Dietary Nitrate and Nitrite: Friend or foe?

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

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

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

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

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

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

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

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

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

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

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

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