HIGH-RESOLUTION MASS SPECTROMETRY BASED METABOLOMICS REVEALS ACYLCARNITINES AS KEY METABOLITES DISCRIMINATING RED FROM WHITE MEAT COLONIC DIGESTION

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Abstract – Epidemiological research has demonstrated that the consumption of red meat is an important risk factor for the development of colon cancer. The main hypothesis states that haem iron can act as a catalyst in the formation of toxic *N*-nitroso compounds (NOCs) and in lipid peroxidation. However, further research is required to elucidate the relation between the consumption of red meat and the development of colon cancer. In this study, an innovative untargeted Ultra High Performance Liquid Chromatography - High Resolution Mass Spectrometry (UHPLC-HRMS) based metabolomics approach was used to create a red meat associated metabolic fingerprint. For this purpose, the human gastrointestinal digestion of chicken and beef was simulated. After multivariate statistical analysis 5 out of 10 red meat associated metabolites were identified: 3 acylcarnitines, 1 dipeptide and DL-2-aminooctanoic acid. In literature, acylcarnitines have been associated with several disorders, e.g. cardiovascular disease. Dipeptides and amino acids can be endogenously nitrosated to NOCs and can thus be situated in the haem pathway. Although the correlation between all these compounds and colon cancer has not yet been thoroughly investigated, it is possible that these metabolites in particular are involved in the red meat-colon cancer pathway, which warrants further research.

Key Words – Colorectal Cancer (CRC) - Fecal Metabolomics – Orbitrap Mass Spectrometry

I. INTRODUCTION

Colon cancer has a high prevalence in the population; men and women have a risk of 5% and 3.3%, respectively, to develop the disease before the age of 75 [1]. The CRC incidence significantly increased in especially Western countries and epidemiological research has revealed red meat to be an important risk factor [2-4]. The main hypothesis states that haem iron can act as a catalyst in the formation of lipid peroxidation products (e.g. malondialdehyde and 4-hydroxynonenal) and NOCs [5,6]. The haem-derived components can induce mutations in specific genes (K-RAS, APC, TP53) by the production of reactive oxygen species (ROS) and DNA adducts (e.g. O^6-carboxymethylguanine) [5,7]. However, the involvement of other red meat associated metabolites is very likely and thereby worth to investigate. An innovative way to map these metabolites lies in the use of an untargeted metabolomics approach.

II. MATERIALS AND METHODS

Meat preparations

Two meat preparations with fresh beef diaphragma (local slaughterhouse) and chicken breast (local butcher) were produced. The meat samples were chopped into cubes of approximately 1-2 cm^3. Subcutaneous pork fat was added to the meat samples to obtain a total fat content of 20%. The meat samples were first minced using a grinder (omega T-12) equipped with a 10 mm plate, followed by grinding through a 3.5 mm plate. The meat samples were heated in a hot water bath for 30 minutes after the core temperature had reached

90°C. Finally the meat samples were homogenized with a food processor and stored at -20°C.

In vitro digestions

For the simulation of the colon digestion, fecal material was obtained from two volunteers at three different points in time to perform three incubations. Both volunteers were young adult males with no history of antibiotics for at least six months and no known gastro-intestinal diseases. Fecal material was individually processed and the fecal inocula were prepared as described in literature [8]. The *in vitro* simulation of the gastrointestinal digestion consisted of an enzymatic digestion (mouth, stomach and duodenum), followed by colonic fermentation. Preparation of digestion fluids and execution of the incubations were carried out as described earlier [8]. Each incubation was performed in triplicate and digestion samples were stored at -80°C until analysis.

UHPLC-Orbitrap-HRMS

The digestion samples were centrifuged (21161 g, 5 min) and the supernatant was filtered through a polyvinylidene fluoride membrane (0.22 µm, 33 mm ⌀, Millex, USA). The filtrate was then diluted (1/5) with ultrapure water (UP) (Millipore, Brussels, Belgium) and transferred to an LC-MS vial. Quality control samples, made from a pool of all the individual samples, were also obtained for column conditioning and normalization of the data. An Acquity Waters HSS T3 column (150 mm x 2.1 mm, 1.8 µm, Thermo Fisher Scientific, San Jose, USA) was used for compound separation. The column temperature was set at 45°C and the temperature of the autosampler at 10°C. As mobile phases 0,1% formic acid in UP water and 0.1% formic acid in acetonitrile were used in a gradient elution program with a flow rate of 0.4 ml min^{-1}.

The Orbitrap Exactive™ mass analyzer (Thermo Fisher Scientific, San Jose, USA) was equipped with heated electrospray ionization (HESI II), which was used in polarity switching mode. Processing of the data was performed with Xcalibur ™ 2.1 (Thermo Fisher scientific, San Jose, USA). The instrument was operated in full scan modus with a resolution of 100 000 full width at half maximum (FWHM).

Statistical data treatment

Statistical analysis of raw data was done for each volunteer and for each ionization mode separately with Sieve™ 2.1 (Thermo Fisher Scientific, San Jose, USA) and Simca™ 13 (Umetrics, Malmo, Sweden). Peak alignment was bypassed after visual check of the obtained chromatographic peaks. Filtering of metabolites was based on ratio (<0.66 or >1.5), P-value (<0.05) and isotopic peaks (C12). The residual metabolites were then imported in Simca™ to select those metabolites, which are specific for the digestion of beef compared with chicken. A PCA-X model was created to look for potential outliers. Validation of the dataset was performed in the PLS and OPLS model with a permutations test and CV-ANOVA (P-value<0.05), respectively. Finally an S-plot was created of the OPLS model to select those metabolites that are specific for the digestion of beef. A VIP plot was used to evaluate the importance of a certain metabolite (VIP-value>0.8: metabolite retained). To be sure that the selected metabolites were specific to colonic digestion, a comparison was made with the statistically analyzed duodenal samples.

III. RESULTS AND DISCUSSION

The raw data from the beef and chicken digestion samples for each volunteer and each ionization mode were analyzed separately in Sieve. In table 1 the number of frames before and after filtering are presented for the colon digestion samples.

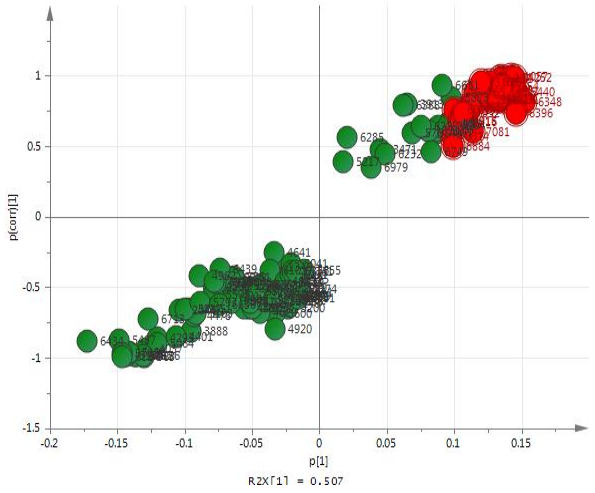
Table 1 Number of frames in Sieve™ before and after filtering for the colon digestion

Volunteer	Ionization mode	Before filtering	After filtering
P1	+	14 424	133
P1	-	4 639	31
P2	+	13 057	387
P2	-	4 930	97

In Simca™ all plots were validated with CV-Anova (P-value<0.05) and the permutations test. Validity parameters (preferential>0.5) of the models for the different analyses were as follows: R2X>0.61 (PCA-X), R2Y>0.98 (OPLS) and

Q2Y>0.98 (OPLS). An example of an S plot (OPLS) is given in Figure 1.

Fig.1 S-plot (P1, + ionization mode, colon)



Each dot in a S-plot represents a metabolite. The metabolites in the left lower quadrant are specifically associated with the digestion of chicken and the ones in the right upper quadrant are specifically associated with the digestion of beef (= metabolites of interest). A cut off value was used to select the most specific metabolites for the digestion of beef (red dots) and only those with a VIP-value>0.8 were retained. In negative ionization mode, one of the selected metabolites was common for both volunteers. In positive ionization mode 10 metabolites were common for both volunteers, of which 3 metabolites were identified as acylcarnitines based on the online Human Metabolome Database [9] (Table 2).

Table 2 Common metabolites in the colon for the two volunteers

m/z	IM	RT	Δppm	Putative identification
146.11738	+	1.5	1.1	3-dehydroxycarnitine
160.13284	+	1.6	2.2	DL-2-aminooctanoic acid
180.07907	-	1.0		Unknown
239.13870	+	7.0		Unknown
241.15411	+	2.0		Unknown
245.14875	+	1.7	3.3	isoleucyl-hydroxyproline, hydroxyprolyl-leucine leucyl-hydroxyproline, hydroxyprolyl-isoleucine
262.12789	+	1.7	2.3	methylmalonylcarnitine
264.13223	+	1.5		Unknown
330.26324	+	9.0	1.9	4,8-dimethylnonanoylcarnitine
344.24240	+	0.7		Unknown

RT=retention time, IM=ionization mode

These metabolites were not found in the duodenum, which implicates the potential involvement of the gastrointestinal microbiome during their production in the colon.

Acylcarnitines are formed during fatty acid metabolism when long-chain acyl groups are transferred by coenzyme A to carnitine. Subsequently, the complex can be transported into the mitochondrial matrix for further oxidation to acetyl-CoA, an important component in the citric acid cycle [10]. Acylcarnitines are also described as biomarkers in several disorders, e.g. autism, cardiovascular and inflammatory diseases [11,12]. In literature, the presence of acylcarnitines in urine has been related to the consumption of red meat [13]. Since these metabolites were only detected in the colonic digestion samples, bacterial involvement is assumed. 3-dehydroxycarnitine (also known as gamma-butyrobutaine) is identified as an intermediate metabolite in the intestinal bacterial catabolism of L-carnitine, which is higher in red meat compared with white meat, to trimethylamine. The latter metabolite can be converted into trimethylamine-N-oxide (TMAO) in the liver. TMAO has been linked to atherosclerosis [12]. Additionally it is possible that the toxic TMAO is involved in the red meat-colon cancer pathway, e.g. TMAO in plasma was associated with colorectal cancer in postmenopausal women in a prospective cohort study [14]. Bacterial production of methylmalonylcarnitine and 4,8-dimethylnonanoylcarnitine in particular have not yet been described in literature. In addition, 4,8-dimethylnonanoylcarnitine is believed to be exclusively formed in peroxisomes of eukaryotic cells from phytanic acid. Nevertheless, it can be interesting to investigate also bacterial production of 4,8-dimethylnonanoylcarnitine in the colon [15]. Phytanic acid, bacterially produced in the rumen and present in meat of ruminants, is associated to Non-Hodgkin lymphoma through the production of ROS, oxidative stress and DNA damage [15].

Besides the acylcarnitines, DL-2-aminooctanoic acid was also detected in the colon digestion samples. This amino acid has not been described in literature as a specific metabolite of the digestion of red meat, but it has been identified as a biomarker for insulin resistance [16]. Insulin can

act as a growth factor and tumor promoter [17]. In addition, amino acids (also present in dipeptides) can be nitrosated with the formation of carcinogenic NOC's, e.g. N-nitrosopyrrolidine can be formed from proline [18].

IV. CONCLUSION

The used metabolomics approach with UHPLC-HRMS in this study provides a new insight in the association between red meat and colorectal cancer. The simulation of the *in vitro* digestion allows to investigate the direct and local effect of red meat in the colon through the production of associated metabolites. Nevertheless further identification of the detected metabolites and validation of their importance as biomarkers for red meat digestion is warranted.

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