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Review

Nutritional epidemiology in the context of nitric oxide biology: A risk–benefit evaluation for dietary nitrite and nitrate

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ABSTRACT

The discovery of the nitric oxide (NO) pathway in the 1980s represented a critical advance in understanding cardiovascular disease, and today a number of human diseases are characterized by NO insufficiency. In the interim, recent biomedical research has demonstrated that NO can be modulated by the diet independent of its enzymatic synthesis from L-arginine, e.g., the consumption of nitrite- and nitrate-rich foods such as fruits, leafy vegetables, and cured meats along with antioxidants. Regular intake of nitrate-containing food such as green leafy vegetables may ensure that blood and tissue levels of nitrite and NO pools are maintained at a level sufficient to compensate for any disturbances in endogenous NO synthesis. However, some in the public perceive that dietary sources of nitrite and nitrate are harmful, and some epidemiological studies reveal a weak association between foods that contain nitrite and nitrate, namely cured and processed meats, and cancer. This paradigm needs revisiting in the face of undisputed health benefits of nitrite- and nitrate-enriched diets. This review will address and interpret the epidemiological data and discuss the risk–benefit evaluation of dietary nitrite and nitrate in the context of nitric oxide biology. The weak and inconclusive data on the cancer risk of nitrite, nitrate and processed meats are far outweighed by the health benefits of restoring NO homeostasis via dietary nitrite and nitrate. This risk/benefit balance should be a strong consideration before there are any suggestions for new regulatory or public health guidelines for dietary nitrite and nitrate exposures.

Introduction

Today, we are bombarded with media reports of studies relating diet to a number of chronic diseases, including coronary heart disease, cancer, type 2 diabetes and osteoporosis. For the past several decades, observational studies of diet and cancer have yielded many inconsistent results [1,2]. Given the limited variation in dietary intakes within many study populations and the seemingly weak diet–cancer associations that have been observed, results of such studies depend critically on an accurate assessment of dietary exposure [3,4]. Measurement error in exposure can lead to serious errors in the reported relative risks of cancer for dietary intakes and can substantially reduce the statistical power to detect true existing relationships [3,4]. Extreme caution is required when interpreting associations, or the lack thereof, between dietary factors and disease.

Overall, the media does a fairly good job of reporting the limitations of the studies and the fact that the published results are merely expressions of risk probability. However, a recent, prominent *Perspective* article in the New England Journal of Medicine [5] noted that all too frequently, what is conveyed about health and disease by many journalists is wrong or misleading, especially when they ignore complexities or fail to provide context. When this happens, the public health messages conveyed are inevitably distorted or inadequate. Therefore, the news media need to become more knowledgeable and to more fully embrace their role in delivering accurate, complete and balanced messages about health.

This is especially needed when the results being communicated to the public are diet-health associations, due mainly to the complexity of the diet compared to a drug/placebo clinical trial. There has been a dramatically increased interest in nutrition and health over the past decade. What we eat or don't eat is constantly being linked to various diseases, and there is a constant flow of anxiety-provoking media headlines on television, radio, print and more recently the Internet. "Carcinogen-of-the-month" reporting has become very alarming to consumers, and dietary epidemiological

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studies always seem to be contradicting each other, leading to much nutrition nonsense and food faddism. Because of these fears, consumers become sporadic or chronic avoiders of specific foods and ingredients, such as salt, fat, soft drinks, artificial sweeteners, carbohydrates in general, coffee/caffeine and meat products.

In a recent issue of Archives of Internal Medicine, Sinha et al. [6] reported in a large prospective study that red and processed meat intakes were associated with modest increases in total mortality, cancer mortality, and cardiovascular disease mortality. Like many other studies, it failed to completely consider several additional factors that can contribute to chronic disease, including participants' behavior as to alcohol and tobacco use, exercise, weight and access to health care. It also failed to recognize the role of beef, pork and other red meat in providing essential and under-consumed nutrients. In response to such reporting deficiencies, many individuals become either confused or alarmed about their own personal situation. There are also calls by numerous public health and consumer organizations to change our lifestyles as a matter of public policy. Therefore, it is important for participants in the social debate to also understand the strengths and limits of epidemiological research. We will review the science of epidemiology, introduce the criteria for interpretation of the data and then discuss some published reports on diet and risk of disease. Since foods are heterogenous and complex in terms of their composition and contribution to the overall diet, ascribing an individual component of food as "good" or "bad" is fraught with difficulty. We will present a cursory review of current epidemiological data, but we will also focus primarily on the context of nitrite and nitrate in foods and what is reported about them, in order to present a balanced view of dietary sources of essential nutrients and a potential risk–benefit evaluation.

Epidemiology as a scientific discipline

Modern epidemiology is the branch of medicine that deals with the study of the causes, distribution and control of disease frequency in human populations. Historically, epidemiology began as the study of epidemics of infectious disease. Epidemiology essentially looks for patterns of disease (time, place, exposures, personal characteristics). Nothing affects our health more than what we choose to eat. Many studies relate the association between processed meats and cancer to their nitrite and nitrate contents. The primary concern for exposure to dietary nitrite (and nitrate as a precursor to nitrite) is its propensity to form potentially carcinogenic *N*-nitrosamines and their consequent potential to cause human cancer [7,8]. In terms of human cancer risk and diet, most of the publications, discussion and media attention have focused on data from epidemiological studies. Therefore, it is important to understand the different types of dietary epidemiological studies, while at the same time noting that epidemiological associations reported between dietary components, specific foods (or food groups) and chronic disease are rarely sufficient to establish cause and effect relationships. The results of epidemiological investigations must also be evaluated through other types of supportive studies (animal studies, mechanistic studies, metabolic studies, human clinical intervention trials, etc.) before persuasive causal relationships can be firmly established [9,10].

There are several types of epidemiological studies, each with their own strengths and weaknesses [10]. Ecologic/descriptive studies are the simplest and least persuasive type. They characterize differences between large and diverse populations by simple generalizations and can help formulate hypotheses; however, they cannot control for potential confounding factors, i.e., factors that are known risk factors for the disease. Case-control studies focus on individuals and provide stronger evidence for an association than ecologic studies. "Recalled" past diets of individuals diagnosed with a disease (cases) are compared to those of individuals without the disease (controls) in a retrospective case-control study. Many researchers rely on this type of study because of lower cost, smaller sample size and ability to study many potential factors. However, such retrospective studies are subject to recall bias and unavailable or incomplete data particularly accurate dietary exposure data. There may also be questions regarding adequacy of the "control" group. Follow-up (prospective cohort) studies, on the other hand, are considered to provide the most definitive information and are the most persuasive study design. In these studies a cohort (group) of individuals, who do not yet have a specific disease, are selected and followed over a period of time while collecting specific information regarding diet and other factors related to the development of the disease. However, prospective studies are more costly and require more time and larger numbers of subjects. Following on the results of these types of epidemiological study, it is critical to conduct human, randomized clinical intervention studies in order to ultimately establish cause–effect relationships.

Interpretation of epidemiological studies demands causation criteria

To evaluate research findings in any area of scientific investigation, certain scientific standards, established by experts in each field, need to be applied. This is especially true when trying to determine the health effects of the inclusion or exclusion or varying levels of components in the diets of humans. The eminent British biostatistician and epidemiologist A. Bradford Hill published a seminal paper in 1965 [11] offering a number of interpretation criteria that would be useful when interpreting the statistical results observed in epidemiological studies. The goal of these criteria was to guide epidemiologists in inferring causation (or establishing "causal inference") from the associations observed in such studies. In effect, Bradford Hill tried to provide a framework to judge whether associations observed in a body of epidemiological work could be determined to be causal. Since the time of his publication, these criteria (which Bradford Hill urged others to call "viewpoints" and not true criteria) have become a de facto standard to evaluate the statistical associations found in epidemiological research.

Bradford Hill pointed out, however, that none of these nine viewpoints provide indisputable evidence for or against a cause and effect hypothesis. What he claimed they could do, with greater or less strength, was to help answer the fundamental question of whether there is any other way of explaining the set of observed facts, i.e., is there any other answer equally, or more likely than cause and effect? His criteria are still very relevant to the scientific and public discussion of diet and disease relationships. Bradford Hill's causation criteria are described below, with examples given to help illustrate each of the criteria.

The interpretation criteria for epidemiological associations presented by A. Bradford Hill in 1965.

1. Strength of association	Magnitude of the effect
2. Temporality	Exposure must precede the disease
3. Consistency	Similar findings in many studies without contradictory results
4. Theoretical plausibility	Biological/Physiological/Metabolic knowledge supports the findings
5. Coherence	No alternate hypotheses
6. Specificity	No confounding factors are found
7. Dose–response	Higher risk with higher exposure
8. Experimental evidence	Animal and *in vitro* experiments show the effect
9. Analogy	Similar findings in other situations can apply

1. *Strength of association:* The stronger the relationship between the independent variable (the risk factor) and the dependent variable (the disease), the less likely it is that the relationship is due to an extraneous variable (a confounder). For example, the relative risk of smokers developing lung cancer is around 10 or above (i.e., a 10-fold higher risk than for non-smokers). The relative risk associated with consumption of grains containing mycotoxins and liver cancer is around 6. These are quite strong associations, and by contrast, modest relative risks on the order of 2 or less should be viewed with skepticism due to the likelihood of many potential confounders. This was recognized in 1994 when the U.S. National Cancer Institute publicly indicated that relative risks values less than 2 were not strong enough to use for public policy pronouncements [12] (this document is available through the National Cancer Institute's CancerFax and CancerNet services, and in the News Section of the NCI's PDQ database. To get the document from CancerFax, dial +1 301 402 5874 from the handset on your fax machine and follow the recorded instructions to receive the contents list: U.S. National Cancer Institute Press Release, Oct 16, 1994).

2. *Temporality:* The exposure must precede the disease by a reasonable amount of time, i.e., a cause must precede an effect in time. For example, cigarette smoking over a period of years is well-established to increase lung cancer risk. While this relationship seems obvious, there have been published findings that have occasionally violated this criterion.

3. *Consistency:* Multiple observations of an association, with different people under different circumstances and with different measurement instruments, increase the credibility of a causal finding. Different methods (e.g., ecological, cohort and case-control studies) should produce the same conclusion. The relationship should also hold for different groups of people (in males and females, in different populations on different continents). This criterion is greatly debated, however, because consistency is often in the eye of the beholder. Some reviewers can conclude that six of ten studies with a statistically significant association represent a consistent finding, while others can see the same set of data as inconsistent.

4. *Theoretical plausibility:* It is easier to accept an association as causal when there is a rational and theoretical basis for such a conclusion supported by known biological and other facts. While our knowledge of physiology today is vast, there is still much that is unknown about the complex interactions of ingested foods and food components and their metabolism, interactions and potential adverse effects.

5. *Coherence:* A cause-and-effect interpretation for an observed association is clearest when it does not conflict with what is known about the variables under study and when there are no plausible competing theories or rival hypotheses. In other words, the association must be coherent with other existing knowledge. The conclusion that smoking causes lung cancer, based on decades of epidemiologic, laboratory animal, pharmacokinetic, clinical and other biological data, showed that all available facts stuck together as a coherent whole.

6. *Specificity in the causes:* In the ideal situation, the effect has only one cause. In other words, showing that a disease outcome is best predicted by one primary factor adds credibility to a causal claim. But this is often not the norm. High consumption of one food, food ingredient or nutrient can be covariate with low consumption of another food or ingredient, and it is often difficult to determine which of the two is more important. Other non-dietary confounders must also be considered. For example, chronic *Helicobacter pylori* infection is a strongly suspected risk factor for gastric cancer [13]. Nutritional epidemiological studies that fail to account for the incidence of this infection in the population studied should therefore be viewed with caution.

7. *Dose–response relationship:* There should be a direct biological gradient (or dose–response) between the risk factor (the independent variable) and people's status on the disease variable (the dependent variable). Many dietary epidemiological studies report a significant statistical trend for dose–response, but on closer examination, the data can be highly inconsistent. The data may only span a very narrow range of dietary intakes or in some cases such a wide range of intakes that upper intakes are representative of a grossly unbalanced diet. Calculated relative risks may sometimes decline to non-significant levels with higher intakes, or in some cases one quartile of intake may have a high enough calculated risk to skew the overall results. Such data patterns, when deviating from the linear dose–response, need to be considered as unlikely dose–response relationships and also be considered as part of the consistency criterion.

8. *Experimental evidence:* Any related research (animal, *in vitro*, etc.) that is based on experiments and supports the conclusions of epidemiological studies will make a causal inference more plausible. This must be tempered with the understanding that animals are not people, and results from lifetime, chronic feeding bioassays, where rodents are dosed at levels up to and sometimes exceeding maximum tolerated doses (MTDs), in such testing programs as the U.S. National Toxicology Program, may often be very useful but are not definitive by themselves.

9. *Analogy:* Sometimes a commonly accepted phenomenon in one area can be applied to another area. For instance, a newly discovered *N*-nitrosamine may be considered to be a carcinogen if it has structural similarities to other well-known carcinogenic *N*-nitrosamines. However, analogy is an obtuse criterion, and thus is considered to be a weaker form of evidence. This makes the application of the analogy consideration even more uncertain than the application of considerations on plausibility and coherence.

Bradford Hill developed his list of "criteria" that continues to be used today. When using them, it's important not to forget Hill's own advice: "None of these nine viewpoints can bring indisputable evidence for or against a cause and effect hypothesis... What they can do, with greater or less strength, is to help answer the fundamental question – is there any other way of explaining the set of facts before us, is there any other answer equally, or more, likely than cause and effect?" Phillips and Goodman [14] have recently provided observations on the use of Hill's "causal criteria." They argued that the uncritical repetition of Hill's criteria is probably counterproductive in promoting sophisticated understanding of causal inference, but they set out a simplified list of the key Hill considerations that they thought is worthy of repeating:

- "Statistical significance should not be mistaken for evidence of a substantial association.
- Association does not prove causation (other evidence must be considered).
- Precision should not be mistaken for validity (non-random errors exist).
- Evidence (or belief) that there is a causal relationship is not sufficient to suggest action should be taken.
- Uncertainty about whether there is a causal relationship (or even an association) is not sufficient to suggest action should not be taken."

The authors pointed out that while these points may seem obvious when stated so briefly and bluntly, causal inference and health policy decision-making would benefit tremendously if they were considered more carefully and more often.

The International Food Information Council (IFIC) Foundation has addressed the issue of "How to Understand and Interpret Food and Health-Related Scientific Studies" [15]. They discuss the types

of epidemiological research studies and what journalists, educators and health professionals should look for when critically reviewing scientific studies. When evaluating an epidemiological report, IFIC suggested that the following questions can be asked:

- Could the study be interpreted to say something else?
- Are there any methodological flaws in the study that should be considered when making conclusions?
- Are the study's results generalizable to other groups?
- How does the work fit with the body of research on the subject?
- What are the inherent limitations of this type of study and does the research design fit the stated purpose of the study?
- Has the author omitted important points in the background section which could have a meaningful effect on the study design or interpretation of the results?
- Are there any major design flaws in the study and are the data collection measures appropriate to answer the study questions?
- Were methodological limitations acknowledged and discussed and what influence might these have had on the results?
- What is the real and statistical significance of the results?
- To whom do the results apply and how do the results compare to those of other studies on the subject?
- Are the conclusions supported by the data?
- Are the conclusions of the study related to the stated purpose of the study? If not, does the study design and results support the secondary conclusions?

A recent Commentary addressed the important issue of false-positive results that are inherent in the testing of hypotheses concerning cancer and other human illnesses [16]. The authors point out that epidemiology has been increasingly criticized for producing results that are often sensationalized in the media and sometimes fail to be upheld in subsequent studies. They suggested general guidelines or principles, including editorial policies requiring the prominent listing of study caveats, which may help reduce the reporting of misleading results. The authors also urged increased humility by study authors regarding findings and conclusions in epidemiology, noting that this would go a long way toward diminishing the detrimental effects of false-positive results in several areas: (1) the allocation of limited research resources; (2) the advancement of knowledge of the causes and prevention of cancer and (3) the scientific reputation of epidemiology. They concluded that such efforts would help to prevent oversimplified interpretations of results by the media and the public.

Diet and cardiovascular disease

Now with these epidemiological criteria in mind, we will present the available data on certain foods and risk of specific diseases, namely cardiovascular disease (CVD) and cancer. Nothing is more important to our health than our diet, and there are well-established and recognized dietary patterns that confer health benefits. Numerous dietary epidemiological data have generally indicated an inverse relationship between dietary intake of fruits and vegetables and incidence of both CVD and cancer. The specific constituents of fruits and vegetables that afford protection from CVD have and continue to be widely researched and debated. Although there are many dietary components which may have protective effects, antioxidants appear to be one of the groups of phytochemicals that play a significant role.

One group of antioxidants present in fruits and vegetables is known as "polyphenols" or "polyphenolics" and is believed to neutralize free radicals formed in the body, thus minimizing or preventing damage to cell membranes and other cell structures. Although antioxidants are generally credited with improving states of oxidative stress, epidemiological studies which have evaluated the efficacy of supplementation with a high dose of an antioxidant alone, such as vitamin E, have shown no apparent improvement and in some cases a decrease in cardiovascular protection [17]. Yet, when these antioxidants are consumed through the diet in the form of fruits and vegetables, there is a significant degree of protection, best represented by the decrease in mortality rates from CVD of those consuming the popular Mediterranean diet [18]. Although Mediterranean diets vary by country, seasonal availability of ingredients and traditional cooking habits, they all tend to be rich in fruits, vegetables and monounsaturated fatty acids while low in saturated fat and meat. Epidemiological studies, such as the Seven Countries Study [19–25], have led to theories describing why and how the Mediterranean diet may promote a lower incidence of CVD, and antioxidants are emerging as a key component. However, since antioxidant supplementation alone does not confer the cardiovascular benefits, there must be more to explain the cardioprotection afforded by fruits and vegetables.

The data and emerging story presented in this context provide a convincing argument for nitrite and nitrate as probable protective components [26,27]. The Seven Countries Study constitutes the first nutritional epidemiological investigation that provided solid data for cardiovascular disease rates in different populations. The Seven Countries Study is the prototypical comparison study of populations, made across a wide range of diet, risk, and disease experience. It was the first to explore associations among diet, risk, and disease in contrasting populations (ecologic correlations). Central chemical analysis of foods consumed among randomly selected families in each area, plus diet-recall measures in all the men, allowed an effective test of the dietary hypothesis. The study was unique for its time, in standardization of measurements of diet, risk factors, and disease; training its survey teams; and central, blindfold coding and analysis of data. In this study the results for all-cause death rates in Greece, Japan and Italy were quite favorable compared with the USA, Finland, the Netherlands and the former Yugoslavia; the results also showed a lower incidence of CVD after a 5-year follow-up for the same countries that exhibited low mortality. The diet consumed by the Mediterranean cohorts studied was associated with a very low incidence of CVD and was called the *Mediterranean diet* by Keys [28]. Following the Seven Countries Study, the Mediterranean diet has oftentimes been singled out as a healthy diet. Additional studies later confirmed the association of the Mediterranean diet with decreased incidence and prevalence of chronic diseases, mainly CVD, in countries where it was consumed.

There are food peculiarities for the different populations in the Mediterranean basin. However, beyond the apparent differences, there are nutritional characteristics common to all or most of the diets in the Mediterranean region. The Mediterranean diet is characteristically low in saturated and high in monounsaturated fats (olive oil), low in animal protein, rich in carbohydrates, and rich in vegetables and leguminous fiber. People consuming a Mediterranean diet eat a relatively large amount of fish and white meat, abundant fruits and vegetables and a low amount of red meat, but they also drink moderate amounts of red wine [29]. The health benefits of Mediterranean diets have been attributed, at least in part, to the high consumption of antioxidants provided by fruit, vegetables and wine and to the type of fat, rich in monounsaturated and ω–3 fatty acids from vegetables and fish, and especially to a balanced ω–6/ω–3 fatty acid ratio, as is found in the traditional diet of Greece prior to 1960 [29]. Emerging data reveal that part of the health benefits may be mediated through their nitrite/nitrate content since diets rich in fruits and vegetables, i.e., Mediterranean diet are enriched in nitrite and particularly nitrate [26,27]. In fact, based on a convenience sample from each, a typical Mediterranean diet may contain as much as 20 times higher nitrite and nitrate than a typical western diet [30].

Diet and cancer

In contrast to the evidence on diet and CVD, epidemiological data on the consumption of meats and the risk of cancer sometimes reveal a slightly increased risk. Since dietary factors, which can be numerous and complex, may yield both positive and negative risk associations, they are of great interest to the research community, public health agencies and to the public. A total of 1,479,350 new cancer cases and 562,340 deaths from cancer are projected to occur in the U.S. in 2009 [31]. The U.S. National Institutes of Health estimate overall costs of cancer in 2007 at $219.2 billion. Smoking, poor nutrition, and physical inactivity are important risk factors for cancer. There is evidence that dietary patterns, foods, nutrients, and other dietary constituents are closely associated with the risk for several types of cancer. And while it is not yet possible to provide quantitative estimates of the overall risks, it has been estimated that up to 35 percent of cancer deaths may be related to dietary factors [32]. Many epidemiologic studies have shown that populations that eat diets high in vegetables and fruits and low in animal fat, meat, and/or calories have reduced risk of some of the most common cancers. Coincidently, fruits and vegetables are enriched with nitrite and nitrate from the soil. Colorectal cancer is the third leading cause of cancer-related deaths for both males and females in the U.S. Consumption of specific food components has been associated with risk of colorectal cancer. Dietary factors associated with increased colorectal cancer risk, include red meats and processed meats, while dietary fiber consumption is associated with decreased risk in low risk populations.

The consumption of red meat and, in particular, processed or preserved or cured meats (i.e., meats treated with nitrite as a food additive, including ham, bacon, hot dogs, etc.), has been related to the incidence of colorectal cancer since 1975 in several epidemiological studies. A worldwide recommendation for moderation in the consumption of preserved meats, such as sausages, salami, bacon and ham, was launched by the World Health Organization in 2003 [33]. However, a 2007 report by the World Cancer Research Fund and the American Institute of Cancer Research (WCRF/AICR) has presented a recommendation to "avoid processed meats" [34], based on a meta-analysis of a limited number of selected cohort studies showing increased risk of colorectal cancer with increased intake of processed meats. The summary estimate of relative risk was determined to be 1.21 (95% confidence interval = 1.04–1.42) per 50 g intake/day and was supported by case-control studies. A separate Swedish meta-analysis of 14 cohort studies reported a slightly lower summary hazard ratio estimate of 1.09 (95% confidence interval = 1.05–1.13) per 30 g intake/day [35]. However, these findings must be viewed with skepticism, considering that a relative risk ratio of 1.0 indicates no increase in risk and anything less than 2.0 should not be used for public policy recommendations, according to the U.S. National Cancer Institute [12].

According to the WCRF/AICR summary estimate of relative risk, a decrease of 50 g/day in processed meat consumption may then lower the total number of colorectal cancer cases by approximately 20%. A relative risk estimate of <1.3 would normally receive little attention in the epidemiological community. However, exposure to processed meats is so widespread, even a modest association, if proven causal, may have considerable public health consequences [36]. Curiously, the same literature review by the WCRF/AICR found a statistically significant 26% protective effect against rectal cancer for the highest meat consumption level, an important finding not referenced in the WCRF/AICR report or the press release that accompanied the report. However, there are also large epidemiologic studies showing no association between colorectal cancer and exposure to red or processed meats [37,38], and a recent meta-analysis published since the release of the WCRF/AICR report does not appear to support an independent association between animal fat intake or animal protein intake and colorectal cancer [39]. In addition, the findings of another recent meta-analysis showed no support for an independent relation between red or processed meat intake and kidney cancer [40]. Although some of the summary results were positive, all were weak in magnitude, most were not statistically significant, and associations were attenuated among studies that adjusted for key potential confounding factors, as has often been seen in many cancer epidemiology studies of meat intake.

The International Agency for Research on Cancer (IARC), a United Nations/World Health Organization body headquartered in Lyon, France, has the mission to coordinate and conduct research on the causes of human cancer and the mechanisms of carcinogenesis and to develop scientific strategies for cancer control. The agency is involved in both epidemiological and laboratory research and disseminates scientific information through publications, meetings, courses, and fellowships. IARC Director Peter Boyle and his colleagues responded to the media confusion caused by the release of the WCRF/AICR in an editorial in the *Annals of Oncology* [2]. They strongly objected to the report's downplaying the causative role of tobacco smoking and second-hand smoke in cancer causation and also criticized the report's conclusions on overweight, obesity and diet as major cancer causation factors. Boyle and colleagues specifically cast doubt on the rationale used to classify as "convincing" the evidence linking high meat intake to colorectal cancer risk. In fact, they argued that this conclusion on meat and cancer raised questions about the WCRF/AICR evaluation process and about the robustness of the classification system. Pointing out the fragile grounds on which the conclusions of the WCRF report were based, the editorial's authors felt that the information to the media should have been more cautious and less confusing, and that after decades of dietary research activity, we still do not know how we need to change what we eat to reduce our cancer risk.

Firm evidence of cancer causation in humans is lacking for dietary nitrite and nitrate. A comprehensive review [41] could find no epidemiological evidence linking stomach, brain, esophageal and nasopharyngeal cancers to dietary intake of nitrate, nitrite or *N*-nitroso compounds. This conclusion was further supported by a study showing that cured meat consumption was not linked to adult or childhood brain cancer in the U.S. [42]. The epidemiological data on meats, particularly cured and processed meats, becomes important in terms of exposure because such products are known to contain nitrite and nitrate. There are many other confounding factors in meats (saturated fats for example), but the direct implications for nitrite and nitrate content are far from conclusive. Early reports implicated nitrite and nitrate in processed meats as the culprit. Since the 1950s, when the potential to form carcinogenic *N*-nitrosamines from the reaction of nitrous acid with secondary amines was recognized, the use of nitrite salts as food preservatives has been under intense scrutiny. Numerous case-control studies have been conducted worldwide to determine if there is a link between gastric cancer and nitrate intake [41,43,44].

It is well known that elevated dietary nitrate intake leads to elevated salivary nitrate levels and, after reduction by oral bacteria, higher levels of ingested nitrite [45–47]. Studies in Canada, Italy, Sweden and Germany involving thousands of study subjects have failed to show an association or demonstrated an inverse association between estimated nitrate intake and gastric cancer, perhaps because much of the nitrate was from vegetables [44]. Occupational exposure to very high levels of nitrate occurs in fertilizer workers, who have elevated body burdens of nitrate and elevated

salivary nitrate and nitrite levels show no increased incidence of gastric cancers.[44]. Case-control studies attempting to link nitrate and nitrite consumption to brain, esophageal, and nasopharyngeal cancers have also been inconclusive [41]. In other studies published over two decades, the relationship between the consumption of cured meats during pregnancy and the risk of brain and other tumors in offspring was examined [43]. In a review of 14 epidemiological studies, 13 of which were case-control studies, Blot et al. [43] could not conclude a relationship between cured meat consumption during pregnancy and brain or any other cancers. It may be that in the limited number of epidemiological studies linking nitrate, nitrite or cured meats to a specific cancer site, other as-yet uncharacterized dietary or environmental factors may be involved.

Thus, the concern for the use of added nitrite in processed meats (added nitrate is only used in a very limited number of products outside the U.S.) has waxed and waned as numerous studies were published and independent review and food regulatory panels have been convened to make determinations about nitrite for use as a food additive and public policy. In 2000, the results of a comprehensive battery of rodent carcinogenicity and mutagenicity studies by the U.S. National Toxicology Program (NTP), including a standard, 2-year chronic cancer bioassay of sodium nitrite in rats and mice, were presented to the NTP Technical Reports Review Subcommittee for evaluation. The final NTP Technical Report [48] 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. In short, any suspicion of sodium nitrite's carcinogenicity in rodents was not supported by this state-of-the-art cancer bioassay study. Shortly thereafter, in 2000, nitrite was also reviewed and evaluated for potential listing as a developmental and reproductive toxicant under the Proposition 65 Statute in California. A review of 99 studies on sodium nitrite led the state's Developmental and Reproductive Toxicant Identification Committee of eight independent scientists to conclude that sodium nitrite should not be listed as a developmental toxicant or as a male or female reproductive toxicant under California's Proposition 65 law. The literature and activities associated with the above events were reviewed and published by Archer [49].

The latest review of ingested nitrate and nitrite carcinogenicity was conducted in June 2006 by an expert working group convened by IARC [50]. The IARC working group made a decision to classify nitrate and nitrite for their potential as human carcinogens as follows:

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

The expert group found that nitrate/nitrite was weakly associated with human stomach cancer only. They also appeared to disregard or misinterpret the findings of the NTP cancer rodent bioassay, which showed sodium nitrite was not carcinogenic in rats and mice. The above conclusion, which in a very narrow sense may be accurate, has in a broader biological sense questionable and minimal practical application. In simple terms, this overall evaluation means that the ingestion of food and water that contain nitrate or nitrite (e.g., spinach and other green leafy vegetables, root vegetables, bread, beer, cured meats), in combination with amines and amides commonly found in food, can react in the stomach to form *N*-nitrosamines and *N*-nitrosamides, which are known animal carcinogens already classified by IARC.

Antimicrobial benefits of nitrite in the food supply

Despite the very weak associations sometimes reported between dietary nitrite/nitrate and cancer, we must not forget the essential nature of these "curative" salts in the safety of the food we eat. The antibotulinal properties of nitrite have long been recognized. The use of nitrite to preserve meat has been employed either indirectly or directly for thousands of years. Nitrite inhibits outgrowth of *Clostridium botulinum* spores in temperature-abused (i.e., non-refrigerated) meat products. The mechanism for this activity was extensively investigated and results from inhibition of iron–sulfur clusters essential to energy metabolism in this obligate anaerobe [51–53]. Importantly, the broad antimicrobial effects of nitrite and implications for human health are still being researched. Commensal bacteria that reside within and on the human body can reduce nitrate, thereby supplying a large and alternative source of nitrite. Thus, ingested nitrite is also derived from reduction of salivary nitrate [54]. About 25% of orally ingested available nitrate is actively secreted into the saliva. This nitrate is partially converted to nitrite by oral bacteria and then disproportionates with formation of NO after entering the acidic environment of the stomach, helping to reduce gastrointestinal tract infection, increase mucous barrier thickness and increase gastric blood flow [54].

Humans, unlike prokaryotes, are thought to lack the enzymatic machinery to reduce nitrate back to nitrite. However, recent discoveries reveal a functional mammalian nitrate reductase [55]. Lundberg and Govoni demonstrated that plasma nitrite increases after consuming nitrate [45]. Therefore, dietary and enzymatic sources of nitrate are potentially large sources of nitrite in the human body. The nitrite in saliva has significant antimicrobial benefits when it is swallowed and converted to nitrous acid and other nitrogen oxides in the intestinal tract. The bactericidal effects of gastric fluids are significantly enhanced by the presence of ingested nitrite. This has been demonstrated for known foodborne pathogens such as *Escherichia coli 0157:H7* and *Salmonella* [56,57]. Nitrite and nitric oxide are also effective bacteriocidal agents against other microorganisms associated with diseases such as *H. pylori*, which has been linked to gastric cancer [58] and skin pathogens [59–61]. As a food additive, nitrite is also important in controlling potential growth of *Listeria monocytogenes* in processed meats. Models that estimate the effects of ingredients on microbial growth show dramatic reductions when nitrite is included [62–68]. The use of such models has enabled formulations of nitrite-cured processed meat products that will not support growth of *L. monocytogenes*. To date, this has not been achieved for uncured counterparts where the only ingredient difference is nitrite. Therefore, addition of nitrite appears essential and requisite for ensuring food safety.

The United States Department of Agriculture (USDA) has done extensive research to develop models to predict growth of pathogens under a variety of conditions [69]. In their models, incorporation of nitrite at current levels significantly inhibits growth of *Listeria, E. coli, Staphylococcus aureus*, and *Bacillus cereus*. By way of illustration, the following predictions were calculated from the USDA Pathogen Modeling program 7.0 model [69] and imply significant pathogen risk reduction when nitrite is included in products (see Table 1).

A second illustration from the same USDA model shown below is in agreement with the reports of enhanced bactericidal effects of nitrite in gastric fluid (see Table 2).

The tradition of curing meats and more recent knowledge surrounding the use of sodium nitrite to address risk of pathogens has been embodied in modern food manufacturing systems. The use of these approaches in cured meats has been estimated to sig-

Table 1
Predicted growth time (hours) for pathogenic organisms from log 3.0 CFU/mL to log 6.0 cfu/mL under anaerobic conditions in broth culture at pH 6.0 in the presence of 2.0% sodium chloride and different levels of sodium nitrite.

Organism	Temperature (°C)	No nitrite	Sodium nitrite (60 ppm)	Sodium nitrite (120 ppm)
Listeria monocytogenes	4	208	283	383
E. coli 0157:H7	9	217	238	284
S. aureus	12	195	230	284
B. cereus	10	144	230	274

Table 2
Predicted 6.0 log reduction time (hours) for pathogenic organisms in broth culture at pH 4.0, 25 °C in the presence of 2.0% sodium chloride and different levels of sodium nitrite.

Organism	No nitrite	Sodium nitrite (60 ppm)	Sodium nitrite (120 ppm)
Listeria monocytogenes	781	158	60
E. coli 0157:H7	147	77	–[a]
S. aureus	1572	780	295
Salmonella	6.3	3.7	2.2

[A] Outside valid parameter range.

nificantly reduce the risk of listeriosis ascribed to such products [70]. If the products were not cured (i.e., no nitrite was used), the risk reduction would be greatly diminished and major efforts would be needed to develop systems to produce equivalently safe products.

Risk–benefit evaluation

Nitrite is now known to be an intrinsic signaling molecule [71,72] capable of producing NO under appropriate conditions as well as forming nitrosothiols [71,73]. Nitrite has been shown to increase regional blood flow [74], increasing oxygen delivery to hypoxic tissues. Enhancing nitrite availability through therapeutic intervention by administering bolus nitrite prior to cardiovascular insult has shown remarkable effects in reducing the injury from myocardial infarction, ischemic liver and kidney injury, stroke and cerebral vasospasm [75–80] in animal models. These first reports on the efficacy of nitrite in cytoprotection have led to nine current clinical trials for the use of nitrite and/or nitrate in both healthy volunteers and patients with specific cardiovascular complications (www.clinicaltrials.gov). Most recently, nitrite has been shown to precondition the myocardium when given 24 h prior to ischemic insult due to the modulation of mitochondrial electron transfer [81] as well as augment ischemia-induced angiogenesis and arteriogenesis [82]. Nitrite also presents remarkable efficacy in promoting regional blood flow in sickle cell patients [83]. Plasma nitrite levels increase in response to exercise in healthy individuals, whereby in aged patients with endothelial dysfunction there is no increase in nitrite from exercise [84]. Nitrite has also been shown to predict exercise capacity [85] and enhance exercise efficiency in humans [86]. Physical activity can even prevent age-related impairment in NO availability in elderly athletes [87]. We now know that nitrite is just as efficacious when given orally at restoring NO biochemistry [88], reversing hypertension from NOS inhibition [89], protecting from myocardial ischemia–reperfusion injury [90], inhibiting microvascular inflammation, reversing endothelial dysfunction and reducing levels of C-reactive protein [91]. This provides proof of concept that dietary sources of nitrite have important physiological functions.

According to the World Health Organization, cardiovascular disease (CVD) is the number one killer of both men and women in the U.S. These deaths represent a staggering 40% of all deaths. Close to 1 million people die each year and more than 6 million are hospitalized. The cost of CVD, in terms of health care and lost productivity, is over $270 billion and increasing as the baby boom population ages. Ischemic heart disease, as the underlying cause of most cases of acute myocardial infarction, congestive heart failure, arrhythmias, and sudden cardiac death, is the leading cause of morbidity and mortality in all industrialized nations. In the United States, ischemic heart disease causes nearly 20% of all deaths (~600,000 deaths each year), with many of these deaths occurring before the patient arrives at the hospital. Heart disease is very likely the result of a dysfunctional endothelium.

One of the most important substances released by our body is NO. The discovery that the human body makes NO from L-arginine revolutionized science and medicine. Continuous generation of NO is essential for the integrity of the cardiovascular system and a decreased production and/or bioavailability of NO is central to the development of cardiovascular disorders. It is also important for communication in our nervous system and a critical molecule which our immune system uses to kill invading pathogens, including bacteria and cancer cell. Reduced NO availability is a hallmark of a number of disorders, including CVD. Understanding strategies to enhance and restore NO homeostasis is critical to developing treatments to cardiovascular disease and more importantly strategies to prevent disease from developing.

The notion that there may be certain foods that can enrich NO within our body is revolutionary. Therapeutics is the branch of medicine concerned with the remedial treatment of disease. It is prudent at this juncture to take a step back and look at nitrite and nitrate as a means of prevention of a number of diseases associated with NO insufficiency. Early intervention to restore NO/nitroso homeostasis through natural dietary means may prove to be a cost-effective and natural means to prevent disease. It is becoming increasingly clear that a deficiency in NO is a hallmark of a number of disease conditions. It is highly unlikely that nature devised a singular pathway which requires a complex five-electron oxidation of a semi-essential amino acid requiring multiple co-factors and prosthetic groups. There is, by design, enormous redundancy in physiology where there are multiple pathways and regulatory processes for critical biological functions. This redundancy ensures that if one pathway is absent or dysfunctional, there is an alternative to produce and regulate critical signaling molecules and pathways. Maintenance of NO homeostasis by nitrite may be the redundant backup system in NO biology. A simple one-electron reduction of nitrite to form NO or bimolecular reaction with thiols to form nitrosothiols is a viable and effective system for recapitulating NO biochemistry. Since at least half of our body's pool of nitrite is derived from what we eat, we can at will, affect NO biochemistry through dietary means. Discovery and recognition of this pathway is likely to affect public health and strategies to prevent and/or treat disease. We are currently faced with an enormous public health crisis as our aging baby boomers succumb to disease, particularly CVD. Treatment and care of our greatest generation is a tremendous economic burden, and developing strategies to prevent disease or reduce injury from a cardiovascular accident will improve patient outcome and enhance quality of life. Recognizing a natural and inexpensive regimen of foods rich in ni-

trite and nitrate to restore NO homeostasis can have profound effects on public health. It is time for health care professionals, clinical nutritionists, dieticians, food scientists and epidemiologists to begin discussion and appreciate contemporary views of nitrite and nitrate in the context of indispensable nutrients.

As with any remedy or treatment, a risk–benefit reward evaluation should be considered. A 2009 Expert Report published by the Institute of Food Technologists (IFT) provided a comprehensive review of the safety and regulatory processes for making decisions about the risks of chemicals in foods [92]. This report described the need for scientists, health professionals and public health authorities to evaluate the health benefits of a specific food or food group when also assessing the health risks that may be posed by the food or food group. Numerous case examples were cited in the report, specifically the weighing of the risks of methyl mercury contamination of certain fish compared to the nutritional benefits of fish consumption, the cancer-reducing effects of coffee consumption even with the presence of trace levels of animal carcinogens in roasted coffee, and the potential cancer risks of heat-induced carcinogens (known to occur at trace levels) in otherwise healthy and nutritional foods and beverages. One of the key conclusions of this expert report was that the risks and benefits of the whole food should be evaluated instead of simply focusing on the risks of individual chemical components of the food.

Using the concepts discussed in the IFT Expert Report, we predict that the benefits of dietary nitrite and nitrate will strongly outweigh any potential risks, particularly for patients with conditions of NO insufficiency. The now recognized and undisputed benefits of dietary nitrite and nitrate should be put in context to the very weak associations to its potential risks. In an editorial comment, Katan [93] eloquently put this point in perspective: "...evidence for adverse effects of dietary nitrate and nitrite is weak, and intakes above the legal limit might well be harmless. This is not unusual in regulatory toxicology. Many chemicals and contaminants might well be safe at intakes above their legal limit. Authorities willingly accept that possibility; erring on the safe side with many chemicals is justified if it keeps just one true carcinogen out of the food supply. But the trade-off changes when excessive caution deprives us of beneficial substances, as claimed by Hord et al. for nitrate [26]. In that case, the evidence for harm needs to be weighed against the potential benefit."

Conclusion

A long history of safe use as a food additive, minimal endogenous production of *N*-nitrosamines and natural metabolism of ingested nitrite all argue that nitrite as currently used in foods is a safe food additive and even beneficial to human health. Dietary intake of nitrate is a well-known marker of a health-promoting fruit and vegetable diet. In addition, nitrite and nitrate per se, as individual chemical compounds, have never been shown to be carcinogens in animal or human studies. Nitrate itself is not capable of reacting with amines to form *N*-nitrosamines. One could ask the question then, how simultaneous ingestion of nitrite/nitrate and nitrosatable amines/amides could be prevented or reduced by public policy considerations stemming from IARC's "probably carcinogenic to humans" conclusion, when the majority of ingested (swallowed) nitrite is endogenously produced in saliva and the major source of nitrate is the consumption of health-promoting fruits and vegetables. Thus, to eat is to ingest nitrite, nitrate, amines and amides, regardless of the specific diet. Specifically, the consumption of processed, cured meats would be no more or less risky given the low amount of residual nitrite in such products ready to consume (approximately 10 ppm in individual servings, and sometimes even undetectable).

There will very likely be considerable debate about the emerging health benefits of dietary nitrite and nitrate in light of the fact that we have been told to limit their intake. However, when considered in the context of evolving research about the biological function of all nitrogen oxides (including nitrite and nitrate) and their metabolism, any changes to current food regulations on nitrite are simply unwarranted, as are any regulatory implications for the unavoidable presence of nitrate in fruits and vegetables. The use of nitrite as a direct food additive represents only a small addition to the body burden of endogenously produced nitrogen oxides. It is hard to believe that the ingestion of nitrite from cured meats or nitrate from fruits and vegetables could have any potential adverse toxic outcomes. As more is understood about the human metabolic nitrogen oxide cycle, it will become apparent that nitrite is a safe and appropriate food additive providing many more benefits to society than risks, and nitrate naturally occurring in fruits and vegetables and some drinking waters poses insignificant risks. In fact, the inherent nitrate content of Traditional Chinese Medicines and their ability to convert nitrite to NO in essence provide over 5000 years of phase 1 safety data in humans with known curative properties for certain ailments and conditions [94].

Consumers should avoid getting caught up in fads to the point where they might ignore sound science and common sense. Dietary fads seem to have varying lifetimes, sometimes 3 months to even 3 years and, often in retrospect, some common sense critical thinking at the early stages of dietary research reporting could avoid a lot of poor dietary advice. We have seen a variety of diet vs. disease fads, some spanning decades, such as the low-fat craze which, according to some observers, has led to significant increases in the incidence of both obesity and type 2 diabetes. Such fads may have negative consequences where they not only fail to improve public health, but actually result in unintended adverse health effects. The importance of dietary variety, balance and moderation should be stressed along with the importance of protective factors (including adequate consumption of fruits and vegetables) in the total diet, combined with a physically active lifestyle. We believe that the weak and inconclusive data on nitrite and nitrate cancer risks described here are far outweighed by the health benefits of restoring NO homeostasis as described by the volumes of published work over the past 10 years [72,95–101]. The risk/benefit balance should be a strong consideration before there are any suggestions for new regulatory or public health guidelines for dietary nitrite and nitrate exposures.

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