Toxicogenomic responses to N-Nitroso compound exposure in relation to human colorectal cancer risk

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ISBN 978-94-6159-016-9
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Omslagillustratie: Jurby Jumawan
Lay-out: Dennie G.A.J. Hebels
Omslagrealisatie en druk: Datawyse Boekproducties, Maastricht

The studies presented in this thesis were performed at the Nutrition and Toxicology Research Institute Maastricht (NUTRIM), which participates in the Graduate School VLAG (Food Technology, Agrobiotechnology, Nutrition, and Health Sciences), accredited by the Royal Netherlands Academy of Arts and Sciences.
Financial support by the *Orbis Medisch en Zorgconcern* (Sittard) and *Greiner Bio-One B.V.* (Alphen a/d Rijn) for the printing of this thesis is gratefully acknowledged.

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PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Maastricht,
op gezag van de Rector Magnificus, Prof. mr. G.P.M.F. Mols
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen
op donderdag 16 december 2010 om 10.00 uur

door

Dennie Geert Anne Jozef Hebels

geboren te Heerlen op 4 januari 1983

UNIVERSITAIRE PERS MAASTRICHT

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The research described in this thesis was funded by the European Network of Excellence on Environmental Cancer Risk, Nutrition, and Individual Susceptibility (ECNIS, FOOD-CT-2005-513943) and the EU Integrated Project NewGeneris, 6th Framework Programme, Priority 5: Food Quality and Safety (FOOD-CT-2005-016320).

“There is a theory which states that if ever anyone discovers exactly what the Universe is for and why it is here, it will instantly disappear and be replaced by something even more bizarre and inexplicable.

There is another which states that this has already happened.”

Douglas Adams

CONTENTS

Abbreviations

APC	Adenomatous polyposis coli
ATNC	Apparent total nitroso compounds
BRM	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2
BUB3	Budding uninhibited by benzimidazoles 3 homolog
cAMP	Cyclic AMP
CCMO	Clinical Trial Center Maastricht
CCR	Carbon centered radicals
CFTR	Cystic fibrosis transmembrane conductance regulator
CI	Confidence interval
CIN	Chromosomal instability
COX-2	Cyclooxygenase 2
CRC	Colorectal cancer
CREB	cAMP response element-binding
Cy	Cyanine
CYP	Cytochrome P450
DMSO	Dimethylsulfoxide
EDG3	Sphingosine-1-phosphate receptor 3
EDNRA	Endothelin receptor type A
EGFR	Epidermal growth factor receptor
ENU	N-ethyl-N-nitrosurea
EPR	Electron paramagnetic resonance
ESR	Electron spin resonance
FAP	Familial adenomatous polyposis
FBXW7	F-box and WD repeat domain containing 7
FOX	Forkhead box
Fpg	Formamidopyrimidine-DNA glycosylase
GC-MS	Gas chromatography-mass spectrometry
GEO	Gene Expression Omnibus
GEPAS	Gene Expression Profile Analysis Suite
GI	Gastro-intestinal
GO	Gene ontology
$H_2NO_2^+$	Protonated nitrous acid
HBSS	Hank's balanced salt solution
HCA	Heterocyclic amine
HCA	Hierarchical clustering analysis
HFSC	Hyperfine splitting constant
HNO_2	Nitrous acid
HNPCC	Hereditary non-polyposis colorectal cancer
HP1-BP74	Heterochromatin protein 1 binding protein
HPLC	High pressure liquid chromatography
HTT	Huntingtin
IARC	International Agency for Research on Cancer
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IFN	Interferon
IL-8	Interleukin 8
iNOS	Inducible NO synthase
MGMT	O^6-alkylguanine DNA-alkyltransferase

MIF	Macrophage migration inhibitory factor
MMR	Mismatch repair
MN	Micronuclei
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
MNU	N-methyl-N-nitrosurea
MSI	Microsatellite instability
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N_2O_3	Dinitrogen trioxide
N_2O_4	Dinitrogen tetroxide
NCBI	National Center for Biotechnology Information
NCR	Nitrogen centered radicals
NDEA	N-nitrosodiethylamine
NDELA	N-nitrosodiethanolamine
NDMA	N-nitrosodimethylamine
NMOR	N-nitrosomorpholine
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NNN	N-nitrosonornicotine
NO/$^{\bullet}$NO	Nitric oxide
NOC	N-nitroso compound
NOCP	N-nitroso compound precursor
NPIP	N-nitrosopiperidine
NPYR	N-nitrosopyrrolidine
O^6-meG	O^6-methylguanine
OR	Odds ratio
ORF	Open reading frame
PAH	Polycyclic aromatic hydrocarbon
PBS	Phosphate buffered saline
PC	Principal component
PCA	Principal component analysis
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKA	Protein kinase A
POR	P450 oxidoreductase
PRR6	Centromere protein V
ROS	Reactive oxygen species
SMAD3	SMAD family member 3
TCF/LEF	Transcription factor T-cell factor/Lymphoid enhancer factor
TF	Transcription factor
TGF-β	Transforming growth factor β
TM	Tail moment
Wnt	Wingless

Chapter 1

General introduction

1 Colorectal cancer

1.1 Cancer

Cancer is an affliction that affects millions of people each year and thus represents a significant burden of disease in addition to the economic burden, including cost of illness, phase-specific and long-term costs [1]. Establishing knowledge on the causes and possible treatments of this disease has therefore been a high priority of the scientific community for many decades. Extensive research on the pathophysiology, molecular mechanisms and risk factors behind cancerous growths has led to important progress in the field of cancer treatment and prevention. Nonetheless, cancer is still one of the leading causes of death worldwide and the World Health Organization has projected it to become the leading cause of death in 2010 [2]. Much research is therefore still needed to improve treatment, identify other risk factors, and create preventive strategies. Such strategies have especially high priority since it has been estimated that 30% of cancer deaths can be prevented by modifying exposure to known risk factors [3,4].

1.2 Colorectal Cancer Epidemiology

Every organ in the human body is susceptible to cancerous growth although some organs to a larger degree than others. For example, cancers of the lung, prostate, breast, and colon/rectum are responsible for a disproportionate share of yearly cancer incidence and mortality rates [1]. Causes for these differences can be found in genetic predisposition, environmental factors, and lifestyle choices since these will more readily affect some organs than others with regard to genetic susceptibility and degree of exposure. This is especially true for the gastro-intestinal (GI) tract where the digestion of food and the absorption of nutrients lead to a continuous exposure to a wide array of compounds, many of which have carcinogenic properties. This makes the cells of the GI tract a potential target for mutagenic lesions which could ultimately give rise to tumor development. Indeed, GI cancers as a group account for the majority of global cancer cases, with bowel cancer, also generally called colorectal cancer (CRC), being the most prevalent form of cancer within this group [2,5].

CRC is the fourth most common form of cancer in men and the third most common cancer in women worldwide [5] and the second leading cause of cancer-related death in the Western world, causing an estimated 655,000 deaths worldwide each year [6,7]. Most CRC cases occur between the sixth and seventh decade of life and occurrence is slightly higher in men than in women and increases with age. CRC has a yearly incidence of approximately 1 million cases with incidence rates varying strongly worldwide, up to approximately 25-fold, and being highest in

Europe, North America, and Oceania [2,7,8]. Incidence rates in most countries are still rising, although the United States are now witnessing a decline in CRC incidence [8].

Implementation of new treatments and prevention strategies for CRC is thus likely to result in a considerable decrease in incidence and death rates. The goal of this thesis is to contribute to the understanding of causative factors in CRC development, which may contribute to the optimization of CRC prevention. In the following paragraphs, the biological and molecular background of CRC development and associated risk factors are described, which will lead to the rationale and aims of the studies described in the rest of this thesis.

1.3 The Carcinogenic Process

The development of cancer is usually divided into three distinct steps [9]. The first step, initiation, results from exposure to mutagens and results in little or no observable changes in the cellular or tissue morphology but does confer an irreversible alteration of the genetic material giving rise to a permanent increase in susceptibility to cancer formation. At this stage, alterations with the greatest initiating effect occur in proto-oncogenes and tumor-suppressor genes involved in regulation of cell growth and differentiation. Such mutations can result from exposure to dietary components, tobacco smoking, environmental carcinogens and chronic inflammatory states but also inborn genetic aberrations. Initiated cells have the potential to develop into a clone of pre-neoplastic cells. The second step, tumor promotion, is characterized by an alteration in genetic expression and clonal growth of the initiated cell population. It requires a non-mutagenic stimulus, such as tissue disruption by wounding or inflammation, resulting in an enhanced cellular proliferation and eventual formation of a non-malignant tumor which may regress without further stimulus. During this phase additional genetic and epigenetic changes can occur, further stimulating pre-neoplastic growth. In the final progression step, the tumor transitions to limitless, invasive growth, characterized by changes in the number and/or arrangement of chromosomes. Model simulations demonstrate that this growth is the result of adaptation to the hypoxic and acidic environment that limited growth after the promotion phase [10]. Subsequent tumor development and malignant transformation requires some additional tissue disruption although much of cellular evolution in this phase seems to require no external stimulus.

As already mentioned, the continuous exposure of the colon to a large number of potentially hazardous compounds is thought to be an important factor contributing to this multi-step carcinogenic process in CRC. However, the proliferative potential of the colon epithelium is also likely to play an important role. In the next paragraph the development of CRC will be described in more detail, starting with a short overview of colon anatomy and histology and subsequently focusing on genetic alterations associated with the stages of initiation, promotion, and progression.

1.3.1 Anatomy and Histology of the Colon in Relation to CRC

The large intestine forms the last part of the human GI tract and consists of the cecum, appendix, colon, rectum, and anal canal. The large intestine is often used synonymously for the colon, which is due to the fact that the colon makes up the largest part of the large intestine. The colon is divided in four sections: the ascending colon, the transverse colon, the descending colon, and the sigmoid colon which connects to the rectum. CRC most commonly develops in the lower part of the descending colon, the sigmoid colon, and the rectum [11].

Important functions of the large intestine include the absorption of water and electrolytes from the fecal effluent, maintaining the body's fluid balance, and providing a location for flora-aided fermentation of indigestible carbohydrates (fibers) and production of certain vitamins which are subsequently absorbed [12]. This absorption process is controlled by the colonic wall, which comprises four layers: the mucosa, the submucosa, the muscularis externa and the serosa (Figure 1). The mucosa consists of the epithelium, which lines the colon lumen, a deeper loose connective tissue layer known as the lamina propria involved in support of the mucosal epithelium and containing numerous cells with immune function, and the muscularis mucosae, a smooth muscle layer involved in expelling the contents of glandular crypts and enhancing contact between the epithelium and the contents of the colon lumen. Nodules of lymphatic tissue present in the mucosa extend into the submucosa, which is composed almost entirely of a network of collagen fibers vital to the strength and distensibility of the colon during colon peristalsis. Peristalsis is coordinated by the muscularis externa which results in the propulsion of a food bolus through the colon. The serosa is the outer layer of the colon and secretes a lubricating fluid used to reduce friction from muscle movement and is connected to the peritoneum [12]. Most cases of CRC are adenocarcinomas (95%) which develop from the epithelium and during progression infiltrate the muscularis mucosae, the submucosa and thence the muscularis externa. This is followed by invasion of the subserosa and peritoneum or adjacent organs, and subsequent metastasis [13].

The colon mucosa is highly specialized in absorption of water and remaining nutrients and mucus production to accommodate bolus movement. It contains tubular glands, called crypts of Lieberkühn, which extend down to the muscularis mucosae and are lined by four types of cells: enteroendocrine cells, absorptive cells (or enterocytes), goblet cells, and stem cells. The rectum strongly resembles the colon from a histological point of view, but the crypts are generally deeper and number fewer per unit area [12]. Enteroendocrine cells are located in the lower portions of the intestinal crypts and are specialized in the secretion of hormones that influence GI secretion or motility. The absorptive cells are the most numerous cell type and have a striated apical border consisting of a dense array of microvilli which, together with crypt formation, increases the absorptive surface area. Goblet cells are specialized in secretion of mucus, which facilitates passage of material

through the bowel. The absorptive and goblet cells are continuously replenished by dividing stem cells, which line the bottom walls of the crypts. Stem cells have a relatively low rate of proliferation but they give rise to a population of more rapidly dividing transit amplifying cells [14]. As a result, cell proliferation occurs in up to two-thirds of the length of the crypt while cells differentiate and lose the ability to divide during a continuous upward flow to the surface epithelium where they eventually undergo apoptosis and are shed in the lumen of the intestine. There is an increasing amount of evidence that these stem cells represent the main target of tumorigenic mutations due to both their long life and their capacity for self renewal [15,16].

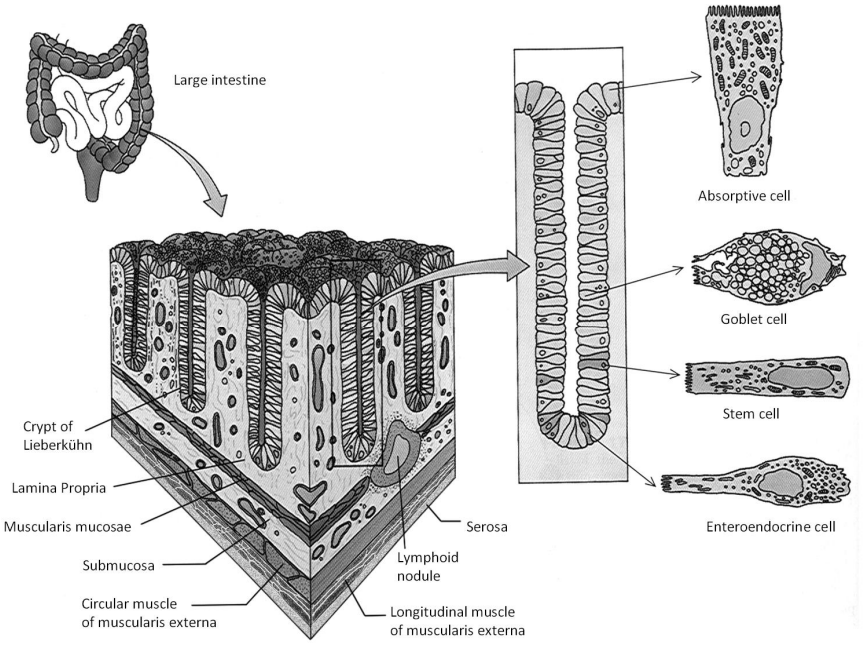

Figure 1: Schematic overview of the colon wall layers and the cell types associated with the crypts of Lieberkühn. Adapted from ref. [12].

1.3.2 Genetic Alterations in CRC Development

The continuous mitotic process of (stem) cells in the crypts results in a rapid mucosal surface turnover throughout life, with the entire epithelial lining being replaced every 3–5 days. A population of intestinal stem cells is responsible for the generation of more than 10^{10} differentiated cells everyday [16]. Consequently, this mitotic environment provides the ideal circumstances for the development of cancer since

the entire GI tract is constantly under exposure to dietary and environmental factors increasing the risk of precancerous lesions to progress along the carcinogenesis pathway into a malignant tumor. As with any type of cancer, CRC originates from cells undergoing sequential mutations in specific DNA sequences that disrupt normal mechanisms of proliferation and self-renewal thereby driving the transition from healthy colonic epithelia to increasingly dysplastic adenoma and finally to CRC.

Studies on hereditary cancer syndromes have contributed extensively to the understanding of cancer biology, which in case of CRC accounts for 5–10% of cases [17–20]. The overall majority of CRC cases seem to arise in a non-hereditary, sporadic fashion. The two most well known hereditary CRC syndromes are familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC). FAP is an autosomal dominant syndrome associated with mutations in the adenomatous polyposis coli (APC) gene, resulting in the development of hundreds of polyps by the third decade of life. HNPCC, with an incidence of 4–6% (versus <0.5% for FAP), is caused by mutations in any of the DNA mismatch repair (MMR) genes. Studies on FAP and HNPCC allowed for the identification of two alternative pathogenic mechanisms for colorectal tumorigenesis, i.e. chromosomal instability (CIN) and microsatellite instability (MSI), which have both advanced our understanding of the molecular basis of CRC [16,18,21].

Fearon and Vogelstein [22] developed a genetic model for CRC development based on genetic alterations in hereditary CRC syndromes, which proposes that CRC develops as a result of the progressive accumulation of genetic and epigenetic changes in colon tissue (Figure 2). This applies to the pathogenic mechanism of both CIN and MSI. CIN encompasses the majority of sporadic CRC tumors (85%) and is marked by mutations in APC and subsequent mutations in other genes resulting in gross chromosomal abnormalities. Mutations in APC, which are suspected to be the initiating event in many cases of sporadic CRC, lead to over-activation of the Wingless (Wnt) signaling pathway, resulting in a higher rate of tumor initiation due to the abrogation of the “gatekeeper” function of this gene. APC, in a complex with axin and glycogen synthase kinase 3, normally associates with β-catenin, resulting in the latter’s degradation. Mutated forms of APC are unable to bind to β-catenin, allowing it to move to the nucleus and initiate transcription of genes involved in cell proliferation [16,23–25].

K-ras mutations appear to occur after APC mutations and are associated with the transitory state from an early to an intermediate adenoma. Mutated forms of K-ras result in a prolonged activation of the Ras protein allowing it to transduce mitogenic signals that regulate cell cycle progression and proliferation. There is evidence that in some cases of CRC, K-ras, instead of APC, is the initiating event in CRC tumorigenesis [16,23–25].

SMAD proteins are part of the transforming growth factor beta (TGF-β) pathway which is involved in apoptosis, differentiation and the negative regulation of growth

in intestinal epithelial cells. Mutational inactivation has been found in SMAD2 and 4, which both have tumor-suppressor properties. The Wnt and TGF-β pathways have common intermediates in the regulation of the cell cycle. When both pathways are deregulated a synergistic effect ensues, leading to a definite cell cycle enhancement and growth advantage which plays an important role in the promotion phase of tumor development and the formation of a late adenoma [16,23–25].

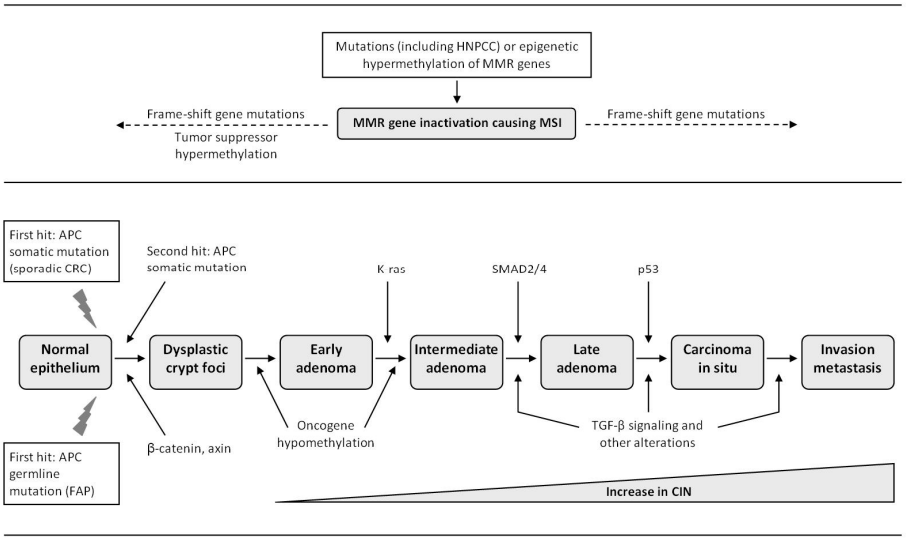

Figure 2: Scheme of key genetic events in colorectal tumorigenesis shown for the MSI (top) and CIN (bottom) pathogenic mechanisms of CRC.

Mutation of the p53 tumor-suppressor gene appears to be a relatively late event in the carcinogenic process and plays a decisive role in the progression of adenoma to carcinoma. With loss of p53 function, several cell cycle checks, apoptosis mechanisms, and proliferative balances are disrupted, which facilitates the formation of gross chromosomal aberrations and aneuploidy. Cells at this point are able to rapidly accumulate genetic alterations, which can eventually allow metastasis of the tumor [16,23–25].

MSI comprises the remaining cases of sporadic CRC (15%) and features mutations or epigenetic hypermethylation of “caretaker” MMR genes. Hypermethylation results in the formation of inactive chromatin and hence transcriptional repression and is mainly associated with tumor suppressor genes [26–28]. In case of MMR gene inactivation, DNA mismatch repair is compromised which subsequently results in an increase in MSI-associated frame-shift mutation frequency thereby greatly increasing tumor initiation [16,22,25,27,29]. As a result, MSI is characterized by a

different mutation pattern than CIN with regard to the type of mutations and the target genes involved. Dozens of MSI target genes have been identified, most of which are involved in cell proliferation, apoptosis, and DNA repair [30,31]. Although MSI can be an initiating event, it can occur at any point in the adenoma-carcinoma sequence [32].

Epigenetic changes have also been found in the CIN pathway of CRC, where the opposite effect of DNA hypomethylation results in the activation of oncogenes [26,27,33,34]. DNA hypermethylation and hypomethylation both occur in the early stages of colorectal tumorigenesis and are mainly associated with MSI and CIN, respectively [33,35,36]. Epigenetic modifications are therefore increasingly being recognized as a crucial mechanism in cancer development in both pathogenic CRC mechanisms [26,37–39].

The mutational spectra covering the three phases of carcinogenesis consequently result in the large majority of colorectal tumors to progress through a series of histopathological stages ranging from dysplastic crypts through small benign adenomas and ultimately malignant tumor formation with metastatic properties [40]. Years of research, focused on finding the causes that drive this process, has led to the identification of a number of important CRC risk factors as described in the next paragraph.

1.4 CRC Etiology

With the high proliferative potential of the colon epithelium and the continuous exposure to potentially carcinogenic agents that can initiate events early in the Vogelstein model, it is not surprising that CRC is one of the most common neoplastic diseases in the world. Possible CRC risk factors have therefore been extensively investigated and certain elements in human lifestyle have been implicated as crucial to the development of CRC.

1.4.1 The Vital Role of Lifestyle

There is an especially strong trend towards an increased CRC incidence in economically transitioning countries, including many Eastern European countries, most parts of Asia and select countries in South America, which most likely reflects the adoption of Western lifestyles and behaviors [41]. In Japan, for example, a developed country with a strong economy, age-standardized rates have increased dramatically between 1959 and 1992, a period during which Western-type foods in the diet increased and energy expenditure decreased as many people adapted to a sedentary lifestyle [42]. The role of environmental and lifestyle factors is further supported by migrant studies which show that migrants moving from low to high risk countries or

regions eventually adopt the cancer risk of the host country within one or two generations [43–45].

There are also strong indications that changes in lifestyle, such as modifications in nutrition and more exercise, can decrease cancer incidence [46,47]. For example, it has been estimated that 70% of CRC deaths is avoidable through dietary change [48,49]. However, the worldwide increase in CRC incidence points toward a failed early detection and prevention strategy as well as failure to address lifestyle and dietary challenges of urbanization [50]. In the next paragraph an overview is given of CRC risk factors which provide potential targets for CRC prevention strategies.

1.4.2 Lifestyle-associated CRC Risk Factors

With respect to lifestyle, compelling evidence indicates that avoidance of smoking and heavy alcohol use, prevention of weight gain, and maintenance of a reasonable level of physical activity are associated with markedly lower risks of colorectal cancer [51]. Studies in the United States have estimated that approximately 15–20% of CRC cases can be attributed to smoking and similar associations have been found in other countries [52,53]. An alcohol intake of more than 2 drinks per day is associated with a significantly increased CRC risk [52,54]. The risk associated with obesity, and particularly central adiposity, is most likely related to insulin resistance, but pro-inflammatory conditions may also play a role [52]. Since regular physical activity can prevent obesity, it is not surprising that risk reductions are observed with an increase in physical activity frequency and intensity [52]. It is estimated that physically active individuals have a 20–30% lower CRC risk compared to less active individuals [55]. All of these risk factors are associated with a Western lifestyle, which is a plausible explanation for the higher CRC incidence in the Western world. Associations between CRC risk and nutrition typically found in Western diets have also been studied and meat intake is consistently identified as an important contributor [51,52]. Population-based case-control studies have shown, for example, that red meat intake is associated with an increased risk of p53 mutated cancers [56]. Several cohort and meta-analyses have concluded that meat consumption is associated with relative CRC risks, varying from 1.28 to 1.71 for red meat and 1.20 to 1.84 for processed meat [57–60]. It has been estimated that if average red meat intake is reduced to 70 grams per week, colorectal cancer risk would hypothetically decrease with 7–24% [60].

1.4.3 Meat Consumption and CRC: Possible Causes

The fat content of meat has been investigated as a possible cause for the association between meat intake and CRC but most studies have not supported a specific relation nor was dietary iron intake found to be associated with colorectal adenoma

[52,61–63]. Meat is also a rich source of dietary protein which can increase fecal ammonia concentrations as a result of bacterial fermentation, deamination, and decarboxylation processes. Although high luminal ammonia levels are associated with pro-carcinogenic modifications in colon tissue, convincing epidemiological evidence linking ammonia to CRC is lacking [64].

Another explanation for the relation between CRC and meat intake is the formation of carcinogenic heterocyclic amines (HCAs) in meat following prolonged frying, grilling, or broiling at high temperatures [65–67]. Several studies, using different approaches, have implicated HCAs in CRC risk and it seems likely these compounds play a part in the observed relationship between red meat and CRC risk [52,68]. It is, however, remarkable that no such relation exists between fish or poultry (white meat) consumption and CRC even though HCAs are also formed during the cooking process of these foods [69–72]. Diets high in chicken, for example, result in an increased exposure to HCAs in humans [73]. In fact, long-term consumption of fish or poultry is inversely associated with risk of CRC [57,74].

Although polycyclic aromatic hydrocarbons (PAHs) present in the diet are linked to cancer risks, they can be formed in any type of meat, including white meat, as a result of incomplete combustion processes during cooking [75–77]. Moreover, PAHs are mostly found in smoked and charred foods [76,78] and the contribution of PAHs from the diet to CRC risk remains uncertain because of difficulties in quantifying individual intake [64]. Other compounds present in red meat, but not in white meat, are therefore more likely to be responsible for the association with CRC risk.

A group of compounds that could explain the relation between red or processed meat, but not white meat, and CRC are the N-nitroso compounds (NOCs) since diets high in red or processed meat result in significantly increased levels of fecal NOCs, in contrast to white meat diets [79–83]. Diets high in red and processed meat are also consistently linked with CRC incidence [75,84,85]. Seeing that NOCs are well known for their carcinogenicity in animals [86], this could be an important contributor to CRC development, and it is this group of compounds that will be the focus of this thesis.

2 N-Nitroso Compounds and Carcinogenicity

2.1 Discovery of NOCs as Carcinogenic Agents

NOCs are a class of organic chemicals characterized by a nitrogen-bound nitroso group (-N=O) and were first reported in 1863 [87]. NOCs were not known to have adverse biological effects until 1937, when a case of accidental poisoning was reported but this publication remained largely ignored [88]. Only in 1954, when the first results on toxicity testing of the NOC N-nitrosodimethylamine (NDMA) were

published, did NOCs gain wider attention [89]. NDMA was soon found to be carcinogenic [90], which led to the testing of a number of analogs of NDMA and the subsequent publication of a paper in 1967 on the organotrophic carcinogenic effect of 65 NOCs in test animals [91]. Additional research has so far revealed that out of the approximately 300 NOCs tested for carcinogenicity, about 90% are carcinogenic in animal species in a variety of sites [86].

Unlike other large groups of chemicals, like PAHs, NOCs were initially not considered as being relevant in human carcinogenesis. However, in the 1970s it was realized that the ease of NOC formation from dietary precursors gave them a relevance heretofore unsuspected [92]. The metabolism and formation of NOCs has since received much attention and has led to important insights into the toxicological properties of these compounds, which is crucial to understanding the carcinogenic risk posed by NOC exposure in humans. In the next paragraphs these properties will be described in relation to human exposure and the associated cancer risk.

2.2 NOC Exposure in Humans

The two main classes of NOCs are formed by the nitrosamines and nitrosamides, which are both implicated in human exposure and differ in their mode of genotoxicity. As described later in more detail, nitrosamines are relatively stable compounds and require enzymatic activation to become genotoxic while the unstable direct acting nitrosamides do not. Sub-classifications of both NOC classes include dialkyl, cyclic, and aromatic nitrosamines for the nitrosamine group and alkylnitrosamides, nitrosoureas, and nitrosocarbamates for the nitrosamide group [86]. Nitrosoguanidines are also included in the nitrosamide category, although principally they form a separate group. The general chemical structures of nitrosamines and nitrosamides are $R1CH_2N(NO)CH_2R2$ and $R1CH_2N(NO)C(=O)R2$, respectively. R1 can be an alkyl, aryl or hydrogen moiety, while R2 can have various structures which are often (related to) alkyl, aryl or hydrogen moieties. For certain groups of nitrosamides nitrogen (N) or oxygen (O) is part of the R2 moiety and forms the connection with C(=O). The genotoxic properties of these compounds are conferred by the $R1CH_2$ moiety. Some examples of frequently investigated NOCs are shown in Figure 3.

Human exposure to NOCs mostly includes compounds from the dialkyl, cyclic, and aromatic nitrosamine groups and the nitrosoureas, while the other groups are less relevant. Exposure occurs both exogenously, i.e. preformed NOC from a variety of different sources, and as a result of endogenous formation processes which mostly take place in the GI tract.

2.2.1 Exogenous NOC Exposure and Human Carcinogenic Risks

Average NOC exposure from exogenous sources has been estimated to be approximately 1.10 µmol/day and mainly consists of NOCs found in the diet (72%), occupational exposure (25%), cigarette smoke (2%), and miscellaneous minor sources, such as pharmaceutical products, cosmetics, and indoor and outdoor air (1%) [93–95]. Dietary products that contain NOCs include cured meat products, beer, Scotch whisky, cheese, and fish, some of which have been linked to the etiology of nasopharyngeal, esophageal, stomach, colorectal, and liver cancer [58,96–101]. Dietary NOCs most commonly found in these dietary items are the nitrosamines NDMA, N-nitrosodiethylamine (NDEA), N-nitrosopiperidine (NPIP), and N-nitrosopyrrolidine (NPYR), all of which are carcinogenic in test animals [86,99]. Occupational exposure has mainly been associated with workers in rubber, leather, and metal industries, who are also at an increased risk of several types of cancer, including cancer of the bladder, esophagus, stomach, lung, and leukemia [93,102,103]. This has been related to exposure to NDMA, NDEA, N-nitrosomorpholine (NMOR), and N-nitrosodiethanolamine (NDELA). Tobacco use accounts for the vast majority of NOC exposure in smokers and snuff users and mostly consists of exposure to the nitrosamines N-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Exposure to NNN and NNK could explain the higher incidence of lung, esophageal, pancreatic, and nasopharyngeal cancer in these groups [93,97].

Exposure from exogenous sources used to be considerably higher, especially dietary and occupational exposure, but a number of safety measures implemented in the 1980s led to a significant decrease in daily exposure levels [95]. NOC exposure from exogenous sources has only been linked to the occurrence of nitrosamines. Because of the unstable nature of nitrosamides, it is almost impossible for these chemicals to exist in the diet or other sources [98]. Most of the human nitrosamide exposure might originate from (intragastric) endogenous nitrosation processes, which also contribute significantly to endogenous nitrosamine formation, as described next.

2.2.2 Endogenous NOC Formation and Human Carcinogenic Risks

Although NOC exposure from exogenous sources can be high, especially in tobacco users, it has been estimated that endogenous NOC formation can account for as high as 75% of total human NOC exposure [94]. This can be attributed to the ease of formation of these compounds. The classical reaction resulting in the formation of NOCs is a relatively simple interaction between nitrite and a NOC precursor (NOCP) under acidic conditions, leading to NOCP nitrosation, i.e. addition of a nitroso group (Figure 4). For nitrosamines this involves the formation of N_2O_3, from nitrous acid (HNO_2, the acidic form of nitrite), or N_2O_4 (in combination with dissolved oxygen) which then reacts with nitrosamine precursors. Most of these precursors are secon-

dary amines, although primary and tertiary amines can also become nitrosated. Nitrosamides are formed through reaction of the protonated form of nitrous acid ($H_2NO_2^+$) with nitrosamide precursors, such as alkylamides and ureas [86,93,104,105]. Initially, NOCs were only thought to be formed in the acidic environment of the stomach. However, nitrosation in less acidic or neutral conditions, such as in the duodenum, small intestine and colon, is also possible and can be stimulated by relatively high concentrations of nitrate or nitrite and the presence of denitrifying bacteria that convert nitrate to nitrite and nitric oxide (NO). NO reacts with dissolved oxygen to form N_2O_3 or N_2O_4 which can subsequently nitrosate NOCPs [93,106–112].

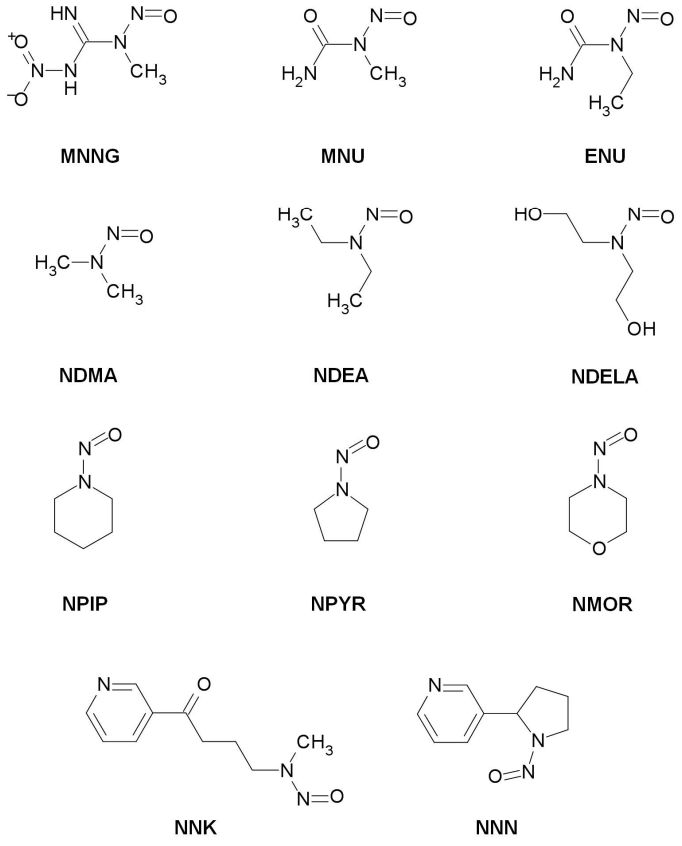

Figure 3: Molecular structures of a selection of frequently investigated NOCs, showing three nitrosamides (top row) and eight nitrosamines. Full names are given in paragraphs 2.1, 2.2.1, and 2.3.

Endogenous NOC exposure in humans is known to occur on a regular basis following the consumption of food that contains the necessary precursors for nitrosation processes to take place in the GI tract. This includes foods with added nitrates and nitrites, such as salt-preserved meat, and foods rich in amines or amides [94,95,113]. As mentioned before, diets high in red or processed meat, but not diets high in white meat, result in significantly increased levels of endogenously formed fecal NOCs [79–83]. Although white meat is also a source of NOCPs, there are indications that the heme content in red meat is an additional factor involved in the endogenous nitrosation process, catalyzing the formation of NOCs [85]. Given that red meat is a richer source of heme iron than white meat, such an effect might go part way towards explaining why the epidemiological correlations are more convincing with red as compared with white meat [75,84,85]. Similarly, the high nitrate and nitrite content of certain processed meats could result in stimulation of endogenous nitrosation processes in the stomach [83,93,114,115]. Diets containing fish in combination with high, but not low, nitrate levels have also been shown to increase urinary NOC excretion [116,117]. Further evidence implicating endogenous NOC formation as a contributor to CRC risk comes from epidemiological studies showing that more than 10 years of exposure to an average nitrate level of >5 mg/l results in an increased risk in subgroups with a high theoretical nitrosation [118,119]. In groups with a low vitamin C intake the odds ratio (OR) for CRC was 2.0 (95% confidence interval [CI]: 1.2–3.3) and for groups with a high meat intake the OR was 2.2 (CI: 1.4–3.6).

Given the nitrosating potential of NO in the GI tract, it is not surprising that the production of NO during inflammation by inducible NO synthase (iNOS) has also been found to result in an increased nitrosation in non-acidic environments [93,120–126]. In people with a medical history of bowel inflammation, CRC risk is also increased (OR: 2.2, CI: 1.1–4.3) [118]. Inflammatory bowel disease (IBD), which is characterized by a persistent state of chronic inflammation of the large intestine, is associated with a strongly increased CRC risk of approximately 5-fold compared to the general population [127–129]. Although there is a large amount of evidence that increased oxidative stress levels associated with chronic inflammation contribute to neoplastic transformation in IBD patients [130], endogenous NOC formation stimulated by NO production may also play a significant part. Inflammatory conditions have indeed been shown to increase NOC formation, both *in vitro* and *in vivo* [131,132]. This notion is supported by the discovery of increased NOC levels in feces of IBD patients [133].

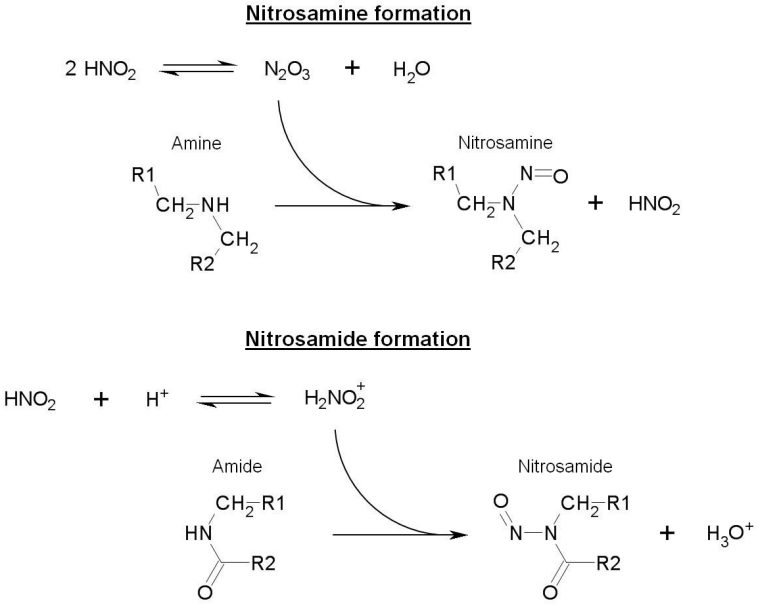

Figure 4: Reaction schemes of nitrosamine and nitrosamide formation.

2.3 Metabolism of NOCs and Molecular Targets in Relation to Human Cancer

The carcinogenicity of NOCs in animals and the cancer risk associated with NOC exposure in humans is generally attributed to their DNA alkylating genotoxic properties. Both nitrosamides and nitrosamines alkylate by formation of a highly reactive alkyldiazonium ion which can react with DNA, RNA, and proteins. However, the formation of this ion differs between both classes. For nitrosamides it is formed through a dealkylation pathway, the first step of which involves alkaline catalysis, which is a spontaneous hydrolysis reaction resulting in the formation of an alkyldiazohydroxide (Figure 5). This molecule further decomposes into the alkyldiazonium ion [93,134]. Dealkylation can be augmented by presence of thiol compounds, such as glutathione and cysteine, although this depends on the type of nitrosamide [135–138]. A denitrosation pathway for nitrosamides has also been described but is not thought to result in genotoxicity [139–142]. The unstable nature of nitrosamides makes them suitable agents to induce tumor formation and nitrosamides like N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), N-methyl-N-nitrosurea (MNU), and N-ethyl-N-nitrosurea (ENU) have frequently been used in animal carcinogenicity studies [143,144]. However, nitrosamines, such as NDMA and NDEA, can also be highly effective carcinogenic agents depending on the metabolic activity of the exposed tissue [106].

Nitrosamine metabolism is dependent on cytochrome P450 (CYP) enzymes which catalyze the formation of α-hydroxynitrosamines (Figure 5). These are unstable compounds which decompose to alkyldiazohydroxides and subsequently follow the same dealkylation path as the nitrosamides [145,146]. A competing denitrosation pathway also exists for the nitrosamines resulting in non-genotoxic detoxification [146,147]. Several different CYP enzymes have been described to activate nitrosamines by α-hydroxylation, the most important of which is CYP2E1, mostly involved in the activation of dialkylnitrosamines [148,149]. Activation of other, more complex, nitrosamines occurs through the activity of several more CYP enzymes, including CYP2A4, 2A5, 2A6, 2A13, 2B1, 2C8, 2C9, 2C19 and 3A4 [148–153]. Activity of these enzymes is organ specific and the specificity of each CYP enzyme differs per nitrosamine which explains the organ specific tumor induction of nitrosamines whereas nitrosamides mostly cause tumors at the site of administration [86,134,154].

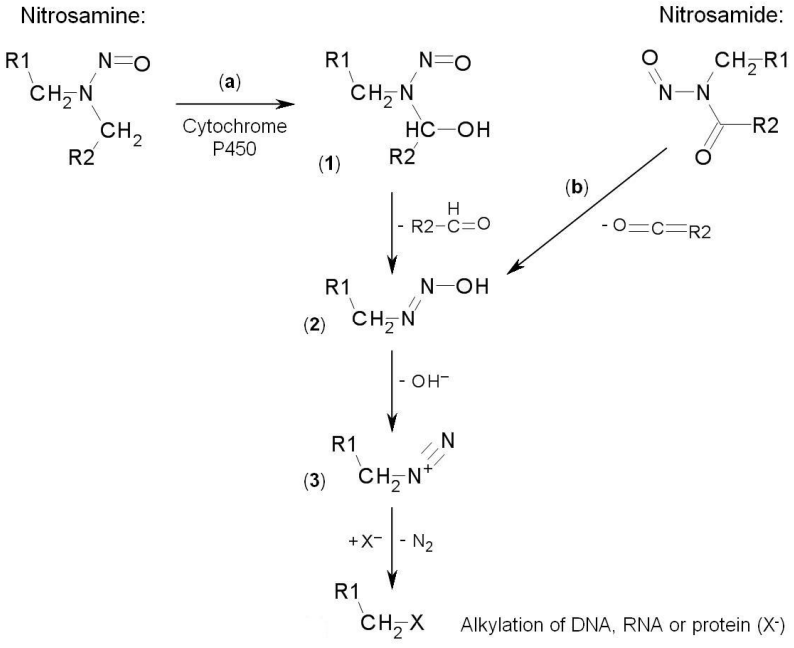

Figure 5: Reaction scheme of nitrosamine (**a**) and nitrosamide (**b**) metabolism. Different metabolites are shown by: (**1**), α-hydroxynitrosamine; (**2**), alkyldiazohydroxide; (**3**), alkyldiazonium ion.

The reactive nature of the alkyldiazonium ion results in most of the ions being detoxified by reaction with water and only a small fraction eventually alkylates nu-

cleophilic molecules, such as DNA [134,155]. Alkylation of DNA has been described for all four DNA bases, but the most important alkylations from a mutagenic and carcinogenic perspective are the O^6 position of guanine and the O^4 position of thymine [134,154,156–159]. Formation of O^6-alkylguanines results in G:C→A:T transitions, while O^4-alkylthymines give rise to T:A→G:C transitions. Genotoxic and mutagenic effects related to the induction of these adducts have been described for most NOCs in a number of different assays, including the sister chromatid exchange assay, hypoxanthine-guanine phosphoribosyl transferase assay, and the Ames test [86,160]. Although alkylation of the N^7 position of guanine is quantitatively the most important (approximately 10–100 times higher as other adducts), it is not associated with carcinogenesis [95,161]. N^7-alkylguanine is, however, often used as a marker of exposure to alkylating agents because of its high induction rate in DNA and because it is repaired relatively slowly [162–166]. O^6-alkylguanines are repaired much more efficiently by the O^6-alkylguanine DNA-alkyltransferase (MGMT) repair enzyme and, although they are present in DNA in smaller amounts, they are also commonly used as a marker of NOC exposure in both animals and humans [164,167–171].

In addition to the well established alkylating effect of NOCs, both nitrosamides and nitrosamines have also been described to have oxidative stress-inducing properties which is related to their metabolism and can lead to the induction of oxidative DNA lesions, such as 8-oxodeoxyguanosine [172–175]. Oxidative damage may also contribute to NOC carcinogenicity, although alkylation is probably the most relevant type of NOC-induced DNA damage from a human carcinogenic perspective [145]. For example, human NOC exposure through tobacco smoke and nitrate-cured meat is associated with increased levels of methylation adducts [176–179]. There is also evidence to suggest that elevated levels of O^6-methylguanine (O^6-meG) occur in the regions of the large intestine where most neoplasms occur, i.e. the sigmoid colon and rectum [180]. Furthermore, low MGMT activity has been associated with colorectal tumors in humans [181–186].

Further evidence that NOC exposure-associated alkylation plays an important role in human cancer etiology comes from the extrapolation of animal data to humans. NOC carcinogenicity has been demonstrated in more than 40 different animal species, including primates, which already makes it highly unlikely that humans are not susceptible to these effects [86]. This is supported by a few cases of NDMA poisoning in humans where acute liver toxicity and formation of O^6-meG were some of the observed symptoms, comparable to toxicity effects found in experimental animals, thereby suggesting that human NOC exposure results in a similar toxicological response, which may include cancer [187–189]. Patas monkeys, which closely resemble humans with regard to CYP metabolism [190,191], display NDMA-induced O^6-meG lesions in a wide spectrum of tissues, including colorectal tissue [167] and these animals are also susceptible to NOC-induced cancer [192]. Additional evi-

dence that test animals and humans display similar responses with respect to DNA alkylation was given by Souliotis *et al*. [193] and Kyrtopoulos [168] who found that human susceptibility to accumulation of O^6-meG in blood leukocytes at chronic low doses of an alkylating agent is comparable to that of the rat. Extrapolation of these data also suggested that the alkyl adduct levels observed in blood DNA from healthy non-smoking individuals would have to be derived from endogenous formation since these levels were higher than what could be expected to come from exogenous sources, as based on data on exogenous exposure in the rat studies. Chronic exposure to low nitrosamine concentrations in rats is also known to result in carcinogenicity [194,195] and, under the assumption that the comparison between rats and humans is justified, chronic exposure in humans to low NOC concentrations may well have carcinogenic consequences.

Overall, there is much evidence available that humans are exposed to considerable amounts of NOCs through exogenous exposure and endogenous nitrosation and that most of these exposures are indirectly linked to cancer risks in a variety of organs. In populations with a high red and processed meat consumption and bowel inflammation CRC risks are increased, which can be linked to a higher rate of endogenous exposure in the colon. Although epidemiological research and extrapolation of animal data are compatible with a carcinogenic role of NOCs in humans, this evidence is far from conclusive. Moreover, there is very little information available on the toxicological properties of NOCs in exposed humans which is vital to arrive at a more accurate risk assessment. Since carcinogenicity studies in humans are not possible, additional research will have to focus on modifications in molecular mechanisms that can be linked to NOC exposure in humans and which are indicative of a potential carcinogenic effect. For this reason there is a need to add molecular biological methods to the assessment of NOC exposure-associated effects on man, with particular emphasis on carcinogenesis in the colon. Whole genome based microarray analysis, which can provide a large amount of data on molecular mechanisms associated with exposure to toxicological compounds, is a valuable tool to address this issue.

3 Whole Genome Based Microarray Analysis in Toxicology and Nutrition

Gene expression analysis using microarrays is a high-throughput technology that allows for the simultaneous analysis of the expression level of thousands of genes. The principle of microarray analysis is based on the hybridization of a nucleic acid sample from cells or a tissue to a large set of oligonucleotide probes, which are spotted onto a glass slide (the microarray) to detect variations in expression. For

this, mRNA is isolated from cells or tissue and amplified into fluorescent labeled cRNA or cDNA, depending on the type of array. Each cRNA or cDNA hybridizes to its complementary sequence probe on the microarray and the fluorescent signal of all spotted genes is subsequently measured in a scanner. By labeling nucleic acids from a control and an exposed sample differently and hybridizing them to the same slide, differences in transcription levels can de detected. Alternatively, a one-color approach can be used where all samples are hybridized against separate arrays and compared with each other [196].

The different levels of transcribed genes at a particular moment in time, the transcriptome, constitute a gene expression profile which provides information on individual genes and how these relate with regard to bio-molecular pathways. Since these expression levels can be subject to change, for example as a result of exposure to a toxicological compound, this provides a way of monitoring differentially regulated genes and pathways which is helpful in understanding the mechanism of toxicity of the compound under investigation (toxicogenomics) [197,198]. Similarly, in nutrition research effects of certain diets or supplements can be investigated which is based on the concept that dietary chemicals directly or indirectly modulate the expression of genes (nutrigenomics) [199,200]. This can be directed towards the identification of detrimental gene expression changes following the consumption of particular diets, which could explain the progression from a healthy phenotype to a disease phenotype associated with these diets. Alternatively, beneficial gene expression modulations induced by a dietary intervention can also be investigated.

The identification of patterns of differential regulation could also serve as a transcriptomic biomarker for the involvement of specific bio-molecular pathways. For example, the determination of gene expression profiles induced by compounds in an *in vitro* model system could be used as a biomarker to identify similar profiles in tissues of interest of human subjects exposed to these compounds through the diet. In a different approach, pivotal gene expression changes between health maintenance and disease progression could be determined [199,201]. This could be reached by determining biomarkers of exposure to toxicological compounds in humans, like blood metabolite or urinary excretion levels, and linking these to transcriptomic profiles in surrogate or target tissues. If such transcriptomic profiles are also found in the disease state (e.g. cancer) or linked to a marker predicting disease state than these profiles could be promising as biomarkers for exposure-associated disease.

Research on whole genome gene expression modifications induced by NOC exposure has been very limited. Identifying modified genes and their involvement in bio-molecular pathways could therefore lead to a new level of insight into the toxicological properties of NOCs and possible implications for their carcinogenic risk in humans. Based on the applications outlined above, several study designs will be used in this thesis to further assess the role of NOCs in human carcinogenesis.

4 Aim and Outline of the Thesis

Many tests on *in vitro* mutagenicity and animal carcinogenicity have linked NOC exposure with cancer development. However, it is still not clear whether NOC exposure is also accompanied by any detrimental health effects in humans. Many studies have convincingly demonstrated that human exposure to NOCs in the colon occurs on a daily basis, either directly through food or indirectly through endogenous nitrosation of NOCPs. Furthermore, the increased CRC risk associated with a high red meat intake suggests a possible link with NOC exposure, but there have been no studies in humans that link NOC exposure to markers of a carcinogenic effect. Therefore, this thesis aims to improve the understanding of the molecular mechanisms of NOC-induced toxicity in humans in relation to CRC.

The introduction of microarray-based whole genome gene expression analysis has led to a high throughput monitoring of changes in gene expression level of multiple genes simultaneously. Given the large amount of data that can be generated from this technology, microarrays can be helpful in answering the question whether NOCs are relevant in human carcinogenicity in a number of different ways. Extrapolation of results from a toxicogenomics analysis of NOC exposure in an *in vitro* system could reveal molecular mechanisms of NOCs that play a role in the carcinogenic process. Furthermore, NOC-specific profiles may be identified that signify exposure in human tissues. *In vivo* transcriptomic analyses directed at finding gene expression modifications associated with markers of NOC exposure could also provide valuable insights in NOC-induced mechanisms in tissue from human individuals which could influence carcinogenic pathways. Similarly, a genomics approach focused on finding differentially modified genes or molecular pathways following dietary intakes that increase NOC exposure, such as red meat, may offer an explanation for the increased cancer risk associated with such diets.

Therefore, we hypothesize that whole genome microarray-based gene expression analysis will further elucidate the role of NOCs in human CRC development. The objective of this thesis is to use gene expression analysis, in combination with markers of NOC-induced effects, to gain more insight in the possible carcinogenic potential of NOCs in humans, with particular emphasis on colon carcinogenicity.

To reach this objective, a combination of *in vitro* and *in vivo* studies is used to address the issue of NOC exposure-induced molecular events in relation to possible implications for colon carcinogenesis in humans. Since very little is known on NOC-induced gene expression modifications in general, the first half of this thesis focuses on determining the genomic response to NOC exposure in an *in vitro* model for the human colon. For this, the human colon adenocarcinoma cell line Caco-2 is first exposed for 24 hours to genotoxic concentrations of six different NOCs after which microarray analyses is performed. To distinguish between possible differences in mechanism of action of the two most important classes of NOCs, i.e. nitrosamides

and nitrosamines, two nitrosamides are tested, i.e. MNNG and MNU, and four nitrosamines, i.e. NDMA, NDEA, NPIP, and NPYR (see also Figure 3). These initial experiments are described in **Chapter 2**. Since knowledge on radical mechanisms associated with NOC exposure in living tissue is limited and these mechanisms may contribute to the genotoxic and carcinogenic properties of NOCs, the radical generating properties of the six NOCs analyzed in the first study are subsequently investigated. As described in **Chapter 3**, by means of electron spin resonance (ESR) analysis, differences between nitrosamines and nitrosamides with respect to radical generation in a cellular system are compared and coupled with gene expression modifications at 1 and 24 hours of exposure to determine the development of associated transcriptomic responses over time. The time-dependent development of gene expression levels induced by the six selected NOCs is analyzed in more detail in **Chapter 4**, with the emphasis on identifying nitrosamide-induced gene expression changes at earlier time points (1 and 6 hours) in light of their unstable and reactive nature. Moreover, transcriptomic profiles specifically linked to methylation levels induced by MNNG, MNU, and NDMA are investigated to link gene expression changes with pro-carcinogenic alkyl-adduct formation.

The second half of this thesis focuses on three transcriptomics-based *in vivo* studies to assess NOC-associated carcinogenic risks in humans. The first of these studies, presented in **Chapter 5**, aims at investigating the relationship between human NOC exposure and MN formation, as a marker of carcinogenic effect, in relation to associated gene expression changes, using lymphocytes as a surrogate tissue. This is studied in human individuals not known to have experienced a higher than average level of endogenous nitrosation and who thus represent the general population with regard to NOC exposure levels. Particular emphasis is given to the identification of genes which could be suitable as mechanistic-based transcriptomic biomarkers of NOC exposure in relation to MN formation.

Human subjects expected of having a higher than average rate of endogenous nitrosation are investigated in the next two chapters. As described in **Chapter 6**, colon tissue from patients diagnosed with IBD is compared with tissue from control subjects to find gene expression modulations that are indicative of NOC exposure by linking the genomics response to endogenous nitrosation levels in the colon. The final study, described in **Chapter 7**, focuses on the effect of a diet high in red meat on NOC formation and fecal water genotoxicity in relation to transcriptomic changes induced in colonic tissue of IBD and control subjects to clarify the role of NOCs in red meat consumption-associated CRC risk in humans.

Chapter 8 presents a summary and general discussion of the major findings of all studies presented in the thesis and focuses on the implications of this research with regard to the carcinogenic potential of NOCs in humans and possibilities for future research.

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Chapter 2

Molecular signatures of N-nitroso compounds in Caco-2 cells: implications for colon carcinogenesis

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Toxicological Sciences 2009;108(2):290-300

Abstract

N-nitroso compounds (NOCs) are genotoxic, carcinogenic to animals, and may play a role in human cancer development. Since the gastro-intestinal tract is an important route of exposure through endogenous nitrosation, we hypothesize that NOCs exposure targets genetic processes relevant in colon carcinogenesis. To investigate these genomic responses, we analysed the transcriptomic effects of genotoxic concentrations of two nitrosamides, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG, 1µM) and N-methyl-N-nitrosurea (MNU, 1mM), and four nitrosamines, N-nitrosodiethylamine (NDEA, 50mM), N-nitrosodimethylamine (NDMA, 100mM), N-nitrosopiperidine (NPIP, 40mM), and N-nitrosopyrrolidine (NPYR, 100mM), in the human colon carcinoma cell line Caco-2. Gene Ontology gene group, consensus motif gene group and biological pathway analysis revealed that nitrosamides had little effect on gene expression after 24 hours of exposure, whereas nitrosamines had a strong impact on the transcriptomic profile. Analyses showed modifications of cell cycle regulation and apoptosis pathways for nitrosamines which was supported by flow cytometric analysis. We found additional modifications in gene groups and pathways of oxidative stress and inflammation, which suggest an increase in oxidative stress and pro-inflammatory immune response upon nitrosamine exposure, although less distinct for NDMA. Furthermore, NDEA, NPIP and NPYR most strongly affected several developmental motif gene groups and pathways, which may influence developmental processes. Many of these pathways and gene groups are implicated in the carcinogenic process and their modulation by nitrosamine exposure may therefore influence the development of colon cancer. In summary, our study has identified pathway modifications in human colon cells which may be associated with cancer risk of nitrosamine exposure in the human colon.

Introduction

More than 5 decades ago it was discovered that N-nitroso compounds (NOCs) were carcinogenic in test animals [1], raising the question whether exposure to this class of compounds might also be relevant for the development of human cancer. Additional research demonstrated the occurrence of NOCs in the environment and certain types of food, such as beer, fish, and nitrite-preserved meat [2]. Furthermore, multiple bacterial mutagenicity studies and animal experiments resulted in dozens of NOCs being classified by the National Toxicology Program as "reasonably anticipated to be a human carcinogen" [3]. NOCs can also be formed endogenously by nitrosation of dietary precursors, a process that occurs predominantly in the gastrointestinal tract [4,5]. As a consequence, measures have been taken to reduce dietary and environmental exposure to NOCs [6].

NOCs can be divided into two main sub-classes, nitrosamines and nitrosamides [7], which can both induce alkylating DNA damage by formation of a highly reactive diazonium ion [2,5]. DNA bases are alkylated by diazonium ion species at the N^7 and O^6 positions of guanine and the O^4 position of thymine. O^6-alkylguanine adducts have been identified as the main pre-mutagenic lesion and cause GC-AT transition mutations [5]. O^4-alkylthymine results in TA-GC transitions, while N^7-alkylguanine adducts have not been shown to be mutagenic [8].

There are, however, several indications for differences in modes-of-action between nitrosamines and nitrosamides. Cyclic nitrosamines can also form similarly reactive cyclic oxonium ions [9]. Further, while nitrosamides can spontaneously decompose to form the diazonium ion, nitrosamines require metabolic activation through α-hydroxylation by several P450 enzymes [5]. Additionally, nitrosamines may release nitric oxide radicals ($^{\bullet}NO$) under certain conditions and generate formaldehyde during metabolism, which may ultimately lead to deamination and hydroxymethyl adduct formation [10,11].

Evidence of NOC involvement in human cancer development is mostly based on epidemiological studies and data on endogenous nitrosation. However, further studies are required to establish whether or not NOCs are true human carcinogens [5,12]. While the genotoxic and carcinogenic effects of NOCs are usually attributed to their DNA damaging properties, little is known about other molecular events caused by these compounds which may also be relevant in the carcinogenic process in humans.

Microarray-based gene expression analysis provides a valuable tool for identifying different modes of action and molecular responses to NOC exposure. Although there are a few animal studies investigating gene expression modulation in NOC-induced tumor or pre-neoplastic tissues, little is known on gene expression modifications after NOC exposure in human cells or tissues [13,14]. Therefore, the analysis of such genomic responses may be indicative of the role of NOCs in human carcino-

genesis. Since the gastro-intestinal tract presents the first target of dietary or endogenously formed NOCs and the colon has been identified as an important site for endogenous nitrosation it is of relevance to investigate gene expression modifications in colon cells after NOC exposure [4]. Within this respect, previous animal studies have implicated nitrosamides in the development of colon cancer [15], while nitrosamines are known to be genotoxic in intestinal cell lines [16].

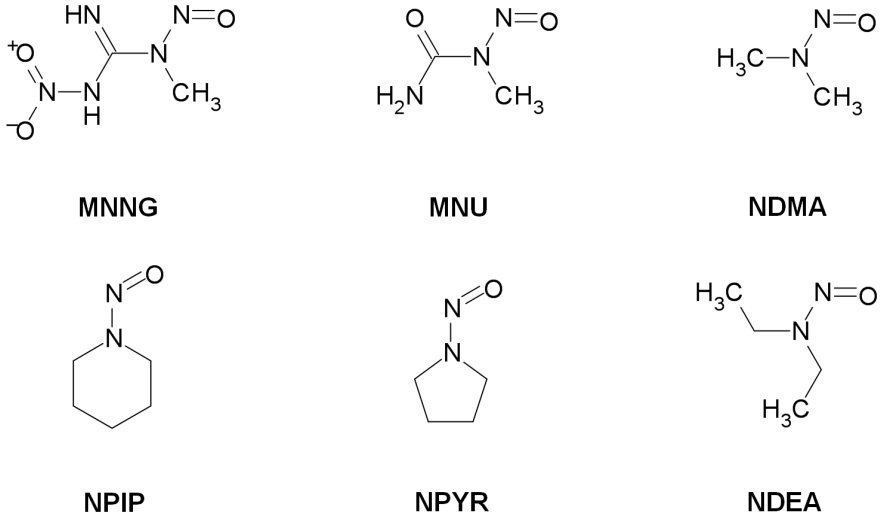

Figure 1. Molecular structures of the six NOCs used in this study.

We therefore hypothesize that analysis of molecular pathways modified by NOCs will help to determine the role of these compounds in human colon carcinogenesis. To investigate this, we analysed genome-wide gene expression in the human colon adenocarcinoma cell line Caco-2 after exposure to different NOCs. Caco-2 cells were exposed to six NOCs known to be relevant for human exposure (Figure 1): the nitrosamines N-nitrosodiethylamine (NDEA), N-nitrosodimethylamine (NDMA), N-nitrosopiperidine (NPIP), and N-nitrosopyrrolidine (NPYR) and the nitrosamides N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and N-methyl-N-nitrosurea (MNU) [2]. By comet assay analysis, we selected non-cytotxic, equally genotoxic concentrations of these NOCs to identify corresponding modifications of transcriptomic levels which may reveal genetic processes relevant in the carcinogenic process and also may discriminate between different NOCs. Various data analysis techniques were used to identify NOC-induced modulations of gene groups. Individual gene expression data were also linked with levels of apoptosis and cell

cycle distribution as phenotypic markers of effect to functionally anchor gene sets differentially modified by NOC exposure.

Material and methods

Cell culture

The human colon adenocarcinoma cell line Caco-2 was cultured in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, Zwijndrecht, The Netherlands) with 4.5g/L glucose, L-glutamine, $NaHCO_3$ and pyridoxine HCl supplemented with 1% (v/v) non-essential amino acids, 1% Na-pyruvate, 1% penicillin/streptomycin and 10% (v/v) heat-inactivated foetal calf serum, all purchased from Gibco BRL (Breda, The Netherlands). Cell cultures were transferred weekly by trypsinization and incubated at 37°C in a humidified incubator containing 5% CO_2.

Treatment and isolation of cells

Caco-2 cells were treated with increasing concentrations of MNNG, MNU, NDEA, NDMA, NPIP and NPYR or the solvent control (MilliQ or DMSO, final concentration 0.1%) in 6-wells plates or 25 cm^2 culture flasks for 24 hours. All NOCs were obtained from Sigma-Aldrich (the respective product numbers and reported purity were: 15427LO (97%), N4766 (11% H_2O, 3% acetic acid), N0756, N7756, N6007 (99.8%), and 158240 (98.9%)). Experiments were carried out in quadruplicate. Cells were isolated by trypsinization and suspended in PBS and subsequently placed on ice (for comet assay) or fixed in methanol and stored at -20°C (for flow cytometry). Cells for microarray experiments were isolated with TRIzol® Reagent (Invitrogen, Breda, The Netherlands), which stabilizes RNA, and stored at -20°C. Viability of cells was determined with the MTT test as described by Mosmann [17] with minor modifications.

Immunocytochemistry and flow cytometric analysis

Methanol-fixed cells were indirectly stained for cleaved cytokeratin 18 (an early sign of apoptosis) using the primary antibody M30 CytoDeath (Roche, Penzberg, Germany) and subsequently analysed using a FACSort (Becton Dickinson, Sunnyvale, USA) equipped with an Argon ion laser and a diode laser as described by Schutte *et al.* [18]. Data analysis was performed using CellQuest software (version 3.1, Becton Dickinson, San Jose, USA). Data were gated on pulse-processed PI signals to exclude doublets and larger aggregates from analysis. M30 CytoDeath positive and negative signals were sorted in the gated population and displayed as percentage of total cells. In addition, cell cycle distribution was determined using ModFIT LT (version 2.0, Verity Software House, Topsham, USA).

Alkaline single-cell gel electrophoresis (comet assay)

The alkaline comet assay allows for the detection of single and double strand breaks as well as abasic sites and sites where DNA repair is taking place, which are all expected to be induced by NOC exposure. The assay was performed as described by Singh *et al*. [19] with minor modifications. Comets were visualized using a Zeiss Axioskop fluorescence microscope (at 200× magnification). Randomly, 50 cells were analysed using the Comet assay III software (Perceptive Instruments, Haverhill, UK). DNA damage was expressed as tail moment (TM, the product of tail DNA content and mean tail migration distance).

RNA isolation and purification of microarray samples

Test concentrations for microarray experiments were selected based on the induction of genotoxicity as assessed by comet assay, as well as on the absence of cytotoxicity as evaluated by means of MTT tests (Table 1). The lowest concentrations resulting in a significant increase in DNA damage (two-tailed Student's *t*-test, *p*-value <0.05) in the comet assay, and having a TM between 0.5–3.5 (i.e. moderate DNA damage levels) and a viability >90%, were selected. These concentrations were: MNNG (1μM), MNU (1mM), NDEA (50mM), NDMA (100mM), NPIP (40mM) and NPYR (100mM). RNA was isolated from TRIzol® suspended cells according to the manufacturer's protocol with minor modifications, followed by a clean up, using an RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) with DNase treatment. RNA quantity and purity were determined spectrophotometrically. RNA quality was assessed by automated gel electrophoresis on an Agilent 2100 Bioanalyzer (Agilent Technologies, Amstelveen, The Netherlands). All samples were pure and free of RNA degradation.

Target preparation, hybridization and scanning procedure

The Two-Color Microarray-Based Gene Expression Analysis kit from Agilent Technologies (Amstelveen, The Netherlands) was used to generate Cyanine (Cy) labelled cRNA according to the manufacturer's protocol. Two of the quadruplicate test and reference samples were labelled with Cy3 and Cy5 (PerkinElmer, Norwalk, USA), while the remaining two test and reference samples were dye-swapped. Hybridization was carried out on Agilent 4x44K Whole Human Genome microarrays. Dye incorporation rates were used to put appropriate amounts of Cy3 and Cy5 labelled test and reference samples together (i.e. 10 pmol Cy3 vs. 10 pmol Cy5). After hybridization and washing slides were dried applying a controlled N_2 gas flow before scanning.

Slides were scanned on a GenePix® 4000B Microarray Scanner (Molecular Devices, Sunnyvale, USA). Cy3 and Cy5 were excited at wavelengths of 532 and 635

nm, respectively. Laser power was set to 100%. The photo multiplier tube gain was set to a saturation tolerance of 0.02% to minimize background and saturated spots. The images obtained (resolution 5 micron, 16 bit tiff image) were processed with Imagene 8.0.1 software (Biodiscovery, El Segundo, USA) to measure mean signal intensities for spots and local backgrounds followed by a quality control in Microsoft Excel (Microsoft Corporation, Redmond, USA). Poor spots were flagged manually and automatically.

Data preparation and normalization

Data were further processed in GeneSight 4.1 (Biodiscovery). Flagged spots were excluded from analysis. For each spot, mean local background intensity was subtracted from mean signal intensity, and spots with a mean net signal of <10.0 were omitted from analysis. Background corrected mean intensities were log2 transformed. Normalization of expression differences was performed using LOWESS and the replicates of each gene were combined to a mean expression difference with exclusion of outliers (beyond 2 standard deviations). The expression difference for each spot was calculated by subtracting the log2 transformed mean intensity of the control sample from the log2 transformed mean intensity of the treated sample resulting in a log2 ratio.

Data analysis

T-profiler analyses were performed to identify responsive gene groups within the complete gene expression profiles, without the need for selection of significantly modulated genes. T-profiler uses the unpaired *t*-test to score changes in average transcriptomic activity of pre-defined groups of genes within a single expression profile and expresses the significance of the change as a *t*-value [20,21]. Adjusted *p*-values (*E*-values) are calculated and gene groups with an *E*-value of ≤0.05 are considered to be significantly regulated. T-profiler analyses were performed with log2 ratio data to identify gene groups based on Gene Ontology (GO) and consensus motif/transcription factor categorization. GO categories with over 1000 genes were excluded since they provide little relevant information.

For further pathway analysis of significantly modulated genes (two-tailed Student's *t*-test, *p*-value <0.05, unadjusted *p*-values) two repositories of pathways were used. MetaCore™ (GeneGo, San Diego, USA) is an online software suite that identifies and visualizes the involvement of differentially expressed genes in specific cellular pathways, which is subsequently related to the total amount of genes involved in the particular pathway and in all the available pathways combined. Pathways with a *p*-value <0.05 were considered significantly modulated. The second repository of pathways was PathVisio version 1.1 beta [22]. PathVisio calculates a *z*-score, based

on a hypergeometric distribution, for each biological pathway term. Pathways with a *z*-score >2 were considered significantly modulated. The reason for combining MetaCore and PathVisio is that, since both repositories contain different pathways, more information may be retrieved from the database.

The online software suite GenePattern version 3.1 (http://www.broad.mit.edu/cancer/software/genepattern/) was used for hierarchical clustering analysis (HCA) and principal component analysis (PCA) of the tested compounds. HCA was performed using the unweighted average as a clustering method and Pearson correlation as a similarity measure. PCA plots were generated in GenePattern by reducing the data to two dimensions and visualized in Microsoft Excel.

Spearman's rank correlations between gene expression and levels of apoptosis or cell cycle distribution were calculated using the online Gene Expression Profile Analysis Suite (GEPAS, http://gepas.bioinfo.cipf.es/). Ratios of apoptosis or cell cycle phase percentages versus their respective controls were first calculated and subsequently log2 transformed to obtain a data distribution similar to the log2 gene expression values. These transformed values were then correlated with the gene expression data.

A supplementary data Microsoft Excel file is available online and can be found at: http://dl.dropbox.com/u/11005104/Thesis%20Dennie%20Hebels%20Supplementary%20Data%20Chapter%202.xls.

Results

Transcriptomic response at selected genotoxic NOC concentrations

For microarray experiments a suitable concentration of each NOC was first determined by testing a dosis range of 0.1 µM – 100 mM in several assays. The goal was to select a genotoxic concentration, which did not result in a cytotoxic effect in the MTT test. The lowest concentrations resulting in a significant increase in DNA damage in the comet assay, and having a TM between 0.5–3.5 (i.e. moderate DNA damage levels) and a viability >90%, were selected. Final concentrations were 1µM MNNG, 1mM MNU, 50mM NDEA, 100mM NDMA, 40mM NPIP and 100mM NPYR. Corresponding viability and genotoxicity levels are presented in Table 1. Main target organs for carcinogenicity are also reported for each NOC.

Modulation of gene expression by the selected genotoxic NOC concentrations was subsequently analysed on microarrays to elucidate the underlying modes of action of these compounds and to detect and identify differential gene expression profiles. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus [23] and are accessible through GEO Series accession number GSE14284 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE14284). An initial analysis of

differentially expressed genes revealed that a much smaller number of genes was significantly modulated by the nitrosamides MNNG and MNU (357 and 744 resp.) compared to the nitrosamines NDEA, NDMA, NPIP, and NPYR (10271, 5440, 9847 and, 12609 resp.). In fact, the number of genes modulated by these nitrosamides is smaller than the number of false positives that could be expected at a 5% significance level (2200 genes on a 44K array).

Table 1: Main target organs, viability and genotoxicity (mean ± SD) of Caco-2 cells after 24 hours of exposure to NOC concentrations used for microarray testing.

NOC	Main target organs[a]	Concentration	Viability (%)[b]	Genotoxicity (TM)[c]
MNNG	Stomach	1 µM	106	0.89±0.52
MNU	Stomach, nervous system	1 mM	107	1.92±0.98
NDEA	Liver, esophagus, nasal	50 mM	105	0.70±0.43
NDMA	Liver, kidney, lung	100 mM	100	3.03±1.17
NPIP	Esophagus, nasal, liver	40 mM	103	2.96±2.08
NPYR	Liver, lung	100 mM	98	1.34±0.58

[a] From ref. [15]. [b] Viability as compared to control. Viability at NOC concentrations shown did not significantly deviate from control levels. [c] Genotoxicity for blank and DMSO control were 0.05±0.05 and 0.06±0.04 respectively. Genotoxicity at NOC concentrations shown were all significantly increased versus control levels (*p*-value <0.05).

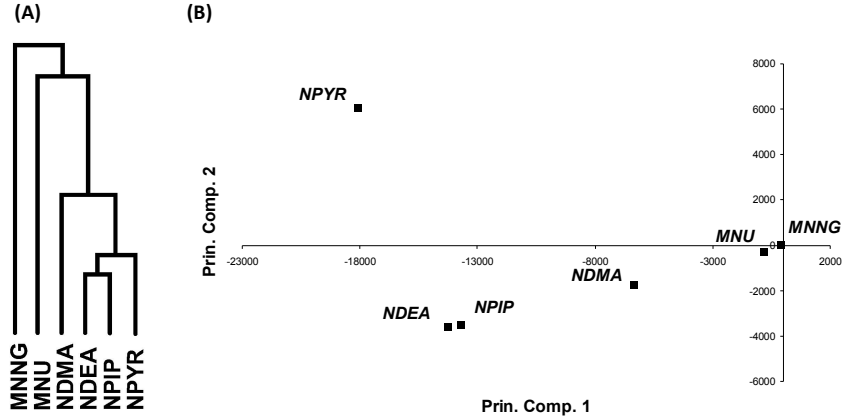

Figure 2: Dendogram of Pearson correlation HCA **(A)** and PCA plot **(B)** for genes significantly modulated for at least one of the six NOCs. The percentage variation for principal components 1 and 2 were 81.22 and 6.87%.

To visualize the differences in gene expression profiles between NOC we first performed a Pearson correlation HCA on genes significantly modulated (*p*-value

<0.05) for at least one of the six NOCs. The clustering dendogram (Figure 2A) shows a distinction between the four nitrosamines and two nitrosamides. MNNG and MNU cluster separately from the nitrosamines and each other. Although the NDMA and NPYR expression patterns cluster within the nitrosamine group, the corresponding PCA plot (Figure 2B) shows that both compounds dissociate from the other two nitrosamines, a pattern that is analogous to the number of significantly modulated genes. The PCA also shows the nitrosamides grouping together near the origin, indicating little effect on gene expression levels.

Gene group and pathway identification

T-profiler, MetaCore and PathVisio were used to identify significantly modulated gene groups and pathways. Here only a selection of results is presented. Complete gene group and pathway lists and additional data can be found in the supplementary data Excel file.

For a functional interpretation of all expressed genes, without pre-selection, we first performed a T-profiler analysis on gene groups. GO categories, significantly modulated in T-profiler for at least one of the six NOCs and with less than 100 genes per group, are listed in Table 2A. The nitrosamides (MNNG and MNU) failed to modulate any GO gene groups, while nitrosamines affected a wide variety of categories. All nitrosamines caused strong up regulations of genes in the cadmium and copper ion binding groups. NPIP also shows strong up regulation of genes involved in protein folding. Genes were mostly down regulated by all nitrosamines in two mitochondrion related gene groups, which mainly contain ribosomal proteins and NADH dehydrogenases and some apoptosis related genes. Effects on protein synthesis were also evident by modulation of the gene groups Nucleolar part, Ribosome biogenesis and assembly, rRNA metabolic process, rRNA processing and tRNA processing. Oxidoreductase activity is modified by NDEA and NPIP, while alkyl/aryl transferase activity is only modified by NPYR. NPYR also uniquely modified genes associated with FAD binding, biosynthesis of glutamine family amino acids and glutathione transferase activity.

GO gene groups significantly regulated (p-value <0.05) for at least one of the six NOCs, generated by T-profiler, were subsequently used for Pearson correlation HCA. As shown in Figure 3A the gene groups again show a discrimination between nitrosamides and nitrosamines, but in comparison to Figure 2A nitrosamines cluster better. The accompanying PCA plot (Figure 3B) also shows the nitrosamines grouping together indicating comparable effects on a functional level, although NDMA is located more distinctly. MNU and MNNG clearly form a separate group.

T-profiler was also used to perform an analysis on motif/transcription factor (TF) associated gene groups, which are defined as genes with a match to a particular consensus motif within 600 base pairs upstream of the open reading frame (ORF)

allowing no overlap with neighbouring ORFs [20]. Most commonly affected motif gene groups, sorted by similarity in biological function, are shown in Table 2B. Most motif gene groups were considered up regulated and the genes in these groups contained motifs specific to TFs that are involved in many different biological functions. The Forkhead box (FOX) TFs, for example, are involved in the regulation of cell cycle, apoptosis, oxidative stress and developmental processes, including embryogenesis. Other TFs, such as CREB1 and the POU family, are implicated in neuronal development, while the ATF family is associated with several aspects of the stress response, including oxidative stress. There were also eight gene groups with binding motifs associated with homeobox containing genes, involved in development. Down regulation of genes with TF motifs for CDC5L, the E2F family and MYC/MAX mainly involved regulation of the G1/S transition, apoptosis and DNA repair. Nitrosamines were again most strongly involved in gene expression differences, while MNNG only affected two gene groups associated with development and homeobox gene expression. MNU was not involved in any gene group modifications. The complete list of modified GO and motif gene groups can be found in the supplementary data (worksheet 1 and 2).

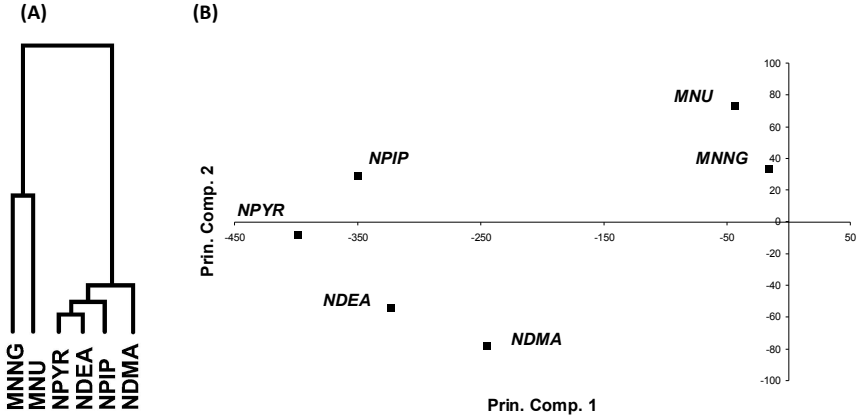

Figure 3: Dendogram of Pearson correlation HCA **(A)** and PCA plot **(B)** for T-profiler GO gene groups significantly regulated for at least one of the six NOCs. The percentage variation for principal components 1 and 2 were 91.59 and 4.39%.

We subsequently performed additional pathway analyses using MetaCore and PathVisio to further elucidate modifications of the underlying pathways and their theoretical effects on cellular processes. Theoretical effects were determined by individually assessing the effects of up and down regulated genes in each pathway. The nitrosamides were excluded from this analysis since the number of significant

genes was beneath the false positive threshold. A complete list of modulated pathways can be found in the supplementary data (worksheet 3 and 4).

Table 2. T-profiler analysis of: **(A)** GO gene groups with <100 genes; **(B)** most common motif gene groups and their associated transcription factors (TF) and biological functions.

(A)

GO gene groups	NOC	*t*-values	No. of genes
Cadmium ion binding	3/4/5/6	13/5.9/5.25/12.12	6
Copper ion binding	3/6	6.08/6.45	57
Response to protein stimulus / unfolded protein	5	6.1	44
FAD binding	6	-4.58	58
Glutamine family amino acid biosynthetic process	6	-4.88	16
Glutathione transferase activity	6	-4.4	19
Glycolysis	5	-4.43	44
Intramolecular oxidoreductase activity	3/5	-4.46/-4.38	38
Mitochondrial lumen / matrix	3/5/6	-4.58/-4.34/-6.05	87
Mitochondrial membrane part	3/4/5/6	-5.09/-4.4/-5.24/-5.05	91
Nucleolar part	3	-4.42	26
Oxidoreductase activity, acting on the aldehyde or oxo group of donors	5	-5.09	28
Ribosome biogenesis and assembly	3/4/6	-5.24/-4.93/-4.39	65
rRNA metabolic process	3/4	-5.07/-4.91	56
rRNA processing	3/4/6	-5.17/-4.82/-4.46	53
Transferase activity, transferring alkyl or aryl (other than methyl) groups	6	-5.08	45
tRNA processing	6	-5.28	57

(B)

Motif gene groups	NOC	*t*-values	TF	Biological functions[a]
TTGTTT_V$FOXO4_01	3/4/5/6	5.45/4.57/5.05/8.07	FOXO4	Cell cycle, cell death, cell metabolism, oxidative stress, embryonic development
RTAAACA_V$FREAC2_01	5/6	4.55/5.44	FOXF2	
V$FOXO3_01	6	4.3	FOXO3A	
V$FOXO1_01	6	4.03	FOXO1A	
V$CREB_Q2_01	3/5	4.38/5	CREB1	Neuronal development, stress response
V$CREB_Q4_01	3/5	4.3/5.8	CREB1	
V$CREB_Q2	3/5	4.19/4.76	CREB1	
V$CREB_01	3/5	4.18/5.09	CREB1	
V$ATF3_Q6	3/5/6	5.09/6.46/4.04	ATF3	Stress response
TGAYRTCA_V$ATF3_Q6	3/5/6	4.94/5.64/5.11	ATF3	
V$CREBP1CJUN_01	5	4.39	JUN/ATF2	
TGACGTCA_V$ATF3_Q6	5	4.38	ATF3	
V$CREBP1_01	6	4.53	ATF2	
V$OCT1_06	6	4.24	POU2F1	Neuronal development, hormone production
V$POU3F2_02	6	4.63	POU3F2	
V$OCT1_03	6	5.19	POU2F1	
V$POU1F1_Q6	1	4.14	POU1F1	

Motif gene groups	NOC	*t*-values	TF	Biological functions[a]
TGACAGNY_V$MEIS1_01	3/6	4.73/6.17	MEIS1	Modulation of ho-
TAATTA_V$CHX10_01	3/6	4.62/6.24	VSX1	meodomain respon-
CGTSACG_V$PAX3_B	3/6	4.21/4.01	PAX3	sive genes involved in
V$TGIF_01	3	4.03	TGIF	development
YTAATTAA_V$LHX3_01	6	4.02	LHX3	
V$LHX3_01	6	4.18	LHX3	
V$NKX25_02	6	4.66	NKX2–5	
V$EN1_01	1	4.12	EN1	
V$CDC5_01	3/6	4.23/5.81	CDC5L	Cell cycle regulation
V$E2F1_Q6	4	-4.02	E2F1	(G1/S transition),
V$E2F1_Q6_01	4	-4.17	E2F1	apoptosis, DNA repair
V$E2F_Q4_01	4	-4.59	E2F/TFDP1	
V$E2F_Q6_01	3	-4.25	E2F/TFDP1	
SGCGSSAAA_V$E2F1DP2_01	3/4/5	-4.32/-4.46/-4.39	E2F1/TFDP1/RB1	
V$MYCMAX_01	3/4	-4.62/-4.1	MYC/MAX	

[a] According to Gene Ontology from http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene and http://www.genecards.org/. NOCs are indicated with: 1 (MNNG), 2 (MNU), 3 (NDEA), 4 (NDMA), 5 (NPIP), 6 (NPYR). Significant results (*E*-value ≤0.05) are reported with *t*-value >4.32 or <-4.32 for GO gene groups and *t*-value >4 or <-4 for motif gene groups. Gene groups with positive and negative *t*-values can, on average, be considered up regulated and down regulated, respectively.

Cellular processes most strongly affected by modified pathways and their theoretical effects are presented in Table 3. Both tools identified a large number of pathways involved in apoptosis, cell cycle regulation and DNA repair after exposure to nitrosamines. Cell cycle progression is inhibited by modifications in the G1/S phase transition, DNA replication, and metaphase checkpoints, while DNA damage recognition and repair pathways, involved in double strand breaks among others, are activated. All four nitrosamines showed modifications of apoptosis response and cell cycle regulation pathways. DNA damage response pathways were modified by NDEA, NPIP and NPYR, but not by NDMA.

Pathways related to oxidative stress appear to be activated by nitrosamine exposure. Glutathion metabolism and ASK1-mediated oxidative stress induced apoptosis are both stimulated. Immune response pathways were strongly activated by NDEA, NPIP and NPYR, but again not by NDMA, resulting in a stimulation of pro-inflammatory genes. Changes in nucleotide metabolism were induced by all four nitrosamines and are indicative of a DNA replication block and modifications of the nucleotide turnover. Lipid metabolism was also affected, resulting in inhibition of fatty acid synthesis and beta oxidation.

MetaCore and PathVisio analysis also showed significant modulations of several pathways involved in development, such as the Notch and EGFR1 signalling pathways (supplementary data worksheet 3 and 4), for NDEA, NPIP and NPYR. These pathways were absent for NDMA exposure.

Table 3. Theoretical effects of pathways significantly modulated by nitrosamines as found in MetaCore and PathVisio analyses for a selection of strongly affected cellular processes.

Cellular process (theoretical effect)	Modulated pathways	NOC	Genes in pathway[a]	
			Significant	Total
Apoptosis (Stimulation of apoptotic process)	Anti-apoptotic TNFs/NF-kB/Bcl-2 pathway	3/5/6	17/21/21	41
	Caspase cascade	3	17	31
	FAS signalling cascades	3	24	41
	NGF activation of NF-kB	5	15	26
	p53-dependent apoptosis	3/5/6	17/16/18	29
	Role of IAP-proteins in apoptosis	3/5	15/10	25
	TNFR1 signalling pathway	3/5/6	23/18/23	38
	Apoptosis mechanisms[b]	4	22	84
	Proteasome degradation[b]	4	19	68
	TGF-beta receptor signalling pathway[b]	5/6	54/65	153
Cell cycle regulation (Cell cycle block in S and G2 phase)	ATM/ATR regulation of G1/S checkpoint	5/6	17/20	32
	Brca1 as a transcription regulator	5	16	30
	ESR1 regulation of G1/S transition	5	17	30
	Initiation of mitosis	5	15	25
	Nucleocytoplasmic transport of CDK/Cyclins	3	12	14
	Regulation of G1/S transition	3/5/6	25/21/28	40
	Role of SCF complex in cell cycle regulation	5	15	28
	Start of DNA replication in early S phase	3/4/5/6	18/15/21/22	31
	The metaphase checkpoint	3/6	3/16/19	36
	Transition and termination of DNA replication	3/4/5	17/12/15	26
	Cell cycle[b]	3/5/6	34/36/44	87
	DNA replication[b]	3/4/5/6	22/15/22/21	42
	G1 to S cell cycle control[b]	3/4/5/6	32/17/34/34	68
DNA damage recognition/repair (Stimulation of DNA repair)	Role of Brca1 and Brca2 in DNA repair	3	14	30
	NHEJ mechanisms of DSBs repair	3/6	11/10	18
	ATM/ATR regulation of G1/S checkpoint	5/6	17/20	32
	Brca1 as a transcription regulator	5	16	30
Response to oxidative stress (Anti-oxidant gene stimulation / Glutathione metabolism inhibition)	Glutathione metabolism[b]	4/6	9/25	36
	Role of ASK1 under oxidative stress	3/5/6	10/7/11	15
Immune response (Stimulation of pro-inflammatory genes)	MIF-mediated glucocorticoid regulation	5/6	10/12	21
	Signalling pathway mediated by IL-6 and IL-1	3/5/6	8/11/16	26
	Bacterial infections in normal airways	5	19	39
	IL1 signalling pathway[b]	3/5/6	16/20/23	33
	MIF in innate immunity response	3/5/6	13/16/18	24
	MIF-JAB1 signalling	5/6	11/10	17
	Toll-like receptor (TLR) ligands and common TLR signalling pathway leading to cell pro-inflammatory response	5	20	37

Cellular process (theoretical effect)	Modulated pathways	NOC	Genes in pathway[a]	
			Significant	Total
Nucleotide metabolism (DNA replication block)	ATP/ITP metabolism	3/4/5/6	46/22/36/44	74
	dATP/dITP metabolism	3/4/5/6	29/20/26/29	53
	dCTP/dUTP metabolism	3/4/5	26/17/25	46
	dGTP metabolism	3/4/5	24/14/22	42
	GTP-XTP metabolism	3/6	34/33	59
	TTP metabolism	3/4/5/6	23/14/22/22	38
Lipid metabolism (Inhibition of fatty acid synthesis and oxidation)	Cholesterol Biosynthesis	4[c]	2	14
	PDGF activation of prostacyclin synthesis	5	8	11
	Regulation of fatty acid synthase activity in hepatocytes	3	10	16
	Fatty Acid Beta Oxidation[b]	3/6	5/6	8
	Fatty Acid Biosynthesis[b]	3/4/6	15/7/14	22
	Triacylglyceride Synthesis[b]	5	15	26

NOCs are indicated with: 3 (NDEA), 4 (NDMA), 5 (NPIP), 6 (NPYR). [a] Significantly modulated genes (*p*-value <0.05 or *z*-score >2) versus total number of genes in pathway. [b] PathVisio pathways (sometimes overlapping with MetaCore pathways). [c] Theoretical effect unclear due to low number of significant genes compared to total.

Phenotypic markers of effect: apoptosis and cell cycle distribution

Since the gene group and pathway analysis tools all identified cell cycle regulation and apoptosis pathways as significantly modulated for the nitrosamines we performed a flow cytometry analysis of the cell cycle distribution and apoptosis levels to see whether these effects could also be found on a phenotypic level. Effects on apoptosis and cell cycle distribution induced by all six NOCs are displayed in Figures 4A and B respectively. The nitrosamides MNNG and MNU do not show any increase in apoptosis as compared to control levels while treatment with the nitrosamines results in a significant increase in the number of apoptotic cells ranging from 8 to 15% (*p*-value <0.01), which is in agreement with the gene group and pathway analyses. Caco-2 cells exposed to MNNG and MNU at these concentrations also do not show effects on cell cycle distribution (Figure 4B). For NDMA there seems to be an accumulation of cells in the S and G2 phases while the other nitrosamines display a decrease in S phase cells and a G2 accumulation, although the G2 accumulation for NPIP and NPYR was not significant.

Correlation of genes with phenotypic markers of effect

In order to link the gene expression changes with the phenotypic effects on apoptosis and cell cycle distribution following NOC exposure, we performed Spearman's rank correlation tests between gene log2 ratios and log2 transformed apoptosis and cell cycle distribution data. Significantly correlating genes (*p*-value <0.05) were subsequently used for a pathway analysis in MetaCore. In the supplementary data

(worksheet 5) the significantly modulated pathways that were related to apoptosis and the S and G2 phases of the cell cycle are shown. Although the G1 phase did correlate with a number of genes, these genes did not return any significant pathways. Genes correlating with apoptosis did not return any apoptosis pathways, but there were several cell cycle, cytoskeleton remodeling and cell adhesion pathways associated as well as a number of immune response pathways. The S phase and G2 phase correlating genes also returned several cell cycle related pathways.

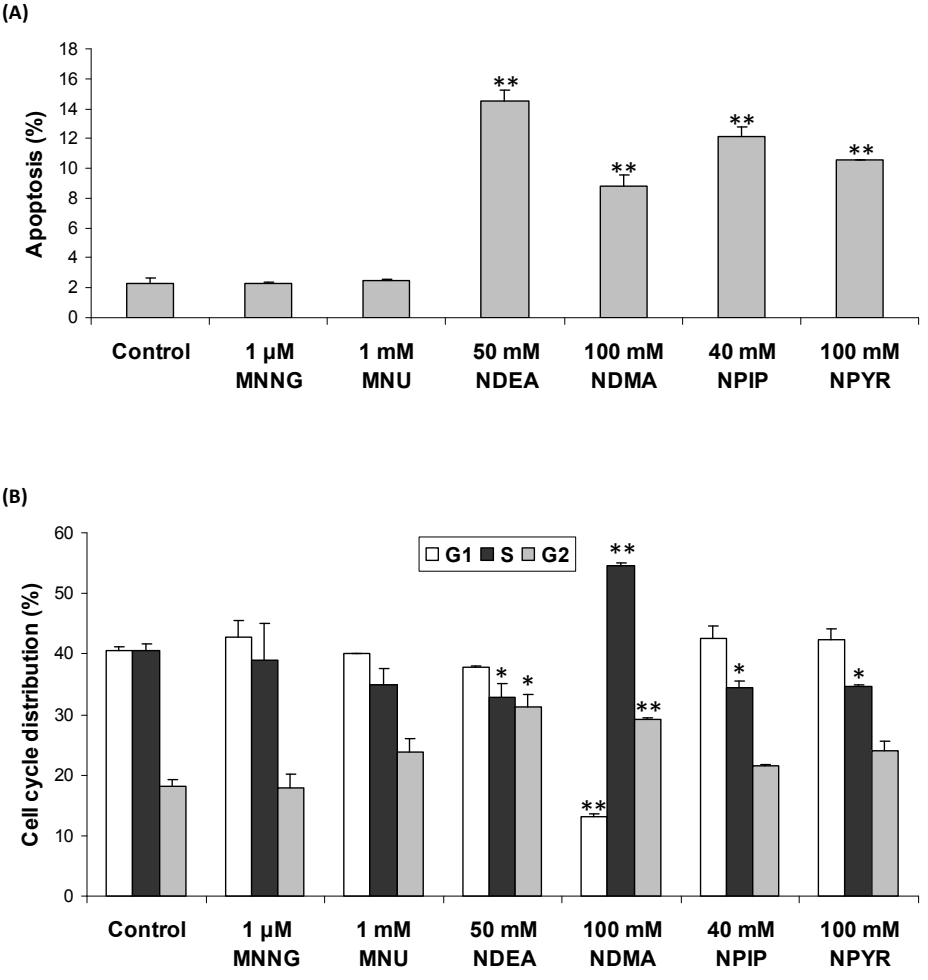

Figure 4. Levels of apoptosis **(A)** and cell cycle distribution **(B)** in Caco-2 cells exposed to genotoxic concentrations of six NOCs for 24hr as measured by flow cytometry analysis. DMSO control is not shown but was identical to normal control. Error bars indicate standard deviation. * *p*-value <0.05; ** *p*-value <0.01

Discussion

In this study, we identified the gene expression modulating effects of six NOCs in the human colon adenocarcinoma cell line Caco-2, in order to gain more insight in their respective molecular mechanisms at genotoxic doses. The non-cytotoxic concentrations we selected for microarray analysis resulted in a comparable level of genotoxicity as measured by comet assay (Table 1). It was quite surprising to find very little effect of the two nitrosamides as compared to the nitrosamines. The number of genes significantly modulated by both nitrosamides was smaller than the number of expected false positives. An HCA of genes significantly modulated by at least one of the NOCs shows a clear separation of nitrosamides and nitrosamines (Figure 2A). The corresponding PCA (Figure 2B) suggests that this discrimination is mainly based on the relatively small transcriptomic effects induced by MNU and MNNG. This was confirmed by functional data on apoptosis and cell cycling which also indicated little if no effects of nitrosamides (Figures 4A and B).

To study these changes in gene expression in more detail we performed T-profiler gene group and MetaCore/PathVisio pathway analyses. Since the transcriptomic response of the nitrosamides was beneath the false positive threshold, these data were excluded from the pathway analysis.

Gene group and pathway analyses revealed a considerable number of processes modified by nitrosamine exposure (Tables 1, 2 and supplementary data worksheet 1–4). Pathway analyses indicated an increase in apoptosis and inhibition of cell cycle progression following nitrosamine exposure. In addition, a number of nucleotide metabolism pathways was modulated which may cause a DNA replication block, while the inhibition of lipid metabolism pathways is associated with apoptosis (Table 3) [24]. The GO gene group analysis revealed gene groups likely to play a role in apoptosis and cell cycle regulation after nitrosamine exposure, such as the ubiquitine cycle, several mitochondria related GO categories, oxidoreductase activity and electron transport chain gene groups (Table 2A and supplementary data worksheet 1). Though less explicit, also DNA repair pathways were modified by NDEA, NPIP and NPYR (Table 2A). NPYR also affected an alkyl/aryl transferase activity gene group that may be involved in alkyl/aryl adduct repair. Motif gene group analysis revealed multiple gene groups that are associated with TFs known to play an important role in these processes (Table 2B), including several Forkhead box TFs and E2F1 [25,26]. Apoptosis, regulation of cell cycle progression and DNA repair are all direct consequences of DNA damage, which activates and regulates these downstream cellular processes through the activation of the p53 tumour suppressor protein [27].

Interestingly, gene groups and pathways associated with oxidative stress were found for all nitrosamine exposures. The cadmium and copper ion binding GO gene groups (Table 2A), for example, contain many metallothioneins which are implicated in free radical scavenging [28]. Furthermore, regulation of oxidative stress path-

ways, resulting in stimulation of anti-oxidant genes, were identified by MetaCore, while motif gene groups responsive to the FOX and CREB/ATF TFs, were also modulated. These TFs are known to play an important role in the cellular oxidative stress response [29,30]. These results indicate an increase in radical formation following nitrosamine exposure, which may be related to the release of $^{\bullet}$NO from nitrosamines or radical formation during nitrosamine metabolism as reported by others [11,31–33]. This suggests an important role for reactive oxygen species (ROS) in the induction of DNA damage by nitrosamines, and this may play a significant part in the carcinogenic process.

NDEA, NPIP and NPYR also strongly affected cellular immune response pathways (Table 3). All three compounds influenced pathways involved in the stimulation of pro-inflammatory cytokines, including the IL-1 and IL-6 signalling pathways. Immune response GO gene groups were also modified after NPIP and NPYR exposures (supplementary data worksheet 1). These gene groups contain many pro-inflammatory genes, including a number of interleukins and tumour necrosis factor genes and their corresponding receptors. Intestinal epithelial cells, including the Caco-2 cell line, are known to excrete pro-inflammatory cytokines in response to different stimuli and also express cytokine receptors [34,35]. Such stimuli include oxidative stress which has been shown to increase cytokine production in Caco-2 cells [36,37]. Cytokine production, especially IL-6, plays an important role in the induction of the acute phase response and stimulation of the intestinal inflammatory response [38]. It could be hypothesized that a high intestinal exposure to these specific nitrosamines, originating either directly from the diet or formed endogenously, incites intestinal inflammation, possibly triggered by increased oxidative stress levels. Such an increase in inflammation could contribute to the carcinogenic process and play a part in NOC exposure related cancers [39].

Another interesting result from the motif gene group analysis was the identification of several TF associated gene groups containing homeodomain responsive genes which are implicated in essential developmental processes, including embryogenesis (Table 2B). These gene groups were mainly identified for NDEA, NPIP and NPYR. Deregulated expression of homeobox genes has also been found in many types of cancer, including colon cancer, and they seem to play a pivotal role in the carcinogenic process [40]. Pathway analyses also showed significant modulations of several pathways involved in development, such as the Notch and EGFR1 signalling pathways, after NDEA, NPIP and NPYR exposure (supplementary data worksheet 3 and 4).

NDEA, NPIP and NPYR thus seem to pose a higher cancer risk than NDMA. NDMA had a smaller effect on gene expression modifications compared to the other nitrosamines and NDMA was absent in pathways involved in the immune response and developmental TF associated gene groups, both of which can be linked to an increased carcinogenic potential. Although the HCA and PCA analyses (Figure 3C and

D) show a rather close grouping of the nitrosamines, suggesting similar effects on a functional level, NDMA does dissociate from the other nitrosamines. NDMA uniquely affected steroid metabolism pathways which are involved in cholesterol, androstenedione and testosterone synthesis and metabolism (supplementary data worksheet 3 and 4): the implications of this, however, are unclear. NDMA also uniquely modified three E2F family related motif gene groups involved in cell cycle regulation among others (Table 2B). Regarding the other three nitrosamines, NPIP and NPYR modulated more pathways than NDEA. This difference is mainly attributable to the larger number of pathways affected by NPIP and NPYR that are involved in the immune response and development (supplementary data worksheet 1, 3 and 4). Exposure to NPIP or NPYR may therefore also imply a higher carcinogenic risk than exposure to NDEA or NDMA. The differences in response between the nitrosamines may be related to the type of adducts generated by each individual compound. NDMA will result in methyl adducts and NDEA in ethyl adducts, while NPIP and NPYR are expected to generate a heterogeneous group of alkyl and aryl adducts [9].

Animal studies with exogenous exposure to NOC have not identified the colon as one of the main target organs of NOCs (Table 1). We, however, hypothesize that the colon is a relevant target tissue, predominantly as a result of high exposures following the endogenous formation of NOCs which is known to occur in the colon [4]. Although our results are based on an intestinal adenocarcinoma cell line, and extrapolation of these results to normal colonic tissue should be done with restraint, Caco-2 cells are generally considered a good model for the human colon [41]. Our findings can therefore be considered as indicative of mechanistic effects following colonic NOC exposure.

Measurements of cell cycle distribution and the level of apoptosis following NOC exposure demonstrated an increased number of apoptotic cells and a shift in cell cycle distribution (Figure 4A and B). The S phase accumulation after NDMA treatment indicates a delay during the replication process, possibly due to the presence of DNA adducts. The decrease in S phase and increase in G2 phase cells following NDEA, NPIP and NPYR exposure, may indicate that DNA adducts are largely repaired and cells are resuming the cell cycle. The difference in NDMA response with regards to cell cycle distribution may be reflected by the three uniquely modified E2F family motif gene groups identified by T-profiler (Table 2B).

While apoptosis, as shown by flow cytometry, did not correlate with apoptosis pathways it was related with several cytoskeleton remodelling, cell adhesion and cell cycle regulation pathways (supplementary data worksheet 5), which is to be expected since apoptosis is associated with such processes. The S and G2 phases were also associated with many of these pathways, in addition to being linked with apoptosis. Pathways involved in the immune response and signalling were also

abundantly linked to apoptosis and cell cycle regulation, which may suggest a role for the immune response in these responses.

The absence of strong gene modulating effects by MNU and MNNG may be related to the direct acting properties of nitrosamides. Since nitrosamides do not require metabolic activation to become genotoxic, this may result in rapid transcriptomic responses that also subside faster. This requires further study where in view of their genomics responses in human Caco-2 cells *in vitro* it is suggested that nitrosamines rather than nitrosamides play a significant role in colon cancer development in humans. This is in contrast to animal studies which implicate nitrosamides in colon cancer development, rather than nitrosamines [15].

The data obtained in this study provide new insights into possible modes of action of nitrosamines and their gene expression effects at genotoxic levels in the human intestinal epithelial cell line Caco-2 and its implications for colon carcinogenesis. We have confirmed the possible relevance of ROS production after NOC exposure and identified modulation of the inflammatory response and developmental pathways as potential NOC targets using different approaches. Where it has to be considered that the gene expression responses *in vitro* have been sampled at rather high incubation concentrations of nitrosamines, identified processes may be of mechanistic importance in assessing carcinogenic risk after NOC exposure in the human colon.

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Chapter 3

Radical mechanisms in nitrosamine and nitrosamide-induced whole genome gene expression modulations in Caco-2 cells

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Toxicological Sciences 2010;116(1):194-205

Abstract

N-nitroso compounds (NOCs) may be implicated in human colon carcinogenesis, but the toxicological mechanisms involved have not been elucidated. Since it was previously demonstrated that nitrosamines and nitrosamides, representing two classes of NOCs, induce distinct gene expression effects in colon cells that are particularly related to oxidative stress, we hypothesized that different radical mechanisms are involved. Using ESR spectroscopy, we investigated radical generating properties of genotoxic NOC concentrations in human colon adenocarcinoma cells (Caco-2). Cells were exposed to nitrosamides (N-methyl-N'-nitro-N-nitrosoguanidine, N-methyl-N-nitrosurea) or nitrosamines (N-nitrosodiethylamine, N-nitrosodimethylamine, N-nitrosopiperidine, N-nitrosopyrrolidine). Nitrosamines caused formation of reactive oxygen species (ROS) and carbon centered radicals, which was further stimulated in presence of Caco-2 cells. N-methyl-N-nitrosurea exposure resulted in a small ROS signal, and formation of nitrogen centered radicals (NCR), also stimulated by Caco-2 cells. N-methyl-N'-nitro-N-nitrosoguanidine did not cause radical formation at genotoxic concentrations, but at increased exposure levels, both ROS and NCR formation was observed. By associating gene expression patterns with ROS formation, several cellular processes responding to nitrosamine exposure were identified, including apoptosis, cell cycle blockage, DNA repair and oxidative stress. These findings suggest that following NOC exposure in Caco-2 cells, ROS formation plays an important role in deregulation of gene expression patterns which may be relevant for the process of chemical carcinogenesis in the human colon, in addition to the role of DNA alkylation.

Introduction

N-nitroso compounds (NOCs) have long been known to be mutagenic in bacterial mutagenicity studies and carcinogenic in test animals [1], which has lead to the classification of dozens of NOCs as potential human carcinogen [2]. NOC have been found in the environment and certain types of food, such as beer, fish, and nitrite-preserved meat [3], but can also be formed endogenously by nitrosation of dietary precursors, a process that occurs predominantly in the gastro-intestinal tract [4–6]. Although a direct link with human carcinogenicity is still lacking, NOCs have been suggested to play a role in the development of several types of cancer, including brain, head and neck, gastric and colorectal cancer [1,7].

NOCs can be divided into two main sub-classes, nitrosamines and nitrosamides [8], which are both capable of inducing alkylating DNA damage by formation of a highly reactive diazonium ion [3,5]. O^6-alkylguanine adducts, for example, have been identified as the main pre-mutagenic lesion and cause GC-AT transition mutations [5]. Cyclic NOCs may also form similarly reactive cyclic oxonium ions, capable of causing heterocyclic DNA adducts [9]. There are, however, important differences in modes-of-action between nitrosamines and nitrosamides. While nitrosamides can spontaneously decompose to form a diazonium or oxonium ion, nitrosamines require metabolic activation through α-hydroxylation by several cytochrome P450 (CYP) enzymes [5].

The genotoxic aspects of NOCs have been studied in detail, but data on gene expression modulation associated with these effects were, until recently, lacking. In a recent toxicogenomics study, we provided a detailed analysis of gene expression changes in the human colon adenocarcinoma cell line Caco-2 following exposure to comparably genotoxic concentrations of six NOCs, and this revealed strong differences in transcriptional responses between nitrosamides and nitrosamines (**Chapter 2**) [10]. An interesting observation was the induction of several oxidative stress and radical scavenging related pathways and gene groups by the nitrosamines, while this response was absent following nitrosamide exposure. These transcriptomic responses suggest a difference in radical generating capability between nitrosamines and nitrosamides. Although both classes of compounds are known to be capable of inducing oxidative stress, the molecular mechanisms may be different [11–13]. Several studies have reported the formation of short-lived radicals from both nitrosamines and nitrosamides by liver microsomes, and upon UV radiation or presence of Fenton reagent, however, only under non-physiological conditions and in absence of a cellular system [12,14–16]. The available data suggest that nitrosamines and nitrosamides form different types of radicals, but it is uncertain whether these two classes of compounds differ with respect to the type of radical formation in a cellular system.

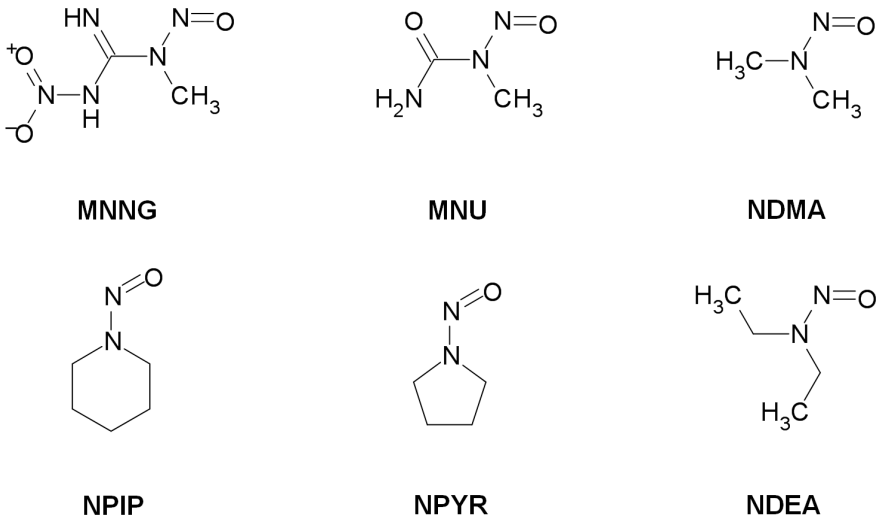

Figure 1: Molecular structures of the two nitrosamides and four nitrosamines used in this study.

Based on the strong discrimination in gene expression modifications between nitrosamides and nitrosamines following exposure in the human Caco-2 cell line as found in our previous study, we hypothesize that these two classes of NOCs display a difference in radical generating ability. To investigate this, we performed electron spin resonance (ESR or EPR) spectroscopy on Caco-2 cells exposed to six NOCs (Figure 1) that have been used frequently in literature: the nitrosamides N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and N-methyl-N-nitrosurea (MNU), and the nitrosamines N-nitrosodiethylamine (NDEA), N-nitrosodimethylamine (NDMA), N-nitrosopiperidine (NPIP), and N-nitrosopyrrolidine (NPYR). These nitrosamines have been demonstrated to occur in the colon, while MNU can be formed under gastric conditions and subsequently reach the colon [3,6]. MNNG is widely used as a model nitrosamide compound, and although it is less relevant in human exposure, it has been used to induce malignant transformation of human colonic epithelial cells [17]. For all compounds, we applied concentrations that induced comparable levels of genotoxicity in the comet assay, to detect differences in radical generation at doses known to result in distinct gene expression patterns (**Chapter 2**) [10]. In addition, equimolar NOC concentrations were tested to determine cellular differences at equal doses. Furthermore, we hypothesize that NOC-induced radical generation is associated with changes in the gene expression pattern. To test this, gene expression modifications at two time points (1 and 24 hours) were separately associated with ESR data to functionally anchor specific pathways to the formation of radicals during the exposure period and to investigate the development of the gene expression response over time. Since the Caco-2 cell line is widely used as an *in vitro*

model for the human colon, this study will clarify the cellular radical generating properties of NOCs that may be relevant for human colorectal cancer development.

Material and Methods

Cell culture

The human colon adenocarcinoma cell line Caco-2 was cultured in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, Zwijndrecht, The Netherlands) with 4.5 g/L glucose, L-glutamine, $NaHCO_3$ and pyridoxine HCl supplemented with 1% (v/v) non-essential amino acids, 1% Na-pyruvate, 1% penicillin/streptomycin and 10% (v/v) heat-inactivated foetal calf serum, all purchased from Gibco BRL (Breda, The Netherlands). Cell cultures were transferred weekly by trypsinization and incubated at 37 ºC in a humidified incubator containing 5% CO_2.

Chemicals and exposure concentrations

Caco-2 cells were exposed to six NOCs, i.e. MNNG, MNU, NDEA, NDMA, NPIP and NPYR, or the corresponding solvent control (MilliQ for the nitrosamines or DMSO [0.1%] for the nitrosamides). All NOCs were obtained from Sigma-Aldrich (the respective product numbers and reported purity were: 15427LO [97%], N4766 [11% H_2O, 3% acetic acid], N0756, N7756, N6007 [99.8%], and 158240 [98.9%]). Cells were exposed to comparably genotoxic concentrations, as determined by comet assay analysis described previously (**Chapter 2**) [10], or equimolar concentrations (1 mM). The comparably genotoxic concentrations were 1 µM (MNNG), 1 mM (MNU), 50 mM (NDEA), 100 mM (NDMA), 40 mM (NPIP) and 100 mM (NPYR). Solutions of the spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO, Sigma-Aldrich) diluted in nitrogen flushed MilliQ water were further purified by charcoal treatment. Stock concentrations of DMPO were determined spectrophotometrically by using the extinction coefficient $\varepsilon = 7700\ M^{-1}\ cm^{-1}$. H_2O_2 (35%, w/v) was obtained form Merck Sharp & Dohme (Haarlem, The Netherlands).

Electron spin resonance spectroscopy measurements

For ESR experiments Caco-2 cells were plated out three days before and grown to 90–100% confluence in 20 cm^2 culture dishes. To guarantee comparability of individual experiments cell numbers were in the same range for all dishes ($3.38 \pm 0.32*10^6$ cells/dish). At the start of the experiments dishes were washed twice with Hank's Balanced Salt Solution (HBSS, Gibco BRL), followed by incubation of the cells in 2 ml 50 mM DMPO in HBSS in a CO_2 incubator at 37°C for 30 minutes. This was followed by adding the required amount of a comparably genotoxic or equimolar

dose of MNNG, MNU, NDEA, NDMA, NPIP or NPYR to the HBSS and incubation for 30 minutes. This incubation time was found to result in the highest DMPO-radical adduct concentration. Longer incubations led to lower signals due to the intracellular depletion of DMPO as the decay of the DMPO-radical adduct does not result in the regeneration of free DMPO. Cells were subsequently harvested by scraping and homogenized by pipetting. For each sample a 100 µl glass capillary (Brand, Wertheim, Germany) was filled with the suspension and sealed. After sealing, the capillary was immediately placed in the resonator of the ESR spectrometer. During exposure and measurement of samples, light exposure was kept to a minimum. ESR spectra were recorded at room temperature on a Bruker EMX 1273 spectrometer equipped with an ER 4119HS high sensitivity resonator and 12 kW power supply operating at X band frequencies. The modulation frequency of the spectrometer was 100 kHz. Instrumental conditions for the recorded spectra were as follows: magnetic field, 3450 G; scan range, 200 G; modulation amplitude, 1 G; receiver gain, $1x10^4$; microwave frequency, 9.85 GHz; power, 50 mW; time constant, 40.96 ms; scan time, 20.97 s; number of scans, 35. Quantification of the spectra was performed by peak surface measurements through double integration of the ESR spectrum using the WIN-EPR spectrum manipulation program (Bruker BioSpin, Wormer, The Netherlands). Spectra were created at identical intensity scale to enable visual comparison of the different conditions in each figure. Number of cells and viability were determined by trypan blue exclusion analysis. Viability (>90%) was not significantly different from control samples. All experiments were performed in triplicate and statistical analysis was performed using an unpaired two-tailed *t*-test with a 95% confidence interval.

Microarray hybridization and data analysis

Gene expression modifications, following a 1 and 24 hour exposure to comparably genotoxic NOC concentrations, were measured to investigate the link with radical formation found after 30 minutes. The 1 hour microarray hybridizations (two biological replicates) were hybridized against Agilent 4x44K Whole Human Genome microarrays (Agilent Technologies, Amstelveen, The Netherlands), as described previously (**Chapter 2**) [10]. The biological replicates of the NOC and reference samples were dye-swapped in the hybridization protocol.

After scanning the microarray slides, using settings described before (**Chapter 2**) [10], the data was flagged manually and automatically using the GenePix Pro software (version 6.0, Molecular Devices, Sunnyvale, CA). Following a quality control in the statistical software environment R (version 2.10.1, The R Foundation for Statistical Computing, Vienna, Austria), regions of lower quality were identified and subsequently flagged in Spotfire DecisionSite (version 9.1, TIBCO, Somerville, MA). LOWESS normalization and subsequent data analysis was performed in ArrayTrack (ver-

sion 3.4, NCTR, Jefferson, AR). The expression difference for each spot was calculated by subtracting the log2 transformed mean intensity of the control sample from the log2 transformed mean intensity of the treated sample resulting in a log2 ratio. Gene expression data, following a 24 hours exposure period were taken from our previous study (**Chapter 2**) [10].

Gene expression data from both the 1 and 24 hour exposures were subsequently used to perform correlation analyses with ROS levels. Ratios of ROS versus their respective controls were first calculated and subsequently log2 transformed to obtain a data distribution similar to the log2 gene expression values. Spearman's rank correlations between gene expression and levels of ROS were calculated using the online Gene Expression Profile Analysis Suite (GEPAS, http://gepas.bioinfo.cipf.es/). Significantly correlating genes (p-value < 0.05) were subsequently analysed using MetaCore™ (GeneGo, San Diego, CA). MetaCore™ is an online software suite that identifies and visualizes the involvement of differentially expressed genes in specific cellular pathways, which is subsequently related to the total amount of genes involved in the particular pathway and in all the available pathways combined. This results in a set of significantly modulated pathways (p-value < 0.05).

The gene expression data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO) and are available through GEO Series accession numbers GSE20993 (for 1hr data) and GSE14284 (for 24hr data), accessible via http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE20993 and http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE14284.

A supplementary data Microsoft Excel file is available online and can be found at: http://dl.dropbox.com/u/11005104/Thesis%20Dennie%20Hebels%20Supplementary%20Data%20Chapter%203.xls.

Results

Radical generation at genotoxic concentrations

To investigate the difference in radical generating properties between nitrosamides and nitrosamines in a cellular system, we first tested the effects of these compounds after exposure of Caco-2 cells to genotoxic concentrations for 30 minutes. DMPO was used as a spin trapping agent since it is widely used for the detection of reactive oxygen species (ROS)-induced ESR signals and can also pick up carbon and nitrogen centered radicals (CCR and NCR respectively), which have been shown to be formed in previous studies [14,15].

Figure 2 shows representative ESR signals induced by NDEA under different conditions. In presence of Caco-2 cells, NDEA induces a characteristic 4-line 1:2:2:1 ESR signal with a hyperfine splitting constant (HFSC) of $a_N = a_H = 14.7$G (Figure 2C),

identified as a ROS-induced DMPO-OH signal [18], and a NOC radical consisting of a clear 6-line pattern (a_N = 15.4 and a_H = 23.6, Figure 2C). Identical signals were generated after exposure with the other three nitrosamines (results not shown). As further explained in the Discussion this 6-line pattern is indicative of a trapped CCR [14]. Although there is also a DMPO-OH and NOC radical pattern visible in the incubation without cells (Figure 2A), incubation with cells clearly increases the signal intensity, indicating a stimulating effect in a cellular system. Caco-2 cells also display a small DMPO-OH signal spontaneously (Figure 2B). The induction of nitrosamine-induced cellular ROS formation was confirmed in control experiments with H_2O_2 (Figure 2D), showing a characteristic ROS signal.

Exposure of Caco-2 cells for 30 minutes to nitrosamides resulted in different ESR spectra as compared to those induced by the nitrosamines. MNU displays a more complex signal, which consists of a DMPO-OH signal, and an additional 18-line MNU-derived NOC radical spectrum, which is visible after incubation both with and without cells (Figure 3A and C). Non-NOC exposed solvent control cells only displayed a small background DMPO-OH signal (Figure 3B). A spectrum was simulated using HFSCs reported in literature [15] for MNU following UV light exposure to identify the NOC radical. We included a DMPO-OH signal with the simulated spectrum which resulted in a composite simulated pattern (Figure 3D) similar to the results obtained from MNU after cellular exposure (Figure 3C). As discussed later on, the NOC radical detected is probably an NCR. MNNG on the other hand only displayed a small DMPO-OH signal, but this does not appear to be higher as compared to the solvent control (Figure 3E). A NOC radical, as observed with MNU, was not present.

Altogether, the ESR study showed that at comparable genotoxic concentrations nitrosamines and nitrosamides strongly differ with respect to radical generation. In Caco-2 cells, all nitrosamines and MNU induced significant ROS formation (Figure 4). Presence of cells significantly increases DMPO-OH levels following nitrosamine exposure. Within the nitrosamine group, NDEA, NPIP and NPYR induce a significantly higher ROS production than NDMA. Without cells, no ROS formation above normal background levels was detected. MNNG, in presence of cells, displays no apparent increase in ROS production compared to its control.

In addition to ROS formation, a compound-specific NOC radical was detected following nitrosamine exposure (Table 1A). NOC radical levels were significantly increased in the presence of cells, again indicating a cellular stimulating effect. The NOC radical formed from MNU was also significantly higher in the presence of cells. No NOC radical signals were found following MNNG exposure.

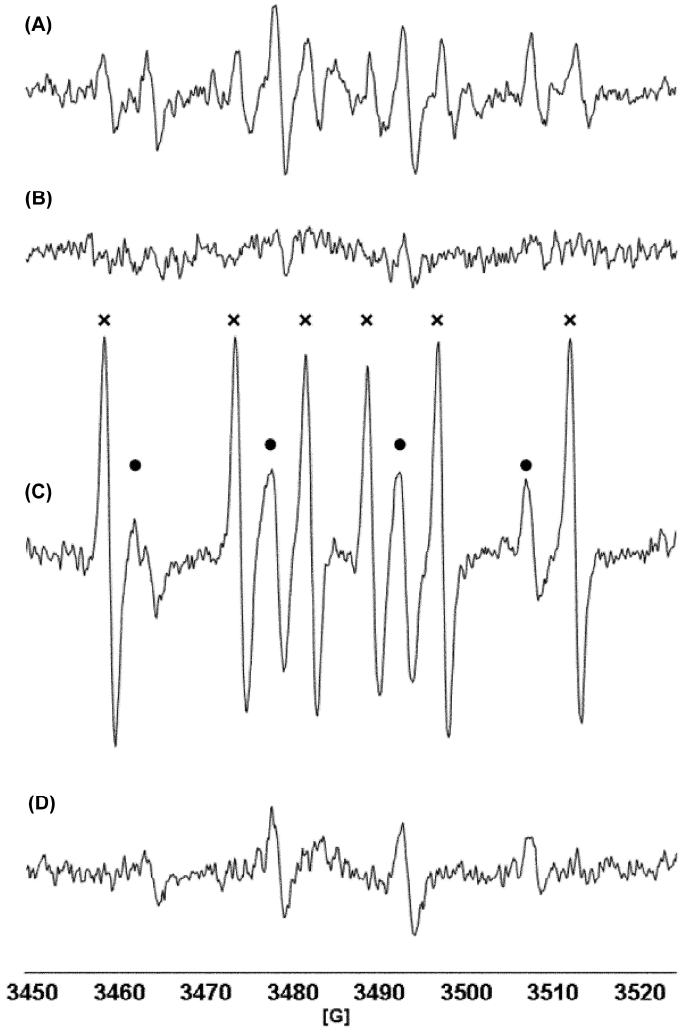

Figure 2: ESR spectra recorded after a 30 minute incubation with 50 mM NDEA and 50 mM DMPO without cells present **(A)**, or incubation in the presence of DMPO pre-treated Caco-2 cells with solvent control **(B)**, with 50mM NDEA **(C)** or with 0.5 mM H_2O_2 **(D)**. Identical signals were generated after exposure with the other three nitrosamines.

Representative spectra of triplicate experiments are shown; Spectra are shown at identical intensity scale; **×** DMPO-trapped CCR signal; ● DMPO-trapped ROS signal.

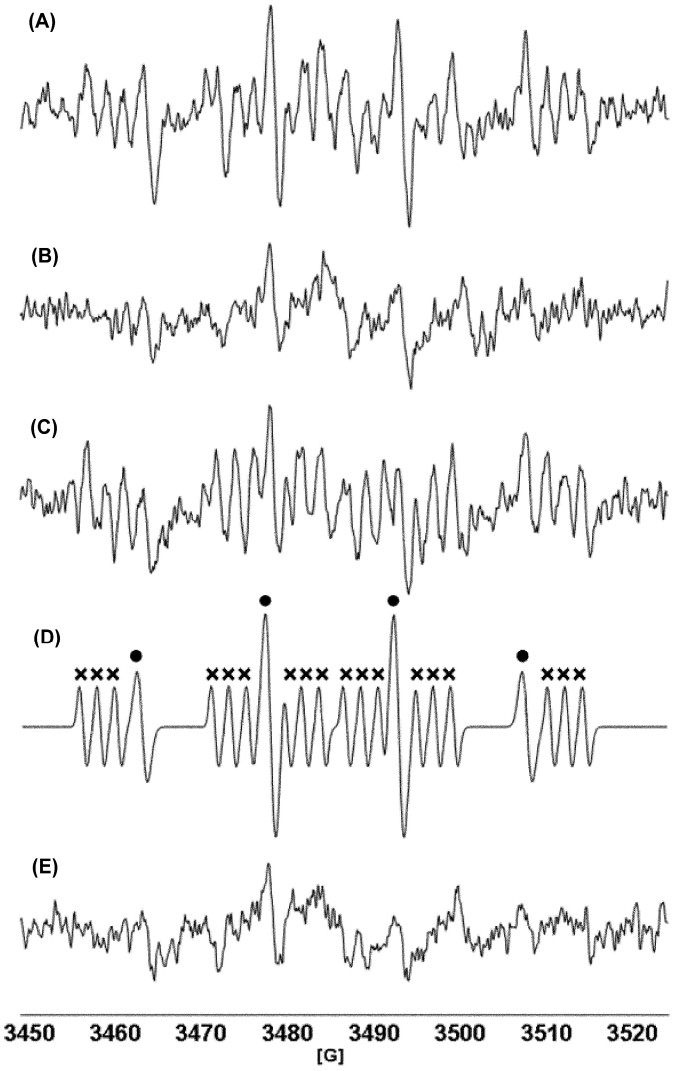

Figure 3: ESR spectra recorded after a 30 minute incubation with 1mM MNU and 50 mM DMPO without cells present **(A)**, or incubation in the presence of DMPO pre-treated Caco-2 cells with solvent control **(B)**, with 1 mM MNU **(C)**, a simulation of an MNU derived NCR (a_{NO} = 15.1G, a_N = 2.0G and a_β^H = 23.4G) in combination with ROS (DMPO-OH, $a_H = a_N$ = 14.7 G) **(D)**, and incubation in the presence of DMPO pre-treated Caco-2 cells with 1 µM MNNG **(E)**.

Representative spectra of triplicate experiments are shown; × DMPO-trapped NCR signal; ● DMPO-trapped ROS signal.

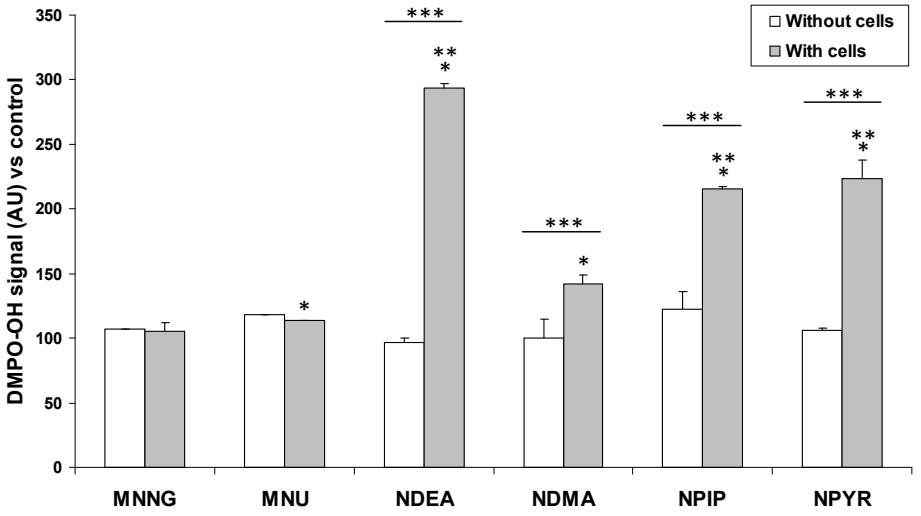

Figure 4: Intensities of the ESR detected ROS formation after 30 minute incubations in presence or absence of Caco-2 cells and relative to solvent control levels.

* Significantly higher versus solvent control level (p-value <0.01), ** Significantly higher versus NDMA ESR signal (p-value <0.001), *** Significantly higher versus without cells condition (p-value <0.05), AU = arbitrary units.

Table 1. Intensities of the ESR detected compound-specific NOC radicals in presence or absence of Caco-2 cells after 30 minute incubations at genotoxic concentrations **(A)** and ROS formation and compound specific NOC radicals in Caco-2 cells after 30 minute incubations at an equimolar concentration versus control levels **(B)**.

(A)

	MNNG-derived NCR	MNU-derived NCR	NDEA-derived CCR	NDMA-derived CCR	NPIP-derived CCR	NPYR-derived CCR
Without cells	n.d.	1.28±0.003	1.30±0.04	0.57±0.08	1.07±0.18	0.61±0.002
With cells	n.d.	1.86±0.02[a,b]	2.71±0.45[a,b]	1.53±0.24[a,b]	2.11±0.04[a,b]	2.20±0.34[a,b]

(B)

	ROS level		MNNG-derived NCR	MNU-derived NCR
	MNNG	MNU		
Solvent control	0.58±0.02	0.55±0.003	n.d.	n.d.
NOC exposed	3.02±0.47[a,c]	0.62±0.001[a]	3.04±0.61[a]	1.86±0.02[a]

Values represent arbitrary units (*10^6), [a] Significantly higher versus solvent control level (p-value <0.01), [b] Significantly higher versus without cells condition (p-value <0.05), [c] Significantly higher MNNG-induced ROS level compared to MNU, n.d. = non detectable

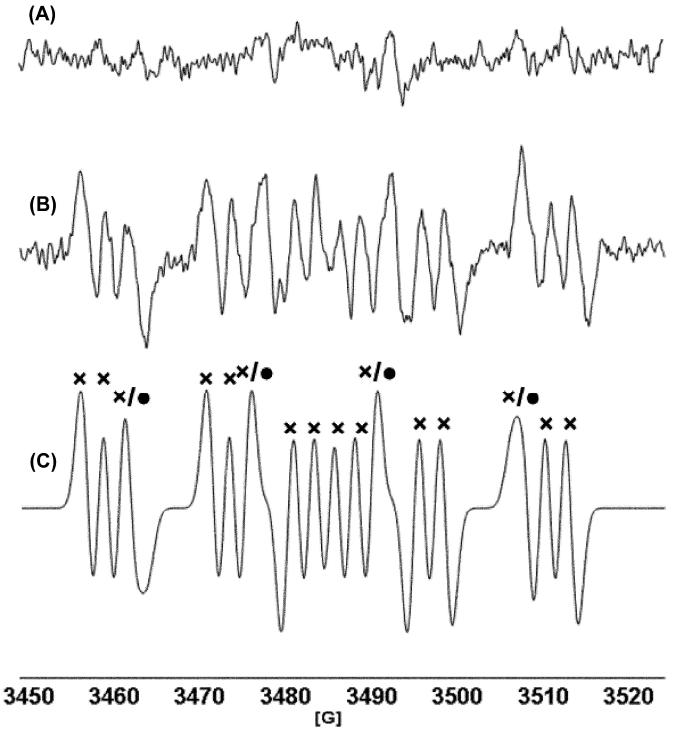

Figure 5: ESR spectra recorded after a 30 minute incubation in Caco-2 cells at an equimolar concentration of 1mM. As exemplified here by the spectrum of NDEA **(A)**, none of the nitrosamines displayed a signal different from solvent control cells (Figure 2B). The pattern displayed by MNNG **(B)** matches a simulation of an MNNG derived NCR (a_{NO} = 15.1G, a_N = 2.5G and a_β^H = 22.1G) together with ROS (DMPO-OH, a_H = a_N = 14.7 G) **(C)**.
Representative spectra of triplicate experiments are shown; × DMPO-trapped NCR signal; ● DMPO-trapped ROS signal.

Radical generation at equimolar concentrations

We subsequently tested all six NOCs at an equimolar concentration to investigate differences in radical formation between these compounds at similar doses. All compounds were tested at a 1 mM exposure concentration for 30 minutes, which corresponded with an increase in concentration from genotoxic levels for MNNG, and a decrease for the four nitrosamines. This concentration was selected since it can be expected that MNNG will generate a detectable ESR spectrum at this concentration, similar to the other nitrosamide, MNU. In addition, nitrosamide concentrations higher than 1 mM are likely to result in cytotoxicity, which needs to be avoided. At 1 mM, all four nitrosamines no longer displayed the spectrum seen at genotoxic concentrations (Figure 5A). Only a small DMPO-OH signal was present, which was not significantly different from control cells (Figure 2B). MNNG on the

other hand showed a 16-line ESR spectrum (Figure 5B). Since the spectrum for MNU at 1 mM turned out to be a composite signal from a compound-specific NOC radical (identified as an MNU NCR) and DMPO-OH signal, we used the same approach to simulate a spectrum for MNNG. Based on HFSCs reported by Grover *et al.* [15] for an MNNG derived NCR we could simulate a comparable composite signal including a DMPO-OH signal (Figure 5C). ROS and NOC radical formation at an equal dose of 1 mM of the nitrosamides is shown in Table 1B. ROS formation is significantly higher following MNNG incubations compared to MNU.

Gene expression correlation analysis

We subsequently investigated changes in the gene expression profile during incubation that can be attributed to the formation of radicals, by measuring the strength of the gene expression response at 1 and 24 hours of exposure. Since the ESR experiments require measurements to be performed at the beginning of the exposure due to instability of the spin trapped adduct, we first tested whether NOC were still present in a sufficient amount after 24 hours of exposure to continue the formation of radicals. NDMA, a model nitrosamine and the most volatile NOC, was shown to be present at 90% of the original concentration after 24 hours. To link differences in ROS formation induced by nitrosamides and nitrosamines, with transcriptomic responses, we performed Spearman's rank correlation analyses with gene expression modulations across the six compounds in two separate analyses for two different time points (1 and 24 hours). Significantly correlating genes (p-value <0.05), i.e. genes whose log2 expression ratios across the six compounds correlate with the induced ROS levels by these compounds, were subsequently used for a pathway analysis in MetaCore. Since the NCR's and CCR's identified by ESR analysis all represent compound-specific radicals, and, thus, are different at the molecular level, gene expression correlation analyses were not performed with NOC radical levels.

Table 2 shows a selection of cellular processes that are correlated with ROS levels following NOC exposure at genotoxic concentrations. Affected cellular processes after 1 hour of exposure include apoptosis, cell cycle regulation, DNA repair, and the oxidative stress response. Because the number of significantly affected genes for each pathway was relatively small at this time point, the expected biological effect for the cellular processes was difficult to assess. At 24 hours, however, mostly the same biological processes were found to be modulated as after 1 hour. The effects were more pronounced, though, as demonstrated by the higher number of significantly modulated genes, allowing for the direction of the expected biological effects (up or down regulation) to be determined for most processes, although the modulated pathways contributing to these processes were different. The total number of gene expression modifications found to correlate with ROS levels observed at the beginning of the exposure period (30 min.), was also higher after 24

hours, corresponding with approximately 23% of the data set (versus 7% at 1 hour). Analogous to the significantly higher ROS levels which we found following nitrosamine exposure compared to nitrosamides, at 24 hours, modulated pathways were only found to be associated with nitrosamine exposure. A full overview of modulated pathways can be found in the supplementary Excel file. Among the most commonly affected cellular processes were apoptosis, cell cycle regulation, nucleotide metabolism, and cytoskeleton remodeling. Apoptosis was mainly stimulated, while cell cycle progression was inhibited during the G1/S phase transition and mitosis. Changes in nucleotide metabolism are indicative of a DNA replication block and modifications of the nucleotide turnover. The cytoskeleton remodeling pathways can be linked to cytoskeletal re-arrangements associated with the processes of apoptosis, cell cycle progression and cell movement and adhesion. In addition, DNA repair and oxidative stress pathways were affected. In most cellular processes all four nitrosamines were involved, except for the apoptosis and oxidative stress processes, which were not modulated by NDMA. Furthermore, a number of immune response, G-protein signaling and developmental pathways were modified (supplemental data), indicating modulations in the inflammatory response, G-protein controlled processes and cell differentiation.

Table 2. A selection of affected cellular processes significantly modulated by a set of ROS-correlating genes found after MetaCore pathway analysis at 1 and 24 hours of NOC exposure.

1hr exposure		**Genes in pathway[c]**		*24hr exposure*		**Genes in pathway[c]**	
Modulated pathways[a]	**NOC[b]**	**Significant**	**Total**	**Modulated pathways[a]**	**NOC[b]**	**Significant**	**Total**
				Apoptosis			
				(Stimulation of apoptotic process)[d]			
Apoptotic TNF-family pathways	4	2	41	Beta-2 adrenergic receptor anti-apoptotic action	3/6	7	15
DNA-damage-induced apoptosis	3	2	15	HTR1A signaling	3/6	12	38
p53-Dependent apoptosis	3	4	29	BAD phosphorylation	3/6	11	35
EPO-induced Jak-STAT pathway	2	3	35	EPO-induced Jak-STAT pathway	3/6	11	35
p53 Signaling pathway	3	8	39				

1hr exposure		Genes in pathway[c]		*24hr exposure*		Genes in pathway[c]	
Modulated pathways[a]	**NOC[b]**	**Significant**	**Total**	**Modulated pathways[a]**	**NOC[b]**	**Significant**	**Total**
				Cell cycle regulation			
				(Cell cycle block in S and G2 phase)[d]			
ESR1 regulation of G1/S transition	1/3	6	32	Spindle assembly and chromosome separation	5/6	11	32
Regulation of G1/S transition (part 2)	3	5	26	Initiation of mitosis	3/5	9	25
Role of SCF complex in cell cycle regulation	3	3	29	Regulation of G1/S transition (part 1)	3/5/6	12	38
p53 Signaling pathway	3	8	39	Transition and termination of DNA replication	4	7	26
				Cell adhesion			
				(diverse effects related to apoptosis and cell cycle regulation)[d]			
Cadherin-mediated cell adhesion	6	5	26	Integrin-mediated cell adhesion and migration	3/5/6	20	45
Endothelial cell contacts by junctional mechanisms	6	3	26	Histamine H1 receptor signaling in the interruption of cell barrier integrity	3/4/5/6	16	37
Ephrins signaling	3	5	41	Integrin inside-out signaling	5	11	44
Histamine H1 receptor signaling in the interruption of cell barrier integrity	1	8	37	Chemokines and adhesion	3	22	93
PLAU signaling	3	3	38				
Role of tetraspanins in the integrin-mediated cell adhesion	6	6	37				

1hr exposure		Genes in pathway[c]		24hr exposure		Genes in pathway[c]	
Modulated pathways[a]	**NOC[b]**	**Significant**	**Total**	**Modulated pathways[a]**	**NOC[b]**	**Significant**	**Total**
				Cytoskeleton remodeling			
				(diverse effects related to apoptosis and cell cycle regulation)[d]			
CDC42 in cellular processes	6	3	22	TGF, WNT and cytoskeletal remodeling	3/4/5/6	35	107
Cytoskeleton remodeling	3	12	96	Cytoskeleton remodeling	3/5/6	31	96
Integrin outside-in signaling	3/6	8	46	Keratin filaments	4/5	10	36
Keratin filaments	1/3/6	9	36	Reverse signaling by ephrin B	3/4/5	9	30
RalA regulation pathway	6	2	26	Neurofilaments	3/4/5/6	12	25
Reverse signaling by ephrin B	3/4/6	9	30	Role of PKA in cytoskeleton reorganisation	3/5/6	13	31
Role Activin A in cytoskeleton remodeling	3	2	20	Regulation of actin cytoskeleton by Rho GTPases	3/4/5	10	23
Role of PKA in cytoskeleton reorganisation	3/4	6	31	Role Activin A in cytoskeleton remodeling	3	7	20
TGF, WNT and cytoskeletal remodeling	3	14	107	FAK signaling	3/5/6	16	47
				Integrin outside-in signaling	3/5/6	14	46
				Slit-Robo signaling	3	10	30
				Alpha-1A adrenergic receptor-dependent inhibition of PI3K	3/5/6	5	12
				DNA damage repair			
				(Stimulation of DNA repair)[d]			
ATM/ATR regulation of G1/S checkpoint	1/3	4	32	NHEJ mechanisms of DSBs repair	3/4/5/6	10	19
Brca1 as a transcription regulator	1/3	6	30	Role of Brca1 and Brca2 in DNA repair	4	6	30
DNA-damage-induced apoptosis	3	2	15				
p53 Signaling pathway	3	8	39				

1hr exposure		Genes in pathway[c]		*24hr exposure*		Genes in pathway[c]	
Modulated pathways[a]	**NOC[b]**	**Significant**	**Total**	**Modulated pathways[a]**	**NOC[b]**	**Significant**	**Total**
				Nucleotide metabolism			
				(DNA replication block / DNA repair)[d]			
TTP metabolism	2	3	38	GTP-XTP metabolism	3/4/5/6	22	59
				CTP/UTP metabolism	3/4/5/6	23	65
				ATP/ITP metabolism	3/4/5/6	28	85
				dCTP/dUTP metabolism	3/5/6	16	46
				dATP/dITP metabolism	3/5/6	18	54
				TTP metabolism	3/5	12	38
				dGTP metabolism	3/5	12	38
				Oxidative stress			
				(Stimulation of anti-oxidant response)[d]			
Delta-type opioid receptor mediated cardioprotection	2/3	5	25	Delta-type opioid receptor mediated cardioprotection	3/5/6	8	25
(L)-selenoaminoacids incorporation in proteins during translation	2/3/4/5/6	2	36	Role of ASK1 under oxidative stress	3/5/6	9	22
				Development			
				(diverse effects)			
25 pathways involved (see supplementary data)	1/2/3/4/6			24 pathways involved (see supplementary data)	3/4/5/6		
				G-protein signaling			
				(diverse effects)			
6 pathways involved (see supplementary data)	1/2/3			7 pathways involved (see supplementary data)	3/5/6		
				Immune response			
				(diverse effects)			
22 pathways involved (see supplementary data)	1/2/3/4/6			7 pathways involved (see supplementary data)	3/4/5/6		

[a] Significantly modulated (p-value <0.05). [b] NOCs involved denoted by 1 (MNNG), 2 (MNU), 3 (NDEA), 4 (NDMA), 5 (NPIP), 6 (NPYR). [c] Significantly modulated genes (p-value <0.05) versus total number of genes in pathway. [d] Expected effects (between parentheses) of cellular processes are only based on pathways found after 24 hours.

Discussion

Although the radical generating capacity of NOCs has received some attention [11,13,19,20], no studies have been performed to establish radical formation processes in a cellular system. Our recently performed toxicogenomics study demonstrated the importance of radical processes in cellular responses to NOC exposures, with nitrosamines and nitrosamides showing different transcriptomic profiles (**Chapter 2**) [10]. In the present study, we established for the first time differences between nitrosamines and nitrosamides with respect to radical generation in a cellular system.

Investigating cellular radical formation at comparably genotoxic concentrations, all 4 nitrosamines displayed a similar ESR spectrum, showing both a typical DMPO-OH and a compound-specific NOC radical signal (Figure 2C, 4 and Table 1A). The generation of DMPO-OH adducts is indicative of ROS formation, which can lead to oxidative DNA damage. It has indeed been demonstrated that apart from the induction of alkylating DNA damage, oxidative damage is an important second type of DNA lesion that may be induced by nitrosamine exposure [19,21]. The origin of ROS following nitrosamine exposure is most likely related to their metabolic activation by CYP enzymes (Figure 6A) [22,23] and particularly CYP2E1, known to be involved in nitrosamine metabolism [24], is capable of forming ROS by redox cycling [23].

The NOC radical signal we detected was identified as originating from a CCR and its formation has been suggested to involve a short-lived intermediate in the α-hydroxylation step (the α-nitrosamino radical, Figure 6A) [22,25,26]. Indeed, formation of CCRs from nitrosamines was observed following incubation of nitrosamines with rat liver microsomes indicating a CYP enzyme catalyzed process being responsible [14]. Moreover, UV light-induced release of $^{\bullet}$NO [15], which shares similarity with the denitrosation pathway (Figure 6), results in the formation of CCRs with similar spectra. We now show that this CCR is also formed upon cellular exposure to nitrosamines. The α-nitrosamino radical can subsequently either dealkylate or denitrosate. The latter metabolic route is usually considered a non-genotoxic alternative [27]. Since all four nitrosamines used in this study are metabolized via hydroxylation of the α-carbon, the α-nitrosamino radical DMPO-adducts we measured gave similar HFSCs, which explains the analogous spectra we found for the nitrosamines.

The observation that higher levels of both ROS and compound-specific CCRs are formed in presence of Caco-2 cells (Figure 4, Table 1A), strongly suggests the involvement of CYP metabolism, known to be present in this cell line [28]. In the absence of cells, spontaneous formation of CCRs by nitrosamines was also observed, albeit at significantly lower levels (Figure 2A, Table 1A). Nitrosamines have been shown to spontaneously denitrosate at low levels, which is similar to UV light-induced denitrosation [15,16,29]. UV light-induced formation of CCRs, as described by Grover *et al.* [15], can not be completely ruled out, although precautions were

taken to minimize light exposure. Trace amounts of iron or other transition metals could also have resulted in a Fenton-induced degradation of nitrosamines [29,30]. Cells lacking strong CYP activity could therefore also be exposed to the CCR which could either denitrosate or dealkylate after reacting with a hydroxyl radical.

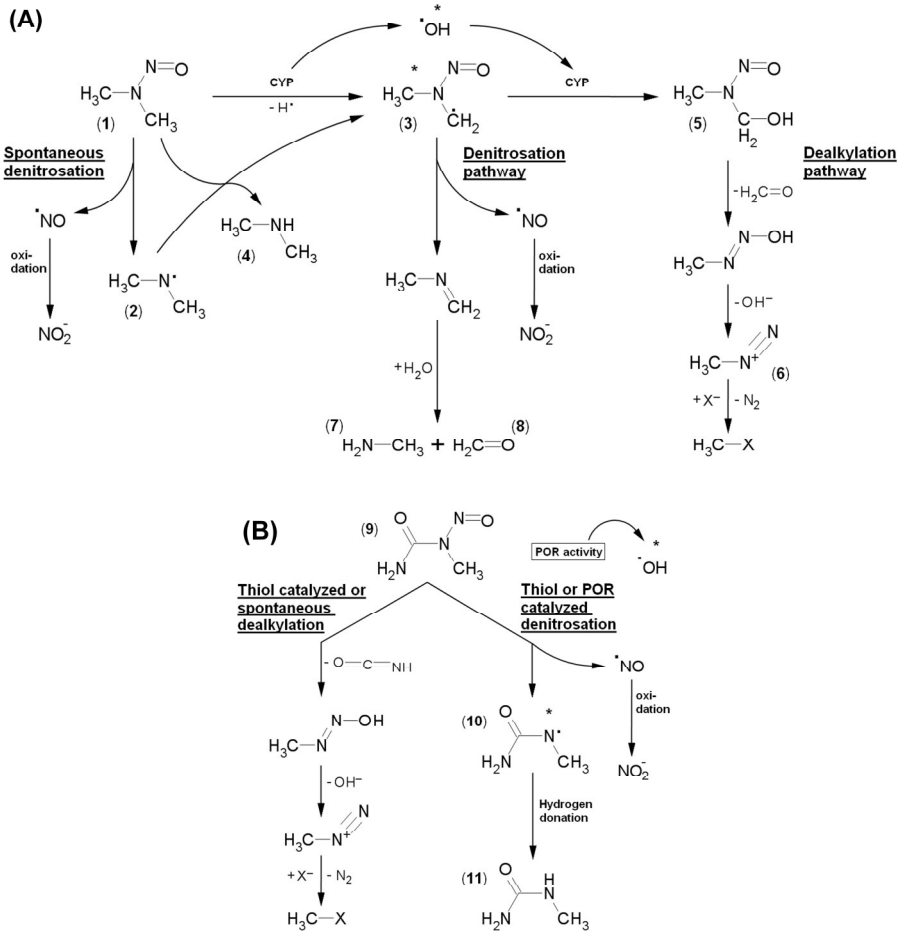

Figure 6: (A) Metabolism of nitrosamines (shown for NDMA (**1**) as an example). Spontaneous denitrosation gives rise to the alkylaminyl radical (**2**), which can react with the parent compound, resulting in the formation of the α-nitrosamino CCR (**3**) and the corresponding amine (**4**). During CYP metabolism, hydroxyl radicals (•OH) and CCR's are formed, which can recombine in the dealkylation pathway and form the α-hydroxynitrosamine (**5**) which decomposes into the highly reactive diazonium species (**6**) that can damage nucleophilic compounds (X^-), such as DNA. Alternatively, denitrosation can occur, which gives rise to •NO, methylamine (**7**) and formaldehyde (**8**). **(B)** Metabolism of nitrosamides (shown for MNU (**9**) as an example). Thiol or POR catalyzed denitrosation results in the formation of an NCR (**10**), which subsequently produces the corresponding amide (**11**). Alternatively, dealkylation can occur (thiol catalyzed or spontaneous) similar to the nitrosamine dealkylation pathway.

***** Radicals trapped by DMPO in this study

At a lower concentration of 1 mM no ROS formation above control levels was observed for nitrosamines and CCRs were entirely absent from the spectra (Figure 5A), clearly indicating that radical formation is nitrosamine concentration dependent and detected only at concentrations that are genotoxic in the comet assay.

The results we found after nitrosamide exposure were quite different from the nitrosamines. At comparably genotoxic doses, both MNU and MNNG produced a small amount of ROS above control levels in Caco-2 cells, although this was only significant for MNU (Figure 4). At a higher dose of 1 mM (equimolar to MNU), MNNG produced a ROS signal in cells that was significantly higher than control levels and also higher than MNU-induced ROS levels (Figure 5B and Table 1B). MNU and MNNG are both known to cause oxidative stress in animal models and cellular systems [12,31,32], and it appears that this ROS formation is dependent on cellular components such as glutathione and the hypoxanthine-xanthine-oxidase system, which may explain the ROS levels seen in the Caco-2 cell incubations.

Incubation of cells with MNU at a genotoxic concentration also generated a compound-specific NOC radical (Figure 3C). We propose that this could be an NCR (the amidyl radical, Figure 6B), which was also found after UV irradiation and the concomitant denitrosation of MNU [15]. Although absent at a genotoxic concentration of 1 µM, at 1 mM, MNNG also generates such a radical (Figure 5B). The formation of these radicals has also been suggested by others [33] and simulations of DMPO-trapped amidyl radicals and DMPO-OH indeed resulted in a composite spectrum that exactly fits our detected spectra (Figure 3D and 5C). The observation that cells enhance the formation of this NCR (Table 1A) suggests a stimulating effect of cells for nitrosamide exposure as well. Although nitrosamides do not require metabolic activation by CYP enzymes to become genotoxic, nitrosamides are known to react with thiols, such as glutathione, which can result either in denitrosation or dealkylation (Figure 6B). The latter is similar to the spontaneous decomposition of nitrosamides. The presence of cellular thiols could therefore catalyze the formation of amidyl radicals. The biological relevance of these radicals is questionable, since they would normally recombine with the hydrogen residue of glutathione to form a harmless amide. Moreover, Romert et al. [34] have shown that under physiological conditions, the thiol mediated denitrosation pathway plays a relatively small role and metabolism is mostly directed towards dealkylation. Additionally, thiol catalyzed denitrosation appears only relevant for MNNG, not MNU [35]. Another explanation for the cellular stimulation of NCR formation from nitrosamides is via microsome dependent denitrosation which may occur as a result of cytochrome P450 oxidoreductase (POR) activity and is augmented by cytochrome P450 [36,37]. Activity of these enzymes could also contribute to the DMPO-OH signal observed following cellular nitrosamide exposure. Denitrosation of nitrosamides is considered as a non-genotoxic metabolic route and is unlikely to result in significant damage to cellular components, although the concomitant ROS formed could lead to oxidative

damage. Since the genotoxicity of nitrosamide and nitrosamine concentrations in our study was similar, and the denitrosation route for nitrosamides appears less important for DNA damage formation, the genotoxic effect of nitrosamides is most likely related to DNA alkylation. It is interesting that MNNG induces genotoxicity at a much lower concentration than MNU [38–40] and this could be related to a higher reactivity of MNNG with thiols or a higher rate of spontaneous dealkylation.

The fact that at comparably genotoxic doses, a higher ROS formation is observed for nitrosamines (Figure 4), whereas alkylating damage appears to be more relevant in the genotoxicity of MNNG and MNU [34], does not necessarily mean that nitrosamine exposure-related alkylating damage is less important. CCR levels clearly indicate the formation of α-nitrosamino radicals that are likely to further react to form alkylating diazonium ion species. The fact that nitrosamine exposure does not show radical formation at a concentration of 1 mM, indicates that at equal dose nitrosamides are likely to be more genotoxic than nitrosamines, as also demonstrated by the higher concentrations needed to induce a similar level of DNA damage in the comet assay. Although the comet assay may be more sensitive to ROS-induced DNA breaks, thereby detecting ROS related effects more readily, the alkaline comet assay is also known to respond to alkylating damage as a result of DNA breaks induced by alkyl adduct repair mechanisms and alkali labile sites [41]. Since MNNG and MNU exposure also resulted in a significant level of DNA damage, whereas ESR analysis showed (almost) no ROS formation above background at these genotoxic concentrations, the DNA damage level can not be attributed to ROS and can only be ascribed to alkylating damage. Consequently, the DNA damage level induced by nitrosamines at genotoxic concentrations also has to be (partly) attributed to alkylating damage. Alkylating damage can be expected after exposure to 1 mM nitrosamides [42] and the formation of DMPO-OH (Table 1B) suggests oxidative damage is also a likely outcome, whereas the absence of nitrosamine-induced ROS and CCR signals at this concentration suggests that these types of damage are not likely to occur with nitrosamines in Caco-2 cells. Of course this depends on the metabolic CYP activity of cells, which in the Caco-2 cell line is lower than in, for example, liver tissue [28]. A higher metabolic rate could result in a higher nitrosamine-related radical production and genotoxicity compared to nitrosamides. A higher level of genotoxicity is, however, not automatically associated with a greater carcinogenicity as shown by Brambilla *et al.* [43]. In view of the complex relationship between radical formation, genotoxicity and human carcinogenicity as well as the multiple mechanisms underlying the carcinogenic process, it is difficult to assess, based on our data, which compounds would pose a higher carcinogenic risk in the human colon, using Caco-2 cells as an *in vitro* model.

To establish the link between ROS formation and the transcriptomic response, we identified gene expression changes that significantly correlated with ROS levels (Table 2). Although we identified a number of pathways that were affected after 1

hour of exposure, the relatively low number of significantly affected genes in each pathway makes it difficult to assess the direction of the biological effect of these modified pathways. The correlation between ROS formation and gene expression data after 24 hours, however, shows much more pronounced effects on the same cellular processes, including apoptosis, cell cycle regulation, DNA repair and the oxidative stress response. In view of the residual amount of NDMA after 24 hours, radicals should be formed continuously during the exposure. This indicates that the gene expression modifications associated with ROS formation become more enhanced over time. The difference in the number of significantly modulated genes and molecular pathways between nitrosamines and nitrosamides, shows a similarity with the ROS-induced DMPO-OH signal, being much higher following nitrosamine exposure. The lower ROS level of NDMA compared to the other three nitrosamines is also reflected by the absence of a number of cellular processes following NDMA exposure after 24 hours, again suggesting the level of ROS has a determinant effect on the extent of gene expression changes. Oxidative stress pathways are most strongly associated with exposure to NDEA, NPIP and NPYR, which also display the highest levels of ROS formation. The correlations that were found also indicate that ROS may be partially responsible for the cell cycle blocks and increased apoptosis levels we previously observed (**Chapter 2**) [10], in addition to the well known effect of alkyl-adducts on cell cycle blockage and apoptosis[44]. This is likely to be a direct consequence of the DNA damaging properties of ROS, since DNA damage repair is also correlated with ROS levels (Table 2). Other processes like cytoskeleton remodeling and nucleotide metabolism are likely related to cytoskeletal changes associated with apoptosis and cell cycle regulation and changes in the nucleotide pool as a result of the DNA replication blockage during S phase. A number of significantly modulated pathways involved in the immune response were also found. As nitric oxide has important immunoregulatory functions and oxidative stress is known to stimulate cytokine production in Caco-2 cells [45–47], these modulated pathways could be related to $^{\bullet}$NO formed after denitrosation of nitrosamines or oxidative stress caused by nitrosamine-induced ROS formation. In the supplemental data, a complete overview of modulated pathways can be found, which in addition to the cellular processes already mentioned, also shows a number of significantly modulated pathways involved in G-protein signaling and cellular development. Modulations in G-protein signaling and developmental pathways, many of which are involved in crucial parts of cell differentiation, could influence the cancer risk and contribute to the genotoxic aspects of ROS in the carcinogenic process, in addition to NOC-induced alkylating damage and its associated gene expression changes. Although correlation analyses could not be performed with the NOC radicals, it is likely they contribute to these ROS-associated gene expression changes since metabolism of NOCs results in a concomitant production of both ROS and NOC radicals.

In summary, we have demonstrated for the first time NOC-induced ROS-formation and compound-specific NOC radicals in colon cells. By analyzing both nitrosamides and nitrosamines, differences in radical generation mechanisms were found, which are helpful in explaining the observed difference in genotoxicity in the Caco-2 cell line between these two classes of potentially carcinogenic compounds. The data indicate that the levels of ROS generated by NOCs may contribute to the genotoxic effects of alkylation and that they strongly influence pivotal gene expression pathways which increase in effect over time. Therefore, at comparably genotoxic concentrations, nitrosamines not only display a stronger concomitant radical production than nitrosamides, they also affect several molecular pathways much stronger than nitrosamides. In addition to the carcinogenic risk associated with NOC-induced DNA alkylation, the identified ROS-related mechanisms may also contribute to the development of human colon cancer. Overall, it is concluded that radical formation by NOCs, i.e. both CCRs and ROS, may play an important role in health effects associated with colonic NOC exposure and that nitrosamides and nitrosamines induce distinct molecular responses.

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Chapter 4

Time-series analysis of gene expression profiles induced by nitrosamides and nitrosamines elucidates modes-of-action underlying their genotoxicity in human colon cells

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Abstract

N-nitroso compounds (NOCs), a class of well-established animal carcinogens, may represent a potential carcinogenic risk to humans since they are present in the diet and can be formed endogenously. We used the colon adenocarcinoma cell line Caco-2 to investigate gene expression changes at three time points (1, 6, and 24 hours) following exposure to genotoxic concentrations of six different NOCs (two nitrosamides, four nitrosamines) with the purpose of identifying biological processes that may play a part in the carcinogenicity of these compounds. This is especially important for nitrosamide exposure, where in light of their high reactivity and unstable nature, important gene expression modifications may take place early in the exposure. We also analyzed NOC-induced O^6-methylguanine adducts in relation to transcriptomics since these adducts may influence the expression of genes pivotal in NOC-associated carcinogenicity. Many modified pathways appeared related to DNA damage, cell cycle, apoptosis, growth factor signaling and differentiation, which are linked with carcinogenicity. Nitrosamides showed the strongest response at 1 hour of exposure, while nitrosamines had the strongest effect at 6 and 24 hours. The gene expression response was also confirmed by phenotypic anchoring in relation to effects on apoptosis and cell cycle distribution. Additionally, methylation was strongly associated with processes that may contribute to the carcinogenic risk. In summary, we have found that NOC-induced gene expression changes vary over time and that many of the modified pathways and processes indicate a carcinogenic risk associated with NOC exposure since they involve pathways implicated in crucial developmental signaling cascades.

Introduction

N-nitroso compounds (NOCs) have long been recognized as genotoxic and carcinogenic agents in animal models and therefore may also be implicated in human carcinogenesis [1]. The gastro-intestinal tract seems to be a particularly important target since NOC are known to occur in certain types of food, such as beer, fish and nitrite-preserved meat [2]. In addition, endogenous nitrosation of NOC precursors, particularly in the stomach and colon, can significantly increase the colonic NOC exposure [3,4]. The endogenous formation of NOCs may also explain the link between meat consumption and colorectal cancer (CRC) risk since CRC incidence rates are strongly correlated with average meat consumption levels [5,6] and meat consumption results in increased levels of endogenous nitrosation [3]. To investigate the possible role of NOCs in human colon carcinogenesis, we previously explored the transcriptomic effects of genotoxic NOC concentrations following a 24 hour exposure in the human colon adenocarcinoma cell line Caco-2 (**Chapter 2**) [7]. We distinguished between two main classes of NOCs, nitrosamines and nitrosamides. An interesting observation was the absence of strong gene expression effects following nitrosamide exposure. Since nitrosamides, in contrast to nitrosamines, can decompose without the need for metabolization to form genotoxic intermediates, particularly alkylating diazonium ion species, we hypothesize that modifications in critical molecular processes may occur earlier in the exposure period. Furthermore, although the alkylating potential of NOCs has been studied extensively and the role of alkylation in the carcinogenic process is well documented [8–10], it is of interest to analyze gene expression changes associated with NOC-induced alkylation over time. Alkylation of DNA, and methylation in particular, by alkylating agents has previously been linked to the modulation of gene expression. For example, O^6-methylguanine (O^6-meG) has been shown to both inhibit and promote the action of maintenance methylases on adjacent cytosine bases which is likely to result in chromatin modifications that can influence the level of gene transcription [11,12]. In addition, O^6-meG can inhibit transcription factor binding [13,14]. Investigating methylation-associated gene expression changes could thus lead to the identification of critical pathways which may underlie the carcinogenic mechanism of action of methylating agents.

In order to establish time-dependent gene expression responses, we analyzed genome-wide gene expression in Caco-2 cells exposed at 1, 6 and 24 hours to comparably genotoxic concentrations of 2 different nitrosamides (N-methyl-N'-nitro-N-nitrosoguanidine [MNNG] and N-methyl-N-nitrosurea [MNU]) and 4 nitrosamines (N-nitrosodiethylamine [NDEA], N-nitrosodimethylamine [NDMA], N-nitrosopiperidine [NPIP], and N-nitrosopyrrolidine [NPYR]). These NOCs can be formed in the gastro-intestinal tract [2,15] or induce malignant transformation of human colonic epithelial cells [16]. We subsequently identified cellular processes that were modi-

fied over time by these NOCs, and applied separate per time point analysis to determine at what time points relevant pathways or cellular processes were most strongly modulated. Individual gene expression data were linked with levels of apoptosis and cell cycle distribution as phenotypic markers of effect to functionally anchor gene sets differentially modified by NOC exposure. To investigate the gene expression modulations associated with methylation, we further explored the transcriptomic response of the three methylating NOCs (MNNG, MNU, and NDMA). To do this, we correlated gene expression levels with O^6-meG adduct levels, one of the most frequently occurring pre-mutagenic DNA lesions found after exposure to methylating agents [17,18], and analyzed the correlating gene set for involvement in pathways or cellular processes. Our overall goal is to establish a transcriptomic overview of NOC-induced and methylation-associated responses at a biological pathway level over time that can be used for further analysis on the carcinogenic potential of these compounds.

Material and methods

Cell culture

The human colon adenocarcinoma cell line Caco-2 was cultured in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, Zwijndrecht, The Netherlands) with 4.5g/L glucose, L-glutamine, $NaHCO_3$ and pyridoxine HCl supplemented with 1% (v/v) non-essential amino acids, 1% Na-pyruvate, 1% penicillin/streptomycin and 10% (v/v) heat-inactivated foetal calf serum, all purchased from Gibco BRL (Breda, The Netherlands). Cell cultures were transferred weekly by trypsinization and incubated at 37 ºC in a humidified incubator containing 5% CO_2.

Treatment and isolation of cells

Caco-2 cells were treated with MNNG, MNU, NDEA, NDMA, NPIP or NPYR or the corresponding solvent control (MilliQ or DMSO, final concentration 0.1%) at comparably genotoxic concentrations, as established previously by comet assay analysis (**Chapter 2**) [7]. These concentrations were: MNNG (1µM), MNU (1mM), NDEA (50mM), NDMA (100mM), NPIP (40mM) and NPYR (100mM). Exposures were carried out in 25 or 75 cm^2 culture flasks for 1, 6 or 24 hours. All NOCs were obtained from Sigma-Aldrich (the respective product numbers and reported purity were: 15427LO [97%], N4766 [11% H_2O, 3% acetic acid], N0756, N7756, N6007 [99.8%], and 158240 [98.9%]). Experiments were carried out in duplicate. Cells were isolated by trypsinization and washed in PBS and subsequently stored at -20°C (for methylation assay) or fixed in methanol and stored at -20°C (for flow cytometry). Cells for

microarray experiments were isolated with TRIzol® Reagent (Invitrogen, Breda, The Netherlands) and stored at -20 ºC. Viability of cells was determined with the MTT test in ELISA plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) as described by Mosmann [19] with minor modifications. All exposures resulted in a viability of more than 90%.

Immunocytochemistry and flow cytometric analysis

Methanol-fixed cells were indirectly stained for cleaved cytokeratin 18 (an early sign of apoptosis) using the primary antibody M30 CytoDeath (Roche, Penzberg, Germany) and subsequently analysed using a FACSort (Becton Dickinson, Sunnyvale, USA) equipped with an Argon ion laser and a diode laser as described by Schutte *et al.* [20]. Data analysis was performed using CellQuest software (version 3.1, Becton Dickinson, San Jose, USA). Data were gated on pulse-processed PI signals to exclude doublets and larger aggregates from analysis. M30 CytoDeath positive and negative signals were sorted in the gated population and displayed as percentage of total cells. In addition, cell cycle distribution was determined using ModFIT LT (version 2.0, Verity Software House, Topsham, USA).

Determination of O^6-meG in DNA by ELISA

For the conduct of the newly developed ELISA, a 96-well microtiter plate was coated with anti-rabbit-IgG by the addition of 100 µl of antiserum (10 µg/ml) in coating buffer (Na_2CO_3 0.015M, $NaHCO_3$ 0.035M; pH 9.6) and overnight incubation at 4^{o}C. After washing three times with water, non-specific binding sites were blocked with 310 µl casein (0.25%) in PBS/0.5% Tween (PBST) for 1.5 h at 37°C, followed by washing with two cycles of PBST.

Triplicate samples of DNA for analysis (unknown or standards 1–11 µg DNA dissolved in 55 µl buffer) were exhaustively digested with Msp1 (5x2 units/µg DNA) by overnight reaction in microcentrifuge tubes at 37^{o}C using the supplier's buffering conditions. The digested DNA was then made single-stranded denatured by heating to 95^{o}C for 10 minutes, snap frozen in liquid nitrogen and placed on ice. Subsequently, anti-O^6-meG antibodies were allowed to react with adduct-containing DNA fragments by the addition of an equal volume of rabbit anti-O^6-meG antiserum (diluted 3,750-fold in 2xPBS, 1% Tween, 0.5% casein) and incubation at 37°C for 1.5 hours in a shaking water bath. After this incubation, 100 µl of each DNA-antiserum mixture were transferred to a well of the microplate prepared as described above and incubated for 1.5h at room temperature to allow binding of the antibody-adducted DNA complexes, followed by washing with five cycles of PBST. Two sequential steps then followed: 1) addition of semi-purified monoclonal anti-ssDNA mouse antibody diluted 100 fold in PBST, 0.25% casein and 2) addition of goat anti-

mouse IgG conjugated with alkaline phosphatase diluted also in PBST, 0.25% casein. Each step was followed by washing with five cycles of PBST. Finally, two extra washing cycles with Tris buffer (20 mM Tris, 1 mM $MgCl_2$; pH 9.5) were performed. A chemiluminescence signal was finally generated by the addition to the wells of CDP-Star substrate containing Emerald II enhancer (100 µl) and incubation of the plates at room temperature for 30 min. Chemiluminescence was measured using a Safire II Microplate Luminometer (TECAN, Männedorf, Switzerland) at 542 nm.

For the quantitation of O^6-meG in unknown DNA samples, a standard curve consisting of DNA standards with known O^6-meG content was constructed with each microwell plate. These standards were prepared by mixing HeLa DNA, methylated with MNU and accurately analyzed by HPLC (O^6-meG content: 1 adduct/13,000 nucleotides), with unmodified HeLa DNA extracted from O^6-meG-DNA methyltransferase-overexpressing cells as described previously [21].

Microarray hybridization and data analysis

Microarray hybridization was performed as described previously (**Chapter 2**) [7]. In short, RNA was isolated from QIAzol® suspended cells, following a 1, 6 or 24 hour exposure to comparably genotoxic NOC concentrations (two biological replicates) and dye-labeled cRNA (Cy3 or Cy5) was synthesized. Samples were hybridized on Agilent 4x44K Whole Human Genome microarrays (Agilent Technologies, Amstelveen, The Netherlands) against their vehicle control, applying a dye-swap between the biological replicates.

After scanning the microarray slides, using settings described before (**Chapter 2**) [7], the data was flagged manually and automatically using the GenePix Pro software (version 6.0, Molecular Devices, Sunnyvale, CA). Following a quality control in the statistical software environment R (version 2.10.1, The R Foundation for Statistical Computing, Vienna, Austria), regions of lower quality were identified and subsequently flagged in Spotfire DecisionSite (version 9.1, TIBCO, Somerville, MA). LOWESS normalization and subsequent data analysis was performed in ArrayTrack (version 3.4, NCTR, Jefferson, AR). The expression difference for each spot was calculated by subtracting the log2 transformed mean intensity of the control sample from the log2 transformed mean intensity of the treated sample resulting in a log2 ratio.

ArrayTrack was used to find significantly modulated genes (two-tailed Student's *t*-test, p-value < 0.05) with a minimum absolute log2 ratio of 0.5 for each compound and time point which were subsequently imported in MetaCore™ (GeneGo, San Diego, CA). MetaCore™ is an online software suite that identifies and visualizes the involvement of differentially expressed genes in specific cellular pathways, which is subsequently related to the total number of genes involved in the particular path-

way and in all the available pathways combined. This results in a set of significantly modulated pathways (p-value < 0.05).

The online software suite GenePattern version 3.1 (http://www.broad.mit.edu/cancer/software/genepattern/) was used for principal component analysis (PCA) of the tested compounds. PCA plots were generated in GenePattern by reducing the data to two dimensions and visualized in Microsoft Excel. Time-course effects were further investigated, by ANOVA analysis in ArrayTrack to determine which genes changed their expression profile significantly over time. These genes were also analyzed in MetaCore to determine their involvement in pathways.

Gene expression data were subsequently used to perform Spearman's rank correlation analyses with levels of apoptosis, cell cycle phase distribution, and O^6-meG levels using the online Gene Expression Profile Analysis Suite (GEPAS, http://gepas.bioinfo.cipf.es/). Significantly correlating genes (p-value < 0.05) were subsequently further analyzed in MetaCore.

The gene expression data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO) and are available through GEO Series accession number GSE20993 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE20993).

A supplementary data Microsoft Excel file is available online and can be found at: http://dl.dropbox.com/u/11005104/Thesis%20Dennie%20Hebels%20Supplementary%20Data%20Chapter%204.xls.

Results

Time-course analysis: PCA and ANOVA

In order to investigate the transcriptomic effects of NOCs over time we performed whole genome microarray analysis at three time points for each of the six selected NOC at comparably genotoxic concentrations, as determined previously [7]. PCA plots were generated to visualize the differences in gene expression profiles between NOCs on different time points. The three non-methylating NOCs (NDEA, NPIP, and NPYR) show a similar response compared to each other, and simultaneously demonstrate a time-dependent development of the gene expression response (Figure 1). Although NDMA is closely associated with these nitrosamines at 6 hours of exposure, it shows a different response after 24 hours, which we also found in our previous study (**Chapter 2**) [7]. To look at each individual time point in more detail, PCA plots were also generated per time point (Suppl. data, sheet 1). While these plots show a similar pattern as Figure 1, they also reveal that MNU and MNNG respond differently at each time point. In accordance with the number of significantly modulated genes and pathways, MNU develops the most differentiating response at 1 hour of exposure, while MNNG generates such a response at 6 hours.

After 24 hours of exposure both nitrosamides are grouped closely to the origin of the plot indicating little effect.

The time-course development of the gene expression profile was further investigated using an ANOVA analysis to determine which cellular processes contribute most to the changes in expression profile over time. In Table 1, an overview of processes for each of the six compounds found by MetaCore analysis is presented. In the Suppl. data Excel file (sheet 2) a more comprehensive overview can be found which includes the names of the GeneGO pathways modified in each of the cellular processes. Amino acid metabolism, apoptosis and survival, and cystic fibrosis are significantly modified over time by all compounds, except MNU. The development, G-protein signaling, and immune response processes are modified by all six compounds and are likely to play an important part in the time-course development of the gene expression profile, especially since the number of pathways involved in these processes is, overall, relatively high. Other strongly modified processes comprise cell cycle, steroid metabolism, and transcription. When comparing the nitrosamide and nitrosamine-induced time-dependent changes, in general, more processes and pathways seem to be modified over time during nitrosamine exposure. The percentages of involved genes are also higher for the nitrosamine group, which is in agreement with the PCA plots. The Venn diagram in Figure 2A shows the overlap between the two nitrosamides and the nitrosamines as a group. MNNG and MNU uniquely change the expression of a considerable number of pathways over time. For MNNG these pathways are mainly involved in apoptosis and survival, glutathione metabolism, G-protein signaling and immune response, while for MNU cell cycle, development, and DNA damage responses are mainly affected (Suppl. data, sheet 3). The pathways uniquely modified by the nitrosamines are involved in similar processes but often regulate different parts of the process. For example, the apoptosis and survival pathways include different signaling cascades and several development pathways are involved in transforming growth factor beta (TGF-β) signaling. There are also a number of pathways involved in processes not modified by nitrosamides, such as nucleotide metabolism and several other metabolic pathways. The Venn diagram in Figure 2B shows that within the nitrosamine group, a total of 35 pathways are uniquely modified by NDMA, which includes a number of pathways involved in apoptosis and survival, cell cycle regulation, development, and DNA damage (Suppl. data, sheet 4).

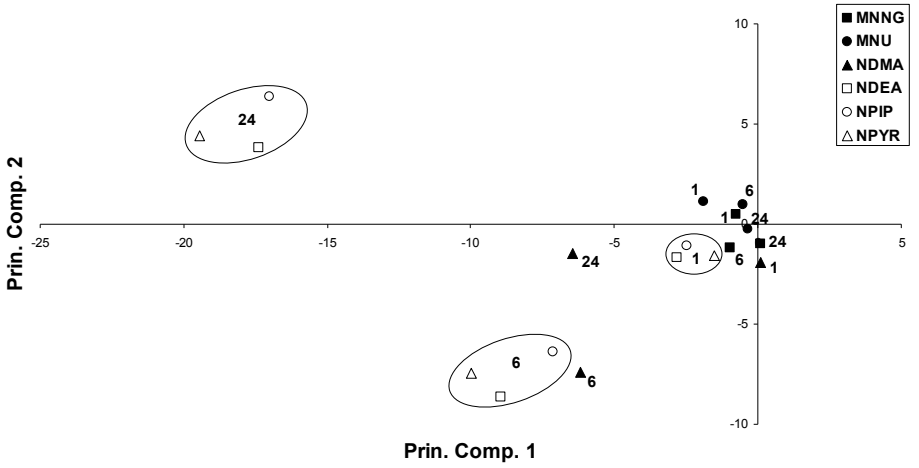

Figure 1: PCA plot of genes present for all NOCs at the three time points tested. The percentage variation for principal components 1 and 2 were 61.06 and 14.83%.

Table 1: MetaCore analysis of ANOVA data resulting in the identification of cellular processes modified over time for each NOC following exposure at genotoxic concentrations.

Cellular process	MNNG	MNU	NDMA	NDEA	NPIP	NPYR
Amino acid metabolism	5(30)		2(48)	4(56)	1(56)	5(78)
Apoptosis and survival	3(26)		12(52)	5(44)	3(48)	8(63)
Butanoate metabolism					1(55)	
Cell adhesion					2(62)	
Cell cycle	1(25)	11(38)	8(55)		1(50)	7(68)
Cystic fibrosis	1(26)		1(65)	1(53)	4(68)	2(69)
Cytoskeleton remodeling		1(20)			2(52)	
Development	2(26)	9(28)	13(49)	2(49)	7(48)	3(61)
DNA damage	1(24)	3(34)	5(54)	1(46)		
Folic acid metabolism			1(60)			
Fructose metabolism		2(26)	2(49)			
Galactose metabolism					2(59)	1(69)
Glutathione metabolism and oxidative stress	3(21)		1(67)			1(78)
Glycolysis and gluconeogenesis			1(60)	1(53)	2(57)	1(67)
G-protein signaling	2(49)	1(36)	2(52)	1(50)	1(56)	2(65)
Heme metabolism					1(48)	
Immune response	10(31)	6(31)	4(55)	3(58)	7(54)	1(67)
Neurodisease						1(72)
Neurophysiological process			1(58)	1(58)		
N-Glycan biosynthesis				1(47)		
Nucleotide metabolism			2(53)			2(53)
Oxidative phosphorylation				1(37)	1(46)	1(54)
Propionate metabolism						1(62)
Proteolysis					1(58)	1(86)
Regulation of lipid metabolism	1(3)		1(67)		1(67)	1(65)
Reproduction		1(21)				

Cellular process	MNNG	MNU	NDMA	NDEA	NPIP	NPYR
Signal transduction		1(26)			1(48)	1(59)
Steroid metabolism	1(29)		2(56)	3(57)	2(68)	2(69)
Sulfur metabolism	1(39)			1(50)	1(56)	
Transcription	1(23)	1(26)	3(50)		3(55)	2(67)
Transport	1(20)		1(42)			2(66)
Tricarboxylic acid cycle					1(56)	
Ubiquinone metabolism					1(46)	1(64)
Urea cycle					1(52)	

Values represent the number of significantly modified pathways for each cellular process followed by the average percentage of significantly modulated genes across the three time points in these pathways. Absence of a value means that the cellular process was not differentially modified over time for that particular compound.

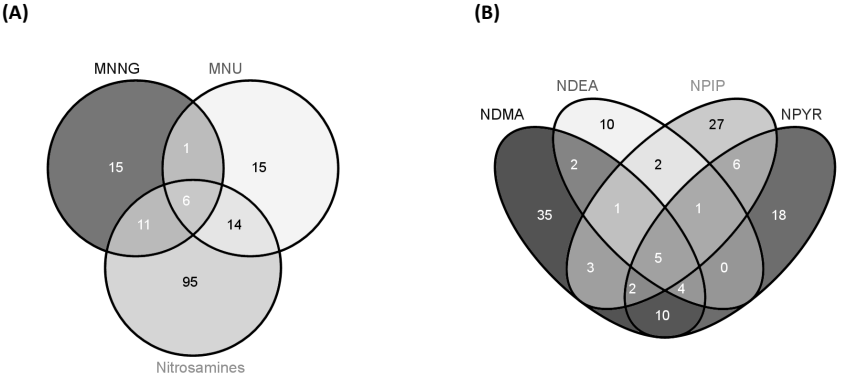

Figure 2: Venn diagrams showing the number of overlapping ANOVA pathways between MNNG, MNU, and the nitrosamines as a group **(A)** and between the four individual nitrosamines **(B)**.

Transcriptomic analysis per time point

To investigate transcriptomic changes per time point for each compound, significantly modulated genes (p-value < 0.05) with a minimum absolute log2 ratio of 0.5 were selected and subsequently used for a pathway analysis in MetaCore. An overview of the cellular processes modified by each of the six compounds per time point and a more comprehensive overview, which includes the names of the GeneGO pathways modified in each of the cellular processes, is presented in the Suppl. data (sheets 5–8). The percentage of significantly modulated genes in each of the pathways and the average per process is also presented there. A total of 39 cellular processes was found to be modified by at least one of the compounds and for most processes the percentage of involved genes for the associated pathways increases with time. Some of the processes most strongly represented include apoptosis and survival, cell cycle, cytoskeleton remodeling, development, DNA damage, G-protein

signaling, immune response, regulation of lipid metabolism, and transcription. Although some cellular processes are significantly modulated by the same compound at all three time points, most only come up once or twice. The development and immune response processes, for example, are represented at every time point for nearly every compound. Cell cycle and DNA damage processes, on the other hand, are mostly modified at 6 hours. To visualize the changes over time, in Figure 3 the total number of pathways per condition for all processes combined is shown with the total average of involved genes. The percentage of involved genes stays relatively constant over time for the nitrosamides. The number of pathways, however, is highest at 1 hour of exposure for both nitrosamides and MNU even shows the highest number of pathways of all NOC across all time points. Although the nitrosamines modulate different numbers of pathways across time and also influence many pathways at 1 hour, the average percentage of genes involved in these pathways is highest at 6 and 24 hours. Overall, the nitrosamides appear to induce the strongest response at 1 hour, while the nitrosamines tend to have the most pronounced effects at later time points.

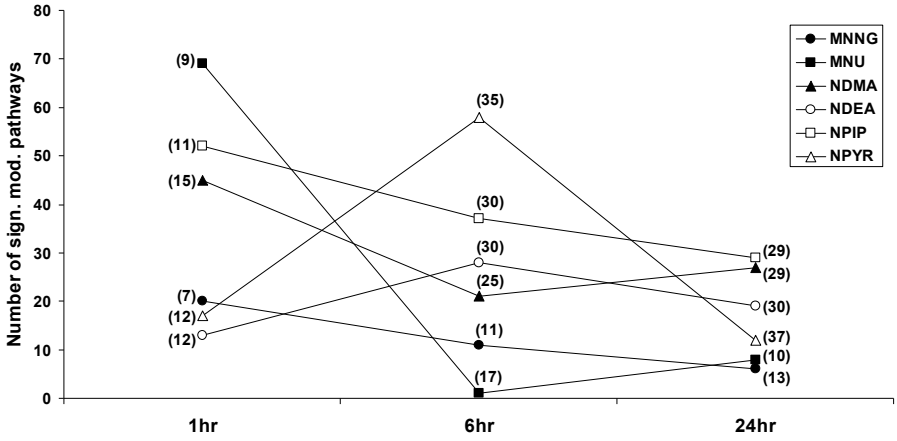

Figure 3: Time-course dependent effect of NOC exposure on the number of significantly modulated GeneGO pathway maps (p-value < 0.05) and the average percentage of significantly modulated genes involved in these pathways per condition (between parentheses).

Apoptosis and cell cycle distribution correlation analyses

We subsequently associated gene expression data from all time points and compounds with levels of apoptosis and cell cycle distribution as phenotypic markers of effect to functionally anchor gene sets differentially modified by NOC exposure over time. Levels of apoptosis and effects on cell cycle distribution were determined by

flow cytometry as shown in Figures 4A and 2B, respectively. The nitrosamines show a time-dependent increase in the level of apoptosis, although this increase is only significantly different from control conditions at the 24 hours nitrosamine exposure. Cell cycle distribution after 24 hours of exposure was significantly affected by all compounds, except MNNG. At earlier time points, no effects were observed compared to control distribution. MNU, NDEA, NPIP, and NPYR exposure resulted in an S-phase accumulation of cells, which for MNU, NPIP, and NPYR was also associated with a decrease in G1-phase cells, while NDEA shows a concomitant decrease in G2-phase cells. NDMA exposure results in a G1-phase accumulation of cells and a decreased S-phase distribution.

Genes significantly correlating with the level of apoptosis or cell cycle phase distribution (p-value <0.05) across all time points and compounds were used for a pathway analysis in MetaCore. As shown in Table 2, these genes are indeed involved in apoptosis and cell cycle related cellular processes. A complete overview of correlating processes can be found in the Suppl. data (sheet 9).

O^6-meG correlation analysis

To investigate which gene expression modifications are associated with the methylating properties of NOCs, we performed a correlation analysis considering effects of the three methylating NOCs. O^6-meG adducts were measured by HPLC analysis, which revealed a time-dependent decrease in the number of adducts for all three compounds, although NDMA seems to reach a steady-state level between 6 and 24 hours (Figure 5).

Table 2: Selection of cellular processes correlated with apoptosis and cell cycle phase distribution as found by MetaCore analysis.

Phenotypical marker	Cellular process					
	Apoptosis and survival	Cell adhesion	Cell cycle	Cytoskeleton remodeling	Nucleotide metabolism	Proteolysis
Apoptosis	7(67)	3(68)	2(68)	2(66)	5(68)	2(72)
G1-phase		1(50)	1(33)			
S-phase			1(27)		1(17)	
G2-phase		2(19)	1(21)	2(19)		

Values represent the number of significantly modified pathways for each cellular process followed by the average percentage of significantly modulated genes across the three time points and six compounds in these pathways.

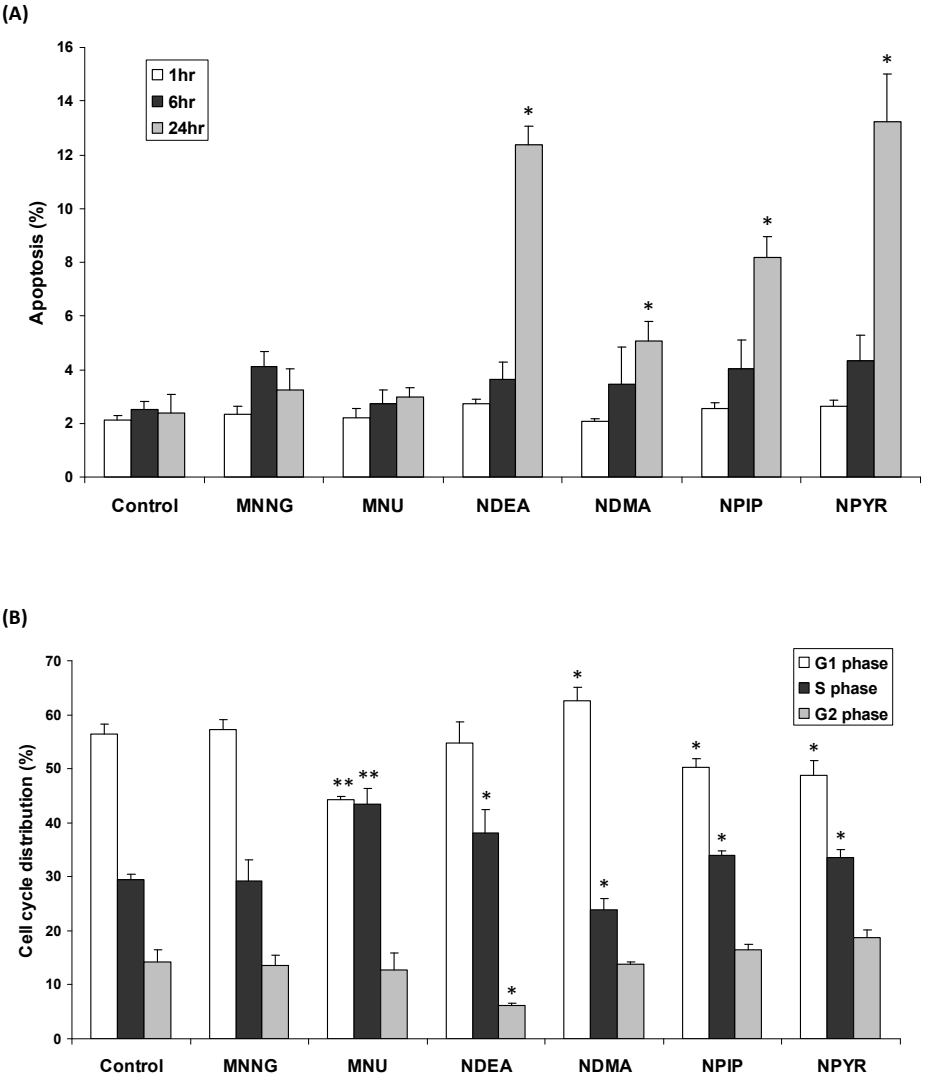

Figure 4: Flow cytometric analysis of **(A)** the level of apoptosis in Caco-2 cells exposed to genotoxic concentrations of six NOCs for 1, 6 or 24 hours and **(B)** cell cycle distribution at 24 hours of exposure. At 1 and 6 hours, cell cycle distribution was not significantly different from control. DMSO control is not shown but was identical to normal control. Error bars indicate standard deviation. Student's *t*-test versus control: * *p*-value <0.05; ** *p*-value <0.01

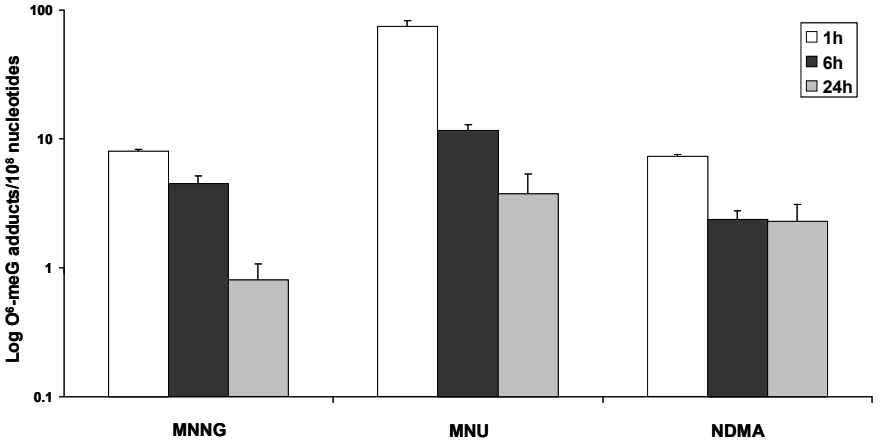

Figure 5: Level of O^6-meG in DNA from Caco-2 cells following exposure to genotoxic concentrations of MNNG, MNU or NDMA. Values are corrected for background levels.

Table 3: Cellular processes correlated with O^6-meG levels in DNA as found by MetaCore analysis.

Cellular process	Correlating pathways
Apoptosis and survival	7(24)
Cell cycle	1(21)
Chemotaxis	1(25)
Cytoskeleton remodeling	2(20)
Development	24(26)
DNA damage	2(23)
G-protein signaling	6(26)
Immune response	10(25)
Neurophysiological process	3(32)
Nucleotide metabolism	1(33)
Regulation of lipid metabolism	4(31)
Riboflavin metabolism	1(40)
Signal transduction	1(29)
Transcription	4(26)
Translation	1(19)

Values represent the number of significantly modified pathways for each cellular process followed by the average percentage of significantly modulated genes in these pathways.

Genes found to significantly correlate (p-value <0.05) with O^6-meG adducts induced by MNNG, MNU, and NDMA across all time points were analyzed in MetaCore to identify which pathways and processes were most strongly associated with methylation levels. Processes correlated with the level of O^6-meG adducts are presented in Table 3, which includes several apoptosis and survival, cell cycle regulation, cytoskeleton remodeling, and DNA damage related pathways. Other strongly modified processes are involved in development, immune response, G-protein sig-

naling, regulation of lipid metabolism, and transcription. The average percentage of genes in these pathways is relatively high, indicating a strong involvement with the methylating damage induced by these compounds. A complete overview of all the pathways involved in these processes can be found in the Suppl. data (sheet 10).

Discussion

Investigating gene expression changes at multiple time points not only allows for the analysis of genes and cellular processes that change their expression over time, it also provides information on the gene expression responses that might otherwise be missed by analyzing just one time point. By doing so, crucial biological processes can be identified that may play a part in the carcinogenicity of these compounds. This is especially important for nitrosamide exposure, where in light of their high reactivity and unstable nature, important gene expression modification may take place early in the exposure. Furthermore, investigating the role of alkylation in the induction of gene expression changes could contribute to our understanding of the carcinogenic effect of this type of DNA damage. In this study, these queries were explored using a number of different analysis techniques.

Nitrosamide induced gene expression response

In the PCA plots (Figure 1 and Suppl. data, sheet 1) the nitrosamides shift position over time and always group separately from the nitrosamines with MNNG and MNU uniquely changing the expression of many pathways over time as compared to the nitrosamines (Figure 2A and Suppl. data, sheets 2 and 3). Although the total average percentage of significantly modulated genes stays relatively constant over time for both nitrosamides (Suppl. data, sheet 5 and Figure 3), the genes modulated after 1 hour of exposure are involved in a higher number of pathways which demonstrates the importance of interpreting data at a functional level. As demonstrated by Figure 2A and the affected cellular processes per time point (Suppl. data, sheets 5–8), MNNG and MNU have different effects on the expression of genes at the tested concentrations. Modifications in cell cycle pathways, for example, are observed after 1 hour of MNU exposure, whereas MNNG exposure does not seem to affect the progression of the cell cycle at all. As later discussed in more detail, induction of methyl adducts in DNA by MNU after 1 hour is significantly higher than for MNNG (Figure 5) at the concentrations used, which could explain the absence of a cell cycle block following MNNG exposure. In addition, MNU and MNNG induce different protein adducts, either through carbamoylation (MNU) or guanidinylation (MNNG), and histones are known targets which could differently influence the gene expression profile [22]. Nuclear protein methylation is also known to be induced by alkylat-

ing agents [23–25] and the level of MNNG-induced methylation (protein or DNA) is determined by intracellular thiol concentrations which could all play an important part in the transcriptomic changes [26]. It is also interesting that carcinogenic methylating agents methylate proteins in a different pattern than non-carcinogenic methylating agents which could contribute to their carcinogenic potency [23]. Modifications in apoptosis and cell cycle regulation pathways for MNNG and MNU exposure respectively, are generally in agreement with flow cytometry data showing a significantly different MNU-induced cell cycle phase distribution at 24 hours, whereas MNNG shows a (non-significant) peak in apoptosis at 6 hours (Figure 4A and B). Other modified processes that contribute strongly to the transcriptomic time-course development include development, DNA damage, G-protein signaling, immune response and transcription and as shown by the per time point analysis (Table 1 and Suppl. data, sheets 5–8), these modifications appear to take place early in the exposure.

Both nitrosamides also modulate a considerable number of pathways involved in developmental processes that regulate cell growth and proliferation, such as the WNT signaling pathway and growth factor/hormone regulated pathways (Suppl. data, sheets 5–8). These types of modifications are especially interesting when the carcinogenic properties of nitrosamides are considered as the carcinogenicity of these compounds is often only attributed to their DNA damaging capability. Alterations in WNT signaling and growth regulation play an important role in colorectal carcinogenesis [27,28] and could contribute to cancer development. In addition to these developmental effects, immune response pathways are also strongly affected (Table 1 and Suppl. data, sheets 5–8), and include several interleukin and macrophage migration inhibitory factor (MIF) signaling pathways. Nitrosamides have been reported to have immunosuppressive effects [29,30] and intestinal epithelial cells, including the Caco-2 cell line, excrete pro-inflammatory cytokines and express cytokine receptors [31,32]. Interleukin-6, for example, plays an important role in the induction of the acute phase response and the intestinal inflammatory response [33] and also has immunosuppressive properties [34], while MIF is associated with colorectal cancer development [35].

Nitrosamine induced gene expression responses

With regard to the nitrosamines, especially at the 6 and 24 hours time points, a separate group is formed in the PCA plot (Figure 1), although NDMA forms a deviating response at 24 hours. Based on the changes in the percentage of genes and the number of pathways over time (Figure 3), the highest response seems to be induced at the 6 hour time point, even though NDMA and NPIP form an exception with regard to the number of pathways. One hour of exposure already affects many pathways, although the average percentage of involved genes is considerably lower than

at 6 or 24 hours, which is to be expected considering the lower numbers of significantly modulated genes. The deviating response of NDMA is most likely due to the 35 uniquely modified pathways found in the ANOVA analysis (Figure 2B and Suppl. data, sheets 2 and 4), which are involved in processes like apoptosis and survival, cell cycle, development, and DNA damage. Based on these specific processes it could be argued that the type of DNA damage induced by NDMA plays a role in its response. NDMA-induced methyl adducts, which are different from the larger alkyl adducts induced by the other nitrosamines, are repaired more efficiently by O^6-methylguanine-DNA methyltransferase, which is one the most important enzymes associated with alkyl-adduct repair, and this will also influence the cell cycle blockage and apoptosis process [9,36,37]. NDMA indeed causes the lowest increase in apoptosis and a different cell cycle blockage effect compared to the other nitrosamines (Figure 4A and B).

A large number of pathways involved in development are regulated at every time point by the nitrosamines, where nitrosamide-induced modifications are limited to the 1 hour exposure. The response seems to be strongest after 6 and 24 hours since the percentage of involved genes is highest at these time points (Figure 3). Many of these pathways are differentially regulated over time (Table 1) and include several pathways involved in growth factor signaling and differentiation, such as TGF-β and the WNT and Notch signaling pathways (Suppl. data, sheet 2). From a carcinogenesis perspective, this could be very important for human nitrosamine exposure where modifications in these pathways could influence the level of differentiation and cell division in the colonic epithelium. The WNT and Notch signaling pathways, for example, are known to interact in the maintenance of progenitor cell populations in the colon [27]. Interestingly, Abdel-Aziz *et al*. [38] also found modifications in the WNT signaling pathways in mouse lung tumors induced by the cigarette-smoke derived nitrosamine 4-(methylnitrosamino)-1-(3-pyridyle)-1-butanone. It seems that the nitrosamines, as well as the nitrosamides, investigated in this study are all capable of modifying essential developmental processes, which could work in concert with the DNA damaging capabilities of NOCs in the carcinogenic process.

Nitrosamine exposure also strongly affects the immune response as demonstrated by the large number of pathways involved in this process across time and at every time point (Table 1 and Suppl. data, sheets 2 and 5–8). Although the nitrosamides also affected the immune response throughout the exposure period, the nitrosamine-induced response is much stronger, especially at 6 and 24 hours, with more involved pathways and a higher percentage of modulated genes. Nitrosamines have immunosuppressive effects [39] and this may influence the innate immune response of colonic epithelial cells. Since the immune response also plays an important role in the development of colon cancer, especially in the promotion phase of the carcinogenic process, this indicates an additional way for nitrosamines to influ-

ence the colon cancer risk [28]. As shown in the Suppl. data (sheets 6–8), several cytokine signaling pathways, such as MIF and interleukin-1, 6, 12, 22 and 27 signaling are modified, many of which are implicated in colon cancer development [28,35]. The four nitrosamines do not always affect the same pathways and the net effect on immune modulation may not always be the same for all nitrosamines, although the PCA plots imply that especially NDEA, NPIP, and NPYR display a similar response. Our findings for both the nitrosamides and nitrosamines with regard to the immune response suggest further research into the immunomodulatory properties of NOCs.

Other interesting observations are the modifications in pathways involved in regulation of lipid metabolism, cystic fibrosis, and steroid metabolism which are regulated at all time points, although not for every nitrosamine (Suppl. data, sheets 6–8). Lipid metabolism regulation could be associated with the apoptotic process [40], but it may also play a part in the cystic fibrosis process. The pathways belonging to this process are involved in ion channel conductance regulation and lipid synthesis and transportation and are known to play a very important role in intestinal fluid and ion concentration regulation [41]. The steroid metabolism pathways could be explained in the light of nitrosamine metabolism which is known to be influenced by steroid signaling pathways [42,43].

Phenotypic anchoring of apoptosis and cell cycle effects

Taken together, the ANOVA and time point analyses of NOC-induced gene expression modifications confirm the changes in levels of apoptosis and cell cycle distribution found by flow cytometry for both the nitrosamides and nitrosamines which already shows that a time series analysis of transcriptomic data can reflect the phenotypic effects of NOC exposure. The association between the flow cytometry results and the apoptosis and cell cycle regulation processes is further confirmed by a correlation analysis (Table 2, Suppl. data, sheet 9), which shows that genes significantly correlating with apoptosis or cell cycle phase distribution are, in turn, involved in these very same processes and closely related processes like cell adhesion, cytoskeleton remodeling, nucleotide metabolism, and proteolysis.

Methylation-related gene expression modulations

Since there are indications that methylating damage, such as that induced by methylating NOC, may influence the gene expression response, this could contribute to the carcinogenic effect associated with this type of damage. The potential epigenetic effect of methylating compounds is not based on the classic mechanism of cytosine methylation since these agents are not know to induce cytosine adducts. Rather, guanine methyl adducts, and possibly others, can influence transcription

factor binding and the action of maintenance methylases on adjacent cytosine bases, which could both have important consequences for gene expression modifications in relation to carcinogenesis [11–14].

The analysis of O^6-meG levels shows that NDMA induces a relatively low level of methylation compared to the nitrosamides which is somewhat surprising, especially since the concentration of NDMA used in this study is much higher than for the nitrosamides. Animal studies have shown that NDMA is, on a per orally administered dose basis, by far the most efficient agent capable of generating O^6-meG in blood cell DNA when compared with other known methylating agents, such as MNU [44,45]. However, this does not seem to be true for Caco-2 cells, and possibly the human colon, since we found MNU to be the most efficient methylating agent, while MNNG generated methyl adducts at approximately the same amount as NDMA (Figure 5). Since the alkylating properties of nitrosamines are highly dependent on their metabolic activation by CYP450, this is likely to strongly influence the formation of O^6-meG adducts. Although Caco-2 cells and the human colon are both known to express CYP enzymes involved in nitrosamine metabolism [46,47], this is lower than in, for example, liver tissue. A combination of the CYP450-dependent biotransformation and higher stability of nitrosamines allows for a longer continuous exposure which is likely to influence gene expression more extensively and explain the transcriptomic difference between nitrosamines and nitrosamides found in this study. The decrease in O^6-meG adducts following NDMA exposure indeed levels out between 6 and 24 hours suggesting a continuous formation of reactive diazonium ions, whereas both nitrosamides show an incessant decrease over time.

A correlation analysis of transcriptomics results across all time points reveals a number of processes that are associated with methylation induced by MNNG, MNU, and NDMA (Table 3). Apoptosis and survival, cell cycle regulation and related processes, such as cytoskeleton remodeling and nucleotide metabolism, are involved and would also be expected to be associated with methylation levels, since this type of damage is a well-known inducer of apoptosis which is accompanied by cell cycle blockage and cytoskeleton modifications [9]. DNA damage pathways are indeed associated with methylation levels showing that the level of adduct formation correlates with the expression levels of genes involved in DNA repair. The results also suggest that methylation levels influence development, G-protein signaling and the immune response, since many pathways are involved in these processes. Especially development is strongly represented and includes many pathways involved in growth factor signaling often involving the JAK-STAT cascade, while the G-protein signaling process includes a number of RAS signaling pathways (Suppl. data, sheet 10). These pathways are often implicated in the carcinogenic process and it is possible that the modifications in these pathways represent an epigenetic component of methylation-associated gene expression changes, thereby providing a way to influence crucial carcinogenic mechanisms in parallel to the genotoxic mode of action

[48–50]. Whether other types of alkylation, such as adducts induced by NDEA, NPIP, and NPYR, are also linked to gene expression modifications is not known, but would be worth investigating.

It is an interesting observation that MNU, which causes the highest O^6-meG levels after 1 hour of exposure, also affects the highest number of pathways at this time point (Figure 3). In general the time-dependent decrease in methylation level induced by these three investigated compounds is paralleled by a decrease in the number of significantly modulated pathways. Although the processes found in the correlation analysis were also found in the ANOVA and per time point analyses, the pathways involved in each of the processes are not always the same or not that strongly represented for the individual compounds in the time point analysis (see Suppl. data, sheets 2 and 6–8). Especially the nitrosamides are not strongly involved in some of the processes found in the correlation analyses, such as the apoptosis and DNA repair processes. This may be a result of the filtering criterion used in the time point analysis, whereas the correlation analyses uses all significantly correlating genes regardless of log2 ratio or *p*-value per time point. The pathways found in the correlation analyses, which combines all time points in the analysis, therefore probably represent a more subtle effect that is difficult to pick up per time point.

In summary, we have found that NOC-induced gene expression changes vary over time and that nitrosamides induce the strongest response early in the exposure. Many of the pathways and processes modified may indicate a carcinogenic risk associated with NOC exposure since they involve pathways implicated in crucial developmental signaling cascades. Methylation is also associated with processes that could contribute to the carcinogenic risk following colonic NOC exposure, suggesting that methylating mechanisms can play a role in the carcinogenic process through gene expression modulations. Further research is needed to determine if these modifications also play a role in cancer development in the human colon.

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Chapter 5

Whole genome gene expression modifications associated with nitrosamine exposure and micronucleus frequency in human blood cells

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Abstract

N-nitroso compounds (NOCs) are suspected human carcinogens and relevant in human exposure. NOCs also induce micronuclei (MN) formation *in vivo*. 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. Moreover, investigating transcriptomic responses in relation to NOC exposure may provide crucial information on underlying molecular mechanisms of action. We therefore aimed to establish a relationship between human NOC exposure under daily life conditions and MN formation in association with transcriptomic changes, using lymphocytes as a surrogate tissue for analyzing carcinogenic events in target organs. Gene expression levels and MN frequency were analyzed in lymphocytes from adult females participating in the pan-European biomarker research project NewGeneris. To assess NOC exposure, urine samples were analyzed for marker nitrosamines. NOC excretion levels and MN frequency were subsequently linked to peripheral blood transcriptomics. We demonstrate 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. The modified genetic processes and genes found in this study may be of interest for future investigations into the carcinogenic risk associated with NOC exposure in humans. We believe that with this study we have created a model for in-depth investigations of environmental cancer risks among carcinogen-exposed humans.

Introduction

Research on human exposure to N-nitroso compounds (NOCs) has received much attention since it was shown in the 1960's that many NOCs are carcinogenic in rats [1]. Most NOCs have been classified as probable or possible human carcinogens by the International Agency for Research on Cancer [2], and many have been identified in foodstuffs, such as cured meat and beer, particularly NOCs belonging to the nitrosamine class. Policy measures to decrease the level of NOCs in these products have led to a significantly lower dietary exposure [3]. NOCs may also be formed endogenously in the stomach and colon out of the reaction between nitrate and amines or amides; this may actually result in higher colonic exposure than caused by food-borne NOC and may thus represent an important carcinogenic risk [4,5]. It remains difficult, however, to assess the actual human cancer risk as human exposure is relatively low and information on the possible (pre-) carcinogenic effects of NOCs in humans is sparse. Although there have been numerous reports on genotoxic and mutagenic properties of NOCs *in vitro* in, for example, the sister chromatid exchange assay and Ames test [6], 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 [7]. In several epidemiological studies, human NOC exposure has been associated with increased cancer risks of the stomach, esophagus, bladder, and colon [8,9], in particular in association with dietary intake of food items with relatively high levels of NOC precursors or nitrosating agents [4,10,11]. There is, however, still no consensus on whether NOCs actually are human carcinogens.

In an attempt to assess the potential carcinogenic risk of NOCs in humans, with particular interest for NOC-induced molecular mechanisms in the colon, we previously investigated gene expression changes in the human colon adenocarcinoma cell line Caco-2 (**Chapters 2**, **3**, and **4**) [12,13]. 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. Therefore, in the present study, we have evaluated whether NOC exposure in human subjects induces gene expression responses which may provide insights in actual human cancer risk. We have previously used genomic analyses to improve our understanding of childhood cancer risk associated with exposure to air pollution [14] and have also investigated differences in gene expression levels in adults subject to environmental burdens where associations were found of gene expression with blood and urinary measures of environmental carcinogens [15]. In this study, we used a similar approach by analyzing the excretion of two marker nitrosamines, i.e. N-nitrosodimethylamine (NDMA) and N-nitrosopiperidine (NPIP) [16], in urinary samples from pregnant mothers participat-

ing in the Danish pilot study of the multidisciplinary research project NewGeneris (http://www.newgeneris.org/) [17,18]. Both nitrosamines are known to be carcinogenic in test animals, may occur in several foodstuffs, and can be formed endogenously [19-21]. Moreover, since NOCs cause micronuclei (MN) formation *in vivo* and MN represent a well-validated biomarker of effect with regard to carcinogenic risk [22-24], MN analysis in peripheral lymphocytes provides an interesting marker to associate with human NOC exposure. NOC excretion levels and MN frequency were subsequently linked to peripheral blood transcriptomics to gain mechanistic insight into the relationship between human NOC exposure and MN formation in relation to human carcinogenesis.

Material and methods

Study population

Potential study participants were identified from the pregnancy medical records from the University Hospital of Copenhagen and the inclusion criteria were: (i) no chronic health complications e.g. diabetes; (ii) older than 18 years; (iii) no private commercial banking of cord blood; (iv) signed written informed consent; and (v) capacity to understand the informed consent. Eligible participants were: (i) non-smoking pregnant women, (ii) with no residential environmental tobacco smoke exposure, and (iii) who spent the majority of the time at home. The Ethics Committee of the Capital Region of Denmark (Reference No. J. Nr. H-KF-01-327603) and the Danish Data Protection Agency (Reference No. J. Nr. 2007-41-0415) reviewed and approved the studies prior to initiation. Further details on characteristics of study population have been described before [18].

Biological sample collection

Blood samples (~50 mL) were drawn in heparinized tubes (Becton Dickinson, Oxford, UK) from 30 pregnant women by venipuncture in the morning hours, between 7 and 11 a.m., 1-2 hrs before their planned Caesarean section. The women had been fasting for approximately 6 hrs. To stabilize RNA, 0.4 mL of whole blood was mixed with 1.2 mL RNAlater (Ambion, Naerum, Denmark) and stored at -20°C for the first 24 hrs followed by longer term storage at -80°C until use. Twenty-four hour urine was collected in 2L containers 1-5 days before child delivery and approximately 10 g of NaOH was added to the urine to avoid degradation of NOC. Aliquots of 50 mL were first stored at -20°C and subsequently at -80°C until analysis.

GC-MS analysis of NDMA and NPIP in urine

NDMA and NPIP were determined by gas chromatography-mass spectrometry (GC-MS) as previously described [25]. In short, urine samples from donors were thawed and extracted with dichloromethane (750 µL/14 mL urine). Then 1 µL of the extracted dichloromethane solution was injected into the GC-MS system. Molecular ions were detected using a resolution of 3000 and quantification was performed by a calibration curve for each of the two nitrosamines, using standards dissolved in urine and extracted similarly to adjust for a possible loss of nitrosamines during work-up. Recovery of NOC was 40% (NDMA) and 90% (NPIP) (detection limit: 0.05 ng/mL urine). Total urinary NOC levels (NDMA + NPIP) and NDMA and NPIP separately were expressed in nmol/mmol creatinine. Creatinine was measured by the Jaffe's kinetic alkaline picrate method. Creatinine and picric acid were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Creatinine concentrations were determined spectrophotometrically at a wavelength of 500 nm in ELISA plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands).

Micronucleus assay

The MN frequencies per 1000 once-divided binucleated cells were analyzed using the cytokinesis-block micronucleus assay. Approximately 6 hrs after collection from donors, whole blood cultures were initiated in duplicate. At 72 hrs following phytohaemagglutinin stimulation, cells were harvested, fixed, applied to slides and Giemsa-stained as described before [18,26] and subsequently scored (~2000 binucleated cells per donor) using a light microscope at 400× magnification.

Microarray hybridization and data analysis

RNA was isolated from RNAlater stabilized whole blood according to the manufacturer's protocol. Microarray hybridization was performed as described previously (**Chapter 2**) [12]. In short, dye-labeled cRNA (Cy3) was synthesized following the Agilent one-color Quick-Amp labeling protocol (Agilent Technologies, Amstelveen, The Netherlands). Samples were hybridized on Agilent 4x44K Whole Human Genome microarrays. After scanning the microarray slides, using settings described before (**Chapter 2**) [12], bad and empty spots were flagged in GenePix Pro (version 6.0, Molecular Devices, Sunnyvale, CA). Quality control was performed in R (version 2.10.1, The R Foundation for Statistical Computing, Vienna, Austria). Quantile normalization and data processing was performed in ArrayTrack (version 3.4, NCTR, Jefferson, AR). Log2 transformed spot intensities or ratios were used for further analyses.

ArrayTrack was used to find significantly modulated genes (two-tailed Welch *t*-test, $p < 0.05$) by comparing the highest and lowest tertile of subjects based on total

urinary NOC excretion or MN frequency. Genes with minimal expression differences (absolute log2 ratio <0.5) were filtered out and only genes present in >70% of subjects were used. Genes were subsequently imported into MetaCore™ (GeneGo, San Diego, CA) to identify the involvement of differentially expressed genes in specific cellular pathways. Pathways with a p <0.05 were considered significantly modulated.

Gene expression data (log2 transformed spot intensities) were also used to perform Spearman's rank correlation analyses with levels of urinary NOC excretion and MN frequency using the online Gene Expression Profile Analysis Suite (GEPAS, http://gepas.bioinfo.cipf.es/). Only genes present in at least 70% of subjects were used without further pre-selection. Prior to correlation analysis, missing values were imputed in GenePattern (http://www.broad.mit.edu/cancer/software/genepattern/, version 3.1) by finding the k nearest neighbors (k = 15, Euclidean metric) and imputing missing elements by averaging non-missing elements of its neighbors. Significantly correlating genes (p <0.05) were further analyzed in MetaCore for pathway involvement. A gene network based on a select number of genes was created in MetaCore using Dijkstra's shortest paths algorithm.

The gene expression data discussed in this publication have been deposited in NCBI's (National Center for Biotechnology Information) Gene Expression Omnibus (GEO) and are available through GEO Series accession number GSE23919 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE23919).

Statistical analysis

Associations between urinary NOC excretion levels and MN frequencies were investigated using Pearson correlation analysis after log2 transformation of urinary excretion values to obtain a normally distributed data set. Normality of the individual parameters was checked with the Kolmogorov-Smirnov test. A p <0.05 was considered significant.

Results

Correlation analysis

To link NOC exposure with carcinogenic risk, we first determined whether there was a relationship between urinary NOC excretion (i.e. NDMA + NPIP), as a marker of exposure, and lymphocytic MN frequency as a biomarker of effect. Although there was a linear positive trend between MN and urinary total NOC excretion, this did not achieve statistical significance (r = 0.32, p = 0.09, results not shown). Next, we performed a similar correlation analysis using data on urinary excretion of NDMA

and NPIP separately. NPIP showed a positive association with MN frequency, though not significant (results not shown). NDMA excretion levels on the other hand were positively and significantly correlated with MN frequency (r = 0.36, p <0.05, Figure 1).

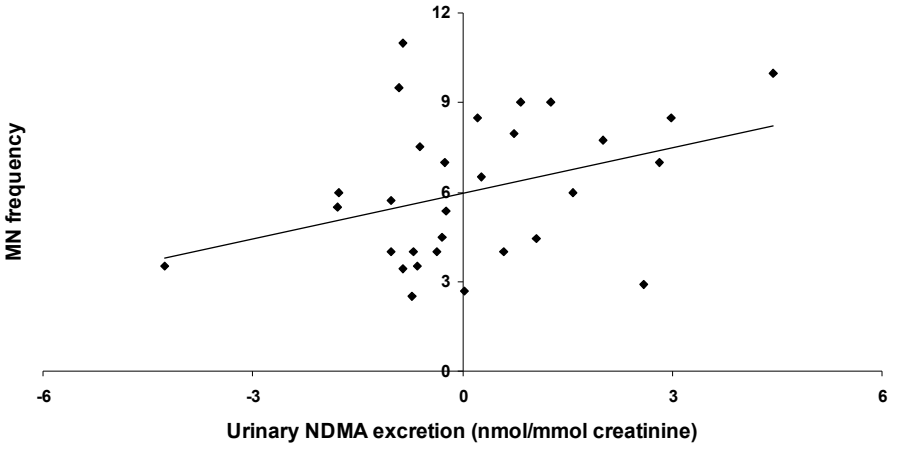

Figure 1: Pearson correlation plot of log2 transformed urinary NDMA excretion versus MN frequency in lymphocytes (R = 0.36, p < 0.05, n = 30).

NOC exposure-related gene expression

Next, we investigated transcriptomic responses in lymphocytes to identify gene expression modulations that can be related to NOC exposure which may enable identifying underlying molecular modes-of-action. We started by studying the involvement of significantly modulated genes between high and low total urinary NOC excretion (NDMA + NPIP) groups in biological pathways. Since NDMA and NPIP both showed a positive association with MN frequency (as shown above), both nitrosamines were used to determine tertiles in the gene expression analysis. In Table 1, significantly modulated pathways (p <0.05) between the highest and lowest tertile groups are presented. Differences in levels of NOC excretion are mainly associated with modifications in amino acid metabolism, apoptosis and survival, cell adhesion, and a few other signaling and metabolism pathways.

Table 1: GeneGO pathways significantly modulated by genes differentially regulated between highest and lowest tertile of subjects based on total urinary NOC excretion as exposure marker.

Process	Pathways involved	% genes[a]	*p*-value
Amino acid metabolism	Selenoamino acid metabolism	22	0.021
	Glycine, serine, cysteine and threonine metabolism	12	0.026
	Tyrosine metabolism (melanin)	15	0.043
Apoptosis and survival	Endoplasmic reticulum stress response pathway	13	0.003
	Regulation of Apoptosis by Mitochondrial Proteins	11	0.035
	Caspase cascade	11	0.035
	Cytoplasmic/mitochondrial transport of proapoptotic proteins Bid, Bmf and Bim	10	0.045
Blood coagulation	Blood coagulation	14	0.016
Cell adhesion	ECM remodeling	14	0.006
	Plasmin signaling	14	0.016
Development	Thrombopoetin signaling via JAK-STAT pathway	16	0.012
Immune response	IL-2 activation and signaling pathway	10	0.045
Neurophysiological process	GABAergic neurotransmission	20	0.026
	Gamma-aminobutyrate (GABA) biosynthesis and metabolism	15	0.043
Regulation of lipid metabolism	RXR-dependent regulation of lipid metabolism via PPAR, RAR and VDR	15	0.043
Sulfur metabolism	Sulfur metabolism	14	0.050

Tertile group sizes for NOC excretion were $n = 10$.

[a] Percentage of significantly correlated genes compared to the total number of genes in the pathway.

To explore gene expression differences associated with NOC exposure in more detail, we also analyzed Spearman's rank correlations between individual gene expression data and total urinary NOC values. Significantly correlating genes ($p < 0.05$) were analyzed for their involvement in biological pathways using MetaCore (Table 2). NOC excretion was again associated with amino acid metabolism and apoptosis and survival. One pathway involved in cell cycle regulation was also identified. Most of the other modified pathways were involved in cytoskeleton remodeling, development, and G-protein signaling.

Table 2: GeneGO pathways significantly associated with total urinary NOC excretion as found by MetaCore analysis of significantly correlating genes.

Process	Pathways involved	% genes[a]	*p*-value
Amino acid metabolism	Leucine, isoleucine and valine metabolism	36	0.046
Apoptosis and survival	Apoptotic TNF-family pathways	32	0.003
	Caspase cascade	29	0.024
Carbohydrate metabolism	Glycolysis and gluconeogenesis	50	0.006
Cell cycle	Regulation of G1/S transition	27	0.046

Process	Pathways involved	% genes[a]	*p*-value
Cytoskeleton remodeling	ACM3 and ACM4 in keratinocyte migration	44	0.022
	Role of PKA in cytoskeleton reorganisation	33	0.023
	ESR1 action on cytoskeleton remodeling and cell migration	36	0.046
Development	Activation of astroglial cells proliferation by ACM3	33	0.038
	EGFR signaling via PIP3	36	0.046
G-protein signaling	RhoA regulation pathway	44	0.000
	Regulation of CDC42 activity	30	0.019
	Regulation of RAC1 activity	28	0.030
	Rac2 regulation pathway	30	0.039
Immune response	Antigen presentation by MHC class II	57	0.008
Neurophysiological process	Long-term depression in cerebellum	42	0.014
	Netrin-1 in regulation of axon guidance	40	0.033
	Glutamate regulation of Dopamine D1A receptor signaling	36	0.046
Sulfur metabolism	Sulfur metabolism	31	0.049
Transcription	CREB pathway	33	0.038

Group size for NOC excretion correlation analysis was $n = 30$.

[a] Percentage of significantly correlated genes compared to the total number of genes in the pathway.

Table 3: GeneGO pathways significantly modulated by genes differentially regulated between highest and lowest tertile of subjects based on MN frequency.

Process	Pathways involved	% genes[a]	*p*-value
Carbohydrate metabolism	Fructose metabolism	17	0.027
	Glycolysis and gluconeogenesis	15	0.040
Regulation of lipid metabolism	Phospholipid metabolism	38	0.006
	RXR-dependent regulation of lipid metabolism via PPAR, RAR and VDR	21	0.031
	HETE and HPETE biosynthesis and metabolism	20	0.038

Tertile group sizes for MN frequency were $n = 9$.

[a] Percentage of significantly correlated genes compared to the total number of genes in the pathway.

Table 4: GeneGO pathways significantly associated with MN frequency as found by MetaCore analysis of significantly correlating genes.

Process	Pathways involved	% genes[a]	*p*-value
Amino acid metabolism	Aspartate and asparagine metabolism	19	0.042
Carbohydrate metabolism	Fructose metabolism	17	0.025
	Glycolysis and gluconeogenesis	20	0.036
Cytoskeleton remodeling	Role of PKA in cytoskeleton reorganisation	22	0.011
Development	Role of IL-8 in angiogenesis	14	0.028
	Endothelin-1/EDNRA signaling	16	0.033
	EDG3 signaling pathway	18	0.050
Heme metabolism	Heme metabolism	19	0.018

Process	Pathways involved	% genes[a]	*p*-value
Regulation of lipid metabolism	Regulation of fatty acid synthase activity in hepatocytes	38	0.006
	Phospholipid metabolism	38	0.006
	Stimulation of Arachidonic acid production by ACM receptors	14	0.028
	RXR-dependent regulation of lipid metabolism via PPAR, RAR and VDR	21	0.030
	Inhibitory action of Lipoxins and Resolvin E1 on neutrophil functions	20	0.036
Signal transduction	PKA signaling	15	0.038
Steroid metabolism	Cortisone biosynthesis and metabolism	29	0.044
Transcription	CREB pathway	20	0.036
Transport	Regulation of degradation of deltaF508 CFTR in CF	19	0.018
	CFTR folding and maturation (norm and CF)	25	0.019
	Regulation of CFTR activity (norm and CF)	17	0.025
	Regulation of degradation of wt-CFTR	19	0.042

Group size for MN frequency correlation analysis was $n = 28$.

[a] Percentage of significantly correlated genes compared to the total number of genes in the pathway.

Micronuclei frequency-related gene expression

Similar to the analyses performed with urinary NOCs, we associated differentially expressed genes with MN frequencies in the highest versus lowest MN tertile and performed a correlation analysis of MN levels and gene expression data. Significantly modulated genes between the highest and lowest tertiles or significantly correlating genes ($p < 0.05$) were subsequently analyzed in MetaCore. In Table 3 and 4 respectively, the results of the tertile and correlation analyses are presented. The tertile analysis only identified pathways related to carbohydrate and lipid metabolism. Most of these pathways were also identified in the MN frequency correlation analysis in addition to pathways involved in development and transport and a few other cytoskeleton remodeling, signal transduction, transcription, and metabolism-related pathways.

Comparison of Table 1 and 2 with Table 3 and 4, shows that three pathways are in common for the urinary NOC and MN analyses, which are “Role of PKA (protein kinase A) in cytoskeleton reorganisation”, “CREB (cAMP (cyclic AMP) response element binding) pathway”, and “RXR (retinoid X receptor)-dependent regulation of lipid metabolism via PPAR (peroxisome proliferator-activated receptor), RAR (retinoic acid receptor) and VDR (vitamin D receptor)”.

MN formation-associated gene network analysis

We recently presented a network analysis of a list of genes related to MN formation, involved in processes like spindle assembly and DNA damage control, based on

available knowledge from literature [27]. These genes were here compared with the NOC excretion and MN frequency-related gene sets and three genes (out of a total of 27 genes and 3 gene complexes) were found to overlap. One of these genes, identified as FBWX7 (F-box and WD repeat domain containing 7), was found in the MN frequency-related gene set and was represented in both the tertile comparison and the correlation analysis. FBXW7 was significantly up regulated in the upper tertile group compared to the lower tertile group (log2 ratio: 0.8) and was positively correlated with MN frequency. The other two genes were found in the NOC excretion-related group and were identified as BUB3 (budding uninhibited by benzimidazoles 3 homolog) and caspase 2. BUB3 was significantly down regulated in the upper tertile group compared to the lower tertile group (log2 ratio: -1.1) and negatively correlated with NOC excretion levels (p <0.05). Caspase 2 was non-significantly up regulated in the upper tertile group compared to the lower tertile group (log2 ratio: 1.8), but significantly and positively correlated with NOC excretion (p <0.05). MetaCore was subsequently used to integrate FBXW7, BUB3, and caspase 2 into a network to visualize their interactions. In Figure 2A, a network analysis is presented to find the shortest directed paths between genes of interest. The network does not include all known interactions of FBXW7, BUB3, and caspase 2 with other genes since this would generate a too large network. The genes in this network were checked for matches with significantly modulated or correlated genes in the NOC excretion and MN frequency-related gene sets and three matching genes were identified as caspase 8, huntingtin (HTT), and SMAD3 (SMAD family member 3), which were significantly correlated with NOC excretion, thus increasing the potential relevance of the genes in the network in relation to carcinogenicity. Since O^6-methylguanine-DNA methyltransferase (MGMT) is an important gene involved in repair of alkyl adducts, and NDMA-induced O^6-methylguanine (O^6-meG) in particular [19], this gene was screened for significant modification or correlation with NOC excretion and/or MN frequency and found to be significantly up regulated in the MN frequency tertile analysis (log2 ratio: 0.5). Addition of MGMT to the network shows a close interaction with genes already present in the network. In Figure 2B, an overview is presented of possible locations where these seven genes and some of the modified pathways could play a role in the relation between NOC exposure, repair, MN formation, and carcinogenesis.

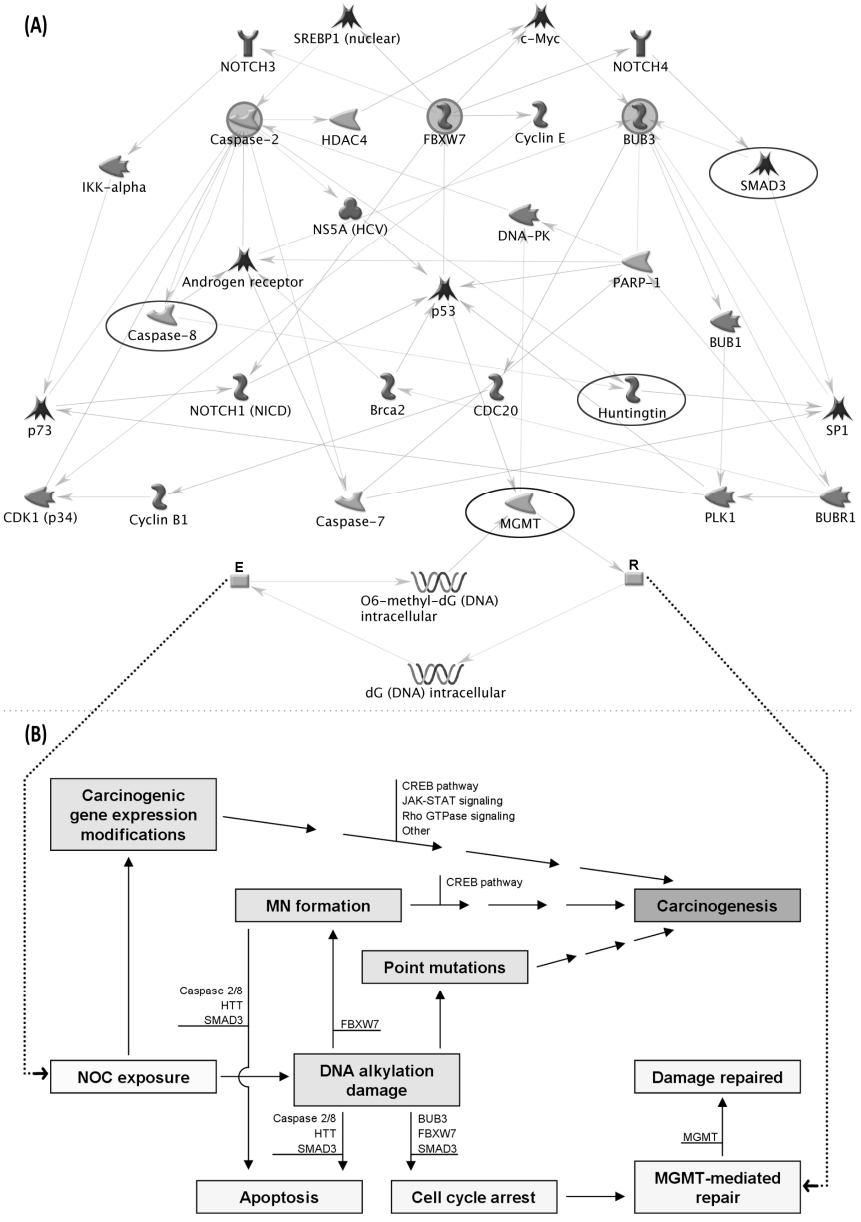

Figure 2. (A) Shortest paths network developed in MetaCore based on genes related to FBXW7, BUB3, and caspase 2 shown in blue circles. Ellipses indicate additional genes associated with urinary NOC excretion (in red, i.e. caspase 8, huntingtin, and SMAD3) and MN frequency (in blue, i.e. MGMT). Exposure to NOC resulting in DNA damage is indicated by "E" and the DNA repair process by "R". Detailed information on the symbols can be found at: http://www.genego.com/pdf/MC_legend.pdf. Colour figure is included separately. **(B)** Possible locations are shown for the involvement of the most important genes in the network analysis and some of the modified pathways presented in Tables 1-4, in the route from NOC exposure to repair or other outcomes, such as apoptosis, MN formation, and carcinogenesis.

Discussion

Analyzing gene expression modulations associated with known markers of exposure or well-validated phenotypic markers of effect provides an innovative way of assessing environmental health risks in chemically-exposed populations of interest. Microarray technology generates a large amount of information which not only makes it an interesting marker of effect but also provides a complete overview of modified genes and processes that can be helpful in understanding the molecular basis of the effects induced by the compounds under investigation. In the present study, we investigated the possible carcinogenicity of NOCs in humans, using whole blood as a surrogate tissue, by linking transcriptomics with urinary NOC excretion and lymphocytic MN frequency as markers of exposure and effect, respectively.

The positive association found between urinary NOC excretion levels and MN frequencies in lymphocytes indicates a relation between NOC exposure and the induction of genotoxicity as a predictor of carcinogenicity. We found that NDMA excretion was significantly correlated with MN frequency (Figure 1), in contrast to NPIP. Excretion levels of NDMA were higher than for NPIP in the majority of subjects (on average approximately 3 times as high, results not shown). Other studies have also implicated NDMA as a likely contributor to human genotoxicity in surrogate tissues. Formation of O^6-meG in human lymphocyte DNA, for example, which is a pre-mutagenic lesion induced by NDMA, was found in a large number of individuals not known to have suffered excessive exposure to methylating agents and the data were attributed to NDMA exposure [28]. The fact that in our study MN frequency was associated with exposure to NOCs, and particularly NDMA, points towards an increased carcinogenic risk associated with NOC exposure since lymphocytic MN frequency is a validated predictor of human carcinogenicity [22].

We subsequently established the transcriptomic response associated with exposure to NOCs in lymphocytes to identify gene expression modifications that may provide insight into the carcinogenicity of these compounds in humans. Two separate analyses were performed, one focusing on the comparison of subjects between the higher and lower tertile of NOC excretion as a marker of exposure (Table 1) and one focusing on genes significantly correlated with NOC excretion across all subjects (Table 2). The first analysis allows for the identification of the strongest effects between high and low exposure groups while the correlation analysis may capture more subtle effects since all genes, regardless of log2 ratio or significance level, are used. Associations with several apoptosis and survival pathways were identified in both analyses indicating that cell survival is affected by NOCs in humans. A closer examination of the genes involved in these pathways revealed that several pro-apoptotic genes were up-regulated in the upper exposure tertile of subjects thus indicating that higher NOC exposure may be related with an increase in apoptosis. This may demonstrate a direct consequence of the genotoxic potency of these

compounds. Although *in vitro* exposure of cultured cells requires high millimolar concentrations of NOCs to induce DNA damage or apoptosis (**Chapter 2**) [12,29,30], human lymphocytes become apoptotic at micromolar NOC concentrations which are physiologically more relevant [23]. This may be related to a relatively high rate of cytochrome P450 (CYP)-catalyzed NOC metabolism in lymphocytes [31].

The analyses also revealed a cell cycle regulation pathway to be associated with NOC excretion (Table 2) in addition to several pathways involved in cell adhesion and cytoskeleton remodeling (Table 1 and 2). These are often linked with the cell cycle regulation and apoptosis process, providing further evidence for NOC-induced genotoxicity since DNA damage, and in particular MN formation, affects the progression of the cell cycle [32]. The modifications in apoptosis- and cell cycle-related pathways associated with NOC excretion, in addition to the relation found with MN frequency, indicate that NOC exposure levels found in humans at daily life conditions may contribute to the carcinogenic process.

A relatively large number of pathways appear involved in development and G-protein signaling processes. These pathways are of interest since they involve signaling cascades that are important in the regulation of differentiation and cell proliferation. Signaling pathways that were found to be modulated were implicated in the JAK-STAT signaling cascade and epidermal growth factor receptor (EGFR) signaling and regulation of a number of Rho GTPases. All of these pathways play important roles in cell growth, differentiation, cell death, DNA synthesis and cell proliferation which are crucial in cancer cell development [33-35]. These modifications may also be relevant with regard to the NewGeneris objective of identifying prenatal exposures that possibly influence the development of childhood cancer, especially since NDMA can cross the placental barrier, thereby leading to fetal exposure [17,36]. In relation to this, the pathways belonging to neurophysiological processes could be important in neural tube defects that arise during fetal development since this has been associated with NOC exposure [37]. Other pathways related to NOC excretion include a number of amino acid metabolism pathways, which can probably be attributed to translation-associated protein synthesis, and two immune response pathways, that are indicative of immunomodulation and could be relevant in light of the immunosuppressive effects described for NOCs in rodents [38].

Similar tertile and correlation analyses on gene expressions changes and in relation to MN frequencies were performed to investigate whether MN frequency itself was associated with pathways of interest. In the tertile comparison analysis (Table 3), only pathways belonging to two cellular processes, i.e. carbohydrate and lipid metabolism, were identified. Most of these pathways were also identified in the correlation analysis (Table 4) and they may be related to three other modified pathways that are more easily interpreted with regard to MN levels, which are the cytoskeleton remodeling pathway describing the role of PKA, the PKA signal transduction pathway itself and the CREB transcription pathway. PKA is dependent on

the level of cAMP in the cell, and both cAMP and PKA have been implicated in MN formation in blood cells [39]. Moreover, PKA signaling plays an important role in the regulation of glycogen, glucose, and lipid metabolism with CREB being an important downstream transcription factor target of PKA [40].

A few developmental pathways involving interleukin 8 (IL-8), endothelin receptor type A (EDNRA), and sphingosine-1-phosphate receptor 3 (EDG3) were also identified and are of special interest since they are all involved in angiogenesis [41-43]. The relevance of the transport pathways involving the cystic fibrosis transmembrane conductance regulator (CFTR), which functions as a chloride channel and controls the regulation of transport pathways, is difficult to assess.

It is interesting that two of the three overlapping pathways between NOC excretion and MN frequency are associated with PKA and CREB signaling, which are implicated in MN formation as discussed above, and may therefore represent an intermediate mechanism in the link between NOC exposure and MN formation. The continuum between exposure markers, effect markers, and disease and the identification of marker-associated transcriptomic responses, presents the possibility of using gene expression modifications as mechanistic biomarkers, especially if similar transcriptomic modifications are found in the disease. This constitutes the Meet-in-the-Middle concept as proposed by Vineis and Perera [44]. It is therefore especially interesting that the CREB pathway is now being recognized as an important player in human cancer [45].

Recently, we published a transcriptomic network analysis of MN formation-related genes based on available peer-reviewed literature [27]. In the present study, the genes included in this previously published network were screened for matches with both the NOC excretion- and MN frequency-associated expression sets. Three genes were found to match and a network dedicated to find the shortest paths between these genes was created (Figure 2A). As could be expected, FBXW7, which was identified in the MN frequency related gene set, is an important regulator in cell cycle and DNA damage processes, but also in aneuploidy and NOTCH signaling (Table 5) which is intimately linked with MN formation [46]. BUB3 and caspase 2, which were both identified in the NOC excretion related gene set, are mostly involved in cell cycle regulation and apoptosis and DNA damage, respectively. FBXW7 and caspase 2 are also implicated in the neoplastic process, stressing their importance in relation to MN formation-associated carcinogenic risk. Although FBXW7, BUB3, and caspase 2 are not directly linked with each other in the network, they have many upstream and downstream genes in common all of which are important in the processes described in Table 5. The network was subsequently screened for additional matches with the gene sets used in this study and three more genes (caspase 8, HTT, and SMAD3) were found to be present in the gene set correlating with NOC excretion. All three genes had direct interactions with BUB3 or caspase 2 (Figure 2A) and were involved in processes presented in Table 5, particularly apoptosis,

cell cycle, DNA damage, and neoplasm/carcinoma formation. MGMT was added separately to this network since it is one of the most important DNA repair enzymes involved in repairing alkyl adducts and because it was found to be significantly up regulated in the MN frequency tertile analysis. As could be expected, MGMT interacts with the genes already present, including p53. The network, and these seven genes in particular, are thus likely to influence each other in response to NOC exposure and/or MN formation. The network as such presents a transcriptomic interactome that integrates gene expression modifications in response to NOC exposure, MGMT-mediated repair, and MN formation. An overview of where these genes and some of the modified pathways could be implicated in the route from exposure to disease is presented in Figure 2B.

Since this is one of the first studies focused on identifying gene expression modifications in surrogate tissue, further research is needed to validate these results and possibly add other relevant genes or processes to the list. The effects which we observed in whole blood may be of relevance for molecular events in target tissues exposed to NOCs, such as the gastro-intestinal tract or the liver, which would normally be difficult to investigate due to their inaccessibility. Although it is important to note that extrapolation of results found in surrogate tissues to target tissues should be done with caution, blood transcriptomics are known to reflect effects induced in target tissue [47].

In summary, we have for the first time found a link between urinary excretion of NOCs by individuals under daily life circumstances and lymphocytic MN frequency as a marker of effect indicating elevated human cancer risk. Gene expression changes associated with both markers revealed modifications in pathways which indicate molecular responses related to genotoxicity and carcinogenicity. This suggests an increased cancer risk for humans exposed to NOCs through the diet or as a result of endogenous nitrosation processes. The genetic processes and genes found to be modified in this study may be of great relevance in future investigations into NOC-associated carcinogenicity in humans.

Table 5: Biological processes regulated by FBXW7, BUB3, and Caspase 2.

Genes in MN frequency related gene set	Genes in total urinary NOC excretion related gene set	
FBXW7	BUB3	Caspase 2
Aneuploidy	Cell cycle	Apoptosis and survival
Cell cycle	Mitotic cell cycle checkpoint	DNA damage
DNA damage	Spindle assembly checkpoint	Neoplasm/Carcinoma formation
Neoplasm/carcinoma formation		Proteolysis
NOTCH signaling		
Proteolysis		

Information on genes was obtained from Entrez Gene of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene).

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Chapter 6

Transcriptomic profiles in colon tissue from inflammatory bowel diseases patients in relation to N-nitroso compound exposure and colorectal cancer risk

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Abstract

N-nitroso compounds (NOC) have been suggested to play a role in human cancer development but definitive evidence is still lacking. In this study we investigated gene expression modifications induced in human colon tissue in relation to NOC exposure to gain insight in the relevance of these compounds in human colorectal cancer (CRC) development. Since there are indications that inflammation stimulates endogenous NOC formation, the study population consisted of patients with inflammatory bowel disease (IBD) and irritable bowel syndrome patients as controls without inflammation. Strong transcriptomic differences were identified in colonic biopsies from IBD patients and compared to controls that enhance the understanding of IBD pathophysiology. However, fecal NOC levels were not increased in IBD patients, suggesting that inflammation did not stimulate NOC formation. By relating gene expression changes of all subjects to fecal NOC levels, we did, however, identify a NOC exposure-associated transcriptomic response that suggests that physiological NOC concentrations may induce genotoxic responses and chromatin modifications in human colon tissue, both of which are linked to carcinogenicity. 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 the molecular basis of NOC-induced carcinogenesis. We conclude that NOC exposure is associated with gene expression modifications in the colon that may increase CRC risk in humans.

Introduction

N-nitroso compounds (NOC) have long been suspected to play a role in human carcinogenesis since many NOC possess genotoxic and mutagenic properties and are known animal carcinogens [1,2]. Human exposure to NOC occurs almost entirely through food and endogenous nitrosation of NOC precursors (NOCP) in the gastrointestinal tract [3] and, following absorption into the bloodstream, NOC may have deleterious effects in many tissues. Epidemiological studies have indeed linked human (endogenous) NOC exposure to several types of cancer, including esophagus, stomach, colorectal and bladder cancer [4–7], but proof of a causal association between NOC and human cancer is still missing.

In several recent *in vitro* studies in a human colon cell line, we have explored the transcriptomic effects of NOC in relation to their genotoxicity in order to elucidate potentially important gene expression modifications that may play a role in the carcinogenic process (**Chapters 2**, **3**, and **4**) [8,9]. We identified many NOC-modulated biological pathways that regulate crucial processes with regard to carcinogenesis, such as DNA damage control, proliferation, and differentiation. These modulations may act in concert with the DNA-damaging properties of NOC and increase the carcinogenic risk associated with exposure. Furthermore, we have previously identified gene expression changes in NOC-exposed humans in relation to micronucleus formation as a marker of carcinogenic risk, using lymphocytes as a surrogate tissue (**Chapter 5**).

As a result of endogenous nitrosation processes in the intestinal lumen, highest exposure levels may occur in the human colon and therefore it is an important target tissue to investigate concerning NOC exposure-induced gene expression modulations. Since there are indications that colon inflammation also stimulates endogenous NOC formation [10,11], individuals suffering from inflammatory bowel disease (IBD) present an interesting population to study NOC-exposure related transcriptomic effects, especially since this group is at increased risk of colorectal cancer (CRC) [12,13]. Gene expression modifications as a result of an increased NOC exposure may partially explain the colorectal cancer risk in this group of patients, as well as in NOC-exposed individuals in general.

We therefore hypothesize that NOC exposure in the human colon results in gene expression modifications in colon tissue that indicate carcinogenic properties of these compounds in humans. To investigate this, we studied transcriptomic differences in colon biopsies obtained from IBD patients and control subjects with irritable bowel syndrome (IBS) in relation to fecal NOC levels as a marker of exposure. With this study, we aim to identify molecular pathways changed upon colonic NOC exposure which may contribute to the carcinogenic process in the human colon.

Material and methods

Subjects and study design

The study population was recruited in the Netherlands at the Department of Gastroenterology in the Orbis Medical Center in Sittard and the Academic Hospital Maastricht. A total of 81 patients were recruited, of which 44 were IBD patients (29 male, 15 female, mean age 48.8 ± 14.4, range 23–81 years) and 37 were control patients (15 male, 22 female, mean age 56.2 ± 15.3, range 21–78 years) who were diagnosed with IBS. Eligible IBD patients had a history of ulcerative colitis with a moderate exacerbation of the inflammatory state at the time of inclusion as based on an anamnesis and the scheduled colonoscopy. Crohn's disease patients were excluded from participation in the study as well as patients who had to be admitted to hospital or had a history of colorectal adenomas. Only IBS patients proven free (normal colonoscopy) from colorectal disease and other gastrointestinal disorders were included as controls. Food diaries were recorded for three consecutive days after which subjects collected a feces sample. This was immediately frozen (-20 °C) upon collection. A smaller group of participants from both patient groups (19 IBD and 13 IBS) also participated in a colonoscopic examination during which six biopsies were taken from mucosal tissue in visually non-inflamed regions of the colon (in most cases from the sigmoid and descending colon) which were immediately frozen in liquid nitrogen and stored at -80 °C until use. The colonoscopy was performed as part of either surveillance or suspected gastrointestinal disorders. Use of additional anti-inflammatory medication in IBD patients for treating the exacerbated colon inflammation was postponed until the end of the study. During three days before the colonoscopy, subjects followed their normal dietary habits, with a few modifications. To keep nitrate intake between subjects similar, participants were asked to use low-nitrate mineral water when preparing food or drinks and to avoid vegetables with high nitrate concentrations, such as spinach, lettuce, and celery. A high fruit consumption, especially fruit rich in vitamin C, and use of vitamin supplements also had to be avoided as this has been reported to inhibit endogenous nitrosation [14].

This study was approved by the Medical Ethical Committees Atrium Orbis Zuyd and Clinical Trial Center Maastricht (CCMO registration number NL13359.096.06) and written informed consent was obtained from the participants prior to the start of the study.

Fecal calprotectin

Calprotectin (in μg/g feces) as a marker of chronic inflammation was determined as described previously [15]. In short, approximately 100 mg of wet feces was diluted

50 times in extraction buffer, homogenized, and centrifuged after which the supernatant was used for analysis of calprotectin using a standard ELISA kit (Hycult Biotech, Uden, The Netherlands) according to the manufacturer's instructions.

Fecal apparent total nitroso compound determination

Apparent total nitroso compounds (ATNC) were analysed in feces as described previously [16], using an Ecomedics CLD 88 Exhalyzer (Ecomedics, Duernten, Switzerland). In short, fecal material was diluted 1:5 in ultrapure water and homogenized for 20 min. Thereafter, 500 µl of a 5% (wt/vol) sulfamic acid solution was added to remove nitrite and samples were injected into a purge vessel kept at 60 °C and filled with a standard tri-iodide reagent (38 mg I_2 was added to a solution of 108 mg KI in 1 ml water; to this mixture, 13.5 ml glacial acetic acid was added) to determine ATNC. Results are presented as nmol/g feces.

Microarray hybridization and data analysis

Three biopsies were separately dissolved in QIAzol® (Qiagen, Venlo, The Netherlands) using a tissue disruptor and subsequently pooled. RNA was isolated according to the manufacturer's protocol. The average RNA integrity number was 7.1 ± 1.0. Microarray hybridization was performed as described previously (**Chapter 2**) [9] with some modifications. In short, dye-labeled cRNA (Cy3) was synthesized following the Agilent one-color Quick-Amp labeling protocol (Agilent Technologies, Amstelveen, The Netherlands). Samples were hybridized on Agilent 4x44K Whole Human Genome microarrays (Agilent Technologies). After scanning the microarray slides, using settings described before (**Chapter 2**) [9], bad and empty spots were flagged using the GenePix Pro software (version 6.0, Molecular Devices, Sunnyvale, CA). Quality control was performed in the statistical software environment R (version 2.10.1, The R Foundation for Statistical Computing, Vienna, Austria). Quantile normalization and subsequent data processing was performed in ArrayTrack (version 3.4, NCTR, Jefferson, AR). Log2 transformed spot intensities were used for further analyses.

The online software suite GenePattern version 3.1 (http://www.broad.mit.edu/cancer/software/genepattern/) was used for principal component analysis (PCA) of the gene expression intensities of all subjects. PCA plots were generated in GenePattern by reducing the data to three dimensions.

ArrayTrack was used to find significantly modulated genes between IBD and IBS groups (two-tailed Welch *t*-test, $p < 0.05$) with a minimum average absolute log2 ratio of 0.5 between groups. Further filtering was performed by selecting only those genes that were present in at least 70% of subjects (both IBD and IBS patients). Genes were subsequently imported in MetaCore™ (GeneGo, San Diego, CA) to iden-

tify the involvement of differentially expressed genes in specific cellular GeneGo pathways or Gene Ontology (GO) processes. Pathways with a p <0.05 were considered significantly modulated.

For Spearman's rank correlation analyses gene expression data were correlated with ATNC levels. Only genes present in at least 70% of subjects were used without further pre-selection. Prior to correlation analysis, missing values were imputed in GenePattern by finding the k nearest neighbors (k was set to 15), using a Euclidean metric, and imputing the missing elements by averaging the (non-missing) elements of its neighbors. The online Gene Expression Profile Analysis Suite (GEPAS, http://gepas.bioinfo.cipf.es/) was used to perform correlation analyses and significantly correlating genes (p <0.05) were subsequently further analyzed in MetaCore. A gene network based on a select number of genes was created in MetaCore using Dijkstra's shortest paths algorithm.

Statistical analysis

Results are presented as mean ± SD. Normality of data sets was tested using the Kolmogorov-Smirnov test. Potential differences between groups were assessed using the Mann-Whitney U test, or Student's t-test (for equal or unequal variances, as based on Levene's test) depending on the normality of data. Spearman's rank test was used for correlation analyses.

Results

Food diaries and fecal calprotectin levels

The results of the food diary analysis are presented in Table 1. Average daily intake during the three days prior to the biopsy sampling was not significantly different between IBS controls and IBD patients, except for total and saturated fat, cholesterol, zinc, folic acid, and vitamin B12. However, as shown in Table 2, the male/female ratio was not equal between these groups and after correcting for gender, the differences in food intake were no longer found to be significant.

Since IBD patients were used as a model for increased nitrosation we determined whether IBD patients had higher fecal calprotectin levels as a measure of inflammation. IBD patients indeed had a significantly higher fecal calprotectin level than control patients, in both the total group and the biopsy subjects group (p <1*10–^{7} and <0.001, respectively), indicating a higher level of inflammation (Table 2).

Table 1: Reported average daily dietary intake before biopsy sampling in IBS controls and IBD patients.

Dietary intake (average/day)	**IBS controls** *n* = 37	**IBD patients** *n* = 44
Energy (kcal)	1818 ± 469	2068 ± 593
Protein (g)	70.5 ± 19.6	79.3 ± 20.9
Animal protein (g)	24.0 ± 16.4	28.7 ± 18.2
Total fat (g)	67.7 ± 21.4	89.4 ± 34.3[a]
Saturated fat (g)	26.0 ± 9.7	32.3 ± 11.5[b]
Cholesterol (mg)	170 ± 79.9	228 ± 113[b]
Carbohydrates (g)	219 ± 66.6	214 ± 60.6
Fibers (g)	17.9 ± 5.8	19.1 ± 5.9
Alcohol (g)	6.6 ± 10.0	10.7 ± 14.6
Water (g)	1803 ± 523	1856 ± 566
Sodium (mg)	2922 ± 774	3318 ± 921
Potassium (mg)	2603 ± 658	2769 ± 812
Calcium (mg)	705 ± 297	813 ± 279
Magnesium (mg)	264 ± 63.3	275 ± 73.0
Iron (mg)	10.2 ± 2.6	11.1 ± 3.5
Selenium (µg)	36.7 ± 12.4	49.9 ± 34.8
Zinc (mg)	7.5 ± 2.3	9.0 ± 2.6[b]
Folic acid (µg)	139 ± 45.4	165 ± 45.1[b]
Vitamin A (µg)	765 ± 469	938 ± 532
Vitamin B1 (mg)	1.0 ± 0.3	1.1 ± 0.5
Vitamin B2 (mg)	1.0 ± 0.4	1.2 ± 0.4
Vitamin B3 (mg)	14.6 ± 4.3	15.5 ± 6.3
Vitamin B6 (mg)	1.4 ± 0.5	1.5 ± 0.5
Vitamin B12 (µg)	2.9 ± 1.7	4.2 ± 3.1[b]
Vitamin C (mg)	51.2 ± 32.1	62.4 ± 43.7
Vitamin D (µg)	3.5 ± 1.8	4.4 ± 3.2
Vitamin E (mg)	13.2 ± 8.4	14.0 ± 8.8

[a, b] Significantly different between IBS and IBD groups at $p < 0.01$ ([a]) or $p < 0.05$ ([b]) but not after correction for gender (Student's *t*-test).

Gene expression changes between IBD patients and controls

To investigate differences in gene expression between IBD patients and controls, we performed whole genome microarray analysis on RNA isolated from colon biopsies. The PCA plot in Figure 1 shows that IBS controls group separately from IBD patients indicating distinct profiles at the gene expression level. These differences were further investigated by selecting significantly modulated genes (p <0.05, Welch *t*-test) between the groups at an absolute log2 ratio cut-off value of 0.5. The results of this analysis are presented in Table 3 and show a high number of significantly modulated pathways (p <0.05) involved in the immune response and in development. Most developmental pathways found to be modified are involved in the JAK/STAT signaling cascade and growth hormone signaling, while the immune response pathways are mostly involved in cytokine signaling, such as interleukins 1, 3, 10, 12, 17,

22, 23, and 27 and interferon (IFN) gamma, and the complement signaling system. Strongly up and down regulated genes in the modified immune response pathways clearly indicate the pro-inflammatory state in IBD patients compared to controls. Significantly modulated genes in the developmental pathways are also mostly involved in downstream signaling cascades linked to the immune response. The remaining modified pathways/processes were involved in arginine metabolism, blood coagulation, cell adhesion, and cytoskeleton remodeling.

Table 2: Population characteristics, calprotectin, and ATNC levels in IBS controls and IBD patients shown for all subjects and the biopsy subgroup.

	All subjects		Biopsy subjects	
	IBS	**IBD**	**IBS**	**IBD**
	n = 37	*n* = 44	*n* = 13	*n* = 19
Age (yrs) {range}	56.2 ± 15.3 {21–78}	48.8 ± 14.4 {23–81}	55.5 ± 13.1 {25–69}	49.8 ± 12.1 {29–72}
Gender (male/female)	15/22	29/15	7/6	15/4
Calprotectin (µg/g feces)	11.0 ± 16.8	37.0 ± 26.1[a]	12.0 ± 17.0	38.8 ± 21.0[b]
ATNC (nmol/g feces)	7.4 ± 8.1	10.1 ± 10.5	7.2 ± 6.1	10.5 ± 11.2

[a] Significantly higher in IBD group compared to IBS group (p <1*10^{-7}, Mann-Whitney U test).

[b] Significantly higher in IBD group compared to IBS group (p <0.001, Student's t-test).

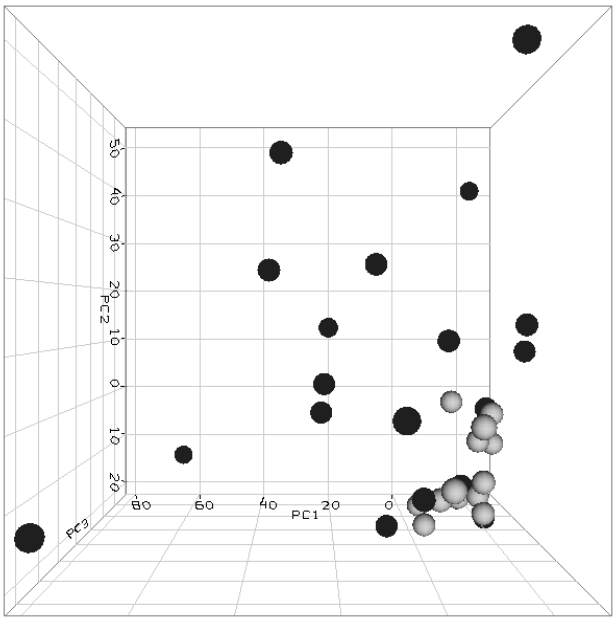

Figure 1: PCA plot of the microarray-based expression profiles showing a separation between IBD patients (black) and IBS controls (gray). Data were described by three principal components (PCs) accounting for 24, 13, and 9% of variation.

Table 3: Significantly modulated GeneGo pathways grouped by cellular process as found by MetaCore analysis of genes differentially regulated between IBD (*n* = 19) and IBS (*n* = 13) patients.

Cellular process	Pathways involved	% genes[a]	*p*-value
Amino acid metabolism	(L)-Arginine metabolism	26	0.017
Blood coagulation	Blood coagulation	42	0.000
Cell adhesion	ECM remodeling	26	0.004
	Cell-matrix glycoconjugates	27	0.014
Cytoskeleton remodeling	Neurofilaments	29	0.036
Development	Angiotensin signaling via STATs	47	0.000
	Leptin signaling via JAK/STAT and MAPK cascades	37	0.001
	Growth hormone signaling via STATs and PLC/IP3	30	0.003
	Thrombopoetin signaling via JAK-STAT pathway	35	0.003
	PDGF signaling via STATs and NF-kB	28	0.022
	EPO-induced Jak-STAT pathway	23	0.023
	Prolactin receptor signaling	21	0.023
	GM-CSF signaling	22	0.027
	Hypoxia-induced EMT in cancer and fibrosis	38	0.033
	Transcription regulation of granulocyte development	25	0.034
	EPO-induced PI3K/AKT pathway and Ca(2+) influx	25	0.034
	Growth hormone signaling via PI3K/AKT and MAPK cascades	20	0.042
Immune response	IL-17 signaling pathways	38	0.000
	Oncostatin M signaling via JAK-Stat in human cells	57	0.000
	Antiviral actions of Interferons	42	0.000
	IL-23 signaling pathway	60	0.000
	Alternative complement pathway	39	0.000
	IL-27 signaling pathway	47	0.000
	MIF-mediated glucocorticoid regulation	44	0.000
	IFN alpha/beta signaling pathway	39	0.001
	Bacterial infections in normal airways	28	0.002
	Cytokine production by Th17 cells in CF	33	0.002
	Oncostatin M signaling via MAPK in human cells	30	0.003
	Lectin induced complement pathway	32	0.003
	Classical complement pathway	32	0.003
	IL-10 signaling pathway	33	0.005
	Bacterial infections in CF airways	25	0.005
	IL-1 signaling pathway	25	0.005
	IL-3 activation and signaling pathway	32	0.006
	Histamine H1 receptor signaling in immune response	28	0.007
	IL-22 signaling pathway	30	0.008
	CD40 signaling	22	0.012
	IL-12 signaling pathway	31	0.013
	IL-12-induced IFN-gamma production	27	0.014
	IFN gamma signaling pathway	23	0.014

Cellular process	Pathways involved	% genes[a]	*p*-value
	MIF in innate immunity response	25	0.021
	PGE2 signaling in immune response	25	0.021
	Mucin expression in CF via IL-6, IL-17 signaling pathways	26	0.028
	Th1 and Th2 cell differentiation	25	0.034
	Sialic-acid receptors (Siglecs) signaling	38	0.033
Reproduction	GnRH signaling	20	0.042
Transcription	CREM signaling in testis	38	0.033

[a] Percentage of significantly modulated genes compared to the total number of genes in the pathway.

Endogenous NOC formation

To investigate whether a higher rate of endogenous nitrosation in IBD patients could contribute to the observed gene expression differences, levels of fecal ATNC were determined in IBD and control patients. Although the average ATNC level is approximately 40% higher in IBD patients than in IBS controls, inter-individual variation is high (Table 2). As a result, no significant difference was found in ATNC between controls and IBD patients in the total group or biopsy subgroup. ATNC levels were not correlated with calprotectin as a marker of inflammation either (results not shown) and a PCA plot distinguishing between the highest versus lowest ATNC patients (IBS or IBD) also did not show any specific group clustering (results not shown). This indicates that there was no clear association between endogenous nitrosation and inflammatory conditions in the colon.

NOC-associated gene expression modifications

Although colonic inflammation did not result in a distinct increase in endogenous NOC formation in this study, relevant gene expression modulations can still be associated with individual fecal NOC levels regardless of the presence of inflammation. To investigate the transcriptomic response specifically linked to NOC formation in more detail we performed a Spearman's rank correlation analysis between ATNC levels and log2 gene expression intensities. Significantly correlating genes (p <0.05) were subsequently analyzed for their involvement in GeneGo pathways and GO processes. In Table 4, all GeneGo pathways and the top ten of GO processes significantly associated (p <0.05) with ATNC levels are presented. The most strongly represented cellular process was transport with pathways mainly involved in clathrin-coated vesicle formation and macropinocytosis. A DNA damage repair pathway was also associated with ATNC levels while the remaining pathways share significantly correlating genes that are involved in cytoskeleton remodeling and signaling pathways related to cell cycle regulation and apoptosis, such as the RAC1 G-protein signaling pathway. The top ten of GO processes significantly associated with ATNC levels were mostly involved in nucleosome/chromatin assembly and RNA process-

ing. In Figure 2, the closest interactions between the significantly correlating genes in the Chromatin assembly GO process are shown. The network contains 14 genes significantly correlating with ATNC levels, consisting of 11 histone genes, the heterochromatin protein 1 binding protein (HP1-BP74) gene, centromere protein V (PRR6), and SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2 (BRM). Most of the histone genes were positively correlated with ATNC levels.

Table 4: GeneGo pathways and the top ten of GO processes significantly associated with ATNC levels as found by MetaCore analysis of significantly correlating genes in biopsy subjects of IBD and IBS groups combined (n = 32).

GeneGo pathways			
Cellular process	**Pathways involved**	**% genes[a]**	***p*-value**
Cytoskeleton remodeling	RalA regulation pathway	17	0.023
Development	PIP3 signaling in cardiac myocytes	15	0.040
DNA damage	NHEJ mechanisms of DSBs repair	29	0.004
G-protein signaling	RAC1 in cellular process	23	0.003
Signal transduction	AKT signaling	15	0.040
Transport	Clathrin-coated vesicle cycle	22	0.000
	wtCFTR and delta508 traffic / Clathrin coated vesicles formation (norm and CF)	23	0.023
	Macropinocytosis regulation by growth factors	14	0.029
	Regulation of degradation of wt-CFTR	19	0.040
GO processes			
GO process name		**% genes[a]**	***p*-value**
Chromatin assembly		20	0.0000
mRNA metabolic process		11	0.0000
Chromatin assembly or disassembly		17	0.0001
Nucleosome assembly		20	0.0001
Protein-DNA complex assembly		18	0.0001
Nucleosome organization		18	0.0002
Cellular biopolymer catabolic process		9	0.0003
DNA packaging		15	0.0004
RNA metabolic process		8	0.0005
RNA processing		9	0.0005

[a] Percentage of significantly correlated genes compared to the total number of genes in the pathway or process.

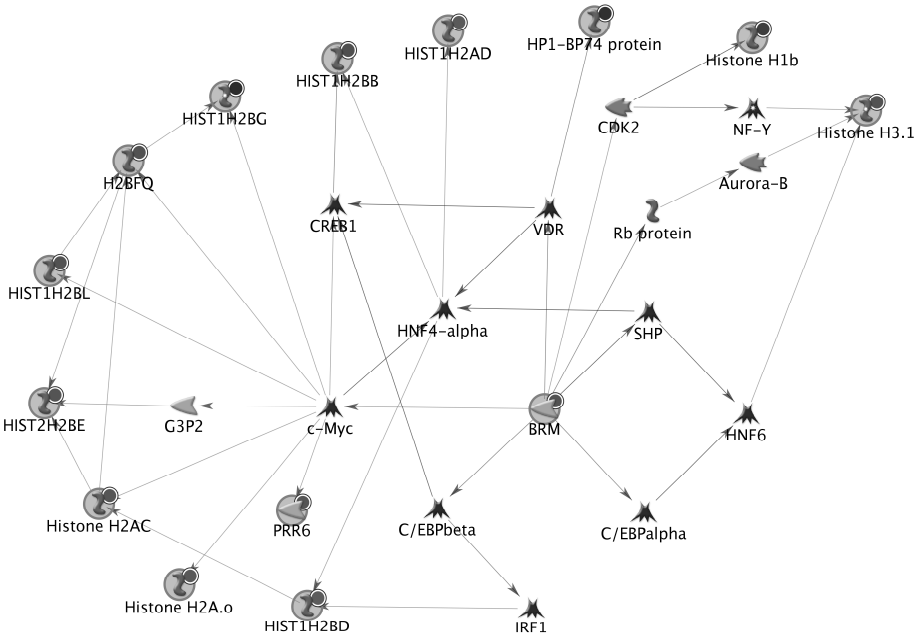

Figure 2: A network analysis of genes in the Chromatin assembly GO process showing the shortest paths between genes significantly correlating with ATNC levels in both biopsy patients groups. Significantly correlating genes are designated by a large blue circle in the background. Positive and negative correlation is indicated with small red and blue circles, respectively. Colour figure is included separately.

Discussion

With this study we aimed to investigate gene expression modifications induced in human colon tissue in relation to NOC exposure. Since the relevance of NOC in human CRC development is still a matter of debate, identifying changes in the expression pattern may be helpful in assessing the carcinogenic risk in humans. A few studies have demonstrated an increased nitrosation in inflammatory environments, including the colon of patients with IBD who thus form an interesting population to study NOC-associated gene expression changes. Inducible nitric oxide synthase (iNOS) activity, as found in IBD patients [17], is associated with an excess production of nitrosating agents, such as NO, nitrogen oxides, and nitrite [18], which could explain the formation of carcinogenic NOC. Although there is a large amount of evidence that increased oxidative stress levels associated with chronic inflammation contribute to neoplastic transformation in IBD patients [19], NOC may also play a significant role.

As demonstrated by the fecal calprotectin levels (Table 2), IBD patients indeed had a higher level of colonic inflammation compared to IBS controls. However, no significant differences were found in fecal ATNC levels between both groups, nor

was there a significant correlation between calprotectin and ATNC levels, indicating that in this study colonic inflammation did not clearly stimulate the formation of fecal NOC. It should be noted that the control subjects used in this study were all diagnosed with IBS and can therefore not be considered as healthy controls. However, IBS is not characterized as an inflammatory condition [20,21], as also demonstrated by the clear difference in inflammation level compared to the IBD subjects, thus making them a suitable control group. Food diaries recorded by all subjects showed that the average intake of most dietary components was not significantly different between IBD and IBS patients, except for total fat, saturated fat, cholesterol, zinc, folic acid, and vitamin B12, which were all significantly higher in the IBD group and can be ascribed to differences in gender ratio between groups (Table 1). It is, however, unlikely that any of the significantly different dietary factors influences NOC formation. The most important dietary components with regard to stimulation or inhibition of endogenous nitrosation are animal protein, vitamin C and E, and possibly iron [22,23], none of which were different between groups. We conclude that interindividual differences in dietary patterns are not accountable for the absence of significantly different fecal ATNC levels between the study populations.

At the gene expression level, IBD patients and controls did form a distinctive response as shown by the PCA plot where IBD and IBS patients form two groups with only a small degree of overlap (Figure 1). This is in agreement with several studies showing that the gene expression pattern in colon biopsies from ulcerative colitis patients is different from non-inflamed controls [24–27]. Similar to what was found in these studies, differentially modified genes between IBD patients and controls are strongly involved in the immune response, and in particular biological pathways regulating the inflammatory response, including many cytokine signaling pathways (Table 3). (L)-Arginine metabolism, which is essential in the production of NO through the activity of iNOS, was also significantly modulated. The iNOS gene, which is activated by cyclooxygenase 2 (COX-2) and many cytokines [28,29], was significantly up regulated in IBD patients, as was COX-2 (results not shown). This is expected to lead to NO production which can again stimulate the formation of pro-inflammatory mediators by increasing the expression of COX-2, thus creating a positive feedback loop [30]. Cytokine production in ulcerative colitis patients is one of the main factors contributing to the ongoing relapsing activation of the mucosal immune system in the gut which fails to be resolved and leads to a persistent state of chronic inflammation [31,32]. Indeed, many pro-inflammatory genes were found to be up regulated in the immune response pathways, including several cytokines and cytokine receptors, which is especially interesting since the biopsies were taken from visually non-inflamed tissue in the colon. This suggests that a much larger part of the colon than just the visually inflamed section is in a (low) state of inflammation. Similar observations were made in tissue from ulcerative colitis patients in remission [24] and it is possible that, even in the absence of an inflammatory state,

the mRNA expression pattern in colon tissue from IBD patients facilitates a relapse in inflammation following the right trigger. In addition to the strong immune response modifications, a large number of development pathways were differentially modulated between IBD patients and controls. JAK/STAT signaling pathways are particularly strongly represented which is not surprising considering the essential role of these pathways in cytokine signaling [33,34]. JAK/STAT signaling also plays an important role in regulating growth, survival, and differentiation and modifications in these pathways could therefore influence the carcinogenic process in IBD patients. Epithelial cell turn-over, for example, is increased in the colonic IBD mucosa compared to normal tissue through the actions of CDKN1A which is regulated by JAK/STAT signaling [35,36]. In combination with the DNA damaging and mutagenic factors present in the inflammatory environment, this provides the ideal circumstances for stimulation of the carcinogenic process.

Although ATNC were detected in almost every fecal sample, the transcriptomic differences identified between IBD patients and controls cannot be attributed to NOC exposure because of the absence of a significant difference in fecal ATNC levels between these two groups. However, by specifically looking for genes whose expression values are significantly correlated with individual ATNC levels, it is still possible to identify NOC exposure-associated gene expression modulations regardless of the presence of inflammation. IBD patients and controls were therefore combined to create an integrated analysis across all biopsy subjects. The set of genes found to be significantly correlated with ATNC levels was involved in several pathways that can be linked to the genotoxic properties of NOC (Table 4). One pathway regulating DNA repair was identified in addition to four signaling cascade pathways regulated by members of the Ras superfamily of small GTPases (RALA and RAC1), phosphatidylinositol (3,4,5)-trisphosphate (PIP3), and serine/threonine protein kinase AKT. Although not grouped under the same cellular process by MetaCore, all of these pathways are implicated in similar mechanisms like cell cycle regulation, apoptosis/survival, and cytoskeleton remodeling. Many of the significantly correlating genes within these pathways are shared and include forkhead box O3, Fas ligand, the 14–3-3 gene, cyclin D3, and actin the first three of which are positively correlated with ATNC levels and the remaining two are negatively correlated (results not shown). This could, in theory, result in stimulation of apoptosis, and inhibition of cell cycle progression, as based on information on these genes obtained from Entrez Gene of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene). Pathways specifically implicated in apoptosis or cell cycle regulation were not identified. This is not surprising considering that the NOC concentrations reached in the human colon are unlikely to cause enough damage to strongly induce a cell cycle block or apoptosis (**Chapter 2**) [9].

ATNC levels were also significantly correlated with genes present in a number of transport pathways involved in cystic fibrosis transmembrane conductance regulator (CFTR) degradation in conjunction with clathrin-coated vesicle formation and macropinocytosis, which are two important endocytosis routes found in most cells [37]. CFTR is a chloride ion channel which controls the level of hydration of the luminal content through ion concentration regulation [38]. The relevance of modifications in these pathways in relation to NOC exposure is not clear.

The set of significantly correlating ATNC genes was subsequently analyzed for its involvement in GO processes that can be linked to colonic NOC exposure. The top 10 of significantly modulated GO processes contained 6 processes involved in chromatin assembly/disassembly and this process thus appears to be important. Since the relevance of these processes is difficult to interpret without the level of interplay between genes provided by a pathway analysis, we explored in depth the significantly correlating genes in the most significant of these 6 processes (Chromatin assembly) in a network analysis to visualize their interactions. As shown in Figure 2, the significantly correlating genes in the Chromatin assembly process all encode histones or proteins involved in chromatin organization. Regulation of the chromatin structure plays an essential role in controlling gene expression and these results suggest that NOC could influence gene expression by histone modifications. NOC have been described to induce chromatin damage, resulting in inhibition of protein and nuclear RNA synthesis, and to cause imbalances in histone and non-histone proteins, but only at high concentrations in animal models [39–42]. NOC-induced alkylation of these proteins could affect their interaction with DNA and influence gene expression levels [39,43]. Histone methylation, for example, promotes heterochromatin formation resulting in gene silencing [44] and it is possible that (methylating) NOC influence the expression of genes through such epigenetic mechanisms. Promotion of heterochromatin formation is supported by our data since all positively correlating histone genes are variants of histone H2A and H2B which are core components of heterochromatic nucleosomes [45]. Histones H1b and H3.1 and the BRM protein are also involved in heterochromatin formation, but they were negatively correlated with ATNC levels, while the function of the negatively correlating gene HP1-BP74 is not known. Possibly related to this is the discovery that alkylating DNA damage, and formation of O^6-methylguanine in particular, is linked to the modulation of gene expression by both inhibiting and promoting the action of maintenance methylases on adjacent cytosine bases which is likely to result in chromatin modifications [46–48]. It is also possible that the up regulation of histone genes represents a compensatory synthesis of new histones to replace damaged ones. Since this would require remodeling of the nucleosome complex, gene expression may also be influenced. In the past two decades, histone modification following NOC exposure has not received much attention in literature and these results may be a starting point for new investigations into the relation between NOC and chro-

matin assembly in relation to gene expression changes. Alterations in the expression of histone and chromatin remodeling genes in relation to NOC exposure has, to our knowledge, never been shown before. Finding these modifications in humans exposed to physiological NOC concentrations under daily life circumstances, could be an important discovery and may have relevance with regard to carcinogenic risks associated with NOC exposure.

It can be concluded that, although inflammation did not provide a statistically reliable model for increased nitrosation in this study, gene expression modifications were identified that could contribute to the understanding of IBD pathophysiology. More importantly, by specifically relating gene expression changes with fecal ATNC levels, we have identified NOC exposure-associated transcriptomic responses that suggest that physiological NOC concentrations cause genotoxicity and chromatin modifications in human colon tissue, both of which are linked with carcinogenicity. This is the first study to analyze the transcriptomic response in the human colon in relation to NOC exposure thereby shedding light on the possible role of NOC in human CRC development.

Acknowledgements

The authors would like to recognize the efforts of the endoscopy department staff at the Orbis Medical Center and the Maastricht University Medical Center and thank the study subjects for their participation. The authors also wish to gratefully acknowledge the technical assistance of Dr. Daisy Jonkers.

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Chapter 7

Red meat intake-induced increases in fecal water genotoxicity correlate with pro-carcinogenic gene expression changes in the human colon

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Abstract

Red meat consumption is associated with an increased colorectal cancer (CRC) risk, which may be due to an increased endogenous formation of genotoxic N-nitroso compounds (NOCs). To assess the impact of red meat intake on potential risk factors of CRC, we investigated the effect of a 7-day dietary red meat intervention in human subjects on endogenous NOC formation and fecal water genotoxicity in relation to transcriptomic changes induced in colonic tissue. In order to evaluate the potential effect of an inflamed colon on endogenous nitrosation, the study population consisted of inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) control subjects without inflammation. The intervention had no effect on fecal NOC formation but fecal water genotoxicity significantly increased in response to red meat intake. Since IBD patients showed no difference in fecal NOC formation or fecal water genotoxicity levels as compared to IBS controls, for transcriptomic analyses, all subjects were grouped together. Genes significantly correlating with the increase in fecal water genotoxicity were involved in biological pathways indicative of genotoxic effects, including modifications in DNA damage, cell cycle, and apoptosis pathways. Moreover, WNT signaling and nucleosome remodeling pathways were modulated that are known to play a part in the carcinogenic process in the human colon. These results are in line with a possible oxidative effect of dietary heme. We conclude that the gene expression changes identified in this study corroborate the genotoxic potential of diets high in red meat and point towards a possible risk of CRC development in humans.

Introduction

Consumption of red meat is associated with a higher risk of colorectal cancer (CRC) and this may be a result of an increased genotoxic stress in the colon since diets high in red meat content have been found to increase fecal water genotoxicity [1–5]. It has been proposed that endogenously formed N-nitroso compounds (NOCs) are responsible for the link between red meat consumption and CRC risk [6] since red meat is known to stimulate NOC formation in the colon [7]. Meat in general is a source of NOC precursors in the form of amines and amides and heme protein present in red meat is thought to catalyze endogenous nitrosation [8,9]. As most NOCs have mutagenic and genotoxic properties, which explain their carcinogenic effect in test animals [10,11], they may also contribute to CRC development in humans. Moreover, gene expression changes associated with NOC exposure could play a part in the carcinogenic process. Indeed, transcriptomic effects following NOC exposure *in vitro* have been found that may influence their carcinogenic effects (**Chapters 2**, **3**, and **4**) [12,13].

We have previously investigated the possible role of NOCs in human CRC development by studying gene expression modifications associated with exposure to these compounds in the inflamed human colon (**Chapter 6**). There are indications that inflammation stimulates endogenous nitrosation which makes the inflamed colon an interesting model to study the effect of NOCs in relation to CRC [14,15]. Although we were unable to detect this stimulatory effect in our previous study, we did find transcriptomic changes in colonic tissue in relation with fecal NOC levels that may contribute to pre-carcinogenic events induced in the colon.

The relation between red meat consumption and increased levels of nitrosation makes a dietary red meat intervention study suitable as a model for investigating NOC exposure-associated gene expression changes in the human colon. Furthermore, an interaction between inflammation and red meat consumption may hypothetically result in an even higher rate of fecal nitrosation. In the present study, we therefore investigated the impact of dietary red meat intake on fecal NOC formation and fecal water genotoxicity in relation to transcriptomic changes induced in colonic tissue obtained from a subset of subjects from our previous study (**Chapter 6**). Gene expression changes identified in this study may be helpful in explaining the role of red meat consumption in human CRC.

Material and methods

Subjects and study design

The study population was recruited in the Netherlands at the Department of Gastroenterology in the Orbis Medical Center in Sittard and the Maastricht University Medical Center and consists of a subset of subjects who participated in our previous study (**Chapter 6**). A total of 12 patients were recruited, of which 6 were inflammatory bowel disease (IBD) patients (5 male, 1 female, mean age 51.2 ± 14.5, range 31–71 years) and 6 were diagnosed with irritable bowel syndrome (IBS) (3 male, 3 female, mean age 55.0 ± 15.0, range 25–65 years). Food diaries were filled out on three consecutive days, after which subjects collected a first feces sample. This was immediately frozen (-20 °C) upon collection. During a colonoscopic examination, six biopsies were taken from mucosal tissue in visually non-inflamed regions of the colon (in most cases from the sigmoid and descending colon) which were immediately frozen in liquid nitrogen and stored at -80 °C until use. The intervention week started immediately after the first colonoscopy exam and involved a high red meat diet consisting of 300 grams of red meat per day for 7 days. Only beef products were consumed, which included round steak, entrecôte, veal schnitzel, veal cutlet, and veal escalope. Participants were instructed to cook the meat medium and were not allowed to eat any other meats during the intervention week. Food diaries were filled out daily during this week. Three days before and during the intervention period subjects followed their normal dietary habits, with a few modifications. To keep nitrate intake between subjects similar, participants were asked to use low-nitrate mineral water when preparing food or drinks and to avoid vegetables with high nitrate concentrations, such as spinach, lettuce, and celery. A high fruit consumption, especially fruit rich in vitamin C, and use of vitamin supplements also had to be avoided as this has been reported to inhibit endogenous nitrosation [16].

After the intervention, a second colonoscopy was performed and six biopsies were taken at the same location as in the first exam. Subjects collected a second feces sample on the last intervention day prior to the colonoscopy which was also immediately frozen (-20 °C) upon collection. The first colonoscopy was performed as part of either surveillance or suspected gastrointestinal disorders, while the second colonoscopy was performed solely for the purpose of this study. Use of additional anti-inflammatory medication in IBD patients to treat the exacerbated colon inflammation was postponed until the end of the study.

This study was approved by the Medical Ethical Committees Atrium Orbis Zuyd and Clinical Trial Center Maastricht (CCMO registration number: NL13359.096.06) and written informed consent was obtained from the participants prior to the start of the study.

Fecal apparent total nitroso compound determination

Apparent total nitroso compounds (ATNC), as a measure for NOCs, were analyzed in feces as described previously [17], using an Ecomedics CLD 88 Exhalyzer (Ecomedics, Duernten, Switzerland). In short, fecal material was diluted 1:5 in ultrapure water and homogenized for 20 min. Thereafter, 500 µl of a 5% (wt/vol) sulfamic acid solution was added to remove nitrite and samples were injected into a purge vessel kept at 60 °C and filled with a standard tri-iodide reagent (38 mg I_2 was added to a solution of 108 mg KI in 1 ml water; to this mixture, 13.5 ml glacial acetic acid was added) to determine ATNC. Results are presented as nmol ATNC/g feces.

Fecal water alkaline single-cell gel electrophoresis (comet assay)

Fecal water samples were prepared from fecal material collected from patients who participated in the red meat intervention. After homogenization of the fecal material for 2 minutes, samples were ultracentrifuged at 50,000 *g* for 2 hours at 10 °C. The supernatant fecal water was aliquoted and stored at -20 °C until use. The human colon adenocarcinoma cell line Caco-2 was used to test fecal water genotoxicity in the standard and formamidopyrimidine-DNA glycosylase (Fpg) comet assay which is specifically directed at oxidized purines and thus creates more DNA strand breaks. Caco-2 cells were cultured in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, Zwijndrecht, The Netherlands) with 4.5 g/L glucose, L-glutamine, $NaHCO_3$ and pyridoxine HCl supplemented with 1% (v/v) nonessential amino acids, 1% Na-pyruvate, 1% penicillin/streptomycin and 10% (v/v) heat-inactivated fetal calf serum, all purchased from Gibco BRL (Breda, The Netherlands). Cell cultures were incubated at 37 ºC in a humidified incubator containing 5% CO_2. For fecal water exposures, cells were harvested by trypsinization and resuspended in growth medium containing 10% fecal water followed by a 30 min. incubation at 37 ºC. After incubation, cells were centrifuged (300 *g*, 5 min.), washed once in phosphate buffered saline, and placed on ice. The standard and Fpg alkaline comet assays were subsequently performed in triplicate as described by Singh *et al.* [18] and Plaum *et al.* [19] with minor modifications. Comets were visualized using a Zeiss Axioskop fluorescence microscope (at 200× magnification). Randomly, 50 cells were analyzed using the Comet assay III software (Perceptive Instruments, Haverhill, UK). DNA damage was expressed as tail moment (TM, the product of tail DNA content and mean tail migration distance). In each experiment, control cells from a batch of frozen H_2O_2 exposed Caco-2 cells (100 µM, 30 min., frozen at -80 ºC in freeze medium containing 10% dimethylsulfoxide) were co-electrophorized and scored along with the fecal water-exposed cells to compensate for any inter-electrophoresis variation.

Microarray hybridization and data analysis

RNA isolation from biopsy material and hybridization on Agilent 4x44K Whole Human Genome microarrays (Agilent Technologies, Amstelveen, The Netherlands) was performed as described previously (**Chapter 6**). After scanning the microarray slides, using settings described before (**Chapter 2**) [13], bad and empty spots were flagged using the GenePix Pro software (version 6.0, Molecular Devices, Sunnyvale, CA). Quality control was performed in the statistical software environment R (version 2.10.1, The R Foundation for Statistical Computing, Vienna, Austria). Quantile normalization and subsequent data processing was performed in ArrayTrack (version 3.4, NCTR, Jefferson, AR). Log2 transformed spot intensities were used for further analyses.

ArrayTrack and Microsoft Excel were used to find significantly modulated genes between before and after intervention groups (paired *t*-test, $p < 0.05$) with a minimum average absolute log2 ratio of 0.5 between groups. Further filtering was performed by selecting only gene pairs (before paired with after) that were present in at least 8 out of 12 subjects. Genes were subsequently imported in MetaCore™ (GeneGo, San Diego, CA) to identify the involvement of differentially expressed genes in specific cellular GeneGO pathways. Pathways with a $p < 0.05$ were considered significantly modulated.

For Spearman's rank correlation analyses, gene expression data from all 12 subjects were correlated with genotoxicity levels (TMs from standard and Fpg comet assays). Only genes present in at least 70% of subjects were used without further pre-selection. Prior to correlation analysis, missing values were imputed in GenePattern (version 3.1, http://www.broad.mit.edu/cancer/software/genepattern/) by finding the *k* nearest neighbors (*k* was set to 15), using a Euclidean metric, and imputing the missing elements by averaging the (non-missing) elements of its neighbors. The online Gene Expression Profile Analysis Suite (GEPAS, http://gepas.bioinfo.cipf.es/) was used to perform correlation analyses and significantly correlating genes ($p < 0.05$) were subsequently further analyzed in MetaCore.

Statistical analysis

Results are presented as mean ± SD. Normality of data sets was tested using the Kolmogorov-Smirnov test. Potential differences between groups were assessed using the Student's *t*-test (for equal or unequal variances, as based on Levene's test), paired *t*-test or Wilcoxon signed-rank test depending on the normality of data and presence of paired samples.

Results

Dietary intake during red meat intervention

The results of the food diary analysis are presented in Table 1 and are consistent with an increased intake of red meat. The average daily dietary intake of protein, animal protein, potassium, and zinc was significantly increased during the red meat intervention as compared to before (p <0.05 or <0.01). All other dietary components were not significantly influenced by the intervention.

Table 1: Reported average daily dietary intake before and after the red meat intervention for all subjects (n = 12).

Dietary intake (average/day)	**Before intervention**	**During intervention**
Energy (kcal)	2072 ± 596	2028 ± 509
Protein (g)	80.0 ± 24.7	121 ± 19.2[a]
Animal protein (g)	32.0 ± 24.9	69.6 ± 11.2[a]
Total fat (g)	85.7 ± 37.8	73.8 ± 23.6
Saturated fat (g)	29.9 ± 12.3	29.7 ± 8.2
Cholesterol (mg)	226 ± 144	274 ± 101
Carbohydrates (g)	199 ± 57.3	196 ± 70.5
Fibers (g)	19.2 ± 6.2	19.9 ± 7.2
Alcohol (g)	19.4 ± 15.5	14.5 ± 13.4
Water (g)	1579 ± 358	1671 ± 467
Sodium (mg)	3305 ± 928	2991 ± 751
Potassium (mg)	2702 ± 596	3408 ± 771[b]
Calcium (mg)	632 ± 241	762 ± 283
Magnesium (mg)	281 ± 68.8	303 ± 90.7
Iron (mg)	11.3 ± 3.6	12.9 ± 4.0
Selenium (µg)	52.2 ± 42.3	47.3 ± 15.6
Zinc (mg)	8.6 ± 2.2	13.7 ± 2.5[a]
Folic acid (µg)	142 ± 31.4	152 ± 35.4
Vitamin A (µg)	881 ± 320	694 ± 194
Vitamin B1 (mg)	1.1 ± 0.3	1.4 ± 0.5
Vitamin B2 (mg)	1.1 ± 0.4	1.1 ± 0.4
Vitamin B3 (mg)	18.5 ± 7.7	23.2 ± 6.2
Vitamin B6 (mg)	1.6 ± 0.5	1.7 ± 0.4
Vitamin B12 (µg)	4.3 ± 4.0	5.6 ± 1.9
Vitamin C (mg)	34.7 ± 15.8	49.5 ± 16.6
Vitamin D (µg)	5.9 ± 4.4	4.0 ± 1.3
Vitamin E (mg)	15.9 ± 11.4	13.3 ± 9.3

[a] Significantly higher after the intervention compared to before at p < 0.01 (paired t-test)
[b] Significantly higher after the intervention compared to before at p < 0.05 (paired t-test)

Fecal ATNC levels

Fecal material of subjects completing the red meat intervention was first analyzed for ATNC content. IBD patient and IBS control groups were initially analyzed separately for covering the possibility of detecting an additional effect of inflammation on endogenous nitrosation during the intervention period. However, neither the IBS nor the IBD patients displayed a significant difference in fecal ATNC levels after the intervention (paired *t*-test) and there were no differences between IBS and IBD patients before or after the red meat intervention period (Student's *t*-test) (Figure 1). Combining all subjects also showed no significant effect of the intervention on fecal ATNC levels.

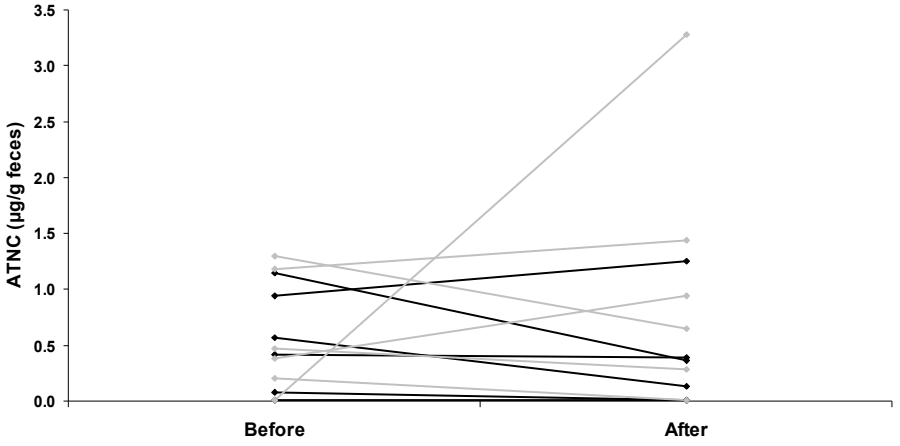

Figure 1: Fecal ATNC levels in IBD (black lines) and IBS patients (gray lines) before and after the red meat intervention. No significant differences were detected between before and after measurements or between IBS and IBD subjects.

Fecal water genotoxicity

Although endogenous NOC formation was not significantly affected by the red meat diet, we investigated *in vitro* fecal water genotoxicity of all subjects before and after the red meat intervention as a broader marker of exposure which reflects damage induced by all genotoxic fecal water constituents and which is known to respond to increased red meat intake. Individual fecal water genotoxicity levels are presented in Figure 2. In 10 out of 12 subjects fecal water genotoxicity appeared to be increased after the meat intervention, while the remaining 2 subjects did not respond to meat intake. Similar to fecal ATNC levels, there was no significant difference in genotoxicity level between IBD patients and IBS controls before or after the intervention (Student's *t*-test) and therefore both groups were combined to determine

the effect of the red meat intervention (Table 2). Since inter-individual variability in fecal water genotoxicity was high, the Wilcoxon signed-rank test was used. Averaged at group level, the intervention resulted in a significant increase in genotoxicity of more than a factor 2 in both the standard and the Fpg comet assay (p <0.05). Incubation with Fpg enzyme led to a significant increase in DNA strand breaks compared to the standard comet assay both before and after the intervention (p <0.01), indicating the presence of oxidized purines.

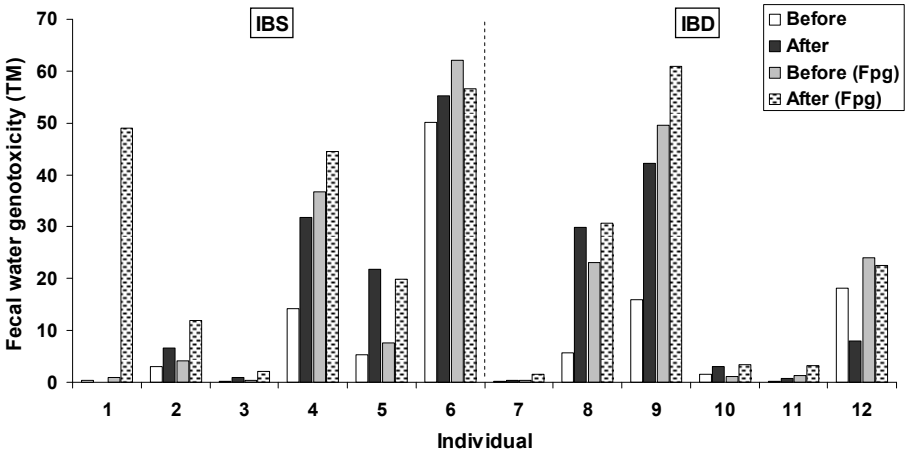

Figure 2: Fecal water genotoxicity level of IBS and IBD subjects before and after the red meat intervention week using the standard and Fpg comet assay. Values were corrected for blank levels. Total n = 12.

Table 2: Fecal water genotoxicity level of all subjects before and after the red meat intervention week (n = 12) using the standard and Fpg comet assay.

	Standard comet assay genotoxicity[a]	Fpg comet assay genotoxicity[a]
Before intervention	10.4 ± 14.8	19.1 ± 22.0[b]
After intervention	18.2 ± 19.2[c]	23.4 ± 22.1[b,c]
Ratio after/before	2.7 ± 1.7	2.4 ±1.4

Measurements were corrected for blank levels. [a] Genotoxicity is quantified as TM, [b] Genotoxicity level significantly higher with Fpg (p <0.01) based on Wilcoxon signed-rank test, [c] Genotoxicity level significantly higher after the intervention (p <0.05) based on Wilcoxon signed-rank test.

Gene expression modifications associated with red meat intervention

Since fecal ATNC and genotoxicity levels were not different between IBS and IBD subjects, for subsequent transcriptomic analyses both groups were combined to find red meat intervention-related gene expression modifications. To analyze whether the intervention influenced the expression of genes in these subjects, a paired t-test was performed and significantly modified genes (with an additional

log2 ratio cut-off of 0.5) were analyzed in MetaCore. A small number of GeneGO pathways were found to be significantly modified by meat consumption (Table 3). Pathways were involved in several processes, including cytoskeleton remodeling, development, and immune response, but the percentages of involved genes for each pathway are low, indicating only small effects.

Table 3: GeneGO pathways significantly modulated in subjects following a 7-day red meat intervention (*n* = 12).

Cellular process	Pathways involved	% genes[a]	*p*-value
Atherosclerosis	Role of ZNF202 in regulation of expression of genes involved in Atherosclerosis	17	0.020
Cytoskeleton remodeling	Fibronectin-binding integrins in cell motility	11	0.042
Development	NOTCH-induced EMT	29	0.000
	NOTCH Signaling Pathway	13	0.002
	NOTCH1-mediated pathway for NF-KB activity modulation	12	0.010
Immune response	CD16 signaling in NK cells	8	0.030
	Murine NKG2D signaling	12	0.038
Transcription	Assembly of RNA Polymerase II pre-initiation complex on TATA-less promoters	14	0.026
Transport	Intracellular cholesterol transport	8	0.036

[a] Percentage of significantly correlated genes compared to the total number of genes in the pathway.

Fecal water genotoxicity-associated gene expression modifications

By specifically looking at gene expression changes associated with the overall increase in fecal water genotoxicity as a phenotypic marker of effect, relevant genes may be identified that are otherwise missed when comparing the before and after measurements with each other. To investigate the transcriptomic response linked to the increase in fecal water genotoxicity we performed a Spearman's rank correlation analysis between TM levels and log2 gene expression intensities. Two separate correlation analyses were performed, using TMs from the standard and the Fpg comet assay. Significantly correlating genes from both analyses were subsequently analyzed in MetaCore for their involvement in GeneGO pathways and the two lists of pathways resulting from this analysis were combined, the results of which are presented in Table 4. Pathways were found to be involved in a wide range of cellular processes. Pathways controlling apoptosis and survival, cell cycle regulation, and DNA damage repair were significantly associated with levels of fecal genotoxicity. Pathways involved in closely related processes like cytoskeleton remodeling, cell adhesion, and proteolysis were also found. Development pathways associated with genotoxicity levels included the strongest affected pathway in the list, the WNT signaling pathway of which more than 50% of genes were correlated with genotoxicity levels. The other development pathways were involved in signaling pathways

linked with cytoskeleton reorganization. Other strongly associated processes include regulation of lipid metabolism, which contains pathways implicated in cholesterol and fatty acid biosynthesis, and transport, which mainly contains pathways regulating endosome formation and transport.

Table 4: GeneGO pathways significantly associated with genotoxicity levels as found by MetaCore analysis of significantly correlating genes in subjects participating in a red meat intervention (n = 12).

Cellular process	Pathways involved	% genes[a]	p-value
Amino acid metabolism	Histidine-glutamate-glutamine metabolism	29	0.036
Apoptosis and survival	Regulation of Apoptosis by Mitochondrial Proteins	21	0.017
Carbohydrate metabolism	Pentose phosphate pathway	50	0.001
	Glycolysis and gluconeogenesis	33	0.043
Cell adhesion	Integrin-mediated cell adhesion and migration	33	0.018
	Histamine H1 receptor signaling in the interruption of cell barrier integrity	24	0.018
	Role of tetraspanins in the integrin-mediated cell adhesion	23	0.022
Cell cycle	The metaphase checkpoint	20	0.015
Cytoskeleton remodeling	Role of PKA in cytoskeleton reorganisation	39	0.007
	Fibronectin-binding integrins in cell motility	29	0.007
	ESR1 action on cytoskeleton remodeling and cell migration	27	0.046
	Thyroliberin in cytoskeleton remodeling	31	0.046
Development	WNT signaling pathway. Part 1. Degradation of beta-catenin in the absence of WNT signaling	54	0.001
	Growth hormone signaling via STATs and PLC/IP3	22	0.014
	Slit-Robo signaling	30	0.029
	Angiotensin signaling via beta-Arrestin	24	0.036
	Role of IL-8 in angiogenesis	18	0.041
DNA damage	Role of SUMO in p53 regulation	47	0.002
Immune response	IL-12 signaling pathway	24	0.036
Neurophysiological process	Thyroliberin in cell hyperpolarization and excitability	33	0.035
Proteolysis	Putative SUMO-1 pathway	38	0.003
	Putative ubiquitin pathway	27	0.023
Regulation of lipid metabolism	Cholesterol Biosynthesis	40	0.003
	Niacin-HDL metabolism	43	0.012
	Unsaturated fatty acid biosynthesis	31	0.046
Signal transduction	Calcium signaling	29	0.032
Transcription	Sin3 and NuRD in transcription regulation	31	0.012
Translation	Non-genomic (rapid) action of Androgen Receptor	20	0.038
Transport	Delta508-CFTR traffic / Sorting endosome formation in CF	37	0.010
	Clathrin-coated vesicle cycle	25	0.013
	Normal wtCFTR traffic / Sorting endosome formation	29	0.018
	RAN regulation pathway	33	0.043

[a] Percentage of significantly correlated genes compared to the total number of genes in the pathway.

Discussion

The underlying mechanism of the correlation between red meat consumption and cancer of the colon and rectum has been investigated for many years and endogenous formation of NOCs has been suggested to play a role in this association. Although red meat intake-induced increases in fecal NOC levels, as measured by ATNC, have been demonstrated in several studies [17,20,21], we were unable to find an increase following a red meat intervention in this study (Figure 1). The presence of inflammation in the colon, which was previously found to stimulate endogenous nitrosation [14], in combination with the red meat diet also did not increase ATNC formation in a subgroup of subjects with IBD. As shown by the food diary analysis (Table 1), animal protein (and total protein) was significantly increased after the intervention, as was potassium and zinc intake, which can all be ascribed to the increase in meat consumption [22]. However, in contrast to other red meat diet intervention studies, food intake was not controlled in this study [20,21,23–25]. In addition, subjects switched from their normal everyday diets to a high red meat diet which may not have constituted a sufficient enough increase. This may have introduced more variation, making it difficult to establish effects on ATNC levels.

Despite the absence of an increase in NOC formation after the red meat intervention, fecal water genotoxicity was significantly higher after the intervention (Figure 2 and Table 2). Red meat consumption is not only associated with a more than two-fold increase in fecal water genotoxicity, but oxidative stress also appears to be of relevance since both before and after the intervention, implementation of Fpg in the comet assay demonstrated significantly increased oxidative DNA damage levels. Although NOCs are capable of generating oxidative stress (**Chapter 3**) [12], it is very unlikely that fecal water NOC concentrations are capable of inducing damage at levels observed in previous *in vitro* experiments where NOC-induced genotoxicity in Caco-2 cells was observed at millimolar concentrations which do not occur *in vivo* (**Chapter 2**) [13]. It has indeed been shown before that fecal water genotoxicity is independent of endogenous NOC formation [23], and therefore DNA damage induced as a result of an increased meat intake does not appear to be related to the presence of NOCs. Although heterocyclic amines (HCAs) and polycyclic aromatic hydrocarbons (PAHs), which are formed during the cooking of meat, also pose a genotoxic and carcinogenic risk [26,27], they are not likely to cause the increase in fecal genotoxicity levels as seen here either. Fecal HCA and PAH concentrations are not expected to be high enough to induce the level of DNA damage found in the comet assay as demonstrated by the low level of HCA and PAH-induced DNA adducts in human colon tissue [28,29]. Moreover, PAHs are mostly formed during cooking of meat at high temperatures and with direct flames which was not performed in this study. Heme protein on the other hand, which is present in red meat

in high levels and associated with an increased CRC risk [26,30–34], has been shown to induce genotoxicity as measured by comet assay at concentrations found in the gut lumen [35]. Apart from the reported heme-stimulated NOC formation [8], which was not observed in our study, heme may act as a catalyst of oxidative stress, which may also explain the increase in oxidative DNA damage in the Fpg comet assay. Heme-catalyzed oxidations can damage lipids, proteins, and DNA and a major pathway involves lipid peroxidation resulting in the formation of lipid alkoxy radicals and heme oxyradicals which can initiate further oxidations [35,36].

Fecal water genotoxicity has previously been related to colon carcinogenesis in animals [37], and the results found here support such a relation in humans as well. Red meat intake-stimulated fecal water genotoxicity may result in a higher level of DNA damage in human colonic epithelium and induce the carcinogenic process. To study the effects of the red meat intervention in more detail with emphasis on possible pro-carcinogenic modifications, we subsequently addressed differences in the transcriptomic response as a result of the intervention, with a particular focus on fecal water genotoxicity. Genes differentially regulated as a result of the red meat intervention were first investigated and found to be involved in a small number of pathways, but the percentage of involved genes was relatively low (Table 3). Although the three NOTCH signaling pathways are interesting in light of their role in gut development and homeostasis in relation to the proliferative potential of intestinal adenomas and adenocarcinomas [38], modifications in these pathways are too limited to accurately assess their biological relevance regarding red meat intake and CRC risk. Effects on the other identified pathways suffer from the same limitation, which prompted us to analyze genes specifically associated with fecal water genotoxicity since this is more directly related with a potential CRC risk. Significantly correlating genes found in this analysis not only were involved in a much larger number of pathways, the percentage of involved genes per pathway was also considerably higher (Table 4). Several of the identified pathways can be related to an increased genotoxic stress. The DNA damage pathway involved in p53 regulation, for example, would theoretically lead to breakdown of the p53 protein since the MDM2 and ubiquitin genes were both positively correlated with fecal water genotoxicity in this pathway. MDM2 and ubiquitin work together in targeting p53 for degradation [39] and this is an indication that, as a result of increased DNA damage, high levels of p53 protein are present. The two proteolysis pathways involving ubiquitin and SUMO-1 serve as an additional indicator that levels of p53 are being regulated since both are intimately involved in modifying p53 stability [40]. Because of p53's central role in the regulation of apoptosis and cell cycle progression [41], it is not surprising that pathways controlling these processes were also found. The identified cell cycle pathway is involved in the metaphase checkpoint while the modulated genes in the apoptosis pathway suggest an inhibition of the apoptotic process, since several pro-apoptotic genes were lowered in expression. This is an indication

that after the intervention the balance between growth arrest (to repair DNA damage) and apoptosis is still favored towards the former in the colon of these subjects. The modified pathways belonging to the cellular processes of cell adhesion and cytoskeleton remodeling all appear to be interrelated since they share a large number of significant genes. The overall theoretical effect that can be deduced from these pathways points towards promotion of stress fiber formation. Stress fibers are formed in response to genotoxic stressors through activation of the small GTPase RhoA, and play a role in delaying cell death which is in agreement with the anti-apoptotic response found in the apoptosis pathway [42]. The expression of RhoA was indeed increased in this pathway as fecal water genotoxicity increased. Stress fiber formation is also supported by modifications in some of the development pathways, including the Slit-Robo and IL-8 signaling pathways, which are involved in cytoskeleton reorganization and display a large degree of overlap with the cytoskeleton remodeling and cell adhesion pathways.

One of the most interesting pathways found to be related to fecal water genotoxicity is the strongly modified WNT signaling pathway which is involved in epithelial proliferation and differentiation. Aberrant activation of WNT signaling represents a major oncogenic process in the development of many epithelial cancers, including CRC [43–46]. Activation of WNT signaling results in β-catenin accumulation which subsequently forms a complex with the transcription factor T-cell factor/lymphoid enhancer factor (TCF/LEF) resulting in the activation of oncogenic target genes. Our results indicate that expression of the TCF/LEF transcription factor in this pathway increases with the level of fecal water genotoxicity. This constitutes a carcinogenic risk and could be an important discovery in explaining the red meat intake-associated cancer risk in humans. Moreover, since the WNT signaling pathway is also targeted by p53 [47], our observation of pathways involving p53 regulation suggests a close link between fecal water genotoxicity, DNA damage control by p53, and WNT signaling. We have also previously found modifications in WNT signaling in Caco-2 cells following exposure to an oxidative environment [48], suggesting that oxidative mechanisms may be responsible for the effects seen here. Interestingly, Brookes *et al.* [49] have demonstrated that iron increases WNT signaling. Since iron plays an essential role in mediating the oxidative genotoxicity associated with heme exposure, this is another indication that heme protein plays a role in the effects observed here. In this line, modifications in genes controlling epithelial proliferation and differentiation have been discovered in rats following an increase in dietary heme intake [50]. It could be hypothesized that the gene expression response associated with red meat intake-induced fecal water genotoxicity, is attributable to the heme content of red meat and this would be a worthwhile topic for future research.

Another interesting discovery is the Sin3 and NuRD transcription regulation pathway. Sin3 and NuRD are nucleosome remodeling and histone deacetylase com-

plexes that control transcriptional repression [51,52] and several genes that form part of these complexes, including histone deacetylase 2, were positively correlated with fecal water genotoxicity levels. NuRD complex-induced gene silencing has also been demonstrated in colon cancer cells [53–55]. These modifications point towards an epigenetic mechanism by which red meat could influence important molecular pathways.

The remaining pathways are suggestive of modifications in carbohydrate and lipid metabolism and endocytosis-mediated transport, which could be important given the role of absorption in the colon, but it is not clear what their relevance could be in relation to fecal water genotoxicity.

In summary, we observed a red meat intake-induced increase in fecal water genotoxicity which was not related to fecal NOC content nor influenced by inflammation in the colon. We propose that heme protein is a likely candidate for this increase, especially since fecal water-induced oxidative damage was higher following the intervention. Furthermore, we have identified a large number of pathway modifications related to red meat intake-induced fecal water genotoxicity some of which could contribute to carcinogenic transformations in the human colon. The gene expression changes identified in this study corroborate the genotoxic potential of a diet high in red meat and point towards a possible risk for CRC development in humans.

Acknowledgements

The authors would like to acknowledge Mr. Herman Vermaas for his advice on the meat intervention. Furthermore, we would like to recognize the efforts of the endoscopy department staff at the Orbis Medical Center and the Maastricht University Medical Center and thank the study subjects for their participation. The authors also wish to acknowledge Liliane Jimenez-Van Hoorn, Tammy Oth, and Kevin van Tilburg for their technical assistance.

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Chapter 8

Summary and general discussion

The human colon is exposed to a wide variety of potentially hazardous compounds and often at much higher levels than most other organs. In combination with the high proliferative potential of the colon epithelium, this creates an environment susceptible to mutagenic lesions with a pro-carcinogenic potential which may explain the high colorectal cancer (CRC) incidence rate observed worldwide [1–5]. Identifying compounds that may be responsible for this high cancer incidence is therefore a high priority. N-nitroso compounds (NOCs) have been suggested as possible candidates based on their genotoxicity, mutagenicity, and carcinogenicity in animals [6,7]. Intestinal NOC-levels are also found to be increased in the colon lumen following the consumption of certain foods, such as red meat, as well as in case of inflammation, both well established risk factors in CRC etiology [8–12]. There is, however, very limited proof of a carcinogenic potential of NOCs in humans, although some studies suggest that extrapolation of animal data on NOC-induced genotoxicity, and possibly carcinogenicity, to humans is warranted [13]. In order to evaluate the carcinogenic potential of these compounds in humans, we need to increase our understanding of the molecular processes associated with NOC exposure. Since transcriptomics research has been founded on the paradigm that gene expression profiles are capable of revealing mechanisms of toxicity, gene expression analysis based on microarray technology provides a valuable tool for identifying molecular biomarkers for colon carcinogenesis associated with NOC exposure in humans. A suitable way to investigate this for the colon is to perform *in vitro* and *in vivo* experiments on human colonic cells and tissue, aiming to link phenotypic markers of genotoxicity or carcinogenicity to transcriptomic changes. Therefore, in this thesis, we evaluate the hypothesis that NOCs induce gene expression changes indicating CRC risk, by identifying molecular pathways that are relevant in the carcinogenic process, and that such gene expression profiles and modulated biological processes are associated with actual NOC exposure levels in human study populations. In order to do so, we have examined gene expression changes in human colon cells after NOC exposure, focusing on *in vitro* time-dependent changes in the expression profiles and on the possible involvement of radical intermediates in this response. Subsequently, whether such gene expression responses occur in human subjects in relation to NOC exposure was first investigated in a healthy population, using lymphocytes as a surrogate tissue and urinary NOC excretion as a marker of exposure. Next, transcripomic profiles were established in human colon tissue in relation to fecal NOC concentrations, applying two different study designs that are expected to influence the level of endogenous nitrosation: a comparison between absence and presence of colonic inflammation and before and after increased red meat consumption in a dietary intervention study.

In vitro studies

In order to identify transcriptomic profiles that are indicative of the potential carcinogenic role of NOCs in the human colon, we first performed a series of *in vitro* experiments described in **Chapters 2**, **3**, and **4**. For these experiments the human colon adenocarcinoma cell line Caco-2 was exposed to comparably genotoxic concentrations of six model compounds, i.e. two nitrosamides (MNNG and MNU) and four nitrosamines (NDEA, NDMA, NPIP, and NPYR). Initial experiments showed a large number of gene expression modulations induced after 24 hours exposure to the nitrosamines, which suggest a carcinogenic risk associated with NOC exposure (**Chapter 2**). Most pathway modifications were indicative of a genotoxic effect and potentially important modifications in development and G-protein signaling cascades involved in cell proliferation and differentiation. Pathways and gene groups suggestive of oxidative stress and a radical scavenging response were also identified and underline the ability of these compounds to generate intracellular damage which possibly affects their carcinogenic potency. Oxidative damage following NOC exposure has indeed been described in literature and could contribute to the carcinogenic potential of these compounds, which may be reflected by transcriptomic responses. Radical mechanisms were therefore further investigated in **Chapter 3**, where nitrosamides and nitrosamines were found to generate distinct radical profiles. Although both nitrosamides and nitrosamines generated reactive oxygen species (ROS), these levels were higher during nitrosamine exposure. Furthermore, nitrosamides generated nitrogen-centered radicals, while nitrosamines gave rise to carbon centered radicals. The radicals generated during nitrosamine exposure were all formed via the dealkylating metabolic route which is directly related to genotoxicity through CYP-induced ROS formation and the eventual formation of alkyl-adducts. In contrast, nitrosamides are expected to induce most of their genotoxic effect through alkylation, which is not associated with radical formation as demonstrated by the metabolic scheme in **Chapter 3**. The results thus showed that radical formation between nitrosamines and nitrosamides is very different and may have contributed to the contrasting gene expression profiles observed in **Chapter 2**. A correlation analysis between the observed gene expression modifications and the radical generating properties of these compounds at 1 and 24 hrs of exposure showed that the modified genes were involved in a large number of processes and this gene expression response became more pronounced over time. Modulations in apoptosis, cell cycle control, and DNA repair, can be regarded as a confirmation of the genotoxic potential of ROS generated during NOC metabolism. A large number of pathways involved in development processes, which often control proliferation and differentiation, can also be linked with a possible pro-carcinogenic effect of ROS. The limited number of pathways that were associated with nitrosamide-induced ROS levels was only found at 1 hr of exposure which is an indication that transcriptomic effects induced by nitrosamides occur early in the exposure.

The study on the temporal effects of NOC-induced gene expression responses presented in **Chapter 4** showed that gene expression changes varied over time and that nitrosamides indeed induced the strongest response early in the exposure at 1 hr. The nitrosamines on the other hand showed the strongest response at 6 and 24 hrs, but also caused a considerable transcriptomic effect at 1 hr. Many of the affected pathways and processes were implicated in crucial developmental signaling cascades, such as the WNT, NOTCH, and TGF-β signaling pathways, some of which represent crucial steps in the colorectal cancer model described by Fearon and Vogelstein [14,15] (**Chapter 1**). It thus seems that all investigated NOC are capable of modifying essential developmental processes, albeit at different moments in the exposure period. Such modifications could work in concert with the DNA damaging capabilities of NOC in the carcinogenic process.

Upon correlating levels of O^6-methylguanine induced by the three methylating NOCs (MNNG, MNU, and NDMA) with gene expression modifications it was shown that DNA-methylation was also associated with relevant molecular changes which may represent an epigenetic component in the gene expression response, such as histon methylation. These modifications were not only related to processes directly associated with their genotoxic properties, such as DNA damage, cell cycle control, and apoptosis regulation, but also with development processes that may contribute to the carcinogenic risk through JAK-STAT and RAS signaling [16–18] (**Chapter 4**). In combination with the processes identified in **Chapter 3** for NOC-induced oxidative stress, these findings indicate that both oxidative and methylating mechanisms are associated with gene expression modulations that may play a role in the carcinogenic process.

Overall, **Chapters 2**, **3**, and **4** provide evidence that NOCs induce molecular mechanisms which may represent a CRC risk in humans. Modified mechanisms indicative of NOC-induced genotoxicity and alterations in proliferation and differentiation are especially important in this risk. If such modifications also occur in intestinal cells following *in vivo* NOC exposure, this may contribute to the carcinogenic process by offering a growth advantage [19,20]. The consequences of these modifications may be greatest in self-renewing stem cell populations in the colon crypts. Stem cells have been suggested to be the driving force behind tumor growth and an increase in genotoxicity in parallel with an altered rate of proliferation and differentiation may be most relevant in these cells with regard to colon carcinogenesis [21].

In vivo studies

Although the genotoxicity and gene expression modifications found in the *in vitro* model point towards a possible carcinogenic risk, neither can be unequivocally associated with human carcinogenicity. The human *in vivo* studies described in this thesis therefore aimed to establish transcriptomic profiles that are related to NOC

exposure, and to evaluate whether or not these gene expression patterns indicate the same or similar molecular mechanisms relevant in carcinogenesis as found earlier in the *in vitro* studies. This was investigated in human lymphocytes as a surrogate tissue (**Chapter 5**) and the human colon as a target tissue (**Chapters 6** and **7**) in relation to NOC exposure.

In the first *in vivo* study, described in **Chapter 5**, the relationship between human NOC exposure and micronuclei (MN) formation as a validated marker of carcinogenic risk [22], was investigated in relation to associated gene expression changes in lymphocytes. This study involved human subjects at normal levels of endogenous nitrosation and can be regarded as representative for the general population. Using urinary excretion of nitrosamines as a marker of NOC exposure, a significant positive correlation was found between urinary excretion of NDMA and lymphocytic MN formation. Since lymphocytic MN represent a validated biomarker of cancer risk in humans, this is a first indication that NOC exposure under normal circumstances may play a role in human cancer development. Although the whole blood transcriptomic response associated with urinary NOC excretion and MN frequency only showed a small degree of similarity with the *in vitro* studies, the modifications that were identified were also related to the carcinogenic process. Molecular pathways associated with urinary NOC excretion indicated a potentially genotoxic response, as also reported in **Chapters 2** and **4**, since several pathways involved in apoptosis, cell cycle, and cytoskeleton remodeling were found. In addition, several protein kinase A (PKA) and cAMP response element-binding (CREB) signaling pathways were identified which were also detected after a 1 hr exposure to a genotoxic concentration of NDMA in **Chapter 4**. These pathways were also found to be associated with lymphocytic MN frequency. This suggests that the PKA and CREB signaling pathways form a common intermediate in the link between NOC exposure and MN. This was further explored by screening the gene lists associated with urinary NOC excretion and MN frequency for overlap with a previously established list of genes involved in MN formation [23]. A network analysis on the overlapping transcripts in these lists led to the compilation of a small set of genes that could be suitable as mechanistic-based transcriptomic biomarkers of NOC exposure as a predictor of carcinogenic risk.

We subsequently investigated the colon as a direct target tissue. For that, we defined two different study designs based on either inflammation stimulated nitrosation, i.e., in patients with inflammatory bowel disease (IBD), and on heme-iron stimulated nitrosation following an increased dietary intake of red meat. In the study described in **Chapter 6**, differences in nitrosation capacity and transcriptomic changes in colon biopsies were compared between IBD patients and control patients without inflammation. Strong differences in gene expression profile were observed between both groups, mostly involving modulations with pro-inflammatory outcomes and regulation of JAK/STAT signaling in IBD patients, but

this was not associated with an increased fecal NOC content as a marker of endogenous exposure. Despite the lack of evidence for strongly stimulated nitrosation as a result of the inflammatory conditions, individual fecal NOC levels were found to correlate with several molecular pathways and processes many of which related to the carcinogenic process. As found in **Chapter 5**, the overall transcriptomic response showed only a small degree of similarity with responses observed the *in vitro* studies, but results were consistent with a possibly carcinogenic effect of NOCs in this target tissue. Several pathways that signal a response to genotoxicity were identified such as activation of DNA repair and some minor modifications in pathways that could influence the cell cycle and apoptosis. The most interesting result might be the discovery of several cellular GO processes involved in chromatin organization which contained a large number of histone genes positively correlated with fecal NOC levels. It is hypothesized that epigenetic mechanisms play a role in the NOC-induced gene expression response and there is some evidence to support this [24,25]. Given the established role of epigenetic modifications in chromatin in the development of cancer [26], this could be very relevant for NOC-associated CRC risk in humans.

The stimulatory effect of red meat consumption on endogenous nitrosation forms the basis for our second model for investigating NOC exposure-associated gene expression changes in the human colon [27–30]. The study presented in **Chapter 7**, describes the impact of dietary red meat intake on endogenous NOC formation and fecal water genotoxicity in relation to transcriptomic changes induced in colonic biopsy tissue from human subjects. Increased meat consumption (300 g per day for 7 days) was not found to result in an increased fecal NOC formation. Although the intake of meat was significantly higher in our subjects after the intervention, as demonstrated by animal protein intake reported in the food diaries, the difference may not have been large enough to find an increase in endogenous nitrosation. Despite the absence of an increased NOC formation, the red meat intervention did result in a significantly increased fecal water genotoxicity, which was also found to correlate with a large number of pathway modifications in the colon tissue. Some of these pathways were indicative of genotoxic effects, and included modifications in DNA damage control, cell cycle regulation, apoptosis and stress fiber formation. This shows that the genotoxicity of the fecal water is reflected by gene expression modifications in response to a genotoxic burden. Since genotoxicity levels induced by fecal water were accompanied by an increase in oxidative damage after the intervention, as measured by the formamidopyrimidine DNA glycosylase comet assay, this suggests that heme protein in meat could have been responsible. Heme has well known oxidative properties which can damage DNA, proteins, and lipids [31,32]. Moreover, dietary heme has also been associated with an increased CRC risk [8,33–37]. The most important pathway modifications discovered in the transcriptomic analysis with regard to colon carcinogenesis were related to WNT

signaling and nucleosome remodeling. As described in the genetic model for colorectal tumorigenesis proposed by Fearon and Vogelstein (**Chapter 1)**, the WNT pathway is strongly involved in CRC development [14,15,21,38,39]. The downstream transcription factor target T-cell factor/lymphoid enhancer factor (TCF/LEF) was found to be positively correlated with fecal water genotoxicity. TCF/LEF activates several oncogenic target genes, including c-Myc, c-Jun/Fra-1, and cyclin D1 [40]. Promotion of the WNT signaling pathway by fecal water may thus lead to stimulation of cell division in an environment which is subject to an increased rate of DNA lesions as a result of the genotoxicity of the fecal matter. So despite the absence of a relation between NOCs and red meat consumption, the latter is linked with transcriptomic modifications that support its association with human CRC.

The *in vivo* studies thus indicate that human NOC exposure modulates molecular pathways involved in CRC although this could not be associated with the consumption of red meat. The degree of similarity between the *in vitro* and *in vivo* transcriptomic responses was, however, limited since the *in vitro* exposures resulted in much more pronounced transcriptomic effects. This indicates that the *in vitro* gene expression profiles are less suitable for predicting responses *in vivo* and stresses the importance of linking *in vivo* transcriptomic responses to markers of NOC exposure in order to more accurately assess carcinogenic risks based on gene expression data. This is not entirely unexpected since the *in vitro* experiments were carried out at rather high incubation concentrations which are unlikely to occur in a physiological situation. Furthermore, although Caco-2 cells display numerous morphological and biochemical characteristics of enterocytes [41], cell lines are a very simple model representation of a much more complex target organ and differ from normal human tissue with regard to genetic makeup and possibly CYP activity. Although this complicates the extrapolation of *in vitro* results to an *in vivo* situation, the consistency of particular transcriptomic responses indicative of a carcinogenic effect, clearly is of interest.

Recommendations for future research

The studies described in **Chapters 6** and **7** were designed to investigate transcriptomic changes in populations theorized to display increased nitrosation levels. However, no increased nitrosation was observed which may have been due to a too high level of variation inherent to the study design since previous more strictly controlled red meat intervention studies reported significant increases in fecal NOC formation following similar daily amounts of meat [42–46]. Nevertheless, the *in vivo* results show that NOC exposure-associated transcriptomic effects could be relevant in human carcinogenesis. It can therefore still be assumed that relatively high exposures constitute a higher risk. Additional studies are thus recommended to investigate a pro-nitrosation model in a more controlled fashion. Since red meat consump-

tion in healthy subjects has been most consistently found to stimulate nitrosation [27,42–49], a strictly controlled study based on a crossover design that switches from low red meat to high red meat diets is most likely to result in significantly increased levels of fecal NOC content. NOC exposure-associated gene expression modifications in colon tissue biopsies should then be present even stronger. Fecal water genotoxicity by comet assay and fecal heme content could also be determined to see whether heme protein is indeed responsible for the increase in genotoxicity observed in **Chapter 7**. Genotoxicity induced by NOC exposure could be investigated by determining alkylation levels in colon biopsy DNA. As described in **Chapter 1**, both O^6-methylguanine and N^7-methylguanine are interesting adducts to measure. Although these adducts only represent damage induced by methylating NOC, methyl adducts are one of the most likely types of adducts to be formed because they have been detected *in vivo* in many studies [13,50–54]. Moreover, NDMA, which is a methylating NOC, is one of the most frequently detected NOCs in feces and has been suggested to be a likely candidate to explain the DNA methylation adduct levels detected in blood from normal populations [9,13,55–57]. Detection of these adducts in human colon biopsy material in relation to NOC exposure would link NOC exposure even more strongly with carcinogenesis in the colon.

Measurement of alkyl adducts in lymphocytes, in addition to MN frequency, is another interesting topic for future research. Alkyl adducts may display a correlation with NOC exposure levels, similar to what was found in **Chapter 5** for MN formation. Such a relation would make these adducts an interesting biomarker, especially when associated with an increased cancer risk. Further validation of the small gene network identified in **Chapter 5** with the potential of serving as a mechanistic-based transcriptomic biomarkers of NOC exposure in relation to MN formation, would then also be possible. This could be coupled with a strictly controlled red meat intervention as suggested above.

Specific dietary phytochemicals have previously been shown to inhibit nitrosamine-induced damage *in vitro* [58,59]. The radical generating properties of NOCs demonstrated in **Chapter 2**, suggest that anti-oxidant molecules could exert their protective effect during NOC metabolism and this would also be worthwhile investigating. Likewise, anti-oxidant supplementation with red meat could affect fecal water genotoxicity levels and possibly counteract some of the pro-carcinogenic gene expression modifications described in **Chapter 7**. It has previously been demonstrated that increased vegetable intake results in modulation of genes with a cancer preventive potential [60]. Combining such a study approach with an investigation into the pro-oxidative effects of heme could result in important insights in red meat intake associated CRC etiology and new possibilities for prevention strategies.

Lastly, the transcriptomic changes regarding chromatin/nucleosome remodeling described in **Chapters 6** and **7** are suggestive of an epigenetic component in gene

expression regulation induced by NOCs and red meat. This could be of importance in carcinogenic pathways and further investigation of these epigenetic mechanisms could lead to the identification of molecular targets for CRC prevention.

Overall conclusions

Both the *in vitro* and the *in vivo* studies described in this thesis support the hypothesis that NOCs influence cellular processes involved in human CRC development. The newly generated overview of NOC-induced gene expression modulations presented in the *in vitro* studies already provides persuading evidence that NOC exposure, either through DNA alkylation or ROS formation, may influence crucial carcinogenic pathways. The most convincing evidence in support of our hypothesis comes from the *in vivo* studies where NOC exposure at physiologically relevant levels is associated with MN formation in lymphocytes and gene expression modulations in both lymphocytes and colon tissue. This does not only reflect the genotoxicity of these compounds but also epigenetic mechanisms-of-action for NOCs are suggested. The CRC risk associated with red meat consumption is supported – although not by increased colonic NOC formation – by an increased fecal water genotoxicity which is linked with several pro-carcinogenic changes in molecular pathways.

The research presented here is the first transcriptomics-based investigation into the role of NOCs in the development of cancer and the results are consistent with the hypothesis that NOCs represent a carcinogenic risk to humans. Although additional research is required to understand the extent of this risk, it may be advised to limit exposure to NOCs by avoiding the regular consumption of foods or combinations of food that are likely to increase endogenous nitrosation. Furthermore, although, based on lack of increased nitrosation in our intervention study, this would not necessarily include red meat, it still seems advisable to limit red meat intake in view of its effects on crucial molecular pathways involved in human colorectal carcinogenesis.

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Samenvatting en algemene discussie

Het menselijk colon (de dikke darm) wordt dagelijks blootgesteld aan een breed scala aan potentieel toxische stoffen en vaak in veel hogere concentraties dan de meeste andere organen. In combinatie met de hoge proliferatie van het colon epitheel, creëert dit een vatbare omgeving voor mutagene schade met een procarcinogeen potentieel wat mogelijk de hoge wereldwijde colonkanker incidentie kan verklaren [1-5]. Het identificeren van verbindingen die verantwoordelijk kunnen zijn voor deze hoge incidentie van kanker is daarom een hoge prioriteit. N-nitroso verbindingen (NOC's) zijn geopperd als mogelijke kandidaten op basis van hun genotoxiciteit, mutageniteit en carcinogeniteit in proefdieren [6,7]. Daarnaast zijn er aanwijzingen dat intestinale NOC niveaus stijgen in het colon lumen na de consumptie van bepaalde voedingsmiddelen, zoals rood vlees, evenals in aanwezigheid van een ontsteking, beide risicofactoren voor het ontstaan van colonkanker [8-12]. Er is echter nauwelijks direct bewijs voor een carcinogeen effect van NOC's in de mens, hoewel sommige onderzoekers stellen dat een extrapolatie van gegevens in proefdierstudies over NOC-geïnduceerde genotoxiciteit, en mogelijk carcinogeniteit, toegestaan is [13]. Om het carcinogene vermogen van deze verbindingen in de mens te kunnen evalueren, hebben we een beter begrip van de moleculaire processen die geassocieerd zijn met NOC blootstelling nodig. Transcriptomics onderzoek is gebaseerd op het paradigma dat genexpressie profielen in staat zijn mechanismen van toxiciteit te verduidelijken. Genexpressie analyse op basis van microarraytechnologie biedt daarom een waardevol instrument voor het identificeren van moleculaire biomarkers voor colonkanker in relatie tot NOC blootstelling in de mens. Dit kan in het colon onderzocht worden door het uitvoeren van *in vitro* en *in vivo* experimenten op humane coloncellen en -weefsels, met als doel om fenotypische markers van genotoxiciteit of carcinogeniteit te associëren met veranderingen in het transcriptoom. Daarom is in dit proefschrift de hypothese geëvalueerd dat NOC-geïnduceerde genexpressie veranderingen indicatief zijn voor een colonkanker risico, door het identificeren van moleculaire pathways die relevant zijn in het carcinogene proces, en door deze genexpressie profielen en gemoduleerde biologische processen in verband te brengen met NOC blootstellingsniveaus in de mens. Hiertoe hebben we genexpressie veranderingen in menselijke darmcellen onderzocht na blootstelling aan NOC's, met nadruk op de *in vitro* tijdsafhankelijke veranderingen in de expressie profielen en de mogelijke betrokkenheid van radicale intermediairen in deze respons. Vervolgens werd gekeken of deze genexpressie veranderingen ook optraden bij menselijke proefpersonen in relatie tot NOC blootstelling. Dit werd eerst onderzocht in een gezonde populatie met behulp van lymfocyten als surrogaat weefsel en urinaire NOC excretie als een marker voor blootstelling. Vervolgens werden transcriptoom profielen vastgesteld in humaan colonweefsel in relatie tot fecale NOC concentraties, door toepassing van twee verschillende studiedesigns die mogelijk het niveau van endogene nitrosatie beïnvloeden: een design gebaseerd op de vergelijking tussen afwezigheid en aanwezigheid van ontsteking in het colon en

een voedingsinterventie design gericht op het effect van een verhoogde consumptie van rood vlees.

In vitro studies

Met het oog op het identificeren van transcriptoom profielen die indicatief zijn voor het potentiële carcinogene effect van NOC's in het menselijk colon, werd eerst een reeks *in vitro* experimenten uitgevoerd, beschreven in **Hoofdstuk 2**, **3** en **4**. In deze experimenten werd de humane colonadenocarcinoom cellijn Caco-2 blootgesteld aan vergelijkbaar genotoxische concentraties van zes modelverbindingen, namelijk twee nitrosamides (MNNG en MNU) en vier nitrosamines (NDEA, NDMA, NPIP en NPYR). De eerste experimenten resulteerden in de inductie van een groot aantal genexpressie modulaties na een 24-uurs blootstelling aan nitrosamines, die wijzen op een mogelijk carcinogeen risico geassocieerd met NOC blootstelling (**Hoofdstuk 2**). De meeste pathway modificaties waren indicatief voor een genotoxische respons en vertoonden potentieel belangrijke veranderingen in celontwikkeling en G-eiwit signaleringscascades die betrokken zijn bij celproliferatie en differentiatie. Pathways en gengroepen die een oxidatieve stress en radicaal “scavenging” respons indiceren werden ook geïdentificeerd en onderstrepen het vermogen van deze verbindingen om intracellulaire schade te genereren die eventueel van invloed is op hun kanker-verwekkend vermogen. Oxidatieve schade als gevolg van blootstelling aan NOC’s is inderdaad eerder in de literatuur beschreven en kan bijdragen aan het carcinogene effect van deze verbindingen, wat mogelijk wordt weerspiegeld door de transcriptoom respons. Radicaal mechanismen werden daarom verder onderzocht in **Hoofdstuk 3**, waar nitrosamides en nitrosamines verschillende radicaal profielen bleken te genereren. Hoewel zowel nitrosamides als nitrosamines reactieve zuurstof species (ROS) genereerden, waren deze niveaus hoger tijdens nitrosamine blootstelling. Bovendien genereerden nitrosamides stikstof-gecentreerde radicalen, terwijl nitrosamines koolstof-gecentreerde radicalen produceerden. De radicalen die ontstonden tijdens nitrosamine blootstelling konden allemaal geïdentificeerd worden als zijnde afkomstig uit de dealkylerende metabole route die rechtstreeks verband houden met genotoxiciteit via cytochroom P450 (CYP)-geïnduceerde ROS vorming en de uiteindelijke vorming van alkyl-adducten. Van nitrosamides daarentegen wordt verondersteld dat het grootste deel van het genotoxische effect wordt geïnduceerd door middel van alkylering, wat niet is geassocieerd met radicaalvorming zoals is af te leiden uit het metabole schema in **Hoofdstuk 3**. Uit de resultaten blijkt dus dat radicaalvorming tussen nitrosamines en nitrosamides sterk verschilt, wat bijgedragen kan hebben aan de contrasterende genexpressie profielen waargenomen in **Hoofdstuk 2**. Een correlatie analyse tussen de waargenomen genexpressie wijzigingen en de radicaal-genererende eigenschappen van deze verbindingen na een 1 en 24-uurs blootstelling, liet zien dat de gemodificeerde genen waren betrok-

ken bij een groot aantal processen en dat deze genexpressie respons zich verder ontwikkelde over de tijd. Modulaties in apoptose, celcyclus regulatie, en DNA herstel, kan beschouwd worden als een bevestiging van de genotoxische effecten van ROS gegenereerd tijdens NOC metabolisme. Een groot aantal pathways die betrokken zijn bij ontwikkelingsprocessen, die vaak proliferatie en differentiatie beïnvloeden, kan ook gekoppeld worden aan een mogelijk pro-carcinogene werking van ROS. Het beperkte aantal pathways dat geassocieerd was met nitrosamide-geïnduceerde ROS niveaus werd alleen gevonden bij een blootstelling van 1 uur, wat een indicatie is dat de transcriptoom effecten in het begin van de blootstelling worden geïnduceerd.

Uit de studie over de tijdsafhankelijke effecten van de NOC-geïnduceerde genexpressie respons in **Hoofdstuk 4** is gebleken dat de genexpressie veranderingen variëren over de tijd en dat nitrosamides inderdaad de sterkste respons induceren in het begin van de blootstelling na 1 uur. De nitrosamines daarentegen vertoonden de sterkste reactie na 6 en 24 uur, maar resulteerden ook in een aanzienlijke effect op het transcriptoom na 1 uur. Veel van de gemodificeerde pathways en processen waren betrokken bij cruciale ontwikkeling signaleringscascades, zoals de WNT, NOTCH, en TGF-β signaleringspathways. Sommige van deze pathways representeren cruciale stappen in het colorectale kanker model beschreven door Fearon en Vogelstein [14,15] (**Hoofdstuk 1**). Het lijkt er dus op dat alle onderzochte NOC's in staat zijn tot het wijzigen van essentiële ontwikkelingsprocessen, zij het op verschillende momenten in de blootstellingperiode. Dergelijke wijzigingen kunnen bijdragen aan de DNA-beschadigende eigenschappen van NOC's in het carcinogene proces.

Na correlatie van de O^6-methylguanine niveaus, geïnduceerd door de drie methylerende NOC (MNNG, MNU en NDMA), met genexpressie wijzigingen bleek dat DNA-methylatie ook was geassocieerd met relevante moleculaire veranderingen die een epigenetische oorzaak kunnen vertegenwoordigen in de genexpressie reactie, waaronder histon methylatie. De geïdentificeerde genexpressie wijzigingen waren zowel gerelateerd aan processen die rechtstreeks verband houden met hun genotoxische eigenschappen, zoals DNA-schade, celcyclus regulatie en apoptose, als aan ontwikkelingprocessen die kunnen bijdragen aan het risico op kanker door middel van JAK-STAT en RAS signalering [16-18] (**Hoofdstuk 4**). In combinatie met de processen die in **Hoofdstuk 3** werden geïdentificeerd voor NOC-geïnduceerde oxidatieve stress, geven deze bevindingen aan dat zowel oxidatieve als methylerende mechanismen zijn geassocieerd met genexpressie modulaties die een rol kunnen spelen in het carcinogene proces.

Samengevat, leveren **Hoofdstuk 2**, **3** en **4** bewijs dat NOC's moleculaire mechanismen beïnvloeden die mogelijk een colonkanker risico voor de mens met zich meebrengen. Modificaties in mechanismen die indicatief zijn voor NOC-geïnduceerde genotoxiciteit en veranderingen in proliferatie en differentiatie zijn bijzonder belangrijk in dit risico. Indien dergelijke wijzigingen ook ontstaan in colon-

cellen in reactie op *in vivo* NOC blootstelling, kan dit aan het carcinogene proces bijdragen door het bieden van een groeivoordeel [19,20]. De gevolgen van deze wijzigingen zouden het grootst kunnen zijn in de zelfvernieuwende stamcelpopulaties in de coloncrypten. Het is gesuggereerd dat stamcellen de drijvende kracht achter de groei van tumoren zijn en een toename van genotoxiciteit parallel met een veranderde proliferatiesnelheid en differentiatie kan het meest relevant in deze cellen zijn met betrekking tot colonkanker [21].

In vivo studies

Hoewel de genotoxiciteit en genexpressie wijzigingen gevonden in het *in vitro* model wijzen op een mogelijk carcinogeen risico, kan dit niet één-op-één worden vertaald naar humane carcinogeniteit. De humane *in vivo* studies beschreven in dit proefschrift waren daarom gericht op het vaststellen van transcriptoom profielen die gerelateerd zijn aan NOC blootstelling, en om te beoordelen of deze genexpressie patronen indicatief zijn voor dezelfde of vergelijkbare moleculaire mechanismen betrokken bij carcinogenese als eerder gevonden in de *in vitro* studies. Dit werd onderzocht in humane lymfocyten als een surrogaat weefsel (**Hoofdstuk 5**) en het menselijke colon als een doelorgaan (**Hoofdstuk 6** en **7**) in relatie tot NOC blootstelling.

In de eerste *in vivo* studie, beschreven in **Hoofdstuk 5**, is de relatie onderzocht tussen menselijke NOC blootstelling en micronuclei (MN) vorming, als een gevalideerde marker van kankerrisico [22], in relatie tot genexpressie wijzigingen in lymfocyten. In deze studie werden proefpersonen onderzocht met een normaal niveau van endogene nitrosering, die dus beschouwd kunnen worden als representatief voor de algemene populatie. Gebruikmakend van urinaire uitscheiding van nitrosamines als een marker voor NOC blootstelling, werd een significante positieve correlatie gevonden tussen de urinaire excretie van NDMA en lymfocytaire MN vorming. Aangezien lymfocytaire MN een gevalideerde biomarker vertegenwoordigen voor kankerrisico in de mens, is dit een eerste indicatie dat NOC blootstelling onder normale omstandigheden een rol zou kunnen spelen in het humane kankerproces. Hoewel de volbloed transcriptoom respons geassocieerd met urinaire NOC excretie en MN frequentie slechts een geringe mate van overlap met de *in vitro* studies vertoonde, waren de veranderde genexpressie profielen ook gerelateerd aan het carcinogene proces. Moleculaire pathways geassocieerd met urinaire NOC excretie zijn indicatief voor een potentiële genotoxische respons, zoals ook beschreven in **Hoofdstuk 2** en **4**, aangezien meerdere pathways werden gevonden die betrokken zijn bij apoptose, celcyclus, en cytoskelet modellering. Daarnaast werden verschillende proteïne kinase A (PKA) en “cAMP respons element-binding” (CREB) pathways geïdentificeerd die ook werden aangetroffen na een 1-uurs blootstelling aan een genotoxische concentratie NDMA in **Hoofdstuk 4**. Deze pathways waren ook geas-

socieerd met lymfocytaire MN frequentie. Dit suggereert dat de PKA en CREB signaalwegen een gemeenschappelijke intermediair vormen in de relatie tussen NOC blootstelling en MN. Dit werd verder onderzocht door het screenen van de genlijsten geassocieerd met urinaire NOC excretie en MN frequentie voor overlap met een eerder samengestelde lijst van genen die betrokken zijn bij MN vorming [23]. Een netwerk analyse van de overlappende transcripten in deze lijsten leidde tot de compilatie van een kleine set genen die geschikt zou kunnen zijn als mechanisme gebaseerde transcriptoom biomarker voor NOC blootstelling als een voorspeller van carcinogeen risico.

Vervolgens werd het colon onderzocht als een direct NOC doelwit weefsel. Daarvoor werden twee verschillende studieopzetten gedefinieerd gebaseerd op ontsteking-gestimuleerde nitrosering, bij patiënten met chronische darmontsteking (IBD), en op heemijzer-gestimuleerde nitrosering door een verhoogde inname van rood vlees. In de studie beschreven in **Hoofdstuk 6**, werden de verschillen in nitroseringscapaciteit en transcriptoom veranderingen in colonbiopten vergeleken tussen IBD-patiënten en controle patiënten zonder ontsteking. Er werden sterke verschillen in genexpressieprofiel waargenomen tussen beide groepen, wat veelal modulaties betrof met een pro-inflammatoir effect en regulering van JAK/STAT-signalering bij IBD patiënten, maar dit was niet geassocieerd met een verhoogd gehalte aan fecale NOC's als een marker voor endogene blootstelling. Ondanks het ontbreken van een sterk gestimuleerde nitrosering als gevolg van inflammatie, bleken individuele fecale NOC niveaus gecorreleerd te zijn met verschillende moleculaire pathways en processen waarvan veel gerelateerd waren aan het carcinogene proces. Vergelijkbaar met wat werd gevonden in **Hoofdstuk 5**, vertoonde de totale transcriptoom respons slechts een geringe mate van gelijkenis met de waargenomen respons in de *in vitro* studies, maar de resultaten waren in overeenstemming met een mogelijk carcinogeen effect van NOC's in dit doelweefsel. Verschillende pathways die een reactie op genotoxiciteit impliceren werden geïdentificeerd, zoals de activering van DNA herstel en enkele kleine wijzigingen in pathways die de celcyclus en apoptose kunnen beïnvloeden. Een van de meest interessante resultaten was de ontdekking van verschillende cellulaire Gene Ontology processen die betrokken zijn bij chromatine-organisatie waarin zich een groot aantal histon genen bevindt die positief gecorreleerd zijn met fecale NOC niveaus. Op basis hiervan kan gehypothetiseerd worden dat epigenetische mechanismen een rol spelen bij de NOC-geïnduceerde genexpressie respons, iets dat ook door enig bewijs in de literatuur wordt ondersteund [24,25]. Gezien de rol van epigenetische modificaties in chromatine in de ontwikkeling van kanker [26], kan dit zeer relevant zijn voor een NOC-geassocieerd colonkanker risico in de mens.

De studie in **Hoofdstuk 7** beschrijft de invloed van rood vlees inname op de endogene vorming van NOC's en fecale water genotoxiciteit in relatie tot transcriptoom veranderingen in colonbiopsie weefsel van proefpersonen. Een toename in

rood vleesconsumptie (300 g per dag gedurende 7 dagen) bleek niet te leiden tot een verhoogde fecale NOC vorming. Hoewel de inname van vlees significant hoger was na de interventie, zoals ook blijkt uit de dierlijke eiwitinname gerapporteerd in de voedingsdagboeken, is het verschil mogelijk niet groot genoeg geweest om te leiden tot een verhoogde endogene nitrosering. Ondanks de afwezigheid van een verhoogde NOC vorming, leidde de rood vleesinterventie wel tot een significant verhoogde fecale water genotoxiciteit, die ook gerelateerd bleek te zijn met een groot aantal pathway modificaties in het colonweefsel. Sommige van deze pathways waren indicatief voor genotoxische effecten, en betroffen modificaties in de DNA schade respons, regulatie van de celcyclus, apoptose en "stress fiber" formatie. Dit wijst er op dat de genotoxiciteit van het fecale water wordt weerspiegeld door genexpressie wijzigingen in reactie op een genotoxische belasting. Aangezien het door fecaal water geïnduceerde genotoxiciteitsniveau gepaard ging met een toename van oxidatieve schade na de interventie, zoals gemeten in de formamidopyrimidine DNA glycosylase comet assay, suggereert dit dat heemeiwit in vlees hiervoor verantwoordelijk kan zijn geweest. Bovendien is heem in de voeding ook in verband gebracht met een verhoogd risico op colonkanker [8,33-37]. De belangrijkste aan colonkanker gerelateerde gemodificeerde pathways ontdekt in de genexpressie analyse waren Wnt signalering en nucleosoom modellering. Zoals beschreven in het genetische model voor colorectale tumorvorming, ontwikkeld door Fearon en Vogelstein (**Hoofdstuk 1**), is de Wnt-pathway sterk betrokken bij colonkanker ontwikkeling [14,15,21,38,39]. Het downstream-transcriptiefactor doelwit "T-cel factor/lymfoïde enhancer factor" (TCF/LEF) bleek positief te zijn gecorreleerd met fecale water genotoxiciteit. TCF/LEF activeert verschillende oncogene doelwitgenen, waaronder c-myc, c-Jun/Fra-1, en cycline D1 [40]. Ondanks het ontbreken van een relatie tussen NOC's en rood vleesconsumptie is de laatste dus wel verbonden met transcriptoom wijzigingen die een associatie met humaan colonkanker ondersteunen.

De *in vivo* studies laten dus zien dat NOC blootstelling in mensen leidt tot modulatie van moleculaire pathways die betrokken zijn bij colonkanker, hoewel deze blootstelling niet kon worden geassocieerd met de consumptie van rood vlees. De mate van overeenstemming tussen de *in vitro* en *in vivo* transcriptoom reacties was echter beperkt aangezien de *in vitro* blootstelling resulteerde in een veel meer uitgesproken transcriptoom effect. Dit is een indicatie dat *in vitro* genexpressie profielen minder geschikt zijn voor het voorspellen van de *in vivo* respons en benadrukt daarnaast het belang van het koppelen van een *in vivo* transcriptoom respons aan markers van NOC blootstelling om nauwkeuriger carcinogene risico's op basis van genexpressie data te bepalen. Dit is niet geheel onverwacht, aangezien de *in vitro* experimenten werden uitgevoerd bij vrij hoge incubatieconcentraties waarvan het onwaarschijnlijk is dat ze in fysiologische situaties bereikt worden. Daarnaast dient vermeld te worden dat, hoewel Caco-2 cellen tal van morfologische en biochemi-

sche kenmerken van enterocyten vertonen [41], cellijnen een zeer eenvoudig model representeren van een veel complexer doelorgaan en afwijken van normale menselijke weefsels met betrekking tot de genetische samenstelling en mogelijk CYP activiteit. Hoewel dit de extrapolatie van *in vitro* resultaten naar een *in vivo* situatie bemoeilijkt, is de consistente waarneming van transcriptoom responsen die indicatief zijn voor een carcinogeen effect, duidelijk van belang.

Aanbevelingen voor toekomstig onderzoek

De studies beschreven in **Hoofdstuk 6** en **7** waren ontworpen om veranderingen in het transcriptoom te onderzoeken in populaties die in theorie een verhoogde nitrosering zouden kunnen vertonen. Er werd echter geen verhoogde nitrosering waargenomen wat te wijten kan zijn aan een te hoge mate van variatie die inherent is aan het studiedesign aangezien eerdere strikter gecontroleerde rood vlees interventiestudies wel een significante stijging van de fecale NOC vorming rapporteerden bij vergelijkbare dagelijkse vleesinname hoeveelheden [42-46]. Desalniettemin tonen de *in vivo* resultaten aan dat transcriptoom effecten geassocieerd met NOC blootstelling relevant kunnen zijn in de humane carcinogenese. Het kan dus nog steeds worden aangenomen dat een relatief hoge vleesinname het risico op colonkanker verhoogt. Additionele studies zijn daarom aan te raden om een pro-nitroseringsmodel te onderzoeken op een meer gecontroleerde wijze. Aangezien rood vlees consumptie in gezonde proefpersonen het meest consistent in verband is gebracht met nitrosering [27,42-49], zou een strikt gecontroleerde studie in deze populatie op basis van een cross-over design dat van een laag rood vlees dieet overschakelt op een hoog rood vlees dieet het meest waarschijnlijk moeten leiden tot aanzienlijk hogere fecale NOC niveaus. NOC blootstelling-geassocieerde genexpressie veranderingen in colonbiopten zouden dan nog sterker aanwezig moeten zijn. Fecale water genotoxiciteit en fecale heem concentraties kunnen dan ook bepaald worden om vast te stellen of heemeiwit inderdaad verantwoordelijk is voor de stijging van de genotoxiciteit waargenomen in **Hoofdstuk 7**. Genotoxiciteit geïnduceerd door NOC blootstelling kan worden onderzocht door het bepalen van alkyleringsniveaus in colonbiopt DNA. Zoals beschreven in **Hoofdstuk 1**, zijn zowel O^6-methylguanine als N^7-methylguanine relevante adducten om te meten. Hoewel deze adducten slechts de schade geïnduceerd door methylerende NOC representeren, behoren methyl-adducten tot de meest waarschijnlijk gevormde adducten aangezien ze in veel *in vivo* studies gedetecteerd zijn [13,50-54]. Bovendien is NDMA een van de meest voorkomende NOC's in feces en is deze methylerende nitrosamine waarschijnlijk verantwoordelijk voor methyl-adduct niveaus in witte bloedcellen van normale populaties [9,13,55-57]. Detectie van deze adducten in humaan colonbiopt materiaal in relatie tot NOC blootstelling zou NOC's nog sterker in verband kunnen brengen met de coloncarcinogenese.

Alkyl-adducten zouden ook een correlatie kunnen vertonen met NOC blootstellingsniveaus, vergelijkbaar met de correlatie die werd gevonden in **Hoofdstuk 5** met MN formatie, en is daarom een ander relevant onderwerp voor toekomstig onderzoek. Een dergelijke relatie zou van deze adducten een interessante biomarker maken, vooral wanneer deze geassocieerd is met een verhoogd risico op kanker. Verdere validatie van het genen netwerk geïdentificeerd in **Hoofdstuk 5** zou dan ook mogelijk zijn waardoor de bruikbaarheid van dit netwerk als een mechanisme gebaseerde transcriptome biomarker van NOC blootstelling wordt verduidelijkt. Dit kan worden gekoppeld aan een strikt gecontroleerde rood vlees interventie zoals hierboven voorgesteld.

Van specifieke voedinggerelateerde phytochemicaliën is eerder *in vitro* aangetoond dat ze nitrosamine-geïnduceerde schade remmen [58,59]. De radicaalgenererende eigenschappen van NOC's, zoals beschreven in **Hoofdstuk 2**, suggereren dat anti-oxidant moleculen hun beschermende werking tijdens NOC metabolisme zouden kunnen uitoefenen en dit is de moeite waard om te onderzoeken. Anti-oxidant toevoeging aan rood vlees zou ook van invloed kunnen zijn op de fecale water genotoxiciteit en mogelijk ook een aantal van de in **Hoofdstuk 7** beschreven pro-carcinogene genexpressie veranderingen kunnen tegengaan. Het is eerder aangetoond dat een verhoogde groente-inname resulteert in een kanker-preventieve modulatie van genexpressie [60]. Het combineren van een dergelijk studiedesign met een onderzoek naar de pro-oxidatieve effecten van heem kan leiden tot belangrijke inzichten in rood vleesinname gerelateerde colonkanker etiologie en zicht geven op nieuwe preventieve strategieën.

Tot slot wijzen de transcriptoom wijzigingen met betrekking tot chromatine/nucleosoom modellering, beschreven in **Hoofdstuk 6** en **7**, op een epigenetische component in de genexpressieregulatie geïnduceerd door NOC's en rood vlees. Dit kan van belang zijn in carcinogene pathways en verder onderzoek naar dergelijke epigenetische mechanismen kan mogelijk nieuwe moleculaire targets identificeren voor de preventie van colonkanker.

Algemene conclusies

Zowel de *in vitro* als *in vivo* studies beschreven in dit proefschrift ondersteunen de hypothese dat NOC's cellulaire processen beïnvloeden die betrokken zijn bij de humane colonkanker ontwikkeling. Het nieuw gegenereerde overzicht van NOC-geïnduceerde genexpressie modulaties op basis van de *in vitro* studies levert al overtuigend bewijs dat NOC blootstelling, hetzij door middel van DNA-alkylering of ROS vorming, cruciale carcinogene pathways kan beïnvloeden. Het meest overtuigende bewijs ter ondersteuning van onze hypothese komt van de *in vivo* studies waar NOC blootstelling op fysiologisch relevante niveaus werd geassocieerd met MN vorming in lymfocyten en genexpressie modulaties in zowel lymfocyten als

colonweefsel. Dit weerspiegelt niet alleen de genotoxiciteit van deze verbindingen, maar suggereert ook epigenetische werkingsmechanismen voor NOC's. Verhoogde consumptie van rood vlees leidde niet tot een toename van NOC vorming in het colon. Het colonkanker risico geassocieerd met de consumptie van rood vlees wordt echter wel ondersteund door een verhoogde fecale water genotoxiciteit, wat bovendien is gerelateerd aan een aantal pro-carcinogene veranderingen in moleculaire pathways.

Het onderzoek beschreven in dit proefschrift is het eerste op transcriptomics gebaseerde onderzoek naar de rol van NOC's in de ontwikkeling van kanker en de resultaten zijn in overeenstemming met de hypothese dat NOC's een carcinogeen risico vormen voor de mens. Hoewel extra onderzoek nodig is om de omvang van dit risico vast te stellen, is het aan te raden om blootstelling aan NOC's te beperken door het voorkomen van een regelmatige consumptie van voedingsmiddelen of combinaties van voedsel die waarschijnlijk de mate van endogene nitrosering verhogen. Hoewel dit niet noodzakelijkerwijs rood vlees betreft, gebaseerd op het ontbreken van een verhoogde nitrosering in onze interventiestudie, lijkt het nog steeds wenselijk om de rood vleesinname te beperken op basis van de gevonden effecten op cruciale moleculaire pathways die betrokken zijn bij de humane coloncarcinogenese.

Dankwoord

Nog één dag en het resultaat van viereneenhalf jaar AIO'en ligt bij de drukker en aangezien van uitstel afstel komt, is het dus nu of nooit voor het schrijven van het dankwoord. Ik heb soms de neiging van een mug een olifant te maken en loop dan ook al dagen te klagen dat ik het dankwoord nog steeds niet geschreven heb. Dat nemen sommige mensen mij niet in dank af. Des te meer reden om dit te compenseren met een dankwoord. Ik heb maar alvast een Leffe Blond ingeschonken ter stimulering van de geest. Het gaat wat ver om de *Abbaye de Leffe* te bedanken, maar dat neemt niet weg dat ik er een zekere mate van inspiratie aan kan toeschrijven tijdens writer's blocks of algehele mind blocks. Gelukkig zijn de concentraties nitrosamines in bier al jaren minder dan ooit het geval was waardoor ik met een gerust hart mijn glas kan leegdrinken en alle mensen kan bedanken die hebben bijgedragen aan dit proefschrift en aan mijn plezierige tijd bij GRAT. Het klinkt ongelooflijk cliché, maar het is zonder twijfel waar dat ik nooit zo ver was gekomen zonder de hulp van een groot aantal mensen.

Allereerst mijn promotor en co-promotores. Prof. dr. Jos Kleinjans, beste Jos, het zal niet als een verrassing komen als ik zeg dat ik jouw begeleiding de afgelopen jaren zeer op prijs heb gesteld. Het geven van de juiste bijsturing, advies en kritiek op de juiste momenten hebben mij altijd weten te motiveren om de schouders eronder te zetten en het onderzoek tot een goed einde te brengen. Ik wil je daarvoor hartelijk bedanken! Dr. Theo de Kok, beste Theo, de afgelopen jaren heb ik met veel plezier met je samengewerkt. Ik verbaas mij er nog steeds over hoe doeltreffend en pragmatisch jij onderzoek aanpakt. Waar ik soms problemen zag, zag jij vaak oplossingen. Humor en wetenschap gaan ook perfect samen, daar ben ik in ieder geval van overtuigd! Zonder jouw uitstekende begeleiding had ik dit nooit kunnen bereiken. Samen met Jos vormen jullie het perfecte *dynamic duo* promotieteam. Natuurlijk ben ik mijn tweede co-promotor, Dr. Leopold Engels, ook zeer dankbaar. Beste Leopold, jouw bijdrage aan onze patiëntenstudie is van essentieel belang geweest en onze professionele samenwerking heb ik altijd zeer weten te waarderen. Dat bleek ook als ik bij nieuwe deelnemers op bezoek ging en zij vol lof spraken over hun dokter!

Het spreekt voor zich dat ik alle GRAT collega's wil bedanken voor de geweldig leuke en leerzame tijd de afgelopen jaren! Mijn kamergenoten moet ik daarbij absoluut apart vermelden. Niet alleen omdat kamer 4.102 nog altijd de leukste AIO-kamer van de hele UM is, maar ook omdat het een hele prestatie is om het viereneenhalf jaar lang met mij uit te houden. Het scheelt daarbij dat niemand die viereneenhalf volledig vol heeft moeten maken; er zijn namelijk nogal wat wisselingen in samenstelling geweest. Naar mijn weten gelukkig nooit als direct gevolg van mijn aanwezigheid. Mijn regelmatige opmerking dat het universum tegen mij is werd nog wel eens als irritant ervaren. Dat doet me denken aan een geweldige quote uit *The*

Hitchhiker's Guide to the Galaxy van Douglas Adams: *"In the beginning the Universe was created. This has made a lot of people very angry and been widely regarded as a bad move."* Joost, Joyce, Marieke, Yvonne (het was trouwens "viëtse"), Linda, Hans en Danitsja, het was een erg leuke tijd! Ik ben er trouwens van overtuigd dat vooral Marieke de target practice met elastiekjes door Joost en mij altijd erg op prijs heeft gesteld.

Ook mijn AIO buren (Marlon, Kimberley, Marten, Wim, Christina en natuurlijk de ex-AIO's Nicole, Nejla, Karen M. en Sabine) hebben hun steentje bijgedragen aan de geweldige werksfeer, ondanks mijn regelmatige bezoekjes om al mijn problemen in detail uit de doeken te doen. Mijn rijkunsten hebben de meesten van jullie ook in (nog steeds) levende lijve mogen meemaken en de reacties zijn nogal gemengd. Misschien moeten we wat meer ritjes maken om tot een definitieve conclusie te komen. De activiteiten van het GRAT animatieteam wil ik nog even apart vermelden. Met name de tripjes naar Durbuy georganiseerd door Karen M. en Sabine waren een groot succes. Karen M., ook nog bedankt voor al je promotietips en hulp tijdens deze laatste AIO dagen!

Kevin (a.k.a. Hochstenbaaaaaaaach!), echt geweldig dat je drieeneenhalf jaar geleden als AIO-overbuurman bent begonnen. Wij kunnen op vrijwel elk niveau met elkaar overweg en dan vooral het niveau van, ja wie sage ich das jetzt, "koek en koffie" en "hemmeneks". Great minds think alike, zullen we maar zeggen. De gezamelijke reis naar Utah was toch wel het hoogtepunt van de afgelopen jaren! Prettaaaaay, prettaaaaay, prettaaaaay good!

Hoewel ik jullie al kort genoemd heb, Karen en Joyce, kom ik onder een aparte vermelding niet uit. Zeker nu ik jullie als paranimfen gerecruteerd heb. Joyce, jij hebt nogal wat te verduren gehad. Ik herinner mij een incidentje met een keiharde pepernoot en een Engels cursusmiddag die uiteindelijk toch geannuleerd bleek te zijn. Karen, jij hebt dat gelukkig niet mee hoeven maken maar je zit dan ook iets verder weg. Je zegt niet voor niks wel vaker "er is geen groter leed dan wat de mens zichzelf aandeed". Het werk wordt toch een stuk leuker als twee van je beste maatjes ook dagelijks aanwezig zijn en daarvoor wil ik jullie bedanken. Ik kan dan natuurlijk niet jullie eega's, Timmeeeeeehhh en Merry, overslaan. Het is altijd weer lachen geblazen als wij bij elkaar zijn. En Merdan, ik weet niet hoeveel alinea's dankwoord je in gedachten had maar de ruimte is helaas beperkt dus misschien moeten we in plaats daarvan maar eens een tripje naar El Paso, Texas boeken. Iemand zei me eens dat "there's more Mexicans there than you can shake a stick at".

De overige leden van de lunch-gestapo (Sandra, Leen, Kristien) mag ik natuurlijk ook niet vergeten. Elke keer als ik weer met tegenzin naar beneden werd gesleurd

kreeg ik toch even de pauze die ik nodig had om weer verder te gaan. Daarnaast wil ik Sandra nog bedanken voor de hulp op het lab, zoals bijv. het uitlenen van je pasje om mijn onwelriekende samples in de ultracentrifuge te kunnen gooien.

Lou en Lucien, samen met Kevin vormen wij toch wel een geweldig muzikaal team. Onze sterk overlappende (en al zeg ik het zelf, zeer goede) muzieksmaak is altijd weer een favoriet onderwerp van discussie. Want de rest heeft toch nog nooit gehoord van The Ramones, Pixies, The Aggrolites, en al die andere bands die de revue hebben gepasseerd. Als “The Debasers” moet het toch mogelijk zijn om ooit nog een keer met de eerste prijs naar huis te gaan!

Edwin, bedankt voor je hulp met de analyses op de MS, een apparaat wat ondertussen hoogbejaard is en in het museum thuishoort. Marcelleke, ik wil je niet alleen bedanken voor alle lab-gerelateerde hulp maar ook voor je geweldige gevoel voor humor en je buitengewoon relaxte reactie op blunders als het waterbad vergeten uit te zetten tijdens het schoonmaken en het laten ontdooien van een heel vat met cellen.

Jacco en Danyel, samenwerking met jullie is een garantie voor succes want het heeft mooi een aantal sterke publicaties opgeleverd! Dat moeten we vaker doen! Simone, toen ik begon met het opzetten van de patiëntenstudie, wat een onmogelijke taak leek, heb ik heel veel gehad aan jouw advies en hulp. Heel erg bedankt! Anneloes, bedankt voor je hulp met het regelen van vanalles en nog wat, met name in de hectische laatste weken voor mijn promotie!

Marie-Claire, ook jou mag ik natuurlijk niet vergeten. Alle hulp en advies van de afgelopen jaren maar ook de vermakelijke gesprekken over zaken variërend van Arola’s tot eBay en van Duitse emails tot Waalse eigenaardigheden heb ik altijd gewaardeerd. Dankeschön!

Ook wil ik een aantal stagiaires bedanken die met hun werk in belangrijke mate hebben bijgedragen aan dit proefschrift. Roongnapa, Liliane, Tammy, Kevin van Tilburg, Mette en Marloes (technisch natuurlijk geen stagiaire, maar je hebt me wel enorm geholpen!), bedankt voor jullie inzet! And Mette, thanks for the steady supply of Tyrkisk Peber!

Uiteraard gaat mijn dank ook uit naar alle deelnemers aan de humane studies, zonder wie dit proefschrift de helft korter zou zijn geweest, en naar Herman Vermaas voor het mij wegwijs maken in de vleesindustrie.

De analisten van het klinisch lab in Orbis, de dames van de receptie endoscopie en het endoscopie team, en Carla in het bijzonder, hebben een buitengewoon belangrijke rol gespeeld bij het plannen en in goede banen leiden van het onderzoek. Jullie vormen een hecht en professioneel team. Mijn bezoekjes aan het ziekenhuis verliepen altijd soepel en ik ben steeds met veel plezier bij jullie langsgeweest.

De extra hulp vanuit het azM om voldoende patiënten te vinden voor ons onderzoek was daarbij ook van belang. Prof. dr. Ad Masclee, Wout Mares, Marieke Pierik, bedankt voor jullie bijdrages en advies! Wout, jou wil ik nog extra bedanken aangezien je ook in Sittard patiënten hebt weten te includeren en mee hebt geholpen bij het organiseren van het hele proces.

Tot slot wil ik mijn ouders en Nancy bedanken omdat zij altijd voor me klaar staan thuis. Misschien dat over enkele weken eindelijk duidelijk wordt wat ik nu precies gedaan heb de afgelopen jaren!

Curriculum vitae

Curriculum vitae

Dennie Geert Anne Jozef was born in Heerlen on January 4th 1983. After finishing pre-university education at the Rolduc College in Kerkrade in 2001, he started with the study Health Sciences at Maastricht University. He did his internship at the Department of Health Risk Analysis and Toxicology (GRAT) where he investigated the possibility of predicting gene expression changes induced by mixtures from individual compound data. He graduated cum laude in 2005 on the sub-specialism Biological Health Sciences. From April 2006 until October 2010, he worked as a PhD student at GRAT under the supervision of Prof. dr. Jos Kleinjans, Dr. Theo de Kok, and Dr. Leopold Engels. The research performed during this period is described in the present thesis, part of which was conducted in close collaboration with the Orbis Medical Concern in Sittard-Geleen and the Academic Hospital in Maastricht. Collaborations were also established with several international partners. He presented his research findings at a number of national and international symposia and conferences. These presentations in addition to the completion of several courses and the attendance of meetings and other activities within the Nutrim School for Nutrition, Toxicology, and Metabolism and the Graduate School VLAG (Advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences) qualify him for a VLAG-certificate. Since November 1st 2010, he is working as a postdoctoral fellow at GRAT.

List of publications and achievements

Full papers

Staal YCM, Hebels DGAJ, van Herwijnen MHM, Gottschalk RWH, van Schooten FJ, van Delft JHM. *Binary PAH-mixtures cause additive or antagonistic effects on gene expression but synergistic effects on DNA adduct formation*. Carcinogenesis. 2007;**28**(12):2632–40.

Hebels DGAJ, Jennen DGJ, Kleinjans JCS, de Kok TMCM. *Molecular signatures of N-nitroso compounds in Caco-2 cells: implications for colon carcinogenesis*. Toxicological Sciences. 2009;**108**(2):290–300.

Hebels DGAJ, Briedé JJ, Khampang R, Kleinjans JCS, de Kok TMCM. *Radical mechanisms in nitrosamine and nitrosamide-induced whole genome gene expression modulations in Caco-2 cells*. Toxicological Sciences. 2010;**116**(1):194–205.

Hebels DGAJ, Brauers, KJJ, van Herwijnen, MHM, Georgiadis P, Kyrtopoulos SA, Kleinjans JCS, de Kok TMCM. *Time-series analysis of gene expression profiles induced by nitrosamides and nitrosamines elucidates modes-of-action underlying their genotoxicity in human colon cells*. In preparation.

Hebels DGAJ, Jennen DGJ, van Herwijnen MHM, Moonen EJC, Pedersen M, Knudsen LE, Kleinjans JCS, de Kok TMCM. *Whole genome gene expression modifications associated with micronucleus formation in blood cells from nitrosamine-exposed humans*. Submitted.

Hebels DGAJ, Sveje KM, de Kok MC, van Herwijnen MHM, Kuhnle GGC, Engels LGJB, Vleugels-Simon CBEM, Mares WGN, Pierik M, Masclee AAM, Kleinjans JCS, de Kok TMCM. *Transcriptomic profiles in colon tissue from inflammatory bowel diseases patients in relation to N-nitroso compound exposure and colorectal cancer risk*. In preparation.

Hebels DGAJ, Sveje KM, de Kok MC, van Herwijnen MHM, Kuhnle GGC, Engels LGJB, Vleugels-Simon CBEM, Mares WGN, Pierik M, Masclee AAM, Kleinjans JCS, de Kok TMCM. *Red meat intake-induced increases in fecal water genotoxicity correlate with pro-carcinogenic gene expression changes in the human colon*. In preparation.

Abstracts

Staal YCM, Hebels DGAJ, van Herwijnen MHM, Gottschalk RWH, van Schooten FJ, van Delft JHM. *Gene expression profiles of polycyclic aromatic hydrocarbon (PAH) mixtures in relation to the individual compound profiles*. Chemico-Biological Interactions. 2006;**161**(2):173. Poster presentation at PhD student meeting of the Netherlands Society of Toxicology and Annual Meeting of the NVT, June 2006, Wageningen, The Netherlands.

Hebels DGAJ, Kleinjans JCS, de Kok TMCM. *Endogenous formation of N-nitrosocompounds and colorectal cancer risk in patients with inflammatory bowel disease*. Chemico-Biological Interactions. 2006;**161**(2):167. Poster presentation at PhD student meeting of the Netherlands Society of Toxicology and Annual Meeting of the NVT, June 2006, Wageningen, The Netherlands.

Hebels DGAJ, Kleinjans JCS, de Kok TMCM. *Endogenous formation of N-nitrosocompounds and colorectal cancer risk in patients with inflammatory bowel disease*. Poster and oral presentation at the Second ECNIS Annual Meeting, February 2007, Maastricht, The Netherlands.

Hebels DGAJ, Kleinjans JCS, de Kok TMCM. *Role of meat consumption in endogenous formation of N-nitroso compounds in patients with inflammatory bowel disease*. Chemico-Biological Interactions. 2007;**169**(2):134-5. Poster presentation at PhD student meeting of the Netherlands Society of Toxicology and Annual Meeting of the NVT, June 2007, Wageningen, The Netherlands.

Hebels DGAJ, Kleinjans JCS, de Kok TMCM. *In vitro genotoxicity and gene expression modulation by N-nitroso compounds*. Poster presentation at the Third ECNIS Annual Meeting, March 2008, Barcelona, Spain.

Hebels DGAJ, Kleinjans JCS, de Kok TMCM. *In vitro genotoxicity and gene expression modulation by N-nitroso compounds*. Poster and oral presentation at the PhD student meeting of the Netherlands Society of Toxicology and Annual Meeting of the NVT, June 2008, Wageningen, The Netherlands.

Hebels DGAJ, Kleinjans JCS, de Kok TMCM. *In vitro genotoxicity and gene expression modulation by N-nitroso compounds*. Poster presentation at the Annual NUTRIM Symposium, November 2008, Maastricht, The Netherlands.

Hebels DGAJ, Jennen DGJ, Kleinjans JCS, de Kok TMCM. *Molecular signatures of N-nitroso compounds in Caco-2 cells: implications for colon carcinogenesis*. Poster

presentation at the 30th anniversary meeting of the Netherlands Society of Toxicology, June 2009, Veldhoven, The Netherlands.

Jiménez van Hoorn LEM, Hebels DGAJ, de Kok TMCM. *Effects of red meat consumption on fecal water genotoxicity in Caco-2 cells: Association with inflammatory bowel disease*. Poster presentation at the 30th anniversary meeting of the Netherlands Society of Toxicology, June 2009, Veldhoven, The Netherlands.

Oth T, Hebels DGAJ, de Kok TMCM. *Influence of red meat consumption and inflamed gastrointestinal tract on endogenous nitrosation*. Poster presentation at the 30th anniversary meeting of the Netherlands Society of Toxicology, June 2009, Veldhoven, The Netherlands.

Hebels DGAJ, Jennen DGJ, Kleinjans JCS, de Kok TMCM. *N-nitroso compounds and colon carcinogenesis: an in vitro toxicogenomics approach*. Poster presentation at the 10th International Conference on Environmental Mutagens, August 2009, Florence, Italy.

Hebels DGAJ, Jennen DGJ, Kleinjans JCS, de Kok TMCM. *N-nitroso compounds and colon carcinogenesis: an in vitro toxicogenomics approach*. Poster presentation at the Annual NUTRIM Symposium, November 2009, Maastricht, The Netherlands.

Hebels DGAJ, Briedé JJ, Khampang R, Kleinjans JCS, de Kok TMCM. *Radical mechanisms in nitrosamine and nitrosamide-induced whole genome gene expression modulations in Caco-2 cells*. Poster presentation at the 49th annual meeting of the Society of Toxicology, March 2010, Salt Lake City, Utah, USA.

Hebels DGAJ, Jennen DGJ, Kleinjans JCS, de Kok TMCM. *N-nitroso compounds and colon carcinogenesis: an in vitro toxicogenomics approach*. Poster presentation at the 49th annual meeting of the Society of Toxicology, March 2010, Salt Lake City, Utah, USA.

Hebels DGAJ, Jennen DGJ, van Herwijnen MHM, Moonen EJC, Pedersen M, Knudsen LE, Kleinjans JCS, de Kok TMCM. *Whole genome gene expression modifications associated with micronucleus formation in blood cells from nitrosamine-exposed humans*. Poster and oral presentation at the 40th Annual Meeting of the European Environmental Mutagen Society, September 2010, Oslo, Norway.

Achievements

Awarded the distinction *Cum Laude* for the Master's degree in Biological Health Sciences in 2005.

Member of the NUTRIM PhD students council from 2007 till 2010, the aim of which is to inform NUTRIM associated PhD students about issues that are discussed at institute level, and to take care that PhD issues are brought to institute council's attention.

Organization of the Annual NUTRIM Symposium in November 2008.

Awarded a travel grant by the Netherlands Society of Toxicology for attendance of the 10th International Conference on Environmental Mutagens, August 2009, Florence, Italy.

Organization of the Annual NUTRIM Symposium in November 2009.

Awarded a travel grant by the Netherlands Society of Toxicology for attendance of the 49th annual meeting of the Society of Toxicology, March 2010, Salt Lake City, Utah, USA.

Co-chair at the 40th Annual Meeting of the European Environmental Mutagen Society, September 2010, Oslo, Norway.

Figure 2, Chapter 6, p. 142

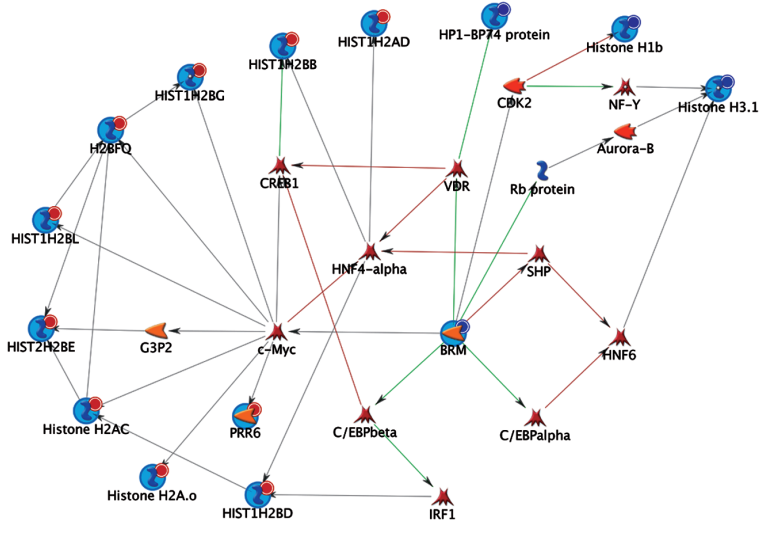

Figure 2A, Chapter 5, p. 124