The concomitant intake of ethanol (in 500 ml beer) prevented CYP 450-mediated NDMA metabolic clearance, which occurs very rapidly in the absence of ethanol [162, 163]. Under these experimental conditions, NDMA formed from AP was protected from CYP 450-dependent metabolic clearance and therefore became detectable in the urine of the volunteers [162, 163]. This confirms the premise that nitrate taken up with the diet undergoes enterosalivary circulation and partial conversion to nitrite in the oral cavity, leading to the endogenous nitrosation of *N*-nitrosatable precursors from food (see Figure 2). NDMA formation from AP was further confirmed by an *ex vivo* study, separately collecting human saliva samples from differently exposed volunteers. From one group of volunteers, saliva was obtained after ingestion of a nitrate-rich vegetable juice containing 200 mg nitrate. From the second group of volunteers, saliva was collected for 0.5 to 5 hours after intake of AP at the recommended daily dose of 500 mg. Saliva samples from both groups were mixed and incubated under simulated gastric conditions (15 min, pH 3, 37°C). NDMA formation in the combined saliva mixture was detected as early as 30 minutes after AP intake, with a maximum yield (980 ng NDMA/ml) 2.5 h after ingestion, still measurable 5 h after ingestion (300 ng NDMA/ml) [163]. This clearly demonstrates that formation of NOC in the gastrointestinal tract is to be expected when high dietary nitrate intake coincides with the ingestion of easily nitrosatable precursors. Enterosalivary recycling of both components with partial reduction of nitrate to nitrite by the oral microbiota, as exemplified in the case of AP, plays a major role (Figure 2).

Potential biomarkers to determine the nitrosation in the gastrointestinal tract need to be developed. The nitrosation of proline to NPRO has been used, due to the fact that endogenously formed NPRO is quantitatively excreted in urine. However, under certain circumstances, e.g. when gastric acid levels are low in the stomach (pH >4), nitrosation of proline does not occur [164], whereas NDMA would still be generated from AP.

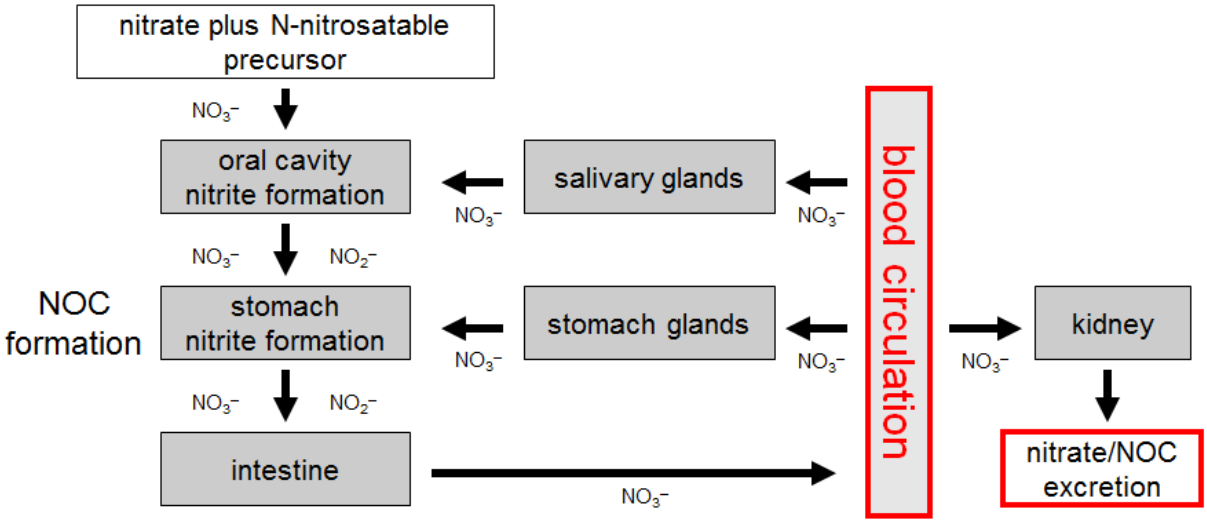


Figure 2: Flow chart of gastrointestinal nitrate circulation.

The exposure of laboratory animals to tobacco constituents may serve as a model to study different aspects related to the carcinogenicity of nitrosamines to humans. The concomitant exposure of rats to nornicotine and nitrite was shown to result in the endogenous formation of *N*-nitrosonornicotine (NNN), a tobacco-specific nitrosamine [165]. NNN is easily and rapidly formed by nitrosation of nornicotine and less readily by nitrosation of the tertiary amine nicotine [166, 167]. NNN, a highly potent tobacco carcinogen, is not found in the diet or in the general environment, except when tobacco smoke is present. Monitoring urinary mercapturic acids as biomarkers provided evidence for the endogenous formation of NNN in humans taking nicotine replacement products [168, 169]. This potential hazard could be addressed, at least in part, by excluding nornicotine contamination from nicotine replacement products or by combining them with nitrosation inhibitors. There is presently no evidence that long-term users of nicotine replacement products are at increased risk of cancer above and beyond that due to their history of smoking. Nevertheless, it would be prudent to avoid exposing the users of these products to NNN.

Evidence from prospective human cohort studies revealed a remarkably strong association between urinary total NNN as biomarker of human NNN intake and the risk of developing esophageal cancer among smokers [170, 171]. This is consistent with evidence from animal carcinogenicity data, showing potent carcinogenic effects of NNN and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in rat esophagus and

lung [172]. NNN and NNK have been classified as “human carcinogens” (group 1) by the International Agency for Research on Cancer (IARC) [173].

Carboxymethylating/methylating agents associated with dietary nitrosating agents may play a potential role in gastrointestinal carcinogenesis. *N*-nitroso glycine derivatives are consistently mutagenic *in vitro* and *in vivo* [174]. As an example, *N*-nitrosoglycocholic acid (NOGC) was found to induce gastric cancer in experimental animals [175]. The formation of certain adducts in human DNA, for example O^6-carboxymethyl-2’-deoxyguanosine (O^6CMdG) and O^6-methyl-2’-deoxyguanosine (O^6MedG), has been assumed to be a consequence of the nitrosation of glycine (or glycine-containing substrates) (Figure 3) [176]. *N*-nitrosation of glycine results in the formation of diazoacetate or its analogues, giving rise to the formation of O^6-carboxymethylguanine (O^6-CMG). It has been reported that O^6-CMG adducts are not repaired by O^6-alkylguanine-DNA-alkyltransferases and may therefore accumulate in the DNA of gastrointestinal tract tissues [176]. The *in vitro* pattern of mutations in the tumor suppressor gene *p53* exposed to potassium diazoacetate has been found to be similar to the pattern of *p53* mutations observed in human gastrointestinal tumors [177]. Antibody-based assays have been developed for the detection of O^6-CMdG, including immunoaffinity-HPLC, immuno-slot-blot assays and immunohistochemistry [178-180]. More recently, sensitive mass spectrometry-based assays have become available [181]. Future prospective studies on colorectal cancer risk may use O^6-CMdG as a potential biomarker.

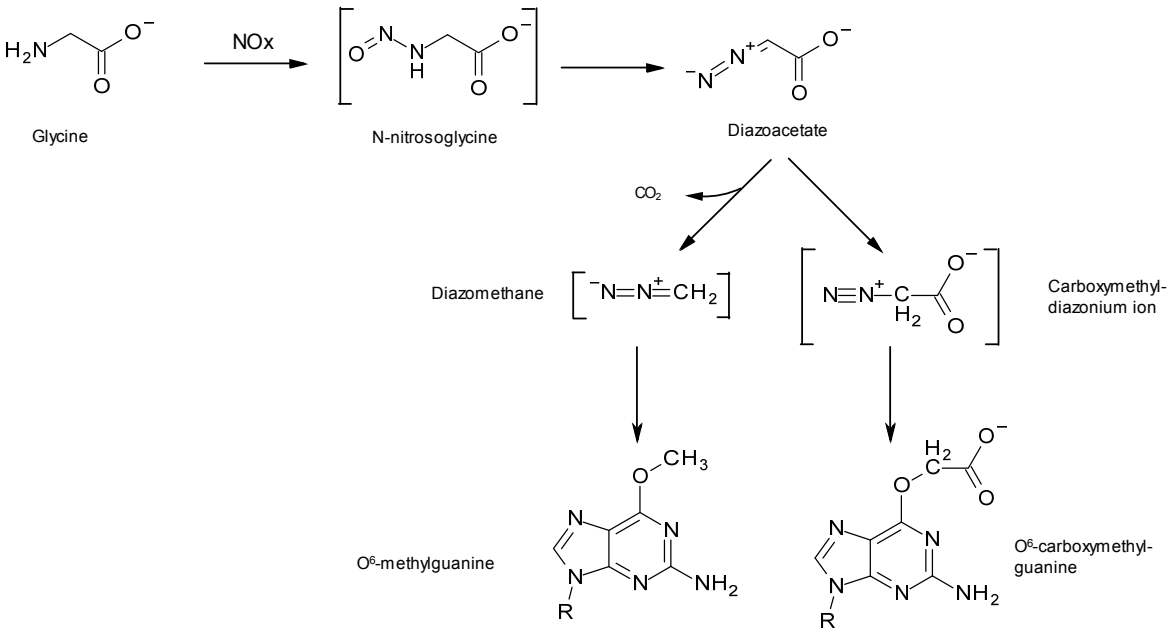


Figure 3: Nitrosation of glycine and formation of guanine adducts [180].

The endogenous nitrosation of 5,6-dihydrouracil (DHU), a physiological metabolite of pyrimidine bases (DNA, RNA) present in human urine and plasma, yields 1-nitroso-5,6-dihydrouracil [182], which is a powerful rat hepatocarcinogen [183]. Furthermore, it has been suggested that this NOC is involved in the formation of the DNA adduct 7-carboxyethylguanine (7-CEG) [184]. Rats fed 5,6-dihydrouracil or β-ureidopropionic acid together with nitrite in drinking water showed a significant increase of 7-CEG levels in hepatic DNA. This is indicative of an endogenous nitrosation, resulting in the formation of direct DNA damaging agents. *N*-methylnitrosamino propionic acid (MNPA) as well as acrylic acid, presumed to be a metabolite of acrolein, have also been proposed to contribute to the formation of 7-CEG adducts in human liver DNA. The latter is a common environmental contaminant and also is an endogenous product of lipid peroxidation [184]. Thus, the endogenous nitrosation of 5,6-dihydrouracil and methylamino propionic acid to the corresponding NOC as well as the endogenous and/or exogenous exposure to acrolein may therefore serve as sources for 7-CEG adducts found in human tissues.

The intragastric formation of NDMA after dietary nitrate intake was also investigated in the *in vitro* dynamic digestion model TIM1 simulating the upper gastrointestinal tract (TNO Nutrition and Food Research, Zeist, The Netherlands). It consists of a computer controlled *in vitro* flow-through system that mimics the physiological processes in the

human stomach and small intestine during digestion. After adding fish as a model food rich in amines together with nitrite to the stomach compartment, formation of NDMA was monitored. Under these *in vitro* conditions, a minimal amount of NDMA was formed. It was assessed to result in a margin of exposure (MOE, see section 5.3) higher than 100.000 [185, 186]. These results suggest that the assessment of nitrosamine formation in such gastrointestinal tract models using food rich in strongly basic amines most likely underestimates the risk associated with endogenous NOC formation, in line with the premise that strongly basic secondary or tertiary amines in food like di- or trimethylamine in fish are not efficiently nitrosated. The mean daily dietary intake of total primary and secondary amines has been reported to be 30 and 7 mg/day, respectively [187]. In addition, there is evidence for contribution of gut microbiota to endogenous NOC formation [40, 41, 188].

5.2 Structure/ activity of N-nitroso compounds

NOCs are a class of potent human carcinogens. The carcinogenic potential of NDMA was already described in 1956 [189], that of the homologue *N*-nitrosodiethylamine (NDEA) only a few years later [190]. In 1967, Druckrey and colleagues published a comprehensive study on the carcinogenicity as well as the structure-activity and dose-response relationship of 65 *N*-nitroso compounds in BD rats [191]. The outstanding carcinogenic potency of NDMA and NDEA has been compellingly established in comprehensive, lifelong animal studies [192-195]. Since bioactivation and interaction with critical cellular targets is similar in animals and humans, NOCs are regarded as presumed human carcinogens. The carcinogenic potential of NOCs depends on their structure. NDMA, NDEA and the tobacco specific NNK are amongst the most potent carcinogens.

Preformed and endogenously formed *N*-nitrosamines are well absorbed in the gastrointestinal tract [196-200]. The first step in the metabolic activation of NOCs is the hydroxylation at the α-C position, mediated by several cytochrome P450 monooxygenases (CYP450). The resulting α-hydroxy-*N*-nitrosamine, a proximal carcinogen, is unstable and rapidly dissociates into an aldehyde and a monoalkylnitrosamine, the latter rearranging into the corresponding diazonium intermediate. The diazonium electrophile can react with cellular macromolecules such as DNA, RNA, or protein, forming covalent adducts with appropriate nucleophilic

centers (see Figure 4). Metabolically generated reactive electrophilic compounds lead to the alkylation of DNA bases, mainly at the N^7, O^6 and N^3 positions of guanine, at the N^1, N^3 and N^7 positions of adenine, and at the O^2 and O^4 positions of thymine. The DNA damaging effect is generally accepted to represent the key event initiating a chain of biological responses finally leading to cancer. Amongst the different DNA base adducts, N^7-alkylguanine, in general, is predominant. However, O^6-alkylguanine as well as O^4- and O^2-alkylpyrimidine adducts, which cause DNA mismatches and miscoding, are more potent mutagenic lesions potentially leading to carcinogenesis.

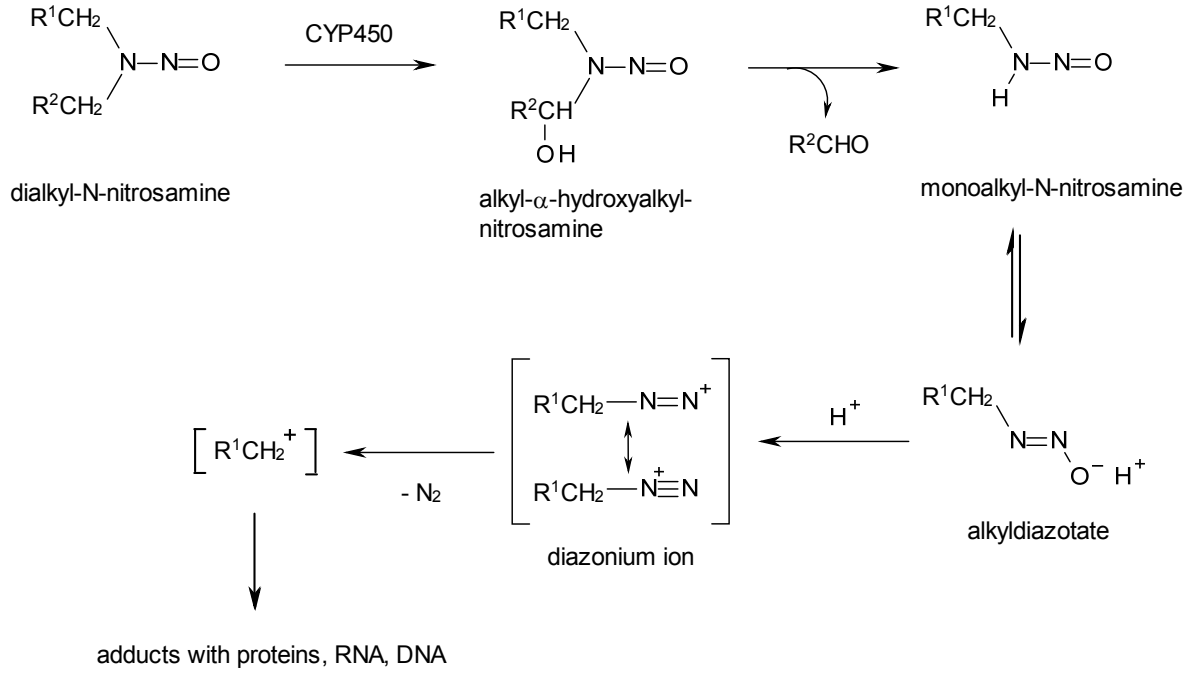


Figure 4: Metabolic activation of dialkyl-*N*-nitrosamine to an electrophilic alkylating agent [154].

The basic structural requirements for the carcinogenicity of NOCs have extensively been reviewed [201, 202]. Since the α-C position is crucial for metabolic activation, branching in this position reduces carcinogenicity. A tertiary substituent in this position completely inhibits carcinogenicity [201]. In addition, the chain length and symmetry of NOCs has a great influence on their organotropy. Symmetric NOCs mainly induce

tumours in the liver, whereas asymmetrically substituted NOCs lead to carcinomas in the oesophagus and/or the urinary bladder (longer side chains) [201, 202].

5.3 Risk assessment of nitrosamines, margin of exposure

Common NOCs have been ranked with respect to their carcinogenic potential by using dose descriptors (BMDL10, T25) prevailing in carcinogenic risk assessment [156, 203]. The Scientific Committee on Consumer Safety (SCCS) of the European Commission has used high quality data [192, 193, 195] in its dose response modelling [156]. The benchmark dose leading to a 10% tumour incidence in rats (BMD10 or BMDL10, taking into account the 95% lower confidence limit) revealed NDEA and NDMA to represent by far the most potent compounds, exhibiting BMDL10 values of 18 and 27 µg/kg b.w./d, respectively [156]. For comparison, BMDL10 values of other potent foodborne carcinogens have been reported to range between 120 (benzo[*a*]pyrene) and 480 µg/kg b.w./d (PhIP) [204]. These may be used to calculate a margin of exposure (MOE), which describes the ratio between human exposure and a dose level inducing a defined tumour response in animals (in this case 10% response). According to EFSA, a MOE of 10,000 or higher would be of low concern for health risk management [205]. Data on occurrence of NOCs in processed foods are rather outdated, being mostly from the 1980s to the 1990s. In these years, a significant reduction of NOC levels in foods and cosmetics was achieved due to appropriate mitigation measures. Human dietary intake of volatile NOCs in Germany has been estimated to be 0.2 – 0.3 µg/person/day [148] (3.3 – 5.0 ng/kg b.w./day based on a body weight of 60 kg). The MOE resulting from NDMA exposure can be assessed to be in the range from 5400 to 8200. Taking into account an additional exposure of 0.4 ng/kg b.w./d NDMA formed endogenously from food components, as estimated from an *in-vitro* gastrointestinal tract model [186], the MOE would marginally decrease to 5000 - 7300. Clearly, there is also a need to update the database on NOC levels in food in order to support assessment of overall exposure and risk. However, dimethylamine or diethylamine as potential precursors of NDMA or NDEA are not considered relevant indicators of endogenous NOC formation from foods. Therefore, adequate biomarkers still need to be identified.

5.4 Nitrate, nitrite, N-nitroso compounds and cancer

5.4.1 Animal Studies

The carcinogenicity of sodium nitrate has been investigated in a 2-year study [206]. Groups of 50 male and 50 female Fischer 344 rats, 8 weeks of age, were fed a diet containing 0, 2.5 or 5% sodium nitrate for 2 years, equivalent to 0, 1250 or 2500 mg sodium nitrate/kg b.w./day or 0, 910 or 1820 mg nitrate ion/kg b.w./day. The incidence of mononuclear cell leukaemia was reduced in treated groups when compared to controls (males: control group, 36%; low-dose group, 4%; high-dose group, 2%; females: control group, 28%; low-dose group, 0%; high-dose group, 2%). No significant differences were observed in the incidence of any other types of tumours. IARC reviewed the carcinogenicity data on nitrate and noted that Fischer 344 rats show a high incidence of spontaneous mononuclear cell leukaemia. It was concluded that there is inadequate evidence in experimental animals for the carcinogenicity of nitrate [207].

The carcinogenicity of sodium nitrite has been investigated by the U.S. National Toxicology Program in a 2-year rat and mouse study [208]. Groups of 50 male and 50 female rats were exposed to 0, 750, 1500 or 3000 ppm sodium nitrite (equivalent to average daily doses of approximately 0, 35, 70, or 130 mg sodium nitrite/kg b.w. in the case of males and 40, 80, or 150 mg sodium nitrite/kg bw in the case of females) in drinking water for 2 years. The NTP study concluded that there was no evidence of carcinogenic activity for sodium nitrite in male or female F344/N rats and in male $B6C3F_1$ mice [208]. Equivocal evidence of carcinogenic activity was reported for sodium nitrite in female $B6C3F_1$ mice, based on the positive trend in the incidences of squamous cell papilloma or carcinoma (combined) in the forestomach [208]. IARC reviewed the carcinogenicity of nitrite and concluded that there is limited evidence in experimental animals for the carcinogenicity of nitrite *per se* [207]. However, IARC also stated that there is sufficient evidence in experimental animals for the carcinogenicity of nitrite in combination with amines or amides [207].

5.4.2 Evidence in humans

The relevance of the endogenous formation of NOCs for human carcinogenesis remains a matter of debate. Experimental models demonstrating that NOCs can induce tumours in the gastrointestinal tract are available, but there is little evidence that exposure to such compounds is directly involved in the induction of such tumors in

humans. Indeed, many NOCs do not directly lead to malignant cell transformation. Instead, they need to be absorbed to become metabolically activated, thereby inducing cancer at sites distant from the site of formation or incorporation. Still, *in vitro* studies have shown that mutations induced by compounds generated by endogenous nitrosation are similar to those found in colorectal tumours, supporting the hypothesis that endogenous nitrosation may be one causative mechanism. IARC concluded that ingested nitrate or nitrite under conditions that result in endogenous nitrosation is probably carcinogenic to humans (Group 2A) [207].

More recent evidence reported a significant association between urinary NOC excretion and micronuclei frequency in human lymphocytes as well as gene expression changes in NOC-exposed Caco-2 cells associated with malignant cell transformation [209, 210]. Whole blood transcriptomes of 30 individuals were screened for transcription patterns correlating with NOC exposure markers and micronuclei frequency in lymphocytes [210]. In a network analysis, various genes were analyzed as potential transcriptomic biomarkers. The association between NOC excretion and micronuclei frequency suggested that an increased cancer risk for humans exposed to NOCs may exist. If confirmed, the identified genes may be used in future studies as genetic markers for NOC exposure. Gene expression analysis in colon biopsies of inflammatory bowel disease and irritable bowel syndrome patients demonstrated a correlation between fecal NOC levels and gene expression changes [211]. Genes associated with chromatin assembly were found to be negatively or positively correlated with the level of apparent total nitroso compounds (ATNC). Intake of red meat was found to be associated with increased fecal water genotoxicity and with gene expression changes of certain potentially relevant signaling pathways. These include protein kinase A, cytoskeleton reorganization, the WNT signaling pathway and small ubiquitin-related modifiers (SUMO), significant regulators of the response to DNA damage [212]. Taken together, the exposure of Caco-2 cells to NOCs leads to changes in gene expression associated with malignant cell transformation, while the incubation of human blood cells with NOCs induced the formation of micronuclei, a biomarker of DNA damage.

5.4.3 Red/processed meat

The consumption of red and processed meat may result in an intake-dependent endogenous formation of ATNC [213, 214]. The main contributors to ATNC formation following the consumption of a high meat diet appear to be nitrosyl heme and nitroso thiols [215]. Major sources for dietary heme are roast beef, steaks, hamburgers, sausages and ham. The amount of ATNC in feces was found to correlate with red meat intake, and additional heme intake further increased the ATNC level [216]. While it is known that free thiol and heme groups are necessary for NOCs to be formed, the mechanisms underlying the meat-induced endogenous formation of NOCs have not yet been completely elucidated. Both nitrosyl heme and nitroso thiols are known to act as NO donors and both can act as nitrosating agents [217, 218]. Therefore, these compounds probably stimulate the formation of NOCs or highly reactive alkylating intermediates, which eventually may lead to DNA damage.

In some human dietary intervention studies, a strong correlation between endogenous NOC formation and the presence of O^6-carboxymethylguanine adducts has been reported [180]. In fact, O^6-CMG adducts were detected in exfoliated colonic cells of volunteers consuming high amounts of red meat and the adduct levels were significantly higher than those of volunteers on a vegetarian diet. In this context it should be noted that *N*-nitrosation of glycine leads to the formation of diazoacetate or its analogues, which may give rise to the formation of O^6-CMG. A study in 185 archived colorectal cancer tumour samples (EPIC Norfolk) observed a strong association between meat intake and GC-to-AT transition mutations [219]. These data support the hypothesis that endogenous nitrosation reactions leading to the formation of alkylating agents and DNA damage are likely to contribute to the association found between the consumption of red and processed meat and colon cancer risk. The potential role of red/processed meat in colorectal cancer development has recently been reviewed [220].

Prospective cohort studies suggest an association of red meat and nitrite-preserved meat intake with an increased risk for colon cancer [215, 221-224]. One of the studies used the outcome of a continuing survey of food intake in the United States to create meat categories for smoked and processed meat. The content of nitrate and nitrite was estimated by using chemical analysis, standard recipes for meat processing and

literature values. The data of a food frequency questionnaire regarding intakes of processed meat and residual nitrate and nitrite were used to estimate the nitrate and nitrite intake [225-227]. In a large prospective cohort study (NIH-AARP) the role of red and processed meat, nitrate and nitrite in cancer development was assessed. Elevated risks were reported for red and processed meat (nitrite) intake in association with colorectal cancer [228], and for nitrate/nitrite intake in association with several types of human cancer including the thyroid gland, ovary, kidney and bladder [227, 229-232]. No associations were reported for other cancer types [222, 229-231, 233, 234]. Findings were controversial for glioma [235-237]. In the EPIC study, endogenous NOCs (calculated from iron intake with meat and fecal ATNC formation) and dietary nitrite were not significantly associated with cancer risk [238, 239].

In conclusion, the epidemiological evidence regarding the role of endogenous NOC formation for human cancer risk is inconsistent. Obviously, there is a need for more elaborate studies, which make use of appropriate biomarkers and also include those determined by applying “omics” technologies, in order to establish causality for the association.

Future comprehensive studies need to take into consideration, among other parameters, nitrate/nitrite exposure and the extent of NOC formation *in vivo*, the nature and relevance of *N*-nitrosatable precursors and the resulting NOCs, the influence of individual dietary and physiologic factors, but also the individual health status, especially with respect to those conditions favoring endogenous NOC formation including inflammatory and/or infectious diseases.

Such comprehensive studies, preferentially in humans, may be integrated into well designed prospective (molecular) epidemiologic studies or be carried out as double blind, randomized controlled intervention studies. Long-term as well as short-term health effects need to be considered.

6 Conclusions, recommendations and research needs with respect to risk/ benefit assessment

The SKLM is of the opinion that there is a need for further research addressing potentially negative and positive health effects associated with dietary nitrate and

nitrite exposure. The available evidence is inadequate to be used as a basis for a comprehensive and reliable assessment of positive as well as negative health effects, especially regarding long-term effects. Human intervention studies should be undertaken, making use of appropriate biomarkers for potentially detrimental or beneficial health effects. Such studies also need to consider individual differences such as age and gender, genetic background and health status as well as the role of infections and inflammatory processes.

6.1 Biomarkers reflecting nitrate/nitrite associated beneficial/adverse effects

To allow an adequate evaluation of the consequences of a dietary exposure to nitrate/nitrite for human health, an array of predictive and reliable biomarkers should be developed. Regarding beneficial effects related to the intake of nitrate/nitrite, monitoring of mean diastolic blood pressure is proposed as a surrogate short-term biomarker predictive for long-term beneficial effects, especially reduced mortality due to cardiovascular diseases (CVD). In addition to blood pressure, further biomarkers related to CVD risk, such as those reflecting effects on circulation/blood flow, platelet adhesion/aggregation and maintenance of vessel tonus, should also be taken into consideration.

At the same time, there is an urgent need to adequately characterize the potential health risk associated with an enhanced dietary nitrate intake and to develop biomarkers indicative of endogenous as well as exogenous NOC exposure. As an easily accessible surrogate biomarker for overall endogenous N-nitrosation, the urinary excretion of non-carcinogenic *N*-nitroso-amino acids may be utilized. However, it needs to be established to what extent monitoring of urinary non-carcinogenic *N*-nitroso amino acids can also be taken as a biomarker for the endogenous formation of carcinogenic NOCs or related genotoxic agents. Potentially negative effects of NOCs are mainly considered to result from genotoxic DNA damage resulting in mutations and in the end in cancer. To corroborate the correlation of such biomarkers with the risk resulting from the *in vivo* generation of carcinogenic NOCs or corresponding genotoxic intermediates, specific DNA adducts as indicators of genotoxic damage by NOCs in human blood leucocytes or biopsy samples need to be monitored. Moreover, specific transcriptomic responses as indicators of genotoxic damage and subsequent biological responses, including DNA repair and mutation induction, should be

investigated in human blood and/or tissue samples and assessed for their value as predictive biomarkers.

The SKLM realizes that the predictive power of these biomarkers will have to be validated in the future by appropriate prospective molecular epidemiology studies.

6.2 Human intervention studies

The SKLM is of the opinion that well designed dietary intervention studies constitute an essential step in the process of accomplishing a reliable risk / benefit assessment with respect to the long-term human health effects of nitrate/nitrite. Such human intervention studies should be performed in subpopulations at an enhanced health risk, for example in slightly hypertensive individuals. It is known that a reduction of 5 mm Hg is sufficient to significantly reduce the long-term risk of developing cardiovascular diseases. An intervention study in which volunteers ingest nitrate/nitrite at a level appropriate to achieve a reliably measurable and significantly beneficial reduction of the mean diastolic blood pressure may be envisaged.

6.3 Specific research needs

The outcome of human studies should constitute an essential part of a comprehensive data base to be established on nitrate/nitrite related beneficial as well as detrimental health effects and their dose dependency. In order to achieve this objective, particular attention should be given to the following issues:

- to deduce potentially minimal effective doses of dietary nitrate that can significantly reduce blood pressure, e.g. from intervention studies in slightly hypertensive subpopulations;
- to develop biomarkers for further potentially beneficial long-term effects;
- to identify specific transcriptomic responses as an indication of short-/long-term human health effects;
- to establish biomarkers that reflect the endogenous formation of carcinogenic NOCs;
- to explore the influence of the health status, especially bacterial infections and inflammatory diseases, on biomarker response;

- to update the database on human dietary intake of nitrate/nitrite and especially NOC;
- to explore the (endogenous) nitrosation kinetics of an array of amino compounds, which reflect the range of chemicals humans are exposed to and can plausibly be expected to act as precursors for carcinogenic NOC and/or to give rise to toxicologically relevant amounts of genotoxic electrophils *in vivo;*
- to more firmly establish the relationship between overall NOC exposure, both from endogenous and exogenous sources, and induction of cancer;
- to establish methods to quantify risks as well as benefits to enable a reliable risk/benefit assessment.

7 References

[1] SKLM, in: Forschungsgemeinschaft, D. (Ed.), *Lebensmittel und Gesundheit: Sammlung der Beschlüsse, Stellungnahmen und Verlautbarungen aus den Jahren 1984 bis 1996*, Senatskommission zur Bewertung der gesundheitlichen Unbedenklichkeit von Lebensmitteln (SKLM), Wiley VCH, Weinheim 1998, pp. 77-86.

[2] WCRF/AICR, Expert Report,Food, Nutrition, Physical Activity and the Prevention of Cancer: A Global Perspective. 2007.

[3] Weitzberg, E., Lundberg, J. O., Novel Aspects of Dietary Nitrate and Human Health. *Annual review of nutrition* 2013.

[4] Gilchrist, M., Shore, A. C., Benjamin, N., Inorganic nitrate and nitrite and control of blood pressure. *Cardiovascular research* 2011, *89*, 492-498.

[5] Hord, N. G., Tang, Y. P., Bryan, N. S., Food sources of nitrates and nitrites: the physiologic context for potential health benefits. *American Journal of Clinical Nutrition* 2009, *90*, 1-10.

[6] Machha, A., Schechter, A. N., Dietary nitrite and nitrate: a review of potential mechanisms of cardiovascular benefits. *Eur J Nutr* 2011, *50*, 293-303.

[7] Lidder, S., Webb, A. J., Vascular effects of dietary nitrate (as found in green leafy vegetables and beetroot) via the nitrate-nitrite-nitric oxide pathway. *Brit J Clin Pharmaco* 2013, *75*, 677-696.

[8] Greer, F. R., Shannon, M., Infant methemoglobinemia: the role of dietary nitrate in food and water. *Pediatrics* 2005, *116*, 784-786.

[9] EFSA, Opinion of the Scientific Panel on Contaminants in the Food chain on a request from the European Commission to perform a scientific risk assessment on nitrate in vegetables. *The EFSA Journal* 2008, *689*, 1-79.

[10] European Commission, E. C., Opinion on nitrate and nitrite. *Reports of the Scientific Committee for Food (SCF)* 1992, *26*, 21-28.

[11] European Commission, E. C., Opinion on nitrate and nitrite. *Reports of the Scientific Committee for Food (SCF)* 1997, *38*.

[12] FAO/WHO, Nitrate (and potential endogenous formation of N-nitroso compounds). *In: WHO Food Additive series World Health Organosation, Geneva* 2003, *50*.

[13] EFSA, Statement on nitrites in meat products. *EFSA Journal* 2010, *8*, 12.

[14] FAO/WHO, Nitrite (and potential endogenous formation of N-nitroso compounds). *In: WHO Food Additive series World Health Organosation, Geneva* 2003, *50*.

[15] Caulfield, J. L., Wishnok, J. S., Tannenbaum, S. R., Nitric oxide-induced deamination of cytosine and guanine in deoxynucleosides and oligonucleotides. *J Biol Chem* 1998, *273*, 12689-12695.

[16] Burney, S., Caulfield, J. L., Niles, J. C., Wishnok, J. S., Tannenbaum, S. R., The chemistry of DNA damage from nitric oxide and peroxynitrite. *Mutation research* 1999, *424*, 37-49.

[17] Eiserich, J. P., Patel, R. P., O'Donnell, V. B., Pathophysiology of nitric oxide and related species: free radical reactions and modification of biomolecules. *Molecular aspects of medicine* 1998, *19*, 221-357.

[18] Gal, A., Tamir, S., Kennedy, L. J., Tannenbaum, S. R., Wogan, G. N., Nitrotyrosine formation, apoptosis, and oxidative damage: relationships to nitric oxide production in SJL mice bearing the RcsX tumor. *Cancer research* 1997, *57*, 1823-1828.

[19] Green, L. C., Deluzuriaga, K. R., Wagner, D. A., Rand, W., *et al.*, Nitrate Biosynthesis in Man. *P Natl Acad Sci-Biol* 1981, *78*, 7764-7768.

[20] Tannenbaum, S. R., Fett, D., Young, V. R., Land, P. D., Bruce, W. R., Nitrite and Nitrate Are Formed by Endogenous Synthesis in Human Intestine. *Science* 1978, *200*, 1487-1489.

[21] Weitzberg, E., Lundberg, J. O., Nonenzymatic nitric oxide production in humans. *Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society* 1998, *2*, 1-7.

[22] Reutov, V. P., Sorokina, E. G., NO-synthase and nitrite-reductase components of nitric oxide cycle. *Biochemistry. Biokhimiia* 1998, *63*, 874-884.
[23] Lundberg, J. O., Govoni, M., Inorganic nitrate is a possible source for systemic generation of nitric oxide. *Free radical biology & medicine* 2004, *37*, 395-400.
[24] Zhang, Z., Naughton, D., Winyard, P. G., Benjamin, N., *et al.*, Generation of nitric oxide by a nitrite reductase activity of xanthine oxidase: a potential pathway for nitric oxide formation in the absence of nitric oxide synthase activity. *Biochemical and biophysical research communications* 1998, *249*, 767-772.
[25] Gladwin, M. T., Cosby, K., Partovi, K., Martyr, S., *et al.*, Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilalates the human circulation. *Circulation* 2003, *108*, 20-21.
[26] Marletta, M. A., Yoon, P. S., Iyengar, R., Leaf, C. D., Wishnok, J. S., Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. *Biochemistry* 1988, *27*, 8706-8711.
[27] Leaf, C. D., Wishnok, J. S., Tannenbaum, S. R., L-arginine is a precursor for nitrate biosynthesis in humans. *Biochemical and biophysical research communications* 1989, *163*, 1032-1037.
[28] Wennmalm, A., Benthin, G., Edlund, A., Jungersten, L., *et al.*, Metabolism and Excretion of Nitric-Oxide in Humans - an Experimental and Clinical-Study. *Circ Res* 1993, *73*, 1121-1127.
[29] Van den Brandt, P. A., Willett, W. C., Tannenbaum, S. R., Assessment of dietary nitrate intake by a self-administered questionnaire and by overnight urinary measurement. *International journal of epidemiology* 1989, *18*, 852-857.
[30] Moncada, S., Higgs, A., The L-arginine-nitric oxide pathway. *The New England journal of medicine* 1993, *329*, 2002-2012.
[31] Hattori, R., Sase, K., Eizawa, H., Kosuga, K., *et al.*, Structure and function of nitric oxide synthases. *International journal of cardiology* 1994, *47*, S71-75.
[32] Schaus, R., Griess' nitrite test in diagnosis of urinary infection. *Journal of the American Medical Association* 1956, *161*, 528-529.
[33] Ohshima, H., Bandaletova, T. Y., Brouet, I., Bartsch, H., *et al.*, Increased nitrosamine and nitrate biosynthesis mediated by nitric oxide synthase induced in hamsters infected with liver fluke (Opisthorchis viverrini). *Carcinogenesis* 1994, *15*, 271-275.
[34] Bartsch, H., Ohshima, H., Pignatelli, B., Calmels, S., Endogenously formed N-nitroso compounds and nitrosating agents in human cancer etiology. *Pharmacogenetics* 1992, *2*, 272-277.
[35] Stuehr, D. J., Marletta, M. A., Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to Escherichia coli lipopolysaccharide. *Proceedings of the National Academy of Sciences of the United States of America* 1985, *82*, 7738-7742.
[36] Hofseth, L. J., Hussain, S. P., Wogan, G. N., Harris, C. C., Nitric oxide in cancer and chemoprevention. *Free radical biology & medicine* 2003, *34*, 955-968.
[37] Hussain, S. P., He, P., Subleski, J., Hofseth, L. J., *et al.*, Nitric oxide is a key component in inflammation-accelerated tumorigenesis. *Cancer research* 2008, *68*, 7130-7136.
[38] Srivatanakul, P., Ohshima, H., Khlat, M., Parkin, M., *et al.*, Opisthorchis viverrini infestation and endogenous nitrosamines as risk factors for cholangiocarcinoma in Thailand. *International journal of cancer. Journal international du cancer* 1991, *48*, 821-825.
[39] Calmels, S., Ohshima, H., Crespi, M., Leclerc, H., *et al.*, N-nitrosamine formation by microorganisms isolated from human gastric juice and urine: biochemical studies on bacteria-catalysed nitrosation. *IARC scientific publications* 1987, 391-395.
[40] Calmels, S., Ohshima, H., Rosenkranz, H., McCoy, E., Bartsch, H., Biochemical studies on the catalysis of nitrosation by bacteria. *Carcinogenesis* 1987, *8*, 1085-1088.
[41] Leach, S. A., Thompson, M., Hill, M., Bacterially catalysed N-nitrosation reactions and their relative importance in the human stomach. *Carcinogenesis* 1987, *8*, 1907-1912.

[42] Tricker, A. R., Kalble, T., Preussmann, R., Increased urinary nitrosamine excretion in patients with urinary diversions. *Carcinogenesis* 1989, *10*, 2379-2382.
[43] Tricker, A. R., Stickler, D. J., Chawla, J. C., Preussmann, R., Increased urinary nitrosamine excretion in paraplegic patients. *Carcinogenesis* 1991, *12*, 943-946.
[44] Correa, P., Haenszel, W., Cuello, C., Zavala, D., *et al.*, Gastric precancerous process in a high risk population: cross-sectional studies. *Cancer research* 1990, *50*, 4731-4736.
[45] Akuta, T., Zaki, M. H., Yoshitake, J., Okamoto, T., Akaike, T., Nitrative stress through formation of 8-nitroguanosine: insights into microbial pathogenesis. *Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society* 2006, *14*, 101-108.
[46] Dedon, P. C., Tannenbaum, S. R., Reactive nitrogen species in the chemical biology of inflammation. *Archives of biochemistry and biophysics* 2004, *423*, 12-22.
[47] Sawa, T., Ohshima, H., Nitrative DNA damage in inflammation and its possible role in carcinogenesis. *Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society* 2006, *14*, 91-100.
[48] Spiegelhalder, B., Eisenbrand, G., Preussmann, R., Influence of dietary nitrate on nitrite content of human saliva: possible relevance to in vivo formation of N-nitroso compounds. *Food and cosmetics toxicology* 1976, *14*, 545-548.
[49] Tannenbaum, S. R., Weisman, M., Fett, D., Effect of Nitrate Intake on Nitrite Formation in Human Saliva. *Food and cosmetics toxicology* 1976, *14*, 549-552.
[50] Qin, L., Liu, X., Sun, Q., Fan, Z., *et al.*, Sialin (SLC17A5) functions as a nitrate transporter in the plasma membrane. *Proceedings of the National Academy of Sciences of the United States of America* 2012, *109*, 13434-13439.
[51] Eisenbrand, G., Spiegelhalder, B., Preussmann, R., Nitrate and nitrite in saliva. *Oncology-Basel* 1980, *37*, 227-231.
[52] Eisenbrand, G., in: Nicolai, H. V., Eisenbrand, G., Bozler, G. (Eds.), *The Significance of N-Nitrosation of Drugs*, Gustav Fischer Verlag, Stuttgart, New York 1990, pp. 47-69.
[53] Lundberg, J. O., Weitzberg, E., Lundberg, J. M., Alving, K., Intragastric nitric oxide production in humans: measurements in expelled air. *Gut* 1994, *35*, 1543-1546.
[54] Benjamin, N., O'Driscoll, F., Dougall, H., Duncan, C., *et al.*, Stomach NO synthesis. *Nature* 1994, *368*, 502.
[55] Govoni, M., Jansson, E. A., Weitzberg, E., Lundberg, J. O., The increase in plasma nitrite after a dietary nitrate load is markedly attenuated by an antibacterial mouthwash. *Nitric Oxide-Biol Ch* 2008, *19*, 333-337.
[56] Jansson, E. A., Huang, L., Malkey, R., Govoni, M., *et al.*, A mammalian functional nitrate reductase that regulates nitrite and nitric oxide homeostasis. *Nature chemical biology* 2008, *4*, 411-417.
[57] Gladwin, M. T., Cosby, K., Partovi, K. S., Patel, R. P., *et al.*, Novel function of human hemoglobin as a nitrite reductase regulates nitric oxide homeostasis and hypoxic vasodilation. *Blood* 2003, *102*, 255a-255a.
[58] Gladwin, M. T., Schechter, A. N., Kim-Shapiro, D. B., Patel, R. P., *et al.*, The emerging biology of the nitrite anion. *Nature chemical biology* 2005, *1*, 308-314.
[59] van Faassen, E. E., Bahrami, S., Feelisch, M., Hogg, N., *et al.*, Nitrite as regulator of hypoxic signaling in mammalian physiology. *Medicinal research reviews* 2009, *29*, 683-741.
[60] Shiva, S., Huang, Z., Grubina, R., Sun, J. H., *et al.*, Deoxymyoglobin is a nitrite reductase that generates nitric oxide and regulates mitochondrial respiration. *Circ Res* 2007, *100*, 654-661.
[61] Cosby, K., Partovi, K. S., Crawford, J. H., Patel, R. P., *et al.*, Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. *Nature medicine* 2003, *9*, 1498-1505.

[62] Nagababu, E., Ramasamy, S., Abernethy, D. R., Rifkind, J. M., Active nitric oxide produced in the red cell under hypoxic conditions by deoxyhemoglobin-mediated nitrite reduction. *J Biol Chem* 2003, *278*, 46349-46356.
[63] Baliga, R. S., Milsom, A. B., Ghosh, S. M., Trinder, S. L., *et al.*, Dietary Nitrate Ameliorates Pulmonary Hypertension Cytoprotective Role for Endothelial Nitric Oxide Synthase and Xanthine Oxidoreductase. *Circulation* 2012, *125*, 2922-2932.
[64] Millar, T. M., Stevens, C. R., Blake, D. R., Xanthine oxidase can generate nitric oxide from nitrate in ischaemia. *Biochem Soc Trans* 1997, *25*, 528S.
[65] Huang, Z., Shiva, S., Kim-Shapiro, D. B., Patel, R. P., *et al.*, Enzymatic function of hemoglobin as a nitrite reductase that produces NO under allosteric control. *The Journal of clinical investigation* 2005, *115*, 2099-2107.
[66] Moncada, S., Palmer, R. M., Higgs, E. A., Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacological reviews* 1991, *43*, 109-142.
[67] Rees, D. D., Palmer, R. M. J., Moncada, S., Role of Endothelium-Derived Nitric-Oxide in the Regulation of Blood-Pressure. *Proceedings of the National Academy of Sciences of the United States of America* 1989, *86*, 3375-3378.
[68] Ceccatelli, S., Lundberg, J. M., Fahrenkrug, J., Bredt, D. S., *et al.*, Evidence for involvement of nitric oxide in the regulation of hypothalamic portal blood flow. *Neuroscience* 1992, *51*, 769-772.
[69] Palmer, R. M., Ferrige, A. G., Moncada, S., Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 1987, *327*, 524-526.
[70] Radomski, M. W., Palmer, R. M., Moncada, S., Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium. *Lancet* 1987, *2*, 1057-1058.
[71] Radomski, M. W., Palmer, R. M., Moncada, S., The anti-aggregating properties of vascular endothelium: interactions between prostacyclin and nitric oxide. *British journal of pharmacology* 1987, *92*, 639-646.
[72] Rand, M. J., Nitrergic transmission: nitric oxide as a mediator of non-adrenergic, non-cholinergic neuro-effector transmission. *Clinical and experimental pharmacology & physiology* 1992, *19*, 147-169.
[73] Bredt, D. S., Snyder, S. H., Nitric oxide, a novel neuronal messenger. *Neuron* 1992, *8*, 3-11.
[74] Garthwaite, J., Boulton, C. L., Nitric-Oxide Signaling in the Central-Nervous-System. *Annu Rev Physiol* 1995, *57*, 683-706.
[75] Larsen, F. J., Schiffer, T. A., Weitzberg, E., Lundberg, J. O., Regulation of mitochondrial function and energetics by reactive nitrogen oxides. *Free radical biology & medicine* 2012, *53*, 1919-1928.
[76] Ceccatelli, S., Hulting, A. L., Zhang, X., Gustafsson, L., *et al.*, Nitric oxide synthase in the rat anterior pituitary gland and the role of nitric oxide in regulation of luteinizing hormone secretion. *Proceedings of the National Academy of Sciences of the United States of America* 1993, *90*, 11292-11296.
[77] Langrehr, J. M., Hoffman, R. A., Lancaster, J. R., Simmons, R. L., Nitric-Oxide - a New Endogenous Immunomodulator. *Transplantation* 1993, *55*, 1205-1212.
[78] Wei, X. Q., Charles, I. G., Smith, A., Ure, J., *et al.*, Altered Immune-Responses in Mice Lacking Inducible Nitric-Oxide Synthase. *Nature* 1995, *375*, 408-411.
[79] Green, S. J., Scheller, L. F., Marletta, M. A., Seguin, M. C., *et al.*, Nitric oxide: cytokine-regulation of nitric oxide in host resistance to intracellular pathogens. *Immunology letters* 1994, *43*, 87-94.
[80] Vielma, A. H., Retamal, M. A., Schmachtenberg, O., Nitric oxide signaling in the retina: what have we learned in two decades? *Brain Res* 2012, *1430*, 112-125.
[81] Carnovale, C. E., Ronco, M. T., Role of nitric oxide in liver regeneration. *Annals of hepatology* 2012, *11*, 636-647.

[82] Liu, Y., Feng, Q., NOing the heart: role of nitric oxide synthase-3 in heart development. *Differentiation; research in biological diversity* 2012, *84*, 54-61.
[83] Heinrich, U. R., Helling, K., Nitric oxide--a versatile key player in cochlear function and hearing disorders. *Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society* 2012, *27*, 106-116.
[84] Steinberg, A., Nilsson Remahl, A. I., Role of nitric oxide in cluster headache. *Current pain and headache reports* 2012, *16*, 185-190.
[85] Grasemann, H., Ratjen, F., Nitric oxide and L-arginine deficiency in cystic fibrosis. *Current pharmaceutical design* 2012, *18*, 726-736.
[86] Kroncke, K. D., Fehsel, K., Suschek, C., Kolb-Bachofen, V., Inducible nitric oxide synthase-derived nitric oxide in gene regulation, cell death and cell survival. *International immunopharmacology* 2001, *1*, 1407-1420.
[87] Messmer, U. K., Brune, B., Nitric oxide-induced apoptosis: p53-dependent and p53-independent signalling pathways. *Biochem J* 1996, *319 (Pt 1)*, 299-305.
[88] Burke, A. J., Sullivan, F. J., Giles, F. J., Glynn, S. A., The yin and yang of nitric oxide in cancer progression. *Carcinogenesis* 2013, *34*, 503-512.
[89] Mocellin, S., Nitric oxide: cancer target or anticancer agent? *Current cancer drug targets* 2009, *9*, 214-236.
[90] Xu, W., Liu, L. Z., Loizidou, M., Ahmed, M., Charles, I. G., The role of nitric oxide in cancer. *Cell research* 2002, *12*, 311-320.
[91] Morcos, E., Carlsson, S., Weitzberg, E., Wiklund, N. P., Lundberg, J. O., Inhibition of cancer cell replication by inorganic nitrite. *Nutrition and cancer* 2010, *62*, 501-504.
[92] Dejam, A., Hunter, C. J., Gladwin, M. T., Effects of dietary nitrate on blood pressure. *New Engl J Med* 2007, *356*, 1590-1590.
[93] Kapil, V., Milsom, A. B., Okorie, M., Maleki-Toyserkani, S., *et al.*, Inorganic Nitrate Supplementation Lowers Blood Pressure in Humans Role for Nitrite-Derived NO. *Hypertension* 2010, *56*, 274-U174.
[94] Larsen, F. J., Ekblom, B., Sahlin, K., Lundberg, J. O., Weitzberg, E., Effects of dietary nitrate on blood pressure in healthy volunteers. *The New England journal of medicine* 2006, *355*, 2792-2793.
[95] Gladwin, M. T., Shelhamer, J. H., Schechter, A. N., Pease-Fye, M. E., *et al.*, Role of circulating nitrite and S-nitrosohemoglobin in the regulation of regional blood flow in humans. *Proceedings of the National Academy of Sciences of the United States of America* 2000, *97*, 11482-11487.
[96] Lundberg, J. O., Weitzberg, E., NO generation from nitrite and its role in vascular control. *Arteriosclerosis, thrombosis, and vascular biology* 2005, *25*, 915-922.
[97] Maher, A. R., Thomas, P., Anderson, S., Abozguia, K., *et al.*, Intra-arterial nitrite causes an enduring vasodilation in man. *Journal of the American College of Cardiology* 2008, *51*, A284-A284.
[98] Velmurugan, S., Pearl, V., Davies, S., Bahra, M., *et al.*, Inorganic nitrate supplementation attenuates platelet reactivity in healthy male volunteers. *Nitric Oxide-Biol Ch* 2012, *27*, S27-S28.
[99] Jung, K. H., Chu, K., Ko, S. Y., Lee, S. T., *et al.*, Early intravenous infusion of sodium nitrite protects brain against in vivo ischemia-reperfusion injury. *Stroke* 2006, *37*, 2744-2750.
[100] Webb, A., Bond, R., McLean, P., Uppal, R., *et al.*, Reduction of nitrite to nitric oxide during ischemia protects against myocardial ischemia-reperfusion damage. *Proceedings of the National Academy of Sciences of the United States of America* 2004, *101*, 13683-13688.
[101] Duranski, M. R., Greer, J. J., Dejam, A., Jaganmohan, S., *et al.*, Cytoprotective effects of nitrite during in vivo ischemia-reperfusion of the heart and liver. *The Journal of clinical investigation* 2005, *115*, 1232-1240.

[102] Tripatara, P., Patel, N. S. A., Webb, A., Rathod, K., *et al.*, Nitrite-derived nitric oxide protects the rat kidney against ischemia/reperfusion injury in vivo: Role for xanthine oxidoreductase. *J Am Soc Nephrol* 2007, *18*, 570-580.
[103] Hendgen-Cotta, U. B., Luedike, P., Totzeck, M., Kropp, M., *et al.*, Dietary nitrate supplementation improves revascularization in chronic ischemia. *Circulation* 2012, *126*, 1983-1992.
[104] Dejam, A., Hunter, C. J., Tremonti, C., Pluta, R. M., *et al.*, Nitrite infusion in humans and nonhuman primates - Endocrine effects, pharmacokinetics, and tolerance formation. *Circulation* 2007, *116*, 1821-1831.
[105] Heiss, C., Meyer, C., Totzeck, M., Hendgen-Cotta, U. B., *et al.*, Dietary inorganic nitrate mobilizes circulating angiogenic cells. *Free radical biology & medicine* 2012, *52*, 1767-1772.
[106] Webb, A. J., Patel, N., Loukogeorgakis, S., Okorie, M., *et al.*, Acute blood pressure lowering, vasoprotective, and antiplatelet properties of dietary nitrate via bioconversion to nitrite. *Hypertension* 2008, *51*, 784-790.
[107] Aboud, Z., Misra, S., Warner, T., Miall, P., *et al.*, The enterosalivary bioconversion of nitrate to nitrite underlies the blood pressure (BP) lowering and anti-platelet effects of a dietary nitrate load. *Brit J Clin Pharmaco* 2008, *65*, 999-999.
[108] Richardson, G., Hicks, S. L., O'Byrne, S., Frost, M. T., *et al.*, The ingestion of inorganic nitrate increases gastric S-nitrosothiol levels and inhibits platelet function in humans. *Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society* 2002, *7*, 24-29.
[109] Carlstrom, M., Persson, A. E., Larsson, E., Hezel, M., *et al.*, Dietary nitrate attenuates oxidative stress, prevents cardiac and renal injuries, and reduces blood pressure in salt-induced hypertension. *Cardiovascular research* 2011, *89*, 574-585.
[110] Bjorne, H. H., Petersson, J., Phillipson, M., Weitzberg, E., *et al.*, Nitrite in saliva increases gastric mucosal blood flow and mucus thickness. *The Journal of clinical investigation* 2004, *113*, 106-114.
[111] Petersson, J., Phillipson, M., Jansson, E. A., Patzak, A., *et al.*, Dietary nitrate increases gastric mucosal blood flow and mucosal defense. *American journal of physiology. Gastrointestinal and liver physiology* 2007, *292*, G718-724.
[112] Carlstrom, M., Larsen, F. J., Nystrom, T., Hezel, M., *et al.*, Dietary inorganic nitrate reverses features of metabolic syndrome in endothelial nitric oxide synthase-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America* 2010, *107*, 17716-17720.
[113] Fernandez, M. L., Ruiz, R., Gonzalez, M. A., Ramirez-Lorca, R., *et al.*, Association of NOS3 gene with metabolic syndrome in hypertensive patients. *Thrombosis and haemostasis* 2004, *92*, 413-418.
[114] Huang, P. L., eNOS, metabolic syndrome and cardiovascular disease. *Trends in endocrinology and metabolism: TEM* 2009, *20*, 295-302.
[115] Moncada, S., Palmer, R. M., Higgs, E. A., The discovery of nitric oxide as the endogenous nitrovasodilator. *Hypertension* 1988, *12*, 365-372.
[116] Modin, A., Bjorne, H., Herulf, M., Alving, K., *et al.*, Nitrite-derived nitric oxide: a possible mediator of 'acidic-metabolic' vasodilation. *Acta Physiol Scand* 2001, *171*, 9-16.
[117] Rodriguez, J., Maloney, R. E., Rassaf, T., Bryan, N. S., Feelisch, M., Chemical nature of nitric oxide storage forms in rat vascular tissue. *Proceedings of the National Academy of Sciences of the United States of America* 2003, *100*, 336-341.
[118] Webb, A. J., Milsom, A. B., Rathod, K. S., Chu, W. L., *et al.*, Mechanisms Underlying Erythrocyte and Endothelial Nitrite Reduction to Nitric Oxide in Hypoxia Role for Xanthine Oxidoreductase and Endothelial Nitric Oxide Synthase. *Circ Res* 2008, *103*, 957-U114.
[119] Petersson, J., Carlstrom, M., Schreiber, O., Phillipson, M., *et al.*, Gastroprotective and blood pressure lowering effects of dietary nitrate are abolished by an antiseptic mouthwash. *Free radical biology & medicine* 2009, *46*, 1068-1075.

[120] Kapil, V., Haydar, S. M., Pearl, V., Lundberg, J. O., *et al.*, Physiological role for nitrate-reducing oral bacteria in blood pressure control. *Free radical biology & medicine* 2013, *55*, 93-100.
[121] Zweier, J. L., Wang, P., Kuppusamy, P., Direct measurement of nitric oxide generation in the ischemic heart using electron paramagnetic resonance spectroscopy. *J Biol Chem* 1995, *270*, 304-307.
[122] Zweier, J. L., Wang, P., Samouilov, A., Kuppusamy, P., Enzyme-independent formation of nitric oxide in biological tissues. *Nature medicine* 1995, *1*, 804-809.
[123] Duranski, M. R., Greer, J. J. M., Dejam, A., Jaganmohan, S., *et al.*, Cytoprotective effects of nitrite during in vivo ischemia-reperfusion of the heart and liver. *Journal of Clinical Investigation* 2005, *115*, 1232-1240.
[124] Kapil, V., Okorie, M., Akram, F., Rehman, F., *et al.*, Oral inorganic nitrate lowers blood pressure and protects against endothelial ischaemia-reperfusion injury in humans. *Nitric Oxide-Biol Ch* 2009, *20*, S37-S37.
[125] Okorie, M., Akram, F., Loukogeorgakis, S., Webb, A., *et al.*, Oral Inorganic Nitrate Protects against Endothelial Ischaemia-Reperfusion Injury in Humans. *Basic Clin Pharmacol* 2009, *105*, 26-26.
[126] Ignarro, L. J., Buga, G. M., Wood, K. S., Byrns, R. E., Chaudhuri, G., Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proceedings of the National Academy of Sciences of the United States of America* 1987, *84*, 9265-9269.
[127] Khan, M. T., Furchgott, R. F., Similarities of Behavior of Nitric-Oxide (No) and Endothelium-Derived Relaxing Factor in a Perfusion Cascade Bioassay System. *Faseb J* 1987, *46*, 385-385.
[128] Srihirun, S., Sriwantana, T., Unchern, S., Kittikool, D., *et al.*, Platelet inhibition by nitrite is dependent on erythrocytes and deoxygenation. *PloS one* 2012, *7*, e30380.
[129] Joshipura, K. J., Ascherio, A., Manson, J. E., Stampfer, M. J., *et al.*, Fruit and vegetable intake in relation to risk of ischemic stroke. *Jama-J Am Med Assoc* 1999, *282*, 1233-1239.
[130] Joshipura, K. J., Hu, F. B., Manson, J. E., Stampfer, M. J., *et al.*, The effect of fruit and vegetable intake on risk for coronary heart disease. *Ann Intern Med* 2001, *134*, 1106-1114.
[131] Hung, H. C., Joshipura, K. J., Jiang, R., Hu, F. B., *et al.*, Fruit and vegetable intake and risk of major chronic disease. *Journal of the National Cancer Institute* 2004, *96*, 1577-1584.
[132] Lundberg, J. O., Carlstrom, M., Larsen, F. J., Weitzberg, E., Roles of dietary inorganic nitrate in cardiovascular health and disease. *Cardiovascular research* 2011, *89*, 525-532.
[133] Webb, A. J., Patel, N., Loukogeorgakis, S., Okorie, M., *et al.*, Acute blood pressure (BP) lowering and vasoprotective effects of beetroot juice: relationship to nitrite derived from dietary nitrate. *J Hum Hypertens* 2007, *21*, 834-835.
[134] Kapil, V., Inorganic Nitrate Supplementation Lowers Blood Pressure in Humans: Role for Nitrite-Derived NO (vol 56, 274, 2010). *Hypertension* 2010, *56*, E37-E39.
[135] Siervo, M., Lara, J., Ogbonmwan, I., Mathers, J. C., Inorganic nitrate and beetroot juice supplementation reduces blood pressure in adults: a systematic review and meta-analysis. *The Journal of nutrition* 2013, *143*, 818-826.
[136] Sobko, T., Marcus, C., Govoni, M., Kamiya, S., Dietary nitrate in Japanese traditional foods lowers diastolic blood pressure in healthy volunteers. *Nitric Oxide-Biol Ch* 2010, *22*, 136-140.
[137] Bailey, S. J., Fulford, J., Vanhatalo, A., Winyard, P. G., *et al.*, Dietary nitrate supplementation enhances muscle contractile efficiency during knee-extensor exercise in humans. *J Appl Physiol* 2010, *109*, 135-148.
[138] Bailey, S. J., Winyard, P., Vanhatalo, A., Blackwell, J. R., *et al.*, Dietary nitrate supplementation reduces the O2 cost of low-intensity exercise and enhances tolerance to high-intensity exercise in humans. *J Appl Physiol* 2009, *107*, 1144-1155.

[139] Lansley, K. E., Winyard, P. G., Fulford, J., Vanhatalo, A., *et al.*, Dietary nitrate supplementation reduces the O2 cost of walking and running: a placebo-controlled study. *J Appl Physiol* 2011, *110*, 591-600.
[140] Vanhatalo, A., Bailey, S. J., Blackwell, J. R., DiMenna, F. J., *et al.*, Acute and chronic effects of dietary nitrate supplementation on blood pressure and the physiological responses to moderate-intensity and incremental exercise. *American journal of physiology. Regulatory, integrative and comparative physiology* 2010, *299*, R1121-1131.
[141] Kenjale, A. A., Ham, K. L., Stabler, T., Robbins, J. L., *et al.*, Dietary nitrate supplementation enhances exercise performance in peripheral arterial disease. *Journal of Applied Physiology* 2011, *110*, 1582-1591.
[142] Collins, R., Peto, R., MacMahon, S., Hebert, P., *et al.*, Blood pressure, stroke, and coronary heart disease. Part 2, Short-term reductions in blood pressure: overview of randomised drug trials in their epidemiological context. *Lancet* 1990, *335*, 827-838.
[143] Lv, J., Neal, B., Ehteshami, P., Ninomiya, T., *et al.*, Effects of intensive blood pressure lowering on cardiovascular and renal outcomes: a systematic review and meta-analysis. *PLoS medicine* 2012, *9*, e1001293.
[144] Barter, P. J., Caulfield, M., Eriksson, M., Grundy, S. M., *et al.*, Effects of torcetrapib in patients at high risk for coronary events. *The New England journal of medicine* 2007, *357*, 2109-2122.
[145] Sander, J., Burkle, G., Induction of Malignant Tumors in Rats by Simultaneous Feeding of Nitrite and Secondary Amines. *Zeitschrift fur Krebsforschung* 1969, *73*, 54-&.
[146] Ohshima, H., Bartsch, H., Quantitative estimation of endogenous nitrosation in humans by monitoring N-nitrosoproline excreted in the urine. *Cancer research* 1981, *41*, 3658-3662.
[147] Ohshima, H., Bereziat, J. C., Bartsch, H., Measurement of endogenous n-nitrosation in rats and humans by monitoring urinary and faecal excretion of N-nitrosamino acids. *IARC scientific publications* 1982, 397-411.
[148] Janzowski, C., Hemm, I., Eisenbrand, G., in: Wichmann, H.-E., Schlipköter, H. W., Fülgraff, G. (Eds.), *Handbuch der Umweltmedizin* ecomed Verlagsgesellschaft, Landsberg, Germany 2000.
[149] WCRF/AICR, Systematic Literature Review Continuous Update Project Report: The Associations between Food, Nutrition ans Physical Activity and the Risk of Colorectal Cancer. 2010.
[150] Ridd, J. H., Nitrosation, Diazotisation, and Deamination. *Q Rev Chem Soc* 1961, *15*, 418-441.
[151] Mirvish, S. S., Formation of N-Nitroso Compounds - Chemistry, Kinetics, and Invivo Occurrence. *Toxicol Appl Pharm* 1975, *31*, 325-351.
[152] Keefer, L. K., Roller, P. P., N-Nitrosation by Nitrite Ion in Neutral and Basic Medium. *Science* 1973, *181*, 1245-1247.
[153] Loeppky, R. N., Bao, Y. T., Bae, J. Y., Yu, L., Shevlin, G., in: Loeppky, R. N., Michejda, C. J. (Eds.), *Nitrosamines and Related N-Nitroso Compounds: Chemistry and Biochemistry*, American Chemical Society, Washington, DC 1994, pp. 52-65.
[154] Habermeyer, M., Eisenbrand, G., in: Stadler, S., Lineback, D. (Eds.), *Process-Induced Food Toxicants and Health Risks*, John Wiley & Sons,, Hoboken, New Jersey 2008.
[155] Tricker, A. R., Kubacki, S. J., Review of the Occurrence and Formation of Nonvolatile N-Nitroso Compounds in Foods. *Food Addit Contam* 1992, *9*, 39-69.
[156] SCCS, EU Scientific Committee on Consumer Safety (SCCS) 2012, p. 46.
[157] SCCS, EU Scientific Committee on Consumer Safety (SCCS) 2012, p. 43.
[158] Vermeer, I. T. M., Pachen, D. M. F. A., Dallinga, J. W., Kleinjans, J. C. S., van Maanen, J. M. S., Volatile N-nitrosamine formation after intake of nitrate at the ADI level in combination with an amine-rich diet. *Environ Health Persp* 1998, *106*, 459-463.

[159] Kuhnle, G. G. C., Story, G. W., Reda, T., Mani, A. R., *et al.*, Diet-induced endogenous formation of nitroso compounds in the GI tract. *Free Radical Bio Med* 2007, *43*, 1040-1047.
[160] Tricker, A. R., Kumar, R., Siddiqi, M., Khuroo, M. S., Preussmann, R., Endogenous Formation of N-Nitrosamines from Piperazine and Their Urinary-Excretion Following Antihelminthic Treatment with Piperazine Citrate. *Carcinogenesis* 1991, *12*, 1595-1599.
[161] Lijinsky, W., Taylor, H. W., Snyder, C., Netteshe.P, Malignant-Tumors of Liver and Lung in Rats Fed Aminopyrine or Heptamethyleneimine Together with Nitrite. *Nature* 1973, *244*, 176-177.
[162] Spiegelhalder, B., in: Nicolai, H. V., Eisenbrand, G., Bozler, G. (Eds.), *The Significance of N-Nitrosation of Drugs*, Gustav Fischer Verlag, Stuttgart, New York 1990, pp. 199-212.
[163] Spiegelhalder, B., Preussmann, R., In vivo Nitrosation of Amidopyrine in Humans - Use of Ethanol Effect for Biological Monitoring of N-Nitrosodimethylamine in Urine. *Carcinogenesis* 1985, *6*, 545-548.
[164] Adam, B., Schlag, P., Friedl, P., Preussmann, R., Eisenbrand, G., Proline is not useful as a chemical probe to measure nitrosation in the gastrointestinal tract of patients with gastric disorders characterised by anacidic conditions. *Gut* 1989, *30*, 1068-1075.
[165] Carmella, S. G., Borukhova, A., Desai, D., Hecht, S. S., Evidence for endogenous formation of tobacco-specific nitrosamines in rats treated with tobacco alkaloids and sodium nitrite. *Carcinogenesis* 1997, *18*, 587-592.
[166] Hecht, S. S., Chen, C.-H. B., Ornaf, R. M., Jacobs, E., *et al.*, Chemical studies on tobacco smoke. 52. Reaction of nicotine and sodium nitrite: formation of nitrosamines and fragmentation of the pyrrolidine ring. *The Journal of Organic Chemistry* 1978, *43*, 72-76.
[167] Mirvish, S. S., Sams, J., Hecht, S. S., Kinetics of nornicotine and anabasine nitrosation in relation to N'-nitrosonornicotine occurrence in tobacco and to tobacco-induced cancer. *Journal of the National Cancer Institute* 1977, *59*, 1211-1213.
[168] Stepanov, I., Carmella, S. G., Briggs, A., Hertsgaard, L., *et al.*, Presence of the carcinogen N'-nitrosonornicotine in the urine of some users of oral nicotine replacement therapy products. *Cancer research* 2009, *69*, 8236-8240.
[169] Stepanov, I., Carmella, S. G., Han, S., Pinto, A., *et al.*, Evidence for endogenous formation of N'-nitrosonornicotine in some long-term nicotine patch users. *Nicotine & tobacco research : official journal of the Society for Research on Nicotine and Tobacco* 2009, *11*, 99-105.
[170] Stepanov, I., Sebero, E., Wang, R., Gao, Y. T., *et al.*, Tobacco-specific N-nitrosamine exposures and cancer risk in the Shanghai cohort study: Remarkable coherence with rat tumor sites. *International journal of cancer. Journal international du cancer* 2013.
[171] Yuan, J. M., Knezevich, A. D., Wang, R., Gao, Y. T., *et al.*, Urinary levels of the tobacco-specific carcinogen N'-nitrosonornicotine and its glucuronide are strongly associated with esophageal cancer risk in smokers. *Carcinogenesis* 2011, *32*, 1366-1371.
[172] Hecht, S. S., Biochemistry, biology, and carcinogenicity of tobacco-specific N-nitrosamines. *Chemical research in toxicology* 1998, *11*, 559-603.
[173] IARC, *Smokeless Tobacco and Some Tobacco-specific N-Nitrosamines*, World Health Organization, Lyon, France 2007.
[174] Puju, S., Shuker, D. E. G., Bishop, W. W., Falchuk, K. R., *et al.*, Mutagenicity of N-Nitroso Bile-Acid Conjugates in Salmonella-Typhimurium and Diploid Human-Lymphoblasts. *Cancer research* 1982, *42*, 2601-2604.
[175] Busby, W. F., Shuker, D. E. G., Charnley, G., Newberne, P. M., *et al.*, Carcinogenicity in Rats of the Nitrosated Bile-Acid Conjugates N-Nitrosoglycocholic Acid and N-Nitrosotaurocholic Acid. *Cancer research* 1985, *45*, 1367-1371.
[176] Shuker, D. E. G., Margison, G. P., Nitrosated glycine derivatives as a potential source of O-6-methylguanine in DNA. *Cancer research* 1997, *57*, 366-369.

[177] Gottschalg, E., Scott, G. B., Burns, P. A., Shuker, D. E. G., Potassium diazoacetate-induced p53 mutations in vitro in relation to formation of O-6-carboxymethyl- and O-6-methyl-2 '-deoxyguanosine DNA adducts: relevance for gastrointestinal cancer. *Carcinogenesis* 2007, *28*, 356-362.
[178] Harrison, K. L., Fairhurst, N., Challis, B. C., Shuker, D. E. G., Synthesis, characterization, and immunochemical detection of O-6-(carboxymethyl)-2'-deoxyguanosine: A DNA adduct formed by nitrosated glycine derivatives. *Chemical research in toxicology* 1997, *10*, 652-659.
[179] Cupid, B. C., Zeng, Z. T., Singh, R., Shuker, D. E. G., Detection of O-6-carboxymethyl-2 '-deoxyguanosine in DNA following reaction of nitric oxide with glycine and in human blood DNA Using a quantitative immunoslot blot assay. *Chemical research in toxicology* 2004, *17*, 294-300.
[180] Lewin, M. H., Bailey, N., Bandaletova, T., Bowman, R., *et al.*, Red meat enhances the colonic formation of the DNA adduct O-6-carboxymethyl guanine: Implications for colorectal cancer. *Cancer research* 2006, *66*, 1859-1865.
[181] Vanden Bussche, J., Moore, S. A., Pasmans, F., Kuhnle, G. G. C., Vanhaecke, L., An approach based on ultra-high pressure liquid chromatography-tandem mass spectrometry to quantify O-6-methyl and O-6-carboxymethylguanine DNA adducts in intestinal cell lines. *J Chromatogr A* 2012, *1257*, 25-33.
[182] Mirvish, S. S., Formation of N-Nitroso Compounds - Chemistry, Kinetics, and In vivo Occurrence. *Toxicol Appl Pharm* 1975, *31*, 325-351.
[183] Bulay, O., Mirvish, S. S., Garcia, H., Pelfrene, A. F., *et al.*, Carcinogenicity Test of 6 Nitrosamides and a Nitrosocyanamide Administered Orally to Rats. *Journal of the National Cancer Institute* 1979, *62*, 1523-1528.
[184] Cheng, G., Wang, M., Villalta, P. W., Hecht, S. S., Detection of 7-(2'-carboxyethyl)guanine but not 7-carboxymethylguanine in human liver DNA. *Chem Res Toxicol* 2010, *23*, 1089-1096.
[185] Krul, C. A., Zeilmaker, M. J., Schothorst, R. C., Havenaar, R., Intragastric formation and modulation of N-nitrosodimethylamine in a dynamic in vitro gastrointestinal model under human physiological conditions. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* 2004, *42*, 51-63.
[186] Zeilmaker, M. J., Bakker, M. I., Schothorst, R., Slob, W., Risk assessment of N-nitrosodimethylamine formed endogenously after fish-with-vegetable meals. *Toxicological sciences : an official journal of the Society of Toxicology* 2010, *116*, 323-335.
[187] Pfundstein, B., Tricker, A. R., Theobald, E., Spiegelhalder, B., Preussmann, R., Mean daily intake of primary and secondary amines from foods and beverages in West Germany in 1989-1990. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* 1991, *29*, 733-739.
[188] Engemann, A., Focke, C., Humpf, H. U., Intestinal formation of N-nitroso compounds in the pig cecum model. *Journal of agricultural and food chemistry* 2013, *61*, 998-1005.
[189] Magee, P. N., Barnes, J. M., The Production of Malignant Primary Hepatic Tumours in the Rat by Feeding Dimethylnitrosamine. *British journal of cancer* 1956, *10*, 114-&.
[190] Schmahl, D., Preussmann, R., Hamperl, H., Leberkrebs-Erzeugende Wirkung Von Diathylnitrosamin Nach Oraler Gabe Bei Ratten. *Naturwissenschaften* 1960, *47*, 89-89.
[191] Druckrey, H., Preussmann, R., Ivankovic, S., Schmahl, D., [Organotropic carcinogenic effects of 65 various N-nitroso- compounds on BD rats]. *Zeitschrift fur Krebsforschung* 1967, *69*, 103-201.
[192] Berger, M. R., Schmahl, D., Edler, L., Implications of the carcinogenic hazard of low doses of three hepatocarcinogenic N-nitrosamines. *Japanese journal of cancer research : Gann* 1990, *81*, 598-606.

[193] Berger, M. R., Schmahl, D., Zerban, H., Combination experiments with very low doses of three genotoxic N-nitrosamines with similar organotropic carcinogenicity in rats. *Carcinogenesis* 1987, *8*, 1635-1643.
[194] Peto, R., Gray, R., Brantom, P., Grasso, P., Dose and time relationships for tumor induction in the liver and esophagus of 4080 inbred rats by chronic ingestion of N-nitrosodiethylamine or N-nitrosodimethylamine. *Cancer research* 1991, *51*, 6452-6469.
[195] Peto, R., Gray, R., Brantom, P., Grasso, P., Effects on 4080 rats of chronic ingestion of N-nitrosodiethylamine or N-nitrosodimethylamine: a detailed dose-response study. *Cancer research* 1991, *51*, 6415-6451.
[196] Gombar, C. T., Harrington, G. W., Pylypiw, H. M., Jr., Anderson, L. M., *et al.*, Interspecies scaling of the pharmacokinetics of N-nitrosodimethylamine. *Cancer research* 1990, *50*, 4366-4370.
[197] Gombar, C. T., Harrington, G. W., Pylypiw, H. M., Jr., Bevill, R. F., *et al.*, Pharmacokinetics of N-nitrosodimethylamine in swine. *Carcinogenesis* 1988, *9*, 1351-1354.
[198] Gombar, C. T., Pylypiw, H. M., Jr., Harrington, G. W., Pharmacokinetics of N-nitrosodimethylamine in beagles. *Cancer research* 1987, *47*, 343-347.
[199] Mico, B. A., Swagzdis, J. E., Hu, H. S., Keefer, L. K., *et al.*, Low-dose in vivo pharmacokinetic and deuterium isotope effect studies of N-nitrosodimethylamine in rats. *Cancer research* 1985, *45*, 6280-6285.
[200] Streeter, A. J., Nims, R. W., Anderson, L. M., Heur, Y. H., *et al.*, Single-dose toxicokinetics of N-nitrosomethylethylamine and N-nitrosomethyl (2,2,2-trideuterioethyl)amine in the rat. *Archives of toxicology* 1990, *64*, 109-115.
[201] Preussmann, R., in: Nicolai, H. V., Eisenbrand, G., Bozler, G. (Eds.), *The Significance of N-Nitrosation of Drugs*, Gustav Fischer Verlag, Stuttgart, New York 1990, pp. 3-18.
[202] Lijinsky, W., Structure-activity relations in carcinogenesis by N-nitroso compounds. *Cancer metastasis reviews* 1987, *6*, 301-356.
[203] O'Brien, J., Renwick, A. G., Constable, A., Dybing, E., *et al.*, Approaches to the risk assessment of genotoxic carcinogens in food: a critical appraisal. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* 2006, *44*, 1613-1635.
[204] Benford, D., Bolger, P. M., Carthew, P., Coulet, M., *et al.*, Application of the Margin of Exposure (MOE) approach to substances in food that are genotoxic and carcinogenic. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* 2010, *48 Suppl 1*, S2-24.
[205] EFSA, Opinion of the Scientific Committee on a request from EFSA related to A Harmonised Approach for Risk Assessment of Substances Which are both Genotoxic and Carcinogenic *The EFSA Journal* 2005, *3*, 31.
[206] Maekawa, A., Ogiu, T., Onodera, H., Furuta, K., *et al.*, Carcinogenicity studies of sodium nitrite and sodium nitrate in F-344 rats. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* 1982, *20*, 25-33.
[207] IARC, *Ingested nitrate and nitrite, and cyanobacterial peptide toxins*, World Health Organization, Lyon, France 2010.
[208] NTP, National Institutes of Health 2001, p. 274.
[209] Hebels, D. G., Brauers, K. J., van Herwijnen, M. H., Georgiadis, P. A., *et al.*, Time-series analysis of gene expression profiles induced by nitrosamides and nitrosamines elucidates modes of action underlying their genotoxicity in human colon cells. *Toxicol Lett* 2011, *207*, 232-241.
[210] Hebels, D. G., Jennen, D. G., van Herwijnen, M. H., Moonen, E. J., *et al.*, Whole-genome gene expression modifications associated with nitrosamine exposure and micronucleus frequency in human blood cells. *Mutagenesis* 2011, *26*, 753-761.

[211] Hebels, D. G., Sveje, K. M., de Kok, M. C., van Herwijnen, M. H., *et al.*, N-nitroso compound exposure-associated transcriptomic profiles are indicative of an increased risk for colorectal cancer. *Cancer letters* 2011, *309*, 1-10.
[212] Hebels, D. G., Sveje, K. M., de Kok, M. C., van Herwijnen, M. H., *et al.*, Red meat intake-induced increases in fecal water genotoxicity correlate with pro-carcinogenic gene expression changes in the human colon. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* 2012, *50*, 95-103.
[213] Hughes, R., Cross, A. J., Pollock, J. R. A., Bingham, S., Dose-dependent effect of dietary meat on endogenous colonic N-nitrosation (vol 22, pg 1999, 2001). *Carcinogenesis* 2001, *22*, 685-685.
[214] Cross, A. J., Pollock, J. R., Bingham, S. A., Haem, not protein or inorganic iron, is responsible for endogenous intestinal N-nitrosation arising from red meat. *Cancer research* 2003, *63*, 2358-2360.
[215] Bastide, N. M., Pierre, F. H. F., Corpet, D. E., Heme Iron from Meat and Risk of Colorectal Cancer: A Meta-analysis and a Review of the Mechanisms Involved. *Cancer Prev Res* 2011, *4*, 177-184.
[216] Cross, A. J., Pollock, J. R. A., Bingham, S. A., Haem, not protein or inorganic iron, is responsible for endogenous intestinal N-nitrosation arising from red meat. *Cancer research* 2003, *63*, 2358-2360.
[217] Bonnett, R., Charalambides, A. A., Martin, R. A., Sales, K. D., Fitzsimmons, B. W., Reactions of Nitrous-Acid and Nitric-Oxide with Porphyrins and Hemes - Nitrosylhemes as Nitrosating Agents. *J Chem Soc Chem Comm* 1975, 884-885.
[218] Butler, A. R., Rhodes, P., Chemistry, analysis, and biological roles of S-nitrosothiols. *Analytical biochemistry* 1997, *249*, 1-9.
[219] Gay, L. J., Mitrou, P. N., Keen, J., Bowman, R., *et al.*, Dietary, lifestyle and clinicopathological factors associated with APC mutations and promoter methylation in colorectal cancers from the EPIC-Norfolk study. *The Journal of pathology* 2012, *228*, 405-415.
[220] Oostindjer, M. A., J.; Amdam, G. V.; Andersen, G.; Bryan, N. S.; Chen, D.; Corpet, D. E.; de Smet, S.; Dragsted, L. O.; Haug, A.; Karlsson, A. H.; Kleter, G.; de Kok,T. M.; Kulseng, B.; Milkowski, A. L.; Martin, R. J.; Pajari, A. M.; Paulsen, J. E.; Pickova, J.; Rudi, K.; Sødring, M.; Weed, D. L.; Egelandsdal, B., The role of red and processed meat in colorectal cancer development: A review, based on findings from a workshop. *Meat science* 2014.
[221] Sandhu, M. S., White, I. R., McPherson, K., Systematic review of the prospective cohort studies on meat consumption and colorectal cancer risk: a meta-analytical approach. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* 2001, *10*, 439-446.
[222] Cross, A. J., Freedman, N. D., Ren, J., Ward, M. H., *et al.*, Meat consumption and risk of esophageal and gastric cancer in a large prospective study. *The American journal of gastroenterology* 2011, *106*, 432-442.
[223] Chan, D. S. M., Lau, R., Aune, D., Vieira, R., *et al.*, Red and Processed Meat and Colorectal Cancer Incidence: Meta-Analysis of Prospective Studies. *PloS one* 2011, *6*.
[224] Sinha, R., Cross, A. J., Graubard, B. I., Leitzmann, M. F., Schatzkin, A., Meat intake and mortality: a prospective study of over half a million people. *Archives of internal medicine* 2009, *169*, 562-571.
[225] Ward, M. H., Cantor, K. P., Riley, D., Merkle, S., Lynch, C. F., Nitrate in public water supplies and risk of bladder cancer. *Epidemiology* 2003, *14*, 183-190.
[226] Ward, M. H., Cerhan, J. R., Colt, J. S., Hartge, P., Risk of non-Hodgkin lymphoma and nitrate and nitrite from drinking water and diet. *Epidemiology* 2006, *17*, 375-382.

[227] Kilfoy, B. A., Zhang, Y., Park, Y., Holford, T. R., *et al.*, Dietary nitrate and nitrite and the risk of thyroid cancer in the NIH-AARP Diet and Health Study. *International journal of cancer. Journal international du cancer* 2011, *129*, 160-172.
[228] Cross, A. J., Ferrucci, L. M., Risch, A., Graubard, B. I., *et al.*, A large prospective study of meat consumption and colorectal cancer risk: an investigation of potential mechanisms underlying this association. *Cancer research* 2010, *70*, 2406-2414.
[229] Dubrow, R., Darefsky, A. S., Park, Y., Mayne, S. T., *et al.*, Dietary components related to N-nitroso compound formation: a prospective study of adult glioma. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* 2010, *19*, 1709-1722.
[230] Aschebrook-Kilfoy, B., Ward, M. H., Gierach, G. L., Schatzkin, A., *et al.*, Epithelial ovarian cancer and exposure to dietary nitrate and nitrite in the NIH-AARP Diet and Health Study. *Eur J Cancer Prev* 2012, *21*, 65-72.
[231] Dellavalle, C. T., Daniel, C. R., Aschebrook-Kilfoy, B., Hollenbeck, A. R., *et al.*, Dietary intake of nitrate and nitrite and risk of renal cell carcinoma in the NIH-AARP Diet and Health Study. *Br J Cancer* 2013, *108*, 205-212.
[232] Ferrucci, L. M., Sinha, R., Ward, M. H., Graubard, B. I., *et al.*, Meat and components of meat and the risk of bladder cancer in the NIH-AARP Diet and Health Study. *Cancer* 2010, *116*, 4345-4353.
[233] Aschebrook-Kilfoy, B., Cross, A. J., Stolzenberg-Solomon, R. Z., Schatzkin, A., *et al.*, Pancreatic cancer and exposure to dietary nitrate and nitrite in the NIH-AARP Diet and Health Study. *American journal of epidemiology* 2011, *174*, 305-315.
[234] Daniel, C. R., Cross, A. J., Graubard, B. I., Park, Y., *et al.*, Large prospective investigation of meat intake, related mutagens, and risk of renal cell carcinoma. *The American journal of clinical nutrition* 2012, *95*, 155-162.
[235] Huncharek, M., Kupelnick, B., Wheeler, L., Dietary cured meat and the risk of adult glioma: a meta-analysis of nine observational studies. *Journal of environmental pathology, toxicology and oncology : official organ of the International Society for Environmental Toxicology and Cancer* 2003, *22*, 129-137.
[236] Michaud, D. S., Holick, C. N., Batchelor, T. T., Giovannucci, E., Hunter, D. J., Prospective study of meat intake and dietary nitrates, nitrites, and nitrosamines and risk of adult glioma. *The American journal of clinical nutrition* 2009, *90*, 570-577.
[237] Terry, M. B., Howe, G., Pogoda, J. M., Zhang, F. F., *et al.*, An international case-control study of adult diet and brain tumor risk: a histology-specific analysis by food group. *Annals of epidemiology* 2009, *19*, 161-171.
[238] Loh, Y. H., Jakszyn, P., Luben, R. N., Mulligan, A. A., *et al.*, N-Nitroso compounds and cancer incidence: the European Prospective Investigation into Cancer and Nutrition (EPIC)-Norfolk Study. *The American journal of clinical nutrition* 2011, *93*, 1053-1061.
[239] Jakszyn, P., Bingham, S., Pera, G., Agudo, A., *et al.*, Endogenous versus exogenous exposure to N-nitroso compounds and gastric cancer risk in the European Prospective Investigation into Cancer and Nutrition (EPIC-EURGAST) study. *Carcinogenesis* 2006, *27*, 1497-1501.

Supplementary material to the SKLM opinion "Nitrate and Nitrite in the Diet: An approach to assess Benefit and Risk for Human Health"

Round table meeting, November 27th 2012, German Research Foundation (DFG), Kennedyallee 40, 53170 Bonn, Germany

Structure and activity of N-Nitroso Compounds (NOC) and overview on endogeneous nitrosation (bio)chemistry

Gerhard Eisenbrand – Chairman of the DFG-SKLM

University of Kaiserslautern, Department of Chemistry, Division of Food Chemistry and Toxicology, Erwin-Schroedinger-Str., 67663 Kaiserslautern, Germany

History

More than 50 years ago N-nitrosodimethylamine (NDMA) has been reported by Peter Magee and his colleague M.J. Barnes (1956) to induce primary liver tumours in rats. This finding originated from an investigation into the potential causes of hepatotoxicity in workers in the rubber industry, where the compound had been explored for suitability as a solvent in the vulcanization process (Barnes and Magee, 1954). Sporadic case reports on accidental (Freund, 1937) or intentional fatal intoxications (Cooper& Kimbrough, 1980; Fussgänger & Ditschuneit,1980) by NDMA published before or later described human postmortem findings, especially acute liver necrosis and haematological toxicity similar to those seen in experimental animals at lethal dosage.

NDMA is a quite polar, low molecular weight water soluble compound, significantly different from the lipophilic carcinogens of higher molecular mass known at that time such as polycyclic aromatic hydrocarbons (PAH), aromatic amines or pyrrolizidines. The seminal finding by Magee & Barnes (1956) was confirmed by Schmähl & Preussmann (1959) who also showed the next homologue, N-nitrosodiethylamine (NDEA) to be at least as potent a carcinogen as NDMA (Schmähl et al., 1960). Subsequently, research into biological activities of N-nitroso compounds (NOC) rapidly gained momentum and a wide range of NOC, including certain nitrosamides such as N-methyl-N-nitrosourea or N-methyl-N-nitrosorurethane (Schoental, 1960; Druckrey et al., 1961), were identified as potent carcinogens. The group headed by Hermann Druckrey in Freiburg, Germany published a seminal and comprehensive study on structure-activity and dose-response relationship (Druckrey et al., 1967) that soon became a “Citation classic”. A specific feature of NOC early on gained much attention, namely their propensity to selectively induce tumours in organs of various animal species, irrespective of the application route. This was systematically extended by William Lijinsky and his colleagues (summarized 1992) and provided the basis for the establishment of highly reproducible experimental tumour models for clinical therapy studies.

The pivotal step in bioactivation of many NOC consists in metabolic generation of electrophilic ultimate carcinogens, in general through cytochrome P450 (CYP) dependent α-hydroxylation, as first shown by Magee and coworkers (1962).

Research on biological effects of NOC in the subsequent years was driven by trying to understand mechanisms of biological and molecular events responsible for the biological effects observed.

Exposure to preformed NOC

Already in the early sixties, the suspicion was raised that NOC might be formed in the human environment at certain conditions (Druckrey & Preussmann, 1962). Ender et al. (1964) in Norway described poisoning of sheep, with clear indication of liver toxicity, after feeding nitrite-treated fish meal. Although appropriate analytical methods were not at hands yet, the group identified NDMA, obviously present at rather high concentrations, as the most probable causative agent. This triggered an avalanche of analytical research to uncover presence of NOC in food.

Formation of NOC: basic considerations

Virtually any situation where nitrosating agents encounter N-nitrosatable amino compounds can give rise to NOC. The classical situation would reflect the reaction of an amine with nitrous acid, in aqueous solution (Ridd, 1961; Mirvish, 1975). Nitrite and nitrous acid under proton catalysis generate the actual nitrosating species, dinitrogen trioxide (N_2O_3) or tetroxide (N_2O_4) or nitrous acidium ion $NO^+ \bullet H_2O$. The acidity of the medium determines the relative prevalence of the nitrosating species. In addition, the basicity of the nitrosatable amine precursor is of great influence for nitrosation in aqueous solution, because only non-protonated nitrogen atoms are available for nitrosation. Therefore, strongly basic amines like simple dialkylamines (pka < 9,5) are not considered to exhibit nitrosation rates that favour substantial NOC formation in aqueous media. Because acid-catalyzed nitrosation is inappreciable at pH > 5, NOC in food or certain consumer products or in specific working place situations most likely arise from exposure to gaseous NO_x.

Such conditions are to be met, for instance, in a variety of food production situations, including smoke treatment or direct drying and kilning technology. Addition of nitrate/nitrite, e.g. in the curing of meat is also of some relevance. Carbonyl compounds present or generated during food treatment can act as nitrosation catalysts as shown for formaldehyde, a reaction discovered by Keefer and Roller (1973).

Nitrosamines most frequently found in food are NDMA, N-nitrosopyrrolidine (NPYR), N-nitrosopiperidine (NPIP) and N-Nitrosothiazolidine (NTHZ). Nonvolatile N-nitroso compounds consist mainly of N-nitrosated amino acids, including the N-nitroso products of sarcosine (NSAR), 3-hydroxyproline and proline (NPRO), thiazolidine-4-carboxylic acid (NTCA), oxazolidine-4-carboxylic acid (NOCA) and N-Nitroso-2-methyl-nitroso thiazolidine-4-carboxylic acid (NMTCA) as well as the oxazolidine analog (NMOCA). NPRO and NTCA are most frequently found in foods, the other compounds have been reported only sporadically. Of note, with the exception of NSAR, which is a relatively weak carcinogen, N-nitrosated amino acids are not mutagenic and not carcinogenic. Average NOC exposure has been calculated for various countries, mostly from dietary surveys (Table 1).

Table 1: Dietary intake of volatile NOC: Germany 1980-1990 (Janzowski et al., 2000)

NDMA µg/person/day	**NPyr + NPIP** µg/person/day	**∑ NOC**	**Year (Country)**	**ng/kg b.w.**
0.1 – 1.0	0.1 – 0.4	0.2 - 1.4	before 1989/90 (D, NL, FIN, UK, F, SW)	1.7 – 23.3
0.2 – 0.3	0.03	0.2 - 0.3	1989/90 (D)	1989/90

A margin of exposure (MOE) may be calculated to arrive at some basis for priority decisions concerning risk management. The MOE describes the ratio between human exposure and a dose level inducing a certain tumour response. As benchmark either the T25, reflecting the dose rate in mg/kg/d which will give 25% tumours at a specific site, or the lower limit of the 95% confidence interval of a dose resulting in a 10% tumour response is used. The thus calculated MOE of dietary NOC exposure from these relatively old data is 5,400-8,200. According to EFSA, a MOE of 10,000 or higher would be of low concern for risk management (EFSA Opinion, 2005).

Formation of NOC in vivo:

Amine contents in foods have been studied in a comprehensive study by Pfundstein et al. (1991). According to these data, the mean daily per capita intake of amines from food was about 30-37 mg of primary and 6-8 mg of secondary amines. Thus in principle, NOC can be expected not only to form under environmental, technical or household conditions favouring the reaction of nitrosatable amines in food with nitrosating agents, but also after ingestion, e. g. during stomach passage.

In the aqueous-acidic medium of the stomach, the nitrosation rate is primarily governed by the protonation of the amine nitrogen and the pH dependent availability of N_2O_3. Therefore the pK-value of the amine determines the rate of NOC formation at a given acidic pH. Mirvish (1975) has investigated the kinetics of amine nitrosation under such conditions, reflecting significant differences in rates. Thus, a weakly basic amine is nitrosated at a given pH-value at a rate about 5 orders of magnitude faster than a strongly basic one, such as piperidine or diethylamine (pK > 11). Accordingly, no tumour formation was observed after feeding nitrite and such secondary amines to rats. In contrast, when weakly basic amines like N-methylbenzylamine and morpholine were given together with nitrite, the same tumours were induced as observed with the corresponding nitrosamines, as first shown by Sander & Bürkle (1969).

The drug amidopyrine (AP) had been withdrawn from the European market in the eighties for various reasons, including its inherent risk of NDMA formation. AP has been found to give rise to liver tumours when fed to rats with nitrite at quite low concentrations (Lijinski, 1975).

Biomarkers of exposure to NOC formed in vivo

In human volunteers, an increased risk for endogenous formation of NDMA after oral intake of 1 tablet of AP (500 µg) and of nitrate (200 mg), taken up in a vegetable juice, had been demonstrated by Spiegelhalder (1990). NDMA excretion in the urine became detectable by blocking it's metabolism

with concurrent application of ethanol (500 ml beer). Earlier studies had shown that concurrent ethanol administration (in 500 ml beer containing 60 ppb NDMA) results in / up to 2.5% NDMA being excreted unmetabolized in urine. From this, an in-vivo formation up to 1.8 mg NDMA was estimated in 1/3 volunteers. In probands saliva the drug was present in concentrations reflecting plasma values, together with nitrate that was reduced in part in the oral cavity to nitrite. When saliva samples were acidified to model the situation after being swallowed into the acidic stomach, NDMA rapidly formed at substantial rates (Spiegelhalder, 1990; Tricker, 1997). Urinary excretion of N-nitrosated metabolites was also used to measure exposure at working places or as a result of anthelmintic medication in the case of piperazine (Bellander, 1990; Tricker et al., 1991)

Although it has been clearly demonstrated that carcinogenic NOC might easily be formed when the appropriate precursors are being taken up, endogenous formation of carcinogenic NOC has not really been evaluated adequately as a process of relevance to human cancer. Apart from selected drugs of high reactivity towards nitrosation endogenous formation of NOC occuring under normal nutritional and physiological conditions needs to be adequately addressed. Nitrosated amino acids such as NPRO, because they are noncarcinogenic and practically quantitatively excreted in the urine, have often been utilized as surrogate biomarkers for overall endogenous nitrosation. It is well known, that there is endogenous formation of nitrosating agents and that certain disorders, such as inflammatory diseases, bacterial, viral or parasite infections and the like can substantially increase endogenous formation of nitrosating agents, thus enhancing the risk of forming carcinogenic NOC in vivo. It is still not clearly established yet, whether NPRO is a valid biomarker for endogenous formation of NOC other than those arising from nitrosation of amino acids. Within nitrosated amino acid derivatives, evidence from data presented by Shuker (Shuker & Margison, 1997; Harrison et al., 1999) suggests that for example N-nitrosoglycocholic acid (and other nitrosated glycine derivatives) form several DNA adducts, including O^6-carboxymethylguanine and, concomitantly = O^6 methylguanine. It is thus very important to develop in the future appropriate biomarkers that might allow a realistic estimate of in vivo formation of carcinogenic NOC. A possible way forward may be to exploit gene expression responses in animals and humans in an exposure related way. This might open new research avenues towards a better understanding of nitrate / NO_x -related biological effects with respect to both, potential health benefits and risks.

References

Barnes JM, Magee PN (1954) Some toxic properties of dimethylnitrosamine. *Brit. J. Ind. Med.* 11: 167-174

Bellander T (1990) Nitrosation of piperazine after oral intake or inhalation exposure, in Eisenbrand et al., The Significance of N-Nitrosation of Drugs, Gustav-Fischer-Verlag, p 213-233

Cooper SW, Kimbrough RD (1980) Acute dimethylnitrosamine poisoning outbreak. *J. Forensic. Sci.* 25: 874-882

Druckrey H, Preussmann R, Schmähl D, Müller M (1961) Erzeugung von Magenkrebs durch Nitrosamine an Ratten. *Naturwissen.* 48: 165

Druckrey H, Preussmann R (1962) Zur Entstehung cancerogener Nitrosamine am Beispiel des Tabakrauchs. *Natruwissen.* 49: 498

Druckrey H, Preussmann R, Ivankovic S, Schmähl D (1967) Organotrope carcinogene Wirkungen bei 65 verschiedenen N-Nitroso-Verbindungen an BD-Ratten. *Z. Krebsforsch.* 69: 103-201

EFSA (2005) Opinion of the Scientific Committee on a request from EFSA related to A Harmonised Approach for Risk Assessment of Substances Which are both Genotoxic and Carcinogenic. *The EFSA Journal* 282: 1-31

Ender F, Havre G, Helgebostad A, Koppang N, Madsen R, Ceh L (1964) Isolation and identification of a heptatoxic factor in herring meal produced from sodium nitrite preserved herring. *Naturwisssen.* 51: 637-638

Freund HA (1937) Clinical manifestations and studies in parenchymatous hepatitis. *Ann. Internal. Med.* 10: 1144-1155

Fussgänger RD, Ditschuneit H (1980) Lethal exitus of a patient with N-nitrosodimethylamine poisoning, 2,5 years following the first ingestion and signs of intoxication. *Oncology* 37: 273-277

Harrison KL, Jukes R, Cooper Dp; Shuker EG (1999) Detection of Contaminant Formation of O^6-Methyl-2'-deoxyguanosine in DNA Exposed to Nitosated Glycine Derivatives Using a Combined Immunoaffinity/HPLC Method, *Chem. Tox. Res., 12, 106-111*

Janzowski C, Hemm I, Eisenbrand G (2000) Organische Verbindungen – Nitrosamine, Kap. VI-4, In *Handbuch der Umweltmedizin* (Wichmann, H.-E., Schlipköter, H.W., Fülgraff, G., eds) 20. Ergänzungslierferung, ecomed Verlagsgesellschaft, Landsberg, Germany

Keefer LK, Roller PP (1973) N-Nitrosation by nitrite ion in neutral and basic medium. *Science* 181: 1245-1246

Lijinsky W (1975) Structure Activity Relations in Reaction of Drugs with Nitrite, in Eisenbrand et al. The Significance of N-Nitrosation of Drugs, Gustav Fischer Verlag, 93-107

Lijinsky W (1992) Chemistry and biology of N-nitroso compounds. Cambridge *Monographs on Cancer Research, Cambridge University Press*, Cambridge, GB

Magee PN, Barnes JM (1956) The production of malignant primary hepatic tumours in the rat by feeding dimethylnitrosmine. *Brit. J. Cancer* 10: 114-122

Magee PN, Farber E (1962) Toxic liver injury and carcinogenesis methylation of rat liver nucleic acids by dimetylnitrosamine *in vitro. Biochem. J.* 83: 114-124

Magee PN, Hultin T (1962) Toxic liver injury and carcinogenesis methylation of proteins of rat liver slices by dimethylnitrosamine *in vitro. Biochem. J.* 83: 106-113

Mirvish SS (1975) Formation of N-nitroso compounds. Chemistry, kinetics and in vivo occurrence. *Toxicol. Appl. Pharmacol.* 31: 325-351

Pfundstein B, Tricker AR, Theobald E, Preussmann R, Spiegelhalder B (1991) Mean daily intake of primary and secondary amines from foods and beverages in the Federal Republic of Germany in 1989-1990. *Food Chem. Toxicol.* 29: 733-739

Ridd JH (1961) Nitrosation diazotisation and deamination: Quaterly Review of the Chemical society London 15, 418-425

Sander J, Bürkle G (1969) Induktion maligner Tumoren bei Ratten durch gleichzeitige Verfütterung von Nitrit und sekundären Aminen. *Z. Krebsforsch.* 73: 54-66

Schmähl D, Preussmann R (1959) The carcinogenic effect of nitrosodimetylamine in Rats. *Naturwissen.* 46: 175

Schmähl D, Preussmann R, Hamperl H (1960) Leberkrebserzeugende Wirkung von Diaethylnitrosamin nach oraler Gabe bei Ratten. *Naturwissen.* 47: 89-99

Schoental R (1960) Carcinogenic action of diazomethane and nitroso-N-methyl urethane. *Nature (London)* 188: 420-421

Shuker DE, Margison P (1997) Nitrosated Glycine Derivativesas a Potential Source of O^6-Methylguanine in DNA, *Cancer Research,* 57, 366-369

Spiegelhalder B (1990) Influence of Dietary Nitrate on in vivo Nitrosation of Amidopyridine in Humans; Use of „Ethanol Effect“ for Biological Monitoring of N-Nitrosdimethylamine in Urine, in Eisenbrand et al. The Significance of N-Nitrosation of Drugs, Gustav-Fischer-Verlag, 199-212

Tricker AR, Kumar R, Siddiqi M, Khuroo MS, Preussmann R (1991) Endogenous formation of N-nitrosamines from pierazine and their urinary excretion following antihelmintic treatment with piperazine citrate. *Carcinogenesis* 12: 1595-1599

Tricker AR (1997) N-Nitroso compounds and man: sources of exposure, endogenous formation and occurrence in body fluids. *Eur. J.*

Recent Studies on the Endogenous Formation of *N*-Nitroso-Compounds

Stephen S. Hecht, Irina Stepanov, and Mingyao Wang

Masonic Cancer Center, University of Minnesota

Endogenous formation of *N*-nitroso- compounds from the reaction of amines or amides with nitrite in the body is a potential source of human carcinogen exposure. In our recent studies, we have investigated the endogenous formation of the tobacco-specific carcinogen *N'*-nitrosonornicotine (NNN) in people using nicotine replacement therapy [1;2]. In separate investigations, we have detected 7-(2-carboxyethyl)guanine in human hepatic DNA [3]. Endogenous nitrosation is one possible source of this DNA adduct. This presentation will summarize data from these projects.

NNN induces oral cavity, nasal cavity and esophageal tumors in rats, and tumors of the respiratory tract in hamsters and mice. NNN is present in all unburned tobacco products such as smokeless tobacco, as well as in cigarette smoke. All tobacco users are exposed to considerable amounts of NNN, considered a human carcinogen by the International Agency for Research on Cancer [4]. NNN is easily and rapidly formed by nitrosation of nornicotine in vitro, and less readily by nitrosation of nicotine [5;6]. Our earlier studies also showed that NNN can be formed in vivo in rats treated with nornicotine and nitrite, and that this endogenous nitrosation can be blocked by ascorbic acid [7;8].

In our recent studies, we have examined the formation of NNN in people who use nicotine replacement products. These products are commonly used by ex-smokers to help maintain a state of smoking cessation. Several types of products are available including nicotine lozenges, nicotine gum, and nicotine patch. In the studies reported here, we investigated urinary levels of NNN in subjects who had stopped smoking with the help of these nicotine replacement therapy products. We observed that several metabolites derived from tobacco smoke constituents such as NNK, pyrene, 1,3-butadiene, acrolein, ethylene oxide, and others decreased smoothly in the days and weeks following smoking cessation [9]. However, this was not the case for NNN. Rather than observing the expected smooth decrease of this tobacco-specific compound after cessation of cigarette smoking, we observed spikes of increased urinary levels of NNN at various times after cessation. The spikes were particularly evident in subjects who used oral nicotine replacement products such as the nicotine lozenge or gum. These results could not be explained by artifactual formation of NNN during collection or storage of the urine samples, or during the analysis for NNN. The results were consistent with endogenous formation of NNN in some subjects, resulting from nitrosation of nornicotine, most likely in the mouth or stomach. In support of this proposal, we have recently examined the nitrosation of [D_4]nornicotine in human saliva [10]. [D_4]Nornicotine was used in this study to eliminate the possibility of sources of NNN other than endogenous formation. Incubation of [D_4]nornicotine with saliva from 10 non-smoking volunteers resulted in the formation of [D_4]NNN in samples from 8 of these individuals, without the addition of any other substance to the saliva. We did not observe [D_4]NNN in saliva samples incubated with [D_4]nicotine. [D_4]NNN, identified by mass spectrometry in these experiments, resulted from the reaction of salivary nitrite with [D_4]nornicotine. These results are entirely consistent with earlier chemical studies demonstrating the rapid nitrosation of nornicotine. Collectively, these results provide convincing evidence for the endogenous formation of NNN in some people using nicotine replacement products. The source of nornicotine in these studies can be the product itself or metabolism of nicotine to nornicotine. This

potential hazard could be addressed, at least in part, by excluding nornicotine from nicotine replacement products or by formulating them with an inhibitor of nitrosation such as ascorbic acid.

Nicotine replacement therapy demonstrably improves smoking cessation. The benefit of cessation far outweighs the risk of NNN exposure during use of these products, because exposure to multiple carcinogens is decreased. There is presently no evidence that long term users of nicotine replacement products have an increased risk of cancer above and beyond that due to their history of smoking. Nevertheless, it would be prudent to avoid NNN exposure in users of these products.

In the second group of studies to be described here, we have analyzed human liver samples for two DNA adducts – 7-(2′-carboxyethyl)guanine (7-CEG) and 7-carboxymethylguanine (7-CMG) [3]. A total of 24 samples were analyzed by liquid chromatography-mass spectrometry. 7-CEG was detected in all samples, mean 373 fmol/μmol G, but 7-CMG was not detected in any sample. The levels of 7-CEG are comparable to those of N^2-ethylidene-dG, formed from acetaldehyde. This adduct has the potential to depurinate spontaneously leading to apurinic sites and mutagenesis.

We considered three possible sources of 7-CEG in human liver DNA. The first would be exposure to 3-(methylnitrosamino)propionic acid (MNPA), which has been detected in some human urine samples. Metabolism of this compound would be expected to produce intermediates that could react with DNA resulting in formation of 7-CEG . We consider this explanation to be fairly unlikely because human exposure to the related nitrosamine *N*-nitrososarcosine is far more common, based on studies of its occurrence in urine, yet we did not detect the corresponding expected DNA adduct 7-CMG in any human liver sample. The second explanation involves reaction of acrylic acid with DNA, which is known to produce 7-CEG, albeit slowly and in low yield. Acrylic acid is a metabolite of acrolein, a common environmental contaminant and endogenous product of lipid peroxidation. All humans appear to have acrolein-DNA adducts in their white blood cells and some other tissues. The third possible explanation involves endogenous nitrosation. 1-Nitroso-5,6-dihydrouracil (NDHU), the nitrosation product of 5,6-dihydrouracil, is a powerful hepatocarcinogen in the rat and reacts readily with DNA to produce 7-CEG. NDHU could be formed endogenously from nitrosation of 5,6-dihydrouracil, a normal pyrimidine metabolite present in human urine and plasma. 5,6-Dihydrouracil is further metabolized to β-ureidopropionic acid and β-alanine, both of which are found in human urine and could also be nitrosated to ultimately produce 7-CEG. We are currently exploring the potential formation of 7-CEG in rats treated with nitrite and 5,6-dihydrouracil or β-ureidopropionic acid. The results of these studies could provide new insights on causes of hepatic DNA damage and possibly hepatocarcinogenesis in humans.

Reference List

1. Stepanov,I., Carmella,S.G., Briggs,A., Hertsgaard,L., Lindgren,B., Hatsukami,D.K., and Hecht,S.S. (2009) Presence of the carcinogen *N′*-nitrosonornicotine in the urine of some users of oral nicotine replacement therapy products. *Cancer Res.*, **69**, 8236-8240.

2. Stepanov,I., Carmella,S.G., Han,S., Pinto,A., Strasser,A.A., Lerman,C., and Hecht,S.S. (2009) Evidence for endogenous formation of *N′*-nitrosonornicotine in some long term nicotine patch users. *Nicotine Tob.Res.*, **11**, 99-105.

3. Cheng,G., Wang,M., Villalta,P.W., and Hecht,S.S. (2010) Detection of 7-(2'-carboxyethyl)guanine but not 7-carboxymethylguanine in human liver DNA. *Chem.Res.Toxicol.*, **23**, 1089-1096.

4. International Agency for Research on Cancer (2007) Smokeless tobacco and tobacco-specific nitrosamines. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, v. 89*. IARC, Lyon, FR, pp 41-583.

5. Mirvish,S.S., Sams,J., and Hecht,S.S. (1977) Kinetics of nornicotine and anabasine nitrosation in relation to *N*'-nitrosonornicotine occurrence in tobacco and to tobacco-induced cancer. *J.Natl.Cancer Inst.*, **59**, 1211-1213.

6. Hecht,S.S., Chen,C.B., Ornaf,R.M., Jacobs,E., Adams,J.D., and Hoffmann,D. (1978) Reaction of nicotine and sodium nitrite: Formation of nitrosamines and fragmentation of the pyrrolidine ring. *J.Org.Chem.*, **43**, 72-76.

7. Carmella,S., Borukhova,A., Desai,D., and Hecht,S.S. (1997) Evidence for endogenous formation of tobacco-specific nitrosamines from nicotine and other tobacco alkaloids in rats. *Carcinogenesis*, **18**, 587-592.

8. Porubin,D., Hecht,S.S., Li,Z.Z., Gonta,M., and Stepanov,I. (2007) Endogenous formation of *N'*-nitrosonornicotine in F344 rats in the presence of some antioxidants and grape seed extract. *J.Agric.Food Chem.*, **55**, 7199-7204.

9. Carmella,S.G., Chen,M., Han,S., Briggs,A., Jensen,J., Hatsukami,D.K., and Hecht,S.S. (2009) Effects of smoking cessation on eight urinary tobacco carcinogen and toxicant biomarkers. *Chem.Res.Toxicol.*, **22**, 734-741.

10. Knezevich,A., Muzic,J., Hatsukami,D.K., Hecht,S.S., and Stepanov,I. (2012) Nornicotine nitrosation in saliva and its relation to endogenous synthesis of *N'*-nitrosonornicotine in humans. *Nicotine Tob Res*.

Physiological effects of nitrate, nitrite and nitric oxide: An Overview on recent insights

Jon Lundberg MD, PhD

Karolinska Institutet

Stockholm, Sweden

Summary: Inorganic nitrate (NO_3^-) and nitrite (NO_2^-) are part of the nitrogen cycle in nature. To toxicologists and the general public these anions are generally known as undesired residues in the food chain with potentially carcinogenic effects (1). Among NO biologists, these inorganic anions have merely been viewed as inert oxidative end products of endogenous nitric oxide (NO) metabolism (2). However, more recent studies surprisingly show that nitrate and nitrite can be metabolized in vivo to form nitric oxide (NO) and other bioactive nitrogen oxides (3-5). This represents an important alternative source of NO especially during hypoxia when the oxygen-dependent L-arginine-NO pathway can be dysfunctional (6). These novel lines of research suggest important biological functions of the nitrate-nitrite-NO pathway with profound implications in relation to the diet and cardiovascular homeostasis. Nitrate in dietary doses reduces blood pressure in healthy volunteers as well as in hypertensives (7-9). In addition nitrate increases the efficiency of mitochondrial respiration (10), inhibits platelet aggregation (11) and improves peripheral blood flow in peripheral artery disease (12). Moreover, an increasing number of studies suggest a therapeutic potential for nitrate and nitrite in diseases such as myocardial infarction, stroke, hypertension, renal failure and gastric ulcers (13, 14). A theory is emerging suggesting nitrate as an active component in vegetables contributing to the beneficial health effects of this food group (15), including protection against cardiovascular disease and type-2 diabetes. Toxicologists and authorities advocating merely negative health effects of nitrate, must now seriously take these opposing new data into consideration when attempting to judge the overall impact of dietary nitrate on human health.

The Nitrate-Nitrite-NO Pathway: Mammalian NOS-independent NO generation from nitrate was first demonstrated in 1994 by two independent groups (16, 17). Nitrate from endogenous (NOS) and exogenous (diet) sources circulates in blood and is actively taken up by the salivary glands and excreted in saliva. In the oral cavity nitrate is reduced to nitrite by commensal bacteria and the nitrite formed is continuously swallowed into the acidic lumen of the stomach (18, 19). Large amounts of NO and other reactive nitrogen oxides are immediately formed in the stomach upon acidification and these species have profound effects on the gastric mucosa, including stimulation of mucosal blood flow, mucus generation and inhibition of pathogen growth (13). Remaining nitrite is absorbed systemically and numerous non-enzymatic and enzymatic pathways help to further reduce the nitrite to NO in blood and tissues (5). Oral bacteria are essential for the bioactivation of dietary nitrate and removal of these bacteria by an antiseptic mouthwash effectively blocks nitrite formation and the biological effects of nitrate (20, 21).

Cardiovascular and metabolic effects of dietary nitrate: In 2006 Larsen and colleagues showed that blood pressure is reduced in healthy volunteers after ingestion of nitrate at doses easily achievable with a diet rich in vegetables (7). Webb and colleagues then noted substantial BP-lowering effects of a natural nitrate source (beetroot juice) (8) and these studies have since been confirmed by numerous groups (22). NO formation from nitrate also has other effects on the cardiovascular system including increases in peripheral blood flow, inhibition of platelet function and improvement of vascular function (measured as increases in flow-mediated dilation, FMD).

Interestingly, dietary nitrate also has metabolic effects. Larsen et al. demonstrated that the amount of oxygen consumed during physical exercise is decreased by nitrate (23), a finding that has been reproduced in numerous studies (24-26). The mechanism of this remarkable effect seems to be a nitrate-induced increase in mitochondrial efficiency so that more ATP is generated for every oxygen molecule consumed thereby making muscular work more efficient (10). Studies now also show that athletic performance is increased by these mechanisms (27). Although not yet studied in detail, this could be of importance not only for competitive athlese but also for certain groups of patients characterized by oxygen deficiency, including those with peripheral artery disease (PAD), mitochondrial diseases and chronic obstructive lung disease. Animal studies have also shown other interesting metabolic effects of nitrate including reversal of the metabolic syndrome-like state seen in mice lacking endothelial NO synthase (28), and stimulation of insulin release from pancreatic islets (29).

Summary and future perspectives

During the last decade we have witnessed a veritable paradigm shift in how we are viewing dietary nitrate in relation to human health. For > 50 years this inorganic anion, abundant in vegetables, has been considered to be solely a toxic unwanted residue in our food chain. Then with the discovery of the L-arginine/NO pathway, scientists began to view nitrate merely as an inert end product of NO metabolism. Now the picture is beginning to change again with the discoveries of potential beneficial effects of nitrate, especially in relation to cardiovascular function. These effects of depend on nitrate bioactivation by oral bacteria which generate the more reactive nitrite anion. Nitrite is partly absorbed systemically and can be further metabolised to NO and other bioactive nitrogen oxides in blood and tissues. Nitrate still has a terrible reputation world wide which is mainly based on the proposed relationship with nitrosamine formation and development of cancer. Importantly however, such effects of nitrate have not been substantiated despite immense efforts over the past five decades. With the emergence of new data showing possible positive effects of nitrate on the cardiovascular system, we need to balance the discussion and also consider such effects when judging the overall impact of nitrate on human health. It is of outmost importance that toxicologists, biochemists, physiologists and other researchers begin to communicate and cooperate with each other to finally settle the role of dietary nitrate in health and disease. Regulatory authorities should be involved in these discussions and ultimately the goal would be to establish solid recommendations regarding allowable levels of nitrate in food and drinking water, acceptable (or recommended) daily intake, and general advice on how nitrate affects human health.

References

1. Tannenbaum SR & Correa P (1985) Nitrate and gastric cancer risks. *Nature* 317(6039):675-676.

2. Moncada S & Higgs A (1993) The L-arginine-nitric oxide pathway. *N Engl J Med* 329(27):2002-2012.

3. Lundberg JO*, et al.* (2009) Nitrate and nitrite in biology, nutrition and therapeutics. *Nature Chemical Biology* 5(12):865-869.

4. Lundberg JO, Weitzberg E, & Gladwin MT (2008) The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. *Nature Reviews Drug Discovery* 7(2):156-167.

5. Gladwin MT*, et al.* (2005) The emerging biology of the nitrite anion. *Nat Chem Biol* 1(6):308-314.

6. van Faassen EE*, et al.* (2009) Nitrite as regulator of hypoxic signaling in mammalian physiology. *Med Res Rev* 29(5):683-741.

7. Larsen FJ, Ekblom B, Sahlin K, Lundberg JO, & Weitzberg E (2006) Effects of dietary nitrate on blood pressure in healthy volunteers. *N Engl J Med* 355(26):2792-2793.

8. Webb AJ*, et al.* (2008) Acute blood pressure lowering, vasoprotective, and antiplatelet properties of dietary nitrate via bioconversion to nitrite. *Hypertension* 51(3):784-790.

9. Ghosh SM*, et al.* (2013) Enhanced vasodilator activity of nitrite in hypertension: critical role for erythrocytic xanthine oxidoreductase and translational potential. *Hypertension* 61(5):1091-1102.

10. Larsen FJ*, et al.* (2011) Dietary Inorganic Nitrate Improves Mitochondrial Efficiency in Humans. *Cell metabolism* 13(2):149-159.

11. Richardson G*, et al.* (2002) The ingestion of inorganic nitrate increases gastric S-nitrosothiol levels and inhibits platelet function in humans. *Nitric Oxide* 7(1):24-29.

12. Kenjale AA*, et al.* (2011) Dietary Nitrate Supplementation Enhances Exercise Performance in Peripheral Arterial Disease. *Journal of applied physiology*.

13. Lundberg JO & Weitzberg E (2012) Biology of nitrogen oxides in the gastrointestinal tract. *Gut*.

14. Lundberg JO & Weitzberg E (2005) NO generation from nitrite and its role in vascular control. *Arterioscler Thromb Vasc Biol* 25(5):915-922.

15. Lundberg JO, Feelisch M, Bjorne H, Jansson EA, & Weitzberg E (2006) Cardioprotective effects of vegetables: is nitrate the answer? *Nitric Oxide* 15(4):359-362.

16. Lundberg JO, Weitzberg E, Lundberg JM, & Alving K (1994) Intragastric nitric oxide production in humans: measurements in expelled air. *Gut* 35(11):1543-1546.

17. Benjamin N*, et al.* (1994) Stomach NO synthesis. *Nature* 368(6471):502.

18. Lundberg JO & Govoni M (2004) Inorganic nitrate is a possible source for systemic generation of nitric oxide. *Free Radic Biol Med* 37(3):395-400.

19. Lundberg JO, Weitzberg E, Cole JA, & Benjamin N (2004) Nitrate, bacteria and human health. *Nat Rev Microbiol* 2(7):593-602.

20. Kapil V*, et al.* (2012) Physiological role for nitrate-reducing oral bacteria in blood pressure control. *Free Radic Biol Med*.

21. Petersson J*, et al.* (2009) Gastroprotective and blood pressure lowering effects of dietary nitrate are abolished by an antiseptic mouthwash. *Free Radic Biol Med* 46(8):1068-1075.

22. Siervo M, Lara J, Ogbonmwan I, & Mathers JC (2013) Inorganic Nitrate and Beetroot Juice Supplementation Reduces Blood Pressure in Adults: A Systematic Review and Meta-Analysis. *The Journal of nutrition* 143(6):818-826.

23. Larsen FJ, Weitzberg E, Lundberg JO, & Ekblom B (2007) Effects of dietary nitrate on oxygen cost during exercise. *Acta Physiol (Oxf)* 191(1):59-66.

24. Bailey SJ*, et al.* (2010) Dietary nitrate supplementation enhances muscle contractile efficiency during knee-extensor exercise in humans. *Journal of applied physiology* 109(1):135-148.

25. Bailey SJ*, et al.* (2009) Dietary nitrate supplementation reduces the O2 cost of low-intensity exercise and enhances tolerance to high-intensity exercise in humans. *Journal of applied physiology* 107(4):1144-1155.

26. Vanhatalo A*, et al.* (2010) Acute and chronic effects of dietary nitrate supplementation on blood pressure and the physiological responses to moderate-intensity and incremental exercise. *American journal of physiology. Regulatory, integrative and comparative physiology*.

27. Lansley KE*, et al.* (2011) Acute Dietary Nitrate Supplementation Improves Cycling Time Trial Performance. *Med Sci Sports Exerc*.

28. Carlstrom M*, et al.* (2010) Dietary inorganic nitrate reverses features of metabolic syndrome in endothelial nitric oxide synthase-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America* 107(41):17716-17720.

29. Nystrom T*, et al.* (2012) Inorganic nitrite stimulates pancreatic islet blood flow and insulin secretion. *Free Radical Biology and Medicine* 53(5):1017-1023.

A 'green' approach to cardiovascular disease: recycling inorganic nitrate and nitrite

Prof Amrita Ahluwalia

William Harvey Research Institute

Barts& The London School of Medicine & Dentistry

Queen Mary University of London

Fruit and vegetable-rich diets reduce blood pressure[1] and risk of ischaemic stroke[2] and ischemic heart disease[3]. Whilst the cardioprotective effects of a fruit and vegetable-rich diet are unequivocal, the exact mechanisms of this effect remain uncertain. Recent evidence has highlighted the possibility that dietary nitrate, an inorganic anion found in large quantities in vegetables (particularly green leafy vegetables), may have a role to play. This beneficial activity lies in the processing in vivo of nitrate to nitrite and then nitrite to the pleiotropic molecule nitric oxide (NO). NO in turn exerts a range of beneficial effects on the cardiovascular system including lowering of blood pressure, suppression of inflammation and prevention/retardation of atherosclerosis. This range of bioactivity essentially endows NO with anti-hypertensive and anti-atherosclerotic activities that protects individuals from hypertension and associated cardiovascular disease[4]. The possibility that provision of nitrate may offer a mechanism for elevating beneficial NO that might, in particular, lower blood pressure is therefore an exciting prospect.

Despite the substantial advances made in anti-hypertensive pharmacotherapy, it is estimated that globally by 2025 there will be 1.5 billion people with hypertension[5]. The significance of this observation is fully appreciated when one considers that ~50% of all heart attacks and ~60-70% of all strokes occur as a direct consequence of raised blood pressure. Indeed, at a population level it has been estimated that as little as a 2mmHg increase in systolic blood pressure increases mortality due to ischaemic heart disease and stroke by 7% and 10% respectively[6]. Furthermore, of considerable concern is that, over the past 3 decades, the numbers of patients with uncontrolled essential hypertension is rising[7]. Thus, whilst pharmacotherapy plays an important role in the therapeutics of hypertension, there is a pressing need for identification of alternative but also preventative strategies that are both effective and easily achievable. This imperative has renewed interest in identifying approaches that take advantage of the beneficial effects of diets rich in fruit and vegetables[8], particularly since epidemiological[9], cohort[2,3] and trial data[1,10] demonstrate that increased consumption of a vegetable-rich diet protects against various forms of CVD, including hypertension[1]. In addition the focus, in part, stems from a perception that dietary interventions may be more acceptable and achievable for many patients. In particular, such an option may provide an alternative that could prove useful in the ~30-40% of treated hypertensives that remain with elevated blood pressures despite their pharmacotherpy[11]. Importantly, whilst some of these individuals likely have what is termed 'resistant hypertension' (recent estimates suggest ~9% in the US[12]), for many of these individuals it is thought that compliance plays a significant contribution[13].

Large-scale clinical trials of several different nutrients found in vegetables including antioxidant vitamins and folate have failed to show a beneficial cardiovascular effect[14-16]. As such attention has focused on other components of vegetables that may have a role, including inorganic nitrate[17,18]. Interestingly, the first published evidence of potential positive effects of inorganic nitrate on blood pressure date back to 1927 when Stieglitz published his observations that bismuth subnitrate lowered blood pressure in individuals with raised blood pressure[19]. Stieglitz published further observations confirming his original findings in specific cohorts[20], however the concept appeared to lose favour with publication of some dissenting observations[21]. In 2006, however, the concept was revived by Lundberg and Weitzberg and colleagues, who conducted a well-controlled clinical study i.e. double blind placebo control, that enabled clear cut assessment of potential bioactivity. This group demonstrated that supplementation of healthy volunteers with sodium nitrate (0.1mmol/kg/day) for 3 days resulted in a decrease in diastolic (DBP) of ~3mmHg[22].

More recently, we extended the above observations to determine whether dietary nitrate might offer similar bioactivity to nitrate salt supplementation. We used beetroot as our vehicle to deliver a dietary nitrate dose. Beetroot is a high nitrate-containing vegetable and, indeed, in our study a relatively high dose of 22.5 mmol was delivered, and a number of outcomes measured over the following 24 hours. A single dose of dietary nitrate lowered both SBP and DBP with the peak effect of 10/8 mmHg occurring approximately 2.5-3 hours following ingestion and blood pressure remaining lowered at the 24 hour time-point[23]. We then conducted a second study assessing the dose-response relationship as well as demonstrating that nitrate salt supplementation exerted an almost identical profile of bioactivity both in terms of the magnitude of the effect and dose-response relationship[24]. Importantly, we discovered that the threshold dose for blood pressure lowering in healthy volunteers was ~4 mmol which equates to just above the recommended daily amount of 4.2 mmol per day in a 70 kg individual (3.7mg/kg/day[25]). These effects of nitrate upon blood pressure are thought to be mediated by its sequential reduction to nitrite and then NO. NO in turn activates the enzyme soluble guanylate cyclase elevating cGMP levels, ultimately resulting in the relaxation of vascular smooth muscle, vasodilation and therefore decrease in blood pressure. Involvement of this pathway in the blood pressure lowering effects of dietary nitrate is supported by observations of nitrate-induced rises in circulating cGMP levels after either dietary or salt supplementation and by increases in vascular compliance, reflecting smooth muscle relaxation[26]. In addition to lowering blood pressure, we also demonstrated that both dietary nitrate ingestion or nitrate salt supplementation provided protection of the endothelium from ischaemia-reperfusion (IR)-induced endothelial damage as well as a suppression of platelet reactivity assessed ex vivo[23]. Together, such a profile of activity would suggest that dietary nitrate might prove useful in the therapeutics of cardiovascular disease.

The activity of orally ingested inorganic nitrate lies in its conversion to nitrite by facultative bacteria found on the dorsal surface of the tongue[27,28], the same pathways that have been proposed to underlie the potential detrimental effects of nitrate[29]. The swallowing of nitrite-rich saliva permits entry of nitrite into the circulation via the stomach and then, once within the circulation, nitrite can be converted to the potent vasodilator nitric oxide (NO)[24,30-32].Indeed, in our work with beetroot we

demonstrated that preventing this 'enterosalivary' circuit, by asking volunteers not to swallow their saliva for 3 hours following ingestion of beetroot, completely prevented the rise in circulating nitrite associated with nitrate ingestion as well as block of the decrease in blood pressure[23]. These findings are in accord with previous observations demonstrating that destruction of oral bacteria using an antibacterial mouthwash abolishes conversion of nitrate to nitrite in the oral cavity as well as blocks the consequent rise in circulating nitrite levels following sodium nitrate salt supplementation[33,34].

An important consideration is that the beneficial effects of inorganic nitrate result in modest rises in circulating nitrite i.e. no more than a doubling of circulating nitrite levels. Since baseline circulating nitrite is thought to lie somewhere in the region of 20-400nM (for review[35]), the biological effects of nitrate are evident with circulating nitrite concentrations, at the very most, in the low μM realm. Importantly, acutely such rises in circulating nitrite are not associated with any overt toxicity such as met-haemoglobinaemia[36]. Certainly, searches of the literature suggest no evidence that consumption of high nitrate containing vegetables is associated with met-haemoglobinaemia or any other negative aspects of activity that have been purported to be associated with inorganic nitrate ingestion either. Whether long term ingestion of a daily dietary nitrate load might result in any toxicity is uncertain, although to date no clear cut prospective evidence supporting the view that inorganic nitrate expresses a toxicity profile at the doses falling within the range tested in healthy volunteers exists[25].

Perhaps, our recent observations in hypertensive patients might go some way to allay concerns regarding potentially appropriate dosing regimes. We have recently demonstrated that despite 4mmol having little to no effect upon blood pressure in healthy volunteers, that in individuals with Grade 1 hypertension (i.e. SBP ≥ 140 mmHg and/or DBP ≥ 90 mmHg) a single dose just below this level (i.e.~3.5mmol) causes an average decrease in blood pressure of ~12/10 mmHg. Such a dose of nitrate clearly sits at the recommended daily consumption amounts and therefore poses perhaps less concern regarding potential, albeit unlikely, toxicity. As in healthy volunteers, the peak effect occurred at 2.5-3 h in parallel with the rise in circulating nitrite concentration, but also with significant blood pressure lowering still evident at 24h (~60% of the peak effect)[37]. This increased potency is in part due to an upregulation in the expression and activity of a key nitrite reductase in hypertensive patients, an enzyme called xanthine oxidoreductase[37]. We have hypothesised that this upregulation results in a greater conversion of circulating nitrite to NO within the blood vessel. We have just completed a double-blind placebo controlled study investigating the effect of this same once a day dose for 4 weeks in patients with hypertension. To date the dietary nitrate has been well-tolerated with no adverse effects reported. The results of this study will determine whether the effects of dietary nitrate on blood pressure can be sustained for longer periods of time (Clinicaltrials.gov identifier: NCT01405898).

Since, we published our initial observations with dietary nitrate there have been a number of studies that have confirmed the blood pressure lowering effects of an acute dietary nitrate load in various cohorts ranging from elite athletes to patients with peripheral vascular disease[38,39]. In addition, a particularly intriguing series of findings demonstrating that inorganic nitrate might also improve mitochondrial oxygen utilisation[38,40] raise the prospect that in addition to the potential beneficial

effects for hypertensives, individuals with conditions such as heart failure may benefit from improved dietary nitrate intake. Such observations provide encouraging support for the thesis that dietary nitrate expresses a range of attributes that need to be considered when assessing the risk:benefit ratio of dietary ingestion.

In sum, the demonstration that dietary nitrate expresses a range of activities that result in positive outcomes upon the cardiovascular system support the need for a reappraisal of risk:benefit ratio in determining acceptable levels of dietary intake.

Reference List

1. Appel LJ, Moore TJ, Obarzanek E, Vollmer WM, Svetkey LP, Sacks FM, Bray GA, Vogt TM, Cutler JA, Windhauser MM, Lin PH, Karanja N. A clinical trial of the effects of dietary patterns on blood pressure. DASH Collaborative Research Group. *N Engl J Med*. 1997;336:1117-1124.

2. Joshipura KJ, Ascherio A, Manson JE, Stampfer MJ, Rimm EB, Speizer FE, Hennekens CH, Spiegelman D, Willett WC. Fruit and vegetable intake in relation to risk of ischemic stroke. *JAMA*. 1999;282:1233-1239.

3. Joshipura KJ, Hu FB, Manson JE, Stampfer MJ, Rimm EB, Speizer FE, Colditz G, Ascherio A, Rosner B, Spiegelman D, Willett WC. The effect of fruit and vegetable intake on risk for coronary heart disease. *Ann Intern Med*. 2001;134:1106-1114.

4. Moncada S, Higgs A. The L-arginine-nitric oxide pathway. *N Engl J Med*. 1993;329:2002-2012.

5. Kearney PM, Whelton M, Reynolds K, Muntner P, Whelton PK, He J. Global burden of hypertension: analysis of worldwide data. *Lancet*. 2005;365:217-223.

6. Lewington S, Clarke R, Qizilbash N, Peto R, Collins RCPS. Age-specific relevance of usual blood pressure to vascular mortality: a meta-analysis of individual data for one million adults in 61 prospective studies. *The Lancet*. 2002;360:1903-1913.

7. Chobanian AV. The Hypertension Paradox-- More Uncontrolled Disease despite Improved Therapy. *New England Journal of Medicine*. 2009;361:878-887.

8. Appel LJ, Brands MW, Daniels SR, Karanja N, Elmer PJ, Sacks FM. Dietary approaches to prevent and treat hypertension: a scientific statement from the American Heart Association. *Hypertension*. 2006;47:296-308.

9. Sacks FM, Rosner B, Kass EH. Blood pressure in vegetarians. *Am J Epidemiol*. 1974;100:390-398.

10. Rouse IL, Beilin LJ, Armstrong BK, Vandongen R. Blood-pressure-lowering effect of a vegetarian diet: controlled trial in normotensive subjects. *Lancet*. 1983;1:5-10.

11. Egan BM, Zhao Y, Axon RN. US Trends in Prevalence, Awareness, Treatment, and Control of Hypertension, 1988-2008. *JAMA: The Journal of the American Medical Association*. 2010;303:2043-2050.

12. Persell SD. Prevalence of Resistant Hypertension in the United States, 2003–2008. *Hypertension*. 2011;57:1076-1080.

13. Ahmed MI, Calhoun DA. Resistant Hypertension. *Hypertension*. 2011;57:1045-1046.

14. Vivekananthan DP, Penn MS, Sapp SK, Hsu A, Topol EJ. Use of antioxidant vitamins for the prevention of cardiovascular disease: meta-analysis of randomised trials. *Lancet*. 2003;361:2017-2023.

15. Bjelakovic G, Nikolova D, Gluud LL, Simonetti RG, Gluud C. Mortality in randomized trials of antioxidant supplements for primary and secondary prevention: systematic review and meta-analysis. *JAMA*. 2007;297:842-857.

16. Bjelakovic G, Nikolova D, Gluud LL, Simonetti RG, Gluud C. Antioxidant supplements for prevention of mortality in healthy participants and patients with various diseases. *Cochrane Database Syst Rev*. 2012;3:CD007176.

17. Lundberg JO, Feelisch M, Bjorne H, Jansson EA, Weitzberg E. Cardioprotective effects of vegetables: is nitrate the answer? *Nitric Oxide*. 2006;15:359-362.

18. Sobko T, Marcus C, Govoni M, Kamiya S. Dietary nitrate in Japanese traditional foods lowers diastolic blood pressure in healthy volunteers. *Nitric Oxide*. 2010;22:136-140.

19. Stieglitz EJ. Bismuth subnitrate in the therapy of hypertension. *J Pharmacol Exp Ther*. 1927;32:23-35.

20. Stieglitz EJ. BIsmuth subnitrate in the treatment of arterial hypertension. *Journal of the American Medical Association*. 1930;95:842-846.

21. Ayman D. BIsmuth subnitrate in the treatment of arteriolar (essential) hypertension. *Journal of the American Medical Association*. 1932;98:545-548.

22. Larsen FJ, Ekblom B, Sahlin K, Lundberg JO, Weitzberg E. Effects of dietary nitrate on blood pressure in healthy volunteers. *N Engl J Med*. 2006;355:2792-2793.

23. Webb AJ, Patel N, Loukogeorgakis S, Okorie M, Aboud Z, Misra S, Rashid R, Miall P, Deanfield J, Benjamin N, MacAllister R, Hobbs AJ, Ahluwalia A. Acute blood pressure lowering, vasoprotective, and antiplatelet properties of dietary nitrate via bioconversion to nitrite. *Hypertension*. 2008;51:784-790.

24. Kapil V, Milsom AB, Okorie M, Maleki-Toyserkani S, Akram F, Rehman F, Arghandawi S, Pearl V, Benjamin N, Loukogeorgakis S, MacAllister R, Hobbs AJ, Webb AJ, Ahluwalia A. Inorganic nitrate supplementation lowers blood pressure in humans: role for nitrite-derived NO. *Hypertension*. 2010;56:274-281.

25. FAO/WHO. Nitrate (and potential endogenous formation of N-nitroso compounds). In: WHO Food Additive series 50. 2003. World Health Organisation, Geneva.

26. Bahra M, Kapil V, Pearl V, Ghosh S, Ahluwalia A. Inorganic nitrate ingestion improves vascular compliance but does not alter flow-mediated dilatation in healthy volunteers. *Nitric Oxide*. 2012;26:197-202.

27. Spiegelhalder B, Eisenbrand G, Preussmann R. Influence of dietary nitrate on nitrite content of human saliva: possible relevance to in vivo formation of N-nitroso compounds. *Food Cosmet Toxicol*. 1976;14:545-548.

28. Tannenbaum SR, Weisman M, Fett D. The effect of nitrate intake on nitrite formation in human saliva. *Food Cosmet Toxicol*. 1976;14:549-552.

29. Tannenbaum SR, Correa P. Nitrate and gastric cancer risks. *Nature*. 1985;317:675-676.

30. Modin A, Bjorne H, Herulf M, Alving K, Weitzberg E, Lundberg JO. Nitrite-derived nitric oxide: a possible mediator of 'acidic-metabolic' vasodilation. *Acta Physiol Scand*. 2001;171:9-16.

31. Cosby K, Partovi KS, Crawford JH, Patel RP, Reiter CD, Martyr S, Yang BK, Waclawiw MA, Zalos G, Xu X, Huang KT, Shields H, Kim-Shapiro DB, Schechter AN, Cannon RO, III, Gladwin MT. Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. *Nat Med*. 2003;9:1498-1505.

32. Dejam A, Hunter CJ, Tremonti C, Pluta RM, Hon YY, Grimes G, Partovi K, Pelletier MM, Oldfield EH, Cannon RO, III, Schechter AN, Gladwin MT. Nitrite Infusion in Humans and Nonhuman Primates. Endocrine Effects, Pharmacokinetics, and Tolerance Formation. *Circulation*. 2007;116:1821-1831.

33. Govoni M, Jansson EA, Weitzberg E, Lundberg JO. The increase in plasma nitrite after a dietary nitrate load is markedly attenuated by an antibacterial mouthwash. *Nitric Oxide*. 2008;19:333-337.

34. Kapil V, Haydar SM, Pearl V, Lundberg JO, Weitzberg E, Ahluwalia A. Physiological role for nitrate-reducing oral bacteria in blood pressure control. *Free Radic Biol Med*. 2013;55:93-100.

35. Kapil V, Webb AJ, Ahluwalia A. Inorganic nitrate and the cardiovascular system. *Heart*. 2010;96:1703-1709.

36. Pluta RM, Oldfield EH, Bakhtian KD, Fathi AR, Smith RK, DeVroom HL, Nahavandi M, Woo S, Figg WD, Lonser RR. Safety and Feasibility of Long-term Intravenous Sodium Nitrite Infusion in Healthy Volunteers. *PLoS ONE*. 2011;6:e14504.

37. Ghosh SM, Kapil V, Fuentes-Calvo I, Bubb KJ, Pearl V, Milsom AB, Khambata RS, Maleki-Toyserkani S, Yousuf M, Benjamin N, Webb AJ, Caulfield MJ, Hobbs AJ, Ahluwalia A. Enhanced vasodilator activity of nitrite in hypertension: critical role for erythrocytic xanthine oxidoreductase and translational potential. *Hypertension*. 2013;In press.

38. Bailey SJ, Winyard P, Vanhatalo A, Blackwell JR, DiMenna FJ, Wilkerson DP, Tarr J, Benjamin N, Jones AM. Dietary nitrate supplementation reduces the O2 cost of low-intensity exercise and enhances tolerance to high-intensity exercise in humans. *Journal of Applied Physiology*. 2009;107:1144-1155.

39. Lansley KE, Winyard PG, Fulford J, Vanhatalo A, Bailey SJ, Blackwell JR, DiMenna FJ, Gilchrist M, Benjamin N, Jones AM. Dietary nitrate supplementation reduces the O2 cost of walking and running: a placebo-controlled study. *Journal of Applied Physiology*. 2011;110:591-600.

40. Larsen FJ, Schiffer TA, Borniquel S, Sahlin K, Ekblom B, Lundberg JO, Weitzberg E. Dietary Inorganic Nitrate Improves Mitochondrial Efficiency in Humans. *Cell Metabolism*. 2011;13:149-159.

Ingestion of dietary nitrate and nitrite and cancer risk

Rashmi Sinha, Mary H. Ward, Amanda J. Cross
Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, Department of Health and Human Services, Bethesda, MD 20892

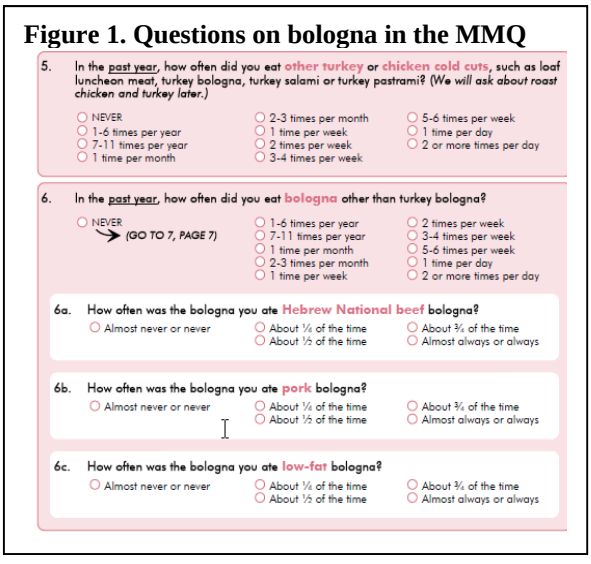
In 2007, the World Cancer Research Fund/American Institute for Cancer Research (WCRF/AICR) report concluded that the "evidence on red meat and processed meat is stronger than it was in the mid-1990s", and there is "convincing" evidence that red and processed meats increase the risk of colorectal cancer. However, the report also stated "epidemiological evidence on other methods of preserving and preparing meats and other animal foods is sparse; the overall evidence remains suggestive, at best."[1]

Nitrite and nitrate are added to most processed meats to prevent bacterial growth and to produce the characteristic red-pink color of cured meats. In 2006, a working group at the International Agency for Research on Cancer (IARC) reviewed nitrate and nitrite exposures and concluded that nitrate and nitrite ingested under conditions that cause endogenous nitrosation are probable human carcinogens.[1-3]

Using USDA food consumption data, we find that red meat composes the largest proportion of meat consumed in the U.S., and nearly a quarter of the meat consumed is processed. One of the proposed mechanisms relating red and processed meat to carcinogenesis is the formation of *N*-nitroso compounds (NOCs); these compounds can induce tumors in all species, at a variety of anatomic sites, and in a wide range of target cells.[4]

The complexity of estimating exposure to nitrate, nitrite, and NOCs makes population-based studies challenging. Many classifications of processed meat include disparate methods such as mincing, smoking, and preserving with salting and other preservation methods. The resulting food groups have many compounds with varying carcinogenic properties. In some cases, processed meat may refer only to processed red meat, rather than all meats that have been processed/ preserved. In spite of the fact that a high proportion of meat consumed in the U.S. and Europe is processed or preserved, most food frequency questionnaires (FFQs) have limited questions about intake of these meats. The typical approach of combining all of these meats in one line-item is not conducive to studying potential mechanisms.

Figure 1. Questions on bologna in the MMQ


5. In the past year, how often did you eat other turkey or chicken cold cuts, such as loaf luncheon meat, turkey bologna, turkey salami or turkey pastrami? *(We will ask about roast chicken and turkey later.)*

○ NEVER ○ 1-6 times per year ○ 7-11 times per year ○ 1 time per month ○ 2-3 times per month ○ 1 time per week ○ 2 times per week ○ 3-4 times per week ○ 5-6 times per week ○ 1 time per day ○ 2 or more times per day

6. In the past year, how often did you eat bologna other than turkey bologna?

○ NEVER → *(GO TO 7, PAGE 7)* ○ 1-6 times per year ○ 7-11 times per year ○ 1 time per month ○ 2-3 times per month ○ 1 time per week ○ 2 times per week ○ 3-4 times per week ○ 5-6 times per week ○ 1 time per day ○ 2 or more times per day

6a. How often was the bologna you ate Hebrew National beef bologna?

○ Almost never or never ○ About ¼ of the time ○ About ½ of the time ○ About ¾ of the time ○ Almost always or always

6b. How often was the bologna you ate pork bologna?

○ Almost never or never ○ About ¼ of the time ○ About ½ of the time ○ About ¾ of the time ○ Almost always or always

6c. How often was the bologna you ate low-fat bologna?

○ Almost never or never ○ About ¼ of the time ○ About ½ of the time ○ About ¾ of the time ○ Almost always or always

At the National Cancer Institute, we developed the Meat Mutagen Questionnaire (MMQ) with a comprehensive set of questions on processed meat. Using the Continuing Survey of Food Intakes by Individuals (CSFII 1994-96), we identified 104 smoked and processed meat food codes consumed in the U.S. during 1994-96.[5] We then reviewed the meat items for frequency of consumption and grouped them on the basis of the type of meat and the food additive composition. This resulted in the following meat categories: bacon, sausage, bologna, hot dogs, smoked fish, deli ham, turkey ham, other turkey or chicken cold cuts, salami, pepperoni, beef luncheon meats, other cold cuts, corned beef, baked ham, liverwurst, smoked turkey, and meat spread/potted meat. We used these categories to develop the MMQ for epidemiologic studies; Figure 1 shows an example for bologna.

In order to develop a database for nitrate, nitrite, and NOCs for red and processed queried in the MMQ, we identified 10 meat types that constituted 90 percent of the total number of mentions of processed meats in the CSFII 1994-1996. The 10 meat types were linked to the USDA Food and Nutrient Database. For each meat type, a composite was prepared using brand name meats that were available from grocery stores in one area in the U.S.[6] We found large variability in the nitrate and nitrite content of different processed meats, although NOC levels were below the limit of detection. In addition to measuring the nitrate and nitrite values in processed meats, we also determined nitrate and nitrite values for all foods included in the 124 line-item FFQ used in the National Institutes of Health (NIH)-AARP Diet and Health Study using values from articles published between 1967 and 2008. If more than one value was available, we calculated means of the published measurement or we used one value even if multiple values were available if it best represented the timeframe for the NIH-AARP Study. We used standardized recipes for foods from the CSFII for mixed foods including vegetable soup, beef stew, chicken pot pie, salads and sandwiches, meatloaf, chicken parmesan, etc. We added this data to a Windows-based application, CHARRED (www.charred.cancer.gov), to provide researchers the ability to estimate nitrite and nitrate intake from meats.

Processed Meat, Dietary Nitrate and Nitrite in the NIH-AARP Study

NCI investigators have evaluated the role of red and processed meat, as well as nitrate and nitrite for mortality and numerous cancers in a large prospective cohort. The NIH-AARP Study is a cohort of 566,402 men and women aged 50-71 years at enrollment, residing in one of 6 U.S. states or 2 metropolitan areas who completed an extensive baseline questionnaire in 1995–1996.

Mortality: Meat intake varies substantially around the world, but the impact of consuming high levels of meat in relation to chronic disease mortality is ambiguous. We investigated the association between types of meat and cause-specific mortality in the NIH-AARP Study. During 10 years of follow-up, we identified 47,976 male deaths and 23,276 female deaths.[7] Men and women in the highest versus the lowest quintile of red and processed meat intake had elevated risks for cancer and cardiovascular disease mortality.[7]

All cancers: During 8 years of follow-up, we ascertained 53,396 cancers (36,907 male cases and 16,489 female cases). Individuals in the highest, versus lowest, quintile of processed meat intake had an elevated risk for colorectal and lung cancer.[8] We also conducted detailed analyses for each cancer separately adjusting for more cancer-specific confounders. Out findings are summarized below:

Esophageal and gastric cancer: After 10 years of follow-up, there were 215 esophageal squamous cell carcinomas, 630 esophageal adenocarcinomas, 454 gastric cardia adenocarcinomas, and 501 non-cardia gastric adenocarcinomas.[9] Nitrite intake from processed meat increased the risk for esophageal adenocarcinoma (P-trend = 0.029). Processed meat, nitrite, and nitrate from processed meats were not associated with gastric cardia or noncardia cancer. Additionally, there was no association between usual adult total dietary intake of dietary nitrate and nitrite and risk of esophageal squamous cell carcinoma, esophageal adenocarcinoma, gastric cardia adenocarcinoma, or distal gastric adenocarcinomas.

Colorectal cancer: During 7 years of follow-up, we ascertained 2,719 colorectal cancer cases. When comparing the fifth to the first quintile, processed meat and nitrate from processed meats were both positively associated with colorectal cancer (hazard ratio, HR=1.16, 95% confidence interval, CI: 1.01-1.32; P-trend=0.017; HR=1.13, 95% CI: 0.97-1.32; P-trend= 0.009). Nitrate from processed meats was also positively associated with rectal cancer (HR=1.26, 95% CI: 0.97-1.63; P-trend=0.006).[10]

Pancreatic cancer: After 10 years of follow-up, we identified 1,728 incident pancreatic cancer cases making this the largest cohort study to date to evaluate this hypothesis.[11] There was no association

between total nitrate or nitrite intake across all foods and pancreatic cancer in men or women. However, men in the highest quintile of summed nitrate/nitrite intake from processed meat had a somewhat elevated risk for this malignancy (HR=1.18; 95% CI: 0.95-1.47; P-trend= 0.11).

Thyroid cancer: After 7 years of follow-up, there were 370 incident thyroid cancer cases (170 men, 200 women).[12] Among men, nitrate intake was positively associated with thyroid cancer risk (highest vs. lowest quintile HR=2.28, 95% CI: 1.29–4.04; P-trend<0.001); however, we observed no trend with intake among women. We found positive associations between nitrate intake and both papillary (highest vs. lowest quintile HR=2.10, 95% CI: 1.09-4.05; P-trend<0.05) and follicular thyroid cancer (HR=3.42; 95% CI: 1.03–11.4; p-trend<0.01) among men. Nitrite intake was associated with an increased risk of follicular thyroid cancer (highest quintile HR= 2.74; 95%CI: 0.86-8.77; p-trend=0.04) among men.

Bladder cancer: During 7 years of follow-up, 854 transitional cell bladder-cancer cases were identified. Individuals in the top compared to the bottom quintile of total dietary nitrite had an increased risk of bladder cancer (HR=1.28; 95% CI: 1.02-1.61; P-trend=0.06), as did those in the highest quintile of nitrate plus nitrite intake from processed meat (HR=1.29; 95% CI: 1.00-1.67; P-trend =0.11). [13]

Glioma: During follow-up, 585 participants were diagnosed with glioma. We found significant positive trends between nitrite intake from plant sources and risk of glioma (HR for quintile 5 versus quintile 1, 1.59; 95% confidence interval, 1.20-2.10; P for trend = 0.028). Examination of interactions between dietary intakes (e.g., nitrite and vitamin C) and a limited analysis of diet at ages 12 to 13 years provided no support for the NOC hypothesis.[14]

Ovarian cancer: 709 incident epithelial ovarian cancer cases were identified after 11 years of follow-up. Women in the highest intake quintile of dietary nitrate had a 31% increased risk (95% CI: 1.01-1.68; P-trend=0.06) of epithelial ovarian cancer, compared with those in the lowest intake quintile.[15] There was no association for total dietary nitrite overall; however, those in the highest intake category of animal sources of nitrite had a 34% increased risk of this malignancy (95% CI: 1.05-1.69; P-trend=0.02).

Renal cell carcinoma: Over 9 years of follow-up, we identified 1,814 cases of RCC (498 clear cell and 115 papillary adenocarcinomas). We found no association between intake of nitrate and/or nitrite from processed meats and risk of renal cell carcinoma.[16] However, further analyses by histologic type showed that those in the highest quintile of nitrite intake from animal sources had an elevated risk of the clear cell subtype (HR=1.68, 95% CI, 1.25–2.27). We found no association for nitrite intake from plant sources or nitrate intake overall.

We have developed methods for estimating nitrate and nitrite in epidemiologic studies. Using these tools we have evaluated the associations between red and processed meat, as well as nitrate and nitrite for multiple cancers in a very large prospective cohort. Further work is needed to improve exposure estimates and develop biomarkers that can easily be incorporated into population-based studies. The role of oral and gut bacteria in the metabolism of nitrate and nitrite has not been addressed in this abstract but is essential to consider. [17]

References:

1. World Cancer Research Fund / American Institute for Cancer Research. Food, Nutrition, Physical Activity, and the Prevention of Cancer: a Global Perspective Washington, DC: AICR; 2007.
2. Grosse Y, Baan R, Straif K, Secretan B, El Ghissassi F, Cogliano V. Carcinogenicity of nitrate, nitrite, and cyanobacterial peptide toxins. Lancet Oncol 2006;7:628-9.

3. IARC. Ingested Nitrates and Nitrites, and Cyanobacterial Peptide Toxins. Lyon, France; 2010.
4. Bogovski P, Bogovski S. Animal Species in which N-nitroso compounds induce cancer. Int J Cancer 1981;27:471-4.
5. Anderson KE, Kadlubar FF, Kulldorff M, et al. Dietary Intake of Heterocyclic Amines and Benzo(a)Pyrene: Associations with Pancreatic Cancer. Cancer Epidemiology Biomarkers & Prevention 2005;14:2261-5.
6. Sinha R, Cross A, Curtin J, et al. Development of a food frequency questionnaire module and databases for compounds in cooked and processed meats. Mol Nutr Food Res 2005;49:648-55.
7. Sinha R, Cross AJ, Graubard BI, Leitzmann MF, Schatzkin A. Meat intake and mortality: a prospective study of over half a million people. Arch Intern Med 2009;169:562-71.
8. Cross AJ, Leitzmann MF, Gail MH, Hollenbeck AR, Schatzkin A, Sinha R. A Prospective Study of Red and Processed Meat Intake in Relation to Cancer Risk. PLoS Med 2007;4:e325.
9. Cross AJ, Freedman ND, Ren J, et al. Meat Consumption and Risk of Esophageal and Gastric Cancer in a Large Prospective Study. Am J Gastroenterol 2010.
10. Cross AJ, Ferrucci LM, Risch A, et al. A large prospective study of meat consumption and colorectal cancer risk: an investigation of potential mechanisms underlying this association. Cancer Res 2010;70:2406-14.
11. Aschebrook-Kilfoy B, Cross AJ, Stolzenberg-Solomon RZ, et al. Pancreatic cancer and exposure to dietary nitrate and nitrite in the NIH-AARP Diet and Health Study. Am J Epidemiol 2011;174:305-15.
12. Aschebrook-Kilfoy B, Sabra MM, Brenner A, et al. Diabetes and thyroid cancer risk in the National Institutes of Health-AARP Diet and Health Study. Thyroid 2011;21:957-63.
13. Ferrucci LM, Sinha R, Ward MH, et al. Meat and components of meat and the risk of bladder cancer in the NIH-AARP Diet and Health Study. Cancer 2010;116:4345-53.
14. Dubrow R, Darefsky AS, Park Y, et al. Dietary components related to N-nitroso compound formation: a prospective study of adult glioma. Cancer Epidemiol Biomarkers Prev 2010;19:1709-22.
15. Aschebrook-Kilfoy B, Ward MH, Gierach GL, et al. Epithelial ovarian cancer and exposure to dietary nitrate and nitrite in the NIH-AARP Diet and Health Study. Eur J Cancer Prev 2012;21:65-72.
16. Daniel CR, Schwartz KL, Colt JS, et al. Meat-cooking mutagens and risk of renal cell carcinoma. Br J Cancer 2011;105:1096-104.
17. DellaValle C DC, Aschebrook-Kilfoy B, et al. Dietary intake of nitrate and nitrite and risk of renal cell carcinoma in the NIH-AARP Diet and Health Study,. Br J Cancer In Press.

Carboxymethylating/methylating agents associated with dietary nitrosating agents: potential role in gastrointestinal carcinogenesis

David E G Shuker

The Grainstore Workshop, Coney Lodge, Park Farm Rd., Birling, West Malling, Kent ME19 5JZ, UK E-mail: david.shuker@btconnect.com Tel: +44 (0)7970 276070

From the point of view of a chemical toxicologist one of the most intriguing aspects of nitrosation reactions is their ability to transform innocuous molecules into agents that display a wide range of biological activity. The archetypical example is the conversion of dimethylamine (DMA) into N-nitrosodimethylamine (NDMA): DMA displays little biological activity but NDMA is a potent liver carcinogens. Over the past fifty years the mechanism by which this occurs has been intensively studied and involves the generation of a highly reactive methylating agent that modifies DNA giving rise to adducts which, if left unrepaired, lead to miscoding sequences that can lead to functional mutations. There are many variations on the theme of N-nitroso compounds (nitrosamines, nitrosamides, nitrosoureas, nitrosoguanidines, and so on) but a common thread running through this area of research is that nitrosation leads to the formation of an N-nitroso derivative which can either decompose to give a DNA-damaging (alkylating) agent, or, do so after some sort of metabolic activation.

This view of nitrosation has given rise to several notions: 1. N-nitroso compounds can be formed in foods or other consumer products and may pose a cancer risk to humans, and, 2. Eliminating or minimising exposure to N-nitroso compounds would lead to a reduction in cancer risk. Some cancer risks have been associated with exposure to N-nitroso compounds – as, for example, with industrial reagents and solvents and tobacco use. However in other areas of human cancer risk such links have proved elusive. Most notably, the role of N-nitroso compounds in gastrointestinal cancer has proved very difficult to resolve. There are many experimental models in which N-nitroso compounds (such as MNNG and the N-nitroso bile acid conjugates) can be used to induce cancers of the GI tract but there is little evidence that exposure to such materials is directly involved in the induction of human cancers. However, experimental models have given us a number of clues which allow a more subtle view of the involvement of N-nitrosation in human GI tract cancers.

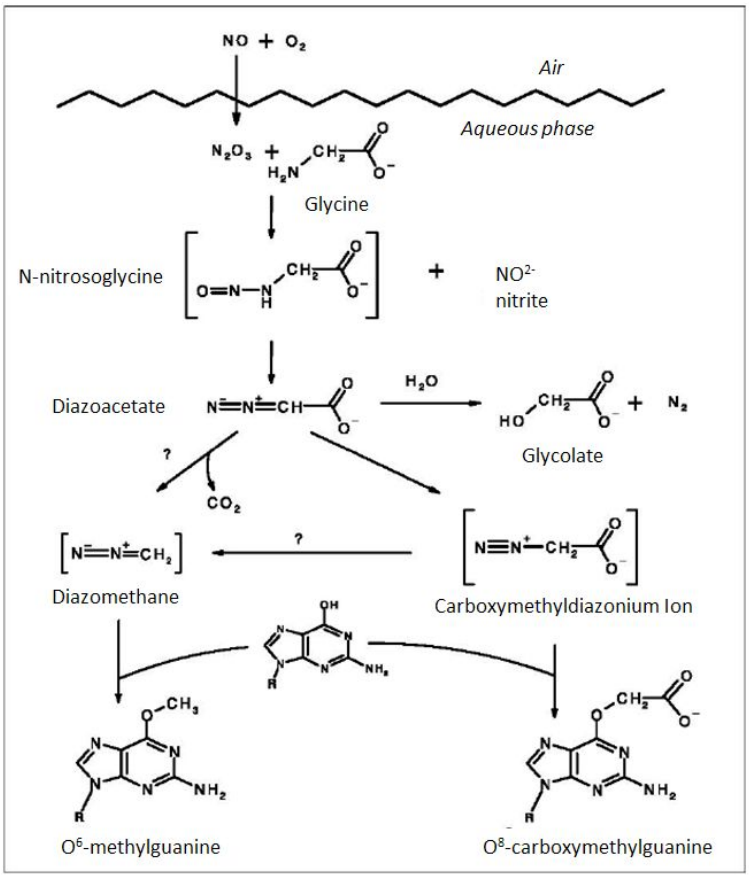
N–Nitrosoglycocholic acid (NOGC) was first synthesised in 1979 as part of a programme in Steve Tannenbaum's group at MIT[1]. NOGC was found to not only induce gastric cancers in experimental animals but also the precancerous lesions typical of the human disease[2]. Early studies on DNA adducts formed by NOGC led to the identification of N-7-carboxymethylguanine, which had also been found by Longnecker and colleagues in DNA treated with azaserine[3]. This result suggested that the carcinogenic properties of NOGC were driven predominantly by the fact that it was an N-nitroso

glycine derivative rather than a bile acid. Azaserine is actually an N-nitrosoglycine derivative in disguise. Initial studies focussed on the formation of carboxymethyl adducts as these were thought to be characteristic enough to be the basis of biomonitoring methodology. However, it became apparent that DNA exposed to a range of nitrosated glycine derivatives also contained methyl adducts. The ability to form both carboxymethyl- and methyl-DNA adducts turns out to be general property of nitrosated glycines. The nitrosated glycines comprise a class of compounds that include N-nitrosamines (e.g. N-nitrososarcosine), N-nitrosamides (e.g. NOGC, N-nitrosopeptides) and diazoacetates (e.g O-diazoacetyl-L-serine [azaserine]). In fact the simplest form of nitrosated glycine is diazoacetate (usually in its potassium salt known as KDA) first reported in 1908[4] but little studied in the ensuing century.

The formation of methyl DNA adducts by nitrosated glycines is intriguing as it suggests that promutagenic adducts such as O6-methyl-2'-deoxyguanosine (O6MedG) can come from sources that are not obvious methylating agents. This might explain why it has proved so difficult to consistently relate levels of O6MedG in human tissues to particular dietary or environmental risk factors. However, the major DNA modifications formed by nitrosated glycines are the carboxymethyl adducts and these have formed the basis of biomonitoring assays. Thus antibody-based assays including immunoaffinity-HPLC[5] and immunoslotblot assays[6] and immunohistochemistry[7] have all been developed and used to quantitate the characteristic adduct, O6-carboxymethyl-2'-deoxyguanosine (O6CMdG) in various studies. More recently, sensitive mass spectrometry-based assays have become available and these too have confirmed the presence of O6CMdG in human tissues[8].

An early observation was that O6CMdG appeared to be resistant to the various O6-methylgaunine methyl transferases that exist in bacterial and mammalian cells to remove this toxic methyl lesion[2].

How might carboxymethyl adducts be formed in vivo? The following scheme shows how glycine can be transformed into both methylating and carboxymethylating agents. The key point here is that the obligatory imtermediates – N-nitrosoglycine, diazoacetate, carboxymethyl-diazonium ion and diazomethane are all very short-lived species at physiological pH. Thus the two O6-alkyl adducts (along with the corresponding N7-alkylguanine adducts) are the 'smoking gun' which shows that the nitrosation reaction had occurred.



The large body of work on endogenous nitrosation carried out by Bartsch, Ohshima and colleagues[9] demonstrated that the human body had plenty of potential to carry out the type of nitrosation described in the scheme. In those studies the detectable surrogate for endogenous nitrosation was L-proline leading to formation of N-nitroso-L-proline which is stable and non-carcinogenic being quantitatively excreted in urine.

What is the evidence that carboxymethylation and methylation from glycine is likely to be of significance for human health? N-nitroso glycine derivatives are consistently mutagenic and carcinogenic is both *in vitro* and *in vivo* assays[10]. The mutation spectrum induced by KDA in a functional p53 mutational assay is quite distinct from that induced by MNU suggesting that carboxymethylation is contributing substantially to the biological activity[11]. Moreover, the spectrum of mutations in p53 exposed to KDA in vitro is very similar to the spectrum of p53 mutations observed in human GI cancers. Site-specific incorporation of O6CMdG into plasmids resulted in mispairing upon replication with a propensity for GC-AT transitions and GC-TA transversions[12]. Whether this activity is driven by the bulk of the O6-carboxymethyl group or its charge, or a combination of the two, is not yet known. Other carboxymethyl adducts formed by KDA have been identified and display distinctive mutagenic activity[13].

Studies in human volunteers consuming controlled amounts of red meat showed that levels of O6CMdG were raised in exfoliated colonic cells of those subjects who consumed the highest levels of red meat. The simplest explanation is that increased consumption of meat protein resulted in increased levels of glycine leading to increased levels of DNA-carboxymethylation. Whether this is predictive of the increased risk of colorectal cancer awaits the outcome of the large prospective studies such as EPIC where stored DNA could be analysed for carboxymethyl adducts. Here the technology has let us down in that efforts to produce a monoclonal antibody for O6CMdG have failed at several attempts. There was no problem in making a polyclonal serum5 so this seems surprising.

It might seem curious that the most common and simplest amino acid glycine should be capable of being converted in a potent mutagen and carcinogen by a simple nitrosation reaction. Can it really be the case that this pathway contributes substantially to the risk of developing gastrointestinal cancer? If so, why hasn't this been seen a long time ago? It is probably the fact that this pathway is so prevalent that explains why it hasn't come to the fore. Background levels of O6MedG have been seen in human DNA for many years and exposure to known methylating agents such as NDMA did not seem to explain this. If the source of O6MedG is nitrosated glycine then this would not have been at all obvious - least of all to myself, who did not spot it until several years of research in the area. If nitrosated glycine is contributing to the burden of mutagenic DNA damage can we do anything about it? The risk of cancer from this pathway is affected not only by the unavoidable consumption of glycine but also by the extent of endogenous nitrosation , which is itself influenced by consumption of nitrate as well as endogenous synthesis of reactive nitrogen species during inflammation. If one adds into this equation the role of DNA repair (or lack of it) as well as other factors then it is perhaps not surprising that it would be difficult to identify a dietary question or parameter that could measure this pathway. This said, is something like O6CMdG well enough characterised to merit further investigation as a biomarker? Are the available methods sensitive and robust enough to be used in a prospective molecular epidemiological study using EPIC samples (or other stored DNA)? Looking further ahead, could such a marker (or set of related markers) be useful in intervention studies (reducing meat/nitrate intake or raising vegetable intake)? These are the questions to address in the course of our discussions.

Acknowledgements

It has been my good fortune to have had the assistance of excellent research students and postdoctoral fellows over the years and I am very grateful for their efforts. Their names appear in the cited references. This presentation is dedicated to the memory of Professor Sheila (Bingham) Rodwell (1947-2009) whose research did so much to focus our minds on the role of dietary factors in gastrointestinal cancer and who was an enthusiastic, thoughtful and patient collaborator of mine for many years.

[1] Shuker DEG, Tannenbaum SR, Wishnok JS. N-nitrosobile acid conjugates 1. Synthesis, chemical reactivity and mutagenic activity. *J Org Chem* 1981;46:2092-6

[2] Shuker DEG, Margison GP. Nitrosated glycine derivatives as a potential source of O6- methylguanine in DNA. *Cancer Res* 1997;57:366-9

[3] Zurlo J, Curphey TJ, Hiley R, Longnecker DS. Identification of 7-carboxymethylguanine in DNA from pancreatic acinar cells exposed to azaserine. *Cancer Res*. 1982, 42:1286-8.

[4] Müller E. Uber pseudo diazoessigsaüre (On pseudo-diazoacetic acid). *Chem Ber* 1908;41:3116-39

[5] Harrison KL, Fairhurst N, Challis BC, Shuker DEG. Synthesis,characterization, and immunochemical detection of O6-(carboxymethyl)-2'-deoxyguanosine: a DNA adduct formed by nitrosated glycine derivatives. *Chem Res Toxicol* 1997;10:652-9

[6] Cupid B, Zeng Z, Singh R, Shuker DEG. Detection of O6-carboxymethyl-2'-deoxyguanosine in DNA following reaction of nitric oxide with glycine and in human blood DNA using a quantitative immunoslot blot assay. *Chem Res Toxicol* 2004;17:294-300

[7] Lewin,M.H., Bailey,N., Bandaletova,T., Bowman,R., Cross,A.J., Pollock,J., Shuker,D.E.G. and Bingham,S.A. Red meat enhances the colonic formation of the DNA adduct O6-carboxymethyl guanine: implications for colorectal cancer risk. *Cancer Res*., 2006, 66, 1859–1865

[8] Vanden Bussche J, Moore SA, Pasmans F, Kuhnle GG, Vanhaecke L. An approach based on ultra-high pressure liquid chromatography-tandem mass spectrometry to quantify O^6-methyl and O^6-carboxymethylguanine DNA adducts in intestinal cell lines. *J Chromatogr A*. 2012, 1257:25-33

[9] Ohshima H, Bartsch H. Quantitative estimation of endogenous nitrosation in humans by monitoring N-nitrosoproline excreted in the urine. *Cancer Res*. 1981;41:3658-62.

[10] Puju, S., Shuker, D. E. G., Bishop, W. W., Falchuk, K. R., Tannenbaum, S. R., and Thilly, W. G. Mutagenicity of N-nitroso bile acid conjugates in *Salmonella typhimurium* and diploid human lymphoblasts. *Cancer Res*., 1982,42: 2601-2604: Busby, W. F., Jr., Shuker,D. E. G., Charnley, 0., Newberne, P. M., Tannenbaum,
S. R., and Wogan,G. N. Carcinogenicityof the nitrosatedbile acid conjugates, N-nitroso-glycocholic acid and N-nitrosotaurocholic acid. *Cancer Res.,* 1985,45:1367-1371,

[11] Gottschalg E, Scott GB, Burns PA, Shuker DE. Potassium diazoacetate-induced p53 mutations in vitro in relation to formation of O6-carboxymethyl- and O6-methyl-2'-deoxyguanosine DNA adducts: relevance for gastrointestinal cancer. *Carcinogenesis*. 2007;28:356-62

[12] Ponnada S, Burns PA,, Xu Y-Z, Shuker DEG. O6-carboxymethylguanine: A potent mutagenic adduct characterised using an Amplification Refractory Mutation System –Polymerase Chain Reaction assay following site-specific incorporation into a p53 cDNA sequence. Unpublished manuscript

[13] Swanson, AL Wang, JS Wang, YS. In Vitro Replication Studies of Carboxymethylated DNA Lesions with *Saccharomyces cerevisiae* Polymerase eta. *Biochemistry*. 2011, 50, 7666-7673

Effect of dietary meat and fish on endogenous nitrosation and colorectal cancer

Gunter G. C. Kuhnle
Department of Food & Nutritional Sciences, University of Reading, UK

1 Introduction

Colorectal cancer (CRC) is one of the most common types of cancer with more than 30,000 new cases, and 14,000 deaths, per year in Germany[1]. Diet has been identified as an important contributing factor, in particular red and processed meat (RR 1.22 (95% CI 1.11 – 1.34) per 100 g/d) [1]. Several hypotheses have been proposed to explain this association, for example the formation of heterocyclic amines (HCA) and polycyclic aromatic hydrocarbons (PAH), the pro-carcinogenic activity of haem [2] or the endogenous formation of nitroso compounds (NOC) [3]. The exposure to HCA and PAH does not explain the differences observed between red and white meat, whereas haem content varies considerably [4], and it is therefore a more likely contributing factor. It has cytotoxic and haemotoxic effects, and can increase colonic epithelial proliferation in rats [5]. However, most of these effects are only observed at haem concentrations equivalent to a daily consumption of more than 600 g red meat. Haem-induced lipid-peroxidation or haem-promoted formation of nitroso compounds are the most-likely mechanisms explaining the association between red meat and colorectal cancer [2]. Lipid-peroxidation results in the formation of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) [6], which can promote cancer [7]. Endogenous nitrosation reactions result in the formation of carcinogenic *N*-nitroso compounds and the subsequent formation of DNA adducts. Previous *in vitro* studies have shown that mutations induced by compounds linked to endogenous nitrosation are similar to those found in colorectal tumours [8], supporting the hypothesis that endogenous nitrosation, rather than lipid peroxidation, is the mechanism underlying the link between meat and cancer.

2 Endogenous formation of nitroso-compounds

Red and processed meat consumption results in an intake-dependent endogenous formation of nitroso compounds, which are commonly referred to as *apparent total* N-*nitroso compounds* (ATNC) [9]. Although details of the chemical nature of ATNC was initially unknown, it was assumed that due to their similarity with *N*-nitroso compounds they have carcinogenic properties. Using refined analytical methods [10], it was possible to show that the main contributors to ATNC following a high meat diet were nitrosyl-haeme and nitroso thiols, while only small amounts of other nitroso compounds were detected (Figure 1).

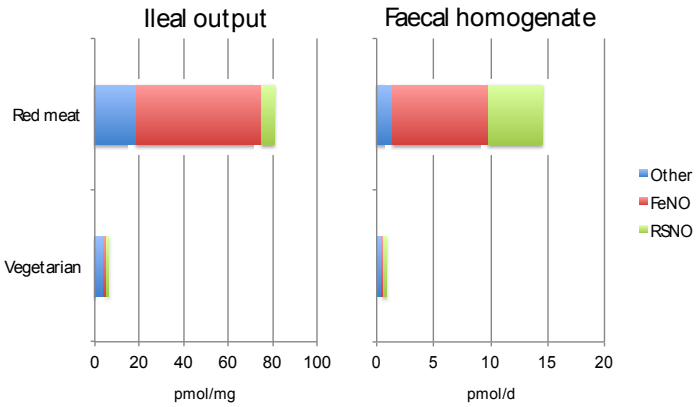


Figure 1 Different types of faecal nitroso compounds following a vegetarian or a high meat in ileal output and faecal homogenates following either a high meat or a vegetarian control diet. Data from Kuhnle *et al.* (2007)

[1] WHO/IARC data (GLOBOCAN) for 2008

2.1 Detection of endogenously formed nitroso compounds

Most methods used for the detection of endogenously formed NOC use chemiluminescence to detect chemically released NO. This method, without any pre-treatment of the sample except for stabilisation and removal of nitrite yields the total amount of NOC present, referred to as ATNC. Nitroso thiols and nitrosyl haem can be detected by using selective chemical denitrosation with mercury(II)chloride (for nitroso thiols) and potassium ferricyanide (for nitrosyl haem). Although this method is generally very reliable, some nitroso compounds – in particular nitroso-thiols – are very sensitive to the presence of metal ions or exposure to UV light, and careful sample handling is important to obtain reliable results.

2.2 Underlying mechanism

The mechanisms underlying the meat-induced endogenous formation of nitroso compounds have not yet been completely elucidated. Data from dietary intervention studies suggested that dietary haem can promote the formation of nitroso compounds [11], however, *in vitro* [10] studies showed that a combination of haem and free thiol groups are necessary for endogenous nitrosation reactions to occur. Indeed, while the removal of the haem group from haemoglobin resulted only in a reduction of NOC formation by 24%, blocking free thiol groups reduced their formation by more than 80%; the addition of free haem as haemin increased the formation of nitroso-thiols by 50%.

The rapid formation of nitroso thiols under acidic conditions can be explained by the large rate constant ($k=465{,}000\ M^{-2}s^{-1}$) for acid catalysed nitrosation, in particular when compared to the constant for N-nitrosation ($k=4600\ M^{-2}s^{-1}$) [12]. The nitrosylation reaction of haem is more complicated and it is therefore difficult to compare rates [13]. However, the reductive and anaerobic environment in the small intestine helps to maintain the haem iron in its ferrous state and facilitates its nitrosylation by nitrite or NO [14]. Both nitrosyl haem and nitrosothiols are known to act as NO donors [15] and both can act as nitrosating agent [16]. It is therefore likely that these compounds promote the formation of *N*-nitroso compounds or other highly reactive alkylating agents, which can result in the formation of DNA adducts. A summary of this putative pathway is shown in Figure 2. Furthermore, these compounds could promote a state of low-grade inflammation, as risk factor of cancer [17], either directly [18] or by causing oxidative and nitrative damage [19].

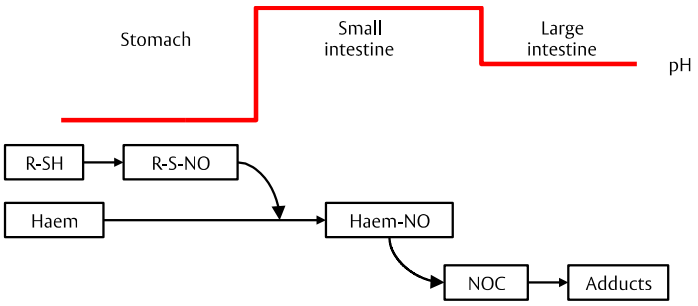


Figure 2: Putative mechanism of meat-induced endogenous formation of nitroso compounds and subsequent formation of DNA adducts.

2.3 Red meat, fish and inflammation

Fish intake has been associated with a reduced risk of colorectal cancer [20], although the underlying mechanism is not known. ?-3 fatty acids might have an effect, e.g. by reducing inflammatory processes, but data from observational studies are inconclusive [21]. In a dietary intervention study, no statistically significant difference in inflammation markers between meat and oily fish diets have been observed [22]. This study also showed that oily fish did not affect the formation of NOC, and the reduction observed can be explained by the reduction in dietary meat.

2.4 Endogenous nitrosation, DNA adducts and mutations

Endogenously formed nitroso compounds can react with DNA bases which results in the formation of DNA adducts and can lead to mutations. Diazoacetate (nitrosated glycine), which cannot be detected using the methods described above, has been shown to react with guanine to form two different adducts, O^6-methylguanine (O^6-mG) and O^6-carboxymethylguanine (O^6-CmG) [23]. In human dietary intervention studies, a strong correlation between endogenous NOC formation and O^6-CmG adducts has been found in exfoliated colonocytes [24] and *in vitro* studies have shown that the pattern of mutation induced by diazoacetate is similar to those found in gastro-intestinal tumours [8]. Furthermore, a recent study in 185 archived CRC tumour samples from EPIC Norfolk showed a strong association between meat intake and GC-to-AT mutation (OR 1.68, 95% CI 1.03–2.75 per 19g/day) [25]. This data supports the hypothesis that endogenous nitrosation reactions, leading to the formation of alkylating agents and DNA adducts, are a likely explanation for the association between red and processed meat and cancer.

3 Summary and future work

Dietary intervention studies, as well as animal and *in vitro* studies, have confirmed that red and processed meat intake results in the endogenous formation of NOC and that these can lead to the formation of DNA adducts and mutations. However, there is still a paucity of data to confirm that endogenous NOC formation is the mechanism underlying the association between red and processed meat and cancer. To establish this link, it is important to address the following:

- The underlying mechanisms are still not completely understood. While it is known that free thiol and haem groups are necessary for NOCs to form, the mechanism of their formation *in vivo*, and subsequent DNA adduct formation, remains unknown. Understanding these mechanisms is not only important to establish the link between red and processed meat and CRC, but also to investigate methods to control NOC formation.
- It is currently not possible to investigate NOC formation in observational studies. A previous study investigating the association between endogenous nitrosation and CRC risk relied on estimate exposure [26], using the iron content from meat to predict endogenous nitrosation. The development of a reliable serum or urinary biomarker of endogenous nitrosation would allow to investigate associations between cancer risk and endogenous nitrosation in epidemiological studies.

References

1. Chan DSM, Lau R, Aune D, Vieira R, Greenwood DC, Kampman E, et al. Red and Processed Meat and Colorectal Cancer Incidence: Meta-Analysis of Prospective Studies. PLoS One 2011;6(6):e20456.
2. Bastide NM, Pierre FHF, Corpet DE. Heme iron from meat and risk of colorectal cancer: a meta-analysis and a review of the mechanisms involved. Cancer Prev Res (Phila) 2011;4(2):177–84.
3. Kuhnle GGC, Bingham SA. Dietary meat, endogenous nitrosation and colorectal cancer. Biochemical Society Transactions 2007;35(5):1355–7.
4. Schwartz S, Ellefson M. Quantitative fecal recovery of ingested hemoglobin-heme in blood: comparisons by HemoQuant assay with ingested meat and fish. Gastroenterology 1985;89(1):19–26.
5. Sesink ALA, Termont DSML, Kleibeuker JH, van der Meer R. Red meat and colon cancer: the cytotoxic and hyperproliferative effects of dietary heme. 1999;59(22):5704–9.
6. Marnett LJ. Oxyradicals and DNA damage. Carcinogenesis 2000;21(3):361–70.
7. Pierre F, Tache S, Guéraud F, Rerole AL, Jourdan M-L, Petit C. Apc mutation induces resistance of colonic cells to lipoperoxide-triggered apoptosis induced by faecal water from haem-fed rats. Carcinogenesis 2007;28(2):321–7.

8. Gottschalg E, Scott GB, Burns PA, Shuker DEG. Potassium diazoacetate-induced p53 mutations in vitro in relation to formation of O6-carboxymethyl- and O6-methyl-2'-deoxyguanosine DNA adducts: relevance for gastrointestinal cancer. Carcinogenesis 2006;28(2):356–62.
9. Hughes R, Cross AJ, Pollock JR, Bingham S. Dose-dependent effect of dietary meat on endogenous colonic N-nitrosation. Carcinogenesis 2001;22(1):199–202.
10. Kuhnle GGC, Story GW, Reda T, Mani AR, Moore KP, Lunn JC, et al. Diet-induced endogenous formation of nitroso compounds in the GI tract. Free Radic Biol Med 2007;43(7):1040–7.
11. Cross AJ, Pollock J, Bingham SA. Haem, not protein or inorganic iron, is responsible for endogenous intestinal N-nitrosation arising from red meat. Cancer Res 2003;63(10):2358–60.
12. Williams DL. Nitrosation Reactions and the Chemistry of Nitric Oxide. Amsterdam: Elsevier; 2004.
13. Tejero J, Basu S, Helms C, Hogg N, King SB, Kim-Shapiro DB, et al. Low NO concentration dependence of reductive nitrosylation reaction of hemoglobin. J. biol. Chem 2012;287(22):18262–74.
14. Chiodi H, Mohler J. Effects of exposure of blood hemoglobin to nitric oxide. Environmental Research 1985;37(2):355–63.
15. Butler A, Rhodes P. Chemistry, analysis, and biological roles of S-nitrosothiols. Anal Biochem 1997;249(1):1–9.
16. Bonnett R, Charalambides A, Martin R, Sales K, Fitzsimmons B. Reactions of Nitrous Acid and Nitric Oxide with Porphyrins and Haems. Nitrosylhaems as Nitrosating Agents. Journal of the Chemical Society: Chemical Communications 1975;:884–5.
17. Aggarwal BB, Shishodia S, Sandur SK, Pandey MK, Sethi G. Inflammation and cancer: how hot is the link? Biochem Pharmacol 2006;72(11):1605–21.
18. Martin GR, Wallace JL. Gastrointestinal inflammation: a central component of mucosal defense and repair. 2006;231(2):130–7.
19. Sawa T, Ohshima H. Nitrative DNA damage in inflammation and its possible role in carcinogenesis. Nitric Oxide 2006;14(2):91–100.
20. Norat T, Bingham SA, Ferrari P, Slimani N, Jenab M, Mazuir M, et al. Meat, fish, and colorectal cancer risk: the European Prospective Investigation into cancer and nutrition. J Natl Cancer Inst 2005;97(12):906–16.
21. Gerber M. Omega-3 fatty acids and cancers: a systematic update review of epidemiological studies. Br J Nutr 2012;107 Suppl 2:S228–39.
22. Joosen AMCP, Lecommandeur E, Kuhnle GGC, Aspinall SM, Kap L, Rodwell SA. Effect of dietary meat and fish on endogenous nitrosation, inflammation and genotoxicity of faecal water. Mutagenesis 2010;25(3):243–7.
23. Shuker DEG, Margison G. Nitrosated glycine derivatives as a potential source of O6-methylguanine in DNA. Cancer Res 1997;57(3):366–9.
24. Lewin M, Bailey N, Bandaletova T, Bowman R, Cross AJ, Pollock J, et al. Red meat enhances the colonic formation of the DNA adduct O6-carboxymethyl guanine: implications for colorectal cancer risk. Cancer Res 2006;66(3):1859–65.
25. Gay LJ, Mitrou PN, Keen J, Bowman R, Naguib A, Cooke J, et al. Dietary, lifestyle and clinicopathological factors associated with APC mutations and promoter methylation in colorectal cancers from the EPIC-Norfolk study. J. Pathol. 2012;228(3):405–15.
26. Jakszyn P, Bingham S, Pera G, Agudo A, Luben R, Welch A, et al. Endogenous versus exogenous exposure to N-nitroso compounds and gastric cancer risk in the European Prospective Investigation into Cancer and Nutrition (EPIC-EURGAST) study. Carcinogenesis 2006;27(7):1497–501.

Dietary Nitrate and Nitrite: Friend or foe?

De Kok, T.M.

Dept. Toxicogenomics, Maastricht University, Maastricht (NL)

Humans are exposed to nitrate primarily through diet and drinking water, with vegetables contributing the largest amount of dietary nitrate per serving (ward et al 2005; Walters 1991). Nitrate is inherently present in all plant materials, especially vegetables and forage crops, and accumulate when the plant matures in a nitrate rich environment (Ashton 1970). Nitrate in drinking water is often the result of contamination of ground water by fertilizer and animal or human waste.

The interest in nitrate consumption is due to the subsequent conversion of nitrate to nitrite, which is of greater concern in the formation of potentially carcinogenic *N*-nitroso compounds (NOC). The endogenous conversion of nitrate to nitrite is a significant source of exposure to nitrites; approximately 5% of ingested nitrates in food and water are converted to nitrite in the saliva (Choi, 1985). Cured meats, baked goods and cereals are other notable sources of nitrite. Nitrite salts are added to meats, poultry, and fish as a means of preservation. Dietary sources of nitrosamines include cured meats, beer, and smoked fish; these foods may contain preformed nitrosamines as the result of cooking and/or preservation methods (Lijinski 1999; Hotchkiss 1987). The vast majority of more than 300 NOC that have been tested in animal studies were shown to be carcinogenic (Lijinski, 1999; Knekt et al 1999).

On the other hand, the role of nitrate and nitrite as healthy dietary components has been reconsidered. Nitrate and nitrite-rich food sources may play a physiological role in vascular and immune function. Higher intakes are suggested to be associated with lower blood pressure and better cardiovascular function (Lundberg 2006, 2009). Furthermore, nitrite is used in most of the meat products to guarantee food safety, as it is important to control pathogenic microbes. Additionally, it helps to control oxidation and rancidity and to ensure a desired pink meat colour. As such it should be considered as a factor contributing positively to human health.

Still, in 2006 new EU regulations limited the use of nitrite and nitrate in meat products to maximum 150 ppm each, and the use and control of nitrite and nitrate in meat was recently evaluated (Honnikel 2008). As a consequence, the EU meat processing industry is becoming increasingly under pressure to reduce the use of nitrite as it has been indicated as one of the critical factors in the observed association between meat consumption and cancer risk. The World Cancer Research Fund (WCRF) repeatedly stated that there is convincing evidence that particularly colon cancer is associated with the consumption of red and processed meat (WCRF 1997, 2007). It has been estimated that the risk of developing colon cancer increases by 49 % when consuming 25 grams of processed red meat per day (Shandu et al 2001). The proposed mechanism to explain this association involves the formation of NOC in the intestinal tract which is stimulated by the combination of nitrite with haem iron, both present in red and processed meat (Vermeer et al, 1998; Cross et al, 2003).

There is a growing body of evidence that phytochemicals in the diet, particularly coming from the consumption of fruits and vegetables, improve human health and reduce the risk of developing colon cancer (Akkeson et al 2005). There is ample evidence to support the anti-cancer effects of specific phytochemicals in plants and herbs, like vitamin C, tocopheroles, flavonoids, carotenoids, glycol alkaloids and others. Different types of phytochemicals may exert their beneficial action via different mechanisms, including effects at the level of formation and kinetics of carcinogenic compounds in the colon, such as NOC, and at the level of cellular protection, for instance by inducing antioxidant or metabolic enzyme systems. Due to synergistic interactions, specific combinations may be more effective than single compounds (de Kok et al 2008). This implies that health risks associated with nitrate or nitrite intake from vegetables may be considerably different from those associated with intake via other dietary sources, such as meat.

In view of these complex interactions between different dietary factors, and a potential relationship with both beneficial and adverse impacts it remains difficult to assess the overall human health impact of nitrate and nitrite intake. Limitations of the available human studies lie in the fact the outcome measurements only reflect short term or acute effects such as reduced blood pressure or early markers of genotoxicity, rather than the occurrence of the ultimate chronic disease. Also, the effects of nitrate and nitrite on different endpoints have never been evaluated in one and the same study, making a risk-benefit evaluation under comparable conditions impossible. With the advent of genomics techniques, new possibilities arise to find answers to these questions, as for instance whole genome transcriptomics analyses provide gene expression profiles reflecting changes in molecular processes involved in both carcinogenesis and cardiovascular health simultaneously (de Kok et al 2012).

We have evaluated the potential of these genomics techniques to advance our understanding of the involvement of NOC and human carcinogenesis in a series of investigations. Although there have been numerous reports on genotoxic and mutagenic properties of NOCs *in vitro* this does not necessarily imply a carcinogenic risk for intact humans, especially since it is difficult to determine the relationship between NOC-induced genotoxicity or mutagenicity and the associated carcinogenicity of these compounds. In several epidemiological studies, human NOC exposure has been associated with increased cancer risks of the stomach, esophagus, bladder, and colon, in particular in association with dietary intake of food items with relatively high levels of NOC precursors or nitrosating agents. There is, however, still no consensus on whether NOCs actually are human carcinogens. In an attempt to assess the potential carcinogenic effect of NOCs in humans, we investigated gene expression changes in the human colon adenocarcinoma cell line Caco-2. Indeed, we identified a large number of NOC-modified molecular pathways involved in processes that may contribute to the carcinogenic potential of NOCs in humans, including pathways crucial in differentiation and proliferation (Hebels et al 2011a).

Subsequently, we have evaluated whether NOC exposure in human subjects induces gene expression responses which provide insights in their possible human carcinogenicity. In order to do so, we establish a relationship between human NOC exposure under daily life conditions and micronucleus (MN) formation in association with transcriptomic changes (Hebels 2011b). Since lymphocytic MN

represent a well-validated biomarker of human cancer risk, establishing a link between NOC exposure and MN frequency in humans may provide evidence for a carcinogenic risk. Gene expression levels and MN frequency were analysed in lymphocytes from adult females participating in the pan-European biomarker research project NewGeneris. To assess NOC exposure, urine samples were analysed for marker nitrosamines. NOC excretion levels and MN frequency were subsequently linked to peripheral blood transcriptomics. We demonstrated an association between MN frequency and urinary NOCs, indicating that NOC exposure under daily life circumstances may impose a cancer risk. We identified modifications in cell cycle and apoptosis pathways which indicate a response to NOC-induced genotoxicity. Moreover, we established a network of genes involved in processes relevant in carcinogenesis. A gene set has been identified that may be used as a transcriptomics biomarkers in future epidemiological studies.

Finally, we analysed whole genome gene expression modifications in human colon biopsies in relation to faecal NOC exposure. We had a particular interest in patients suffering from intestinal inflammation as this may stimulate endogenous NOC formation. Inflammatory bowel disease (IBD) patients diagnosed with ulcerative colitis and irritable bowel syndrome patients without inflammation, serving as controls, were recruited. By associating gene expression levels of all subjects to faecal NOC levels, we identified a NOC exposure-associated transcriptomic response that suggests that physiological NOC concentrations may potentially induce genotoxic responses and chromatin modifications in human colon tissue, both of which are linked to carcinogenicity (Hebels et al 2011c; 2012). In a network analysis, chromatin modifications were linked to 11 significantly modulated histone genes, pointing towards a possible epigenetic mechanism that may be relevant in comprehending NOC-induced carcinogenesis. In addition, pro-inflammatory transcriptomic modifications were identified in visually non-inflamed regions of the IBD colon. We conclude that NOC exposure is associated with gene expression modifications in the human colon that may suggest a potential role of these compounds in colorectal cancer development.

Overall, we conclude that the studies described above provide a proof of principle that transcriptomics responses can be used to identify molecular processes involved in chronic diseases that may help to link specific exposers to adverse health outcomes. This implies that genomics biomarkers could be applied in molecular epidemiological studies aiming to establish relationships between dietary intake of nitrate and nitrite and carcinogenic risk. Additionally, gene expression profiles can be established that are indicative for improved cardiovascular health and can be evaluated in the same studies. This proposed approach may contribute to an improved risk-benefit evaluation of dietary intake of nitrate and nitrite, and to find the answer to the question whether these compounds should best be regarded as friend or foe.

References

Ward MH, De kok T, Levallois P, Brender J, Gulis G, Nolan BT, VanDerslice J: Workgroup report: Drinking-water nitrate and health – recent findings and research needs. Environ Health Perspect 2005, 113(11):1607-1614.

Walters CL: Nitrate and nitrite in foods. New York: Ellis Horwood; 1991.

Ashton MR: The occurrence of nitrates and nitrites in food. The British Food Manufacturing Industries Research Association: Literature Survey No 7 1970, 1-32.

Choi BC: N-nitroso compounds and human cancer: a molecular epidemiologic approach. Am J Epidemiol 1985, 121(5):737-743.

Hotchkiss JH: A review of the current literature on N-nitroso compounds in foods. Adv Food Res 1987, 31:53-115.

Lijinsky W: N-nitroso compounds in the diet. Mutat Res 1999, 443(1–2):129-138.

Knekt P, Järvinen R, Dich J, Hakulinen T. Risk of colorectal and other gastro-intestinal cancers after exposure to nitrate, nitrite and N-nitroso compounds: a follow-up study. Int J Cancer. 1999 Mar 15;80(6):852-6.

Lundberg, JO, Feelisch, M, Bjorne, H, Jansson, EA and Weitzberg, E. 2006. Cardioprotective effects of vegetables: is nitrate the answer? Nitric Oxide, 15: 359–362.

Lundberg, JO, Gladwin, MT, Ahluwalia, A, Benjamin, N, Bryan, NS, Butler, A, Cabrales, P, Fago, A, Feelisch, MFord, PC. 2009. Nitrate and nitrite in biology, nutrition and therapeutics. Nat Chem Biol, 5: 865–869.

Honikel KO. The use and control of nitrate and nitrite for the processing of meat products.
Meat Sci. 2008 Jan;78(1-2):68-76.

World Cancer Research Fund. Food, Nutrition, Physical Activity, and the Prevention of Cancer: a Global Perspective. First expert panel report. 1997. AICR, Washington DC.

World Cancer Research Fund / American Institute for Cancer Research. Food, Nutrition, Physical Activity, and the Prevention of Cancer: a Global Perspective. Second expert panel report. 2007. AICR, Washington DC.

Sandhu MS, White IR, McPherson K. Systematic review of the prospective cohort studies on meat consumption and colorectal cancer risk: a meta-analytical approach. Cancer Epidemiol Biomarkers Prev. 2001;10(5):439-46.

Cross AJ, Pollock JR, Bingham SA. Haem, not protein or inorganic iron, is responsible for endogenous intestinal N-nitrosation arising from red meat. Cancer Res. 2003;63(10):2358-60.

Vermeer IT, Pachen DM, Dallinga JW, Kleinjans JC, van Maanen JM. Volatile N-nitrosamine formation after intake of nitrate at the ADI level in combination with an amine-rich diet. Environ Health Perspect. 1998;106(8):459-63.

Akesson, B. and Mercke, P. (Eds) Biomarkers on dietary exposure to anticarcinogenic food components and their links to mechanisms of action. ECNIS Report, NIOM, Lods, Poland, 2005.

de Kok TM, van Breda SG, Manson MM. Mechanisms of combined action of different chemopreventive dietary compounds: a review. Eur J Nutr. 2008;47 Suppl 2:51-9.

De Kok, T.M., van Breda, S.G. and Briedé, J.J. (2012) Genomics-based identification of molecular mechanisms behind the cancer preventive action of phytochemicals: potentials and challenges. Curr. Pharmaceutical Biotechnol. 13, 255-264.

Hebels, D.G., Brauers, K.J., van Herwijnen, M.H., Georgiadis, P.A., Kyrtopoulos, S.A., Kleinjans, J.C. and de Kok, T.M. (2011a) Time-series analysis of gene expression profiles induced by nitrosamides and nitrosamines elucidates modes of action underlying their genotoxicity in human colon cells. Toxicol Lett.,207, 232-241.

Hebels, D.G., Jennen, D.G., van Herwijnen, M.H., Georgiadis, P.A., Moonen, E.J., Pedersen, M., Knudsen, L.E., Kleinjans, J.C. and de Kok, T.M. (2011b) Whole-genome gene expression modifications associated with nitrosamine exposure and micronucleus frequency in human blood cells. Mutagenesis, 26, 753-761.

Hebels, D.G., Sveje, K.M., de Kok, M.C., van Herwijnen, M.H., Kuhnle, G.C., Engels, L.G., Vleugels, C.B., Mares, W.G., Pierik, M., Masclee, A.A., S.A., Kleinjans, J.C. and de Kok, T.M. (2011c) N-nitroso compound exposure-associated transcriptomic profiles are indicative of an increased risk for colorectal cancer. Cancer Lett. 309:1-10.

Hebels, D.G., Sveje, K.M., de Kok, M.C., van Herwijnen, M.H., Kuhnle, G.G., Engels, L.G., Vleugels-Simon, C.B., Mares, W.G., Pierik, M., Masclee, A.A., Kleinjans, J.C., de Kok, T.M. (2012) Red meat intake-induced increases in fecal water genotoxicity correlate with pro-carcinogenic gene expression changes in the human colon. Food Chem Toxicol. 50:95-103.

Intragastric formation of N-nitrosodimethylamine after fish-with-vegetable meals using an in-vitro digestive model

Zeilmaker[1], M.J., Krul[2], C., Havenaar[2], R., Schothorst[1], R., Bakker[1], M.I. and W. Slob[1]

[1]National Institute for Public Health and the Environment

P.O. Box 1, 3720 BA Bilthoven, The Netherlands

[2]TNO

P.O. Box 360

3700 AJ Zeist, The Netherlands

Corresponding author: M.J. Zeilmaker (e-mail: marco.zeilmaker@rivm.nl)

N-nitrosodimethylamine (NDMA) is an acute and chronic genotoxic carcinogen (Driver *et al.*, 1987; Peto *et al.*, 1991a,b). The human exposure to NDMA consists of direct intake from food (regular exposure) and endogenous synthesis after a meal of vegetables and fish (infrequent exposure)(Krul *et al.*, 2004).

The endogenous synthesis of NDMA starts with the intake of nitrate from vegetables and/or drinking water. The nitrate taken in quickly enters the blood from which is actively secreted to the saliva (Wagner *et al.*, 1983). In saliva nitrate is converted to nitrite by bacterial fermentation (Spiegelhalder *et al.*, 1976). In the stomach swallowed nitrite may react under acidic conditions with the fish component dimethylamine (DMA) to NDMA (Mirvish *et al.*, 1975).

Though *in vivo* experiments in human volunteers have indicated that NDMA can be formed in the stomach after a meal of nitrate rich vegetables and different types of amine-rich fish (Vermeer *et al.*, 1998; van Maanen *et al.*, 1998) only a crude estimate can be given of the actual amount. It can be argued that a more accurate estimate might be obtained by *in vivo* measurement of NDMA formation. However, for practical and ethical reasons a study of NDMA formation in the stomach of human volunteers is very difficult.

We therefore used an alternative way to estimate the formation of NDMA in the human stomach after a vegetable/fish meal, i.e. with a dynamic *in vitro* gastrointestinal model (Minekus *et al.,* 1995; Krul *et al.*, 2004). The (computer-controlled) *in vitro* system mimics the physiological processes in the human stomach, i.e. body temperature, pH profile after food intake, peristaltic movements, secretion of digestive enzymes, etc.

In the gastrointestinal model the nitrosation was investigated between nitrite and DMA and codfish (30 – 100 grams) under different gastric pH conditions (slow and rapid pH decrease). To simulate realistically the swallowing of nitrite-containing containing oral fluid the formation of nitrite was quantified with the aid of a toxicokinetic model for nitrate and nitrite in humans. With this model the flow of nitrite-containing oral fluid into the stomach was calculated for the intake of different levels of nitrate (0.1 – 10 times the ADI) and incorporated in the gastrointestinal model. The formation of NDMA was investigated for a total of 55 different nitrate/codfish combinations. The analysis of these data resulted in a quantitative relationship for NDMA formation as a function of nitrate intake from rich vegetables and concomitant codfish consumption (codfish calibration curve, Krul *et al.*, 2004).

Besides codfish the formation of NDMA was investigated for a variety of frequently consumed fish species such as herring, mackerel, plaice, pollack and salmon with the (concomitant) exposure to nitrate from vegetables. In these fish species the formation of NDMA relative to codfish was 0.39 (herring), 0.33 (tuna), 0.29 (schrimp), 0.44 (fish-fingers), 0.17 (pollack), 0.02 (mackerel) and 0.00 (salmon). Furthermore the (inhibitory) effect of adult-/children food matrices in the stomach on DMNA formation was determined. This resulted in a 75% inhibition (range: 53-89 %).

In order to estimate NDMA formation as occurring during actual meals the codfish calibration curve, its scaling to other fish species and the inhibitory effect of food in the stomach were combined with the concomitant consumption of fish and nitrate from vegetables as reported in the Dutch National Food Consumption Survey-3 (DNFCS-3, Kistenmaker *et al.*,1998). This resulted in 105 DNFCS-3 participants who consumed a meal with fish and nitrate-rich vegetables on at least 1 day.

The estimated amounts of NDMA (per kilogram body weight) for these participants of theDNFCS-3 were analyzed by the Statistical Exposure Model for Incidental Intakes

(STEM.II; Slob, 2006). In short, this model takes into account both the frequency of exposure days and the magnitude of the exposure on these days. This analysis resulted in the acute and long-term exposure to NDMA after a meal of nitrate rich vegetables and fish. The calculated exposures were compared with reference values for the acute and long-term carcinogenic potency of NDMA. Cancer reference values were obtained by analyzing acute and chronic carcinogenicity data ((Driver *et al.*, 1987; Peto *et al.*, 1991a,b) with the BenchMarkDose (BMD) approach. The BMD is the dose corresponding to a specified Benchmark Response (BMR), e.g., a 5 or 10% extra cancer risk, as calculated from a dose-response relationship which is fitted through the data. The lower limit of the

confidence interval around the BMD, i.e., the Benchmark Dose Lower bound (BMDL), represents the dose where the effect is smaller than the BMR with 95% confidence. For fitting of dose-response relationships, the software of PROAST (Slob, 2002; www.proast.nl) was used.

As measure for cancer potency the Margin Of Exposure(MOE) approach was applied. The MOE measures the distance (ratio) between human exposure and some toxicity measure, denoted as the PoD (Point of Departure) or RP (Reference Point). See Barlow et al. (2006) or O'Brien *et al*. (2006) for further

discussions of this approach. The current view is that the BMDL10 is the preferable PoD (Benford *et al.,* 2010; ILSI, 2009).

Using a BMDL10 for (total liver) chronic tumor incidence (0.029 mg/kg-bw) and the 95th percentile of the long-term NDMA exposure distribution (4.1 and 0.40 ng/kg-bw for children of 1 year of age and adults, respectively) resulted in MOEs of 7000 for children 1 year of age and 72,500 for adults.

Similarly, given the incidental high-peak exposure to endogenous NDMA formation in the human population, a risk characterization based on the results from the acute carcinogenicity study by Driver *et al.* (1987) is relevant. For this study a BMDL10 of 11 mg/kg-bw was calculated. The exposure analysis showed that most of the estimated amounts of NDMA in the participants of the food survey were below 0.10 µg/kg. Hence, the MOE for acute NDMA exposure after a fish and vegetable meal would be greater than 100,000.

Both the acute and the chronic MOEs indicate that the combined consumption of fish and nitrate-rich vegetables appears to lead to marginal increases of (additional) cancer risk.

References

Barlow, A. G., Renwick, J., Kleiner, J. W., Bridges, L., Busk, E., Dybing, L., Edler, G., Eisenbrand, J., Fink-Gremmels and, A. and R. Knaap, R., et al. (2006). Risk assessment of substances that are both genotoxic and carcinogenic. Report of an International Conference organized by EFSA and WHO with support of ILSI Europe. Food Chem. Toxicol. 44, 1636–1650.

Benford, D., Bolger, P. M., Carthew, P., Coulet, M., DiNovi, M., Leblanc, J. C., Renwick, A. G., Setzer, W., Schlatter, J., Smith, B., Slob, W., Williams, G., and Wildemann, T. (2010) Application of the margin of exposure (MoE) approach to substances in food that are genotoxic and carcinogenic. Food Chem. Toxicol 48.(Suppl. 1), S1–S112.

Driver, H. E., White, I. N. H., Steven, F. S. and W.H. Butler (1987) A possible mechanism for the dose-response relationship observed for renal mesenchymal tumors induced in the rat by a single dose of Nnitrosodimethylamine. In: "The Relevance of N-nitroso Compounds to Human Cancer, Exposure and Mechanisms" (H. Bartsch, I. O'Neill, and R. Schulte-Hermann, Eds.), pp. 253–255. IARC Scientific Publications No. 84, International Agency for Research on Cancer, Lyon, France.

International Life Sciences Institute (ILSI). (2009) Application of the Margin of Exposure Approach to Compounds in Food Which Are Both Genotoxic and Carcinogenic. (Constable, A., and Barlow, S., Eds.). Summary of a workshop held in October 2008. ILSI Europe, Brussels, Belgium.

Kistemaker, C., Bouman,M., and K. F. A. M Hulshof (1998) In: "De consumptie
van afzonderlijke producten door de Nederlandse bevolkingsgroepen Voedselconsumptiepeiling 1997-1998". TNO report V98.812, Zeist, The Netherlands (In Dutch).

Krul, C.A.M., Zeilmaker, M.J., Schothorst, R.C. and R. Havenaar (2004) Intragastric formation and modulation of N-nitrosodimethylamine in a dynamic in vitro gastrointestinal model under human physiological conditions. Food Chem. Tox., 42, 51–63.

Minekus, M., Marteau, P., Havenaar, R. and J.H.J Huis in 't Veld (1995) A multicompartment dynamic computer-controlled model simulating the stomach and small intestine, ATLA, 23, 197 – 209.

Mirvish, S.S. (1975) Formation of N-nitroso compounds: chemistry, kinetics, and in vivo occurrence. Tox. Appl. Pharm. 31, 325–351.

O'Brien, J., Renwick, A. G., Constable, A., Dybing, E., Müller, D. J. G., Schlatter, J., Slob, W., Tueting, W., van Benthem, J., Williams, G. M., et al. (2006) Approaches to the risk assessment of genotoxic carcinogens in food: a critical appraisal. Food Chem. Toxicol. 44, 1613–1635.

Peto, R., Gray, R., Brantom, P., and P. Grasso (1991a) Dose and time relationships for tumor induction in the liver and esophagus of 4080 inbred rats by chronic ingestion of N-nitrosodiethylamine or N-nitrosodimethylamine. Cancer Res., 51, 6452–6469.

Peto, R., Gray, R., Brantom, P., and P. Grasso (1991b) Effects on 4080 rats of chronic ingestion of N-nitrosodiethylamine or N-nitrosodimethylamine: a detailed dose-response study. Cancer Res. 51, 6415–6451.

Slob, W. (2002) Dose-response modeling of continuous endpoints. Toxicol. Sci. 66, 298–312.

Slob, W. (2006). Probabilistic dietary exposure assessment taking into account
variability in both amount and frequency of consumption. Food Chem. Toxicol. 44, 933–951.

Spiegelhalder, B., Eisenbrand, G. and R. Preussmann (1976) Influence of dietary nitrate on nitrite content of human saliva: possible relevance to in vivo formation of N-nitroso compounds. Food Cosmet. Toxicol. 14, 545–548.

Van Maanen, J.M.S., Pachen, D.M.F.A., Dallinga, J.W. and J.C.S. Kleinjans (1998) Formation of nitrosamines during consumption of nitrate- and amine-rich food and the influence of mouthwashes. Cancer Detect. Prev., 22, 204-212.

Vermeer, I.T.M., Pachen, D.M.F.A., Dallinga, J.W., Kleinjans, J.C.S. and J.M.S. van Maanen (1998) Volatile N-nitrosamine formation after intake of nitrate at the ADI level in combination with an amine-rich diet. Env. Health Perspect., 106, 459-463.

Wagner, D., Schultz, D.S., Deen, W.M. Young, V.R. and S.R. Tannenbaum (1983) Metabolic fate of an oral dose of ^{15}N-labeled nitrate in humans: Effect of diet supplementation with ascorbic acid. Cancer Res., **43**, 1921-1925.

Zeilmaker, M.J., Bakker, M.I., Schothorst, R. and W. Slob (2010) Risk assessment of N-nitrosodimethylamine formed endogenously after fish-with-vegetable meals. Toxicol. Sci., 116(1), 323–335.