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***Basic Study***

**Fecal metabolomic profiles: A comparative study of patients with colorectal cancer *vs* adenomatous polyps**

Nannini G *et al*. Comparative study of fecal metabolomic profiles

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**Abstract**

BACKGROUND

Colorectal cancer (CRC), the third most common cause of death in both males and females worldwide, shows a positive response to therapy and usually a better prognosis when detected at an early stage. However, the survival rate declines when the diagnosis is late and the tumor spreads to other organs. Currently, the measures widely used in the clinic are fecal occult blood test and evaluation of serum tumor markers, but the lack of sensitivity and specificity of these markers restricts their use for CRC diagnosis. Due to its high sensitivity and precision, colonoscopy is currently the gold-standard screening technique for CRC, but it is a costly and invasive procedure. Therefore, the implementation of custom-made methodologies including those with minimal invasiveness, protection, and reproducibility is highly desirable. With regard to other screening methods, the screening of fecal samples has several benefits, and metabolomics is a successful method to classify the metabolite shift in living systems as a reaction to pathophysiological influences, genetic modifications, and environmental factors.

AIM

To characterize the variation groups and potentially recognize some diagnostic markers, we compared with healthy controls (HCs) the fecal nuclear magnetic resonance (NMR) metabolomic profiles of patients with CRC or adenomatous polyposis (AP).

METHODS

Proton nuclear magnetic resonance spectroscopy was used in combination with multivariate and univariate statistical approaches, to define the fecal metabolic profiles of 32 CRC patients, 16 AP patients, and 38 HCs well matched in age, sex, and body mass index.

RESULTS

NMR metabolomic analyses revealed that fecal sample profiles differed among CRC patients, AP patients, and HCs, and some discriminatory metabolites including acetate, butyrate, propionate, 3-hydroxyphenylacetic acid, valine, tyrosine and leucine were identified.

CONCLUSION

In conclusion, we are confident that our data can be a forerunner for future studies on CRC management, especially the diagnosis and evaluation of the effectiveness of treatments.

**Key Words:** Colorectal cancer; Adenomatous polyps; Nuclear magnetic resonance metabolomics; Fecal samples; Fecal metabolomics

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**Core Tip:** Colorectal cancer (CRC) isthe third leading cause of cancer-related death worldwide. Fecal occult blood and serum tumor markers are indicators currently used in the clinic, but their lack of sensitivity and precision limit their use for CRC diagnosis. Colonoscopy is the gold-standard screening technique for CRC, but it is costly and invasive. Using readily accessible biological samples such as stool specimens, in conjunction with high-throughput molecular profiling techniques, could significantly contribute to diagnosing and understanding the patient’s relationship with CRC. We compared with healthy subjects the fecal nuclear magnetic resonance metabolomic profiles of patients with CRC or adenomatous polyposis.

**INTRODUCTION**

Colorectal cancer (CRC) is globally the third most common cause of cancer-related death in both men and women[1]. Many CRC cases are attributable to changeable (and therefore potentially preventable)[2] risk factors including diet, smoking, high alcohol consumption, physical inactivity, and being overweight. If diagnosed at an early stage, CRC shows a good response to therapy and usually a better prognosis, while survival decreases when the diagnosis is late, and the tumor spreads to other organs[3]. In addition, it is well documented that about 95% of CRCs are adenocarcinomas and begin as colonic adenomatous polyps (AP)[4] or adenomas. A series of molecular alterations and mutations induce CRC development. Tumors can be prevented with polyps excision and adequate treatment[5]. The fecal occult blood test and evaluation of serum tumor markers are commonly used in the clinic[6]; however, the lack of sensitivity and specificity of these markers limits their application in CRC diagnosis[6-8]. Currently, colonoscopy represents the gold-standard screening procedure for CRC due to its high sensitivity and specificity, but it is an expensive and especially invasive procedure[9,10]. Therefore, it is highly desirable to introduce custom-made methodologies combining minimal invasiveness, safety, and reproducibility. The screening of fecal samples has many advantages with respect to other screening techniques. In fact, stool screening is certainly non-invasive and primarily reflects the colorectal status. Moreover, the use of easily accessible and non-invasive biological samples, such as stool specimens, combined with high-throughput molecular profiling techniques, can significantly contribute to the diagnosis of CRC and to the understanding of its interaction with the patient.

Metabolomics is an omics science that is an efficient approach to characterizing the change of metabolites in living systems as a response to pathophysiological stimuli, genetic modifications, and environmental factors. Metabolites are low molecular weight organic molecules that take part (as substrates or products) in the biochemical processes essential for sustaining life. Thus, the comprehensive evaluation of metabolites and their changes are fundamental to observe and measure the response of the organism to diverse conditions.

Nuclear magnetic resonance (NMR) spectroscopy is one of the most useful high-throughput techniques to obtain metabolomics information from biological samples[11,12]. NMR-based metabolomics has been successfully applied for disease classification[13-18] and prognosis. Starting from these premises, we compared the fecal NMR metabolomic profiles of patients with CRC or AP with those of healthy controls (HCs) to characterize the differences among the groups and possibly identify diagnostic markers.

**MATERIALS AND METHODS**

***Patients and biological samples***

A total of 86 patients including 32 CRC patients, 16 AP patients, and 38 HCs were enrolled for different studies between January 2016 and February 2019 at the Careggi Hospital and University of Florence, Italy. The Ethics Committee Area Vasta Toscana Centro (Italy) approved the studies. All fecal samples were taken at diagnosis, before starting any treatment (*e.g.,* surgical resection, chemotherapy, probiotic intake). Moreover, patients with evidence of serious illness, immunodeficit, autoimmune or infectious diseases were excluded. CRC patients, AP patients, and HCs were well matched in age, sex and body mass index. Table 1 summarizes the clinical characteristics of the enrolled patients.

Stool samples were collected in pre-labeled collection cups. Fecal water was extracted to ratios of 1:2 (g/mL, weight of unthawed feces-to-buffer) in 0.75 M phosphate-buffered saline (PBS, pH 7.4)[19]. The buffered samples where homogenized by whirl mixing for 30 s and sonicated for 15 min. Then each sample was centrifuged at 10000 g for 10 min at 4 °C, and 700 μL supernatant was transferred to 1.5 mL Eppendorf tubes and centrifuged again at 14000 rpm for 5 min at 4 °C. The clear supernatant was used for NMR analyses.

***NMR sample preparation and analyses***

A total of 70 μL buffer solution (1.5 M KH2PO4/d2O, pH 7.4; 2 mmol/L NaN3; 0.1% TMSP) was added to 630 μL of each fresh fecal water sample, and a total of 600 μL of this mixture was transferred to a 5 mm NMR tube.

One-dimensional proton NMR (1H-NMR) spectra for all samples were acquired using the Bruker 600 MHz spectrometer (Bruker BioSpin srl; Rheinstetten, Germany) operating at 600.13 MHz proton Larmor frequency and equipped with a 5 mm PATXI 1H-13C-15N and 2H-decoupling probe including a *z* axis gradient coil, an automatic tuning-matching, and an automatic and refrigerated sample changer (SampleJet, Bruker BioSpin srl; Rheinstetten).

The BTO 2000 thermocouple served for temperature stabilization at the level of approximately 0.1 K at the sample. Before measurement, samples were kept for at least 3 min inside the NMR probe head for temperature equilibration.

Two one-dimensional 1H-NMR spectra, namely one-dimensional (1D) NOESY and Carr-Purcell-Meiboom-Gill (CPMG), were acquired at 310 K with different pulse sequences: a standard nuclear Overhauser effect spectroscopy pulse sequence 1D NOESY PRESAT (noesygppr1d.comp; Bruker BioSpin) pulse sequence, using 64 scans, 98304 data points, a spectral width of 18028 Hz, an acquisition time of 2.7 s, a relaxation delay of 4 s, and a mixing time of 0.1 s; and a standard spin echo CPMG[20] (cpmgpr1d.comp; Bruker BioSpin) pulse sequence applied to a standard 1D sequence, with 64 scans, 73728 data points, a spectral width of 12019 Hz, and a relaxation delay of 4 s.

***Spectral processing and statistical analysis***

Free induction decays were multiplied by an exponential function equivalent to 0.3 Hz line-broadening factor before applying Fourier transform. Transformed spectra were automatically corrected for phase and baseline distortions and fecal spectra calibrated to TMSP singlet at 0 ppm using the TopSpin version 4.1.0 (Bruker BioSpin GmbH).

NMR spectra were segmented into bins of 0.02 ppm in the spectral range between 0.2 and 10.00 ppm. Regions containing residual water signal (between 4.6 and 4.9 ppm) were removed from the binning. The spectral intensity within each bin was integrated using Assure NMR 2.2, and the corresponding area was calculated to obtain the variable used as input for the statistical methods.

Before analysis of the generated data matrix, probabilistic quotient normalization[21] normalization and mean centering of the variables were performed.

Statistical analyses of the data were performed using R[22]. On processed NMR spectra, multivariate data analyses were performed. Principal component analysis (PCA) was used as unsupervised technique for exploratory analyses to check the homogeneity of the acquired spectra and to visualize the presence of outliers. As a supervised technique, orthogonal projections to latent structures-discriminant analysis (OPLS-DA) was applied. The OPLS-DA method is a multivariate projection approach that is commonly used to model spectroscopic data. Compared to PCA or partial least squares projection to latent structures (PLS), OPLS is able to distinguish between “response-related” and “response-orthogonal” fluctuations in data, delivering benefits in terms of model interpretation[23]. All of the accuracies reported and the confusion matrix for different classifications were assessed by means of 100 cycles of the Monte Carlo cross-validation scheme (MCCV, R script developed in-house). In this case, 90% of the data were randomly chosen at each iteration as a training set to build the model. Then the remaining 10% was tested, and the sensitivity, specificity, and accuracy of the classification were assessed. Metabolite identification was performed manually based on previous literature[19,24], the human metabolome database public database, and a library of pure organic compounds (BBIOREFCODE; Bruker BioSpin). The relative metabolite concentrations (expressed in arbitrary units) were calculated integrating and calculating the peaks area[25].

To determine the discriminating molecules among all classes under study, the Wilcoxon test was chosen to infer differences between two groups of subjects[26]. False discovery rate (FDR) correction was applied using the Benjamini & Hochberg method, and adjusted *P* < 0.05 was considered statistically significant[27]. The effect size, using the Cliff’s delta (Cd) formulation[28], was also calculated to aid in the identification of the meaningful signals giving an estimation of the magnitude of the separation between the different groups. The magnitude was assessed using the thresholds provided in Romano and Coll[29], *i.e.* |Cd| < 0.147 “negligible,” |Cd| < 0.33 “small,” |Cd| < 0.474 “medium,”and otherwise “large.”

Changes in metabolite levels were calculated as the log2 (fold change) ratio of the normalized median intensities of the corresponding signals in the spectra of the two groups. MetaboAnalyst 4.0 free online software was used for pathway analysis[30].

**RESULTS**

***Metabolic fingerprint of CRC and AP patients***

The NMR spectra of 86 fecal extract samples (32 CRC, 16 AP, and 38 HC) were acquired. Because of the suboptimal shimming quality of 7 spectra, only 79 spectra (1D NOESY: 26 CRC, 15 AP and 38 HCs; CPMG: 27 CRC, 14 AP, and 38 HCs) were used in subsequent analyses.

PCA was initially carried out to generate an overview of the variation among the different groups of patients (CRC, AP, and HCs) using bucketed spectra of fecal extracts. Some trends could be detected in the score plots of the first two principal components as shown in Supplementary Figure 1. Indeed, both score plots reveal that CRC and AP patient groups tend to spread in the plots more than HCs that are more grouped. However, no net clustering seems to appear in the metabolomic profiles of the groups of patients neither with respect to HCs using this unsupervised approach.

Comparative analyses among the groups have been performed to test the capability of 1H-NMR fecal water spectra to classify the samples of the patients according to the diagnosis. Several models have been built using a MC cross-validated supervised OPLS-DA approach. First, all three groups (CRC, AP, and HCs) were used in the same model to test the accuracy of the approach in the prediction of the healthy or the pathological state using a single bucketed NMR fecal spectrum (Supplementary Figure 2). As is shown in the score plots of Supplementary Figure 2, the AP samples occupied the middle metabolomic space between HCs and CRC. Indeed, the resulting true AP-positive percentages of the OPLS-DA NOESY and CPMG models were 37.7% and 18%, respectively (Supplementary Figure 2), and most of the AP patients were misclassified within the metabolomic space of HCs or CRC.

Prognostic data about patients are not available therefore was impossible to assess whether the AP patients predicted within the metabolomic space of CRC were more predisposed to developing cancer.

The capability to correctly classify HCs provides another important challenge for clinical screening. Indeed, other OPLS-DA models have been attempted to distinguish the fecal water spectra of HCs compared to AP + CRC patients yielding an overall predictive accuracy of 85.3% using 1D NOESY binned spectra (Table 2, Supplementary Figure 3A).

Furthermore, separated models were established comparing separately the 1HNMR binned spectra of HCs *vs* CRC, HCs *vs* AP, and CRC *vs* AP patients (Table 2, Supplementary Figure 3B-D). As reported in Table 2, all models built on 1D NOESY bucketed spectra are better performing than those built on CPMG spectra.Supplementary Figure 3 shows the score plots related to the higher predictive accuracy among the models listed in Table 2. The reported models suggest the existence of a metabolomic fingerprint in fecal extracts of CRC and AP compared with HCs, confirming what was previously suggested by PCA and the literature available[24,31-33].

In most biofluids, low mass metabolites coexist with high mass biomolecules such as lipids, proteins, and lipoproteins. Here, two NMR pulse sequences were used to selectively observe the different components: 1D NOESY pulse sequence yields a spectrum in which both signals of metabolites and high molecular weight molecules (*e.g.*, lipids, lipoproteins, and albumin) are visible; the CPMG pulse sequence enables the selective observation of small molecule components in solutions containing macromolecules (*via* T2 filtering). Representative one-dimensional 1H-NMR spectra of fecal extracts obtained with the mentioned pulse sequences are shown in Supplementary Figure 4. Indeed, NOESY experiments, which are sensitive to both low and high molecular weight compounds, resulted more accurate classifiers of all of the cases considered in this study and are described in Table 2 (HCs *vs* AP and CRC: sensitivity 84.9%, specificity 85.7%, predictive accuracy 85.3%; HCs *vs* CRC: sensitivity 85.0%, specificity 88.6%, accuracy 86.8%; HCs *vs* AP: sensitivity 71.7.8%, specificity 83.8%, predictive accuracy 77.8%: AP *vs* CRC: sensitivity 87.4%, specificity 71.6%, predictive accuracy 79.5%).

***Metabolic profiles of CRC and AP patients***

With the aim of identifying metabolite-level variations characteristic for each group, univariate analyses were applied to the identified fecal metabolites. Marked changes were observed in the metabolic profiles of fecal samples between CRC patients and HCs. Indeed, the first were characterized by a significantly lower content of 3-hydroxyphenylacetate, methanol, galactose, acetate, xylose and isobutyrate and a higher content of glycerol and phenylalanine (Figure 1A). Compared to HCs, AP patients had significantly lower amounts of 3-hydroxyphelylacetate, butyrate, acetate, propionate, isobutyrate and lactate+threonine (considered together because of the overlapping doublets at 1.33 ppm) as reported in Figure 1C. In CRC fecal extract profiles, when compared to AP patients, only leucine, tyrosine, and valine remained statistically significant and were present in higher amounts (Figure 1B). The complete list of fecal extract metabolites identified is reported in Supplementary Table 1. The most relevant pathways identified are reported inTable 3 and Supplementary Figure 5.

The top six identified metabolomic pathways in CRC (*P* < 0.05) were aminoacyl-tRNA biosynthesis, phenylalanine, tyrosine and tryptophan biosynthesis, valine, leucine and isoleucine biosynthesis, phenylalanine and galactose metabolism and valine, leucine and isoleucine degradation. Among them, valine, leucine and isoleucine biosynthesis, aminoacyl-tRNA biosynthesis, valine, leucine and isoleucine degradation and phenylalanine, tyrosine and tryptophan biosynthesis pathways were also altered in AP patients (Table 3).

**DISCUSSION**

In this study, we evaluated the fecal metabolomic profiles of CRC and AP patients with respect to HCs using NMR spectroscopy. The metabolome is a quantitative collection of low molecular weight compounds generated by metabolism[34]. Metabolomics is an emerging field of research downstream from genomics, proteomics, and transcriptomics. The metabolic screening of stool samples, which reflects the colorectal status, might contribute to the development of non-invasive screening tests[35,36]. The power of metabolomics consists in the capacity to detect and characterize tumors because cellular metabolism alterations represent a key hallmark of cancer[37]. To date, studies evaluating fecal metabolic changes associated with CRC are still lacking. Notably, no study has described fecal metabolomic changes associated with adenoma. Despite the high variability in fecal water profiles, reliable metabolic differences between patients and HCs were observed by preliminary analyses of the spectra. First, the PCA of the 1H-NMR spectral data was carried out to identify some trends and outliers, showing some separation among cancer, adenoma, and HC groups. However, this unsupervised approach does not seem to clearly characterize the groups. These results were not surprising in light of the inter-individual variability introduced with diet, lifestyle, sex *etc.*

To optimize the separation among groups, we used OPLS-DA MCCV models, which were effective in discriminating the fecal metabolomic fingerprints of CRC patients and HCs (overall predictive accuracy of 93.7%). From the above analyses we can conclude that both low and high molecular weight molecules, visible using the 1D NOESY pulse sequence, are important to characterize at metabolic level the two pathological profiles. Moreover, the capability to accurately predict HCs when compared to patients with colonic AP or adenomas and CRC patients (showed predictive accuracy of 85.3%) using 1H-NMR fecal water spectra, could be tested on a larger number of subjects to develop fast screening following a positive fecal occult blood test in order to spare colonoscopy in some patients who have bleeding due to other reasons[38,39].

In detail, the short-chain fatty acids (SCFAs) were found to be significantly decreased in CRC and AP patients, and in particular, lower levels of acetate were observed with respect to the HCs. SCFAs are microbial-derived metabolites, produced through gut bacteria fermentation of complex carbohydrates. SCFAs can be absorbed by the colonic epithelium, suppling energy and playing a crucial role in the regulation of fatty acids, glucose, and cholesterol metabolism[40-43]. A decrease in SCFA abundance is strictly linked to an unhealthy gut microbiota status, and alterations in the fecal SCFA profile may be the result of gut microbiota dysbiosis, inflammatory changes, or both[44]. Our data confirmed what has been previously reported by other studies. In particular, the analyses by Lin *et al*[45] demonstrated a high diagnostic performance of fecal acetate in CRC patients with respect to HCs.

In contrast with previous data, we did not find decreased levels of butyric acid in CRC patients with respect to HCs; however, we observed significantly decreased levels in AP. Butyric acid has an important homeostatic role in the human colon, and *in vitro* and *in vivo* studies have demonstrated its ability in preventing CRC[46-48]. However, other studies have showed contrasting results, suggesting a pro-cancer role of butyric acid, and low levels of butyrate usually seem to be linked with CRC development[31,35]. This double-edged role is named “butyrate paradox”[49,50]. Our results could be very interesting in this respect, suggesting that low levels of butyric acid in adenoma could lead to CRC development, while “normal” levels of butyrate in CRC could support cancer progression and promote the differentiation of regulatory T cells, which show a pro- tumorigenic role[51], especially in advanced CRC.

In addition, we found significantly low levels of 3-hydroxyphenylacetic (3-HPAA) acid in both CRC and AP patients. 3-HPAA acid is a weak acid and one of the most abundant products of polyphenol degradation in the large intestine[52,53]. Food polyphenols are broken down into other phenolic compounds by colonic bacteria action. In this way, poorly absorbable large-size polyphenols are converted to small-sized bioavailable metabolites, including 3-HPAA, which could be more biologically active[54,53]. Polyphenolic colon metabolites could be important endogenous antioxidants able to scavenge excess of free radicals, suppressing their effects on protein, lipid, and DNA damage[55]. A recent study demonstrated that polyphenol metabolites produced by colonic microbiota reduce some enzymatic activities involved in human tumorigenesis[56]. 3-HPAA can act as a CRC preventive agent by inhibition of cyclooxygenase-2 (COX-2)[57], a mediator of inflammation that is significantly overexpressed in a variety of human malignancies. Moreover, some studies have reported that COX-2 inhibitors not only prevent tumor formation but also decrease the number of already established polyps in patients with familial AP[58]. Accordingly, our results suggest that low levels of 3-HPAA are insufficient to inhibit COX-2, and consequently COX-2 expression can promote the development, tumor growth, immune suppression, angiogenesis, and metastasis of cancer cells.

Furthermore, higher amounts of amino acids such as leucine, tyrosine and valine, were present in the stool of CRC patients (compared to AP), probably resulting from malabsorption due to large epithelial inflammation and damage associated with CRC[59]. Previous metabolomic studies on fecal water have suggested that amino acid concentrations mirror the malabsorption of nutrients caused by the malfunction of epithelium barrier protection[60]. In agreement with previous studies, we documented higher levels of amino acids in the fecal water of CRC patients[31,35,61]. Alterations of amino acid levels can be associated with altered cancer cell activities, including the synthesis of proteins or catabolism to provide energy and/or other metabolite substrates.

**CONCLUSION**

In summary, our NMR metabolomics investigation revealed for the first time that fecal sample profiles can discriminate among CRC AP, AP patients and HCs, and some discriminatory metabolites were identified including acetate, butyrate, propionate, 3-HPAA acid, valine, tyrosine, and leucine. These altered metabolites suggest that changes in CRC and adenoma are associated with different pathway perturbations including valine, leucine and isoleucine biosynthesis, aminoacyl-tRNA biosynthesis, valine, leucine and isoleucine degradation phenylalanine, tyrosine and tryptophan biosynthesis, phenylalanine metabolism and galactose metabolism. In conclusion, we are confident that our data can be a forerunner for future studies on CRC management, especially the diagnostics and evaluation of the effectiveness of the treatments.

**ARTICLE HIGHLIGHTS**

***Research background***

Colorectal cancer (CRC) is globally the third most common cause of death. If diagnosed at an early stage, CRC shows a good response to therapy and usually a better prognosis. Unfortunately, despite its invasiveness, colonoscopy represents the gold-standard screening procedure for CRC.

***Research motivation***

Considering that colonoscopy is an expensive and invasive procedure, it seems to be essential to introduce custom-made methodologies combining minimal invasiveness, safety, and reproducibility. Fecal sample screening has many advantages with respect to other screening techniques.

***Research objectives***

The main objective of our study was to compare with HCs (HCs) the fecal nuclear magnetic resonance (NMR) metabolomic profiles of patients with CRC or adenomatous polyposis (AP) to characterize the variations among the groups and potentially identify some diagnostic markers.

***Research methods***

In order to define the fecal metabolic profile of 32 CRC, 16 AP patients and 38 HCs we used proton nuclear magnetic resonance spectroscopy in combination with multivariate and univariate statistical approaches.

***Research results***

The NMR spectra of 86 fecal extract samples have been acquired.With the aim of identifying metabolite level variations characteristic for each group, univariate analyses were applied to the identified fecal metabolites. The most marked changes were observed in the metabolic profiles of fecal samples of CRC patient *vs* HCs. AP patients, compared to HCs show significant lower amount of 3-hydroxyphelylacetate, butyrate, acetate, propionate, isobutyrate and lactate+threonine. In CRC fecal extract profiles, when compared to AP patients, only leucine, tyrosine, and valine remained statistically significant and present in higher amounts.

***Research conclusions***

The metabolic screening of stool samples might contribute to the development of non-invasive screening tests. To date, studies evaluating fecal metabolic changes associated with CRC are still lacking. Furthermore, no study has described fecal metabolomic changes associated with adenoma. The short-chain fatty acids were found to be significantly decreased in CRC and AP patients, and in particular, lower levels of acetate were observed with respect to HCs. In contrast with previous data, we did not find decreased levels of butyric acid in CRC patients compared to HCs; however, we observed significantly decreased levels in AP patients. We showed significantly low levels of 3-hydroxyphenylacetic (3-HPAA) acid in both CRC and AP patients. Finally, higher amounts of amino acids such as leucine, tyrosine, and valine were present in the stool of CRC patients (compared to AP), probably resulting from malabsorption due to large epithelial inflammation and damage.

***Research perspectives***

For the first time, we showed that fecal sample profiles can discriminate among CRC patients, AP patients and HCs, and some discriminatory metabolites were identified including acetate, butyrate, propionate, 3-HPAA acid, valine, tyrosine, and leucine. We believe that our data will be a starting point for future studies on CRC management, especially the diagnostics and evaluation of the effective of the treatments.

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**Footnotes**

**Institutional review board statement:** The study was reviewed and approved by the Comitato Etico Regionale per la Sperimentazione Clinica della Regione Toscana, Sezione AREA VASTA CENTRO Institutional Review Board (CE: 11166\_spe and 13080\_oss).

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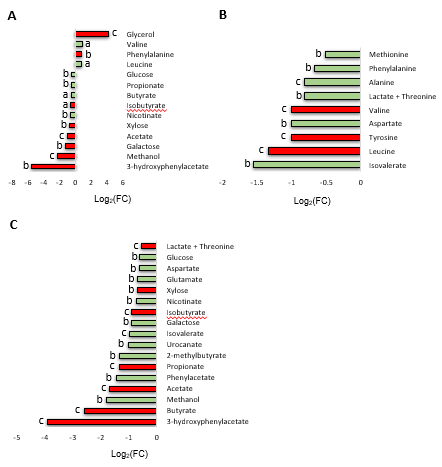
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**Figure Legends**



**Figure 1 Fecal water metabolite levels of colorectal cancer, polyps’ patients and healthy controls.** Boxplots of fold-change (FC) values for the significantly altered metabolites. Red bars represent metabolites levels that remain significant after the false discovery rate (FDR) correction (FDR *P* < 0.05), green bars are the metabolites that are no more significant after the FDR correction (*P* < 0.05). Cliff’s delta effect size is also reported for each metabolites in the comparisons (a: Small effect, b: Medium effect, c: Large effect). A:Comparison between healthy controls (HCs) and colorectal cancer (CRC) patients, negative log2(FC) values mean lower metabolite levels in CRC fecal samples, positive log2(FC) values report higher content in CRC compared to HCs; B: Negative log2(FC) represent higher metabolite levels in CRC patients compared to polyp patients; C: Comparison between HCs and polyp patient-negative log2(FC) values mean lower metabolite levels in polyp patients fecal water.

**Table 1 Clinical characteristics of colorectal cancer patients, adenomatous patients, and healthy controls**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Code** | **Gender ratio m/F** | **Median age, yr** | **Range of age, yr** | **Tumor staging T0/T1/T2/T3/T4 (*n* of patients)** | **Diet** | **Race** |
| CRC | 22 M-10 F; M/F = 2, 2 | 72 | 36-85 | 2/6/10/6/8 | Mediterranean | Caucasian |
| AP | 9M-7F; M/F = 1, 3 | 59 | 41-79 | - | Mediterranean | Caucasian |
| HS | 28M-10F; M/F = 2,8 | 47 | 27-68 | - | Mediterranean | Caucasian |

AP: Adenomatous patients; CRC: Colorectal cancer; HCs: Healthy controls.

**Table 2 Summary of orthogonal projections to latent structures-discriminant analysis models built fecal water spectra acquired using selective one-dimensional proton nuclear magnetic resonance pulse sequences, sensitivity (%), specificity (%) and predictive accuracy (%) assessed following 100 runs of Monte Carlo cross-validation are reported for each model**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **1H NMR fecal extract spectra OPLS-DA cv models** | | | |
| **Group (*n* of samples)** | **Pulse sequences** | **Sensitivity (%)** | **Specificity (%)** | **Predictive accuracy (%)** |
| HS (38) *vs* AP&CRC (41) | 1D noesy; CPMG | 84.9 | 85.7 | 85.3 |
| HS (38) *vs* AP&CRC (41) | 77.5 | 83.8 | 80.5 |
| HS (38) *vs* CRC (26) | 1D noesy; CPMG | 94.2 | 90.2 | 93.7 |
| HS (38) *vs* CRC (27) | 97.3 | 85.2 | 91.0 |
| HS (38) *vs* AP (15) | 1D noesy; CPMG | 90.8 | 76.6 | 87.0 |
| HS (38) *vs* AP (14) | 69.0 | 67.6 | 81.1 |
| AP (15) *vs* CRC (26) | 1D noesy; CPMG | 77.0 | 90.4 | 79.3 |
| AP (14) *vs* CRC (27) | 67.5 | 74.0 | 72.7 |

AP: Adenomatous patients; CPMG: Carr-Purcell-Meiboom-Gill; CRC: Colorectal cancer.

1HNMR: One-dimensional proton nuclear magnetic resonance; HCs: Healthy controls; OPLS-DA: Orthogonal projections to latent structures-discriminant analysis.

**Table 3 Identified pathways from fecal water metabolites**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Pathway analyses fecal water samples** | | | | | | |
| **Fecal metabolites** |  | **Metabolites** | ***P*** | **Holm *P*** | **FDR** | **Impact** |
| AP | Valine, leucine and isoleucine biosynthesis | Leucine, valine | 8.25 × 10-4 | 0.069 | 0.069 | 0.0 |
|  | Aminoacyl-tRNA biosynthesis | Valine, leucine, tyrosine | 0.002 | 0.17 | 0.08 | 0.0 |
|  | Valine, leucine and isoleucine degradation | Leucine, valine | 0.02 | 1 | 0.48 | 0.0 |
|  | Phenylalanine, tyrosine and tryptophan biosynthesis | Tyrosine | 0.02 | 1 | 0.48 | 0.5 |
| CRC | Aminoacyl-tRNA biosynthesis | Phenylalanine, valine, leucine, tyrosine | 2.3 × 10-4 | 0.019 | 0.01 | 0 |
|  | Phenylalanine, tyrosine and tryptophan biosynthesis | Phenylalanine, tyrosine | 2.7 × 10-4 | 0.022 | 0.035 | 1 |
|  | Valine, leucine and isoleucine biosynthesis | Leucine, valine | 0.0012 | 0.1 | 0.035 | 0.0 |
|  | Phenylalanine metabolism | Phenylalanine, tyrosine | 0.0019 | 0.16 | 0.041 | 0.36 |
|  | Galactose metabolism | Galactose, glycerol | 0.014 | 1.0 | 0.2 | 0.05 |
|  | Valine, leucine and isoleucine degradation | Valine, leucine | 0.03 | 1.0 | 0.43 | 0.0 |

An integrated analysis based on MetaboAnalyst 4.0 software built on significantly altered metabolites in colorectal cancer (CRC); and adenomatous patients (AP): View of most contributing pathways; *P* is the original *P* value calculated from the enrichment analysis; Holm *P* is the *P* value adjusted by Holm-Bonferroni method; the false discovery rate is the *P* value adjusted using false discovery rate (FDR); Impact is the pathway impact value calculated from pathway topology analysis.



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