Food Chemistry 230 (2017) 378–387

Contents lists available at ScienceDirect

Food Chemistry

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

DNA adductomics to study the genotoxic effects of red meat consumption with and without added animal fat in rats

Lieselot Y. Hemeryck [a], Thomas Van Hecke [b], Els Vossen [b], Stefaan De Smet [b], Lynn Vanhaecke [a,*]

[a] Laboratory of Chemical Analysis, Department of Veterinary Public Health and Food Safety, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium

[b] Laboratory of Animal Nutrition and Animal Product Quality, Department of Animal Production, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, B-9000 Ghent, Belgium

ARTICLE INFO

Article history:
Received 23 November 2016
Received in revised form 14 February 2017
Accepted 27 February 2017
Available online 28 February 2017

Keywords:
DNA damage
Dietary fat
High resolution mass spectrometry
Lipid peroxidation products
N-nitroso compounds
Red meat

ABSTRACT

Digestion of red and processed meat has been linked to the formation of genotoxic *N*-nitroso compounds (NOCs) and lipid peroxidation products (LPOs) in the gut. In this study, rats were fed a meat based diet to compare the possible genotoxic effects of red vs. white meat, and the interfering role of dietary fat. To this purpose, liver, duodenum and colon DNA adductomes were analyzed with UHPLC-HRMS. The results demonstrate that the consumed meat type alters the DNA adductome; the levels of 22 different DNA adduct types significantly increased upon the consumption of beef (compared to chicken) and/or lard supplemented beef or chicken. Furthermore, the chemical constitution of the retrieved DNA adducts hint at a direct link with an increase in NOCs and LPOs upon red (and processed) meat digestion, supporting the current hypotheses on the causal link between red and processed meat consumption and the development of colorectal cancer.

1. Introduction

DNA adduct formation is the result of the attack of nucleophilic sites in DNA by endo- or exogenous electrophilic molecules. As such, the DNA building blocks, i.e. the guanine (G), cytosine (C), adenine (A) and thymine (T) nucleobases can be altered both structurally and functionally. In the absence of a timely detoxification of the initiating genotoxin and/or repair of the resulting DNA adduct, DNA adduct formation can lead to mutations and chemically induced carcinogenesis (Poirier, 2004). Hence, investigation of DNA adduct formation can provide valuable information on exposure to both environmental and endogenous chemicals with genotoxic, mutagenic and/or carcinogenic properties on the one hand, and their possible adverse health effects on the other. For example, DNA adduct formation is believed to be an intermediate step in hepatocarcinogenesis due to chronic aflatoxin B1 exposure. Aflatoxin B1 is a known human carcinogen that is formed as a secondary metabolite by food and feed contaminating fungi. Its uptake results in the formation of different types of DNA adducts and also leads to a correlated increase in liver cancer risk (Marroquin-Cardona, Johnson, Phillips, & Hayes, 2014). Accordingly, DNA adduct analysis can be very useful to investigate the underlying pathways of several non-hereditary cancers, which comprise the vast majority of cancer cases (Stewart & Wild, 2014).

One of the most prevalent cancer types that mainly occurs due to environmental factors (e.g. diet and lifestyle) is colorectal cancer (CRC). CRC is the third and second most common cancer type in men and women worldwide, respectively, and important influencing factors include adoption of the Western dietary pattern with the excessive consumption of fat, and red and processed meat (Stewart & Wild, 2014). With regard to the observed increase in

Abbreviations: O^6-CM-G, O^6-carboxymethylguanine; O^6-Me-G, O^6-methylguanine; A, adenine; C, cytosine; CRO, crotonaldehyde; Cro-G, Methylhydroxypropanoguanine, the main CRO adduct with G; DNA, DeoxyriboNucleic Acid; G, guanine; Hep-G, heptenal-G; HNE-C, Hydroxynonenal-C; HESI, Heated ElectroSpray Ionisation; HRAM, High Resolution Accurate Mass; HRMS, high resolution mass spectrometry; LPO(s), lipid peroxidation product(s); M_1-acetaldehyde-A, adduct of 1 malondialdehyde and 1 acetaldehyde molecule with A; M_1-G, Pyrimidopurinone, the main malondialdehyde-guanine DNA adduct; M_2-acetaldehyde-A, adduct of 2 malondialdehyde and 1 acetaldehyde molecule(s) with A; M_2-acetaldehyde-G, adduct of 2 malondialdehyde and 1 acetaldehyde molecule(s) with G; M_2-G, adduct of 2 malondialdehyde molecules with G; M_3-C, adduct of 3 malondialdehyde molecules with C; MDA, malondialdehyde; MS, mass spectrometry; MS/MS, Tandem MS; NOC(s), *N*-nitroso compound(s); Oct-G, Octenal-G; OHE-C, oxohexenal-C; OPLS-DA, Orthogonal Partial Least Squares-Discriminant Analysis; PCA, Principal Component Analysis; RT, retention time; T, thymine; U, Uracil; UHPLC, ultrahigh performance liquid chromatography; VIP, Variable Importance in Projection.

* Corresponding author.
E-mail address: Lynn.Vanhaecke@UGent.be (L. Vanhaecke).

http://dx.doi.org/10.1016/j.foodchem.2017.02.129
0308-8146/

CRC risk due to red and processed meat consumption, different research groups have investigated the proposed underlying pathways. Currently, there are several intertwined hypotheses that are still under investigation. A prominent hypothesis states that heme stimulates the formation of both lipid peroxidation products (LPOs) and *N*-nitroso compounds (NOCs) in the gut besides its own direct (cyto)toxicity (Oostindjer et al., 2014). The heme molecule is intrinsically more present in red (e.g. beef) than white (e.g. chicken) meat in the form of myoglobin, which renders this molecule a very potent candidate to help explain the toxicity of red but not white meat. Both exo- and endogenous NOCs may contribute to red and processed meat toxicity. Several types of NOCs (e.g. nitrosamines and nitrosamides) have known carcinogenic properties (Lijinsky, 1999), and the most common route of exposure to NOCs indeed occurs via Western type foodstuffs (Hotchkiss, 1989). However, certain NOCs, i.e. nitrosamines and nitrosamides, can also be formed in the gut during digestion of food. What further supports the NOC hypothesis is the fact that exposure to NOCs has already been linked to an increase in tumor development (Lijinsky, 1999). The same reasoning applies for LPOs; LPOs can originate from both exo- and endogenous processes, and possess known cyto- and genotoxic effects that have been linked to carcinogenesis (De Bont & van Larebeke, 2004; Marnett, 1999).

In previous studies, we were able to link red meat digestion to the increased formation of LPOs (e.g. malondialdehyde), as well as LPO- and NOC-related DNA adducts (e.g. O^6-carboxymethylguanine), (Hemeryck et al., 2016; Van Hecke et al., 2016). The current study aimed to further explore the possible genotoxic effects of red meat consumption *in vivo* since (a) both NOCs and LPOs are prone to DNA adduct formation (De Bont & van Larebeke, 2004) and (b) a shift in DNA adduct profile after beef digestion has been demonstrated previously *in vitro* (Hemeryck et al., 2016).

A state-of-the-art DNA adductomics methodology (Hemeryck, Decloedt, Vanden Bussche, Geboes, & Vanhaecke, 2015), based on accurate mass measurements (HRMS), was employed to map the diet-related DNA adduct profile in tissue from rats on a meat diet. The use of an in-house DNA adduct database and specialized omics software further enabled a focused investigation of possibly relevant diet-related DNA adducts (Hemeryck et al., 2015).

2. Material and methods

2.1. Rat feeding trial

2.1.1. Meat based diets

Four different diets, based on lean chicken (LFCh), fat chicken (lean chicken with added lard; HFCh), lean beef (LFBe) or fat beef (lean beef with added lard; HFBe), were prepared in advance. To this purpose, the *m. pectoralis profundus* of chicken, as a model for white meat, and the *m. pectoralis profundus* of beef, as a model for red meat, were purchased, chopped, minced and ground. Then, the meat (and added lard) was cooked at 70 °C for 70 min, in a hot water bath (cooked to the core, but not overcooked to avoid interference from the formation of genotoxic heterocyclic amines and polycyclic aromatic hydrocarbons), followed by homogenization in a food processor. After this, the 4 different meat based diets were manufactured as is documented in Table S1, vacuum packed and stored at −20 °C.

2.1.2. Rat experiment

For this rat trial (ECD 14/58 (Ghent, Belgium)), 24 male Sprague-Dawley rats (±150 g) were purchased from Janvier laboratories (France). The rats were housed in groups of 4 upon arrival and given a standard laboratory diet (Ssniff R/M-H pellets from Ssniff, Soest, Germany) and water *ad libitum* during the first 10 days. After this adaptation period, all rats were divided at random into 4 groups and housed individually. Then, during 14 consecutive days, each group received a different diet (provided *ad libitum* and refreshed daily), i.e. a diet based on lean chicken (='low fat chicken diet' or 'LFCh'), chicken with added lard (='high fat chicken diet' or 'HFCh'), lean beef (='low fat beef diet' or 'LFBe') or beef with added lard (='high fat beef diet' or 'HFBe'). Following 14/15 days on the experimental diets, all rats were anesthetized with 5% isoflurane gas and euthanized by terminal blood collection from the abdominal aorta, after which the different organs were harvested. Rats were euthanized on 6 consecutive days; one rat of each dietary treatment was sacrificed in a random order each day (a more detailed account of this experiment is provided by Van Hecke et al. (2016)). For this particular study, the liver, duodenal mucosae and colonic mucosae were sampled from each individual rat. Tissues were rinsed with a 0.9% saline solution and stored in 95% of ethanol at −80 °C until further sample processing.

2.2. DNA extraction, DNA hydrolysis and DNA adduct extraction

DNA from liver tissue, duodenal mucosae and colonic mucosae was extracted by means of the NucleoSpin Tissue Machery Nagel DNA extraction kit (Machery Nagel GmbH & Co., Düren, Germany), according to the protocol described by the manufacturer. DNA concentration and purity in each sample were determined with a Nanodrop ND-1000 spectrophotometer (Isogen Lifescience, Ijsselstein, The Netherlands).

The DNA obtained in each individual sample was then subjected to a previously reported and validated DNA adduct extraction protocol (Vanden Bussche, Moore, Pasmans, Kuhnle, & Vanhaecke, 2012). In brief, all DNA samples were hydrolyzed in 0.1 M formic acid at 80 °C during 30 min. After this, sample purification and cleanup was performed with solid-phase extraction (SPE) (Oasis® HLB cartridges (1 cc, 30 mg) Waters (Milford, USA), after which all eluates were evaporated to dryness under vacuum at room temperature. In the final step, all samples were suspended in 100 μl of 0.05% of acetic acid in water and stored at −20 °C until analysis.

2.3. DNA adduct analysis

2.3.1. Reagents and chemicals

Analytical standards for M_1-G, Cro-dG (α-methyl-γ-hydroxy-1, N_2-propano-2′-deoxyguanosine) and their respective internal standards; M_1-G-$^{13}C_3$ and CrodG-^{13}C,$^{15}N_2$, were purchased from Toronto Research Chemicals (Toronto, Canada). O^6-Me-dG (O^6-methyl-2′-deoxyguanosine) and O^6-d_3-Me-dG (internal standard for both O^6-Me-dG and O^6-CM-dG) were obtained from Sigma-Aldrich (St. Louis, USA). O^6-CM-dG (O^6-carboxymethyl-2′-deoxyguanosine) was kindly provided by Prof. S. Moore from Liverpool John Moores University (UK).

O^6-CM-dG, O^6-Me-dG, O^6-d_3-Me-dG, Cro-dG and Cro-dG-^{13}C,$^{15}N_2$ were hydrolyzed to their nucleobase form in 0.1 M formic acid at 80 °C for 30 min. All standards were diluted in methanol and stored at −20 °C in stock and working solutions of 500 ng/ml and 5 ng/ml, respectively.

2.3.2. UHPLC-HRMS analysis

A robust, validated ultrahigh performance liquid chromatography coupled to high resolution mass spectrometry (UHPLC-HRMS) method (Hemeryck et al., 2015) enabled targeted and untargeted DNA adduct analysis. Analysis was performed on 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 as described by Hemeryck et al. (2015). Internal calibration of

the MS detector was performed daily, by infusion of calibration mixtures prepared according to the protocol described in the operations manual (Thermo Fisher Scientific, San José, USA). Instrument control was performed with Chromeleon Xpress and Xcalibur™ 3.0.

Targeted analysis included the detection and quantification of O^6-Me-G, O^6-CM-G (O^6-carboxymethylguanine), M_1-G and Cro-G. Untargeted DNA adduct analysis was enabled by full scan MS acquisition at 100,000 Full Width Half Maximum in a range of 70 to 700 Da.

2.4. Data processing

2.4.1. Xcalibur™

O^6-Me-G, O^6-CM-G, M_1-G and Cro-G were identified by means of an analytical standard and quantified based on a 10-point calibration curve (5, 10, 25, 50, 100, 200, 300, 400 and 500 pg/ml). Xcalibur™ Quan enabled data processing.

2.4.2. ToxFinder™ profiling and GENE-E marker selection

The use of ToxFinder™ software (Thermo Fisher Scientific, San José, USA) and an in-house DNA adduct database (Hemeryck et al., 2015) allowed screening of the full scan HRMS spectra of each sample for the possible presence of known diet-related DNA adducts based on *m*/*z*. The considered inclusion criteria consisted of a minimum signal intensity of 10,000; a maximum mass deviation of 10 ppm, and the presence of a C^{13} isotope. Visualization of the obtained output from ToxFinder™ data processing was enabled by GENE-E matrix (http://www.broadinstitute.org/cancer/software/GENE-E/). Hierarchical clustering of samples was performed by means of 'one minus pearsons correlation', whilst the GENE-E marker selection tool (all possible permutations) was used to search for potential markers. Student's *t*-test was used for statistical interpretation of DNA adduct levels in tissue samples from rats on a different diet (n = 6 per group).

2.4.3. Sieve™ chromatographic peak selection with database lookup

Sieve™ 2.1 (Thermo Fisher Scientific, San José, USA) was implemented to screen for the presence and significance of known diet-related DNA adducts in the different DNA samples. All ions eluting between 0.7 and 5.5 min (of chromatographic analysis) with an *m*/*z* value between 70 and 700 Da were retained. The maximum peak width consisted of 0.5 min and maximum mass deviation was set at 10 ppm. Positive and negative ions were selected in separate experiments. The maximum number of frames was 200,000, whilst minimal peak intensity was set at 50,000 (arbitrary units). After automated processing, Sieve™ reported the *m*/*z* value, retention time and abundance of each detected ion. The database lookup function was used to putatively identify the detected and selected ions by matching the theoretical *m*/*z* values of the diet-related DNA adducts in the in-house database to the *m*/*z* values of the detected ions. To be able to select DNA adducts that are (significantly) higher or lower in tissue from rats that received a different diet, Sieve™ pairwise comparison experiments were executed for each tissue type separately in both the negative and positive ionization mode. Tissue DNA adduct levels were compared for the HFBe and LFBe diet, the HFCh and LFCh diet, the HFBe and HFCh diet, and the LFBe and LFCh diet, resulting in 24 different Sieve™ experiments.

2.4.4. Simca™: Orthogonal Partial Least Squares Discriminant Analysis

Sieve™ was used to select all chromatographic peaks in all samples (= 3 tissue types x 24 rats) simultaneously with the exact same settings as described above (= 2 separate Simca™ experiments, 1 for positive ions, 1 for negative ions). Simca™ 13 (Umetrics AB, Umeå, Sweden) was used to enable processing of multivariate omics data by means of Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA). Automated Simca™ data modelling was performed after correction for the amount of DNA in each sample, logarithmic data transformation and Pareto scaling. R^2 was assessed to ensure goodness of fit (minimal threshold of 0.5) and Q^2 was used to assess the predictive ability of the method (minimal threshold of 0.5). Discriminative/predictive ions were selected based on their excentric position in the S-plot and a Variable Importance in Projection-score (VIP-score) above 1. A VIP above 1 demonstrates a high influence; a VIP below 1, but above 0.8 demonstrates a moderate influence, and a VIP below 0.8 reflects a low influence.

3. Results

3.1. General

The average initial and final body weight of the rats did not differ among the dietary treatments (a more detailed account is provided by Van Hecke et al. (2016). Rats on the diets with added lard had a significantly lower feed intake (−25%) compared to rats on the diets without added lard, but there was no difference ($p = 0.751$) in metabolizable energy intake (data published previously by Van Hecke et al. (2016).

Overall, several diet-related DNA adducts could be retrieved in the DNA obtained from rat liver, duodenal and colonic mucosae by means of UHPLC-HRMS(/MS), which will be discussed in detail below.

3.2. O^6-CM-G, O^6-Me-G, M_1-G and Cro-G DNA adduct levels (Xcalibur™ Quan)

The O^6-CM-G DNA adduct could not be retrieved in any of the samples under investigation. M_1-G could be detected (levels < limit of quantification) in DNA obtained from the duodenal mucosa of 1 rat on a HFBe diet, and in a DNA sample obtained from the colonic mucosa of a rat on a HFCh diet. The O^6-Me-G adduct could be quantified in 6 out of 24 liver samples, 2 out of 24 duodenal samples and 3 out of 24 colon samples. The Cro-G DNA adduct could be detected and quantified in 1 duodenum sample, 2 liver samples and 3 colon samples. There was no clear distinction according to diet, although both O^6-Me-G and Cro-G DNA adduct levels appeared to be lower in liver tissue in comparison to duodenal and colonic mucosal tissue after correction for the amount of DNA in each sample (Tables S2 and S3).

3.3. ToxFinder™ DNA adduct profiling

An overview of the results of ToxFinder™ data processing and GENE-E clustering (pearsons correlation) is presented in the heat map (Fig. 1). Only DNA adduct types that could be retrieved in a vast majority of DNA samples (present in ≥4 out of 6 samples) were included in the heat map.

3.4. Significantly higher or lower DNA adduct levels according to Sieve™ peak integration and database lookup

Different putatively identified DNA adducts could be detected in all tissue types and samples. Two sample differential analysis enabled pairwise comparison of DNA adduct levels in each tissue type according to diet. DNA adduct types that appeared to be distinctly higher in beef *vs.* chicken are of particular interest to this study, due to their possible role in the unknown underlying pathways that causally link red meat consumption and CRC. The same applies for DNA adducts that appear to be higher upon

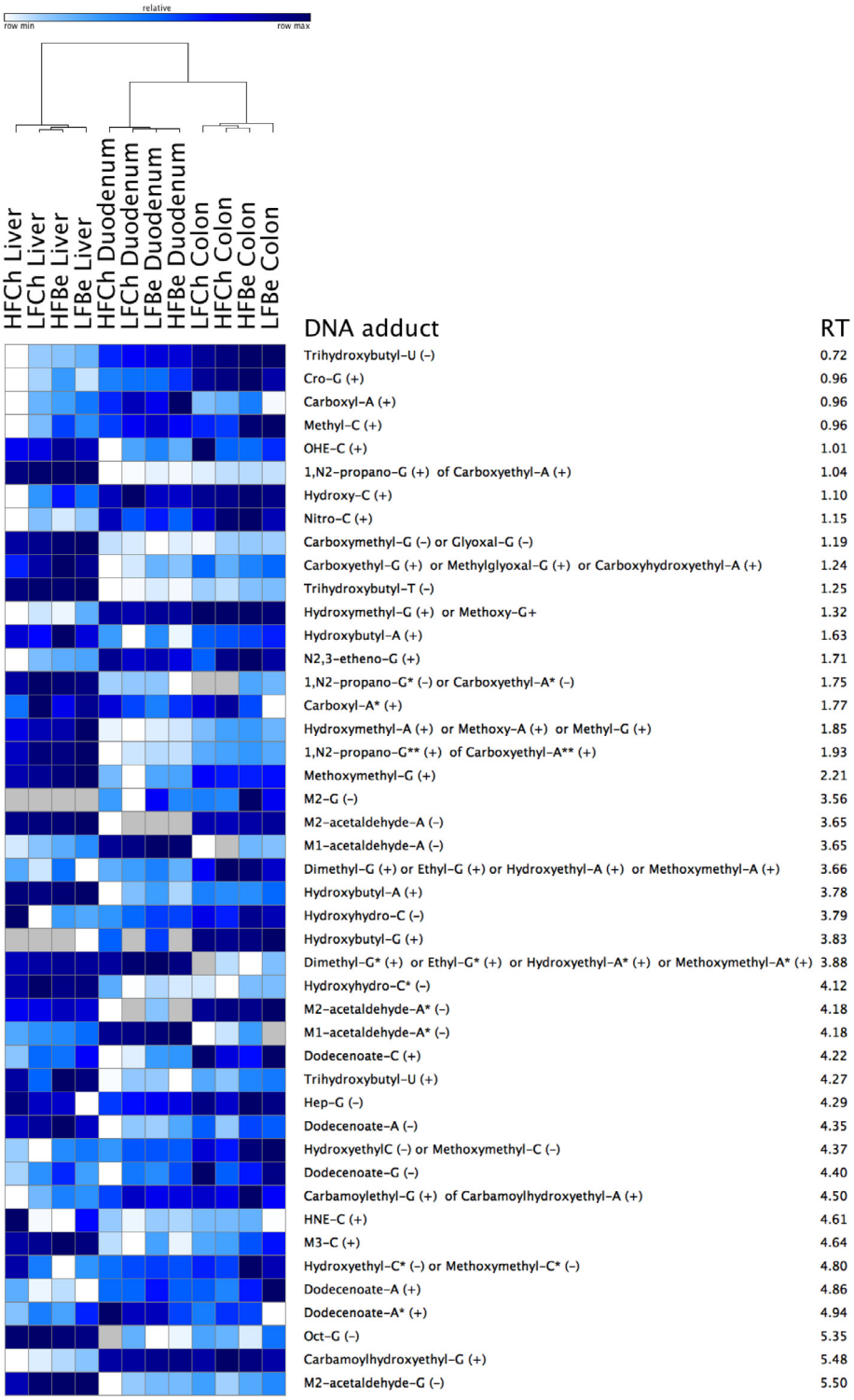

Fig. 1. Heat map of average (n = 6 for each sample type) DNA adduct types and levels in liver, duodenal and colon DNA after correction for the amount of DNA per sample. Darker shades of blue represent higher average DNA adduct levels. HF indicates a high fat content in the diet, LF indicates a low fat content in the diet, Ch stands for chicken and Be stands for beef. RT represents retention time in min, an asterix marks a different isomer of a certain DNA adduct that had already been detected at an earlier RT (different RT = different isomer), and a grey box represents the total absence of DNA adduct detection in those particular samples. The ionization mode in which each DNA adduct type was detected, is provided between brackets after each DNA adduct name. Please consult the abbreviations list for abbreviations for or in DNA adduct names.

digestion of a 'high fat' (HF) diet *vs.* a 'low fat' (LF) diet. Therefore, only those types of DNA adducts will be presented and discussed below (in Tables 1–3). Information on all other DNA adduct types and levels can be consulted as supplementary information (Tables S4–S6).

3.4.1. Influence of fat content in the diet

Different putative DNA adduct types appeared to be higher in rats on a HF diet compared to a LF diet, as is documented in Tables 1–3. None of the DNA adduct types that were significantly ($p < 0.05$) or borderline significantly higher ($p < 0.10$) in one of

Table 1
Significantly higher levels ($p < 0.05$ or (*) $p < 0.10$) of putatively identified DNA adducts (relevant to hypothesis) in liver samples after two sample differential analysis by means of Sieve™ database lookup analysis (RT = retention time in min).

Higher in	DNA adduct	Charge	RT	Δ ppm
HFCh vs. LFCh	Methoxy-A (*)	–	1.5	2.86
	M_1-acetaldehyde-G (*)	+	2.94	5.36
	Methoxy-U	+	3.52	3.45
	Nitro-T (*)	–	3.79	9.44
	Hydroxybutyl-C (*)	–	4.29	6.63
	M_1-acetaldehyde-G	–	4.85	5.97
	Pentenal-G (*)	+	5.11	1.38
HFBe vs. LFBe	Carbamoylhydroxyethyl-G	+	0.82	1.86
	Formyl-U (*)	+	1.29	2.15
	Methyl-C (*)	+	1.49	3.48
	M_1-C	–	3.01	3.77
	Carboxymethyl-C	+	3.88	2.51
	Heptenal-G	+	3.94	1.15
	Pentenal-G	+	5.31	4.75
	Trihydroxybutyl-T	+	5.33	8.49
HFBe vs. HFCh	Butyl-G (*)	+	0.71	5.00
	Formamidopyrimidine-A (*)	+	5.00	9.25
	Oxohexenal-C (*)	+	5.11	0.51
LFBe vs. LFCh	Methoxy-U (*)	+	1.21	2.37
	Carboxymethyl-T or Carboxyethyl-U	+	1.35	1.72
	Heptenaletheno-A (*)	–	1.39	3.22
	Heptenaletheno-A	+	4.11	4.10

Table 2
Significantly higher levels ($p < 0.05$ or (*) $p < 0.10$) of putatively identified DNA adducts (relevant to hypothesis) in duodenal mucosae samples after two sample differential analysis by means of Sieve™ database lookup analysis (RT = retention time in min).

Higher in	DNA adduct	Charge	RT	Δ ppm
HFCh vs. LFCh	Glyoxal-G	–	1.17	0.69
	Hydroxy-A	+	1.35	3.33
	Heptenaletheno-C	+	2.43	9.46
	Methoxymethyl-G or Hydroxyethyl-G	+	2.77	7.62
	M_2-acetaldehyde-A	+	2.83	5.52
	Dihydro-T (*)	+	3.97	3.99
HFBe vs. LFBe	Crotonaldehyde-G	+	0.99	0.71
	Octenal-G (*)	+	4.33	6.01
HFBe vs. HFCh	Carboxyethyl-T	+	0.71	5.42
	Trimethyl-G (*)	–	0.86	8.40
	Pentenal-G	–	0.94	4.71
	Crotonaldehyde-G	+	0.96	0.71
	Heptenaletheno-C	+	0.99	2.39
	Oxohexenal-A (*)	+	1.04	5.25
	Hydroxybutyl-C (*)	+	1.07	3.29
	Dimethyl-T or Ethyl-T	+	1.40	7.91
	Nitro-U	+	2.04	2.16
	Butyl-G	+	2.18	6.55
	Oxohexenal-G	+	2.38	6.31
	Butyl-G (*)	+	3.63	6.62
	M_2-acetaldehyde-A (*)	–	4.18	2.78
	Oxohexenal-G	+	5.03	3.89
	Oxohexenal-G (*)	+	5.50	4.50
LFBe vs. LFCh	$1,N^2$-propano-G (*)	–	1.04	7.17
	Hydroxyhydro-C (*)	–	4.12	4.77

the three tissue types under investigation also appeared to be (borderline) significantly higher ($p < 0.10$) in one of the other two tissue types.

3.4.2. Effect of beef vs. chicken meat digestion

Digestion of a beef diet resulted in a significantly different DNA adduct profile in liver, duodenum and colon (see Tables 1–3). The formyl-U DNA adduct (RT of approximately 1.90 min) was significantly higher in both liver ($p < 0.05$) and duodenal ($p < 0.10$) DNA. A significantly higher level ($p < 0.10$) of both methyl-C (RT of approximately 1.96) and dimethyl-A or ethyl-A (RT of approximately 4.86) could be retrieved in the small and large bowel.

3.5. Discriminating DNA adducts (Simca™ modelling)

An acceptable OPLS-DA model that enables a clear distinction between samples from rats in different dietary groups could not always be constructed. DNA adducts in colon and liver samples could not be modelled according to diet, although samples from different tissue types could easily be distinguished and modelled at all times (Fig. S1). DNA adducts in duodenal DNA could be modelled under certain conditions; the effect of the HFBe diet could be modelled *vs.* the HFCh diet in positive ionization mode. The same applies for samples from rats on a HFCh diet *vs.* rats on a LFCh diet. In negative ionization mode, the beef diet could be modelled *vs.* the

Table 3
Significantly higher levels ($p < 0.05$ or (*) $p < 0.10$) of putatively identified DNA adducts (relevant to hypothesis) in colonic mucosae samples after two sample differential analysis by means of Sieve™ database lookup analysis (RT = retention time in min).

Higher in	DNA adduct	Charge	RT	Δ ppm
HFCh vs. LFCh	Trimethyl-G	+	0.79	5.10
	$N^{2,3}$-etheno-G	+	2.10	4.11
	Butyl-G (*)	+	2.16	6.18
	Methoxy-A (*)	+	4.70	8.85
HFBe vs. LFBe	Hydroxyethyl-T (*)	+	1.01	7.19
	Carboxyethyl-T (*)	+	1.10	6.04
	Carboxymethyl-T or Carboxyethyl-U (*)	+	1.10	4.22
	M_1-G (*)	+	1.15	8.86
	Methyl-C-glycol (*)	+	1.37	4.16
	Hydroxynonenal-G (*)	+	1.51	0.89
	Oxohexenal-A (*)	+	4.00	0.57
HFBe vs. HFCh	M_1-C (*)	–	1.36	1.92
LFBe vs. LFCh	1,N^6-etheno-A (*)	+	0.71	9.70
	Carboxy-T (*)	–	0.72	2.42
	M_1-C (*)	–	1.42	2.66
	U-glycol (*)	–	1.89	4.38
	T-glycol (*)	+	2.16	3.60
	Formamidopyrimidine-A	+	4.86	8.05
	Hydroxynonenal-G	+	5.34	4.08

chicken diet regardless of fat content, whilst the HFBe diet *vs.* the LFBe diet could be modelled with the retained negative ions as well. Different putative DNA adducts demonstrated a high VIP score (>1) and an excentric position in the corresponding S-plot (S-plots of valid models are provided in Fig. S2). Table 4 provides an overview of the potentially discriminating DNA adducts of interest (that could be retrieved with Simca™). When calculating the true positive (=sensitivity) and true negative rate (=specificity) of the potential discriminants, none of the retrieved DNA adduct types allowed a perfect discrimination according to the consumption of a specific meat type.

3.6. Selection of DNA adduct types relevant to the proposed red meat hypotheses

Table 5 contains a selection of the obtained ToxFinder™, GENE-E, Sieve™ and Simca™ output. DNA adduct types were selected if they were retrieved with ToxFinder™ and (1) demonstrated significantly higher or lower levels (significant if $p < 0.05$ or borderline significant if $p < 0.10$) for a certain diet according to the student's *t*-test, or (2) demonstrated significantly higher or lower levels (significant if $p < 0.05$ or borderline significant if $p < 0.10$) for a certain diet according to Sieve™, or (3) were singled out as a potential marker by the GENE-E marker selection tool, or (4) were singled out as a potential marker by Simca™. All listed DNA adduct types could be retrieved in liver, duodenum as well as colon DNA (except for M_2-G (RT 3.66), which could not be detected in liver DNA).

4. Discussion

Red meat and animal fat intake related genotoxicity were assessed in male Sprague-Dawley rats. The red meat associated formation of a limited number of diet-related DNA adduct types (e.g. hydroxyguanine (an oxidative DNA lesion) and O^6-Me-G (alkylation DNA adduct)) has been previously investigated in a small number of rodent and human studies (Le Leu et al., 2015; Lewin et al., 2006; Winter et al., 2011). However, to date, there are no (published) untargeted *in vivo* DNA adductomics studies that investigate the possible genotoxic effects of the consumption of red vs. white meat (with or without added lard), rendering this study to be the first in its kind.

4.1. Liver vs. duodenum vs. colon

The described untargeted application of applied UHPLC-HRMS method enabled extensive DNA adduct profiling. As could be expected due to differences in tissue composition and physiology, and also digestion and metabolisation related differences in exposure to (different levels of) diet-related toxins, a different DNA adduct profile could be retrieved in each tissue type. Simca™ analysis allowed modelling of DNA adduct types and levels according to tissue type. In accordance, GENE-E demonstrated a clear clustering of liver samples, duodenal samples and colon samples, also distinctly clustering all bowel samples and thus reflecting a larger resemblance in the obtained DNA adduct profile in duodenum and colon DNA vs. liver DNA. DNA adduct types that are higher in colonic DNA could be relevant to the red meat CRC-hypothesis due to the fact that red meat consumption has primarily been linked to the development of cancer of the colon, but not liver and/or duodenum. Hence, DNA adduct types that are higher in colon vs. duodenum or liver may reveal important clues on the underlying mechanism. Unfortunately, the relevance (rate of (potential) mutagenic and carcinogenic actions) of the retrieved DNA adduct types cannot be compared objectively at the time

Table 4
Potentially discriminating DNA adducts (with high VIP scores and an excentric S-plot position (Be = beef, Ch = chicken, HF = high fat, LF = low fat)) (Simca™ data).

DNA adduct of interest	DNA adduct type	Charge	RT (min)	Δ ppm	Potentially discriminating marker for	VIP score	Sensitivity (%)	Specificity (%)
M_2-G	Lipid peroxidation	–	0.72	5.78	LF**Be** (vs. LFCh)	1.45	100	0
Hydroxy-A	Oxidation	–	0.89	3.33	**LF**Be (vs. HFBe)	1.21	100	0
Trihydroxybutyl-U	Alkylation & oxidation	+	0.96	0.12	HF**Ch** (vs. HFBe)	2.43	83	83
M_2-G	Lipid peroxidation	+	1.32	7.67	HF**Ch** (vs. HFBe)	2.27	100	50
Trihydroxybutyl-T	Alkylation & oxidation	–	1.39	4.91	LF**Be** (vs. LFCh)	1.95	83	0
M_2-acetaldehyde-A	Lipid peroxidation	–	1.39	7.98	**LF**Be (vs. HFBe)	1.50	83	33
M_2-acetaldehyde-G	Lipid peroxidation	+	3.86	8.06	HF**Ch** (vs. HFBe)	2.65	100	67
					HFCh (vs. LFCh)	1.99	100	33

Table 5
Selection of DNA adduct types that could be relevant to the proposed red meat hypotheses (Be = beef, Ch = chicken, HF = high fat, LF = low fat).

DNA adduct of interest	DNA adduct type	Charge	RT (min)	Δ ppm	Selected as discriminant marker for	Selected in	Selected by	Associated p-value
Trihydroxybutyl-U	Alkylation & oxidation	–	0.72	3.21	HF**Be** vs. HFCh	Duodenum	Student's *t*-test	<0.01
Carboxyl-A	Alkylation	+	0.96	1.50	HF**Be** vs. HFCh	Duodenum	Student's *t*-test	0.02
					HFBe vs. LFBe	Duodenum	Student's *t*-test	0.09
Crotonaldehyde-G	Lipid peroxidation	+	0.96	0.71	**HF**Be vs. LFBe	Duodenum	Sieve™ pairwise comparison	0.03
				1.82			Student's *t*-test	0.01
				0.71	HF**Be** vs. HFCh	Duodenum	Sieve™ pairwise comparison	0.01
				1.82			Student's *t*-test	<0.01
Methyl-C	Alkylation	+	0.96	0.08	(LF & HF) **Be** vs. Ch	All tissues	GENE-E	0.10
Oxohexenal-C	Lipid peroxidation	+	1.01	0.23	HF**Be** vs. HFCh	Liver	Student's *t*-test	0.09
1,N_2-propano-G	Lipid peroxidation	–	1.04	7.17	LF**Be** vs. LFCh	Duodenum	Sieve™ pairwise comparison	0.10
Nitro-C	Nitrosation	+	1.15	0.59	HF**Be** vs. HFCh	Duodenum	Student's *t*-test	0.09
Carboxymethyl-G or Glyoxal-G	Alkylation or lipid peroxidation	–	1.19	4.05	HF**Be** vs. HFCh	Liver	Student's *t*-test	0.04
					HFCh vs. LFCh	Colon	Student's *t*-test	0.02
Carboxyethyl-G or Carboxyhydroxyethyl-A or Methylglyoxal-G	Alkylation and/or lipid peroxidation	+	1.24	1.91	HF**Be** vs. HFCh	Liver	Student's *t*-test	0.02
					HFBe vs. LFBe	Liver	Student's *t*-test	<0.01
					HFCh vs. LFCh	Colon	Student's *t*-test	0.06
Hydroxybutyl-A	Alkylation & oxidation	+	1.63	4.89	**HF**Be vs. LFBe	Liver	Student's *t*-test	0.03
Carboxyl-A	Alkylation	+	1.85	0.48	HF**Be** vs. HFCh	Colon	Student's *t*-test	0.09
Hydroxymethyl-A or Methyl-G or Methoxy-A	Alkylation & oxidation	+	1.85	0.54	**HF**Be vs. LFBe	Liver	Student's *t*-test	0.03
M_2-acetaldehyde-A	Lipid peroxidation	–	3.66	4.95	HF**Be** vs. HFCh	Duodenum	Student's *t*-test	<0.01
M_2-G	Lipid peroxidation	–	3.66	1.65	(LF & HF) **Be** vs. Ch	Colon & duodenum	GENE-E	0.02
					LF**Be** vs. LFCh	Duodenum	Student's *t*-test	0.03
Hydroxyhydro-C	Oxidation	–	3.82	0.71	HF**Be** vs. HFCh	Duodenum	Student's *t*-test	0.01
Hydroxybutyl-G	Alkylation & oxidation	+	3.89	4.33	**HF**Ch vs. LFCh	Duodenum	Student's *t*-test	0.04
Hydroxyhydro-C	Oxidation	–	4.12	4.77	LF**Be** vs. LFCh	Duodenum	Sieve™ pairwise comparison	0.08
				0.41	LF**Be** vs. LFCh	Duodenum	Student's *t*-test	0.03
M_2-acetaldehyde-A	Lipid peroxidation	–	4.18	2.78	HF**Be** vs. HFCh	Duodenum	Sieve™ pairwise comparison	0.10
Heptenal-G	Lipid peroxidation	–	4.29	4.89	HF**Be** vs. HFCh	Liver	Student's *t*-test	0.03
					LF**Be** vs. LFCh	Liver	Student's *t*-test	0.09
Hydroxyethyl-C or Methoxymethyl-C	Alkylation & oxidation	–	4.38	1.45	HF**Be** vs. HFCh	Duodenum	Student's *t*-test	0.09
Carbamoylhydroxyethyl-G	Alkylation & oxidation	+	5.48	0.68	HF**Be** vs. HFCh	Duodenum	Student's *t*-test	0.04
M_2-acetaldehyde-G	Lipid peroxidation	–	5.50	3.71	**HF**Be vs. LFBe	Liver	Student's *t*-test	0.05
					LF**Be** vs. LFCh	Liver	Student's *t*-test	0.09
					HF**Be** vs. HFCh	Duodenum	Student's *t*-test	<0.01

being; i.e. some DNA adduct types are highly mutagenic, some are repaired spontaneously, and some occur endogenously, whilst others are or do not (De Bont & van Larebeke, 2004). Therefore, the DNA adduct types that were distinctly higher or lower in liver, duodenum and/or colon were not explored further in this paper.

4.2. The effect of meat type and fat content in the diet on the DNA adductome

Differences in DNA adduct levels according to diet were investigated separately for each tissue type. Three out of four targeted DNA adducts could be detected (O^6-Me-G, Cro-G and M_1-G) in rat liver, duodenum and colon. Due to the fact that the total amount of DNA in each sample was rather low, we were unable to confirm or refute any possible relation between O^6-Me-G, M_1-G or Cro-G levels and meat type or fat content in the diet. The fourth DNA adduct; i.e. O^6-CM-G, could not be retrieved in this study although its presence has previously been reported in both rats and humans (De Bont & van Larebeke, 2004; Lewin et al., 2006; Terasaki et al., 2008). In the current study, the amount of DNA in the samples could have been too low to detect O^6-CM-G although the detection limit of the utilized method may have been a limiting factor as well (Hemeryck et al., 2015). Alternatively, since rats do not possess an enterosalivary cycle of nitrate, and the meat diets did not contain relevant amounts of nitrite, the formation of O^6-CM-G adducts via the formation of the hypothesized NOC precursors could have been negligible (Chenni et al., 2013). Nevertheless, in future studies, the amount of DNA per sample should be increased ($\geq$ 100 μg) to be able to thoroughly assess the influence of a specific diet on the presence and levels of O^6-Me-G, O^6-CM-G, Cro-G and M_1-G DNA adducts. Even more so since previous research has already pointed out their potential *in vivo* relevance in relation to diet (De Bont & van Larebeke, 2004; Eder, Budiawan, & Schuler, 1996; Lewin et al., 2006; Marnett, 1999; Winter et al., 2011).

Extensive data processing by means of different omics software packages enabled us to single out 22 different putative DNA adduct types that were higher in rat colon, duodenum or liver after digestion of a beef based diet (compared to a diet with chicken) and/or a HF diet (compared to a LF diet). 14 DNA adduct types appeared to be significantly higher after consumption of beef (*vs.* consumption of chicken), 3 DNA adduct types demonstrated an increase upon daily HF consumption (*vs.* LF), and 5 DNA adducts demonstrated an increase after the consumption of a diet with a high fat content (compared to the corresponding LF diet) as well as a beef diet (compared to the consumption of a chicken diet). Since all 22 selected DNA adduct types originate from alkylation, nitrosation and/or oxidation processes, a more in-depth investigation of the retrieved DNA adduct types is highly relevant to the red meat-CRC hypothesis; i.e. NOCs and LPOs rise upon red meat and high fat intake, and are prone to form DNA adducts (De Bont & van Larebeke, 2004; Oostindjer et al., 2014).

4.3. DNA adducts that increased after the daily consumption of beef

Trihydroxybutyl-U (RT of 0.72), carboxyl-A (RT of 1.85 min), methyl-C, oxohexenal-C, 1,N_2-propano-G, nitro-C, M_2-acetaldehyde-A (observed at two different RTs), M_2-G (RT of 3.66 min), hydroxyhydro-C (observed at two different RTs), heptenal-G, hydroxyethyl-C (or methoxymethyl-C) and carbamoylhydroxyethyl-G DNA adducts appeared to be higher in beef fed rats compared to chicken fed rats.

Of these DNA adduct types, only methyl-C, M_2-G and carboxyl-A demonstrate a significant increase in colon DNA, the major tissue type of interest. Methylation of C at its 5th carbon atom induces the formation of 5-methyl-C. 5-methyl-C, also known as the '6th base' of the mammalian genome, plays an important role in gene expression, genomic imprinting and suppression of transposable elements, and is therefore of specific interest in the field of epigenetics (Ito et al., 2011). However, the methyl group in the methyl-C compound that could be detected in this study (and was singled out as a discriminative DNA adduct type for all tissue types according to GENE-E), may be positioned elsewhere, which means the retrieved methyl-C molecule could just as well correspond to N^3-methyl-C, N^4-methyl-C or O^2-methyl-C (Motorin, Muller, Behm-Ansmant, & Branlant, 2007). Direct or indirect addition of a carboxyl group to the adenine nucleobase is an unspecific reaction type that cannot easily be linked to a specific precursor. The gut microbiome may be able to induce the formation of carboxyl-A directly or indirectly (e.g. via nitroso compound formation) although this is merely speculative. Carboxyl-A has not been studied extensively but its C analogue (carboxyl-C) has been detected *in vivo* (genomic DNA of mouse embryonic stem cells and mouse organs) and has been linked to the enzyme mediated demethylation of 5-methyl-C (Ito et al., 2011). Since demethylation of methyl-A has been described as well (=DNA repair pathway) (Begley & Samson, 2003), the mode of action may be similar, possibly resulting in the *in vivo* formation of carboxyl-A. The major malondialdehyde DNA adduct (malondialdehyde (MDA) is an important lipid peroxidation product) is the monomeric M_1-G DNA adduct although multimeric DNA adducts – like the putatively detected M_2-G – can also be formed after polymerization of 2 or more MDA molecules. The formation of multimeric MDA DNA adducts does not occur as fast or frequent as monomeric MDA DNA adduct formation under physiological conditions and is relatively slow at neutral pH. However, according to Marnett (1999), multimeric MDA DNA adduct types can occur in certain – unspecified – *in vivo* circumstances.

Oxohexenal-C and heptenal-G appeared to be significantly higher in liver DNA after daily beef consumption, and for heptenal-G, the effect was observed for both the LF and HF beef diet. Oxohexenal and heptenal are products of lipid peroxidation. Shorter chain LPOs like acrolein and crotonaldehyde are more reactive towards biomacromolecules than the longer chain LPOs like heptenal and oxohexenal, but acrolein and crotonaldehyde primarily originate from ω-3 fatty acids, whilst the longer chain enals appear to originate from ω-6 fatty acids exclusively (Chung et al., 2003). Since dietary ω-6 polyunsaturated fatty acids have been linked to colon tumorigenesis in F344 rats (Rao, Hirose, Indranie, & Reddy, 2001), the retrieval of oxohexenal-C and heptenal-G could be of particular interest.

The tentatively identified trihydroxybutyl-U, 1,N_2-propano-G, nitro-C, M_2-acetaldehyde-A, hydroxyhydro-C, hydroxyethyl-C (or methoxymethyl-C) and carbamoylhydroxyethyl-G DNA adducts were exposed as discriminating DNA adducts for beef vs. chicken digestion in duodenal DNA. The trihydroxybutyl-U molecule has rarely been studied and could just as well correspond to an isomer with the exact same mass (like e.g. trihydroxypropyl-T). Both 1,N_2-propano-G and M_2-acetaldehyde-A are products of lipid peroxidation derived DNA adduct formation. 1,N_2-propano-G is formed due to exposure of G to acrolein (Chung et al., 2003), an ubiquitous and highly reactive LPO, while M_2-acetaldehyde-A can be derived from the interaction of A and a malondialdehyde-acetaldehyde conjugate consisting of 2 MDA molecules and 1 acetaldehyde molecule (Pluskota-Karwatka, Pawlowicz, & Kronberg, 2006). The possible *in vivo* formation of M_2-acetaldehyde protein adducts was confirmed by Tuma, Thiele, Xu, Klassen, and Sorrell (1996), although this might be the first time that the *in vivo* formation of (2 different) M_2-acetaldehyde DNA adducts is suggested. Nitro-C, hydroxyhydro-C, hydroxyethyl-C (or methoxymethyl-C) and carbamoylhydroxyethyl-G are formed upon nitrosation, oxidation and/or alkylation of C and G, which are very unspecific ways of DNA adduct formation that cannot easily be traced to its exact origin/precursor. At the time, more detailed information on nitro-C is not available since it appears that nitro-C has rarely been studied. The available information on hydroxyhydro-C is scarce as well, although this compound has previously been identified in mammalian DNA (Dizdaroglu, 1992). To the best of our knowledge, hydroxyethyl-C and/or methoxymethyl-C have never been detected *in vivo*. Carbamoylhydroxyethyl-G formation on the other hand, has been linked to exposure to acrylamide (used in industry, present in cigarettes and foods processed at high temperatures) in rats, although acrylamide exposure in this study is not very likely (Manière et al., 2005). However, nothing in its chemical structure suggests that carbamoylhydroxyethyl-G formation is strictly limited to the occurrence of acrylamide exposure since the added chemical group is not highly specific; i.e. carbamoylation and alkylation of macromolecules can also be induced by e.g. nitrosourea compounds (a specific group of NOCs) (Wheeler, Bowdon, & Struck, 1975). The latter hypothesis is far more likely following the consumption of beef and the subsequent increased exposure to the NOC formation promoting heme molecule (Oostindjer et al., 2014).

4.4. DNA adducts that increased due to the intake of a high fat diet

Hydroxybutyl-A, hydroxymethyl-A (or methyl-G or methoxy-A) and hydroxybutyl-G significantly increased in liver or duodenal DNA after the daily consumption of a HF meat diet. Airoldi et al., 1994) already documented the *in vivo* formation of hydroxybutyl-G in urothelial and hepatic DNA after administration of a single dose of *N*-nitrosobutyl(4-hydroxybutyl)amine, a carcinogenic NOC, to rats. A similar mechanism may likewise be applicable for hydroxybutyl-A, although additional research is required to support this statement and confirm the detection of hydroxybutyl-A in rat liver DNA. The mass that corresponds to hydroxymethyl-A, methyl-G or methoxy-A could not be identified as O^6-Me-G or N^7-Methyl-G by means of analytical standards (O^6-Me-G elutes at 2.83 min) (Hemeryck et al., 2015) and N^7-methyl-G elutes at 1.50 min (unpublished data). According to literature, N^7-methyl-G is the predominantly formed methyl-G isomer, whilst O^6-Me-G, occurs far less frequently (e.g. 400 N^7-methyl-G molecules compared to 1 O^6-Me-G molecule by the S-adenosylmethionine enzyme, a methyl group donor that contributes to physiological DNA methylation) (De Bont & van Larebeke, 2004). Alternative options for identification include N^1-, and N^3-methyl-G (Di Pietro et al., 2001) or a methoxy-A (Nishio, Ono, Matsuda, & Ueda, 1992) or hydroxymethyl-A (el-Khadem & Sindric, 1974) isomer.

4.5. DNA adducts associated with the intake of a beef as well as a high fat diet

The putatively identified carboxyl-A (RT of 0.96 min), cro-G (RT 0.96 min), carboxymethyl-G (or glyoxal-G),

carboxyethyl-G or (carboxyhydroxyethyl-A or methylglyoxal-G) and M_2-acetaldehyde-A (RT of 5.50 min) were higher in rat DNA after digestion of a beef diet as well as a meat diet with added fat.

In colon DNA, an increase of carboxymethyl-G (or glyoxal-G) and carboxyethyl-G (or carboxyhydroxyethyl-A or methylglyoxal-G) occurred due to the consumption of a HF (chicken) diet (in comparison to a LFCh diet). The carboxymethyl-G compound could not be identified as O^6-CM-G since it did not co-elute with an O^6-CM-G standard (which has a RT of 1.54 min) (Hemeryck et al., 2015). However, two very likely alternatives are N^7-carboxymethyl-G or glyoxal-G for the reason that (a) nitrosated bile salts predominantly form N^7-carboxymethyl-G during reaction with DNA (De Bont & van Larebeke, 2004), and (b) glyoxal-G formation by *N*-nitroso compounds has been documented previously (Loeppky, Cui, Goelzer, Park, & Ye, 1999), and (c) the glyoxal molecule, an endogenously occurring metabolite that is formed during degradation of glucose, glycated proteins and lipid peroxidation, is known to readily react with DNA (Abordo, Minhas, & Thornalley, 1999; Mistry et al., 2003). The latter also applies for methylglyoxal, which is a probable precursor of the methylglyoxal-G DNA adduct; the molecule that may equally correspond to carboxyethyl-G, or carboxyhydroxyethyl-A. Regardless, N^7-carboxymethyl-G (De Bont & van Larebeke, 2004), glyoxal-G (Loeppky et al., 1999), methylglyoxal-G (Vaca, Nilsson, Fang, & Grafstrom, 1998), carboxyethyl-G (Cheng, Wang, Villalta, & Hecht, 2010) as well as carboxyhydroxyethyl-A (Gamboa da Costa et al., 2003) have all been studied and detected *in vitro* and/or *in vivo* in previous DNA adduct studies, rendering all of these DNA adduct types to be valid options for tentative identification.

The carboxyethyl-G (or carboxyhydroxyethyl-A or methylglyoxal-G) and carboxymethyl-G (or glyoxal-G) DNA adducts could also be retrieved in liver DNA where they significantly rose after beef as well as high fat consumption. M_2-acetaldehyde-G also increased upon beef and high fat consumption in liver DNA, also demonstrating a significant increase in duodenal DNA in relation to beef consumption. As was discussed earlier on for its A analogue (M_2-acetaldehyde-A, under section 4.3 *DNA adducts that increased after the daily consumption of beef*), M_2-acetaldehyde-G could originate from the interaction of G and a malondialdehyde-acetaldehyde conjugate, but has also never formerly been detected *in vivo*.

The remaining DNA adducts; carboxyl-A and cro-G, were discriminating for beef *vs.* chicken and HF *vs.* LF beef in the rat duodenum. As was already discussed above, carboxyl-A has not previously been detected *in vivo* although it may be of importance by analogy with carboxyl-C (in relation to methyl-A and 5-methyl-C respectively) (Ito et al., 2011). Since cro-G eluted at RT 0.96 min and not at RT 3.46 min (Hemeryck et al., 2015), we can be certain that this compound does not match with the commercially available cro-G standard that was purchased beforehand. The retrieved cro-G molecule could be an isomer since cro-G does occur in different configurations (Zhang, Villalta, Wang, & Hecht, 2006).

The identity of the putatively identified DNA adducts that were discussed above, were not confirmed by means of analytical standards, which is in part because the currently commercially available number of DNA adduct standards is limited. Nevertheless, there are several valid arguments that can be raised in support of the putative identification of all DNA adduct types; (a) DNA purity was tested and confirmed for each sample after DNA extraction from liver, duodenum and colon tissue samples, (b) DNA was hydrolyzed (in acid at high temperature) and DNA adducts were extracted by means of solid phase extraction, (c) DNA adducts were separated by means of a UHPLC method that was optimized for DNA adduct separation, and (d) Q-Exactive MS analysis allows highly accurate mass measurements. Nevertheless, the findings of this study should be validated by independent follow-up studies.

5. Conclusion

In the past, animal DNA adduct studies have allowed researchers to gain a more thorough understanding of the role of DNA adducts in mutation and carcinogenesis. Even today, animal model studies represent one of the best options to study the relation between dietary exposure to directly or indirectly harmful chemicals, gastro-intestinal formation of genotoxic chemicals, metabolisation (resulting in activation or inactivation) and excretion of genotoxic chemicals, related DNA adduct formation and the onset of disease. This study demonstrated that beef and high fat intake (in comparison with chicken and low fat meat intake) stimulate the formation of certain types of DNA adducts that may be able to help elucidate the red-meat-CRC hypothesis since most of the DNA adduct types that could be retrieved in liver, duodenum and/or colon are the result of DNA alkylation and/or oxidation processes. The exact relevance of these DNA adduct types in relation to the red meat-CRC hypothesis needs to be assessed further in follow-up studies.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. Furthermore, the local ethical committee granted approval for this particular study (ECD 14/58 (Ghent, Belgium)).

Funding

This work was supported by the Federal Public Service Health, Food Chain Safety and Environment, Belgium [Grant RF-11/625 MEATNOX].

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

The authors would like to thank Mieke Naessens (LCA) for her assistance in the lab. S. Coolsaet, E. Claeys, D. Baeyens, G. Verhofsté and J. Vermeiren (LANUPRO) are thanked for their practical assistance during the animal experiment.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2017.02.129.

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