Food and Chemical Toxicology 115 (2018) 73–87

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

DNA adduct profiling of *in vitro* colonic meat digests to map red *vs.* white meat genotoxicity

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ARTICLE INFO

Keywords:
Cancer risk
DNA adductome mapping
Heme
Red meat

ABSTRACT

The consumption of red meat has been linked to an increased colorectal cancer (CRC) risk. One of the major hypotheses states that heme iron (present in red meat) stimulates the formation of genotoxic N-nitroso compounds (NOCs) and lipid peroxidation products (LPOs). By means of DNA adductomics, chemically induced DNA adduct formation can be mapped in relation to e.g. dietary exposures. In this study, this state-of-the-art methodology was used to investigate alkylation and (lipid per)oxidation induced DNA adduct formation in *in vitro* red vs. white meat digests. In doing so, 90 alkylation and (lipid per)oxidation induced DNA adduct types could be (tentatively) identified. Overall, 12 NOC- and/or LPO-related DNA adduct types, i.e. dimethyl-T (or ethyl-T), hydroxymethyl-T, tetramethyl-T, methylguanine (MeG), guanidinohydantoin, hydroxybutyl-C, hydroxymethylhydantoin, malondialdehyde-x3-C, O^6-carboxymethylguanine, hydroxyethyl-T, carboxyethyl-T and 3,N^4-etheno-C were singled out as potential heme-rich meat digestion markers. The retrieval of these DNA adduct markers is in support of the heme, NOC and LPO hypotheses, suggesting that DNA adduct formation may indeed contribute to red meat related CRC risk.

1. Introduction

The vast majority of cancer cases are not hereditary in origin, but are caused by the (chronic) exposure to certain environmental factors. This encompasses exposure to genotoxic chemicals from multiple and highly diverse sources; e.g. heterocyclic amines (HCAs) in meat cooked at high temperatures, mycotoxins in molded food and feed, polycyclic aromatic hydrocarbons (PAHs) in tobacco smoke, diesel exhaust and grilled meat (Stewart and Wild, 2014). Such chemicals can contribute to cancer initiation and development individually and/or synergistically. Moreover, the hence induced DNA adduct formation appears to be key in chemically induced carcinogenesis; covalent binding of genotoxic chemicals to DNA nucleobases can alter genes and induce mutations (Poirier, 2004).

In 2015, the International Agency for Research on Cancer (IARC) and the World Health Organization (WHO) issued that red meat is 'probably carcinogenic to humans' (group 2B) "based on limited evidence that the consumption of red meat causes cancer in humans and strong mechanistic evidence supporting a carcinogenic effect" (IARC, 2015). One of the main hypothetical mechanisms underlying the epidemiological link between red meat consumption and colorectal cancer (CRC) is explained by the 'heme hypothesis'. This hypothesis states that heme, which is intrinsically more present in red meat compared to white meat, stimulates (lipid per)oxidation and N-nitroso compound (NOC) formation in the gut besides affecting direct toxicity (Bastide et al., 2015; Demeyer et al., 2016). NOCs, heme, as well as several known oxidative metabolites (e.g. reactive oxygen species (ROS) and lipid peroxidation products (LPOs)) exert genotoxic effects *via* alkylation and/or oxidation of DNA, as such contributing to chemically induced DNA adduct formation (Hemeryck and Vanhaecke, 2016).

NOC exposure can occur *via* different routes; dietary intake and endogenous formation in the gut. More specifically, nitrosamines and nitrosamides can be formed in the stomach due to the interaction of nitric oxide or nitrite from metabolism, food, saliva and pharmaceutical drugs (Lijinsky, 1992). In the large bowel, microbial fermentation of proteins can lead to the production of amines, which can then be

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https://doi.org/10.1016/j.fct.2018.02.032
Received 21 September 2017; Received in revised form 13 February 2018; Accepted 14 February 2018
Available online 16 February 2018
0278-6915/

List of abbreviations

A	Adenine
C	Cytosine,
CRC	Colorectal Cancer
DNA	DeoxyriboNucleic Acid
G	Guanine,
HESI	Heated ElectroSpray Ionisation
HRMS	High Resolution Mass Spectrometry
LPO(s)	Lipid Peroxidation Product(s)
M_1G	Malondialdehyde-x1-G
M_2G	Malondialdehyde-x2-G
M_3C	Malondialdehyde-x3-C
MDA	Malondialdehyde
MeG	Methylguanine (position of methyl group not specified)
MS	Mass Spectrometry
NOC(s)	N-Nitroso Compound(s)
O^4-eT	O^4-ethylthymine
O^6-CMG	O^6-carboxymethylguanine
O^6-MeG	O^6-methylguanine
OPLS-DA	Orthogonal Partial Least Squares-Discriminant Analysis
PCA-X	Principal Component Analysis
ROS	Reactive Oxygen Species
RT	Retention time
SHIME	Simulator of the Human Intestinal Microbial Ecosystem
T	Thymine
TBARS	Thiobarbituric Acid Reactive Substances
T0	Pre-colonic digestion sampling time point
T48	Post-colonic digestion sampling time point
U	Uracil
UHPLC	UltraHigh Performance Liquid Chromatography
UP	Ultrapure water
VIP	Variable Importance in Projection

transformed to NOCs by means of nitrosation (Louis et al., 2014). Several studies have demonstrated that dietary heme iron, but not inorganic iron and/or meat protein, significantly increases fecal NOC-levels of human volunteers consuming a red meat diet (Cross et al., 2003; Demeyer et al., 2016). Hence, it appears that heme iron catalyzes NOC-formation upon red meat digestion. More specifically, it has been hypothesized that heme can capture NO (e.g. after release by S-nitrosothiols under alkaline conditions in the small bowel), resulting in the formation of nitrosyl heme. Thus, since nitrosyl heme can act as a nitrosating agent, heme iron can promote endogenous NOC formation (Kuhnle and Bingham, 2007). Because NOCs exert DNA-alkylating properties, increased NOC formation can lead to the accumulation of alkylation-induced DNA adducts (Drabløs et al., 2004; Hemeryck and Vanhaecke, 2016).

The heme hypothesis also stipulates a direct and indirect heme iron induced increase of oxidative stress and lipid peroxidation. Through the Fenton reaction, heme iron can stimulate the formation of ROS and LPOs (Kanner, 1994), leading to a cascade of oxidative reactions and resulting in the oxidation of e.g. DNA nucleobases. As such, red meat digestion can increase CRC risk in a twofold manner; i.e. through the induction of oxidative stress and/or by DNA adduct formation (Bastide et al., 2011, 2015; Demeyer et al., 2016; Marnett, 2012).

DNA adduct formation due to red meat consumption could be an important step in the pathophysiology underlying CRC. However, up to date, the exact etiology of red meat induced CRC initiation, promotion and progression lacks full elucidation. This study aimed to further unravel the genotoxic effects of red meat consumption *via* alkylation and/or oxidation induced DNA adduct formation. A multitude of analytical methods can be implemented for the detection of DNA adducts in biological matrices (Farmer and Singh, 2008; Himmelstein et al., 2009) although at the time mass spectrometry (MS) is considered as the gold standard (Balbo et al., 2014; Hemeryck et al., 2016a). More specifically, HRMS is the method of choice to perform untargeted DNA adductomics because it is highly selective, sensitive and most importantly, provides chemical structural information as opposed to e.g. ^{32}P-postlabelling. Different research groups, including ours, have successfully applied HRMS-based DNA adductomics to investigate DNA modifications resulting from inflammation or exposure to dietary and tobacco smoke specific compounds (Balbo and Brooks, 2015; Hemeryck et al., 2015; Hemeryck and Vanhaecke, 2016; Ishino et al., 2015).

In this study, the gastrointestinal digestion of beef diaphragm (a model for red meat) was simulated *in vitro*, and compared to the digestion of chicken breast (a model for white meat). Analysis of any resulting DNA adduct formation was performed by means of a state-of-the-art DNA adductomics platform based on the use of high resolution mass spectrometry, and an in-house DNA adduct database listing all currently known diet-related alkylation and (lipid per)oxidation related DNA adducts (n = 180) (Hemeryck et al., 2015; Hemeryck and Vanhaecke, 2016). In addition, to gain a more profound insight into the underlying mechanisms, additional experiments were performed to assess the possible interfering role of myoglobin, i.e. the heme iron containing protein that is intrinsically more present in red compared to white meat (Bastide et al., 2011).

2. Methods

2.1. Reagents and chemicals

O^6-CMdG (O^6-carboxymethyl-2′-deoxyguanosine) was kindly provided by Dr. S. Moore from Liverpool John Moores University (UK). Deoxyguanosine (dG), O^6-MedG (O^6-methyl-2′-deoxyguanosine) and O^6-d_3-MedG (internal standard for both O^6-MedG and O^6-CMdG) were purchased from Sigma-Aldrich (St. Louis, USA). Analytical standards for M_1G (pyrimido[1,2-*a*]purin-10(1H)-one or 'Malondialdehyde-x1-G'), and its internal standard M_1G-$^{13}C_3$ were obtained from Toronto Research Chemicals (Toronto, Canada).

O^6-CMdG, O^6-MedG, O^6-d_3-MedG, and dG were hydrolyzed to their nucleobase form in 0.1 M formic acid over the course of 30 min at 80 °C. All standards were diluted in methanol to obtain stock and working solutions of 500 ng/mL and 5 ng/mL, respectively.

Myoglobin was obtained from Sigma-Aldrich (St-Louis, Missouri, USA). A stock solution of 10 mg/mL for myoglobin was prepared in ultrapure water (UP) (Millipore, Brussels, Belgium) and stored at −20 °C.

Solvents were of analytical grade (VWR International, Merck, Darmstadt, Germany) when used for extraction and purification steps, and of Optima LC/MS grade for LC−MS (liquid chromatography - mass spectrometry) application (Fisher Scientific UK, Loughborough, UK).

2.2. Meat preparations

Beef diaphragm, chicken breast and subcutaneous pork fat (lard) were obtained from a local slaughterhouse and butcher. The beef and chicken meat were chopped into cubes (1–2 cm^3), after which lard was added to obtain a total fat content of 20%. The meat preparations were minced (with an Omega T-12 (Omega Foodtech, Bologna, Italy) equipped with a 10-mm plate) and ground (with a 3.5-mm plate) thoroughly. Subsequently, the meat preparations were heated in a hot water bath (GFL, Grossburgwedel, Germany) for 30 min after reaching a core temperature of 90 °C. As a final step, the meat preparations were homogenized with a food processor, after which they were stored at −20 °C.

2.3. In vitro gastrointestinal digestion of meat preparations

2.3.1. Collection, storage and pre-cultivation of colonic microbiota

Fresh fecal samples were obtained from 7 male and 3 female non-vegetarian volunteers (age ranging from 22 to 75 yrs old) without any medical history of gastrointestinal disease. None of the solicited volunteers underwent antibiotic treatment during at least 6 months prior to donation. All 10 human donors of fecal material were recruited among the laboratory personnel and their family members through informal announcement, after which all participating volunteers gave their written informed consent. The obtained data and volunteer information were analyzed anonymously and de-identified. The research was approved by the Federal Public Service of Health, Food Chain Safety and Environment, Belgium, but there was no need to submit an application to the ethical committee due to the non-invasive nature of the voluntary donation of fecal samples.

Fresh fecal samples were processed according to a protocol adapted from Molly et al. (1994) as has been described previously (Van Hecke et al., 2014). In short, fresh fecal material was diluted in preheated PBS solution (1:4; w/v) to which sodium thioglycolate (1 g/L) was added as a reducing agent. Subsequently, the fecal slurry was filtered (through a 1 mm metal sieve) and stored at −80 °C on a glycerol stock (20%). Prior to the gastrointestinal digestion of meat, the fecal inocula were pre-cultivated anaerobically for 24 h at 37 °C in brain heart infusion (BHI) broth (obtained from Oxoid Ltd, Hampshire, GB) with added cysteine (37 g/L BHI + 0.5 g/L cysteine) at a 1:9 ratio (v/v). Throughout the manuscript, volunteer samples are labeled as P1-P10.

2.3.2. Simulated gastrointestinal digestion of meat preparations

The use of a well-established *in vitro* gastrointestinal digestion model enabled simulation of stomach, small and large bowel digestion of beef and chicken meat preparations. The utilized model has been described on multiple occasions. Therefore, for all details on the utilized *in vitro* digestion model and the prior preparation of all mimicked gastrointestinal juices, brain heart infusion broth and SHIME ('Simulator of the Human Intestinal Microbial Ecosystem') medium, we refer to previous work (Van Hecke et al., 2014; Vanden Bussche et al., 2014).

For this study, 4.5 g of beef or chicken meat preparations were digested *in vitro* in triplicate, using 10 different fecal inocula (n = 2 × 3 x 10 simulated meat digestions). Samples were taken after simulation of duodenal digestion ("T0" samples, whereby sampling took place immediately after addition of SHIME medium and the fecal inoculum; i.e. just prior to colonic digestion) and at the end of the simulated colonic meat digestion ("T48" samples, whereby sampling took place after 48 h incubation with SHIME medium and fecal inocula; i.e. after the colonic digestion). All meat digestion samples were stored at −80 °C until analysis.

To investigate the role of heme iron in red meat induced genotoxicity, an additional experiment revolving myoglobin addition was performed. The following digestions were performed in triplicate; 4.5 g of beef meat preparation (produced as described previously; 'Meat preparations') without added myoglobin, 4.5 g of beef meat preparation with 5 mg of added myoglobin, 4.5 g of beef meat preparation with 50 mg of added myoglobin, 50 mg of myoglobin (without meat or lard), and 4.5 g of lard with 50 mg of added myoglobin. Samples were obtained at "T0" and "T48" and stored at −80 °C. The fecal inoculum used to perform the colonic digestion was selected ad random (P5).

2.4. DNA adductomics analysis

2.4.1. Sample preparation

DNA adducts in meat digests were extracted and purified according to a protocol previously described by Vanden Bussche et al. (Vanden Bussche et al., 2012) and Hemeryck et al. (2015). In brief; at first, 2 internal standards (O^6-d_3-MeG and M_1G-$^{13}C_3$) were added to each sample. Then, DNA was hydrolyzed in 0.1 M formic acid in UP (30 min, 80 °C) to cleave both adducted and non-adducted DNA nucleobases from all DNA sequences present in the meat digestion samples. Subsequently, sample purification and cleanup was performed by means of solid-phase extraction (SPE) (Oasis® HLB cartridges (1 cc, 30 mg) Waters (Milford, USA)), after which the eluted samples were evaporated to dryness (90 min under vacuum, 20 °C). Finally, the dried residue was re-suspended in 100 µL of 0.05% of acetic acid in UP and stored at −20 °C awaiting analysis.

2.4.2. UHPLC-HRMS analysis

Analysis of DNA adducts in meat digests was enabled by ultrahigh performance liquid chromatography coupled to high resolution mass spectrometry (UHPLC-HRMS) as was described previously by Hemeryck et al. (2015). In brief, chromatographic separation was performed with an Acquity BEH C18 Waters column (1.7 µm, 2.1 × 100 mm; Waters Corporation, Milford, USA). The mobile phases consisted of 0.05% of acetic acid in UP and 100% methanol. The flow of the mobile phases (300 µL per min) and injection of samples was accomplished with a Dionex Ultimate 3000 pump and autosampler (Thermo Scientific, San José, USA), and HRMS DNA adduct analysis was performed by means of a hybrid Quadrupole-Orbitrap High Resolution Accurate Mass Spectrometer (HRAM, Q-Exactive, Thermo Fisher Scientific, San José, USA) coupled to a heated electrospray ionization (HESI-II) source. Internal calibration of the MS system was performed daily by infusion of calibration mixtures that were prepared according to the protocol described in the operations manual (Thermo Fisher Scientific, San José, USA). General instrument control and initial data processing were performed with Chromeleon Xpress and Xcalibur™ 3.0.

2.4.3. Data processing and statistics

2.4.3.1. ToxFinder profiling. The use of ToxFinder 1.0 software (Thermo Fisher Scientific, San José, USA) and an in-house DNA adduct database allowed screening of the full scan HRMS spectra of meat digestion samples for alkylation and/or (per)oxidation induced DNA adducts. Only DNA adducts demonstrating a minimum signal intensity of 20,000, a maximum mass deviation of 10 ppm, recurrence and stable retention time (RT) in replicate samples, and the presence of the naturally occurring C^{13} isotope were retained. The hence obtained output was visualized by means of Morpheus software (https://software.broadinstitute.org/morpheus). Student's t-test was used for statistical interpretation of the detected DNA adduct levels. Tentative identification based on accurate mass was checked manually for each compound (Δ ppm < 10). The identities of O^6-methylguanine (O^6-MeG), O^6-carboxymethylguanine (O^6-CMG) and M_1G were confirmed by means of analytical standards.

2.4.3.2. SIEVE pre-processing. To screen digested meat samples for known alkylation and/or oxidation induced DNA adducts, control compare trend analysis was performed using the database lookup function of SIEVE 2.2 (Thermo Fisher Scientific, San José, USA). Combining the spectral data obtained from the three technical replicate digestion samples assured repeatability of the obtained results. Only ions with an *m/z* between 70 and 700 Da, and eluting between 0.7 and 5.6 min of chromatographic analysis were considered. A mass deviation up to 10 ppm was allowed, whilst the maximum peak width consisted of 0.5 min. Chromatographic peak selection was executed for positive and negative ions separately. The maximum number of frames and minimal peak intensity were set at 200,000 and 20,000 arbitrary units, respectively. After automated processing of all raw files, the database lookup function was enabled to match the retrieved matrix features to DNA adduct identities listed in an in-house diet-related DNA adduct database, enabling tentative DNA adduct identification.

2.4.3.3. SIMCA multivariate statistics. SIMCA 14 software (Umetrics AB,

Umeå, Sweden) was used for multivariate statistics, starting with the importation of the output of SIEVE pre-processing. Overall, data analysis was performed combining the data of all 3 technical replicates at all times to ensure robustness and repeatability.

At first, Principal Component Analysis (PCA-X) was performed to enable preliminary data exploration and detection of possible outliers. Subsequently, logarithmic data transformation and Pareto scaling were performed, followed by automated Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) data modelling. The validity of the obtained OPLS-DA model was checked with permutation testing (n = 100), CV-ANOVA ($p < 0.05$), and assessment of R^2 (must approach 1 (= perfect fit)) and Q^2 (=cross-validated R^2, operated with a minimal threshold of 0.5 to ensure correct prediction). For valid models, discriminative ions were selected based on a Variable Importance in Projection-score (VIP-score) > 0.8 because a VIP > 1 demonstrates a high influence, a VIP > 0.8 (but < 1) demonstrates a moderate influence, but a compound with a VIP < 0.8 merely demonstrates a low influence (Van Meulebroek, 2014).

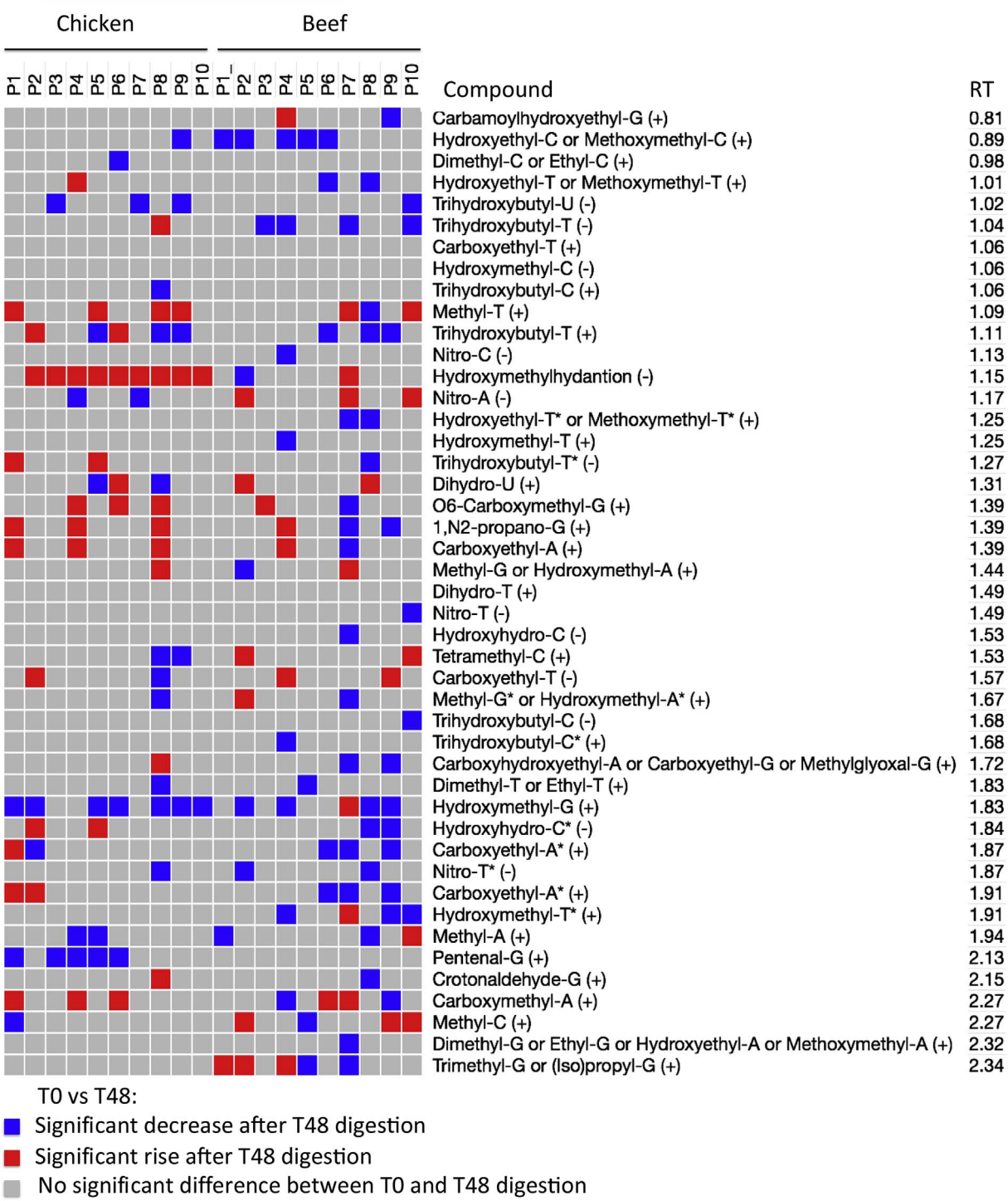

Fig. 1. Heat map displaying the significant ($p < 0.10$) rise or decrease of mean putative DNA adduct levels (different isomers marked with *(*)(*)) during colonic (T48) as opposed to small intestinal (T0) digestion for each of the ten test subjects (P1-P10). "RT" stands for retention time (min). G, C, T, A and U represent guanine, cytosine, thymine, adenine and uracil respectively. (+) or (−) means that specific DNA adduct type was detected in positive or negative ionization mode, respectively.

3. Results and discussion

This study aimed to further unravel the genotoxic effects of red meat consumption due to DNA alkylation and/or oxidation in light of the current hypotheses on the link between red meat consumption and CRC development. It has previously been demonstrated that red meat digestion can promote colon carcinogenesis dependent on heme concentration (Pierre et al., 2004). The exact underlying mechanisms have not been elucidated yet, but it was hypothesized that heme iron readily catalyzes the formation of genotoxic NOCs and LPOs (Bastide et al., 2015). To measure the hence induced DNA adduct formation, an in-house DNA adductomics methodology (Hemeryck et al., 2015) was implemented. The application of this state-of-the-art DNA adductomics platform is highly innovative, allowing us to take the field of DNA adduct research to the next level. Moreover, the DNA adductomics methodology enabled to comprehensively measure DNA adducts and assigned candidate structures that are linked with the consumption of red meat as opposed to white meat, thus helping to further elucidate the mechanisms involved in the red meat - CRC relationship.

Chicken and beef digests (pre-colonic (T0) as well as post-colonic (T48) samples) were screened for the presence of alkylation and/or oxidation induced DNA adducts, enabling comparison of the levels of the retrieved DNA adduct types in different sample types (based on ion abundance; exact quantitation of DNA adduct levels was not executed in light of feasibility).

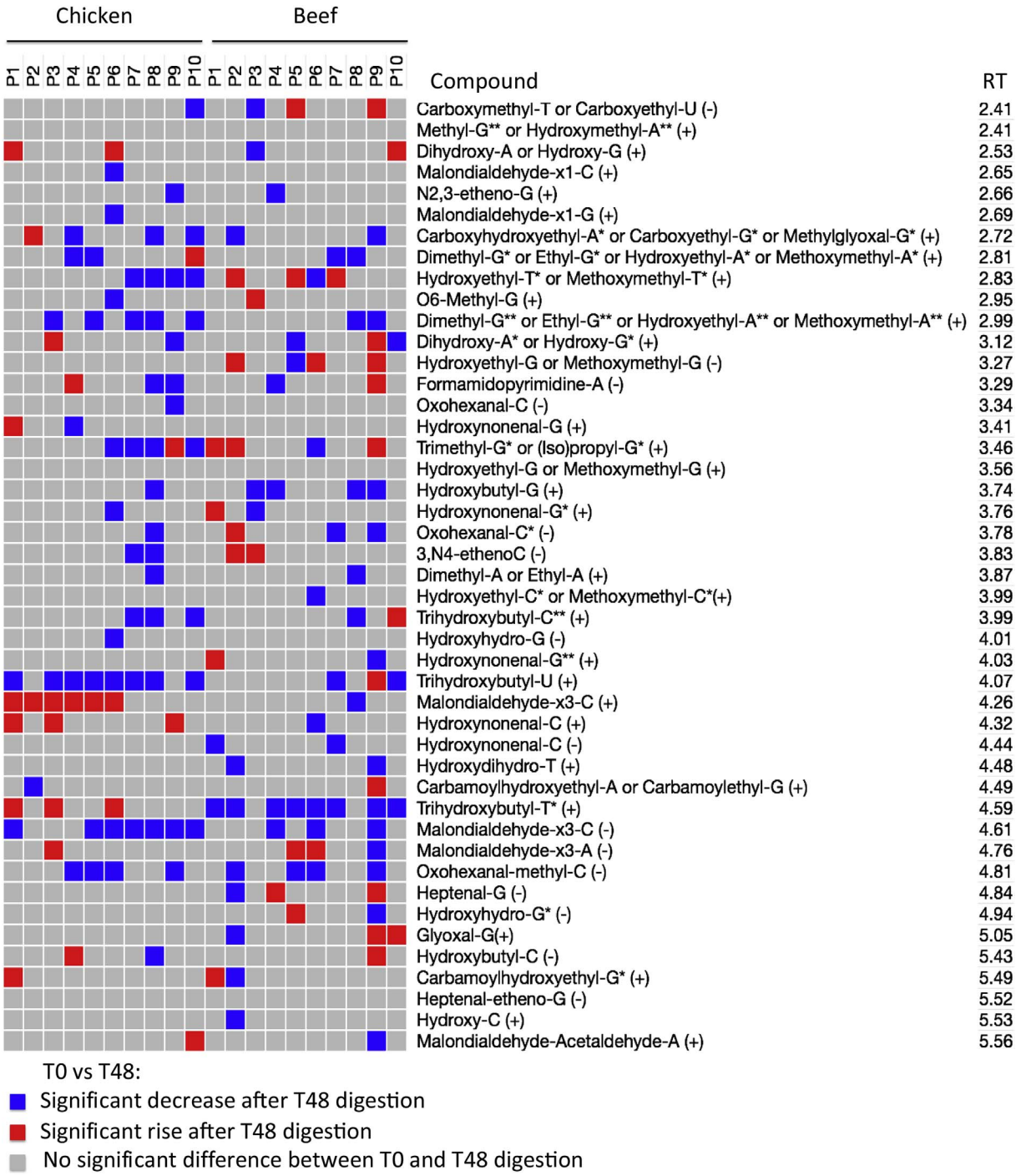

Fig. 1. (*continued*)

3.1. Formation or degradation of DNA adducts during colonic digestion

3.1.1. ToxFinder profiling

The detected abundances of some DNA adduct types increased during colonic digestion, whilst others declined. In supplementary figure 1, the levels of several tentatively identified DNA adduct types in T48 samples are compared to those in T0 samples, this for each test subject separately, by means of a heat map (software.broadinstitute.org/morpheus). Comparison was enabled by subtracting T0 DNA adduct levels from T48 DNA adduct levels (peak areas) after correction for individual sample guanine content, and displaying the difference by color; higher T48 levels (compared to T0) are displayed in red (i.e. the result of the T48 - T0 subtraction is positive), whilst lower T48 levels (compared to T0) are displayed in blue (i.e. the result of the T48 - T0 subtraction is negative). The heat map in supplementary figure 1 displays significant as well as non-significant differences. In Fig. 1, only significant differences are shown (no scale implemented).

As can be observed, DNA adduct profiling demonstrated a clear inter-individual variability with regard to the types and levels of alkylation and/or oxidation induced DNA adducts at the start as well as after *in vitro* colonic meat digestion, which is perfectly in line with previous findings (De Bont and van Larebeke, 2004; Hemeryck et al., 2016b; Lewin et al., 2006; Vanden Bussche et al., 2014). Prior to the start of each colonic digestion, a fecal inoculum is added, resulting in the cultivation of a certain individual's colorectal microbiota in order to mimic colonic meat digestion after enzymatic stomach and small bowel digestion. As such, a pre-colonic digestive sample (T0) can contain DNA adducts because of (a) the interaction between the added fecal DNA (from human, bacterial or dietary origin) and genotoxic molecules formed during the small intestinal digestion of meat, or (b) their presence in the (pre-cultivated) fecal inoculum itself due to prior *in vivo* formation (Vanden Bussche et al., 2014). For example, we have previously demonstrated that the presence of O^6-CMG, an alkylation induced DNA adduct, in meat digests can be linked back to the fecal donor. In other words, some fecal inocula contain and/or lead to the active production of O^6-CMG prior to and/or during colonic digestion, whilst others simply do not (Hemeryck et al., 2016b; Vanden Bussche et al., 2014).

A rise in DNA adduct levels during colonic digestion suggests active formation of its precursor molecules by the colonic microbiota (e.g. O^6-CMG) does no longer rise if the fecal microbiome is neutralized due to autoclavation (Vanden Bussche et al., 2014), whilst a decrease indicates active or passive degradation. Indeed, it has become indisputable that the gut microbiome exerts beneficial as well as detrimental effects on gut and overall human health. For example, with regard to CRC, it has become clear that the gut microbiome actively contributes to cell proliferation, apoptosis, differentiation, and DNA damage. As a result, the gut microbiome, and its metabolic products, strongly influence whether someone develops CRC, or not (Irrazábal et al., 2014; Louis et al., 2014). However, due to the complexity of host-diet-microbiome interactions, a lot of questions still remain (Irrazábal et al., 2014), especially concerning gut microbiome induced DNA adduct formation.

In this study, several oxidation and/or alkylation induced DNA adducts could be detected prior to colonic digestion as well as at the end of digestion. The number of putatively identified DNA adducts that significantly increased during colonic meat digestion exceeds 60, and e.g. includes methyl-T (retention time (RT) of 1.09 min) in test subjects P3, P4, P5, P6 and P9; and methyl-G (or its hydroxymethyl-A isomer, RT 1.44 min) in all 10 test subjects. In contrast, over 40 tentatively identified DNA adduct types demonstrate a significant decline during colonic digestion. The latter DNA adduct types are not of specific interest following colonic digestion, but could be relevant due to their natural *in vivo* occurrence and/or *in vitro* formation following small intestinal meat digestion.

3.1.2. Multivariate statistics

Multivariate statistics (based on SIMCA analysis) revealed a clear discrimination between T0 and T48 samples. More specifically, PCA-X modelling of negative as well as positive ion features showed a distinct grouping of T0 *vs.* T48 samples (supplementary figures 3 and 4), followed by a clear grouping according to test subject (P1 - P10). A valid OPLS-DA model discriminating between T0 and T48 samples could be constructed for each test subject separately and all test subjects combined. OPLS-DA modelling of T0 *vs.* T48 samples confirmed these results, as such demonstrating a distinct difference between both sample types and indicating active formation and/or degradation of DNA adducts during colonic digestion. In Table 1 the characteristics for the different OPLS-DA models are displayed, whereby number of components represents the number of linear combinations of observed variables needed that accounts for a maximal amount of variation in the dataset. OPLS-DA model validity was assessed by (a) R^2X and R^2Y, both goodness-of fit parameters and Q^2Y, a goodness-of-prediction parameter, (b) CV-ANOVA that explains the predictive (variation that is common to both X = predictor and Y = outcome) and orthogonal (variation related to biological and technical factors) differences in the observed variables and (c) permutation tests that explain the total sum of variation in Y. When parameter values are > 0.5 (R^2Y), < 0.01 (b) or are sufficient-excellent (c), good model quality is obtained (Jung et al., 2011; Wiklund et al., 2008). A low R^2X value (e.g. 0.296) is acceptable when the other parameter values meet the set criteria since this value indicates that 29.6% of the variables are used to explain 98.4% (R^2Y) of the observed variances between the two groups, i.e. beef *vs.* chicken T48. Thus, despite a relatively low R^2X, biologically important discriminating DNA adducts could be retrieved from the model (Triba et al., 2015; Zhang et al., 2016).

3.2. DNA adduct profile in red vs. white meat digests

In light of the red meat-CRC hypothesis, DNA adduct types that are more prevalent in beef digests (beef as model for red meat) compared to chicken digests (chicken as a model for white meat) are of specific interest, whilst the increased formation of certain types of DNA adducts due to the digestion of chicken (in comparison to beef) is not. As such, the latter will not be discussed, although all data is reported in figures and tables and can thus be consulted.

Table 1
OPLS-DA model characteristics (T0 *vs.* T48 and Beef *vs.* Chicken).

Model	Charge	Number of components	R^2X	R^2Y	Q^2	CV-ANOVA	Permutation test
T0 *vs.* T48 (beef + chicken samples)	+	1 + 3+0	0.724	0.983	0.963	$p = 0$	Excellent
T0 *vs.* T48 (beef + chicken samples)	–	1 + 3+0	0.760	0.971	0.957	$p = 0$	Excellent
Beef *vs.* chicken (T0 + T48 samples)	+	1 + 9+0	0.830	0.959	0.750	$p < 0.01$	Good
Beef *vs.* chicken (T0)	+	1 + 6+0	0.715	0.986	0.945	$p < 0.01$	Good
Beef *vs.* chicken (T48)	+	1 + 3+0	0.296	0.984	0.615	$p < 0.01$	Good
Beef *vs.* chicken (T0 + T48 samples)	–	1 + 2+0	0.244	0.800	0.236	$p < 0.01$	Good
Beef *vs.* chicken (T0)	–	1 + 5+0	0.245	0.995	0.633	$p < 0.01$	Good
Beef *vs.* chicken (T48)	–	1 + 2+0	0.203	0.880	0.255	$p = 0.014$	Sufficient

3.2.1. ToxFinder profiling

In supplementary figure 2, differences in DNA adduct levels between beef and chicken digests are presented, this for each test subject separately. To enable straightforward comparison, the same method was applied as in section 3.1.1, but instead of substracting T0 from T48 samples, chicken samples were substracted from beef samples. The heat map in supplementary figure 2 displays significant as well as non-significant differences (with color scale). In Fig. 2, only significant differences are shown (no color scale implemented).

Strikingly, a lot of DNA adducts that were significantly up or down regulated following colonic fermentation of both beef and chicken (Fig. 1) have more or less the same abundances in both meat digestion samples (since no significant differences between both meat types could be observed) (Fig. 2). At first glance, this could, to some degree, question red meat specific genotoxicity. Nevertheless, not all DNA adducts are evenly carcinogenic and DNA repair pathways, which involve distinct mechanisms for different types of DNA adducts, can be influenced by genetic and environmental (e.g. dietary) factors (Fahrer and Kaina, 2017). To illustrate this, *in vitro* and *in vivo* studies in rodents have shown that natural antioxidants can induce higher activity and expression of O^6-methylguanine-DNA methyltransferase (MGMT), an enzyme that removes O^6-alkylguanine adducts (Huber et al., 2003;

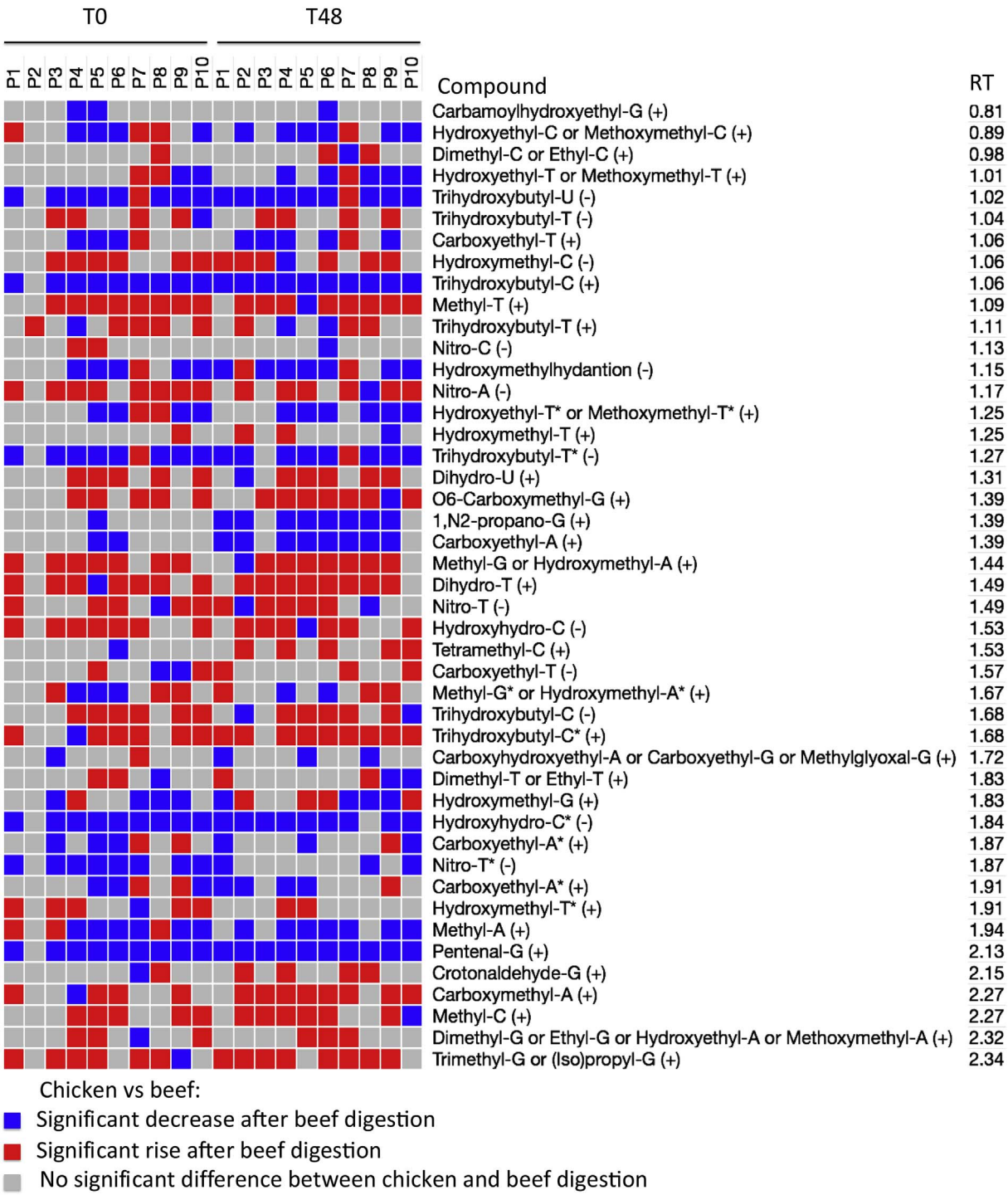

Fig. 2. Heat map displaying the significant ($p < 0.10$) rise or decrease of mean putative DNA adduct levels (different isomers marked with *(*)(*)) during beef as opposed to chicken digestion for each of the ten test subjects (P1-P10) (please consult Fig. 1 for explanation of abbreviations).

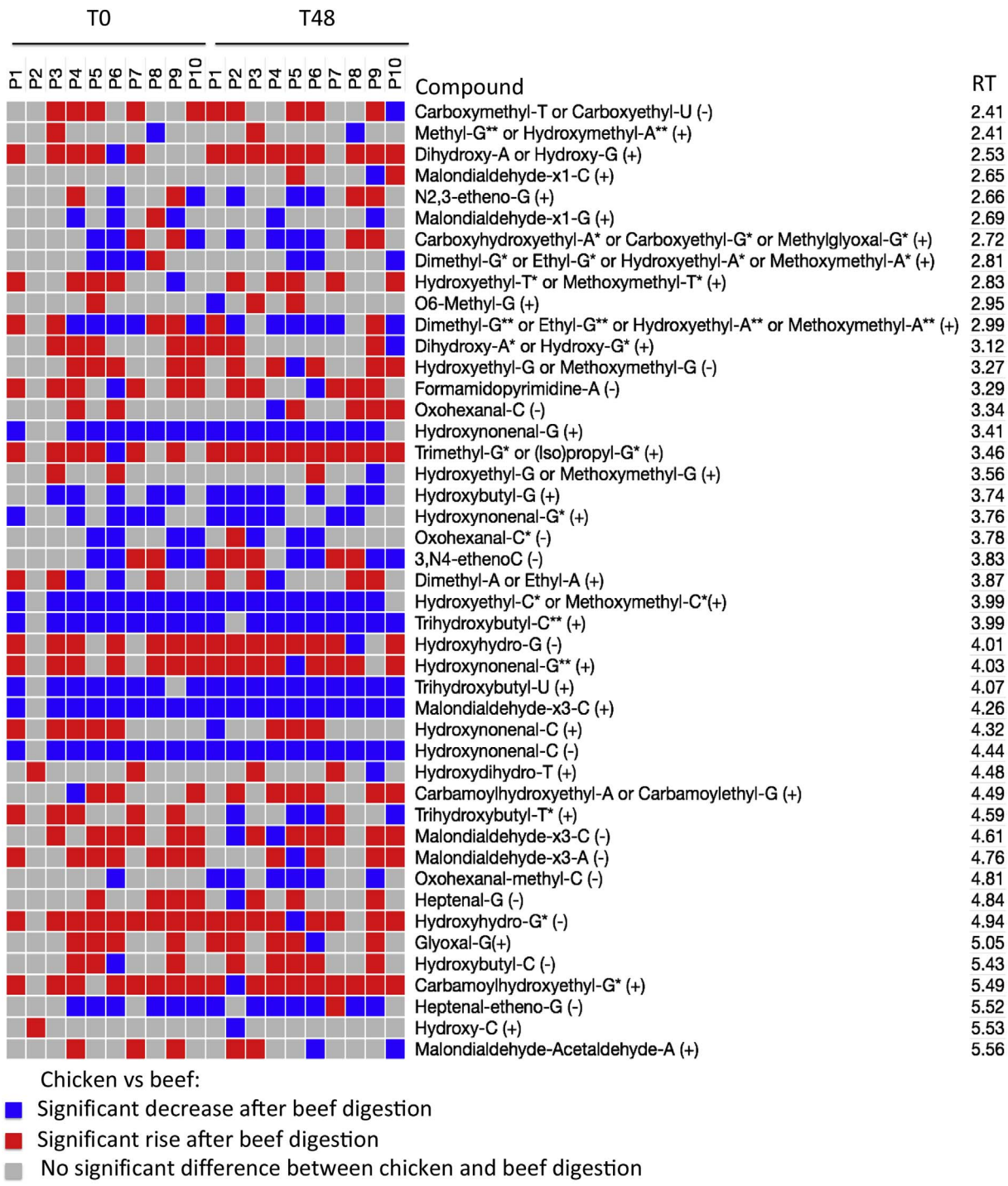

Fig. 2. (*continued*)

Niture et al., 2007). Hence, based on conclusive epidemiological evidence that red and not white meat consumption has been associated with the development of CRC and because of the previously reported differences in biological relevance and repair mechanisms of DNA adducts, it can be assumed that DNA adducts demonstrating 'similar behavior' for both meat types are most likely less relevant towards the development of cancer as opposed to those that are up-regulated after beef digestion only.

3.2.2. Multivariate statistics

SIMCA analysis was performed to investigate whether multivariate statistics could be used to discriminate between beef and chicken digests, as such also enabling the selection of discriminating DNA adduct types.

PCA-X modelling of negative as well as positive ion features did not reveal a distinct grouping of chicken *vs.* beef digests according to DNA adduct profile. As was mentioned previously, it did document a clear distinction between T0 and T48 samples, followed by a clear grouping according to test subject.

During OPLS-DA analysis of positive ion features, a valid model including all test subject digestions could be constructed to discriminate beef digests from chicken digests for T0 and T48 samples combined as well as for T0 and T48 samples separately. With regard to negative ion feature OPLS-DA modelling (using the raw data from all 10 test subject

digestions), discrimination between beef *vs.* chicken digests could only be performed for T0 samples. Valid OPLS-DA models that met all previously set criteria could not be constructed for T0 and T48 combined, and for T48 samples. An overview of the obtained OPLS-DA model characteristics is provided in Table 1.

The valid OPLS-DA model constructed to discriminate between beef and chicken digests in T0 samples (based on positive ion and negative feature data) rendered 4 DNA adduct types with marker potential; i.e. dimethyl-T (or ethyl-T), hydroxymethyl-T, tetramethyl-T, and methylguanine (MeG). Furthermore, guanidinohydantoin and hydroxybutyl-C were retained as discriminating between beef and chicken digestion samples after colonic fermentation (T48) (Table 2). These DNA-adducts will be discussed further on (2.4 Potential red meat digestion markers). The multivariate statistics approach proved to be a powerful complementary platform next to ToxFinder since clear distinct DNA adducts profiles between both meat types prior to colonic fermentation could be successfully obtained, as such revealing potential DNA adduct markers.

3.3. The effect of myoglobin digestion on DNA adduct profile

In total, 5 different experiments were set up to decipher the interfering role of myoglobin in red meat genotoxicity. ToxFinder profiling was performed for each sample; average putative DNA adduct levels in T0 as well as T48 samples are shown in supplementary figure 5 (with color scale). Fig. 3 enables correct statistical interpretation of the observed differences (without color scale).

Based on ToxFinder profiling, a total of 34 different alkylation and/or oxidation induced DNA adduct types significantly ($p < 0.10$) increased in pre-colonic digestion samples upon addition of (5 mg of) myoglobin. Several of these specific DNA adduct types demonstrated significant potential as heme-rich meat digestion markers, and will therefore be discussed in detail below (2.4 Potential red meat digestion markers).

3.4. Potential red meat digestion markers

3.4.1. Hydroxymethylhydantion and malondialdehyde-x3-C

In Fig. 2, it could be observed that the hydroxymethylhydantion and malondialdehyde-x3-C DNA adducts were significantly higher ($p < 0.05$) in pre-colonic beef digests compared to chicken digests for at least 6 test subjects. Hydroxymethylhydantion, a ROS induced thymine alteration (Cooke et al., 2003), was significantly higher after small bowel digestion of beef in comparison to chicken for all 10 test subjects but P1, suggesting that small bowel beef digestion induced oxidative stress and ROS production. A DNA adduct type with a highly similar behavioral pattern is malondialdehyde-x3-C (M_3C, eluting at 4.26 min); i.e. M_3C was significantly higher in pre-colonic beef digests for 6 test subjects. M_3C is a cytosine analogue formed due to the interaction with 3 malondialdehyde molecules, whilst malondialdehyde (MDA) itself is a well-known LPO (Marnett, 1999; Stone et al., 1990). In previous work, we were able to demonstrate that (lipid) peroxidation primarily occurs prior to colonic digestion (Vanden Bussche et al., 2014). Therefore, the retrieval of ROS and/or LPO induced DNA adducts in pre-colonic digestion samples is perfectly in line with expectations. A similar trend for hydroxymethylhydantion and/or M_3C could not be observed in T48 samples, myoglobin addition did not significantly increase M_3C and/or hydroxymethylhydantion levels.

3.4.2. Methylguanine

Methylguanine (MeG) DNA adducts are among the most commonly studied alkylation DNA adduct types. In contrast to our previous *in vitro* meat digestion studies, we were able to detect O^6-MeG in pre- and post-colonic meat digests. More specifically, O^6-MeG significantly increased during colonic meat digestions performed with 4 out of 10 fecal inocula (Fig. 1). A clear trend with regard to red *vs.* white meat digestions could however not be observed (Fig. 2). We were also able to detect 3 methylated guanine residues besides O^6-MeG; MeG isomers eluting at 1.44, 1.67 and 2.41 min respectively. The first isomer (RT 1.44 min) is most striking since it could be detected for all 10 test subjects. At first glance, there was no distinct pattern according to digested meat type. Addition of myoglobin to beef digestion seemed to increase the concentration of this specific MeG isomer, although not significantly ($p > 0.10$) (as can be observed in supplementary figure 5). Nevertheless, multivariate statistics labeled this MeG isomer as a discriminative molecule for beef digestion across all 10 test subjects (Table 2). Because this MeG isomer demonstrated the highest signal intensities, it most likely corresponds to 7-MeG, the most prominently formed DNA alkylation lesion that has previously been detected *in vivo*, but is not promutagenic (De Bont and van Larebeke, 2004; Povey et al., 2002). On the other hand, DNA methylation does regulate gene expression, whilst the presence of 7-MeG in a DNA sequence can also prematurely end DNA replication (De Bont and van Larebeke, 2004), demonstrating the *in vivo* relevance of 7-MeG as a DNA alkylation marker.

3.4.3. Dimethyl-T (or ethyl-T), hydroxymethyl-T and tetramethyl-T

Besides MeG, multivariate statistics delivered dimethyl-T (or ethyl-T), hydroxymethyl-T and tetramethyl-T as potential DNA alkylation (and also oxidation in case of hydroxymethyl-T) red meat digestion markers (Table 2) in T0 samples. Information on the *in vivo* as well as *in vitro* formation of dimethyl-T (or ethyl-T), hydroxymethyl-T and tetramethyl-T in the context of food digestion and/or cancer development is negligible at the time being, apart from dimethyl-T, which can alternatively correspond to ethyl-T. More specifically, e.g. O^4-ethylthymine (O^4-eT) has previously been detected *in vivo* and linked to the daily exposure to ethylating agents (Huh et al., 1988; Kang et al., 1995). We know that O^4-eT can induce DNA miscoding, rendering O^4-eT to be a compound of interest in the context of cancer initiation (De Bont and van Larebeke, 2004). Huh et al. furthermore documented that this compound was significantly more present in malignant liver tumors compared to non-tumoral tissue (Huh et al., 1988).

3.4.4. Guanidinohydantoin and hydroxybutyl-C

In T48 samples, 2 potential red meat digestion markers could be retrieved by means of multivariate statistics, namely guanidinohydantoin and hydroxybutyl-C. Information on the *in vitro* and *in vivo* formation of hydroxybutyl-C, and its potential mutagenic or carcinogenic effects, seems to be non-existent. Its G analogue has however been detected in urothelial and hepatic DNA of rats that were given N-nitrosobutyl(4-hydroxybutyl)amine, a known bladder carcinogen prone to DNA alkylation (Airoldi et al., 1994). Guanidinohydantion on the other hand, is a secondary DNA lesion formed by oxidation of 8-oxoguanine, a primary oxidative G lesion that is rather unstable and prone to further oxidation (Hailer et al., 2005; Sugden and Martin, 2002). Its retrieval suggests the occurrence of oxidative stress and formation of ROS due to colonic red meat digestion, followed by extended and/or extensive oxidation of the G nucleobase.

Table 2
DNA adducts discriminative for beef digests as opposed to chicken digests.

DNA adduct name	Prior to (T0) or after (T48) colonic fermentation	RT (min)	Charge	Δ ppm	VIP-score
Dimethyl-T or ethyl-T	T0	0.72	+	2.92	1.95
Hydroxymethyl-T	T0	0.77	+	3.00	0.89
Methyl-G	T0	1.46	+	3.40	1.23
Tetramethyl-T	T0	2.74	+	3.03	1.66
Guanidinohydantoin	T48	5.36	+	4.88	1.51
Hydroxybutyl-C	T48	4.53	+	4.63	2.46

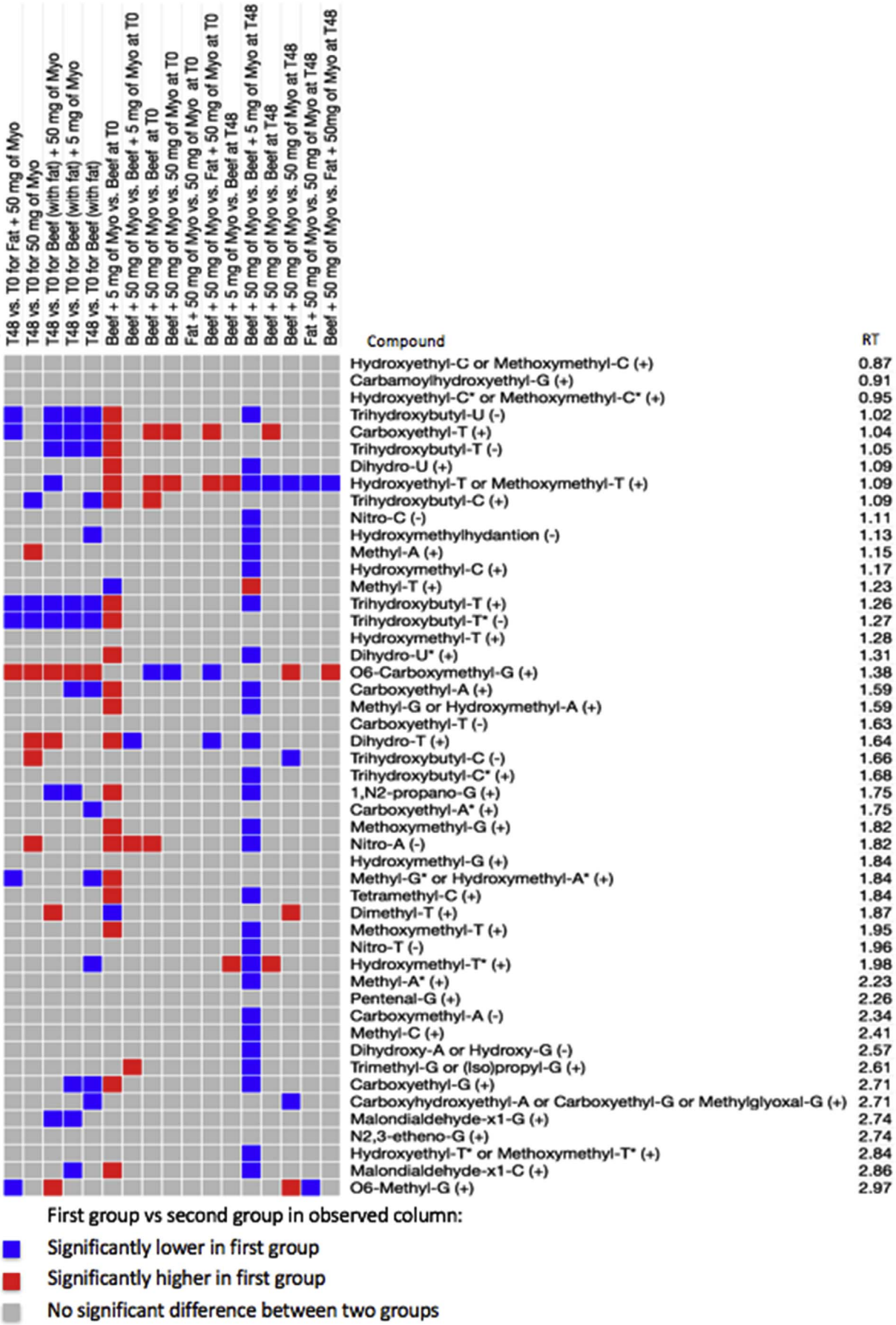

Fig. 3. Heat map (without color scale) displaying significant ($p < 0.10$) differences in putatively detected DNA adduct levels in samples from the different experimental setups (please consult Fig. 1 for explanation of abbreviations). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

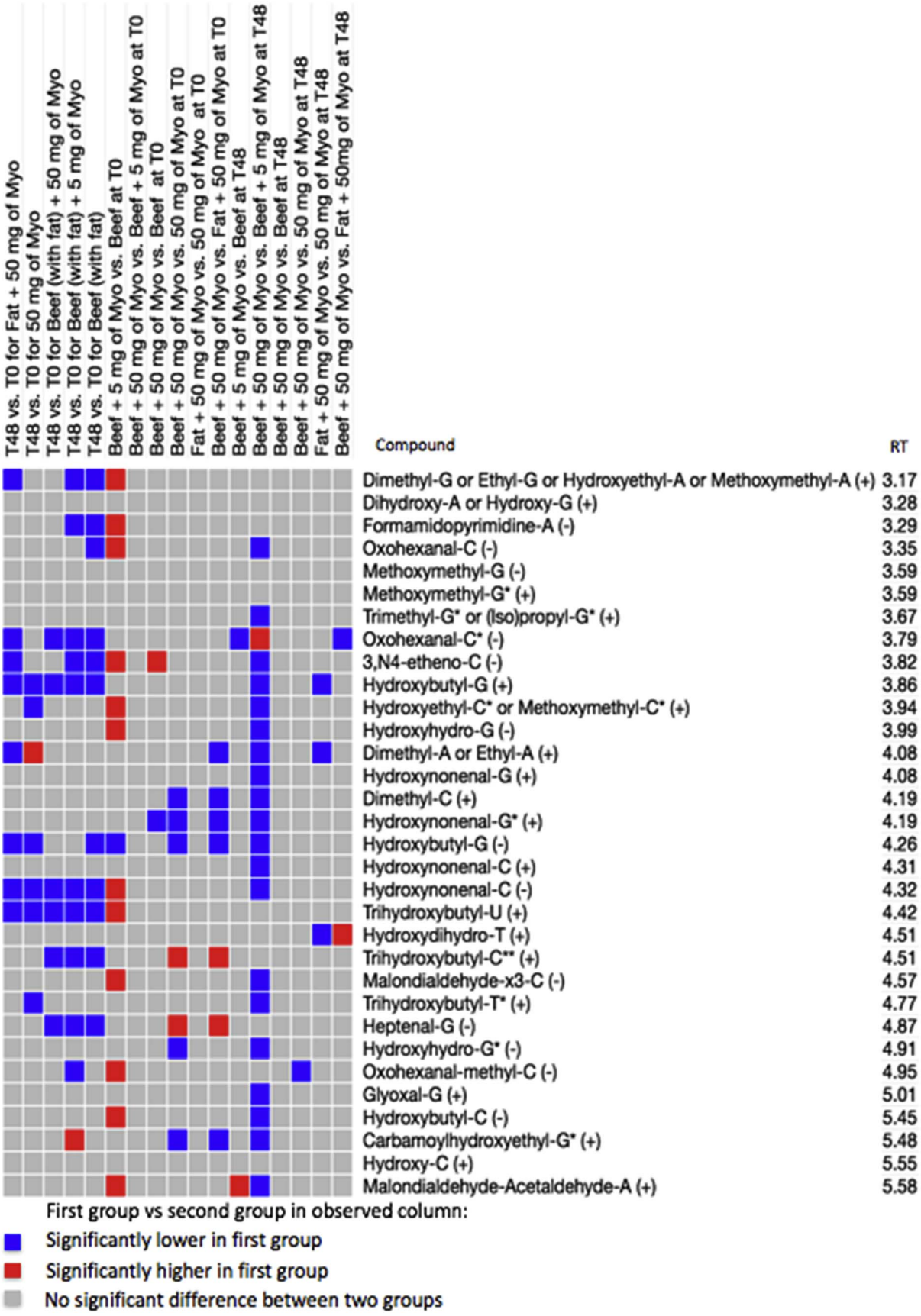

Fig. 3. (*continued*)

3.4.5. O^6-carboxymethylguanine

To the best of our knowledge, O^6-CMG is the only DNA adduct type of which a significant increase has directly been associated with red meat consumption *in vivo* (Lewin et al., 2006). Despite substantial inter-individual variation in its gastrointestinal formation (Lewin et al., 2006; Vanden Bussche et al., 2014), we were able to document that O^6-CMG (RT 1.39 min) significantly rises during the *in vitro* digestion of red meat (Fig. 2), and upon myoglobin addition (Fig. 3), in this as well as previous work (Vanden Bussche et al., 2014). Remarkably, following colonic fermentation of chicken also an increase in O^6-CMG could be observed (Fig. 1). This can be attributed to the presence of alkylating dietary compounds in the fecal inocula. Also, at individual level, a significant increase of O^6-CMG after colonic digestion of chicken is always accompanied by a significant increase after colonic beef fermentation (cfr. P4-P5, P7-P8, P10). Moreover, the opposite is true for P3, P6 and P9, where only a significant difference between colonic and small intestinal digestion for beef but not for chicken was observed. This implies that these results do not show evidence for chicken meat associated formation of O^6-CMG but, more importantly, support the linkage of this DNA adduct with red meat consumption. Since we already know that O^6-CMG is actively formed by the colonic microbiota during colonic meat digestion (Vanden Bussche et al., 2014), the results of this study emphasize the potential relevance of O^6-CMG formation in relation to red vs. white meat digestion and the need for follow-up research.

3.4.6. Hydroxyethyl-T

Hydroxyethyl-T (eluting shortly after 1 min), which might alternatively correspond to methoxymethyl-T (= isomer), appeared to be higher in digests of beef compared to chicken for 5 different fecal inocula ($p > 0.10$ for 4 out of 5 and $p = 0.0003$ for 1 out of 5), and significantly increased due to addition and digestion of myoglobin; $p = 0.034$ for 5 mg of added myoglobin, and $p = 0.007$ for 50 mg of added myoglobin (as can be seen in supplementary Fig. 2 and Figs. 2 and 3, respectively). Hydroxyethyl-T, or its methoxymethyl-T analogue, is best known for its potential antiviral properties (Wang and Seifert, 1996) but has, to the best of our knowledge, never been linked to *in vivo* environmental genotoxicity. In contrast, hydroxyethyl-G has previously been detected *in vivo*, and is furthermore known to originate from several possible sources including lipid peroxidation (De Bont and van Larebeke, 2004). Hydroxyethyl-T may very well have a similar origin.

3.4.7. Carboxyethyl-T

Carboxyethyl-T (eluting shortly after 1 min) was higher ($p > 0.10$) in pre-colonic beef digestion samples (compared to chicken) for 6 out of 10 test subjects (supplementary figure 2), and furthermore significantly rose upon myoglobin addition (Fig. 3, $p = 0.056$ for 5 mg of myoglobin, $p = 0.004$ for 50 mg of myoglobin). For certain fecal inocula/test subjects, this particular DNA adduct type also appeared to be (significantly) higher ($p < 0.10$) in post-colonic beef digests (compared to chicken), although myoglobin addition did not significantly influence post-colonic DNA adduct levels (on display in Figs. 2 and 3, respectively). Carboxyethyl-T, formed by alkylation of T, has not previously been detected *in vivo*, but has been synthetized *in vitro* (Segal et al., 1980).

3.4.8. 3,N^4-etheno-C

3,N^4-etheno-C (RT of 3.83 min) was higher ($p < 0.10$) in beef digests using 4 out of 10 fecal inocula (Fig. 2), and also significantly increased due to the digestion of added myoglobin ($p = 0.003$ for 5 mg, and $p = 0.098$ for 50 mg; on display in Fig. 3). As was the case for carboxyethyl-T, certain fecal inocula/test subjects demonstrated (significantly) higher ($p < 0.10$) 3,N^4-etheno-C levels in post-colonic beef digests (compared to chicken), although myoglobin addition did not significantly influence post-colonic DNA adduct levels (as can be seen in Figs. 2 and 3, respectively). 3,N^4-etheno-C is a known lipid peroxidation induced DNA adduct type that has previously been detected *in vivo* and has furthermore been associated with oxidative stress, base pair substitution mutations and an increased cancer risk (De Bont and van Larebeke, 2004).

3.5. In vitro model vs. in vivo situation and in vivo relevance

Red *vs.* white meat digestion experiments were performed by means of a static *in vitro* digestion model, sequentially exposing the meat to simulated mouth, gastric, small and large intestinal digestion. Said *in vitro* model is very versatile and as such ideally suited for mechanistic explorative work (Hur et al., 2011). Furthermore, the use of an *in vitro* model is preferred over the use of an *in vivo* (e.g. rodent) model due to ethical considerations, whilst the first is also less complex, costly and time-consuming than the latter. In contrast, the employed *in vitro* model demonstrates certain flaws. More specifically, the model does not allow absorption of digestive metabolites and/or interaction with the intestinal wall (Hur et al., 2011). Therefore, genotoxic metabolites formed during simulated digestion of meat, could not directly interact with the intestinal mucosa (and its DNA). In previous work, we added Caco-2 DNA to meat digests to measure the direct interaction between genotoxic meat digestion metabolites and human DNA to remedy this drawback. However, in contrast with expectations, the observed DNA adduct levels did not differ with and without addition of Caco-2 DNA (Vanden Bussche et al., 2014). Human exposure to dietary and/or gastrointestinally formed mutagens could alternatively be studied by other methods such as the frequently used comet assay that assesses DNA strand breaks. More specifically, the comet assay can be employed in colonic cancer cell lines (e.g. HT29, Caco-2, HT29 clone 29A) following exposure to fecal water. Notwithstanding, several studies based on this assay did not observe significant differences between red meat and fish diets or red meat and vegetarian diets (Cross and Sinha, 2004; Joosen et al., 2009). Surprisingly, one study concluded that vegetarian fecal water was more genotoxic as compared to fecal water originating from meat diets (Joosen et al., 2010). All these studies failed to find correlations between fecal NOC concentrations and DNA damage by using the comet assay although NOCs are well known and studied genotoxins (Lijinsky, 1992). Therefore, Gratz et al. (2011) suggested that the comet assay is not suitable to detect genotoxic effects of fecal water or that the combination of genotoxic and genoprotective compounds in fecal water may cover up any possible effects (Gratz et al., 2011). Moreover, the comet assay is not representative for all types of DNA damage since it only monitors DNA strand breaks, whereas e.g. DNA adduct formation does not necessarily induce DNA strand breaks. Therefore, another study applied the polymerase arrest assay to specifically measure DNA adducts induced by fecal water (Greetham et al., 2007). However, for both the comet assay and the polymerase arrest assay, it is necessary to micro-/ultrafilter fecal water before addition to cell lines or incubation with DNA to remove bacteria and DNA degrading products, resulting in a setup that does not directly represent the *in vivo* situation. For example, ± 25% loss of ATNCs could be observed after filtering fecal water (Greetham et al., 2007).

In the hence utilized *in vitro* digestion model, it is assumed that DNA adduct levels in meat digests originate from the direct interaction with bacterial DNA, which is overly abundant in the digestion flasks. By analogy, DNA adducts could also have originated from the interaction with DNA contained in the digested meat, although the exact origin of the DNA adduct containing DNA was not investigated at the time. This results in the fact that, in the utilized *in vitro* model, prokaryote DNA adduct formation is used as a proxy for eukaryote DNA adduct formation. Although prokaryote and eukaryote DNA do demonstrate some distinct differences (e.g. differences in DNA repair functions), the DNA building blocks are chemically identical. Studies on DNA damage and DNA repair very often use prokaryotic DNA as a tool to investigate similar processes in eukaryotic DNA (Bignami et al., 2000) assuming that the overall interaction between genotoxic molecules and the

nucleobases in eukaryotic and prokaryotic DNA are similar, and hence permitting the use of prokaryotic DNA adduct formation as a model for eukaryotic DNA adduct formation. The same applies for DNA adduct repair; e.g. DNA lesions induced by alkylating agents (e.g. NOCs) can be repaired by mechanisms such as direct base repair (methyltransferases or oxidative demethylases), base excision repair (DNA glycosylases) or by nucleotide excision repair mechanisms that exists in prokaryotes and eukaryotes alike. These repair mechanisms have been extensively studied in *E. Coli* whereby the Ada regulon, i.e. a set of genes that is being expressed in response towards alkylating agents (e.g. NOCs), has been crucial in this context. Although the enzymes involved in human DNA repair mechanisms are not entirely the same as those within bacteria, repair of alkylated DNA lesions is highly similar. For example, the bacterial methyltransferase enzyme Ada and the human homologue O^6-alkylguanine-DNA-alkyltransferase (AGT) or O^6-methylguanine-DNA methyltransferase (MGMT) both repair O^6-methylguanine by transferring the methyl group to a cysteine residue of the enzyme (Drabløs et al., 2004). With respect to lipid peroxidation induced DNA damage, DNA adducts such as e.g. M_1G and etheno-dA are primarily repaired by highly similar bacterial and mammalian nucleotide excision repair pathways (Marnett, 2000).

To the best of our knowledge, there is no existing valid *in vitro* digestion model that does allow the direct interaction with human DNA. Therefore, taking all of the above into account, the experimental setup of the current study provides a very good *in vitro* alternative to investigate human exposure to fecal mutagens. Moreover, over the years, the utilized *in vitro* digestion model (or variations thereon) has repeatedly proven to be a valid research tool. Simulation of colonic fermentation is rather complex since *in vivo* microbial communities need to be implemented successfully in an *in vitro* set-up. Nevertheless, Molly et al. (1994) demonstrated that microbial fermentation of polysaccharides and enzymatic processes are in concordance with *in vivo* circumstances (Molly et al., 1994). Additionally, and more specifically, the suitability of the use of *in vitro* digestion models to investigate heme iron induced interferences (e.g. LPO peroxidation and NOCs) has clearly been demonstrated in literature. For example, different studies assessed lipid peroxidation in *in vitro* digestion fluids of different meat preparations (such as beef, chicken, pork and fish) (Steppeler et al., 2016; Van Hecke et al., 2014). In other studies, myoglobin was added to food emulsions to study hydroperoxides and MDA formation after simulated gastric and/or intestinal conditions (Kenmogne-Domguia et al., 2012; Lorrain et al., 2012). In addition, untargeted metabolomics analysis of *in vitro* meat digestion samples revealed interesting red meat associated pathways potentially linked to the development of CRC, cardiovascular diseases and diabetes mellitus (Rombouts et al., 2017). With regard to heme related DNA adduct formation, O^6-CMG was detected after the simulated digestion of red meat and showed a dose-response association with myoglobin (Vanden Bussche et al., 2014). As such, these findings ensure the validity, reproducibility and relevance of *in vitro* digestion models, experiments and hence obtained results.

DNA adduct types demonstrating a significantly higher formation due to the digestion of beef compared to chicken are of specific interest because those particular DNA adduct types could be of importance with regard to CRC initiation through N-nitrosation and lipid peroxidation processes during red meat digestion. However, since not all DNA adduct types are (as) promutagenic and/or procarcinogenic (e.g. 7-methylguanine (7-MeG) is not mutagenic, whilst O^6-MeG is (Povey et al., 2002)), the reported *in vitro* observations require linkage to (intermediate) effects and/or disease outcome *in vivo*. Unfortunately, the DNA adductomics methodology/technology has not been implemented in *in vivo* CRC studies, apart from a recently published rat feeding trial (Hemeryck et al., 2017). Hence, at the time being, information on *in vivo* DNA adduct formation in relation to CRC is quite limited. Nevertheless, it has previously been demonstrated that e.g. normal colonic tissue of CRC patients contains significantly higher DNA adduct levels compared to colonic tissue from healthy controls (Pfohl-Leszkowicz et al., 1995), and that cancerous tissue contains higher DNA adduct levels compared to non-cancerous tissue from healthy volunteers as well as CRC patients (Al-Saleh et al., 2008). Therefore, follow-up research is warranted.

3.6. Observed patterns in pre-vs. post-colonic meat digests

Throughout this study, the genotoxic effects of beef seemed to be more pronounced in pre-colonic digests in comparison to post-colonic digests. We notice that this is most likely due to a larger variety in catabolic as well as anabolic reactions in the (simulated) large bowel. After all, the large bowel is a reaction vessel, subject to a highly diverse range of microbiotic activities (Louis et al., 2014), whilst digestion in the stomach and small bowel mainly consist of purely mechanical and chemical reactions and interactions, which are furthermore assumed to be identical throughout the entire experimental setup in this study. As a result, overall variation is considerably lower in pre-colonic meat digestion samples compared to post-colonic digestion samples, attributing to the fact that the genotoxicity of beef could not always be confirmed in post-colonic meat digestion samples.

3.7. Summary of current and previous findings

The retrieval of several alkylation and/or oxidation induced DNA adduct types in relation to red meat digestion is of importance to the heme, NOC and lipid peroxidation hypotheses, and suggests that the formation of these specific DNA adduct types may contribute to red meat consumption related CRC risk. Specific attention should be given

Table 3
DNA adduct markers assigned to red meat digestion in this and related studies.

DNA adduct name	Source	Context	Test	p-value or VIP-score
O^6-CMG	(Vanden Bussche et al., 2014)	*In vitro* digestion of beef (compared to chicken)	ANOVA	$p = 0.05$
	(Hemeryck et al., 2016b)	*In vitro* digestion of beef (compared to chicken)	Student's t-test	$p < 0.01$
	This study	*In vitro* digestion of beef (compared to chicken)	Student's t-test	$p = 0.05$
Dimethyl-T or ethyl-T	(Hemeryck et al., 2016b)	*In vitro* digestion of beef (compared to chicken)	SIEVE pairwise comparison	$p = 0.02$
	This study	*In vitro* digestion of beef (compared to chicken)	SIMCA analysis	VIP = 1.95
MeG (not O^6-MeG)	(Hemeryck et al., 2017)	Increased *in vivo* formation due to addition of fat	Student's t-test	$p = 0.03$
	This study	*In vitro* digestion of beef (compared to chicken)	SIMCA analysis	VIP = 1.23
Heptenal-G	(Hemeryck et al., 2017)	*In vivo* digestion of beef (compared to chicken)	Student's t-test	$p = 0.03$
	This study	*In vitro* digestion of beef (compared to chicken)	Student's t-test	$p = 0.05$
Carbamoyl-hydroxyethyl-G	(Hemeryck et al., 2017)	*In vivo* digestion of beef (compared to chicken)	Student's t-test	$p = 0.04$
	This study	*In vitro* digestion of beef (compared to chicken)	Student's t-test	$p = 0.03$
M_2G	(Hemeryck et al., 2016b)	*In vitro* digestion of beef (compared to chicken)	SIEVE pairwise comparison	$p = 0.05$
	(Hemeryck et al., 2017)	*In vivo* digestion of beef (compared to chicken)	GENE-E marker selection	$p = 0.02$
M_3C	(Hemeryck et al., 2016b)	*In vitro* digestion of beef (compared to chicken)	SIEVE pairwise comparison	$p < 0.01$
	This study	*In vitro* digestion of beef (compared to chicken)	Student's t-test	$p = 0.01$

to O^6-CMG, MeG, dimethyl-T (or ethyl-T), M_3C, malondialdehyde-x2-G (M_2G) and carbamoylhydroxyethyl-G since we have been able to repeatedly and consistently associate the formation of these DNA adduct types to red meat digestion in this and/or previous *in vitro* and *in vivo* heme-rich meat digestion studies (summarized in Table 3).

O^6-CMG, MeG and dimethyl-T (or ethyl-T) are formed due to DNA alkylation, which may occur due to endogenous as well as exogenous exposure to alkylating chemicals like NOCs (De Bont and van Larebeke, 2004). *In vivo* as well as *in vitro* methylation and ethylation of guanine and thymine has previously been documented, but only O^6-CMG was previously linked to red meat consumption (De Bont and van Larebeke, 2004; Lewin et al., 2006). M_3C and M_2G are DNA adduct types that originate from the interaction between MDA and DNA (Marnett, 1999; Stone et al., 1990). The previously documented rise in the formation of MDA upon heme-rich meat digestion (Vanden Bussche et al., 2014) supports these results although the occurrence of M_2G could not be linked to red meat digestion in the current study. The formation of heptenal-G also aligns with a heme-iron induced increase in lipid peroxidation since heptenal is another well-known LPO (Chung et al., 2003). Carbamoylhydroxyethyl-G has previously been detected *in vivo* in association with acrylamide exposure (Besaratinia and Pfeifer, 2005). Nevertheless, nothing about the carbamoylhydroxyethyl-G structure suggests that the formation of this DNA adduct type is strictly limited to the attack of the guanine nucleobase by acrylamide; i.e. other diet-related genotoxins, including NOCs and/or LPOs, may be able to contribute to its formation.

4. Conclusions

In conclusion, it has been demonstrated that the use of a DNA adductomics platform, implementing an in-house DNA adduct database, allows mapping of diet-related DNA adducts in red *vs.* white meat digests. Different NOC- and LPO-related DNA adduct types could be tentatively identified, which is highly relevant with regard to the red meat-CRC heme hypothesis. Unfortunately, the available information on the human *in vivo* occurrence of a large variety of these DNA adduct types and their relevance in the context of cancer risk, is mostly lacking at the time. Therefore, the *in vivo* relevance of the retrieved DNA adduct types and levels awaits further confirmation. Moreover, absolute quantification of the selected DNA adducts in prospective *in vivo* studies is recommended because more in depth information about relationships between DNA adducts and disease could hence be established. Nevertheless, the results of this study have aided the exploration of red meat and/or heme induced genotoxicity, and can furthermore be used as future reference for *in vivo* DNA adduct profiling studies.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

The authors would like to thank all volunteers for their kind participation and would furthermore like to express their gratitude to D. Stockx, J. Goedgebuer and M. Naessens.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fct.2018.02.032.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.fct.2018.02.032.

References

Airoldi, L., Magagnotti, C., Bonfanti, M., Chiappetta, L., Lolli, M., Medana, C., De Gregorio, G., Fanelli, R., 1994. Detection of O6-butyl- and O6-(4-hydroxybutyl) guanine in urothelial and hepatic DNA of rats given the bladder carcinogen N-nitrosobutyl(4-hydroxybutyl)amine. Carcinogenesis 15, 2297–2301.
Al-Saleh, I., Arif, J., El-Doush, I., Al-Sanea, N., Jabbar, A.A., Billedo, G., Shinwari, N., Mashhour, A., Mohamed, G., 2008. Carcinogen DNA adducts and the risk of colon cancer: case-control study. Biomarkers 13, 201–216.
Balbo, S., Brooks, P., 2015. Implications of acetaldehyde-derived DNA adducts for understanding alcohol-related carcinogenesis. In: Vasiliou, V., Zakhari, S., Seitz, H.K., Hoek, J.B. (Eds.), Biological Basis of Alcohol-induced Cancer. Springer International Publishing, pp. 71–88.
Balbo, S., Turesky, R.J., Villalta, P.W., 2014. DNA adductomics. Chem. Res. Toxicol. 27, 356–366.
Bastide, N.M., Chenni, F., Audebert, M., Santarelli, R.L., Tache, S., Naud, N., Baradat, M., Jouanin, I., Surya, R., Hobbs, D.A., Kuhnle, G.G., Raymond-Letron, I., Gueraud, F., Corpet, D.E., Pierre, F.H., 2015. A central role for heme iron in colon carcinogenesis associated with red meat intake. Canc. Res. 75, 870–879.
Bastide, N.M., Pierre, F.H., Corpet, D.E., 2011. Heme iron from meat and risk of colorectal cancer: a meta-analysis and a review of the mechanisms involved. Canc. Prev. Res. 4, 177–184.
Besaratinia, A., Pfeifer, G.P., 2005. DNA adduction and mutagenic properties of acrylamide. Mutat. Res. Genet. Toxicol. Environ. Mutagen 580, 31–40.
Bignami, M., O'Driscoll, M., Aquilina, G., Karran, P., 2011. Unmasking a killer: DNA O6-methylguanine and the cytotoxicity of methylating agents. Mutat. Res. Rev. Mutat. Res. 462, 71–82.
Chung, F.L., Pan, J., Choudhury, S., Roy, R., Hu, W., Tang, M.S., 2003. Formation of trans-4-hydroxy-2-nonenal- and other enal-derived cyclic DNA adducts from omega-3 and omega-6 polyunsaturated fatty acids and their roles in DNA repair and human p53 gene mutation. Mutat. Res. 531, 25–36.
Cooke, M.S., Evans, M.D., Dizdaroglu, M., Lunec, J., 2003. Oxidative DNA damage: mechanisms, mutation, and disease. Faseb. J. 17, 1195–1214.
Cross, A.J., Pollock, J.R., Bingham, S.A., 2003. Haem, not protein or inorganic iron, is responsible for endogenous intestinal N-nitrosation arising from red meat. Canc. Res. 63, 2358–2360.
Cross, A.J., Sinha, R., 2004. Meat-related mutagens/carcinogens in the etiology of colorectal cancer. Environ. Mol. Mutagen. 44, 44–55.
De Bont, R., van Larebeke, N., 2004. Endogenous DNA damage in humans: a review of quantitative data. Mutagenesis 19, 169–185.
Demeyer, D., Mertens, B., De Smet, S., Ulens, M., 2016. Mechanisms linking colorectal cancer to the consumption of (processed) red meat: a review. Crit. Rev. Food Sci. Nutr. 56, 2747–2766.
Drabløs, F., Feyzi, E., Aas, P.A., Vaagbø, C.B., Kavli, B., Bratlie, M.S., Peña-Diaz, J., Otterlei, M., Slupphaug, G., Krokan, H.E., 2004. Alkylation damage in DNA and RNA—repair mechanisms and medical significance. DNA Repair 3, 1389–1407.
Fahrer, J., Kaina, B., 2017. Impact of DNA repair on the dose-response of colorectal cancer formation induced by dietary carcinogens. Food Chem. Toxicol. 106, 583–594.
Farmer, P.B., Singh, R., 2008. Use of DNA adducts to identify human health risk from exposure to hazardous environmental pollutants: the increasing role of mass spectrometry in assessing biologically effective doses of genotoxic carcinogens. Mutat. Res. Rev. Mutat. Res. 659, 68–76.
Gratz, S.W., Wallace, R.J., El-Nezami, H.S., 2011. Recent perspectives on the relations between fecal mutagenicity, genotoxicity, and diet. Front. Pharmacol. 2, 4.
Greetham, H.L., Bingham, S.A., Burns, P.A., 2007. Adduction of human p53 gene by fecal water: an in vitro biomarker of mutagenesis in the human large bowel. Cancer Epidemiol. Biomark. Prev. 16, 2681–2685.
Hailer, M.K., Slade, P.G., Martin, B.D., Rosenquist, T.A., Sugden, K.D., 2005. Recognition of the oxidized lesions spiroiminodihydantoin and guanidinohydantoin in DNA by the mammalian base excision repair glycosylases NEIL1 and NEIL2. DNA Repair 4, 41–50.
Hemeryck, L.Y., Decloedt, A.I., Vanden Bussche, J., Geboes, K.P., Vanhaecke, L., 2015. High resolution mass spectrometry based profiling of diet-related deoxyribonucleic acid adducts. Anal. Chim. Acta 892, 123–131.
Hemeryck, L.Y., Moore, S.A., Vanhaecke, L., 2016a. Mass spectrometric mapping of the DNA adductome as a means to study genotoxin exposure, metabolism, and effect. Anal. Chem. 88, 7436–7446.
Hemeryck, L.Y., Rombouts, C., Van Hecke, T., Van Meulebroek, L., Vanden Bussche, J., De Smet, S., Vanhaecke, L., 2016b. In vitro DNA adduct profiling to mechanistically link red meat consumption to colon cancer promotion. Toxicol. Res. 5, 1346–1358.
Hemeryck, L.Y., Van Hecke, T., Vossen, E., De Smet, S., Vanhaecke, L., 2017. DNA adductomics to study the genotoxic effects of red meat consumption with and without added animal fat in rats. Food Chem. 230, 378–387.
Hemeryck, L.Y., Vanhaecke, L., 2016. Diet-related DNA adduct formation in relation to carcinogenesis. Nutr. Rev. 74, 475–489.
Himmelstein, M.W., Boogaard, P.J., Cadet, J., Farmer, P.B., Kim, J.H., Martin, E.A., Persaud, R., Shuker, D.E., 2009. Creating context for the use of DNA adduct data in cancer risk assessment: II. Overview of methods of identification and quantitation of DNA damage. Crit. Rev. Toxicol. 39, 679–694.
Huber, W.W., Scharf, G., Nagel, G., Prustomersky, S., Schulte-Hermann, R., Kaina, B., 2003. Coffee and its chemopreventive components Kahweol and Cafestol increase the activity of O6-methylguanine-DNA methyltransferase in rat liver—comparison with phase II xenobiotic metabolism. Mutat. Res. Fund Mol. Mech. Mutagen 522, 57–68.
Huh, N., Satoh, M.S., Shiga, J., Kuroki, T., 1988. Detection of O4-ethylthymine in human

liver DNA. IARC Sci. Publ. 292–295.
Hur, S.J., Lim, B.O., Decker, E.A., McClements, D.J., 2011. In vitro human digestion models for food applications. Food Chem. 125, 1–12.
IARC, 2015. IARC Monographs Evaluate Consumption of Red Meat and Processed Meat. (Lyon).
Irrazábal, T., Belcheva, A., Girardin, Stephen E., Martin, A., Philpott, Dana J., 2014. The multifaceted role of the intestinal microbiota in colon cancer. Mol. Cell 54, 309–320.
Ishino, K., Kato, T., Kato, M., Shibata, T., Watanabe, M., Wakabayashi, K., Nakagama, H., Totsuka, Y., 2015. Comprehensive DNA adduct analysis reveals pulmonary inflammatory response contributes to genotoxic action of magnetite nanoparticles. Int. J. Mol. Sci. 16, 3474–3492.
Joosen, A.M., Kuhnle, G.G., Aspinall, S.M., Barrow, T.M., Lecommandeur, E., Azqueta, A., Collins, A.R., Bingham, S.A., 2009. Effect of processed and red meat on endogenous nitrosation and DNA damage. Carcinogenesis 30, 1402–1407.
Joosen, A.M., Lecommandeur, E., Kuhnle, G.G., Aspinall, S.M., Kap, L., Rodwell, S.A., 2010. Effect of dietary meat and fish on endogenous nitrosation, inflammation and genotoxicity of faecal water. Mutagenesis 25, 243–247.
Jung, J.Y., Lee, H.-S., Kang, D.-G., Kim, N.S., Cha, M.H., Bang, O.-S., Ryu, D.H., Hwang, G.-S., 2011. 1H-NMR-Based metabolomics study of cerebral infarction. Stroke 42, 1282–1288.
Kang, H.-i., Konishi, C., Kuroki, T., Huh, N.-h., 1995. Detection of O6-methylguanine, O4-methylthymine and O4-ethylthymine in human liver and peripheral blood leukocyte DNA. Carcinogenesis 16, 1277–1280.
Kanner, J., 1994. Oxidative processes in meat and meat products: quality implications. Meat Sci. 36, 169–189.
Kenmogne-Domguia, H.B., Meynier, A., Boulanger, C., Genot, C., 2012. Lipid oxidation in food emulsions under gastrointestinal-simulated conditions: the key role of endogenous tocopherols and initiator. Food Digestion 3, 46–52.
Kuhnle, G.G., Bingham, S.A., 2007. Dietary meat, endogenous nitrosation and colorectal cancer. Biochem. Soc. Trans. 35, 1355–1357.
Lewin, M.H., Bailey, N., Bandaletova, T., Bowman, R., Cross, A.J., Pollock, J., Shuker, D.E.G., Bingham, S.A., 2006. Red meat enhances the colonic formation of the DNA adduct O6-Carboxymethyl guanine: implications for colorectal cancer risk. Canc. Res. 66, 1859–1865.
Lijinsky, W., 1992. Chemistry and biology of N-nitroso compounds. Camb. Monog. Canc. Res. 480.
Lorrain, B., Dangles, O., Loonis, M., Armand, M., Dufour, C., 2012. Dietary iron-initiated lipid oxidation and its inhibition by polyphenols in gastric conditions. J. Agric. Food Chem. 60, 9074–9081.
Louis, P., Hold, G.L., Flint, H.J., 2014. The gut microbiota, bacterial metabolites and colorectal cancer. Nat. Rev. Microbiol. 12, 661–672.
Marnett, L.J., 1999. Lipid peroxidation-DNA damage by malondialdehyde. Mutat. Res. 424, 83–95.
Marnett, L.J., 2000. Oxyradicals and DNA damage. Carcinogenesis 21, 361–370.
Marnett, L.J., 2012. Inflammation and cancer: chemical approaches to mechanisms, imaging, and treatment. J. Org. Chem. 77, 5224–5238.
Molly, K., Woestyne, M.V., Smet, I.D., Verstraete, W., 1994. Validation of the simulator of the human intestinal microbial Ecosystem (SHIME) reactor using microorganism-associated activities. Microb. Ecol. Health Dis. 7, 191–200.
Niture, S.K., Velu, C.S., Smith, Q.R., Bhat, G.J., Srivenugopal, K.S., 2007. Increased expression of the MGMT repair protein mediated by cysteine prodrugs and chemopreventative natural products in human lymphocytes and tumor cell lines. Carcinogenesis 28, 378–389.
Pfohl-Leszkowicz, A., Grosse, Y., Carriere, V., Cugnenc, P.H., Berger, A., Carnot, F., Beaune, P., de Waziers, I., 1995. High levels of DNA adducts in human colon are associated with colorectal cancer. Canc. Res. 55, 5611–5616.
Pierre, F., Freeman, A., Tache, S., Van der Meer, R., Corpet, D.E., 2004. Beef meat and blood sausage promote the formation of azoxymethane-induced mucin-depleted foci and aberrant crypt foci in rat colons. J. Nutr. 134, 2711–2716.
Poirier, M.C., 2004. Chemical-induced DNA damage and human cancer risk. Nat. Rev. Canc. 4, 630–637.
Povey, A.C., Badawi, A.F., Cooper, D.P., Hall, C.N., Harrison, K.L., Jackson, P.E., Lees, N.P., O'Connor, P.J., Margison, G.P., 2002. DNA alkylation and repair in the large bowel: animal and human studies. J. Nutr. 132, 3518S–3521S.
Rombouts, C., Hemeryck, L.Y., Van Hecke, T., De Smet, S., De Vos, W.H., Vanhaecke, L., 2017. Untargeted metabolomics of colonic digests reveals kynurenine pathway metabolites, dityrosine and 3-dehydroxycarnitine as red versus white meat discriminating metabolites. Sci. Rep. 7, 42514.
Segal, A., Solomon, J.J., Maté, U., 1980. Isolation of 3-(2-carboxyethyl)thymine following in vitro reaction of β-propiolactone with calf thymus DNA. Chem. Biol. Interact. 29, 335–346.
Steppeler, C., Haugen, J.E., Rodbotten, R., Kirkhus, B., 2016. formation of malondialdehyde, 4-hydroxynonenal, and 4-hydroxyhexenal during in vitro digestion of cooked beef, pork, chicken, and salmon. J. Agric. Food Chem. 64, 487–496.
Stewart, B., Wild, C.P. (Eds.), 2014. World Cancer Report 2014 International Agency for Research on Cancer. WHO.
Stone, K., Uzieblo, A., Marnett, L.J., 1990. Studies of the reaction of malondialdehyde with cytosine nucleosides. Chem. Res. Toxicol. 3, 467–472.
Sugden, K.D., Martin, B.D., 2002. Guanine and 7,8-dihydro-8-oxo-guanine-specific oxidation in DNA by chromium(V). Environ. Health Perspect. 110 (Suppl. 5), 725–728.
Triba, M.N., Le Moyec, L., Amathieu, R., Goossens, C., Bouchemal, N., Nahon, P., Rutledge, D.N., Savarin, P., 2015. PLS/OPLS models in metabolomics: the impact of permutation of dataset rows on the K-fold cross-validation quality parameters. Mol. Biosyst. 11, 13–19.
Van Hecke, T., Vanden Bussche, J., Vanhaecke, L., Vossen, E., Van Camp, J., De Smet, S., 2014. Nitrite curing of chicken, pork, and beef inhibits oxidation but does not affect N-nitroso compound (NOC)-specific DNA adduct formation during in vitro digestion. J. Agric. Food Chem. 62, 1980–1988.
Van Meulebroek, L., 2014. A Metabolomic Framework to Study the Regulating Role of Phytohormones towards Carotenoids in Tomato Fruit. PhD Thesis. Ghent University, Merelbeke, Belgium.
Vanden Bussche, J., Hemeryck, L.Y., Van Hecke, T., Kuhnle, G.G., Pasmans, F., Moore, S.A., Van de Wiele, T., De Smet, S., Vanhaecke, L., 2014. O(6)-carboxymethylguanine DNA adduct formation and lipid peroxidation upon in vitro gastrointestinal digestion of haem-rich meat. Mol. Nutr. Food Res. 58, 1883–1896.
Vanden Bussche, J., Moore, S.A., Pasmans, F., Kuhnle, G.G., Vanhaecke, L., 2012. An approach based on ultra-high pressure liquid chromatography-tandem mass spectrometry to quantify O6-methyl and O6-carboxymethylguanine DNA adducts in intestinal cell lines. J. Chromatogr. A 1257, 25–33.
Wang, G., Seifert, W.E., 1996. Synthesis and evaluation of oligodeoxynucleotides containing 4′-C-substituted thymidines. Tetrahedron Lett. 37, 6515–6518.
Wiklund, S., Johansson, E., Sjöström, L., Mellerowicz, E.J., Edlund, U., Shockcor, J.P., Gottfries, J., Moritz, T., Trygg, J., 2008. Visualization of GC/TOF-MS-based metabolomics data for identification of biochemically interesting compounds using OPLS class models. Anal. Chem. 80, 115–122.
Zhang, Y.J., Wang, F., Zhou, Y., Li, Y., Zhou, T., Zheng, J., Zhang, J.J., Li, S., Xu, D.P., Li, H.B., 2016. Effects of 20 selected fruits on ethanol metabolism: potential health benefits and harmful impacts. Int. J. Environ. Res. Publ. Health 13, 399.