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Regarding: NOIL – nitrite in combination with amines or amides

Dear Office of Environmental Health Hazard Assessment,

I am responding to the notice that the California Environmental Protection Agency's Office of Environmental Health Hazard Assessment (OEHHA) intends to list *nitrite in combination with amines or amides* as known to the State to cause cancer under the Safe Drinking Water and Toxic Enforcement Act of 1986. This action is being proposed under the authoritative bodies listing mechanism and was published by OEHHA on February 7, 2014 on their web site[1]

This notice to list is totally in error for many scientific reasons. I am qualified to comment based on my experience as a researcher in the processed meat industry and at the University of Wisconsin. As a full disclosure, I am also asserting that my comments are my own and have been independently developed by me without input or consultation with any company or trade group. My points are as follows.

[1] http://www.oehha.ca.gov/prop65/CRNR_notices/admin_listing/intent_to_list/noilpkg48cnitrite.html

1. **Scientifically valid data which were not considered by IARC clearly establish that the sufficiency of evidence criteria were not met (Section 25306(f)).**

In 2010, IARC published Volume 94 in the series of IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, entitled *Ingested Nitrate and Nitrite, and Cyanobacterial Peptide Toxins*[2]. This report was developed by a Working Group that met at IARC in Lyon France in the summer of 2006 and reviewed the evidence at that time. A summary containing the conclusions was published in Lancet Oncology[3].

In their evaluation, the Working Group followed established protocols to discuss exposure data, studies of cancer in humans, studies of cancer in animals, mechanisms and other relevant data. The animal toxicology section's discussions included the materials cited by OEHHA in the proposal to list, but they also included significant other discussion concerning the conditions where these compounds could generate carcinogenic N-nitrosamines. This was also discussed by the mechanisms section of the Working Group. Chapter 5 which begins on page 311 of the monograph provides the summary of all the discussion and clearly indicates that the Working Group was combining three factors in their thought process. First was exposure to nitrite and nitrate, second was exposure to nitrosatable amines and amides, and, third was the potential for endogenous nitrosation to form N-nitrosamines, many of which are well established carcinogens. Although most N-nitrosamines are carcinogenic, there is at least one notable exception. N-nitrosoproline which is formed by nitrosation of the common amino acid proline is not carcinogenic as discussed on page 320 of the monograph. It is also notable that these conclusions under IARC rules required **both** the experimental animal model conclusions which OEHHA stresses **and** human cancer data. The IARC working group considered only gastric cancer data in humans as strong

[2] http://monographs.iarc.fr/ENG/Monographs/vol94/mono94.pdf
[3] http://download.thelancet.com/pdfs/journals/1470-2045/PIIS1470204506707896.pdf

enough evidence for their determination, and again it was focused on nitrosation to form N-nitrosamines as a mechanistic hypothesis. The final evaluation is copied below directly from page 323 of

6. Evaluation and Rationale

There is *inadequate evidence* in humans for the carcinogenicity of nitrate in food.

There is *inadequate evidence* in humans for the carcinogenicity of nitrate in drinking-water.

There is *limited evidence* in humans for the carcinogenicity of nitrite in food. Nitrite in food is associated with an increased incidence of stomach cancer.

There is *inadequate evidence* in experimental animals for the carcinogenicity of nitrate.

There is *sufficient evidence* in experimental animals for the carcinogenicity of nitrite in combination with amines or amides.

There is *limited evidence* in experimental animals for the carcinogenicity of nitrite *per se*.

Overall evaluation

Ingested nitrate or nitrite under conditions that result in endogenous nitrosation is *probably carcinogenic to humans (Group 2A)*.

There is an active endogenous nitrogen cycle in humans that involves nitrate and nitrite, which are interconvertible *in vivo*. Nitrosating agents that arise from nitrite under acidic gastric conditions react readily with nitrosatable compounds, especially secondary amines and amides, to generate *N*-nitroso compounds. These nitrosating conditions are enhanced following ingestion of additional nitrate, nitrite or nitrosatable compounds. Some of the *N*-nitroso compounds that could be formed in humans under these conditions are known carcinogens.

the monograph.

Quoting from above, IARC concluded: "There is *limited evidence* in humans for the carcinogenicity of nitrite in food. Nitrite in food is associated with an increased incidence of stomach cancer". They did not find evidence for other cancers, thus a focus on subsequent publications for this cancer site is important. Two major studies were subsequently published by researchers at the National Institutes of Health, and by Europeans workers in the EPIC program which is sponsored by IARC. The Cross et. al. study published in 2011[4] found no association of carcinogenicity of nitrite or nitrate and esophageal or gastric cancer in a very large prospective study of approximately 500,000 people that encompassed ~10 years of follow up. Loh et.

[4] Cross, A.J., Freedman, N.D., Ren, J., Ward, M.H., Hollenbeck, A.R., Schatzkin, A., Sinha, R., Abnet, C.C., 2011. Meat consumption and risk of esophageal and gastric cancer in a large prospective study. American Journal of Gastroenterology 106,432–442.

al.[5] also found no significant association between nitrite or endogenously produced nitroso compounds and cancer in a study of 23,000 people in the UK with a 20 year follow up.

IARC also concluded: "There is *sufficient evidence* in experimental animals for the carcinogenicity of nitrite in combination with amines or amides. There is *limited evidence* in experimental animals for the carcinogenicity of nitrite *per se*" and most importantly the official and summary determination: "Ingested nitrate or nitrite under conditions that result in endogenous nitrosation is *probably carcinogenic to humans (Group 2A)"*.

In making their final classification, IARC did not provide appropriate emphasis to the US National Toxicology Program[6] study of nitrite in rats and mice. This study was recommended by FDA in the 1980's as a follow-up to concern about carcinogenicity of nitrite and nitrate use as food additives. Two reports[7,8] by a select committee of the National Academy of Sciences summarized knowledge at that time and called for more research which prompted the FDA recommendation. The NTP study received extensive peer review and the final Technical Report[6] indicated that the only adverse finding in both rats and mice was an "equivocal evidence" finding that sodium nitrite weakly increased the number of forestomach tumors in female mice but not in male mice or male or female rats. All other organ sites in both rats and mice showed no evidence of carcinogenicity. Thus, the original suspicion of sodium nitrite's carcinogenicity in rodents was not supported by this state-of-the-art cancer bioassay. IARC chose to

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[5] Loh, Y.H., Jakszyn, P., Luben, R.N., Mulligan, A.A., Mitrou, P.N., Khaw, K.T., 2011. N-nitroso compounds and cancer incidence: the European Prospective Investigation into Cancer and Nutrition (EPIC)-Norfolk Study. American Journal of Clinical Nutrition 93, 1053–1061.

[6] National Toxicology Program, 2001 NTP TR495, NTP Technical Report on the toxicology and carcinogenesis studies of sodium nitrite (CAS NO. 7632-00-0)IN F344/N rats and B6C3F1 mice(drinking water studies), , NIH Publication No. 01-3954.

[7] National Academy of Sciences, 1981. The Health Effects of Nitrate, Nitrite and N-Nitroso Compounds. National Academy Press, Washington, DC.

[8] National Academy of Sciences, 1982. Alternatives to the current use of nitrite in foods. National Academy Press, Washington, DC.

discount this information in favor of many small studies where experimental conditions were designed to promote nitrosamine formation and carcinogenesis in animal models. In those studies, the experimental treatments that demonstrated carcinogenicity required high levels of nitrite and specific nitrosatable amines and conditions that favored formation of carcinogenic N-nitrosamine compounds.

I would draw your attention to Table 2 of a peer reviewed discussion[9] of the IARC findings where I was a co-author (attached). It summarizes data from animal studies with important methodological limitations which includes studies published after the IARC review. There are 42 studies listed and 33 show no carcinogenicity of nitrite when added to the control diet; 4 studies show unclear results and 5 report carcinogenicity. Additionally, Table 3 summarizes results of 12 high quality long term animal feeding studies which are now considered to have the most importance in evaluating the safety of compounds. None of these show carcinogenicity of nitrite.

There is no evidence for carcinogenesis in animals which are given varying levels of nitrite and notably the animals are also exposed to significant levels of amines and amides due to the proteins, and vitamins present in their diet.

In summary, the two bases of evidence cited by IARC for their group 2A listing of "nitrite and nitrate under conditions of endogenous nitrosation", have been contradicted by newer epidemiological evidence and by a critical reevaluation of the evidence in animal toxicology studies.

[9] Bryan, N. S. Alexander, D. A., Coughlin, J, R., Milkowski, A. L. Boffetta, P. 2012, Ingested nitrate and nitrite and stomach cancer risk: an updated review, Food *and Chemical Toxicology*, 50, 3646-3665

2. Nitric oxide physiology was not reviewed completely by IARC and S- nitrosation was ignored.

Questions about the carcinogenicity of sodium nitrite arose from the discovery decades ago that most N-nitrosamines are carcinogenic and that humans are exposed to trace levels of them from foods, tobacco, some consumer products and the environment, facts well recognized in existing IARC Monographs. This led to understandable concerns about the reactants (various amines, amino acids and nitrite), and the focus was on nitrate and nitrite because of the recognition that secondary amines were ubiquitous in foods and in the human body, and, that the carcinogenic potential of N-nitrosamines might be controlled by elimination of the nitrite. Unrecognized at the time was the important role that nitrogen oxides play in human physiology. The discovery that nitric oxide is endogenously synthesized and the profound importance of nitric oxide, nitrite and nitrate in human homeostasis have led to the current understanding that there is a metabolic nitrogen oxide cycle. Thus, human exposure to nitrite, nitrate (a nitrite precursor) and secondary amines should now be considered as a normal part of human physiology.

Additionally, since N-nitrosoproline is not carcinogenic and is apparently readily formed in metabolism, one could conjecture that this amine in combination with nitrite is actually protective because it may preferentially form instead of other carcinogenic N-nitrosamines.

We are now learning that the beneficial effects of nitric oxide on human physiology are in many cases modulated via mechanisms involving S-nitrosation. The IARC Working Group did not review or evaluate any of this important S-nitrosation biochemistry. Thus, IARC's classification of "endogenous nitrosation" is actually scientifically inaccurate, since they only focused on N-nitrosation while using the broader term nitrosation. If their review had been more comprehensive, they would have necessarily further qualified

their conclusions regarding endogenous nitrosation to properly specify N-nitrosamine formation. Again I would refer you to the attached review paper[9] for a more complete discussion of the broad class of nitrosation reactions.

3. **The proposal to list is a completely inaccurate extrapolation from the authoritative body (IARC, The International Agency for Research on Cancer) that is cited by OEHHA.**

The overall IARC scientific evaluation was very specific in the reference to both nitrite and nitrate, and somewhat specific regarding nitrosatable amines and conditions where nitrosation endogenously occurs to presumably produce N-nitroso compounds. OEHHA has been grossly inaccurate in: 1) omitting nitrate; 2) broadly including amines and amides; and 3) ignoring the critical qualifier "under the conditions that result in endogenous nitrosation".

Because of these important inaccuracies, I cannot consider the reference to IARC as an authoritative body to be in any way correct, and hence the OEHHA proposal to list is not valid. It goes far beyond what IARC stated.

4. The proposal is inaccurate and impractical from a basic chemical and biological perspective

By incorrectly referencing IARC in the intent to list, the wording of OEHHA's notice lists two broad classes of organic compounds, amines and amides in a totally absurd manner. I raise this point because listing one specific chemical, nitrite in combination with millions of compounds in two broad classes leads to a meaningless listing with no specificity or scientific merit. As written, virtually every biological material will need to be considered under the listing because they have substantial amounts of amines and amides. While this is stated in the part of the intent to list, the ramifications are ignored.

This requires me to make some comments to remind you about some basic organic chemistry and biochemistry. This level of understanding is required for undergraduates to complete standard chemistry proficiency in many scientific disciplines. Apparently this level expertise is lacking among the staff and leadership of OEHHA who drafted, reviewed and released the intent to list. I find it appalling that the wording of the February 7, 2014 OEHHA notice of intent to list reflects such poor basic scientific reasoning.

Morrison and Boyd's Organic Chemistry[10], a textbook used at universities in the 1960-1970's, has two chapters of 53 total pages devoted to amines, which they define as organic compounds that show appreciable basicity and have the general formula RNH_2, R_2NH, or R_3N where R is any alkyl or aryl group. In other words, an amine is any organic compound with a nitrogen atom. This is a class of millions of

[10] Organic Chemistry, Second Edition seventh printing October 1969 , by Robert Thornton Morrison and Robert Nielson Boyd, Allyn and Bacon, Boston, Library of Congress #66-25695

compounds! They can be described as a primary amines, RNH_2, secondary amines RNHR or tertiary amines, R_3N.

Amides are a class of organic compounds where the nitrogen in an amine is covalently attached to a carbon that is part of a carbonyl group. This is also considered to be a peptide bond (as it is known to biologists and biochemists) with a general formula RCO-NH-R. Peptide bonds are what distinguish the polymer chains of amino acids known as peptides and proteins. Proteins, are essential molecules for life and are generally made up of up to 20 different primary amino acids and typically have a terminal NH_2 (amino) group. The word vitamin is a contraction of the term "vital amines". Other compounds in this class are the active components of spices and natural flavorings such as piperine and piperidine in white and black pepper

The essential micronutrient folic acid, a vitamin, is also an amide. There are many amides which are important pharmaceuticals for human therapy. If fact the first antibiotic, sulfanilamide which was discovered by Gerhard Damagk (who received the Nobel Prize in 1939) is classified as an amide as are other important antibiotics such as penicillin. An amide found in a food is capsaicin in chili peppers.

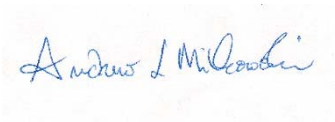
Thus, if the listing as proposed were to go into effect, OEHHA would be faced with the impossible task of determining what levels of one specific compound and two broad classes of chemical compounds would trigger a violation of the 1986 statute.

Bizarre questions then arise. Would pepper served in conjunction with a salad at a restaurant require a warning? Would a person receiving antibiotic therapy need to be warned not to eat any fruits and vegetables? These are only two simple scenarios that could be envisioned. Certainly OEHHA and the courts could be totally overwhelmed by unanticipated legal proceedings from a listing.

In overall summary, I urge you to critically reevaluate this notice of intent to list. There are important scientific issues related to new information not considered by the authoritative body that you cite. Additionally, the errors in how the IARC as the authoritative body was referenced make the current notice of intent to list totally unsupported by any scientific rationale.

Thank you for consideration of my thoughts. I trust that OEHHA will give due consideration to these comments and I would be happy to engage in any follow-up communications should clarification be required.

Respectfully submitted,



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Review

Ingested nitrate and nitrite and stomach cancer risk: An updated review

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ABSTRACT

Nitrite and nitrate are naturally occurring molecules in vegetables and also added to cured and processed meats to delay spoilage and pathogenic bacteria growth. Research over the past 15 years has led to a paradigm change in our ideas about health effects of both nitrite and nitrate. Whereas, historically nitrite and nitrate were considered harmful food additives and listed as probable human carcinogens under conditions where endogenous nitrosation could take place, they are now considered by some as indispensible nutrients essential for cardiovascular health by promoting nitric oxide (NO) production. We provide an update to the literature and knowledge base concerning their safety. Most nitrite and nitrate exposure comes from naturally occurring and endogenous sources and part of the cell signaling effects of NO involve nitrosation. Nitrosation must now be considered broadly in terms of both *S*- and *N*-nitrosated species, since *S*-nitrosation is kinetically favored. Protein *S*-nitrosation is a significant part of the role of NO in cellular signal transduction and is involved in critical aspects of cardiovascular health. A critical review of the animal toxicology literature of nitrite indicates that in the absence of co-administration of a carcinogenic nitrosamine precursor, there is no evidence for carcinogenesis. Newly published prospective epidemiological cohort studies indicate that there is no association between estimated intake of nitrite and nitrate in the diet and stomach cancer. This new and growing body of evidence calls for a reconsideration of nitrite and nitrate safety.
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1. Introduction

For more than 40 years, a highly visible debate regarding the ingestion of nitrate and nitrite and human health has occurred among the media, scientific, regulatory and public health communities. This debate has led ultimately to the examination of mechanisms by which nitrate and nitrite interact within the human body as well as the safety of these compounds in foods.

In the 1960s the safety of human exposure to inorganic nitrate and nitrite began receiving increased scrutiny for a number of reasons. There were documented cases of infantile methemoglobinemia associated with high nitrate in drinking water. Also during this time, atmospheric nitrogen oxides (NO_x) pollution became an environmental concern. In addition, the formation of *N*-nitrosamines, most of which have been shown to be animal carcinogens, in tobacco smoke and in some foods was demonstrated, and this raised the awareness of the potential human health concern and also set the foundation for the debate regarding nitrite and nitrate. During the 1970s and 1980s important research was performed to examine the reactivity of nitrite with nitrosatable amines and to investigate their toxicity using animal models. Simultaneously, processed foods and beverages were investigated for the presence of *N*-nitrosamines and when found, manufacturers introduced processing and ingredient changes to eliminate or minimize their formation (Assembly of Life Sciences (US) Committee on Nitrite and Alternative Curing Agents in Food, 1981; Cassens, 1990; National Academy of Sciences, 1982). Examples of these changes included modification of brewing methods for alcoholic beverages and usage of nitrosation inhibitors in cured and processed meats. There was also considerable public controversy about the use of nitrite and nitrate to cure meat that resulted in changes to regulations in many countries, based on decisions to best balance toxicological risk with the benefits of these two compounds in food preservation and safety assurance (United States Department of Agriculture, 1978). This period of intense scrutiny also resulted in the discovery of nitric oxide (NO) as an endogenously produced metabolite in human physiology with profound biological activity in human physiology (Gladwin et al., 2005).

Although modestly increased associations between consumption of foods containing nitrite and nitrate and certain cancers have been reported in some prospective epidemiologic studies (Larsson et al., 2006a,b; van Loon et al., 1998) overall, findings across studies have been largely inconsistent and equivocal (Cross et al., 2011; Jakszyn et al., 2006; Jakszyn and Gonzalez, 2006; Knekt et al., 1999). Consequently, the overall burden of proof remains inconclusive (Adami et al., 2011; Alexander, 2010; Alexander et al., submitted for publication; Boyle et al., 2008; Cho and Smith-Warner, 2004; Eichholzer and Gutzwiller, 1998; Milkowski et al., 2010; Truswell, 2002). A biologically plausible mechanism for the carcinogenicity of ingested nitrate and nitrite involves endogenous *N*-nitrosation reactions. Although generally considered harmful due to formation of *N*-nitrosamines, biomedical science over the past 20 years has recognized nitrosation reactions as an essential fundamental process in mediated cell signaling (Foster et al., 2003; Stamler et al., 2001).

In 2006, the International Agency for Research on Cancer's (IARC) Monograph Working Group concluded that "Ingested nitrate or nitrite under conditions that result in endogenous nitrosation is probably carcinogenic to humans (Group 2A) (Grosse et al., 2006; World Health Organization, 2006). The final IARC Monograph of this review and classification was not published until 2010 (International Agency for Research on Cancer, 2010). Any classification scheme, such as the one used by IARC, is based on the interpretation and evaluation of the evidence available at that time, and is therefore inherently temporary, and re-evaluations should be done when new evidence becomes available and when it appears that the reviewers may have misinterpreted certain published findings.

For example, acrylonitrile was classified by IARC as "a probable human carcinogen (Group 2A)" in 1986, based on sufficient evidence of carcinogenicity in animals and limited evidence of carcinogenicity in humans (lung cancer). Subsequent results of epidemiologic studies, including a large cohort from the US (Blair et al., 1998) did not confirm the suspected association with lung cancer (Bull et al., 1984a,b; Delzell and Monson, 1982; O'Berg, 1980; Thiess et al., 1980), leading to a re-evaluation by IARC in 1999, which resulted in downgrading of the overall evidence from Group 2A to Group 2B (International Agency for Research on Cancer, 1999).

The legacy of this period of research on nitrate and nitrite, (review and evaluation beginning in the 1960s) is reflected in a dichotomy of current scientific and public attitudes about the occurrence and use of nitrate and nitrite. One group is focused on the evolving knowledge about nitrogen oxide metabolism, its important physiological effects and potential new therapeutic applications for human health. Others focus on the potential human risks associated with the formation of trace levels (low parts per billion) of carcinogenic *N*-nitrosamines in some foods and by their endogenous formation.

Thus, our purpose was to conduct a review of the evidence from experimental animal studies and human epidemiological studies on cancer risk from ingested nitrate or nitrite, with emphasis on studies that were not included in or were published subsequent to the 2006 IARC evaluation. Given the importance of *N*-nitrosation as an underlying mechanism of the possible carcinogenicity of ingested nitrate and nitrite, we also include a detailed review of nitrosation as a fundamental physiological process. This review enhances and updates the current state-of-knowledge pertaining to the toxicological and epidemiological aspects of dietary nitrate and nitrite, with an additional focus on human nitrogen oxide physiology and metabolism.

2. Nitrosation – a fundamental physiological process

The discovery that nitric oxide was the long studied "endothelium-derived relaxing factor or EDRF" resulted in a paradigm shift in the understanding of control of physiological processes. In 1992, Science magazine declared it to be "molecule of the year" (Culotta and Koshland, 1992). There was an explosion in literature in the field and the importance of the finding was recognized with the awarding of the 1998 Nobel Prize in Physiology or Medicine to the pioneering researchers in this field (Mitka, 1998; Smith, 1998; SoRelle, 1998).

Nitrosation is a process of converting organic compounds into *N*- or *S*-nitroso derivatives, i.e., compounds containing the R-NO functionality. Nitrosation, chemically speaking, is the addition of a nitrosonium ion (NO^+) via an electrophilic attack on organic compounds, primarily thiols (S–NO) or amines (N–NO). Primary amines ($R–NH_2$) reacting with nitrite lead to very unstable *N*-nitrosamines that degrade to alcohols. However, secondary amines ($R_1–NH–R_2$) lead to stable *N*-nitrosamines, most of which have been shown to be carcinogens in rodent bioassays after activation by cytochrome P-450 enzymes. The reaction of a nitrogen oxide with a thiol group leads to the formation of an *S*-nitrosothiol (RSNO). Both *N*-nitrosation and *S*-nitrosation reactions can occur at acidic pH, with *N*-nitrosamine formation occurring even at neutral or basic pH. Substances in which the *N*-nitroso group is attached to an oxygen atom are called nitrite esters. When that oxygen is the oxygen atom of water, inorganic nitrite is formed; this is the same nitrite food additive ingredient that is used in cured meats. *N*-nitroso

compounds are usually prepared by the action of nitrous acid or a derivative of it upon a compound containing an easily replaced hydrogen atom, and certain members of the class are obtainable by oxidation of amines or by reduction of nitro compounds.

The simplest example of nitrosation in organic chemistry is probably the nitrosation of thiols, generating *S*-nitrosothiols (RSNO), formerly called thionitrites (reaction 1 below). The reaction is written for nitrous acid nitrosation, which is formed from acidified nitrite, but in principle any of the nitrosating agents will be effective. Secondary amines can also be nitrosated by a similar mechanism (reaction 2 below) although not as readily since amines are poorer nucleophiles than sulfur atoms.

$$RSH + HNO_2 = RS - NO + H_2O \quad \text{thiol nitrosation} \tag{1}$$

$$R_2NH + HNO_2 = R_2N - NO + H_2O \quad \text{secondary amine nitrosation} \tag{2}$$

Since the discovery of the biological properties of NO, intense interest has been aroused in the chemistry of RSNO species, since some are naturally occurring, have powerful biological properties and can release NO (Simon et al., 1993; Stamler et al., 1992b; Upchurch et al., 1995). It has been suggested that RSNO compounds act as storage and transport agents of NO *in vivo* (Stamler et al., 1992a). Furthermore, through transnitrosation reactions, low-molecular weight nitrosothiols can nitrosate proteins as a form of post-translational modification, termed *S*-nitrosation or sometimes referred to as *S*-nitrosylation. The ability of nitrosothiols to act as transducers of NO activity may be one of the most important functions in the human body, and *S*-nitrosation is a fundamental physiological process to convey NO biochemistry. Therefore, the broad term "nitrosation" as defined by IARC, which reflects these essential pathways in the body, has questionable relevance to cancer.

In order to fully appreciate nitrosation chemistry it is first prudent to review the history of nitrosation as it relates to cancer. Prior to the discovery of NO and endogenous formation of nitrosothiols as biological mediators of NO signaling, nitrosation was only associated with formation of *N*-nitrosamines. For many years, *N*-nitrosamine compounds were believed to be produced only during infectious or inflammatory reactions and transplant rejection processes (Grisham et al., 1992; Lancaster et al., 1992) or through ingesting precursors of nitrosation, such as nitrite and nitrate along with nitrosatable low molecular weight amines, although their precise role in these pathologies remains unclear. However, we now know nitrosothiols are also readily formed during inflammation (Jourd'Heuil et al., 2000).

The first report in the 1950s on the hepatocarcinogenic effects of *N*-nitrosodimethylamine (NDMA) (Magee and Barnes, 1956), and the suggestion that *N*-nitrosamines can be formed following nitrosation of various amines (Druckrey and Preussmann, 1962), ignited an enormous interest in *N*-nitrosamines and their possible association with cancer. Direct proof that such *N*-nitrosation reactions can occur was provided by Ender et al. (1964), who identified NDMA in nitrite-preserved fish, and by Sander and Seif (1969), who demonstrated the *in vivo* formation of an *N*-nitrosamine in the acidic conditions of the human stomach. Furthermore Tannenbaum's research group at the Massachusetts Institute of Technology also demonstrated endogenous nitrosation in saliva (Tannenbaum et al., 1978a). Because of the ease of formation of *N*-nitrosamines, potent carcinogenicity, and wide environmental occurrence, considerable efforts have gone into determining their levels in the external and internal human environment, and attempts have been made to assess exposure in order to correlate it with human cancer at specific sites (Bartsch and Montesano, 1984).

Marletta (1988) describe the endogenous formation of carcinogenic *N*-nitrosamines in mammals via the NO pathway, while Tannenbaum's group first described products of nitrosation in the saliva (Tannenbaum et al., 1978a). Excessive nitrite or nitrate intake could potentially generate *N*-nitroso compounds that are carcinogenic (Hecht, 1997; Hill, 1996). However, the discovery of endogenous production of nitrite and nitrate changed the view of these anions as synthetic food additives (Tannenbaum et al., 1978b).

Since the early 1980s there have been numerous reports on the association of *N*-nitrosamines and human cancers (Bartsch and Montesano, 1984; Craddock, 1983), suggesting that limiting environmental and dietary exposure to *N*-nitrosamines could reduce the incidence of diet-related and environmental cancers. However, *in vivo* formation through endogenous NO production may add another dimension to our understanding. More recently, protein *N*-nitrosamines have also been detected in plasma of healthy human individuals (Rassaf et al., 2002), inviting a reassessment of the obligatory carcinogenic role of *N*-nitroso species in man. It is well known that chronic inflammation, particularly in the gut, is associated with an increased risk of malignancy (Collins et al., 1987; Korelitz, 1983; Weitzman and Gordon, 1990). This can be attributed to excess NO production and can modify either DNA directly or through the inhibition of DNA repair enzymes.

Although there is evidence to support a plausible biological mechanism for formation of *N*-nitrosamines, there are also numerous effective inhibitors of *N*-nitrosation reactions in biological systems (d'Ischia et al., 2011). It was discovered that ascorbic acid (vitamin C) very potently inhibits *N*-nitrosamine formation (Mirvish, 1975). Another antioxidant, alpha-tocopherol (vitamin E), has also been shown to inhibit *N*-nitrosamine formation (Mergens et al., 1978; Mirvish, 1996). Ascorbic acid, erythorbic acid and alpha-tocopherol inhibit *N*-nitrosamine formation due to their oxidation–reduction properties. For example, when ascorbic acid is oxidized to dehydroascorbic acid, nitrous anhydride, a potent nitrosating agent formed from sodium nitrite, is reduced to NO, which is not a nitrosating agent. Stoichiometrically, one molecule of ascorbic acid can reduce two molecules of acidified nitrite to NO (Archer et al., 1975; Licht et al., 1988). However, in the presence of dissolved oxygen, NO can be oxidized back to nitrite/nitrous acid (Archer et al., 1975; Licht et al., 1988; Moriya et al., 2002). This recycling means that more than half the molar equivalent of ascorbic acid compared to nitrite is required to prevent formation of *N*-nitroso compounds (Archer et al., 1975; Licht et al., 1988; Moriya et al., 2002). The ratio of ascorbic acid to nitrite is recognized to be a major determinant of the generation of *N*-nitroso compounds within the acidic lumen of the stomach (Archer et al., 1975). Contemporary meat-curing methods use ascorbic acid or erythorbate to prevent *N*-nitrosation reactions and to facilitate the curing process. Most vegetables, which are naturally enriched in nitrate, are also rich in antioxidants such as vitamins C and E and polyphenols, which can act to prevent the undesirable *N*-nitrosation chemistry.

2.1. Current state of nitric oxide science

Concomitant with any amine nitrosative chemistry that may take place through endogenous production of NO or consumption of nitrite and nitrate is thiol nitrosation, which is now considered a fundamental signal transduction pathway in physiology. Protein *S*-nitrosation constitutes a large part of the ubiquitous influence of NO on cellular signal transduction, and accumulating evidence indicates important roles for *S*-nitrosation both in normal physiology and in a broad spectrum of human diseases. *S*-nitrosation is a mechanism for dynamic, post-translational regulation of most or all major classes of protein (Foster et al., 2003; Lima et al., 2010). *S*-nitrosation thereby conveys a large part of the ubiquitous

influence of NO on cellular signal transduction and provides a mechanism for redox-based physiological regulation. This signaling pathway is independent of the well characterized NO-soluble guanylyl cyclase (sGC) pathway, whereby NO binds directly to the heme group of sGC, which then catalyzes the conversion of guanosine triphosphate (GTP) to second messenger cyclic guanosine monophosphate (cGMP). Genetic and biochemical data demonstrate a pivotal role for RSNOs in mediating the actions of NO (Stamler et al., 2001), and RSNOs serve to convey NO bioactivity and to regulate protein function (Foster et al., 2003). This redox-based signal transduction pathway is akin to phosphorylation (Lane et al., 2001), in that both exemplify dynamic regulation of protein function by reversible modification.

S-nitrosation of proteins is increasingly implicated in critical aspects of cardiovascular physiology. In the heart, *S*-nitrosation of essential regulators of β-adrenergic receptor signaling (e.g., G protein receptor kinase 2, β-arrestin 2) and calcium cycling (e.g., the L-type calcium channel and the ryanodine receptor calcium release channel) help to maintain cardiac contractility (Hare, 2003; Ozawa et al., 2008; Sun et al., 2006). In the peripheral vasculature, *S*-nitrosation of caspases (Mannick et al., 1999), dynamin (Kang-Decker et al., 2007) and *N*-ethylmaleimide sensitive factor (Calvert et al., 2007) mitigate inflammation and apoptosis, whereas *S*-nitrosation of hemoglobin regulates blood flow and oxygen delivery (Frehm et al., 2004; Singel and Stamler, 2004). RSNOs have also been implicated in neovascularization and transducing hypoxic signals (Palmer et al., 2007), but their exact roles in these processes have not been elucidated, other than their recognition as essential physiological modifications. The precise regulation of RSNO formation and degradation on specific sites on proteins to affect function is still a subject of intense research. However, Stamler and colleagues have made great strides in our understanding of the regulation of *S*-nitrosation signal transduction (Hess et al., 2005). Most recently nitrite-mediated RSNO formation has been described in physiological systems (Angelo et al., 2006; Bryan et al., 2005; Bryan et al., 2004). Thus, via direct formation of *S*-nitroso products, endogenous or exogenous nitrite (through nitrosative chemistry) may account for some of the biochemistry and pharmacology reactions previously attributed to NO and therefore regulate essential cellular functions through *S*-nitrosation.

Compartmentalization of NO production or nitrite metabolism in a complex with key protein targets of nitrosation may prove to be a predominant way by which specificity is conferred for cellular nitrosation events. Which moiety within a protein that is preferentially *S*-nitrosated and the resulting effects are not yet known. An apparent limitation in *S*-nitrosation biology is the unpredictable instability of such a complex, the nature of the nitrosation product and thus the difficulty in detecting, specifying and quantifying such species.

Denitrosation of cellular proteins is just as important as the *S*-nitrosation events themselves and confirms the complexity and dynamics of NO signaling (Sanghani et al., 2009). It is known that denitrosylation of SNO-proteins in cells can be accomplished by simple chemistry, wherein intracellular glutathione or other thiols act as acceptors and effectively remove nitroso groups via transnitrosation reactions. In this system, the rate of SNO protein decomposition would be modulated by changes in intracellular thiol levels, and conditions that promote glutathione oxidation in cells would enhance steady-state levels of protein *S*-nitrosation. This mechanism would put *S*-nitrosation under redox control within the cell. Most of what is known about cellular signaling events involves only RSNOs.

2.2. Specific conditions dictate nitrosative chemistry

With much of this apparent promiscuous nitrosative chemistry occurring, it is necessary to understand the hierarchy of reactivity. The reaction between NO, thiols and oxygen has been studied in great detail to determine the mechanism of *S*-nitrosothiol formation. *S*-nitrosation through radical and non-radical pathways occurs simultaneously (Keszler et al., 2010) with the radical pathways occurring at near diffusion-controlled limit (Madej et al., 2008). Nitrosation by higher N-oxides of NO, such as N_2O_3, also occurs with the rate-limiting step involving NO reaction with oxygen. Rate constants for this type of reaction for glutathione and several other low molecular weight thiols are in the range of 3–$1.5 \times 10^5\ M^{-1}\ s^{-1}$, and for human serum albumin $0.3 \times 10^5\ M^{-1}\ s^{-1}$ (Kharitonov et al., 1995). In a comprehensive investigation into the targets of nitrosation, it was revealed that cysteine thiols are the primary target for nitrosation reactions followed by tryptophan *N*-nitrosation (Jourd'Heuil et al., 2010). Under the exact experimental conditions, thiol nitrosation is preferred. Interestingly, proline did not undergo *N*-nitrosation under these experimental conditions (Jourd'Heuil et al., 2010).

Broadly assigning nitrosation reactions without any information on the nature of the products further complicates our understanding. Different physiological/pathophysiological conditions may redirect the chemistry and produce an environment conducive to *N*-nitrosation. For example in patients with achlorhydria, due to normal aging process or due to proton pump inhibitors, there can be bacterial overgrowth (Friis-Hansen, 2006; Naylor and Axon, 2003). Under these conditions, bacterial mediated *N*-nitrosation may occur and may be independent from dietary exposures. In this situation, *N*-nitroso compound formation in the achlorhydric stomach must proceed by mechanisms which operate at neutral pH values. One potential mechanism involves the enzymatic catalysis of *N*-nitrosation by a subpopulation of the bacteria and in particular denitrifying organisms colonizing the achlorhydric stomach. This may provide a specific and unique environment for site-specific chemistry, since it is known that nitrite-mediated *N*-nitrosamine formation occurs primarily in neutral or basic pH (Keefer and Roller, 1973), whereas non-enzymatic nitrosation of thiols requires acid pH. Neutral gastric juice contains metabolically active bacteria capable both of generating nitrite from nitrate and catalyzing nitrosation reactions. In this way an intragastric environment suitable for the formation of carcinogenic *N*-nitrosamines exists in the hypochlorhydric and achlorhydric stomach, providing a possible mechanism for the high incidence of gastric cancer in these subjects (Ruddell et al., 1976). This becomes relevant for patients taking proton pump inhibitors. This is also supported by data showing regional differences in gastric pH, ascorbic acid and nitrite concentrations with the high pH occurring at the gastric cardia (Moriya et al., 2002; Suzuki et al., 2003).

The conditions favoring luminal generation of *N*-nitrosamines from ingested nitrate and nitrite are maximal at the most proximal cardia region of the acidic stomach and may contribute to the high incidence of mutagenesis at this site. In the model of gastric carcinogenesis postulated by Correa (Correa et al., 1975), gastric atrophy is an important early stage in the progression to carcinoma which results in the loss of stomach acidity, and colonization of the stomach by bacteria which can also catalyze *N*-nitrosamine formation. As a consequence of the metabolic activity of these bacteria, intragastric nitrite (a precursor to *N*-nitroso compounds) and possibly carcinogenic *N*-nitroso compounds become elevated, which may hasten the progression to carcinoma. Thus, for individuals with achlorhydria associated with *Helicobacter pylori* infection, pharmaceuticals such as proton pump inhibitors or other underlying causes, endogenous *N*-nitrosation in the stomach is a plausible hypothesis.

2.3. Methodological considerations related to nitrosation

Yet another confounding factor in defining nitrosation is the means by which we detect and quantify total nitroso compounds,

sometimes referred to as "apparent total nitroso compounds" (ATNC) (Mirvish, 2008). Historically, analytical methods to determine total nitroso compounds have used reductive de-nitrosation solutions (Walters et al., 1978; Xu and Reed, 1993), which have been shown to reduce all nitroso species, including *S*-nitroso and *N*-nitroso and even nitrite (Rassaf et al., 2002). Without specific methods for discriminating between the two species, very little information can be gained from epidemiological studies or well-controlled studies in animals to be able to determine nitrite-mediated low molecular weight *N*-nitrosamine formation and any association with cancer (Mirvish, 2008; Mirvish et al., 2008; Mirvish et al., 2002). The demonstration of the presence of up to five times higher *N*-nitroso proteins than *S*-nitroso proteins in human plasma (Rassaf et al., 2002) raises additional issues regarding total nitroso compounds, since these are clearly associated with proteins and are not any indication of carcinogenicity or mutagenicity risk. Any method that does not discriminate *S*-nitrosation from *N*-nitrosation or protein-bound from low molecular weight amine reactions should be dismissed. Unfortunately, reviews of methodologies in the literature that quantify ATNC do not provide any such discrimination, so caution should be exercised when interpreting results from such methodologies.

What clearly emerges from our current understanding of nitrosation reactions is as follows: (1) there has to be specificity to the target; (2) one has to define the conditions which favor *N*-nitrosation over *S*-nitrosation; and (3) one has to develop methods to accurately and specifically differentiate between *S*-nitrosation products and *N*-nitrosation products. Furthermore, there should be careful discrimination between protein *N*-nitrosamines (non-carcinogenic) and low molecular weight *N*-nitrosamines (potentially carcinogenic).

Further complicating the question of nitrosation is the recycling of both exogenous and endogenously derived nitrogen oxides via an oral ingestion route which can result in a wide variation in levels of ingestion. The parotid glands actively extract and secrete nitrate into the saliva (Gladwin et al., 2005; Tannenbaum et al., 1976). Overall about 25% of ingested nitrate is re-secreted in the saliva and approximately 25% of salivary nitrate is reduced to nitrite by commensal bacteria in the mouth. Hord et al. (2009) summarized dietary intake estimates of nitrite and nitrate for the "DASH" (Dietary Approaches to Stop Hypertension) diet that included two scenarios for vegetables and fruit consumption. They indicated that a high nitrate intake from these sources could result in as much as 5 mg nitrite ingestion via entero – salivary recycling. An overall summary of sources of ingested nitrite and nitrate that updated prior estimates (Tricker, 1997; White, 1975, 1976) was developed by Milkowski (2011) and is shown in Table 1.

At least two hypotheses for the biological significance of this nitrate/nitrite metabolic recycling have been proposed. The first is that the ingestion of salivary nitrite is a protective mechanism against ingested pathogens (Gilchrist et al., 2010; L'Hirondel and L'Hirondel, 2002), because under acidified conditions in the stomach bactericidal activity of gastric juice is enhanced in the presence of physiological levels of nitrite (Duncan et al., 1997; Dykhuizen et al., 1998; Dykhuizen et al., 1996). The second hypothesis is that nitrate and nitrite serve as reservoirs of NO bioactivity that can be activated under appropriate physiological stimuli. The provision of dietary nitrite and nitrate can restore NO homeostasis under conditions when endogenous NO production from NOS becomes dysfunctional. This redundant compensatory pathway has been shown to beneficially affect a number of diseases and conditions (Bryan, 2006; Bryan and Loscalzo, 2011; McKnight et al., 1994; Suschek et al., 2006).

3. Animal toxicology of nitrite, nitrate and *N*-nitrosamines

Animal toxicology research is an important area of investigation that provides safety data for many chemicals and potential pharmacological agents. The early risk analysis into the safety of nitrate and nitrite as food additives relied heavily on such studies published in the 1960s through the1980s. These studies usually included simultaneous exposures to exogenous nitrosatable amines as part of the study protocols and the carcinogenic responses were often focused on specific tissue sites characteristic of individual *N*-nitroso compounds.

3.1. Methodological considerations in animal toxicology studies

The evaluation of nitrite safety coincided with evolution of methods using animal models for testing the chronic toxicology and carcinogenic potential of chemical substances. Early studies were focused on small numbers of animals tested in each group, limited exposure times, variation in the exposure levels, and examination of only a few target tissues. These early studies only served to establish hypotheses for further testing but did lead the way for the development of large-scale comprehensive evaluation methods embodied in the US National Toxicology Program (NTP) cancer bioassay protocols (Beyer et al., 2011; National Toxicology Program, 2011b; Pastoor and Stevens, 2005).

A few key features of the NTP protocol are: 2-year exposure periods; a broad range of tissues histologically examined in addition to mortality and body mass measures; use of a large number of female and male animals (usually 50 animals per gender per group); multiple-dose exposures to permit trend statistics and to increase the power of the findings; comparisons to historical controls in rat and mouse species in addition to the concurrent

Table 1
Ranges of nitrate, nitrite and nitric oxide exposure from diet, endogenous synthesis and recycling for adult humans expressed as mg/day.

Source	Nitrate	Nitrite	Nitric oxide NO
	NO_3^-	NO_2^-	
From diet (excluding cured processed meat) [a]	50–220	0–0.7	–
From 50 g/day cured processed meat intake [b]	1–4	0.05–0.6	–
Water [c]	0–132	0–10	–
Saliva [d]	>30–1000	5.2–8.6	–
Endogenous Synthesis [e]	–	–	70

From Milkowski (2011).
[a] Based on IARC Table 1.8 (IARC, 2010).
[b] Based on Keeton et al. (2009).
[c] Based on none present to US EPA maximum allowed contaminant level for water of 44 ppm and 2.7 L water consumption/day.
[d] Based on White (1975, 1976) and Hord et al. (2009) and includes both recycling of diet derived nitrate via the enterosalivary route and that from endogenous NO.
[e] Based on 1 mg/kg/day endogenous synthesis for 70 kg adults (Tricker, 1997).

controls in the individual study (National Toxicology Program, 2011a; Rhomberg et al., 2007).

As evidence for human carcinogenicity, interpretation of results in animal models also requires understanding about key physiological and enzymatic differences between the animal and humans. For example, rodents used in these cancer bioassays have forestomachs and Hardarian glands, whereas there are no analogous tissues in humans. Nutritional requirements are also a factor in that dietary essential amino acids, minerals and vitamins vary by species.

Beyond evaluation of compounds in isolation, most cancer bioassays employ extremely high levels of the compound under study, plus potential reactants or interacting substances, in order to produce measurable toxic endpoints as well as to investigate mechanisms and modes of action. However, it is important to note that these often extend to exposures and conditions which do not represent even the extremes of normal free-living human exposures. Consequently, bioassays may truly represent orders of magnitude higher doses than typical human exposure scenarios for low or trace level food ingredients or components.

Many nitrite toxicology and bioassay studies have used administration of highly nitrosatable amines in order to measure the carcinogenic outcomes of the resulting *N*-nitroso compounds. The studies can be considered to have two controls, one without added exogenous nitrite and another with nitrite exposure in the absence of added exogenous nitrosatable amines. Conclusions about potential carcinogenicity need to consider comparisons between the two controls with equal importance to the additional test treatments.

3.2. Animal model toxicology and carcinogenesis studies of sodium nitrite

The 2006 IARC review and evaluation summarized literature conducted over many decades when the standards for conducting such studies were evolving. The preamble to IARC studies (International Agency for Research on Cancer, 2006) indicates long term animal studies are to be given consideration for agents under review and studies that do not meet quality standards may be omitted. Best practice quality parameters for current animal toxicology and carcinogenicity tests include long term, 2 year to lifetime tracking of survival with subsequent histopathology on all tissues on a blinded basis, 50 animals per gender in each treatment, multiple exposure levels, control groups that are concurrently studied, independent peer review of the histopathology and statistical analysis (Derelanko and Hollinger, 2002; Organisation for Economic Co-operation and Development, 2002; US Environmental Protection Agency, 1998). Modern toxicological testing programs, such as those conducted by the NTP, employ such standards (Chhabra et al., 1990). Lack of consideration to the quality of available studies has sometimes led to erroneous carcinogenicity conclusions in the interpretation of the overall evidence for a specific chemical.

IARC correctly concluded that there is no evidence implicating nitrate as an animal carcinogen. However, among the studies referenced in the IARC monograph on nitrite and cancer, there were many experimental deficiencies, including: (1) 10 rat studies and 10 mouse studies that lacked appropriate controls; (2) these studies had small numbers of animals and were of a short duration; (3) these studies had details in the materials and methods sections indicative of significantly inadequate protocols compared to modern standards (Aoyagi et al., 1980; Hirose et al., 2002; Ichihara et al., 2005; Ishii et al., 2006; Ivankovic, 1979; Krishna Murthy et al., 1979; Lijinsky, 1984; Lijinsky et al., 1983; Lin and Ho, 1992; Matsukura et al., 1977; Mirvish et al., 1980; Miyauchi et al., 2002; Olsen et al., 1984; Shank and Newberne, 1976; Yada et al., 2002) (Kitamura et al., 2006a; Kitamura et al., 2006b; Kuroiwa et al., 2007; Kuroiwa et al., 2008a; Kuroiwa et al., 2008b) (Anderson et al., 1985; Borzsonyi et al., 1976; Borzsonyi et al., 1978; Greenblatt and Lijinsky, 1972, 1974; Greenblatt et al., 1971; Greenblatt and Mirvish, 1973; Mirvish et al., 1972). These studies are summarized in Table 2. It is noteworthy that two of these rat studies (Lijinsky, 1984; Lijinsky et al., 1983) were heavily weighted by IARC in concluding that for nitrite there was "sufficient evidence of carcinogenicity" in animal studies. This is an example of inappropriate conclusions being drawn when the quality of the studies was not properly assessed. Overall, where nitrite administered alone was studied without methodological study limitations, there was clearly no carcinogenic evidence in seven rat studies and five mouse studies (Grant and Butler, 1989; Lijinsky et al., 1973; Lijinsky and Reuber, 1980; Maekawa et al., 1982; National Toxicology Program, 2001; Newberne, 1979; van Logten et al., 1972; Hawkes et al., 1992; Inai et al., 1979; Rijhsinghani et al., 1982; Yoshida et al., 1993) (Table 3).

In particular, an early well-publicized study suggesting a carcinogenic effect of sodium nitrite (Newberne, 1979) was reevaluated by an Interagency Working Group (IWG) convened by the US Food and Drug Administration (FDA). The IWG responded to the FDA in 1980 "that no demonstration could be found that the increased incidences of these tumors were induced by the ingestion of sodium nitrite" (Interagency Working Group on Nitrite Research (US) and Food and Drug Administration, 1980). The conclusions of the report indicated that the "nitrite alone" animal studies to that date were not sufficient to make conclusions because they were small and lacked multiple dose levels (National Academy of Sciences, 1981). The controversy around nitrite's possible carcinogenicity at that time, due to the stated deficiencies in the Newberne study, led to FDA commissioning the NTP cancer bioassay of sodium nitrite. It is important to consider the results of the Newberne study in the context of this critical re-evaluation of nitrite carcinogenicity by the NTP.

Sodium nitrite was nominated by the FDA for NTP toxicity and carcinogenesis studies based on its widespread use in foods and the concern about formation of carcinogenic *N*-nitrosamines. The study was initiated in 1989 with 14-week dose ranging studies followed by the 2-year studies conducted from 1995 through 1997. A *Draft NTP Technical Report* was issued in early 2000, peer review was conducted in May 2000 and the final *NTP Technical Report No. 495* was issued in May 2001 (National Toxicology Program, 2001). From the results of the most definitive, chronic carcinogenicity bioassay study of sodium nitrite, the following overall carcinogenicity conclusions were reached after a thorough, public peer review and evaluation:

"Under the conditions of this 2-year drinking water study, there was *no evidence of carcinogenic activity* of sodium nitrite in male or female F344/N rats exposed to 750, 1500, or 3000 ppm. There was *no evidence of carcinogenic activity* of sodium nitrite in male B6C3F1 mice exposed to 750, 1500, or 3000 ppm. There was *equivocal evidence of carcinogenic activity* of sodium nitrite in female B6C3F1 mice based on the positive trend in the incidences of squamous cell papilloma or carcinoma (combined) of the forestomach. Decreased incidences of mononuclear cell leukemia occurred in male and female rats." (National Toxicology Program, 2001, pp. 8–9).

Thus, the only adverse finding in this entire lifetime bioassay of sodium nitrite, fed in drinking water at three doses up to 3000 ppm to both rats and mice (equivalent to average daily doses of approximately 130 mg/kg in male rats, 150 mg/kg in female rats, 220 mg/kg in male mice, and 165 mg/kg to female mice), was the occurrence of combined benign and malignant forestomach tumors in female mice. Consequently, the NTP peer review conducted by the Technical Reports Review Subcommittee concluded in their "Summary" Table (NTP, 2001, p. 10): "Neoplastic effects: None" observed in either male or female rats or mice. The Panel classified the female mouse forestomach tumor findings in their Table as

Table 2
Animal Toxicological Studies of Nitrite Carcinogenicity with Serious Methodological Limitations.

	Duration	# Males	# Females	Nitrite levels (ppm)	Vehicle	Not carcinogenic	Carcinogenic	Study limitation
A. Rat studies								
Shank and Newberne (1976)	130 weeks	96?	?	1000	Rat chow	X	X?	Variable nitrite exposure over study, mixed genders, unclear on multiple generations combined
Matsukura et al. (1977)	16 months	4	0	1600	Pellet diet		X	Few animals, base diet made of fish meal with measured nitrosamines found
Ivankovic (1979)	10 months	0	?	50 mg/kg/d	Feed	X		Short duration, small numbers of animals, only female pregnant rats and offspring
Krishna Murthy et al. (1979)	1 yearr + 120 days	20	20	5000	Feed	X		Small number of animals sacrificed 17 weeks after end of exposure
Aoyagi et al. (1980)	92 weeks	24?	?	800, 1600	Pellet diet	X	X	Follow-up to Matsukura with same fish meal diet. Carcinogenic effect only in high dose group
Mirvish et al. (1980)	Lifetime, 120 weeks max	22	23	3000	Water	?	?	Small numbers of animals, errors in table legends, controls not matched, but from a separate study.
Lijinsky et al. (1983)	2 years	24, 24, 24	24, 24, 24	2000	Diet – 48, water – 24	?	?	Small numbers of animals sacrificed ~20 weeks after end of treatment. Unclear description of histology and data analysis
Lijinsky (1984)	2 years	24	24	2000	Diet	?	?	Small numbers of animals sacrificed ~20 weeks after end of treatment. Preformed nitrosamines in the diet.
Olsen et al. (1984)	132 weeks	70,60, 60,66	70,60, 60,66	200, 1000, 4000 in cured meat formula	Cured meat		X	30 ppb preformed NDMA in diet, authors view results as coming from nitrosamines
Lin and Ho (1992)	10 months	?	?	3000	Squid or wheat based diet	X		Short duration, only 13 animals of unspecified gender
Hirose et al. (2002)	52 weeks	0	10	2000	Water	X		Small study, single gender, only studied mammary tumors
Miyauchi et al. (2002)	36 weeks	10	0	2000	Water	X		Small study, single gender, only studied forestomach tumors
Yada et al. (2002)	28 weeks	5	0	500	Water	X		Small study, single gender, only studied, short duration
Ichihara et al. (2005)	8 weeks	15	0	1000	Water	X		Small study, single gender, short duration, only studied thyroid and kidney tumors
Ishii et al. (2006)	0.5 d–2 weeks	42	0	2000	Water	X		Small study, single gender, short duration, only studied forestomach histology
Kitamura et al. (2006b)	48 weeks	0	10	2000	Water	X		Small study, single gender, only 4 weeks nitrite exposure, only studied forestomach tumors
Kitamura et al. (2006a)	29 weeks	18,20	?	1000, 2000	Water	X		Small study, unknown female animal numbers, short duration
Kuroiwa et al. (2007)	42 weeks	10	0	2000	Water	X		Small study, single gender, only studied forestomach tumors and lesions
Kuroiwa et al. (2008a)	32 weeks	9	0	2000	Water	X		Small study, single gender, only studied surgically induced esophageal lesion from gastric reflux
Kuroiwa et al. (2008b)	12,52,78 weeks	5, 5,15	0	2000	Water	X		Small study, single gender, only studied forestomach tumors and lesions
B. Mouse studies								
Greenblatt et al. (1971)	28 weeks	40	40	1000	Water	X		Short duration
Greenblatt and Lijinsky (1972)	26 weeks	30	30	1000	Water	X		Short duration, 10 weeks delay after treatment before sacrifice,
Mirvish et al. (1972)	28 weeks	40	40	1000	Water	X		Same data as Greenblatt et al. 1971, but reported again for the control and nitrite-treated group
Greenblatt and Mirvish (1973)	25 weeks	40	0	1000	Water	X		Short duration, 13 weeks delay before animal sacrifice
Greenblatt and Mirvish (1973)	20 weeks	40	0	2000	Water	X		Short duration, 10 weeks delay before animal sacrifice
Greenblatt and Lijinsky (1974)	26 weeks	40	40	1000	Water	X		11–12 weeks delay before animal sacrifice

Table 2 (*continued*)

	Duration	# Males	# Females	Nitrite levels (ppm)	Vehicle	Not carcinogenic	Carcinogenic	Study limitation
Borzsonyi et al. (1976)	?	42	40	500 ppm in pregnancy	Water	X		10 pregnant mice exposed, results for offspring kept on unspecified diet and unspecified time
Borzsonyi et al. (1978)	10 + mo	0	19	500 ppm in pregnancy	Water	X		Short exposure of pregnant mice
Borzsonyi et al. (1978)	10 months	62	71	0	–	X		Exposure only *in utero*, offspring of above
Krishna Murthy et al. (1979)	120 days	20	20	5000	Diet	X		Short duration, small number of animals
Anderson et al. (1985)	Life	?	15–20	184, 1840	Water		X	Small number of animals, complicated design of pregnant animals and F1 generation
B. Mouse studies								
Greenblatt et al. (1971)	28 weeks	40	40	1000	Water	X		Short duration
Greenblatt and Lijinsky (1972)	26 weeks	30	30	1000	Water	X		Short duration, 10 weeks delay after treatment before sacrifice,
Mirvish et al. (1972)	28 weeks	40	40	1000	Water	X		Same data as Greenblatt et al. (1971), but reported again for the control and nitrite-treated group
Greenblatt and Mirvish (1973)	25 weeks	40	0	1000	Water	X		Short duration, 13 weeks delay before animal sacrifice
Greenblatt and Mirvish (1973)	20 weeks	40	0	2000	Water	X		Short duration, 10 weeks delay before animal sacrifice
Greenblatt and Lijinsky (1974)	26 weeks	40	40	1000	Water	X		11–12 weeks delay before animal sacrifice
Borzsonyi et al. (1976)	?	42	40	500 ppm in pregnancy	Water	X		10 pregnant mice exposed, results for offspring kept on unspecified diet and unspecified time
Borzsonyi et al. (1978)	10 + months	0	19	500 ppm in pregnancy	Water	X		Short exposure of pregnant mice
Borzsonyi et al. (1978)	10 months	62	71	0	–	X		Exposure only *in utero*, offspring of above
Krishna Murthy et al. (1979)	120 days	20	20	5000	Diet	X		Short duration, small number of animals
Anderson et al. (1985)	Life	?	15–20	184, 1840	Water		X	Small number of animals, complicated design of pregnant animals and F1 generation

"Uncertain findings." Not only were these increased forestomach tumor incidences very weak as a function of dose (1/50, 0/50, 1/50, 5/50 in control, low, middle and high doses, respectively), but the forestomach is not considered to be an appropriate organ for cancer hazard assessment since humans do not even have this organ (Cohen and Arnold, 2011; Hoenerhoff et al., 2009).

The NTP peer review committee reached a unanimous decision to change the *Draft NTP Technical Report's* "equivocal evidence" of female rat mammary tumors to "no evidence" and to change "some evidence" of forestomach carcinogenicity in female mice to "equivocal evidence" in the final Technical Report. The discussion concerning the peer reviewers' changes from the preliminary conclusion of the draft report can be found on pages 13–14 of NTP Report TR495.

Around the time of the initiation of the nitrite bioassay study, NTP had changed its rodent diets to the "NTP-2000 Diet," and there were limited data to suggest that this new diet resulted in a slightly higher spontaneous tumor formation. For the mouse forestomach tumors, there were no tumors in control or lower-dose female mice, yet there were 5 in the 3000 ppm exposure group. In 2010, NTP published a review of the "NTP 2000 Diet" and spontaneous tumor incidence, which showed Squamous Cell Carcinoma or Papilloma Squamous in female mice averaged 1.85%, with a range of 0–6% in 26 studies representing 1298 animals, and male control mice showed slightly higher rates compared to females along with a greater variability from study to study (NTP, 2010b). The continued finding that control animals in these studies have a low and variable level of spontaneous female forestomach tumors also confirms the need for caution in interpreting the results of this target organ.

Since the NTP report was published in 2001, there have been 10 rat studies of nitrite in combination with other agents. They have all been conducted on small numbers of animals for short periods of time and have been focused on specific tumor sites including the forestomach (Hirose et al., 2002; Ichihara et al., 2005; Ishii et al., 2006; Kitamura et al., 2006a; Kitamura et al., 2006b; Kuroiwa et al., 2007; Kuroiwa et al., 2008a; Kuroiwa et al., 2008b; Miyauchi et al., 2002; Yada et al., 2002). The "nitrite only" exposed groups that served as controls in each of these studies have not shown nitrite to present any evidence of tumor formation (Table 3).

4. Epidemiologic studies of ingested nitrate and nitrite and stomach cancer

Numerous epidemiologic studies have been published that examined the potential relationship between nitrate, nitrite, and *N*-nitroso compounds and the risk of cancer. An even larger number of studies have investigated the association between intake of meat, red meat, or processed meat, and risk of cancer. Processed meats are not the primary source of nitrate or nitrite intake (Hord et al., 2009), although they are often inappropriately used as a proxy for dietary exposure. In fact, other foods, in particular vegetables, beer and cereals, can also be important sources of nitrate, nitrite and *N*-nitroso compounds. Furthermore, the concentration of nitrite in meat is highly variable (Buege et al., 2002; Hord et al., 2009; Keeton et al., 2009; Walker, 1990; Walters

Table 3
Summary of Animal Toxicology Studies Involving Nitrite Administration.

	Duration	# Males	# Females	Nitrite levels (ppm)	Vehicle	Not carcinogenic	Carcinogenic
A. Rat studies							
van Logten et al. (1972)	29 months	30	30	200, 5000	40% canned meat diet	X	
Lijinsky et al. (1973)	72 weeks	15	15	2000	Water	X	
Newberne (1979)	26 months	68	68	250, 500, 1000, 2000	Feed	X	
Lijinsky and Reuber (1980)	29 months	20	20	2000	Diet	X	
Maekawa et al. (1982)	2 years	50	50	1250, 2500	Water	X	
Grant and Butler (1989)	115 weeks	50	0	2000, 5000	14.6% prot diet	X	
National Toxicology Program (2001)	2 years	50	50	750, 1500, 3000	Water	X	
B. Mouse studies							
Inai et al. (1979)	109 weeks	50	50	1250, 2500, 5000	Water	X	
Rijhsinghani et al. (1982)	to 110 weeks	30	0	50 μg/g bw single dose	Gavage	X	
Hawkes et al. (1992)	Life	100,200	100,200	2000	Water	X	
Yoshida et al. (1993)	18 months	30	30	10 mg/kg bw/wk	Gavage	X	
National Toxicology Program (2001)	2 years	50	50	750, 1500, 3000	Water	X	

et al., 1979). For these reasons, we have not reviewed in detail the epidemiologic studies of processed meat intake and cancer risk (Cross et al., 2011).

For dietary nitrate and nitrite, the neoplasm which has been most frequently investigated is stomach cancer, and the IARC determination of "limited evidence" for the carcinogenicity in humans of nitrite in food was based on results for this cancer (International Agency for Research on Cancer, 2010). Therefore, we restrict our review of epidemiological studies to stomach cancer. Stomach cancer remains an important neoplasm on a global scale, representing the second most common cause of cancer death. Each year, an estimated 988,000 cases are diagnosed and 736,000 patients die from this disease (Ferlay et al., 2010). The incidence of this disease is higher in low- and medium-income countries than in high-income countries, and in the latter, the rate is higher in low-social class than in high-social class individuals. A striking feature of the epidemiology of stomach cancer is the steady decline in incidence that has taken place since the mid-20th century, when reliable data on cancer registration became available in most countries. The reasons for this trend are not precisely known, but may be attributed to: (i) decreased prevalence of chronic infection with *H. pylori*; (ii) reduced dietary salt intake; (iii) increased intake of fresh fruits and vegetables; and (iv) improvements in refrigeration and food storage in anaerobic packaging which minimizes lipid oxidation (Compare et al., 2010; Crew and Neugut, 2006; Helicobacter and Cancer Collaborative Group, 2001; Shibata and Parsonnett, 2006; Tsugane, 2005).

Established causes of stomach cancer include chronic infection with *H. pylori* (Helicobacter and Cancer Collaborative Group, 2001) and tobacco smoking (Shibata and Parsonnett, 2006). In addition to nitrate, nitrite, *N*-nitroso compounds (the subject of this review) and a related dietary factor, processed meat, a large number of other food groups, specific foods, and food components have been investigated as possible causes of stomach cancer. For none of them, however, is the evidence considered conclusive, but the strongest data pertain to salt and salted foods as risk factors, and fruits and vegetables, including allium vegetables, as protective factors (Compare et al., 2010; Crew and Neugut, 2006; Tsugane, 2005).

A MEDLINE literature search using the PubMed interface was conducted to identify relevant articles published through January, 2012. Unqualified keywords, searched as text words in the title, abstract, and full journal article, were used in a search string for a variety of stomach cancer terms (e.g., stomach or gastric cancer, stomach neoplasm, stomach carcinoma). The dietary search string terms included dietary nitrate and nitrite, including nitroso compounds. In addition, the bibliographies of the WCRF/AICR report on diet and cancer (World Cancer Research Fund (WCRF), 2007) review articles, and meta-analyses pertaining to intake of nitrate/nitrite and stomach cancer were examined in an effort to identify all available literature that may not have been identified by the database searches. We identified and reviewed ecologic, community-based, and case-control studies, although the primary focus of the current review was prospective cohort studies published in English language. We focused on prospective studies because of the inherent limitations in the other designs such as a lack of individual-level data in the ecologic studies and in case-control studies, the potential for recall bias and/or selection bias arising when the exposure distribution of the participating cases or controls is not representative of the population of eligible participants from which the study group was sampled. Studies were excluded that did not report data specifically for intake of nitrate/nitrite and stomach or gastric cancer (i.e., studies of the digestive tract, without specific anatomic identification, were excluded). To be included in the epidemiologic summary, studies were required to report point estimates (i.e., relative risks) and measures of variability (i.e., 95% confidence intervals) for the association between nitrate or nitrite intake and stomach cancer. As indicated previously, the scope of this summary of epidemiologic studies is stomach cancer. Although several cohort studies of nitrate/nitrite and other neoplasms have been published, the majority of evidence involves stomach cancer. Indeed, the evidence of carcinogenicity was reviewed by IARC in 2006 largely in regards to stomach cancer.

Qualitative information and quantitative data were extracted from each study that met the criteria for inclusion. Specifically, information was extracted pertaining to the following: (1) the year of the study, (2) the name and nature of the cohort, (3) geographic location of the study, (4) methods of dietary exposure ascertainment, (5) nitrate and nitrite exposure levels, (6) the number of exposed cases per intake strata, (7) the relative risk estimate and 95% confidence interval for each gender where applicable, and (8) the factors that were adjusted or controlled for in the analysis.

4.1. Summary of epidemiologic studies of intake of nitrate, nitrite, and N-nitroso compounds and stomach cancer

The epidemiologic studies of dietary intake of nitrate/nitrite and stomach cancer generally vary by three overarching factors: (1) study design, i.e., ecologic, case-control and cohort; (2) agent under consideration, i.e., nitrate, nitrite or *N*-nitroso compounds; (3) exposure assessment, i.e., estimates from well water, salivary and urinary measurements, food composition databases and estimates from meat intake based on food frequency questionnaires. In addition, studies vary considerably by population, geographic location, cancer endpoint (incidence or mortality), tumor site (cardia or non-cardia), degree of adjustment for potential confounding factors and methodological quality.

The majority of published epidemiologic data comes from ecologic studies of ingested nitrate and nitrite, however, inference from ecologic studies is limited because of the complex nature of intra-individual variation in endogenous nitrosation, a lack of pertinent exposure and outcome information at the individual level and the potential for variation in other dietary, lifestyle and economic confounding or modifying factors between study subjects due to analyses at the population level. Ecologic studies, if properly conducted and interpreted, may be useful to generate new hypotheses. However, they cannot provide the evidence needed for causal inference. In this specific case, ecologic studies are limited by the nature of the study design, heterogeneity across population groups, lack of individual quantitative exposure estimates, and in the case of nitrate biomarker studies, the fact that recent excretion of nitrate or salivary levels of nitrate does not reflect past dietary exposures (International Agency for Research on Cancer, 2010; Kelm, 1999; Mian et al., 2011). In acknowledgement of these limitations, data from the ecologic studies are inconsistent, with associations observed above and below the null value for both nitrate and nitrite exposure and significant and non-significant correlations for stomach cancer incidence and mortality rates across countries (Barrett et al., 1998; Knight et al., 1990; Lin et al., 2003; Sandor et al., 2001; Zhang et al., 1984).

A number of case-control studies have been conducted on ingested nitrate and nitrite and stomach cancer. Most of these studies estimated exposure based on study subjects' dietary recall on food frequency questionnaires. Collectively, associations tend to differ between nitrate and nitrite exposure among these studies. Statistically significant inverse associations comparing high nitrate exposure to low exposure have been reported in several case-control studies (Hansson et al., 1994; La Vecchia et al., 1994; Palli et al., 2001; Risch et al., 1985; Rogers et al., 1995), and significant inverse trends have been observed as well (Hansson et al., 1994; La Vecchia et al., 1994; La Vecchia et al., 1997; Palli et al., 2001). In contrast, positive associations have been reported in most case-control studies of nitrite exposure and stomach cancer (Buiatti et al., 1990; La Vecchia et al., 1994; La Vecchia et al., 1997), with the strongest associations observed among persons with high nitrite exposure and low vitamin C intake (Engel et al., 2003; Mayne et al., 2001; Rogers et al., 1995). Interestingly, Mayne et al. (2001) observed a significant positive association for nitrite exposure among persons with non-cardia gastric tumors only.

The discordance in associations for nitrate and nitrite exposure may be the result of the underlying dietary habits of the study participants, since exposure is estimated based on dietary recall of specific foods. Thus, if individuals who have nutritionally balanced diets (compared to those with unbalanced diets) are more likely to be categorized as having higher nitrate levels, and if individuals with poor diets are more likely to be classified as having higher nitrite exposures, then the associations between nitrate and nitrite exposure and stomach cancer would likely be confounded or modified by other dietary factors or correlates of diet. In a recent publication of dietary intake of nitrate and nitrite and gastric cancer among residents of Mexico City, inverse associations were reported for the highest categories of both nitrate and nitrite derived from fruits and vegetables, while significant positive associations were observed for the highest categories of nitrate and nitrite derived from animal sources (Hernandez-Ramirez et al., 2009). In a case-control study conducted in Nebraska (Ward et al., 2008), dietary nitrate/nitrite (combined) from animal sources was associated positively, but non-significantly, with distal stomach cancer. In the same study, dietary nitrate, but not nitrite, from plant sources was associated positively with distal stomach cancer.

The most informative epidemiologic results come from the cohort studies (Table 4) which are less prone to bias in evaluating environmental and dietary factors than ecologic and case-control studies. These studies are reviewed in more detail below by chronology of year published.

In a cohort study of gastric cancer incidence among 11,907 Japanese residents of Hawaii, a non-significant inverse association was reported for eight or more servings of nitrate-containing foods (i.e., combined frequency of intake of processed meats, dried fish and pickled vegetables) per week and gastric cancer among men and women (RR = 0.90, 95% CI: 0.5–1.4) based on an average follow-up period of 14.8 years (Galanis et al., 1998). Although the food frequency questionnaire was relatively short, the authors suggested that the findings of this study may be generalized to the Japanese residents of Hawaii because study participants were randomly selected from the general population and the participation rate was high.

In the Netherlands Cohort Study, a prospective cohort of 120,852 men and women, van Loon et al. (1998) evaluated nitrate and nitrite exposure (based on food intake and drinking water) and stomach cancer. Nitrate intake was estimated from a food databank and residential drinking water data. Nitrite intake was assessed solely from the intake of cured meat and was based on food composition values from the TNO Nutrition and Food Research Institute. Non-significant inverse associations for stomach cancer were reported in the third, fourth and fifth quintiles for total nitrate, nitrate from drinking water and nitrate from food. A non-significant elevation in stomach cancer was observed among persons in the highest quintile of nitrite consumption relative to the lowest (RR = 1.44; 95% CI: 0.95–2.18), but no monotonic trend was apparent ($P = 0.24$).

In a prospective follow-up study, men and women enrolled in a multiphasic screening examination cohort in Finland, inverse associations for the highest categories of nitrate (RR = 0.56, 95% CI: 0.27–1.18), nitrite (RR = 0.71, 95% CI: 0.28–1.78) and *N*-nitroso compounds (RR = 0.75, 95% CI: 0.37–1.51) were reported for stomach cancer (Knekt et al., 1999). Exposure to nitrates was derived primarily from vegetables, whereas nitrites were derived mainly from cured meats and sausages, and dietary nitrosamines were derived largely by smoked and salted fish, and cured meats and sausages (Knekt et al., 1999). The duration of participant follow-up in this study was extensive, with a maximum period of 24 years.

In a follow-up study of women enrolled in the Swedish Mammography Cohort, Larsson et al. (2006a) evaluated processed meat consumption and dietary nitrosamines (i.e., nitrosodimethylamine (NDMA)) and stomach cancer. The average frequency of consumption of each food item was multiplied by the NDMA content of age-specific portion sizes to estimate NDMA exposure. Foods included in the calculation of NDMA estimates were specific processed meat products (bacon, side pork, sausages, and ham), smoked fish, caviar and roe, alcoholic beverages and chocolate. The authors reported an approximate 2-fold elevated risk of stomach cancer (HR = 1.96; 95% CI: 1.08–3.58) among women in the fifth quintile of NDMA intake compared to the lowest quintile. There were no significant differences or interactions in associations for NDMA by vitamin C intake or by fruit and vegetable intake. Of note, this study did not specifically evaluate exposure to nitrate or nitrite, and thus, should not be included in the review of evidence specific to nitrate/nitrite and stomach cancer.

The risk of gastric cancer associated with dietary intake of NDMA and endogenous formation of *N*-nitroso compounds (NOCs) was examined in the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort (Jakszyn et al., 2006). This cohort included over 500,000 individuals, and after six years of follow-up, 314 incident cases of gastric cancer were observed. Dietary intake of nitrites and NDMA was estimated by matching food items on country-specific questionnaires with a food database of potential carcinogens, and endogenous NOC exposure was estimated using data on iron intake from meat and fecal ATNC [apparent total

Table 4
Cohort studies of nitrates, nitrites, and *N*-nitroso compounds and stomach cancer.

Author and year	Study location	Exposure ascertainment	Analytical category	Definition (if applicable)	Number of exposed cases	Analytical comparison	Relative risk estimate	95% CI	*P* trend	Continuous data	Statistical adjustment
Cohort studies											
Galanis et al. (1998)	Hawaii (Japanese-American residents of Hawaii)	Short food frequency questionnaire	Nitrate	Combined frequency of intake of dried fish, pickled vegetables and processed meats	Women						
						# of times/week					Age, years of education, Japanese place of birth, gender
					13	0–3 times/week	1.00	Reference	0.62		
					17	4–7 times/week	2.00	1.00–4.00			
					14	8 or more times/week	1.30	0.60–2.8			
					Men						
					26	0–3 times/week	1.00	Reference	0.18		Age, years of education, Japanese place of birth, gender, cigarette smoking, alcohol intake status
					21	4–7 times/week	0.90	0.50–1.60			
					17	8 or more times/week	0.70	0.40–1.20			
van Loon et al., 1998	Netherlands (Netherlands Cohort Study)	FFQ (150 food item)	Nitrate	From drinking-water and foods	Total nitrate						Age, sex, smoking, education, coffee consumption, intake of vitamin C & beta-carotene, family hx of stomach cancer, prevalence of stomach disorders, use of refrigerator or freezer
						Quintiles of intake (mean, mg/day					
					63	I (59.8)	1.00	Reference	0.3		
					67	II (84.7)	1.25	0.84–1.86			
					42	III (104.4)	0.74	0.47–1.15			
					54	IV (127.3)	0.92	0.59–1.44			
					56	V (179.8)	0.90	0.53–1.55			
					Nitrate from drinking-water						
					61	Q1 (0.02)	1.00	Reference	0.39		
					54	Q2 (1.65)	0.93	0.62–1.39			
					53	Q3 (3.85)	0.87	0.51–1.31			
					57	Q4 (6.91)	0.83	0.55–1.24			
					57	Q5 (16.5)	0.88	0.59–1.32			
					Nitrate from foods						
					69	Q1 (55.8)	1.00	Reference	0.18		
					61	Q2 (79.4)	1.02	0.69–1.51			
					45	Q3 (98.7)	0.71	0.46–1.09			
					49	Q4 (120.7)	0.80	0.51–1.25			
					58	Q5 (172.2)	0.80	0.47–1.37			
			Nitrite	Solely from cured meat intake	Total nitrite						
					47	I (0.01)	1.00	Reference	0.24		
					51	II (0.04)	1.20	0.78–1.86			
					58	III (0.09)	1.18	0.77–1.82			
					46	IV (0.16)	0.88	0.56–1.37			

					80	V (0.35)	1.44	0.95–2.18			
Knekt et al., 1999	Finland	Dietary history interview				Quartiles of consumption					Age, sex, municipality, smoking and energy intake
			Nitrate	91.9% provided by vegetables	NR	Q1	1.00	Reference	0.09		
					NR	Q2	1.01	0.56–1.84			
					NR	Q3	0.52	0.25–1.08			
					NR	Q4	0.56	0.27–1.18			
			Nitrite	94.2% derived from cured meats and sausages	NR	Q1	1.00	Reference	0.9		
					NR	Q2	1.10	0.58–2.11			
					NR	Q3	1.88	1.01–3.49			
					NR	Q4	0.71	0.28–1.78			
			NDMA	51.9% derived from smoked or salted fish. 48.1% derived from cured meats and sausages	NR	Q1	1.00	Reference	0.39		
					NR	Q2	1.03	0.55–1.95			
					NR	Q3	0.78	0.39–1.56			
					NR	Q4	0.75	0.37–1.51			
Jakszyn et al. (2006)	European Prospective Investigation into Cancer and Nutrition (EPIC-EURGAST)	Usual diet over previous 12 months measured by country specific validated questionnaires	NDMA	Matching food items on questionnaires with a food database of potential carcinogens (<1 μg on average)	Stomach (Cutpoints for tertiles: men (0.12 and 0.28), women (0.06 and 0.11))						Sex, height, weight, education level, tobacco smoke, cigarette smoking intensity, work and leisure activity, citrus and non-citrus fruits intake, vegetables intake, energy, intake and nitrites
						Tertiles of intake					
					314	2	0.87	0.64–1.20	0.96	1.00 (0.70–1.43)	
					314	3	0.99	0.69–1.41			
					Cardia (Cutpoints for tertiles: men (0.12 and 0.28), women (0.06 and 0.11))						
					92	2	0.74	0.41–1.34	0.29	0.73 (0.30–1.79)	
					92	3	0.68	0.34–1.37			
					Non-Cardia (Cutpoints for tertiles: men (0.12 and 0.28), women (0.06 and 0.11))						
					155	2	1.04	0.66–1.63	0.75	1.09 (0.69–1.73)	
					155	3	1.09	0.65–1.81			
			ENOC	Estimated using data of iron content from meat intake and fecal apparent total NOC formation according to previous studies	Stomach (Cutpoints for tertiles: men (78 and 106), women (65 and 87))						
					314	2	1.12	0.83–1.51	0.10	1.18 (0.99–1.39)	
					314	3	1.32	0.94–1.84			
					Cardia (Cutpoints for tertiles: men (78 and 106), women (65 and 87))						
					92	2	1.43	0.81–2.52	0.50	0.96 (0.69–1.33)	
					92	3	1.29	0.67–2.47			
					Non-Cardia (Cutpoints for tertiles: men (78 and 106), women (65 and 87))						
					155	2	1.22	0.79–1.88	0.04	1.42 (1.14–1.78)	
					155	3	1.61	1.01–2.58			
					Stratified by *H. pylori* infection						Sex, age, center and date of blood draw, height, weight, education, tobacco, cigarette intensity, work&leisure activity, alcohol, energy, nitrites, citrus and non-citrus fruits and vegetable intake, *H. pylori* intake
					111	Infected		*P* for interaction		1.82 (1.32–2.51)	
					12	Non-infected		0.09		0.15 (0.01–4.06)	

(*continued on next page*)

Table 4 (*continued*)

Author and year	Study location	Exposure ascertainment	Analytical category	Definition (if applicable)	Number of exposed cases	Analytical comparison	Relative risk estimate	95% CI	*P* trend	Continuous data	Statistical adjustment
					Stratified by plasma vitamin C						
					54	<40 μmol		*P* for interaction		3.24 (1.77–5.93)	
					55	>40 μmol		0.02		1.10 (0.63–1.93)	
Larsson et al. (2006a)	Swedish Mammography Cohort (18 years of follow-up)	67-item food frequency questionnaire (FFQ)				Quintile of NDMA intake (μg/day)					Age (mos), education (<HS, HS grad or more than HS), BMI (<23.0, 23.0–24.9, 25.0–29.9 or ⩾30) and intakes of total energy (continuous), alcohol (quartiles), fruits (quartiles), vegetables (quartiles)
			NDMA[a]	Calculated intake by multiplying average frequency of consumption of each food item by NDMA content of age-specific portion sizes	28	1 (0.017)	1.00	Reference	0.02		
					28	2 (0.061)	1.03	0.61–1.77			
					37	3 (0.098)	1.66	1.00–2.75			
					32	4 (0.151)	1.60	0.93–2.76			
					31	5 (0.277)	1.96	1.08–3.58			
Cross et al. (2011)	United States (NIH-AARP Diet and Health Study)	124-item FFQ									Age, sex, BMI (<18.5, ⩾18.5 to <25, ⩾25 to <30, ⩾30 to <35, ⩾35 kg m^2 and unknown), ethnicity (non-Hispanic white, non-Hispanic black, Hispanic, other, unknown), education (⩽11yrs, 12yrs or completed HS, post-HS/some college, college grad, post-graduate, unknown), tobacco smoking (never, quit smoking ⩽20 cigs/day, quit smoking >20 cigs/day, current smoker ⩽20 cigs/day, current smoker >20 cigs/day, unknown), alcohol drinking (none, >0 to 1, >1 to 3, >3 drinks/day, unknown), usual physical activity at work (all day sitting, mostly sitting, walking around a lot, lifting/carrying light loads/climbing stairs, heavy work/carrying heavy loads, unknown), vigorous physical activity (never, rarely, 1–3 times/mo, 1–2 times/wk, 3–4 times/wk, ⩾5 times/wk, unknown), daily intake of fruit, vegetables, saturated fat, and calories
			Nitrate	Estimated intake from meats using a database of measured values from 10 types of processed meats, represent 90% of meats consumed in United States	Gastric Cardia						
						Quintiles of intake (median μg per 1000 kcals)					
					39	Q1 (24.2)	1.00	Reference	0.259	0.99 (0.90–1.09)	
					57	Q2 (66.9)	1.17	0.77–1.77			
					36	Q3 (112.7)	0.64	0.40–1.02			
					61	Q4 (174.5)	0.94	0.61–1.45			
					62	Q5 (298.0)	0.81	0.52–1.25			
					Gastric Non-Cardia						
					50	Q1 (24.2)	1.00	Reference	0.578	1.01 (0.92–1.10)	
					48	Q2 (66.9)	0.90	0.60–1.35			
					50	Q3 (112.7)	0.89	0.59–1.33			
					56	Q4 (174.5)	0.91	0.61–1.37			
					73	Q5 (298.0)	1.04	0.69–1.55			
			Nitrite		Gastric Cardia						
						Quintiles of intake (median μg per 1000 kcals)					
					44	Q1 (12.1)	1.00	Reference	0.25	0.89 (0.77–1.03)	
					40	Q2 (34.6)	0.72	0.47–1.11			
					55	Q3 (61.4)	0.88	0.58–1.32			
					61	Q4 (102.9)	0.87	0.58–1.31			
					55	Q5 (199.2	0.71	0.47–1.08			
					Gastric Non-Cardia						
					54	Q1 (12.1)	1.00	Reference	0.615	1.02 (0.91–1.15)	
					44	Q2 (34.6)	0.77	0.51–1.15			

					48	Q3 (61.4)	0.79	0.53–1.18		
					67	Q4 (102.9)	1.04	0.71–1.52		
					64	Q5 (199.2	0.93	0.63–1.37		
Loh et al. (2011)	European Prospective Investigation into Cancer and Nutrition (EPIC)–Norfolk Study (23,363 men and women)	Baseline diet assessed by food frequency questionnaires				Quartile of intake (ng/d)				Age, sex, BMI, cigarette smoking status, alcohol intake, energy intake, physical activity status, education level, menopausal status (in women)
			NDMA[b]	Estimated by matching FFQ food items with food database of potential carcinogens	NR	Q1 (16.8)				
					NR	Q2 (33.4)				
					NR	Q3 (53.1)				
					NR	Q4 (125.9)				
					64**	Stomach cancer	1.13	0.81–1.57	*P*-value: 0.47	
					532*	Combined cases of GI cancer	1.13	1.00–1.28	*P*-value: 0.04	
						Quartile of intake (μg/d)				
			ENOC	Estimated iron content from meat intake and fecal ATNC formation from several human controlled-diet studies	NR	Q1 (64.4)				
					NR	Q2 (71.0)				
					NR	Q3 (76.0)				
					NR	Q4 (78.3)				
					64**	Stomach cancer	1.13	0.88–1.45	*P*-value: 0.34	
					532*	Combined cases of GI cancer	1.02	0.93–1.12	*P*-value: 0.66	
						Quartiles of intake (mg/d)				
			Nitrite[b]	Estimated by matching FFQ Food items with food database of potential carcinogens	NR	Q1 (1.17)				
					NR	Q2 (1.41)				
					NR	Q3 (1.63)				
					NR	Q4 (1.69)				
					64**	Stomach cancer	0.86	0.63–1.19	*P*-value: 0.37	
					532*	Combined cases of GI cancer	0.99	0.89–1.10	*P*-value: 0.83	

* Total number of gastrointestinal cancer cases ($n = 22\,920$).

** Cases of stomach cancer ($n = 22{,}920$).

[a] Foods included in calculations of NDMA intake: specific processed meat products (bacon, side pork, sausages, ham), smoked fish, caviar and roe, alcoholic beverages (light beer, medium-strong beer, strong beer, whiskey) and chocolate.

[b] Data obtained from EPIC-EURGAST study. Data on consumption/intake compiled by conducting a literature search (1980–2003) to identify sources of data on concentrations of nitrosamines in food. Country specific values chosen when available.

NOCs] formation from published studies. The authors reported a null association between NDMA intake and gastric cancer (HR = 1.00, 95% CI: 0.7–1.43). Endogenous NOCs were significantly associated with non-cardia tumors (HR = 1.42, 95% CI: 1.14–1.78), based on an increase of 40 mg/day, but no association with cardia tumors was observed (HR = 0.96, 95% CI: 0.69–1.33). In separate analyses of endogenous NOCs among persons with non-cardia tumors, associations were strongest among persons with *H. pylori* infections and with the lowest levels of plasma vitamin C. In a recent analysis of data from the EPIC-Norfolk, United Kingdom, cohort, Loh et al. (2011) evaluated exposure to *N*-nitroso compounds and total cancer as well as cancer at specific sites, including the stomach. Associations were close to the null value and not statistically significant for NDMA, endogenous NOC and nitrite and total cancer. For stomach cancer, the hazard ratios (per standard deviation) were 1.13 (95% CI: 0.81–1.57), 1.13 (95% CI: 0.88–1.45), and 0.86 (95% CI: 0.63–1.19) for NDMA, endogenous NOC and nitrite, respectively.

Recently, Cross et al. (2011) published a prospective cohort study of meat intake, including nitrate and nitrite, and gastric (i.e., cardia and non-cardia) stomach cancer. This was the largest epidemiologic analysis of nitrate and nitrite and stomach cancer among a US population, and included approximately 500,000 men and women, aged 50–71 years, from six states who were enrolled in the NIH-AARP Diet and Health Study. Dietary information was ascertained at baseline via a 124-item food frequency questionnaire. Nitrate and nitrite exposure were estimated based on the questionnaire responses for processed meat consumption using a database of measured values of 10 types of processed meat. Nitrate (Q5 vs. Q1) was not associated with gastric cardia (HR = 0.81, 95% CI: 0.52–1.25) or gastric non-cardia (HR = 1.04, 95% CI: 0.69–1.55) tumors. Similarly, no significant associations were observed for nitrite exposure, as seven of eight hazard ratios were in the inverse direction across the quintiles of exposure (Q5 vs. Q1, cardia tumors HR = 0.71, 95% CI: 0.47–1.08; non-cardia tumors HR = 0.93, 95% CI: 0.63–1.37). The authors concluded that neither nitrate nor nitrite was associated with gastric cancer.

4.2. Methodological considerations in the epidemiology of stomach cancer

In epidemiologic studies of dietary factors and cancer, particularly studies related to meat intake or correlates of meat intake, such as nitrite exposure derived from processed meats, there are several inherent methodological and analytical considerations. Correlates of dietary intake, such as nitrate, nitrite or *N*-nitroso compounds are not ascertained on FFQs and may not be included in food composition databases because individuals are not able to estimate intake of these compounds, and their levels in foods vary according to multiple factors. Thus, methods of estimating exposure levels are typically based on indirect measurement, which may produce further measurement errors. In addition, exposure to nitrate and/or nitrite is not specific to intake of a certain food, such as processed meat, although many epidemiologic studies estimate exposure to these compounds based on type and frequency of meat intake. In fact, exposure to nitrate and nitrite occurs more frequently through consumption of vegetables and baked and processed cereal products, and most exposure to nitrite occurs endogenously when ingested nitrate is excreted in the saliva and reduced to nitrite by oral bacteria, which is then re-ingested (Dich et al., 1996; Grosse et al., 2006; Honikel, 2008; International Agency for Research on Cancer, 2010). Similarly, exogenous exposure to *N*-nitroso compounds is not specific to processed meat, as they may be found in other dietary sources, such as certain cheeses or malted alcoholic beverages and some distilled whiskey (Alexander et al., 2010; Lijinsky, 1999; Santarelli et al., 2008) as well as from lifestyle choices that include tobacco smoking.

Nitrite exposure estimation is commonly based on frequency of consumption of processed meat from dietary questionnaires. Although the most commonly utilized tools for dietary ascertainment in epidemiologic studies, survey instruments (such as dietary habit assessments and FFQs) are a potential source of both random error and measurement error during the data collection process. In retrospective studies, these errors may be non-differential with respect to case-control status, leading to bias in an unpredictable direction. FFQs may suffer from deficiencies in validity of food intake information, since it is known that past dietary intake is a difficult exposure for study participants to estimate accurately. Compounding the potential limitations in food estimation accuracy is the fact that nitrite (and nitrate, although nitrate is also estimated by drinking water levels) exposure is subsequently estimated from FFQ recall of processed meat intake. Factors involved in metabolic recycling of nitrate result in wide ranges of nitrite ingestion with a potential but unknown covariance with confounding metabolic conditions. Thus, there are many crucial elements of nitrite exposure estimation that may result in important measurement errors. Nitrate and nitrite content of raw foods vary considerably based on cultivar, season and agricultural practices, while in cured processed meats the levels ingested are the result of many factors including: regulations in different countries which have changed significantly over the past 30 years; use of ascorbate/erythorbate which has increased over that time span; time and storage temperature after manufacturing and before ingestion, during which nitrite is depleted (Cassens, 1997; Honikel, 2008; Izumi et al., 1989; Walker, 1990).

The causal nature of the association between chronic *H. pylori* infection, a ubiquitous bacterial infection affecting the stomach, and gastric cancer has been well established. Several prospective and case-control studies have shown significant associations between *H. pylori* seropositivity and gastric cancer risk (RRs ranging from 2.1 to 16.7), and associations may vary by tumor site (Crew and Neugut, 2006). In a meta-analysis of 12 prospective studies, *H. pylori* infection was associated with risk of gastric non-cardia cancers but not with cardia tumors (Helicobacter and Cancer Collaborative Group, 2001). However, only one of the epidemiologic studies on nitrate, nitrite or *N*-nitroso compounds accounted for the potential effect modifying or confounding impact of *H. pylori* infection status among non-cardia tumors only (Jakszyn et al., 2006). Among subjects infected with *H. pylori*, ENOC (endogenous nitroso compounds) was associated with non-cardia cancer (OR = 1.82; 95% CI: 1.32–2.51 for categorical trends), while in non-infected subjects, a non-significant inverse association was observed 0.15 (0.01–4.06) (Jakszyn et al., 2006). The authors also stratified by plasma vitamin C level, and found a statistically significant positive association between ENOC and non-cardia cancer among those with low serum levels of vitamin C only (OR = 3.24; 95% CI: 1.77–5.93). Others have investigated antioxidants and their interactions with the etiology of stomach cancer (Jenab et al., 2006a; Jenab et al., 2006b; Jenab et al., 2006c; Zhang and Farthing, 2005). Salt and salt-preserved foods may increase the risk of *H. pylori* infection and act synergistically to promote gastric cancer (Crew and Neugut, 2006). Several case-control studies have shown that independent associations between high levels of dietary salt or sodium and risk of gastric cancer have ranged between 1.6 and 6.2 (Tsugane, 2005). Thus, it is essential that studies of nitrate/nitrite and stomach cancer account for the likely confounding and modifying impact of *H. pylori* infection, vitamin C and salt intake.

Because of the important role that the stomach plays in food digestion, a wide variety of dietary factors that may be associated with increasing the risk of stomach cancer have been analyzed in

hundreds of epidemiologic studies over the past few decades. Despite volumes of epidemiologic literature on dietary and nutritional factors and stomach cancer, no food group, specific food or nutrient, aside from the positive associations with salt intake, has been conclusively established as increasing or decreasing the risk of this malignancy (Compare et al., 2010; Crew and Neugut, 2006; Tsugane, 2005). Collectively, the epidemiology involving nitrate, nitrite, *N*-nitroso compound or processed meat intake and stomach cancer is no different – associations between studies are inconsistent, there is considerable variation across studies regarding the study designs, populations and methodology, and the two largest cohort studies of higher quality indicate no association. Furthermore, interpretation is complicated due to the inter-correlation of dietary, lifestyle, and socio-economic factors both within and between study populations. Although relatively few studies have stratified analyses by *H. pylori* status, vitamin C level, salt intake or by cardia vs. non-cardia gastric tumors, patterns of associations for nitrate, nitrite and *N*-nitroso compounds appear to be stronger among *H. pylori* infected individuals, persons with nutritionally unbalanced diets or low vitamin C levels and for gastric non-cardia tumors. These potential patterns, particularly for *H. pylori* and low vitamin C, may be attributable to confounding by other dietary or lifestyle factors. Future studies should focus on these important factors.

In summary, the available epidemiologic evidence, and in particular the results of the large prospective studies reported after IARC's review and evaluation in 2006, do not support the hypothesis of an association between ingestion of nitrate or nitrite, and resulting endogenous nitrosation, and stomach cancer. The fact that the results of methodologically weaker studies appear to support an association, which is not confirmed in the most rigorous and informative studies (in particular those of cohort design), strongly points towards bias and confounding as explanations for the former and towards the conclusion of lack of a causal association for stomach cancer. Based on this comprehensive review, the currently available epidemiologic evidence does not support an independent association between nitrate, nitrite or *N*-nitroso compound exposure and stomach cancer. This conclusion is supported by the fact that associations across the cohort studies are generally weak in magnitude, have relative risks above and below the null value with most associations being non-significant, show no consistent evidence of a dose–response relationship and show no associations (with some in the inverse direction) observed in two recently published large prospective studies (Cross et al., 2011; Loh et al., 2011).

5. Conclusions

New information has clearly established that nitrite and nitrate per se are important biological compounds and that nitrosation is an important feature of NO metabolism in human physiology including many nitrosation reactions. *S*-nitrosation may be particularly important to the physiological effects of NO and nitrite. Carcinogenic *N*-nitrosation requires conditions beyond those usually found in normal metabolism. These extraordinary conditions were the focus of concern for exposure of populations to nitrate and nitrite before their role in overall nitrogen oxide metabolism became better understood.

The toxicological and epidemiological evidence on carcinogenicity of nitrate and nitrite share the feature of including studies of variable quality and ability to directly answer questions regarding carcinogenicity. Failure to take into account the strengths and limitations of different groups of studies may lead to conclusions, as exemplified by the 2006 evaluation and classification performed by IARC, which based on additional subsequent studies are no longer supportable. Both in epidemiology and animal carcinogenicity, results of studies of low quality tended to support the hypothesis of a carcinogenic effect of nitrate or nitrite intake, while the results of better designed and conducted studies did not. The evidence from animal studies essentially relies on the NTP bioassay study, which provided only "equivocal evidence" for the carcinogenicity of sodium nitrite in the forestomach of female mice, and "no evidence" of carcinogenicity in any other organs of both sexes of mice and rats. This has been supported by ten subsequent smaller studies where no carcinogenicity in nitrite-only exposed animals was observed.

The results of prospective epidemiologic studies, in particular those of cohort studies reported since 2006, do not consistently suggest an increased risk of stomach cancer from ingested nitrate, nitrite or *N*-nitrosamines. Future epidemiologic studies should account for the likely confounding or effect modifying impact of *H. pylori* infection, Vitamin C and salt intake. In addition, associations between nitrate and nitrite and stomach cancer should be stratified by cardia and non-cardia tumors. Overall, the hypothesis of a risk of cancer in humans from ingested nitrate, nitrite and *N*-nitrosamines, which was proposed on the basis of low-quality studies conducted several decades ago, has not been confirmed in more recent, better-designed animal and epidemiological studies.

The current scientific evidence indicates that usual dietary exposure and endogenous formation of nitrate and nitrite do not entail an increased risk of stomach cancer.

Conflict of Interest

N.S. Bryan and The University of Texas Health Science Centre at Houston have financial interests in Neogenis, a company that develops, produces, and sells nitric-oxide-related products intended to improve health, develops diagnostics for nitric oxide-related metabolites, and performs commercial measurement of nitiric oxide metabolites in biological samples.

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