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N-nitroso compound exposure-associated transcriptomic profiles are indicative of an increased risk for colorectal cancer

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ABSTRACT

Endogenous formation of N-nitroso compounds (NOCs), which are known animal carcinogens, could contribute to human carcinogenesis but definitive evidence is still lacking. To investigate the relevance of NOCs in human colorectal cancer (CRC) development, we analyzed whole genome gene expression modifications in human colon biopsies in relation to fecal NOC exposure. We had a particular interest in patients suffering from intestinal inflammation as this may stimulate endogenous NOC formation, and consequently predispose to CRC risk. Inflammatory bowel disease (IBD) patients diagnosed with ulcerative colitis and irritable bowel syndrome patients without inflammation, serving as controls, were therefore recruited. Fecal NOC were demonstrated in the majority of subjects. By associating gene expression levels of all subjects to fecal NOC levels, we identified a NOC exposure-associated transcriptomic response that suggests that physiological NOC concentrations may potentially induce genotoxic responses and chromatin modifications in human colon tissue, both of which are linked to carcinogenicity. In a network analysis, chromatin modifications were linked to 11 significantly modulated histone genes, pointing towards a possible epigenetic mechanism that may be relevant in comprehending NOC-induced carcinogenesis. In addition, pro-inflammatory transcriptomic modifications were identified in visually non-inflamed regions of the IBD colon. However, fecal NOC levels were slightly but not significantly increased in IBD patients, suggesting that inflammation did not strongly stimulate NOC formation. We conclude that NOC exposure is associated with gene expression modifications in the human colon that may suggest a potential role of these compounds in CRC development.

1. Introduction

N-nitroso compounds (NOCs) have long been suspected to play a role in human carcinogenesis since many NOCs possess genotoxic and mutagenic properties and are known animal carcinogens [1,2]. Most NOCs have therefore been classified as probable or possible human carcinogens by the International Agency for Research on Cancer [3]. Human exposure to NOCs occurs almost entirely through food and endogenous nitrosation of NOC precursors in the gastrointestinal tract [4] and, following absorption into

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the bloodstream, NOCs may have deleterious effects in many tissues. Epidemiological studies have indeed associated human (endogenous) NOC exposure to several types of cancer, including esophagus, stomach, colorectal and bladder cancer [5–8], but proof of a causal relation between NOCs 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 NOCs in relation to their genotoxicity in order to elucidate potentially important gene expression modifications that may play a role in the carcinogenic process [9,10]. 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 NOCs and increase the carcinogenic risk associated with exposure.

As a result of endogenous nitrosation processes in the intestinal lumen, highest NOC exposure levels may occur in the human colon and therefore the colon represents an important target tissue for investigating molecular effects of NOC exposure. Furthermore, there are indications that colon inflammation stimulates endogenous NOC formation since inducible nitric oxide synthase (iNOS) activity, as found in inflammatory bowel disease (IBD) patients [11], is associated with an excess production of nitrosating agents, such as NO, nitrogen oxides, and nitrite [12], which in turn generate NOCs from precursors in the diet [13,14]. Individuals suffering from IBD therefore present an interesting population to study NOC exposure-related adverse effects, especially since this group is at increased risk of colorectal cancer (CRC) [15,16]. We considered a global analysis of gene expression modifications a powerful technology for providing more insight in NOC-mediated modes-of-action in relation to possible pre-carcinogenic events in the human colon.

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 colon patients in relation to fecal NOC levels as a marker of colonic NOC exposure, and compared IBD patients with irritable bowel syndrome (IBS) control subjects. With this study, we aimed to identify changes in molecular pathways which may contribute to the carcinogenic process in the human colon.

2. Material and methods

2.1. Subjects and study design

Subjects were recruited in the Netherlands at the Department of Gastroenterology in the Orbis Medical Center in Sittard and the Academic Hospital Maastricht. A total of 44 IBD patients (29 male, 15 female, mean age 48.8 ± 14.4, range 23–81 years) and 36 control patients diagnosed with IBS (15 male, 21 female, mean age 56.2 ± 15.3, range 21–78 years) were included. 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 as well as patients who had to be admitted to hospital or had a history of colorectal adenomas. Only IBS patients proven free (as evaluated by 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 subset of 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 sigmoid or 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 3 days before the colonoscopy, subjects followed their normal dietary habits, with a few modifications. Namely, to keep nitrate intake among 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 restricted to avoid inhibiting endogenous nitrosation [17].

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

2.2. Fecal apparent total nitroso compound determination

Apparent total nitroso compounds (ATNC) were analyzed in feces by thermal energy analysis as described previously [18], using an Ecomedics CLD88 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.

2.3. Microarray hybridization and data analysis

From each subject, 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 (average RNA integrity number: 7.1 ± 1.0). Microarray hybridization was performed as described previously with some modifications [10]. In short, dye-labeled cRNA (Cy3) was synthesized following the one-color labeling protocol supplied by the manufacturer (Agilent Technologies, Amstelveen, The Netherlands). Samples were hybridized on Agilent 4x44K

Whole Human Genome microarrays. After scanning the microarray slides, using settings described before [10], bad and empty spots were flagged using GenePix Pro (version 6.0, Molecular Devices, Sunnyvale, CA). For each spot, mean local background intensity was subtracted from mean signal intensity, and spots with a mean net signal intensity <10.0 were omitted from analysis. 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 were used for further analyses.

For Spearman's rank correlation analyses gene expression data were correlated with ATNC levels. Only genes present in at least 70% of subjects (both IBD and IBS patients) were used without further pre-selection. Prior to correlation analysis, missing values were imputed in GenePattern by finding the *k* nearest neighbors (*k* set to 15), using a Euclidean metric. Correlation analyses were performed in the Gene Expression Profile Analysis Suite (GEPAS, http://gepas.bioinfo.cipf.es/). Significantly correlating genes ($p < 0.05$) were further analyzed in MetaCore™ (GeneGo, San Diego, CA). A gene network based on a select number of genes was created in MetaCore using Dijkstra's shortest paths algorithm.

GenePattern version 3.1 (http://www.broad.mit.edu/cancer/software/genepattern/) was used for three-dimensional principal component analysis (PCA) of the gene expression intensities of all subjects. With ArrayTrack modulated genes between IBD and IBS groups were identified by combining a log ratio ranking (absolute log2 ratio >0.5) with a non-stringent *p*-value cut-off (two-tailed Welch *t*-test, $p < 0.05$), as suggested by Shi et al. [19]. The false discovery rate at this *p*-value cut-off was 3%, which was calculated using Storey's *q*-value approach [20]. 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 to identify 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.

2.4. Fecal calprotectin

Calprotectin (in μg/g feces) as a marker of chronic inflammation was determined as described previously [21]. 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.

2.5. Statistical analysis

Results are presented as mean ± SD. Normality of data 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.

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 GSE25220 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE25220).

3. Results

3.1. Endogenous NOC formation and associated gene expression modifications

Fecal ATNC levels could be demonstrated above limits of detection (0.01 nmol/g feces) in 65 out of a total of 80 subjects (Table 1). To investigate the transcriptomic response in the colon biopsies specifically linked to this 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 2, all GeneGo pathways and the top 10 of a total of 153 GO processes significantly associated ($p < 0.05$) with ATNC levels are presented. The most strongly represented cellular process was transport with two pathways involved in clathrin-coated vesicle formation and macropinocytosis. Most importantly in view of the established genotoxic properties of NOCs, a DNA damage repair pathway appeared associated with ATNC levels while the remaining pathways share relevant and significantly correlating genes that are involved in signaling pathways related to cell cycle regulation and apoptosis, such as the RAC1 G-protein signaling pathway.

The top 10 of GO processes significantly associated with ATNC levels were mostly involved in nucleosome/chromatin assembly which may relate to epigenomic phenomena. In Fig. 1, the closest interactions between the significantly correlating genes in the *Chromatin assembly* GO process are shown. The network contains 14 genes that significantly correlate (in most cases positively) 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,

Table 1
Population characteristics, fecal ATNC, and calprotectin levels in IBS controls and IBD patients shown for all subjects and the biopsy subgroup.

	All subjects		Biopsy subjects	
	IBS $n = 36$	IBD $n = 44$	IBS $n = 13$	IBD $n = 19$
Age (years) [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)	15/21	29/15	7/6	15/4
ATNC above detection limit	28 of 36	37 of 44	12 of 13	16 of 19
ATNC (nmol/g feces)	7.4 ± 8.1	10.1 ± 10.5	7.2 ± 6.1	10.5 ± 11.2
Calprotectin (μg/g feces)	11.0 ± 16.8	37.0 ± 26.1[a]	12.0 ± 17.0	38.8 ± 21.0[b]

[a] Significantly higher in IBD group compared to IBS group ($p < 1 \times 10^{-7}$, Mann–Whitney *U* test).
[b] Significantly higher in IBD group compared to IBS group at $p < 0.001$ (Student's *t*-test).

Table 2
GeneGo pathways and the top 10 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 ($n = 32$).

Cellular process	Pathways involved	Genes present (significant/total)	% Genes[a]	p-Value
GeneGo pathways				
Cytoskeleton remodeling	RalA regulation pathway	4/23	17	0.023
Development	PIP3 signaling in cardiac myocytes	4/27	15	0.040
DNA damage	NHEJ mechanisms of DSBs repair	4/14	29	0.004
G-protein signaling	RAC1 in cellular process	5/22	23	0.003
Signal transduction	AKT signaling	4/27	15	0.040
Transport	Clathrin-coated vesicle cycle	12/55	22	0.000
	Macropinocytosis regulation by growth factors	5/36	14	0.029

GO process name	Genes present (significant/total)	% Genes[a]	p-Value
GO processes			
Chromatin assembly	14/69	20	0.0000
mRNA metabolic process	39/357	11	0.0000
Chromatin assembly or disassembly	17/103	17	0.0001
Nucleosome assembly	13/66	20	0.0001
Protein–DNA complex assembly	14/76	18	0.0001
Nucleosome organization	13/73	18	0.0002
Cellular biopolymer catabolic process	56/625	9	0.0003
DNA packaging	15/100	15	0.0004
RNA metabolic process	74/898	8	0.0005
RNA processing	48/523	9	0.0005

The false discovery rates for the GeneGo pathways and GO processes presented here were below 17% and 13% respectively.
[a] Percentage of significantly correlated genes compared to the total number of genes in the pathway or process.

actin dependent regulator of chromatin, subfamily a, member 2 (BRM). Most of these genes are involved in the epigenetic regulation of gene expression which thus suggests that NOCs might influence this process.

3.2. Differences between IBD and IBS patients

Although the average ATNC level is approximately 40% higher in IBD patients than in IBS controls, inter-individual variation is high (Table 1). As a result, no significant difference was found in ATNC between controls and IBD patients in the total group ($p = 0.261$) or biopsy subgroup ($p = 0.348$). There also were no significant differences between genders ($p > 0.428$). To check whether IBD patients did display a higher level of inflammation we measured fecal calprotectin levels. IBD patients indeed had a significantly higher fecal calprotectin level than control patients, in both the total group and the biopsy subjects group ($p < 1 \times 10^{-7}$ and $p < 0.001$, respectively), indicating a higher level of inflammation (Table 1). There were no significant differences between genders ($p > 0.735$). ATNC levels were not correlated with calprotectin as a marker of

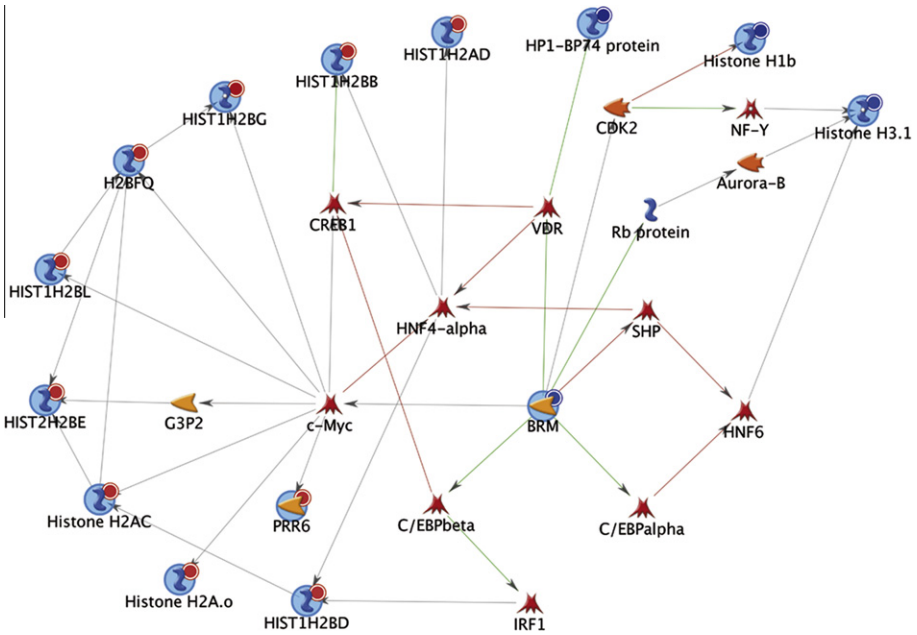

Fig. 1. A network analysis of genes in the *Chromatin assembly* GO process showing the shortest paths between genes significantly correlating with ATNC levels in both biopsy patients groups. Significantly correlating genes are designated by a blue circle in the background. Positive and negative correlation is indicated with small red and blue circles, respectively.

Table 3
Reported average daily dietary intake before biopsy sampling in IBS controls and IBD patients.

Dietary intake (average/day)	IBS controls, $n = 36$	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] Significantly different between IBS and IBD groups at $p < 0.01$ but not after correction for gender (Student's *t*-test).
[b] Significantly different between IBS and IBD groups at $p < 0.05$ but not after correction for gender (Student's *t*-test).

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 strong association between endogenous nitrosation and inflammatory conditions in the colon.

The results of the food diary analysis are presented in Table 3. Average daily intake during the 3 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 1, 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.

3.3. Differential gene expression between IBD patients and controls

We subsequently determined whether gene expression profiles in visually non-inflamed colon tissue provide any additional information on differences in a possible carcinogenic risk between IBD and IBS patients, regardless of ATNC levels. The PCA plot in Fig. 2 shows that IBS controls group separately from IBD patients indicating distinct profiles at the transcriptomic 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. A total of 2266 genes met this criterion and were further investigated in MetaCore. The results of this analysis are presented in Table 4 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 and the complement signaling system. Strongly up and down regulated genes in the modified immune response pathways clearly indicate a pro-inflammatory state in IBD patients compared to controls (results not shown). 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.

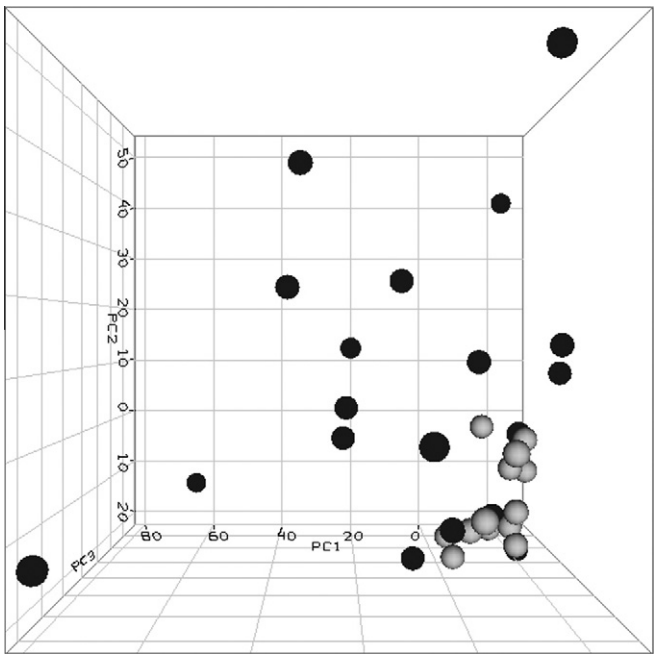

Fig. 2. PCA plot of the microarray-based expression profiles showing a separation between IBD patients (black) and IBS controls (gray). Data were described by three principal components (PCs) accounting for 24%, 13%, and 9% of variation.

Table 4
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 present (significant/total)	% Genes[a]	p-Value
Amino acid metabolism	(L)-Arginine metabolism	6/23	26	0.017
Blood coagulation	Blood coagulation	10/24	42	0.000
Cell adhesion	ECM remodeling	9/35	26	0.004
	Cell–matrix glycoconjugates	6/22	27	0.014
Cytoskeleton remodeling	Neurofilaments	4/14	29	0.036
Development	Angiotensin signaling via STATs	7/15	47	0.000
	Leptin signaling via JAK/STAT and MAPK cascades	7/19	37	0.001
	Growth hormone signaling via STATs and PLC/IP3	8/27	30	0.003
	Thrombopoetin signaling via JAK-STAT pathway	6/17	35	0.003
	PDGF signaling via STATs and NF-kB	5/18	28	0.022
	EPO-induced Jak-STAT pathway	7/31	23	0.023
	Prolactin receptor signaling	8/38	21	0.023
	GM-CSF signaling	7/32	22	0.027
	Hypoxia-induced EMT in cancer and fibrosis	3/8	38	0.033
	Transcription regulation of granulocyte development	5/20	25	0.034
	EPO-induced PI3K/AKT pathway and Ca(2+) influx	5/20	25	0.034
	Growth hormone signaling via PI3K/AKT and MAPK cascades	7/35	20	0.042
Immune response	IL-17 signaling pathways	14/37	38	0.000
	Oncostatin M signaling via JAK-Stat in human cells	8/14	57	0.000
	Antiviral actions of interferons	10/24	42	0.000
	IL-23 signaling pathway	6/10	60	0.000
	Alternative complement pathway	9/23	39	0.000
	IL-27 signaling pathway	7/15	47	0.000
	MIF-mediated glucocorticoid regulation	7/16	44	0.000
	IFN alpha/beta signaling pathway	7/18	39	0.001
	Bacterial infections in normal airways	9/32	28	0.002
	Cytokine production by Th17 cells in CF	7/21	33	0.002
	Oncostatin M signaling via MAPK in human cells	8/27	30	0.003
	Lectin induced complement pathway	7/22	32	0.003
	Classical complement pathway	7/22	32	0.003
	IL-10 signaling pathway	6/18	33	0.005
	Bacterial infections in CF airways	9/36	25	0.005
	IL-1 signaling pathway	9/36	25	0.005
	IL-3 activation and signaling pathway	6/19	32	0.006
	Histamine H1 receptor signaling in immune response	7/25	28	0.007
	IL-22 signaling pathway	6/20	30	0.008
	CD40 signaling	9/41	22	0.012
	IL-12 signaling pathway	5/16	31	0.013
	IL-12-induced IFN-gamma production	6/22	27	0.014
	IFN gamma signaling pathway	8/35	23	0.014
	MIF in innate immunity response	6/24	25	0.021
	PGE2 signaling in immune response	6/24	25	0.021
	Mucin expression in CF via IL-6, IL-17 signaling pathways	5/19	26	0.028
	Th1 and Th2 cell differentiation	5/20	25	0.034
	Sialic-acid receptors (Siglecs) signaling	3/8	38	0.033
Reproduction	GnRH signaling	7/35	20	0.042
Transcription	CREM signaling in testis	3/8	38	0.033

The false discovery rate for the GeneGo pathways presented here was below 36% at p-value 0.05 and below 14% at p-value 0.01.
[a] Percentage of significantly modulated genes compared to the total number of genes in the pathway.

4. Discussion

With this study we aimed to investigate gene expression modifications hypothetically induced in human colon tissue in relation to NOC exposure. Since the relevance of NOCs 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.

NOCs, as measured by ATNC, were detected in most fecal samples (Table 1). Thus, by specifically investigating genes whose expression values are significantly correlated with individual ATNC levels, it appeared possible to identify NOC exposure-associated gene expression modulations in most biopsy subjects by pooling data from IBD and IBS patients. The set of genes found to be significantly correlated with ATNC levels was involved in several pathways that can be attributed to the well-known genotoxic properties of NOCs (Table 2). 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 following the induction of DNA damage by NOCs, as based on information on these genes obtained from Entrez Gene at the 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 NOC levels reached in the human colon are unlikely to cause enough damage to strongly induce a cell cycle block or apoptosis in order to either repair damage or prevent the cell from dividing [10]. However, since these modifications provide evidence for a genotoxic effect of NOCs in colon tissue, it could be hypothesized that prolonged exposure to NOCs results in an accumulation of DNA damage over time. This would be especially relevant in people who display a strong endogenous formation of NOCs in the colon throughout life. In the classic genetic model proposed by Fearon and Vogelstein [22], a continuously high exposure could facilitate the development of CRC by accelerating the carcinogenic transformation of colon cells.

The set of significantly correlating ATNC genes was subsequently analyzed for its involvement in significantly modulated GO processes and the top 10 contained six processes involved in chromatin assembly/disassembly which thus represents an important process. We therefore explored in depth significantly correlating genes in the six most strongly deregulated processes (*Chromatin assembly*) in a network analysis, in order to visualize their interactions. As shown in Fig. 1, 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 NOCs influence gene expression by histone modifications. NOCs 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 [23–26]. NOC-induced alkylation of these proteins could affect their interaction with DNA and influence gene expression levels [23,27]. Histone methylation, for example, promotes heterochromatin formation resulting in gene silencing [28] and it is possible that (methylating) NOCs 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 [29]. 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, as may be caused by NOCs, 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 [30–32]. 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 NOCs and chromatin 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. Given the established role of epigenetic modifications in chromatin in the development of cancer by influencing the expression of tumor-suppressor genes and oncogenes, the discovery of these modifications in humans exposed to physiological NOC concentrations under daily life circumstances, may be of relevance with regard to carcinogenic risks associated with NOC exposure [33]. Since the colon is one of the main targets of NOCs, the histone modifications we identified could represent a novel epigenetic mechanism in the development of CRC following NOC exposure, for instance by providing a growth advantage through the silencing of tumor suppressor genes.

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 [13,14]. Although there is a substantial amount of evidence that increased oxidative stress levels associated with chronic inflammation contribute to neoplastic transformation in IBD patients [34], NOCs may also play a significant role. As demonstrated by the fecal calprotectin levels (Table 1), IBD patients indeed had a higher level of colonic inflammation compared to IBS controls, confirming that IBS patients form a suitable control group [35,36]. However, no significant differences with respect to fecal ATNC levels were found between both groups, nor was there a significant correlation between calprotectin and ATNC levels, indicating that in this study colonic inflammation did not significantly stimulate the formation of fecal NOCs. In a previous study we did find a significant increase in one of the most important excreted fecal NOCs (N-nitrosodimethylamine) in IBD patients compared to healthy controls [13], which may be explained by the inclusion of more severe and hospitalized IBD cases in that particular study. 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 3). It is thus unlikely that these dietary factors influence 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 [37,38], none of which were different between groups. We therefore conclude that inter-individual differences in dietary patterns are not accountable for the absence of significantly different fecal ATNC

levels between the study populations, but IBD severity may be.

At the gene expression level, IBD patients and controls did demonstrate a distinctive response, as shown by the PCA plot where IBD and IBS patients form two groups with only a limited degree of overlap (Fig. 2). This is in agreement with several studies showing that the gene expression profile in colon biopsies from ulcerative colitis patients is different from non-inflamed controls [39–42]. Differentially modified genes between IBD patients and controls were strongly involved in the immune response, and in particular biological pathways regulating the inflammatory response, including many cytokine signaling pathways (Table 4). 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 [43,44], was significantly up regulated in IBD patients, as was COX-2 (results not shown). This is expected to lead to NO production which in turn stimulates the formation of pro-inflammatory mediators by increasing the expression of COX-2, thus creating a positive feedback loop [45]. 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 [46,47]. 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 only the visually inflamed section is in a (low) state of inflammation. Similar observations were made in tissue from ulcerative colitis patients in remission [39] 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 [48,49]. 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 [50,51]. 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.

The difference in the transcriptomic profile between IBD and IBS subjects raises the question whether the correlation analysis with ATNC levels discussed earlier is warranted. As discussed above, the difference in the transcriptomic profile between IBD and IBS patients can most likely be attributed to the difference in colonic inflammatory state since there was no significant difference in fecal ATNC levels between both groups. In addition, both IBD and IBS subjects are exposed to NOCs as demonstrated by their presence in the fecal matter of both groups with similar percentages of samples above the detection limit (Table 1). The transcriptomic difference between IBD and IBS subjects and the absence of a difference in fecal NOC excretion can thus be considered as independent parallel events. Although the gene expression profile associated with IBD status could introduce more noise at the transcriptome level, the NOC excretion-associated transcriptomic profile is likely to represent a gene expression pattern that is NOC-specific regardless of the inflammatory state in the colon.

Overall, we conclude that, although inflammation apparently did not provide a statistically reliable model for increased nitrosation in this study, gene expression modifications were identified that clearly increase the current understanding of IBD pathophysiology. More importantly, by specifically relating gene expression changes to fecal ATNC levels, we have identified NOC exposure-associated transcriptomic responses that indicate that physiological NOC concentrations may potentially induce DNA damage and chromatin modifications in human colon tissue. Since both effects are linked with carcinogenicity, further research into NOC-specific DNA aberrations and chromatin modifications seems relevant and is needed to support these findings. This may be especially important for IBD patients who are already at an increased risk of developing CRC. 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 NOCs in human CRC development.

Conflict of interest

The authors declare no conflict of interest.

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