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Abbreviations: CDV, cardiovascular disease; CRC, colorectal cancer; DM, diabetes mellitus; hccd, human colon chicken digest; hcbd, human colon beef digest; HMDB, Human Metabolome Database; LMSD, LIPID MAPS Structure Database; NOC, N-nitroso compound; Neu5GC, N-glycolylneuraminic acid; OPLS-DA, orthogonal partial least squares discriminant analysis; PCA, principal component analysis; PG, prostaglandin; QC, quality control; ROS, reactive oxygen species; TMAO, trimethylamine-N-oxide; UHPLC-HRMS, ultra-high performance liquid-chromatography high-resolution mass spectrometry.

Abstract

Scope: The consumption of red and processed meat, and not white meat, has been associated with the development of various Western diseases such as colorectal cancer and type 2 diabetes. This work aimed at unravelling novel meat-associated mechanisms that are involved in disease development.

Methods and results: A non-hypothesis driven strategy of untargeted metabolomics was applied to assess colon tissue from rats (fed a high dose of beef *vs.* white meat) and from pigs (fed red/processed meat *vs.* white meat), receiving a realistic human background diet. An increased carnitine metabolism was observed, which was reflected by higher levels of acylcarnitines and 3-dehydroxycarnitine (rats and pigs) and trimethylamine-N-oxide (rats). While 3-dehydroxycarnitine was higher in HT29 cells, incubated with colonic beef digests, acylcarnitine levels were reduced. This suggested an altered response from colon cancer cell line towards meat-induced oxidative stress. Moreover, metabolic differences between rat

and pigs were observed in N-glycolylneuraminic acid incorporation, prostaglandin and fatty acid synthesis.

Conclusion: This study demonstrated elevated (acyl)carnitine metabolism in colon tissue of animals that followed a red meat-based diet, providing mechanistic insights that may aid in explaining the nutritional-physiological correlation between red/processed meat and Western diseases.

1. Introduction

The consumption of red and/or processed meat, and not white meat, has been associated with several diseases, including diabetes mellitus (DM) type II, cardiovascular disease (CDV) and colorectal cancer (CRC).[1,2] In 2015, the International Agency for Research on Cancer concluded that the intake of red meat is 'probably carcinogenic to humans' (Group 2A), whereas processed meat was classified as 'carcinogenic to humans' (Group 1) for CRC.[3] In a meta-analysis, the intake of 100 g per day unprocessed red meat or 50 g per day processed meat increased the CRC risk with 18%.[4] Several red meat-associated metabolites have been proposed as potentially involved in disease pathogenesis, including those that are formed during enzymatic gastro-intestinal digestion or generated upon direct

interaction between the gut microbiome and specific compounds in red meat.[5,6] In this context, heme iron as present in myoglobin has been one of the prime suspects involved in reactions that generate genotoxic and/or cytotoxic metabolites. Indeed, heme iron can act as a catalyst in the gastro-intestinal formation of N-nitroso compounds (NOCs), reactive oxygen species (ROS), lipid peroxidation and protein oxidation products, which may cause DNA damage and aberrant crypt foci, *i.e.* preneoplastic regions in the colon.[3,6] More recent hypotheses propose a role of trimethylamine-N-oxide (TMAO)[7,8], xeno-autoantigens[9,10] and infectious agents[11] in the relationship between red/processed meat intake and disease development. However, the mechanistic exploration of these recent hypotheses remains to be elucidated, as opposed to those of heme-associated metabolites.[5]

Regarding the heme-associated mechanisms, both *in vitro* experiments as well as *in vivo* trials with humans or animals exposed to acute or chronic intake of red meat or heme-rich diets have been conducted. However, the doses of heme iron used in these studies do not often reflect those of normal human exposure. Moreover, it is not straightforward to extrapolate *in vitro* results to an *in vivo* situation, as cell cultures typically lack important conditions of the colon, in particular its anaerobic environment.[12] Next to this, animal models without genotoxic initiation showed that short-term or chronic heme intake may not be sufficient to initiate or promote the progression of CRC.[12] Additionally, the composition of diets used in trials does not always reflect a realistic human dietary intake of meat, for which research is desired that takes into account representative meals, including foods other than meat in order to gain translationally relevant results. This may offer broader insights in the involved red meat-associated metabolites and mechanisms as well as opportunities to mitigate potential damaging effects. Although most studies have focused on heme-associated endpoints, such as NOC formation, lipid peroxidation and specific DNA adducts thereof, this perspective is too narrow as other potential red meat-associated mechanisms remain unexplored.

The aim of this study was to reveal colon tissue metabolites discriminating for red as opposed to white meat intake, among others in the context of representative background diets, by means of untargeted metabolomics and lipidomics. This non-hypothesis driven strategy could aid in the confirmation and/or elucidation of novel red meat-associated metabolites, which may support studies that intend to investigate red meat-associated mechanisms, potentially involved in disease development, particularly CRC.

2. Experimental section

2.1 General overview of the experimental design

Gastro-intestinal digestions were simulated with beef and chicken meat using the fecal inocula of 3 human volunteers for colonic fermentation as described previously.[13] Subsequently, the colon cancer cell line HT29 (mucosa scrapings) was incubated for 24 h with these *in vitro* digestion samples (human colon chicken digest (hccd) and human colon beef digest (hcbd)). This cell line was used since it has commonly been used to study the relationships between diet and disease.[3] Next to this, colon tissue (incl. tunica mucosa, submucosa, and serosa) was sampled from rats that were fed diets supplemented with beef or chicken meat for 3 weeks. Also, colon tissue samples (incl. tunica mucosa, submucosa, and serosa) were obtained from pigs receiving red/processed or white meat, added to two background diets for 4 weeks, *i.e.* Western or recommended.[14] Cell line and animal colon tissue samples were analyzed by ultra-high-performance liquid chromatography high-resolution mass spectrometry (UHPLC-HRMS) for untargeted polar metabolomics and lipidomics. The experimental set-up is presented in Figure 1.

2.2 Rat feeding trial

The study was approved by the Ghent University Ethical Committee (ECD 18/18) and conducted according to the Belgian law and principles of laboratory animal care. Twenty male Sprague-Dawly rats (± 200 g) (Janvier laboratories, France) were introduced at the same time and were fed a control diet (Ssniff R/M-N pellets) (Soest, Germany) for 5 days *ad*

libitum. Afterwards, rats were randomly assigned to a beef or chicken meat diet (10 rats per group), also being provided *ad libitum*. Details on the preparation of these diets can be consulted in the Supplementary Information (Trial Info S1). Every two days, food intake and body weight were monitored and following 21 days of experimental diets, rats were euthanized by administering 5% isoflurane gas and exsanguination. At each of the euthanasia days, two rats per group were sampled randomly. The whole colon tissue layer was removed, rinsed with 0.9% sodium chloride, snap frozen in liquid nitrogen and stored at -80 °C until extraction.

2.3 Pig feeding trial

The study was approved by the Ghent University Ethical Committee (EC 2016/26). Thirty-two 3-weeks old male piglets (Topigs×Piétrain) from 8 different litters were purchased from a commercial farm. Piglets were allocated to 8 pens with 4 piglets per pen, each from a different litter. The four experimental diets were fed in three *ad libitum* feeding periods of 30 min per day, for four weeks after consuming a commercial pre-starter diet for one week. Details on the preparation of the four diets (with red + processed meat or chicken meat as a first factor and Western or prudent background diet as a second factor) can be consulted in the Supplementary Information (Trial info S2). Animals were weighed twice a week and euthanasia was performed at four consecutive days for practical reasons (two piglets from each dietary treatment per day). Piglets were euthanized by intramuscular injection of xylazine (20 mg mL^{-1}, 0.1 mL kg^{-1} bodyweight) and intraperitoneal injection of pentobarbital (60 mg mL^{-1}, 0.8 mL kg^{-1} bodyweight), followed by exsanguination. Colon content and the whole colon tissue layer were sampled, whereby the latter was washed with 0.9% sodium chloride and stored at -80 °C until extraction.

2.4 Cell culture

The human colon cancer cell line HT29 was obtained from ATCC (VA, USA). Dulbecco's Modified Eagle's Medium, supplemented with 10% Fetal Bovine Serum and 1%

penicillin/streptomycin (Life Technologies, Ghent, Belgium), was used to culture cell lines in a humidified incubator at 37 °C and 5% CO_2/95% air. HT29 cells were seeded in 6-well plates at a density of 500,000 cells per well and after 24 h *in vitro* colon meat digests (hccd or hcbd), obtained from 3 human volunteers, were individually added in triplicate at 2.5%. Details on the preparation of the meat digests, being based on either red meat or chicken, can be consulted in the Supplementary Information (Trial Info S3). One day later, cell line samples were extracted for polar metabolomics and lipidomics and stored at -80 °C until analysis. To this end, cell medium was first aspirated and cells were washed with 0.9% sodium chloride. This experiment was repeated three times with a two-week interval resulting in 54 samples treated with *in vitro* hccd or hcbd (= 3 passages x 2 meat types x 3 human volunteers x 3 technical replicates).

2.5 Compositional analysis of the experimental diets

The animal diets were analyzed for dry matter, crude protein and crude fat content according to the ISO 1442–1973, ISO 937–1978 and ISO 1444–1973 methods, respectively (Supplementary Table 1). Analysis of fatty acids and hematin was performed according to Hornsey (1956).[15] Hematin concentrations were converted to amount of heme-Fe using the formula heme-Fe = hematin × atomic weight Fe/molecular weight hematin.[15] All analyses were performed in duplicate by randomly selecting two food aliquots of each experimental diet.

2.6 Polar metabolomics and lipidomics fingerprinting

Untargeted metabolomics and lipidomics were applied to analyze colon cell line and tissue samples, respectively obtained from *in vitro* and *in vivo* experiments. The applied method was successfully validated for these matrices and proven fit-for-purpose in discriminating between the transformed state (cells characterized by increased growth rates and the ability to form malignant tumors) and non-transformed state (cells characterized by normal growth

rates with cellular senescence after a finite number of doublings), as reported by Rombouts *et al.* (2019).[16]

Extraction of polar metabolites and lipids from the colon cancer cell line HT29 and animal colon tissue was performed as described previously,[16] with the extracts being analyzed by lipidomics or polar metabolomics UHPLC-HRMS[17,18]. Details on the respective methods can be consulted in the Supplementary Information (Analytical Procedures). For each of the matrices (HT29 cell line, rat and pig colon tissue), quality control (QC) samples were prepared as pooled samples and run in duplicate after every 10 samples.

For identification, parallel reaction monitoring MS/MS was implemented, applying the following settings for polar metabolomics; mass resolution of 17,500 full width at half maximum, automatic gain control of 2 e^4 ions, maximum injection time of 40 ms and isolation window of 1.0 *m/z* and lipidomics; a mass resolution of 17,500 full width at half maximum, automatic gain control of 2 e^5 ions, maximum injection time of 100 ms and isolation window of 1.0 *m/z*.

2.7 Data analysis

Peak alignment and component extraction

Cellular together with tissue LC-MS data were processed simultaneously with Sieve™ 2.2 software (Thermo Fisher Scientific, CA, USA), for the polar metabolomics and lipidomics experiment. Chromatographic peak alignment and component extraction was performed as described previously.[16] Total ion count normalization was performed to account for differences in cell number and tissue mass between analyzed samples. In addition, blanks were used for subtraction of noise peaks in the samples of interest. Components with a coefficient of variance above 30% in the QC samples were removed from the dataset.[13,16]

Multivariate statistics

Multivariate statistics (SIMCA™ 14.1 software, Umetrics AB, Umea, Sweden) was performed, whereby principal component analysis (PCA-X) and orthogonal partial least

squares discriminant analysis (OPLS-DA) models were established. To this end, data were log-transformed and pareto-scaled. Validity of the OPLS-DA models was evaluated based on R^2Y (> 0.5), Q^2Y (> 0.5), CV-ANOVA (P-value < 0.05) and permutation testing (n = 100).[16] S-plots were constructed to retain those components contributing to the discrimination between red and white meat intake, together with VIP-values (> 1.0) and Jack-Knifed confidence intervals (not including 0).[17,18] For the pig feeding trial, dietary backgrounds were combined for each meat type and hence, data analysis was performed using two dietary treatment groups. This increased the number of samples per meat type, which improved the statistical power to define metabolites that were significantly elevated in colon tissue upon red meat intake.

Multiple univariate statistical analysis

In parallel to the multivariate statistics, multiple independent univariate statistical unpaired t-tests were performed for the untargeted data in MetaboAnalyst 4.0 (www.metaboanalyst.ca) to define components that were significantly (P-value < 0.05) altered between the meat diets. To this extent, data were log-transformed and pareto-scaled. The false discovery rate was controlled by implementing the Benjamini Hochberg P-corrected value.

Identification of components significantly altered between red and white meat diets

Identification of the components of interest was in first instance based on the accurate mass, thereby consulting the Human Metabolome Database (HMDB) and LIPID MAPS Structure Database, applying a mass tolerance of 5 ppm. In addition, the LIPID MAPS Structure Database (LMSD) was applied to obtain "bulk" structures that provide information on the number of carbons and rings/double bounds for the following categories; cholesteryl esters, fatty acyls, glycerolipids, glycerophospholipids, and sphingolipids. To this extent, the accurate masses of significantly altered lipids were introduced in LMSD, whereby the $[M+H]^+$ and $[M-H]^-$ adducts with a mass tolerance of +/- 0.01 *m/z* were evaluated. To have a clear overview of potential shifts in lipid classes, only those putatively identified lipids that could

belong to solely one specific lipid class, based on LMSD, were retained. Next to this, fragmentation profiles of tentatively identified polar metabolites and lipids (including these that could belong to different lipid classes) were scored in SIRIUS and MetFrag software or compared with these of analytical standards. Following this, components were assigned different levels of identification according to Sumner *et al.* (2007).[19]

3. Results

3.1. Multivariate modelling to discriminate red *vs.* white meat exposure

HT29 cell line samples and colon tissue samples obtained from pigs and rats were assessed through polar metabolomics and lipidomics UHPLC-HRMS. Data processing rendered, in positive and negative mode, respectively, 1131 and 1105 components for polar metabolomics and 9966 and 5845 components for lipidomics. PCA-X score plots revealed narrow clustering of QC samples for all matrices, indicating instrumental stability (Supplementary Figures 1 - 3). OPLS-DA models were successfully validated for red *versus* white meat exposure for the polar metabolomics datasets in positive ionization mode for all matrices and for the lipidomics dataset in positive ionization mode for the HT29 cell line samples (Supplementary Table 2). The number of retained discriminative components, based on multivariate selection criteria and univariate statistics, are also presented in Supplementary Table 2.

3.2. Significantly altered polar and lipid components upon red *vs.* white meat exposure

When comparing the polar components that were significantly associated with red or white meat intake, only a small overlap could be observed for the three matrices, *i.e.* 4 and 5 components for white and red meat intake, respectively (Supplementary Figure 4A and 4B).

Additionally, 128 out of the 274 significantly altered polar components and 353 out of the 2402 significantly altered lipids, as obtained from the three different trials, could be assigned putative identities based on the HMDB and/or LIPID MAPS databases (Supplementary

Figure 4). Finally, 55 polar metabolites and lipids were identified based on *in silico* MS/MS data (Tier 2/3) and/or analytical standards (Tier 1) (Supplementary Table 3 and 4).

LMSD bulk search, starting from tentatively identified lipids, was performed to assess general shifts in lipid classes upon red and white meat exposure (Supplementary Figure 5). Cardiolipins were upregulated in the HT29 cell line exposed to hccd. Pigs fed a white as opposed to a red meat diet showed only 6 altered lipids and hence it was more difficult to assess shifts in lipid-related pathways. Next to this, the proportions of mainly fatty acids and ceramides were increased upon red as opposed to white meat digestion/exposure. Furthermore, the ratio of putatively identified saturated fatty acids/mono- or polyunsaturated fatty acids, altered by red meat intake, was much higher in pig colon tissue compared to rat colon tissue and the HT29 cell line.

3.3. (Acyl)carnitine profiles in fecal content, colon cell line and colon tissue samples

In general, L-carnitine and acylcarnitines were the group of metabolites that were most clearly and consistently upregulated after red meat consumption in pig and rat colon tissue (Supplementary Table 4). This with the exception of hexanoylcarnitine, which was found enriched in colon tissue of rats fed with chicken. In contrast to the general finding, HT29 cell line samples contained more of the identified L-carnitine and acylcarnitines (*i.e.* butyrylcarnitine, 2-methylbutyrylcarnitine, and propionylcarnitine) when treated with hccd as opposed to hcbd (Supplementary Table 4). Thus, acylcarnitine profiles were differentially regulated in the HT29 cell line compared to *in vivo* tissue samples upon red and white meat consumption. To gain more insight in the absorption and metabolism of the cited metabolites, we assessed the abundance of the identified acylcarnitines in the *in vitro* colon digests (hccd and hcbd) that were used to treat the HT29 cell line, the untreated HT29 cell line and the colon/fecal content of the pigs and rats.

L-carnitine was significantly (Benjamini Hochberg P-corrected value < 0.05) more abundant in animal colon/fecal content after red as opposed to white meat consumption (Figure 2),

explaining the higher L-carnitine levels in animal colon tissue samples. However, this metabolite was equally abundant in hccd and hcbd, and even more abundant in the HT29 cell line treated with hccd as opposed to hcbd (Figure 2). In concordance with the results for L-carnitine, propionylcarnitine levels were not significantly altered between hccd and hcbd, although an increase after hccd as opposed to hcbd exposure of the HT29 cell line was observed (Figure 2). 3-Dehydroxycarnitine was present in *in vitro* digestive fluids (hccd and hcdb)/colon/fecal content as well as in the cell line and colon tissue samples and was significantly higher after red as opposed to white meat consumption. Aside from rat feces, 3-dehydroxycarnitine levels were significantly more abundant following chicken meat intake, although the opposite was observed in rat colon tissue (Figure 2). Succinylcarnitine levels were higher after red as opposed to white meat intake in both *in vitro* digestive fluids (hccd and hcbd)/colon/fecal content and colon cell line and tissue samples (Figure 2). 2-Methylbutyroylcarnitine and butyrylcarnitine could not be detected in the human *in vitro* colon digests (hccd and hbcd) and animal colon/fecal content, as opposed to the HT29 cell line and animal colon tissue samples (Figure 2), but were detected in pig small intestinal content (data not shown). Hexanoylcarnitine levels were not significantly different between red and white meat diets in pig colon content and rat feces, although they were significantly up- and downregulated in colon tissue after red as opposed to white meat diet in pigs and rats, respectively (Figure 2).

To further explore the cellular metabolism of acylcarnitines in the HT29 cell line, cells were incubated (24 h) with different concentrations of L-carnitine (0-10 mM) and analyzed with the polar metabolomics method. This resulted in a dose-dependent increase of L-carnitine, propionylcarnitine and 2-methylbutyroylcarnitine. Butyrylcarnitine, 3-dehydroxycarnitine, succinylcarnitine and hexanoylcarnitine were not significantly influenced (Figure 3).

4. Discussion

As a major finding, we observed that L-carnitine and six acylcarnitines were more abundant in red (+ processed) meat as opposed to white meat-associated fingerprints in rat and pig colon tissue samples (Figure 4). L-carnitine is more than 6-fold higher in beef compared to chicken meat, implying that this metabolite is a marker of red meat intake. In the human body, L-carnitine is responsible for the bidirectional transport of acyl moieties from cytosol to mitochondria and *vice versa* and thus plays a pivotal role in mitochondrial respiration and fatty acid β-oxidation, resulting in the formation of acylcarnitines through L-carnitine esterification. Acylcarnitines are therefore considered as products from the enzymatic carnitine transfer system, reflecting indirect mitochondrial metabolism (Figure 4).[20]

In various studies, carnitine and acylcarnitine levels in urine and serum have been linked to habitual red and processed meat intake.[21,22] Specifically, levels of propionylcarnitine and 2-methylbutyrylcarnitine were elevated in urine of individuals consuming meat-based diets (from mammals and fish) as opposed to non-meat diets, but no distinction could be made between the different meat sources.[21] Nevertheless, acylcarnitine levels in plasma and urine do not reflect those in tissue as demonstrated by Schooneman *et al.* (2014).[22] Altered acylcarnitine levels could be markers of fatty acid β-oxidation dysregulation and mitochondrial stress, and were found to be predictive for DM type II, CVD and CRC.[23,24,25] In this context, it is important to note that especially long-chain acylcarnitines showed a correlation with these diseases, whilst in our study mainly short and medium-chain acylcarnitines were discriminative. Interesting to note in this perspective, is that succinylcarnitine was the only native acylcarnitine upregulated for red meat in all model systems (Figure 2). With L-carnitine clearly originating from red meat, the origin of succinate in response to red meat intake deserves further exploration. Succinate is not merely a major intermediary of the TCA traditionally considered as an extracellular danger signal in the host, but also a by-product of some bacteria and a primary cross-feeding metabolite between gut resident microbes. Succinylcarnitine accumulation will thus most likely originate from

microbial formation, but with circulating levels of succinate being elevated in several physiological conditions such as obesity and type 2 diabetes and the available evidence suggesting a link between dysbiosis, succinate accumulation in gut, and inflammation, this finding surely warrants further research.[26]

Counter-intuitively, the HT29 cell line showed an opposite trend in L-carnitine and some other acylcarnitine levels compared to the animal colon tissue samples, *i.e.* increased levels of L-carnitine, 2-methylbutyrylcarnitine, butyrylcarnitine and propionylcarnitine were observed upon treatment with hccd as opposed hcbd. L-carnitine and aforementioned acylcarnitine levels were not different between or not detectable in hccd and hcbd, respectively, implying that hccd did not serve as most important source for the relatively high intracellular abundance of these metabolites in the HT29 cell line (Figure 4). Specific cellular processes, genetic predisposition and/or medium exposure may be involved in the production or uptake of increased acylcarnitine levels upon treatment with hccd. As such, cancer- and/or *in vitro*-specific molecular mechanisms could be the underlying causes of increased acylcarnitine formation and/or decreased acylcarnitine degradation upon white as opposed to red meat exposure in cell culture. However, the dietary background of the *in vitro* and *in vivo* experiments was different, which may have affected the outcome. Additionally, it can also be expected that digests have a different effect on epithelial monolayers (*in vitro*, HT29 cell line) *versus* the complex architecture of tissues (*in vivo*, colon tissue).

Aside from succinylcarnitine, 3-dehydroxycarnitine was the only red meat-associated metabolite that showed a significant increase in all investigated matrices, upon red meat intake (Figure 4). Moreover, TMAO, derived from cholines and 3-dehydroxycarnitine, was observed to be elevated in colon tissue of rats subjected to red as opposed to white meat diets (Figure 4). Also, 3-dehydroxycarnitine was significantly elevated in hcbd used to treat the HT29 cell line, in concordance with our previous research,[16] indicating direct cellular absorption of this metabolite, and in colon content of pigs fed red meat diets (Figure 4).

Koeth *et al.* (2014)[27] have demonstrated that 3-dehydroxycarnitine is a major microbial metabolite formed from dietary L-carnitine that is more abundant in red as opposed to white meat. Moreover, the former one can be bacterially metabolized in the gut to trimethylamine that is absorbed in the bloodstream and enzymatically converted to TMAO in the liver.[28] Recently, it has been proposed that TMAO could offer a link between the consumption of red meat and the development of various diseases, including CRC, DM type II and CVD. In this context, Xu *et al.* (2015)[29] demonstrated that TMAO was genetically linked to CRC and identified putative pathways, including WNT signaling and MYC activation, that may provide evidence for that link.[29] TMAO has been described to promote the formation of atherosclerotic plaques in rats and to induce the NLRP3 inflammasome and ROS production in non-transformed colon cell lines.[30,31] To the best of our knowledge, this study is the first to detect increased levels of 3-dehydroxycarnitine in rat and pig colon tissue and TMAO in rat colon tissue, upon red *vs.* white meat intake. The absence of significantly altered TMAO levels in pig colon tissue upon red *vs.* white meat intake could indicate interactions between meat and other ingredients of the complex diets and warrants further research.

This study suggested that cardiolipins in the HT29 cell line were less present upon treatment with hcbd as opposed to hccd (Supplementary Figure 5). Cardiolipins influence the transport of carnitine and acylcarnitines and a disturbed transport may affect mitochondrial processes and hence acylcarnitine formation. Indeed, Battelli *et al.* (1992)[32] and Noel *et al.* (1986)[33] used rat liver mitochondria to assess the importance of cardiolipins for mitochondrial respiration and optimal solubilization of the carnitine-acylcarnitine translocase enzyme, respectively. In both studies, supplementation of cardiolipin-binding agents resulted in decreased mitochondrial respiration and inhibition of translocase activity, respectively.[32,33] Amongst the cellular lipids, cardiolipins are particularly sensitive to ROS and hence oxidative stress, because they are highly unsaturated (90% represented by linoleic acid) and almost exclusively situated within the inner mitochondrial membrane, and thus closely associated

with respiratory chain proteins that act as a major source for ROS production. Therefore, it can be hypothesized that cardiolipins in the HT29 cell line were more affected as a result of oxidative stress upon treatment with hcbd as opposed to hccd in our study, as the hcbd digests have been shown to contain a higher lipid and protein oxidation product content (Figure 4),[34] which may be mediated by TMAO as well (see below). This is further strengthened by Goethals *et al.* (2019)[14], who observed higher levels of oxidative markers in colon content of pigs upon white as opposed to red meat intake due to large differences in fatty acid profiles. Herewith, diets were not corrected for fatty acid differences as opposed to the meat preparations used in the *in vitro* digests and the rat feeding study. In our study, there was no difference in cardiolipin levels in pig colon tissue samples upon red as opposed to white meat intake.

An overall upregulation of ceramides, involved in autophagy, cell cycle arrest and cell death, could be observed after red as opposed to white meat exposure/consumption in HT29 cells and rats, albeit not in pigs (Figure 4). It may be presumed that cellular and tissue *de novo* production of these metabolites occurred in response to stress mechanisms, such as ROS production, upon red meat consumption. Indeed, various conditions known to increase ROS levels have been described to also stimulate ceramide production and induce apoptosis.[35] Hence, it is highly probable that ceramides play a vital role in mitigating the damaging effects (*e.g.* ROS production) occurring upon red meat consumption.

Prostaglandins (PGs) are involved in inflammatory pathways,[36] whereby their production can be stimulated by various factors, including ROS. In this study, three prostaglandins were identified, whereof prostaglandin A1 was associated with red as opposed to white meat diets in rat colon tissue and PG J2 and E2 with white as opposed to red + processed meat consumption in pig colon tissue (Figure 4). PG E2 has been demonstrated to promote gastro-intestinal carcinogenesis by enhancing cell proliferation, survival, angiogenesis and metastasis.[25] Next to this, PG A1 and J2 can induce the expression of the heme oxygenase

enzyme, which is responsible for the degradation of heme to biliverdin and iron.[37] Discrepancy in PG profiles between the two animal studies could be explained by differences in diets, whereby pigs as opposed to rats received red supplemented with processed meat in the context of complex dietary backgrounds. Indeed, studies have shown that differences in dietary lipid composition affect oxidative stress markers and prostaglandin synthesis in mammalian species.[38] Also, fatty acid composition of colon tissue samples was different between rats and pigs upon red (+ processed) meat diets as explained below (Figure 4).

N-glycolylneuraminic acid (Neu5Gc) was increased after consumption of red as opposed to white meat diets in rat colon tissue (Figure 4). Recently, this sialic acid has been described as potentially involved in the development of red meat-associated diseases. Indeed, carnivores such as cats and dogs as opposed to humans are not at high risk for the development of CRC and it has been shown that the former ones are capable of transforming N-acetylneuraminic acid into Neu5Gc, whilst humans cannot.[5] This indicates that humans can only retrieve this metabolite from the diet, whereby beef, pork and lamb are more important sources than poultry.[39] Furthermore, it has been postulated that interaction with circulating anti-Neu5G antibodies could initiate inflammation, one of the hallmarks of cancer and numerous other diseases.[40] Nevertheless, this is the first study that demonstrated the direct link between dietary consumption of beef and the incorporation of Neu5Gc in colon tissue in rats. However, this effect was not observed in HT29 cells and pigs, likely due to, respectively, their human origin and the differences in diets provided. Indeed, the proportion of meat in the whole diet was much higher in the rat as opposed to the pig experiment, most likely resulting in more pronounced effects attributed to red meat consumption. Next to this, mammalian-derived foods other than meat, *e.g.* dairy products, were added to red and white meat diets of pigs and it is known that these are also an

important source of Neu5Gc and may hence mask the difference between red and white meat intake.[40]

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Data Availability Statement:

Data will be shared by the authors upon reasonable request.

Author contributions

C.R.: Formal analysis, Methodology, Investigation, Writing Original Draft, Visualization; **L.V.M.:** Conceptualization, Methodology, Visualization, Funding acquisition; **M.D.S.:** Formal analysis, Investigation, Writing - review & editing **S.G.:** Formal analysis; Investigation, Writing - review & editing**; T.V.H.:** Investigation, Writing - review & editing; **S.D.S.:** Conceptualization, Writing - review & editing, Funding acquisition; **W.H.D.V.:** Conceptualization, Writing - review & editing, Funding acquisition, Supervision; **L.V.:** Conceptualization, Writing - review & editing, Funding acquisition, Supervision.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figure captions

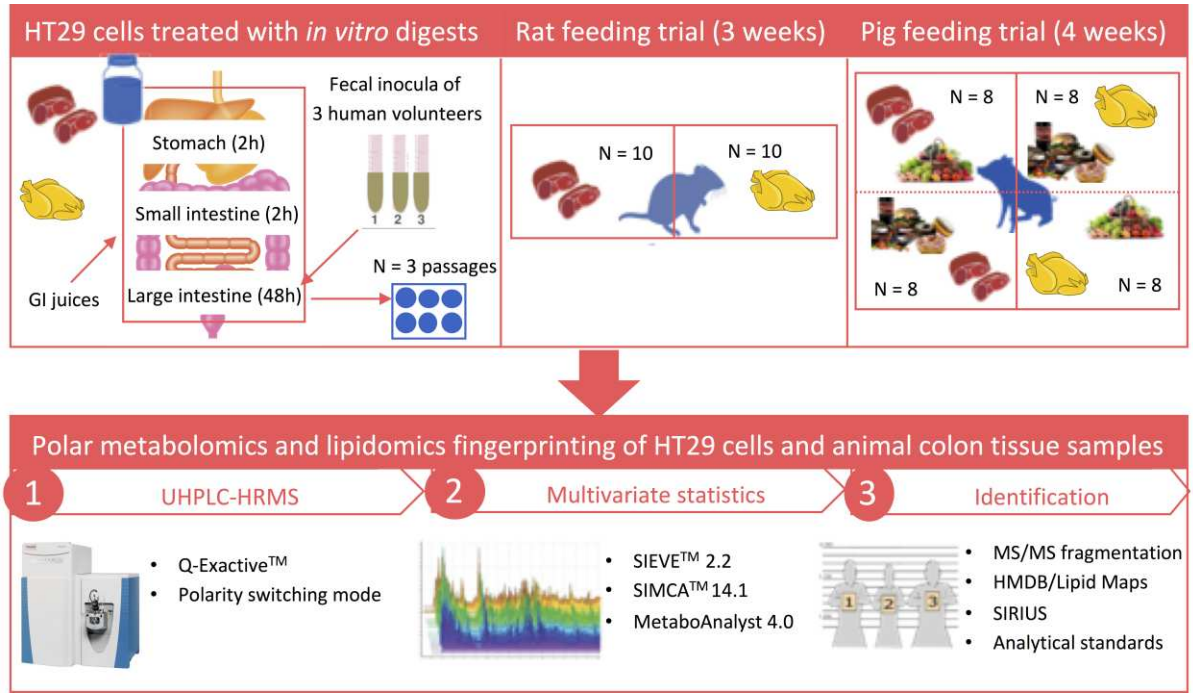

Figure 1. Schematic overview of the experimental set-up, including treatment of HT29 cell lines with *in vitro* meat digests (hccd and hcbd) and dietary trials with rats and pigs. Untargeted metabolomics was applied to HT29 cell line samples and animal colon tissue samples.

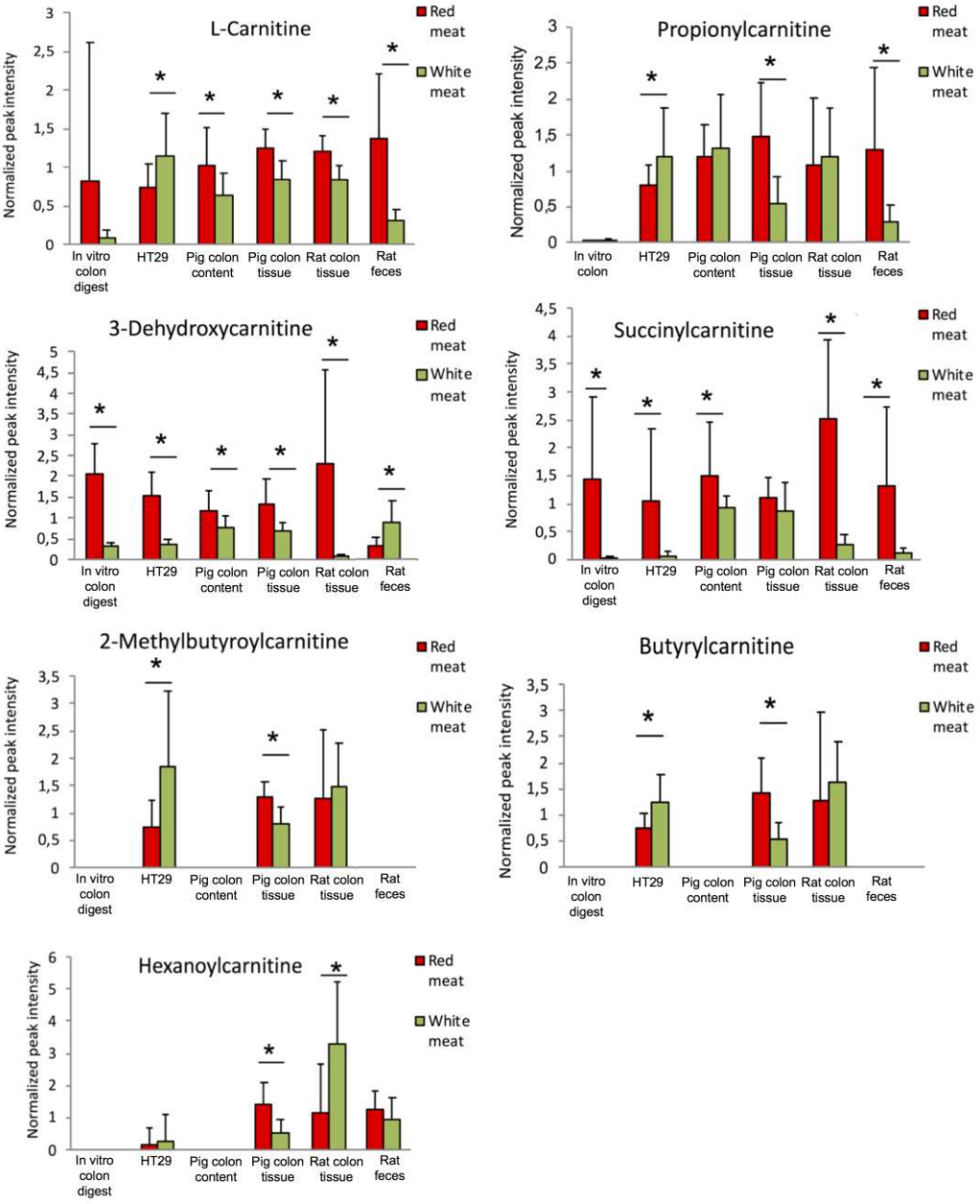

Figure 2. Normalized peak intensities of L-carnitine and various acylcarnitines in human *in vitro* colon meat digestion samples (hccd and hcbd), animal fecal content, HT29 cell line samples and rat and pig colon tissue samples. Normalization was performed by dividing the peak area (in arbitrary units) of a specific compound in a sample by the mean peak area of that compound in the next two QC samples. Compounds with an asterisk were statistically (Benjamini Hochberg P-corrected value < 0.05) different between red and white meat intake. Error bars present standard deviations (*in vitro* digests: n = 3, HT29: n = 9, colon tissue pigs: n = 16, colon content pigs fed chicken meat: n = 14, colon content pigs fed red + processed meat: n = 16, rats: n = 10).

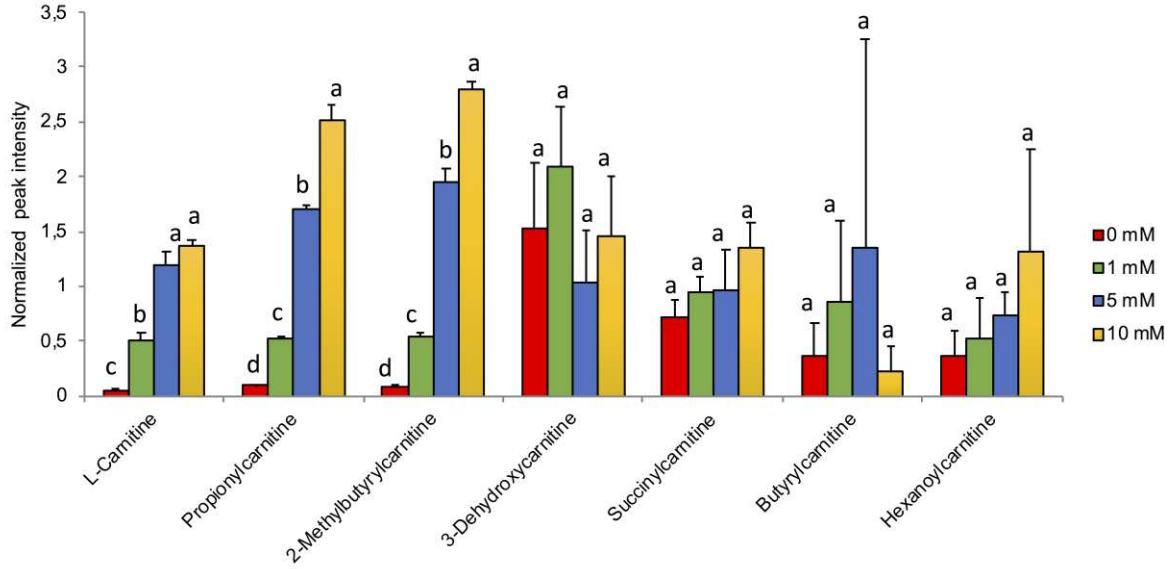

Figure 3. Normalized peak intensities of L-carnitine and various acylcarnitines in the HT29 cell line upon incubation (24 h) with different concentrations of L-carnitine (0-10 mM). Normalization was performed by dividing the peak area (in arbitrary units) of a specific compound in a sample by the mean peak area of that compound in the next two QC samples. Treatments with different letters were statistically (Benjamini Hochberg P-corrected value < 0.05) different for a particular compound. Error bars present standard deviations (n = 3).

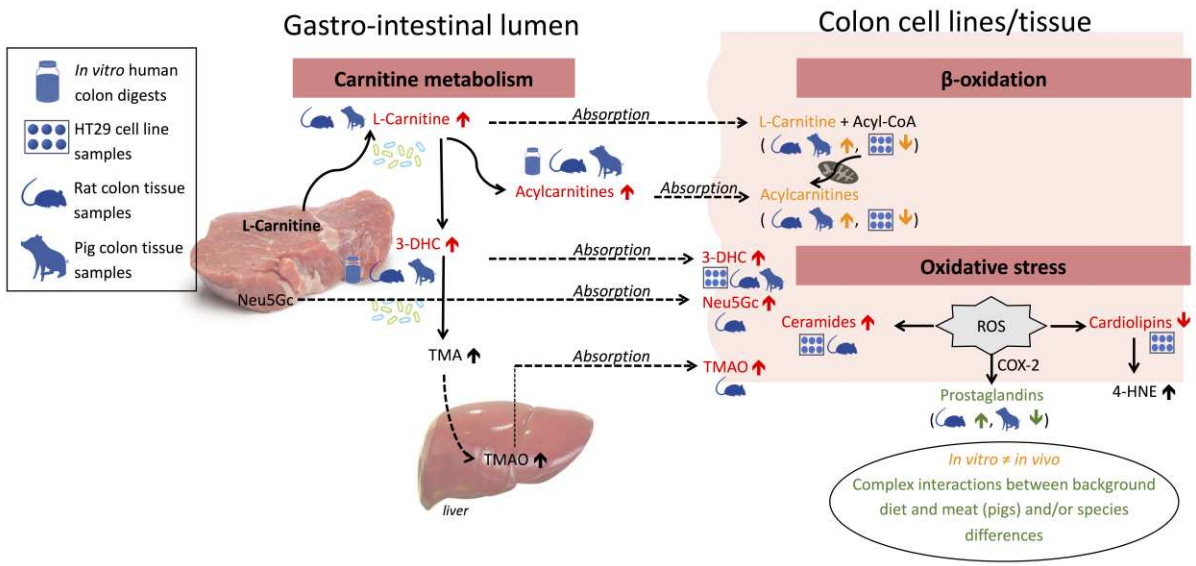

Figure 4. Schematic overview of the interactions between the identified compounds in the gastro-intestinal lumen and colon tissue, and their involvement in red meat-associated pathways. Metabolites in red were elevated (↑) or decreased (↓) upon red *vs.* white meat intake in *in vitro* colon digests (hccd and hcbd), HT29 cell line, rat feces or colon tissue and pig colon content or colon tissue samples. Metabolites in orange and green behaved contradictory between cell line and animal tissue samples or between rat and pig colon tissue samples, respectively. Metabolites and biological processes (e.g. absorption, ROS) in black indicate hypothesized intermediate pathway descriptors, for which no data were available from this study for verification. Acyl-CoA, Acetyl Co-enzyme A; 3-DHC, 3-dehydroxycarnitine; Neu5Gc, N-glycolylneuraminic acid; TMA, trimethylamine; TMAO, trimethylamine-N-oxide; ROS, reactive oxygen species; COX-2, cyclo-oxygenase 2; 4-HNE,4-hydroxynonenal (compound involved in human pathologies, including cancer).

This work aimed at providing new mechanistic insights that may aid in explaining the nutritional-physiological link between red/processed meat and various Western diseases. To this end, metabolomics was applied to assess colon tissue from rats (fed beef *vs*. white meat) and pigs (fed red/processed meat *vs*. white meat), receiving a realistic human background diet. Hereby, an altered metabolism for various acylcarnitines, 3-dehydroxycarnitine and trimethylamine-N-oxide was observed. In addition, the responses of these metabolites were also evaluated in HT29 colon cancer lines.