REVIEW

Metabolomics profile in gastrointestinal cancers: Update and future perspectives

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Abstract

Despite recent progress in diagnosis and therapy, gastrointestinal (GI) cancers remain one of the most important causes of death with a poor prognosis due to late diagnosis. Serum tumor markers and detection of occult blood in the stool are the current tests used in the clinic of GI cancers; however, these tests are not useful as diagnostic screening since they have low specificity and low sensitivity. Considering that one of the hallmarks of cancer is dysregulated metabolism and metabolomics is an optimal approach to illustrate the metabolic mechanisms that belong to living systems, is now clear that this -omics could open a new way to study cancer. In the last years, nuclear magnetic resonance (NMR) metabolomics has demonstrated to be an optimal approach for diseases' diagnosis nevertheless a few studies focus on the NMR capability to find new biomarkers for early diagnosis of GI cancers. For these reasons in this review, we will give an update on the status of NMR metabolomic studies for the diagnosis and development of GI cancers using biological fluids.

Key words: Metabolomics; Nuclear magnetic resonance spectroscopy; Pancreatic cancer; Gastric cancer; Colorectal cancer; Biological fluids

Core tip: Searching for new tumor biomarkers is essential for the early diagnosis of gastrointestinal tumors. Biofluids could give important data, reducing the need for invasive screening and nuclear magnetic resonance-based metabolomics is an optimal approach to understand metabolic dynamics in biofluids.

INTRODUCTION

The continuous increase of the global population, associated with the extended life expectancy, made the cancer one of the main causes of death[1], and for many countries, a very heavy health burden. Despite numerous advances in diagnosis and therapy, gastrointestinal (GI) cancers remain some of the most aggressive cancers for both men and women, as we have previously reported[2]. In particular, the most aggressive types of GI cancers are pancreatic cancer (PC), gastric cancer (GC) and colorectal cancer (CRC). Furthermore, gastric and CRC are respectively in third and fifth place by incidence and even in second and third as regards mortality in both sexes[3]. The GC is one of the most malignant cancers worldwide with a very high rate in Asia[4]. Unfortunately, most cases of GC are diagnosed in the advanced stages with consequent poor prognosis[5]. The epidemiological and molecular feature of GC differ according to the histological type and cancer location. Currently there are several methods for diagnosing GC; however, there are no standardized guidelines[6]. Regarding CRC, it is one of the most diagnosed neoplasms in the world, both among men and women, and is the third most common malignancy[7]. 5-year survival can reach 90% if the tumor is diagnosed at an early stage and is localized, but survival decreases significantly if the tumor is diagnosed late and is spread to other organs[8]. To date, the fecal occult blood and the serum tumor-associated markers are the test commonly used in the clinic; however, the lack of sensitivity and specificity of these markers limits their application in the CRC diagnosis[9,10]. Lastly, PC is one of the most aggressive cancers, with 5-year survival rates of only 5%. PC is currently ranked as the fourth leading cause of cancer-related deaths in the United States and is estimated to be the second leading cause of such fatalities by the year 2020[7]. Mortality is mainly due to late diagnosis because the PC symptoms (such as nausea, weight loss, weariness, abdominal pain) are not disease-specific[11,12]. PC is diagnosed by resonance, computed tomography, endoscopic retrograde cholangiopancreatography and endoscopic ultrasound[13].

For the clinical monitoring of GI cancer, carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 are used as serological tumor-associated markers as well as detection of occult blood in the stool. However, these tests are not currently useful as diagnostic screening as they have low specificity and low sensitivity[14]; but, for the previously reported reasons, the effectiveness of the anti-GI cancers' treatments depends on an early diagnosis. Based on clinical characteristics, different models for various cancers have been developed to assess the causal risk, however although the results may seem significant at the population level, they have a low predictive value when considering the individual patient[15].

A growing number of studies have suggested that the GI metabolites regulate pathogen infection in the different intestinal sections, through genome-based analysis of bacteria and especially by high-throughput metabolomics[16-18]. Many metabolites affect the cell adhesion and biofilm formation; for example, the D-amino acids produced by *Bacillus subtilis* prevent biofilm formation[19]. In addition, recent studies have revealed a major role of metabolites in the regulation of the immune system taking part in the modulation of the adaptive immune cell development, in particular T lymphocytes[20], which have a crucial role in the genesis of the above-mentioned GI tumors. As we have reported in previous studies, the PC as well as gastric and CRC, show an altered specific immune response characterized by a decreased number of effective T cells[21-23].

One of the hallmarks of cancer is dysregulated metabolism, during which cancer cells show increased glucose uptake and produce lactate. This process is named the "Warburg effect", but how and why cancer cells reprogram their metabolic state is not well understood. Several metabolic changes associated with cancer can be linked to cellular growth; in fact, the biosynthesis of lipids, proteins, nucleic acids are required for tumor formation and survival. In most cases the expression of oncogenes or the loss of tumor suppressors lead to changes in the metabolism, by expression, activity or flow of the main metabolic pathways. Numerous components of glucose and glutamine (Gln) metabolism have emerged as important regulators of cancer

metabolism. Considering the importance of metabolic changes in the development and cancer prognosis, the metabolomics represents a fundamental –omics' study, as it can be used to evaluate (assess) the alterations of the main metabolites[14]. It is now clear that the metabolic characteristics of cancer cells change with the disease progression[24,25] and typical metabolic changes include deregulated absorption of amino acids and glucose, increased nitrogen demand and increased use of anabolic metabolic pathways[26]. This metabolic reprogramming can be useful for the diagnosis of tumors at an early stage and biological fluids could give important information, reducing the need for invasive screening. Urine and blood are easily accessible matrix that could be used to identify possible biomarkers associated with cancer risk, presence and prognosis, using nuclear magnetic resonance (NMR) analysis[27]. Blood passes through every organ of the human body, acting as a transport for secreted/excreted molecules (in response to physiological stimuli or stress), while urine contains molecules eliminated by renal filtration[28,29]. Furthermore, there are a lot of evidence suggesting that microbial metabolism by gut microbiota produces a variety of compounds, including fatty acids, indole and vitamin K, many of which have toxic effects on the lumen and contribute to the GI carcinogenesis, especially for CRC. In addition, there are many evidence (essentially in experimental models) that suggest a role of the intestinal microbiome in the PC carcinogenesis. Finally, a growing number of microbiome researchers are recognizing that considerable information could be gained by using a more integrative approach that also includes comprehensive fecal metabolite analysis. Feces contain many molecules that reflect nutrient ingestion, digestion and absorption by gut bacteria and GI tract. The dry fecal matter consists of bacterial biomass (25%-54%), exfoliated colonic epithelial cells, undigested food residues (fiber, protein, DNA, mucopolysaccharides, *etc*.) and small molecules or metabolites such as sugars, organic acids, and amino acids. These small molecules compose fecal metabolome.

There is an increasing interest to use metabolomic based approaches to investigate cancer metabolism. The two majors' instrumental metabolomic techniques are NMR and mass spectrometry (MS). The advantages of these two techniques are intrinsically different. MS platform provides sensitivity and selectivity for metabolomics research, while NMR provides a very high reproducibility, it is quantitative and requires minimal steps for sample preparation allowing to avoid separation or deri-vatization[30]. Due to the possible impact that NMR-based metabolomics performed using easily accessible biofluids could have on the standard clinical practice of cancer diagnosis, prognosis and risk evaluation, this review aims to be a comprehensive overview of the literature available to date in this restricted, but promising field. Conversely, the use of MS based techniques or the metabolomic analysis of cells, tissues and animal models is reported elsewhere[31,32]. Interestingly, while *e.g*., breast cancer has been extensively investigated using NMR-based metabolomics of systemic biofluids (especially for relapse risk prediction)[33], for GI cancers this field still appears in its infancy. In this review, we will give an update on the current status of NMR metabolomics' studies for the diagnosis of GI cancers, discussing the suitability of the different biological samples used and the future perspectives for this analytical approach (Figure 1).

METABOLIC ALTERATIONS IN GI CANCERS

Warburg effect is a shift from adenosine triphosphate (ATP) synthesis by oxidative phosphorylation to ATP generation through glycolysis, also in aerobic condition[34]. Tumor cells obtained a large amount of their energy from aerobic glycolysis, converting glucose to lactate instead of metabolizing it in the mitochondria through oxidative phosphorylation. Therefore, in terms of ATP production per glucose molecule consumed, glycolysis is less efficient than oxidative phosphorylation. This metabolic change forces tumor cells to demand a large glucose amount to satisfy their increased energy, biosynthesis and redox needs. In details, lactate accumulation elicits acidic microenvironment, protective for cancer. The presence of lactic acid induces, in cancer cells, the expression of glycolytic enzymes such as 6-phosphofructokinase 1 (PFK1) to: (1) Increase the ATP provisions; (2) Escape from the cell apoptosis mechanism; and (3) Promote angiogenetic mechanisms, providing in this way a comfortable microenvironment for cancer development and metastasis[35]. Abnormal glucose metabolism in GC, with high levels in serum of 3-hydroxypropionic acid and pyruvic acid, may be involved to tumor proliferation leading to aggressive cancer cell proliferation, which needed a large ATP amount, causing, in turn, abnormal levels of intermediate glucose metabolism[36,37].

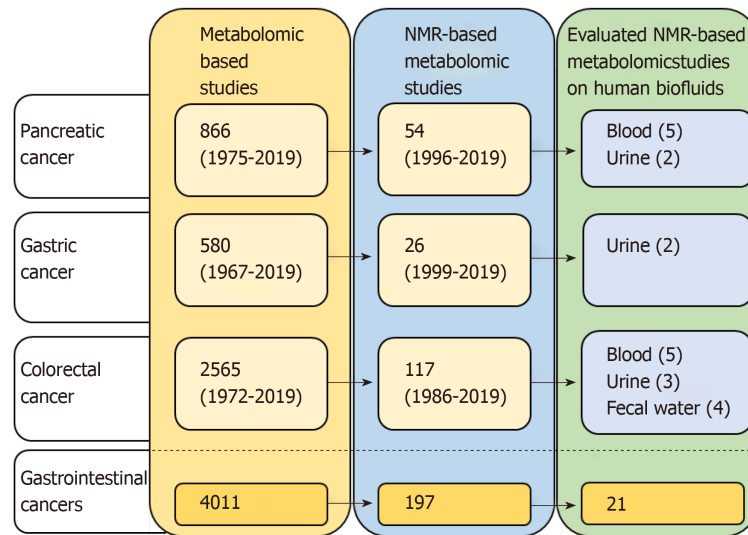In PC, abnormal metabolism depends by cellular factors on the anomalous activity

Figure 1 Selection of discussed nuclear magnetic resonance-based metabolomics review. The figure shows the study workflow. First, we searched for metabolomic-based studies, then we limited our research to nuclear magnetic resonance-based metabolomic studies and finally we only selected 21 nuclear magnetic resonance-based metabolomic studies on human bio-fluids, in particular blood, urine and fecal water. NMR: Nuclear magnetic resonance.

of some oncogenes that change the physiological nutrients consumption[38]. In addition, the metabolic reorganization is carried out by the activation of alterations in genes and oncogenic signaling pathways. In fact, some studies show that mutations of *K-RAS* and other oncogenes (and tumor suppressors) represent the key that leads to an acceleration of PC growth by reprogramming directly cellular metabolism[39,40]. It is now assured that the *K-RAS* gene has a crucial role in PC glucose metabolism. An excessive glucose uptake and overexpression of glycolytic enzymes, including type 1 glucose transporter, hexokinase 1/2, phosphofructokinase and lactate dehydrogenase A[41,42] characterize the PC. For all these reasons, lactate is an important "performer" for tumor-connective tissue and energy trade in cellular compartments around cancer microenvironment. In addition, the acidity of the microenvironment helps to repress immune system by promoting chronic inflammation and by suppressing the adaptive immune response[43] leading by T cells.

Also, in CRC, in response to hypoxia, the expression of the glucose transporter 1 is upregulated in neoplastic cells, inducing the enzymes that metabolize glycogen, including glycogen synthase and glycogen phosphorylase. However, altered glycogen metabolism and its potential impact on the CRC biology remain poorly understood[44]. Increasing evidence demonstrated that Gln is an important metabolic substrate and energy source for tumor cells that need Gln for their growth and survival, a dependence called "glutamine addiction". Recent studies have shown that some cancer cells use Gln to carry out the metabolic processes linked to the cell proliferation and to maintain amino acid levels of the tricarboxylic acid (TCA) cycle, exosamine, nucleotides and other molecules[45,46]. Finally, a new study reports an alternative pathway in the Gln metabolism adopted by PC cells and essential for tumor growth. Usually cells use glutamate dehydrogenase to convert glutamate derived from Gln to α-ketoglutarate in mitochondria in order to use it in the TCA cycle[47,48]. However, the PC feeds the TCA cycles through another pathway, so that the aspartate derived from Gln arrives in the cytoplasm and so, transformed into oxaloacetate *via* aspartatetransaminase (GOT1). The oxaloacetate is then converted into pyruvate at the end of the cycle to increase the ratio between NADPH/NADP + and facilitate the maintenance of the redox state[40].

The growth of cancer cells is strongly based on the possibility of exploiting more autonomous proliferative signaling pathways[49]. PC cells depend strictly on these reactions; in fact, the Gln deprivation or the deactivation of enzymes of this chain of reactions causes an increase in reactive oxygen species and a reduction in reduced glutathione. Finally, the inhibition of the enzymes linked to this cycle reduces the PC growth both *in vitro* and *in vivo*[50].

Lastly, the fatty acid synthase (FASN) is overexpressed in various tumors, showing an important role in cancer onset and progression. Several *in vitro* studies have documented that elevated lipogenesis is correlated with poor prognosis in different tumor types[51]. Lipogenesis is also involved in signal transduction of tumor cells and

is increased in tumor tissue and associated with tumor prognosis. Moreover, FASN reduction can promote apoptosis in tumor cells, inhibiting tumor growth and metastasis. Previous studies have demonstrated that FASN is overexpressed in cancer tissue and serum of GC patients. In fact, usually the tumors present enzymes supporting the production of a large number of lipids for the survival and proliferation of neoplastic cells. The tumors need more lipids as energy sources compared to normal cells. Acetyl-CoA carboxylase, the rate-limiting enzyme for the synthesis of fatty acids, if blocked, inhibits the growth and apoptosis of breast, lung and colon cancer cells[52-55].

METABOLOMICS OF BIO-FLUIDS

Metabolomics is an optimal approach to describe the metabolic dynamics that reflect the response of living systems to pathophysiological stimuli, genetic modifications and environment factors. Indeed, the comprehensive evaluation of metabolites, the low molecular weight organic molecules involved in all biochemical processes as substrates or products with different specific functions, is fundamental to observe and measure the response of the organism to diverse conditions. In recent years, metabolomics has been widely applied to investigate cancer metabolism. Current platforms for metabolomics are NMR and gas, liquid chromatography, ultra-high-pressure liquid chromatography [GC, liver cancer (LC) and UPLC] and more recently capillary electrophoresis, usually hyphenated to MS. The applications of NMR spectroscopy are not limited to liquid and solid samples but extend to intact tissue samples with the use of high-resolution magic angle spinning NMR spectroscopy. NMR is more reproducible, and it not requires laborintensive sample manipulations like fractionation to get quantitative results, while MS is more sensitive than NMR (10^{-12} mol/L *vs* 10^{-6} mol/L). Indeed, these two analytical platforms do have a complementary nature[56,57]. One dimensional (1D) proton (^{1}H) NMR approaches are mostly applied in metabolomics studies, offering several advantages thanks to the natural abundance of the ^{1}H isotope (99.9%) and fast experiments under controlled temperature, giving the possibility to exploit the behavior of many molecules reducing the possibility of sample denaturation. 1D NOESY, CPMG (Carr–Purcell–Meiboom–Gill)[58] and ^{1}H diffusion-edited are the most used pulse sequences in metabolomics studies, which permit namely the observation of both low- and relatively high-molecular weight molecules, the low-molecular-weight compounds selectively, and the selective observation of macromolecular components present in the sample. However, some authors prefer to remove the macromolecular components *via* centrifugation. Platforms for the automatic screening of compounds are always under developing, the B.I. platform (Bruker IVDr, Bruker BioSpin) for analysis and quantification lipids ad lipid subfractions in blood samples.

Ultimately, there are different pre-analytical procedures (which also include the storage of the samples) that could affect the sample and it should be taken into account when comparing the findings of different studies[59,60]. Optimal standard operating procedures for pre-analytical handling of blood and urine for metabolomics' studies and biobanks are well reported by Bernini *et al*[61]. Often 2D spectra for metabolomics studies are required to assign new metabolites, for doubtful cases and metabolite quantification. 2D experiments generally require much longer acquisition times than the standard 1D pulse sequences; therefore, in defining the total acquisition time, one should also consider the stability of the sample under the selected experimental conditions.

Another important aspect of the metabolomic workflow is the use of appropriate statistics to analyze multiparametric data. Most common methods for metabolomic normalization, multivariate and univariate statistical techniques are well reviewed by Vignoli *et al*[56].

NMR based metabolomics has widely demonstrated to be an optimal strategy for diseases' diagnosis[62-65], classification and prognosis[66] and as previously reported we will give an update on the current status of NMR-based metabolomic studies using biological fluids for the diagnosis of GI cancers. In Table 1 we have summarized the different NMR studies evaluated and discussed. Biological samples, are extremely valuable as direct reporters of the diseased region. However, systemic biofluids, such as urine or blood (serum and plasma), but also fecal water, have a slight biochemical correlation with a diseased organ or apparatus, but present two main advantages: The simple, noninvasive or minimally invasive collection, and the ability to reflect the overall response of the patient to the pathological status. On the other hand, urine, blood and fecal water are largely variable in the chemical composition and in number of metabolites, with urine and fecal metabolites being heavily influenced by lifestyle

factors such as food and liquid intake, while blood samples show a better-defined and stable metabolome (Figure 2). Detecting and characterizing cancer-associated biomarkers by metabolomics analysis of bio-fluids could make easy and minimally invasive (reducing the collection of tissue biopsies) the diagnostic approach, representing a valid opportunity of success in the early cancer detection. Altered metabolites in the three different biofluid (blood, urine and stool) identified in 21 NMR-based metabolomic studies, were extracted and summarized respectively in Tables 2-4. A total of 46 metabolites were extracted to be significantly altered in blood samples (serum/plasma), 64 in urine and 28 in fecal water of patients with GI cancers, compared to healthy subjects (or controls). Finally, ten publications report the use of NMR metabolomics to study GI cancers on blood samples, seven are based on urine samples and four on stool samples.

Blood samples

Serum samples are commonly used in clinic to test the presence of tumor markers such as carcinoembryonic antigen and the carbohydrate antigen 19-9, however these tests have good sensitivity but poor specificity due to the presence of false positive results given by other non-neoplastic condition. Thus, the relevance to develop alternative screening tools improving the early detection and fine defining the cancer classification. Blood metabolomics has demonstrated the potential to help GC diagnosis. OuYang *et al*[67], based on a small cohort of 17 PC patients and 23 healthy subjects showed that entire ^{1}H-NMR serum spectra could be used to discriminate the two groups using principal component analysis (unsupervised multivariate statistical approach), identifying altered levels of 3hydroxybutyrate and lactate (Table 2) in PC patients. These alterations were also detected by Zhang *et al*[68] in plasma samples of PC patients (n = 19) in parallel to lower levels of citrate, low-density lipoprotein, high-density lipoprotein, valine, lysine, leucine, isoleucine, histidine, glutamine, glutamate, alanine, and higher levels of NAG (N-acetyl glycoproteins), very-low-density lipoprotein, lipid glyceryl, dimethylamine and acetone (Table 2) compared to healthy subjects. Moreover, the authors identified differences in the plasma metabolomic profile of PC patients also compared to chronic pancreatitis patients (n = 20). More recently, Michálková *et al*[69], compared the plasma samples of 10 PC patients with ten healthy controls, obtaining an impressive discrimination accuracy (94%). However, this study is based on a very limited sample population and the absence of patients being treated is not specified in the exclusion criteria, which could influence the model accuracy.

Bathe *et al*[70], in a well-designed study, demonstrated the possibility to distinguish PC (n = 56) from benign pancreatic conditions (benign masses and chronic pancreatitis) and patients with gallstone disease (n = 43), matched by age, jaundice and incidence of diabetes, by NMR metabolomic analysis of serum (AUROC 0.83). Another more recent paper[71] of the same group reported on a bigger monocentric cohort (n = 157) the difference in the metabolomic profile of malignant and benign pancreatic and periampullary lesions using ^{1}H-NMR and GC-MS. Indeed, it represents an important finding, since, in the clinic, is not always possible to distinguish PC from other non-pancreatic adenocarcinomas such as periampullary adenocarcinomas, especially when located near to the pancreas' head. McConnell *et al*[71], used both ^{1}H-NMR and GC-MS to analyzed the metabolomic profile of serum samples of PC patients. Interestingly, comparing the accuracies for the discrimination among patients and controls obtained using the two approaches, it emerges that NMR-based models are more accurate than GC and then NMR-GC combined models (^{1}H-NMR dataset: Average of 14 metabolites, AUROC 0.74; GC-MS dataset: Average of 18 metabolites, AUROC 0.62; combined CG-MS/^{1}H-NMR datasets: Average of 20 metabolites, AUROC 0.66). A similar approach was proposed by Farshidfar *et al*[72] that using metabolomic data obtained from both ^{1}H-NMR and GC-MS platforms, discriminated serum samples of patients with liver-limited metastasis from local (stage II and III) CRC (NMR AUROC 0.88, GC-MS AUROC 0.87) or extrahepatic metastasis patients (NMR AUROC 0.72, GC-MS AUROC 0.90). In a multicentric study with ^{1}H-NMR metabolomic, Bertini *et al*[73] correctly discriminated the serum profile of metastatic CRC patients (regardless of chemotherapy) from healthy subjects (96.7% accuracy). In addition, the authors used the metabolomic profile as an independent predictor of overall survival (OS) obtaining a hazard ratio of 3.37. In particular, short OS patients were characterized by lower serum level of creatine, lipid (-C=C-CH2-C=C-), lipid (-CH=CH-) and valine and higher levels of lipid (-CH2-OCOR) and NAG. Gu *et al*[74] used serum samples to investigate differential metabolomic profile between CRC patients (n = 40), colorectal polyp patients (n = 32) and healthy controls (n = 38). Patients with colon polyps are at high risk for the development of colon cancer, and compared to the metabolism of healthy controls, they found that the major abnormal metabolic pathways were the pyruvate metabolism, glycerolipid metabolism, Gln and

Table 1 List of evaluated studies

Type of tumor	Ref.	Type of biofluid	Sample size	Mono- or multi-centric study	Cohort allocation	NMR (MHz)	Acquisition temperature and pulse sequences
PC	[70]	S	99 (56 PC; 43 control patients: Benign pancreatic masses, pancreatitis and gallstone disease)	Mono	Calgary, Canada	600	298 K, 1D Noesy: 1024 sc; 2D TOCSY, HSQC
PC	[67]	S	30 (17 PC; 23 HS)	Mono	Fuzhou, China	500	298 K, CPMG: 256 sc
PC	[68]	P	59 (19 PC; 20 chronic pancreatitis; 20 HS)	Mono	Xi'an, China	600	298 K, 1D Noesy: 64 sc
PC	[71]	S	157 (122 PC/periampullary cancer; 35 benign pancreatic/periampullary disease)	Mono	Calgary, Canada	600	298 K, 1D Noesy: 1024 sc; 2D TOCSY, HSQC
PC	[69]	P	20 (10 PC; 10 HS)	Mono	Prague, Czech Republic	500	298 K, CPMG: 128 sc
CRC	[87]	S	57 (38 CRC; 19 HS)	Mono	Birmingham, United Kingdom	800	CPMG: 128 sc; TOCSY: 32 sc; hadamard-TOCSY: 8 scasc
CRC	[73]	S	297 (153 mCRC; 139 HS)	Multi	Denmark	600	310 K, CPMG: 64 sc; JRES: 1 sc
CRC	[72]	S	112 (42 lCRC; 45 liver metast.; 25 extrahepatic metast.)	Mono	Calgary, Canada	600	298 K, 1D Noesy: 1024 sc; 2D TOCSY, HSQC
CRC	[75]	P	70 (40 CRC; 30 liver metastases from CRC)	Multi	Hamburg, Germany	600	300 K, 1D Noesy, CPMG and Diff: 64 sc each
CRC	[74]	S	110 (40 CRC; 32 colorectal polyp patients; 38 HS)	Mono	Xiamen, China	600	298 K, CPMG: 256 sc
PC	[76]	U	87(33 PDAC; 54 HS)	Mono	Verona, Italy	600	300 K, 1D Noesy 32 sc; JRES and HSQC
PC	[77]	U	89 (32 PDAC; 32 benign; 25 HS)	Mono	Alberta, Canada	600	298 K, 1D Noesy: 32 sc
GC	[81]	U	145 (75 GC; 81 HS)	Mono	Seoul, Korea	600	298 K, 1D Noesy: 64 sc
GC	[78]	U	123 (43 GC, 40 benign gastric disease, 40 HS)	Multi	Alberta, Canada	600	298 K, 1D Noesy: 128 sc
CRC	[68]	U	113 (55 CRC; 18 EC; 40 HS)	Mono	Guangdong, China	400	1D Noesy: 256 sc
CRC	[82]	U	62 CRC	Mono	Alberta, Canada	600	298 K, 1D Noesy: 32 sc
CRC	[80]	U	248 (92 CRC; 156 HS)	Mono	Seoul, Korea	500	NA
CRC	[83]	ST	33 (21 CRC; 11 HS)	Mono	Valencia, Spain	600	283 K, CPMG: 256 sc; 2D TOCSY, HSQC
CRC	[84]	ST	100 (68 CRC; 32 HS)	Mono	Guangdong, China	400	298 K, 1D Noesy: 64 sc
CRC	[86]	ST	99 (50 CRC; 49 HS)	Mono	United Kingdom	600	1D Noesy 2816 sc; 2D COSY, HSQC and HMBC
CRC	[85]	ST	140 (70 CRC; 70 HS)	Mono	Guangdong, China	400	298 K, 1D Noesy: 64 sc

glutamate metabolism, and alanine, aspartate and glutamate metabolism. Moreover, they distinguished the metabolomic profile of CRC patients from that of colorectal polyposis (AUROC 0.727). Ghini *et al*[75] raised a very important point about the sample collection by quantifying the effect of preoperative anesthesia on the plasma metabolomic profile of patients with CRC or CRC and LC metastasis. The collection of plasma sample during the preoperative anesthesia is a common procedure that should be avoided in standard metabolomic studies. The authors demonstrated that if compared before the anesthesia CRC *vs* LC can be distinguished using CPMG spectra with an overall classification accuracy of 76.5%, while comparing samples collected during the anesthesia the discrimination accuracy of CRC *vs* LC rose to 90.4%. The increased discrimination was attributed to the authors to the different pharmaceutical treatments administered to CRC patients respect to LC patients.

Urine samples

Some NMR-based metabolomic studies are focused on the characterization of the tumor profile in urine samples sometimes considering heterogeneous group of cases (*e.g.*, patients with different cancer stage, metastatic patients, patients with also other cancer types, *etc*.). If not properly considered, these factors represent important confounding elements.

Napoli *et al*[76] proposed a characteristic urinary metabolomics signature of pancreatic ductal adenocarcinoma (PDAC) in a male cohort. However, the selected PDAC group is very heterogeneous, including 12 patients with liver metastasis, 4 diabetic patients and 3 pancreatitis. These effects, together with gender effect, were not examined in the study. Other studies suggested urine as an excellent bio-fluid to monitor the effect of treatment on patients or for the identification of benign form reducing the need for invasive intervention. Davis *et al*[77] demonstrated that using urine sample is possible to discriminate PDAC patients (*n* = 32) from 25 healthy controls (AUROC of 0.988) and from 32 with benign pancreatic disease (AUROC 0.95) with optimal accuracies. They also evaluate the effect of complete surgical resection on metabolomic profile demonstrating a recovering tendency towards the normal profile. The main study criticism is the limited sample size. The results should be validated on a larger cohort. Similarly, urine profile has been investigated by Chan *et al*[78] in a multicentric study to discriminate a group of GC patients (GC, *n* = 43) from patients with benign gastric diseases (*n* = 40), such as gastritis, ulcer, portal hypertensive gastropathy, gastro-oesophageal reflux disease and polyps, and from a group of healthy subjects (*n* = 40). The study described a characteristic GC profile compared to benign gastric disease subjects and HS. However, despite being one of the few multicentric studies, important confounding factors, such as the presence of patients under neoadjuvant and adjuvant therapy in GC group, and the presence of *Helicobacter pylori* positive patients, are not considered. The possibility to use urine profile for the diagnosis of early stage cancer would be a great opportunity and Wang *et al*[79] showed a characteristic urinary metabolomic fingerprint of stage I and stage II CRC patients (stage I/II *vs* stage III/IV: R2Y = 0.41; Q2 = 0.45). Moreover, in this study authors identified both urinary metabolomic differences in early stage CRC samples respect to esophageal cancer, suggesting that upper and lower GI cancers have different metabolomic profiles, and both overlapping metabolites attributable to shared tumorigenesis pathways (disturbed gut microflora and urea metabolism) associated to tumor cells proliferation/growth. However, Wang *et al*[79] did not focus their research on the characterization of the metabolites that differentiate stage I/II from stage III/IV. A recent study[80] proposed the urine NMR metabolomics as a diagnostic method for pre-invasive CRC patients. The authors evaluated the metabolomic profile of advanced adenoma and stage 0 CRC, revealing a high predictive accuracy in the diagnosis of early colorectal neoplasia patients (CRN *vs* healthy subjects: Specificity 96.2% and sensitivity 95%). However, case and control groups are not sex matched and the groups used for the comparison are numerically unbalanced; thus, the results obtained should be validated on a larger and balanced court of CRN samples. Urine samples have been studied in GCs research also to evaluate the effect of the treatment on patients. Follow-up urine samples, have been analyzed by Jung *et al*[81] to investigate alterations of urinary markers in GC patients underwent curative surgery. The authors showed that the urinary metabolomic profile has a high predictive value for low- and high-stage GC. Moreover, through the analysis of matched tumour and normal stomach tissues, they found results consistent with those obtained from the urine profile, evidencing an up-regulation of lipid

Table 2 Panel of altered metabolites' levels identified in blood samples of gastrointestinal cancer patients *vs* healthy controls

	S			P			S			
	PC[70]	PC[67]	PC[71]	PC[68]	PC[69]	CRC[75]	CRC[87]	CRC[73]	CRC[72]	CRC[74]
2-aminobutyrate									↓²	
2-hydroxyisovalerate									↓	
2-oxoglutarate									↑¹	
3-hydroxybutyrate	↑	↓		↓	↑	↑	↑	↑		
3-hydroxyisovalerate		↓								
Acetate						↓	↑	↑		
Acetoacetate						↑	↑			
Acetone	↑			↑						
Alanine			↓	↓	↑			↓		↓
Arginine						↑				
Asparagine	↓									
Beta-alanine									↓	
Citrate				↓		↑		↓		↓
Creatine	↓		↓					↓	↑	
Creatinine		↑								
Ethanol	↓									
Formate								↑	↓	
Glucose										↑
Glutamate	↑		↑	↓					↓	↑
Glutamine	↓		↓	↓	↑			↓	↑	↓
Glycerol								↑	↑	
HDL				↓						
Histidine				↓					↓	
Hypoxanthine									↑	
Isobutyrate									↓	
Isoleucine		↑		↓					↑	
Isopropanol			↑							
Lactate		↓		↓	↑		↑	↓		
LDL				↓						
Leucine		↑						↓		↓
Lysine	↓		↓	↓						
Mannose	↑		↑						↑	
Myo-inositol			↑							
N-acetyl glycoproteins				↑				↑		
O-phosphocholine									↑	
Ornithine			↓							
Phenylalanine	↑		↑					↑		
Proline			↓					↑		↓
Pyruvate							↑	↓		
Serine										↑
Threonine	↓		↓							
TMAO		↓								
Tyrosine						↓		↓		↓
Urea			↑							
Valine				↓	↑			↓		↓
VLDL				↑						

¹↑Higher metabolite levels in gastrointestinal cancers.
²↓Lower metabolite levels in gastrointestinal cancers. HDL: High-density lipoprotein; LDL: Low-density lipoprotein; TMAO: Trimetlylamine oxide; VLDL: Very-low-density lipoprotein; CRC: Colorectal cancer; PC: Pancreatic cancer.

Advantages	Disadvantages
Blood Minimal sample preparation Less chemical shift variability Information at systemic level Better defined and stable metabolome	Slightly invasive Need for qualified personnel for collection Weak correlation with sick organ
Urine Non-invasive No need for qualified personnel for collection Minimal sample preparation Available in large amount	Variable metabolome (heavily influenced by food and water intake) More chemical shift variability Weak correlation with sick organ
Fecal water Non-invasive No need for qualified personnel for collection Available in large amount Strong correlation with sick organ Correlation with gut microbiota	Variable metabolome (heavily influenced by food and water intake) More chemical shift variability Sample preparation takes longer

Figure 2 Summary of the advantages and disadvantages of various bio-fluids. The figure shows the advantages and disadvantages linked to the use of the three bio-fluids take into consideration for human nuclear magnetic resonance analyses.

oxidation-related metabolites and amino acids in GC patients (Table 3). Even if limited by a small sample size, Dykstra *et al*[82] published an interesting NMR-based metabolomic study on the topic of personalized medicine in CRC patients. In this retrospective study, authors developed predictor model that is moderately accurate in predicting treatment delay, which depends on reactions to chemotherapy, other medical condition and patient choice. Therefore, the possibility to develop a method capable in predicting it could be important to help clinicians planning for future procedures.

Urinary metabolomic could represent a good non-invasive alternative to determine tumor-associated perturbations and despite the good results of these mentioned retrospective studies, new NMR metabolomic based prospective studies should be performed. The latter, if validated with independent and larger cohorts, could demonstrate the importance of NMR metabolomic for the diagnosis of GI cancers using urine samples. Indeed, urine metabolomic analysis could be easily implemented to be used as wide scale population screening. However, in clinics, the biggest drawback of urine metabolomics' profile is the variability of the samples, due to different host (*e.g.*, lifestyle and diet) and environmental factors and finally the pathophysiological status of the patients. More attention should be paid during the experimental design to control these variability factors.

Fecal water
Despite the rising approval of fecal metabolomics, so far there isn't a standardized method to collect, prepare and analyze fecal samples. This deficiency of standardization is intensifying by the fact that this type of matrix is a semi-solid mixture of endogenous and exogenous components, so a quite complicated sample preparation for metabolomic analyses is required. In addition, fecal metabolite analysis has never been examined through a systematic review or a systematic study, differently from urine, serum, plasma, cerebrospinal fluid, and saliva biofluids. Currently 4 studies take into consideration the metabolic analysis with NMR to quantify the concentration of metabolites in fecal extracts in the CRC, while there are even no works investigating the stool metabolic composition of GC and PC patients. The first study conducted by Monleón *et al*[83] on CRC using a ¹H-NMR on a small cohort of 11 controls and 21 CRC patients showed that fecal water extracts have an abundance of small metabolites such as lactate, glucose and amino acids. The spectra exhibited high variability because of the lack of dietary control. Nevertheless, multivariate analysis showed significant differences between the two groups. Similar results were obtained in two different study of Lin *et al*[84,85]. In the first study they investigated the NMR-based fecal metabolomics fingerprinting as predictors of earlier diagnosis in different stages of CRC. In particular, their findings revealed that the fecal metabolic profiles of healthy subjects can be well discriminated from those of even early stage (stage I/II) CRC patients. Moreover, the levels of glucose, lactate, SCFAs, glutamate and succinate at stage I/II differed significantly from those at stage III and IV, giving important molecular information about the staging of CRC. In their second study a total of 70 CRC patients and 70 healthy subjects were enrolled, to rough out the paralleled metabolites of CRC biopsy and the near non neoplastic

Table 3 List of altered metabolites' levels identified in urine samples of gastrointestinal cancer patients *vs* to healthy controls

	U					
	PC[76]	PC[77]	CRC[79]	CRC[80]	GC[78]	GC[81]
1-methylnicotinamide		↑[1]			↓[2]	↓
2-furoylglycine					↑	
2-hydroxyisobutyrate		↑				
2-oxobutyrate						↑
2-phenylacetamide	↑					
3-aminoisobutyrate				↑		↑
3-hydroxyisovalerate	↓			↑		
4-hydroxyphenylacetate		↑				↑
4-pyridoxate		↑				
Acetate						↑
Acetoacetate	↑		↑			
Acetone		↑				↑
Acetylated compounds	↑					
Alanine		↓	↑		↑	↑
Aminobutyrate		↑				
Arginine						↑
Ascorbate				↓		
Asparagine			↓			
Betaine						↑
Choline		↑	↓			
Cis-aconitate		↑	↑			
Citrate	↓			↓		
Creatinine	↓		↓	↓		
Cysteine			↓			
Dimethylamine		↑			↓	
Dimethyl sulfone			↓			
Formate					↑	↑
Fucose		↑				
Glucose	↑	↑				
Glutamine			↑			
Glycerol				↓		
Glycine	↓					↑
Glycolate						↑
Guanido-acetate			↑			
Hippurate	↓		↓	↓		
Histidine						↑
Homocysteine			↑			
Hypoxanthine		↑				↓
Indoxyl sulfate						↑
Isocitrate			↓			
Lactate						↑
Leucine	↑					↑
Mannitol						↑
Methanol		↓				
Methionine						↑
Methylamine			↓			
N-acetyl serotonin					↑	
N-methyl hydantoin						↑
O-acetyl carnitine		↑				↑
Phenylacetyl glycine						↑

Phenylalanine		↓	↑
Putrescine			↑
Succinate			↑
Sucrose		↑	
Taurine	↑	↑	↑
Threonine		↓	
Threonine	↑		
TMAO	↑		
Trans-aconitate	↑	↑	↑
Trigonelline	↓	↓	
Tryptophan	↑		
Tyrosine			↑
Urea		↑	
Valine			↑
Xylose	↑		

[1]↑Higher metabolite levels in gastrointestinal cancers.
[2]↓Lower metabolite levels in gastrointestinal cancers. U: Urine; TMAO: Trimetlylamine oxide; CRC: Colorectal cancer; PC: Pancreatic cancer.

tissues pre- and postoperative fecal samples from the same patients. This work unveiled distinct and discriminatory metabolites across both matrices of CRC patients, but in particular fecal acetate demonstrated the highest diagnostic performance for discriminating CRC from healthy subjects. In the study of Le Gall *et al*[86], presented a list of fecal metabolites expressed in concentration units among 50 CRC patients and 49 controls. Their results showed that there are significant alterations in the metabolite composition of fecal extracts from patients with CRC compared to controls.

DISCUSSION

Early stage GI cancers usually present no symptoms, so are diagnosed at advanced stages with a consequence of poor prognosis. The discovery of predictive biomarkers might lead to early diagnosis with increase in the quality and length of patients' lives. Therefore, the development of low-cost and non-invasive diagnostic techniques is necessary to reduce unfavorable prognosis and medical expenses. In this review, we have reported the results of a series of studies, focusing our survey to NMR-based metabolomic applications in biological fluids, for the discovery of biomarker candidates for GI cancers. The main reason for restricting our analysis to this particular topic stems from the fact that NMR analysis of biofluids is a high throughput, robust, quantitative and reproducible technique that perfectly fits with the concept of large-scale non-invasive population screening. NMR spectra can be easily obtained in a matter of minutes (from 10 to 30 for common fluid samples), and without the need of complex sample pre-treatments. NMR thus offers the possibility to obtain an untargeted and unbiased snapshot of the sample composition, and, with the possibility of quantifying multiple compounds simultaneously, it could become a reference clinical tool for the study of complex biofluids. However, in order to realize in adequate way this approach, it is necessary to standardize both the pre-analytical and the analytical procedures employed for: (1) Sample collection; (2) Handling, transportation; (3)Preparation; and (4) Instrumental analysis. In fact, all these steps could affect the composition of the samples and consequently the analysis' results. The technical specifications for the pre-analytical processes for metabolomics in urine, venous blood serum and plasma have been published by CEN (CEN/TS 16945:2016), following the evidence reported by Bernini *et al*[61]. Unfortunately, these recommendations are still not universally applied. Looking more carefully to the methods employed by the 21 studies considered here, it clearly appears that they are implemented using a variety of machines (spanning from 400 to 800 MHz, being the 600 MHz the most represented), pulse sequences (1D noesy, CPMG, 2D spectra), and number of scans. Therefore, a not perfect match of the results obtained is expected. Furthermore, and more importantly, the cohorts' composition, even for the same cancer kind, is not exactly the same: *i.e*., for PC, in some papers the patients are compared with healthy controls, in some other with patients with benign masses or

Table 4 Panel of altered metabolites' levels identified in fecal water samples of gastrointestinal cancer patients versus healthy controls

	Fw			
	CRC[83]	CRC[84]	CRC[86]	CRC[85]
4-aminohippurate			↓[2]	
Acetate	↓	↓		↓
Alanine		↑[1]	↓	↑
Beta-alanine			↓	
Butyrate	↓	↓		↓
Cholate			↓	
Deoxycholate			↓	
Galactose			↓	
Glucose		↓	↓	
Glutamate		↑		↑
Glutamine		↓	↓	
Glycerol			↓	
Hexose-phosphate			↑	
Isobutyrate			↑	
Isoleucine		↑	↓	↑
Isovalerate			↑	
Lactate		↑		↑
Leucine	↑	↑		↑
Litho deoxycholate			↓	
Methanol			↓	
Ornithine			↓	
Phenylacetate				
Proline	↑	↑		
Propionate		↓		↓
Succinate		↑		↑
Taurine			↓	
Valine		↑		↑
Xylose			↓	

[1]↑Higher metabolite levels in gastrointestinal cancers.
[2]↓Lower metabolite levels in gastrointestinal cancers. Fw: Fecal water; CRC: Colorectal cancer.

with other diseases. The same is true for CRC (where the controls are healthy subjects or patients with polyposis) and for GC (where the controls are healthy subjects and patients with benign lesions). In any case, the main limitation that emerges from the analysis of the selected papers is the small sample size generally employed. The larger study[73] involves 297 participants (153 CRC and 139 HS), the smallest[69] only 20 participants (10 PC and 10 HS), with the others ranging from few tens to a bit more than one hundred participants. Consequently, the statistical power is quite limited, with a not negligible probability of spurious or not reproducible results. Moreover, almost all the studies are monocentric with cohorts recruited in Asia (China), North America (Canada), and Europe (Denmark, Czech Republic, Spain). Only two papers[75,78] involved multicentric cohorts. The consequence is that, due to the known influence of dietary habits and genetic background on the metabolic profile, the results could be not immediately compared due to the broad geographical distribution of the study cohorts. However, all these limitations can be considered also a strength: If from these different study designs and cohorts emerge some similar results or trends, they can be considered robust enough to be further investigated as candidate biomarkers for GI cancers.

In other words, carefully looking at the results of the 21 evaluated studies, we can identify some common alterations (Figure 3).

Three studies on blood reported that 3-hydroxybutyric acid was present in higher amount in CRC cancer[73,75,87] while results on PC are discordant. 3-Hydroxybutyrate is a ketone body and one of its main functions is to provide acetoacetyl-CoA and acetyl-
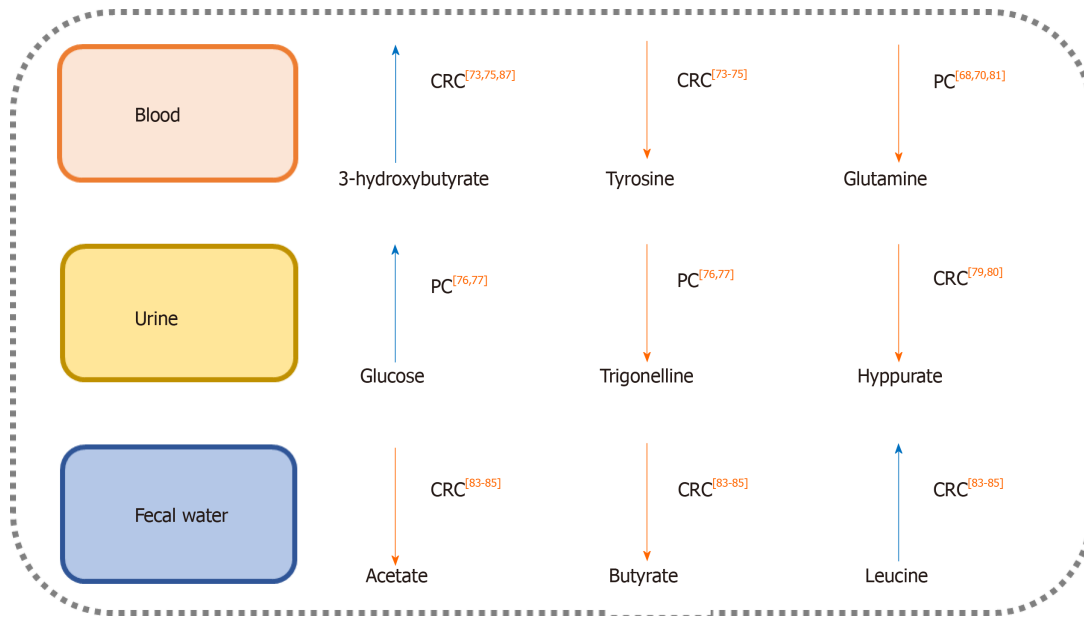
Figure 3 Most significant metabolites identified in the 21 studies analyzed. We have summarized in the outline the most significant metabolites identified in blood, urine and fecal water in the 21 evaluated studies. The green and the red arrows indicate respectively the increase or decrease of the metabolite detected in colorectal cancer or pancreatic cancer in the corresponding references. CRC: Colorectal cancer; PC: Pancreatic cancer.

CoA for the synthesis of cholesterol and lipids. 3-hydroxybutyrate amount in blood increases as oxidation levels increase. Its overexpression could lead to an enhanced lipogenesis promoting tumor growth. Moreover, tyrosine levels in blood samples were concordantly lower in three CRC based studies[73-75]. Several other published studies report the same results; however, the tyrosine[88,89] role has not yet been clarified.

Instead, for what concern the five reported PC cancer studies, only Gln level in blood was found concordant in more than two studies[68,70,81]. Gln plays an important role in regulating redox homeostasis[90]. Cancer cells show an increase Gln demand as the result of a shift from glucose oxidation to "Warburg effect".

Compared to blood, urine has the main advantages of being non-invasive and available in large amounts. Nevertheless, its natural abundance of metabolites and its high variability among patients makes it difficult bio-fluids to analyze. Among all the mentioned studies, it does not emerge a common biomarker(s) describing a metabolic alteration due to GI. This could be ascribed firstly to the sample variability and secondly to the different tumors and disease stage. Several other factors such as gender, age, hormonal status, diet, or physical activity should be taken into account. Moreover, in some cases it is important to evaluate the effect of patients' comorbidities, such as diabetes in the PC, where it may be either a risk factor or a symptom.

Higher glucose level in urine of PC patients, identified by Napoli *et al*[76] and Davis *et al*[77] could be due to the presence of diabetic patients in the group of cases, which were not excluded from both studies.

Trigonelline too, was identified in lower amount in PC by both studies. Its presence may be related to particular dietary products (*e.g.*, coffee, tea, *etc*.) but may also arise from endogenous niacin methylation. However, some of the identified variations in urine could also be attributed to the interaction of the host with the gut-microflora such as lower hippurate levels, identified in urine of CRC patients by Wang *et al*[79] and Kim *et al*[80], supporting what previously seen that CRC is associated with an altered intestinal microbial composition[91].

Fecal water extract, like urine, can be an interesting bio-specimens due to its non-invasive collection. However, at present there are not studies in literature considering the NMR analysis of these bio-fluids in PC and GC patients. Three of the four studies that we have analyzed[83-85] find common alteration of 3 metabolites: Acetate, butyrate, and leucine. Acetate and butyrate are short chain fatty acids (SCFAs), a microbial-derived metabolite, normally produced by the gut bacteria. SCFAs are absorbed by the intestinal epithelium and used as energy sources for intestinal barrier protection[92,93]. The depletion of SCFAs, especially butyrate, in feces could suggest that there has been an intestinal dysbiosis in CRC patients and consequently an alteration in bacterial products[94-96]. Acetate is a precursor molecule for endogenous cholesterol

and can be transformed to acetyl-CoA for lipid biosynthesis. This data confirms that the shift to lipogenesis is a typical change of cancer metabolism. Acetate is probably the most discriminative metabolites of SCFAs in the three studies, and in particular Lin and his group find a link between acetate levels in CRC feces and glucose and myo-inositol levels in colorectal tumor tissues. Significant depletions of glucose and myo-inositol in CRC tissues and decrease of acetate levels in feces could be indicative of an increased energy demand by cancer cells for their growth. Compared to healthy subjects an increase of leucine is reported in the feces of CRC patients, this could be due to the epithelium inflammation that leads to malabsorption of nutrients[97]. However, the amino acid metabolic profile is often very varied as there is no dietary control in patients.

CONCLUSION

Summarizing the results of the selected studies, we can conclude that NMR analysis of bio-fluids could be a high throughput, quantitative and reproducible test that fully fits with the concept of large-scale non-invasive population screening for the GI cancers. To date, there is small the number of studies exploring this opportunity and focusing only on the patients with CRC and in addition using a restricted number of patients and the same country. Therefore, future perspectives are to plan multicentric studies involving a high number of patients and evaluating not only CRC patients but also patients with pancreatic or GC. In addition, a crucial point will have to be the evaluation of interfering factors such as gender, age, hormonal status, diet, physical activity and especially comorbidities and the metabolites associated with gut microbiota, such as the SCFAs. Finally, despite the restricted number of the studies using the stool for the metabolic NMR analysis, we think that the fecal water samples could be an interesting and cheap bio-fluid to explore for future applications.

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