Metabolic Biomarkers of Colorectal Cancer

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Metabolic biomarkers of colorectal cancer (CRC) can be found in several matrices obtained from human body, such as breath, urine, feces, blood, intestinal gas, and tissue. Metabolic CRC biomarkers consist of small molecules, including volatile organic compounds (VOCs), which patterns (profiles) can be acquired by analytical techniques and be used to study the presence and progression of disease in the organism. Gas chromatography-mass spectrometry is a technique that allows to analyze volatiles and other classes of compounds of different chemical groups. Molecular profiles may indicate very specific biochemical ongoing processes in a biological system. Comparisons of metabolic profiles and the processing of this data using statistical tools can potentially enacoloble to distinguish diseased subjects from healthy ones.

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1. Introduction

1.1. Colorectal Cancer Background

According to data regarding cancer burden in 2018 (GLOBOCAN 2018), colorectal cancer (CRC) is currently the third most incident cancer type in the word, with nearly 1.85 million cases and 881 thousand deaths worldwide. In Europe, it occupies the second place in the ranking of cancer occurrence and related deaths, with approximately half a million new cases registered and almost a quarter of a million associated deaths. Moreover, research on cancer progression predicts an increase of 75% in CRC cases over the next 20 years [1]. The global population over time has experienced significant changes in their habits, notably the prevalence of sedentarism, increased intake of dietary fat and processed food, and exposure to carcinogens, all risk factors in CRC [2]. Such context presents a complex perspective on CRC, also a from socioeconomic point of view, emphasizing the need for prevention strategies and promotion of early diagnosis.

It is observed that around 95% of colorectal neoplasms are adenocarcinomas and start as colonic adenomatous polyps [3]. Then, a series of genomic and molecular alterations induce the development of the malignancy in the colon [4]. CRC can be prevented if an intervention occurs leading to excision of the polyps and conduction of proper treatment; therefore, approaches directed towards an early detection of polyps and lesions, before these achieve the malignancy threshold, have substantial importance to reduce both CRC incidence and mortality [3].

1.2. Available Diagnostic Methods

The fecal occult blood test (FOBT), also known as the guaiac test, is generally applied for CRC screening. Nevertheless, this procedure presents relatively low sensitivity, which for this once-only test can be 50% or lower [5][6]. Additionally, FOBT is affected by the presence of interferers, is not specific for distal gut blood and may be insensitive to smaller bleedings. The antibody-based fecal immunochemical test (FIT) for hemoglobin is an improved alternative to FOBT, obtaining a sensitivity greater than 80% [6]. Notwithstanding, the verification of fecal blood can have a low impact on CRC primary assessment and is occasionally indicative of late stage cancer [7]. Currently, colonoscopy is described as the gold-standard screening procedure for CRC as it presents high sensitivity and specificity. However, colonoscopy is a costly and invasive procedure, limiting a patient's access to the examination and resulting in poor compliance rates, aspects that hinder successful implementation of this test in CRC prevention [8][9]. Imaging exams have great reported efficiency, although also carry limitations regarding the cost of procedures and required exposure to radiation [10].

The group of currently available CRC biomarkers can be classified according to the affected biological matrices related to colorectal neoplasm. The most common are tumor, blood and stool biomarkers [11]. Moreover, molecular indicators can be grouped into three classes: prognostic, predictive and diagnostic markers [12]. Prognostic markers indicate the possible progression of the disease, such as: adenomatous polyposis coli (almost 100% of individuals develop CRC with this germ line mutation) [13][14], p53 (tumor suppressor p53 expression) [12], and epidermal growth factor receptor (EGFR; up to 80% over expression in CRC) [15]. Predictive indicators are used to foresee treatment measures to be taken on a patient. They include, e.g., Kirsten rat sarcoma viral oncogene (KRAS; more than 50% of CRC patients carry a mutant allele) [13][16], BRAF (a mutant KRAS gene, which encodes protein B-Raf, found in only 30–40% of the 90% of patients not affected by anti-EGFR therapy) [14][16], and COX-2 (Cyclooxygenase-2; the expression exhibited in 70% of CRC tumors) [12]. Risk stratification and early detection of polyps are provided by diagnostic markers, such as: insulin like growth factor binding protein 2 (IGFBP2; elevated levels in plasma and serum of CRC patients) [12][14], telomerase (an enzyme responsible for synthesizing DNA from chromosome ends for which an increase in activity was noticed for 90% of colorectal tumors) [17], and pyruvate kinase M2 (PKM2; a glycolytic pyruvate kinase isoenzyme increased in the stool of CRC subjects) [16]. Epi proColon® (Epigenomics Inc., San Diego, CA, USA) is a commercially available test relying on the verification of methylated Septin-9 in DNA extracted from blood, by means of polymerase chain reaction (PCR) [18]. This genetic alteration is associated with the presence of CRC tissue. Studies showed that Epi proColon® exam presented sensitivity and specificity ranging from 75 to 81% and from 96 to 99%, respectively [19]. Nevertheless, subsequent clinical trials demonstrated that test sensitivity was insufficient in case of asymptomatic cases and stage I CRC. Cologuard® (Exact Sciences Corporation, Madison, WI, USA) is a stool-based presumptive test for CRC, based on the qualitative detection of fecal DNA markers. This exam presented to be superior to the FIT test, although its rate of detection was around 42% in cases of advanced adenomas [8]. Apart from the displayed limitations, these screening strategies tend to achieve wider acceptance among the population and can indicate the need for further colonoscopic investigation, aiding a more approachable monitoring of CRC.

1.3. Metabolomics Studies on CRC

Metabolomics science emerged as a new approach to study biological systems [20]. In a metabolomics workflow, biological samples are processed and comprehensively analyzed in terms of total metabolites, which can belong to a specific chemical class depending on the envisioned approach and the methodologies selected for sample preparation and pre-concentration. Measurements can involve different analytical platforms, with emphasis given to chromatographic techniques—able to resolve complex mixtures—coupled to mass spectrometry [21], such as gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) [22][23][24].

Among the small metabolites, volatile organic compounds (VOCs) are metabolic products that can elicit diversified patterns that may represent very specific biochemical ongoing processes in the organism. Volatiles' profiles have been studied in the context of several diseases, especially in exhaled breath, using GC-based analyses [25][26][27][28]. In this context, GC analysis is extremely relevant, because it encompasses the group of VOC metabolites, which cannot be properly assessed by LC.

Research on global molecular metabolites as potential markers of diseases is a very interesting approach for the design of methods directed towards the early diagnosis and evaluation of patient's response to therapeutic intervention [20][29]. Molecular profiling presents promising perspectives towards clinical applications. The assessment of a set of metabolites has the possibility to provide information regarding simultaneous metabolic alterations, potentially offering a more accurate and detailed diagnosis, thus, it represents a great advance in personalized medicine [30].

Although contemporary, metabolomics-based methods still face several challenges, such as: the existence of a large body of variables that may impact the metabolic profile; the lack of standardization in workflow protocol and irreproducibility between studies that lead to varied panels of potential biomarkers. Therefore, a deeper inspection is required in order to compare the results reported so far by different research groups concerning the metabolomic investigation in CRC, listing the main developments made to date, and thus offering insights into new aspects to be studied regarding CRC characterization.

2. Studies on Colorectal Cancer Metabolic Biomarkers

2.1. Applied Methodologies

A critical matter involving metabolomics studies is the employment of varied protocols covering sample collection, processing and analysis. In this sense, the selection of specific analytical parameters can deeply influence the set of acquired metabolites, turning valid the discussion on the main aspects prevalent in sample pre-treatment, extraction procedure and analysis in GC-based metabolomics directed towards CRC markers investigation. Several techniques have been employed for the extraction and pre-concentration of the metabolites of interest in different biological samples. The particular characteristics of each matrix determine which sample preparation techniques are required, which in turn, have associated advantages and limitations to be observed by the analyst. Fundamental aspects regarding the selection of biological matrix are the concentration range of the target analytes in the sample, window of detection provided, matrix complexity and involved distribution mechanisms. Sample

preparation techniques to be used should be chosen based on their ability to pre-concentrate the analyte, the availability of specific materials, required processing time and involved costs. Data concerning sample preparations details, study design and statistical approaches employed by the reviewed studies are summarized in Table 1.

Table 1. Table summarizing all 21 studies regarding investigation of biomarkers of CRC in urine, feces, and breath samples.

Reference	Subjects	Sample Preparation and Analytical Technique	Main Analytes	Type of GC Column	Statistical Approach
URINE SAMPLES					
Qiu et al., 2010 [31]	· 60 CRC: Ø stage I: 7 Ø stage II: 23 Ø stage III: 21 Ø stage IV: 9 · 63 HC	solvent extraction with chloroform and derivatization with ECF + GC-MS	SNM: amino acids; organic acids	DB-5MS capillary column (30 m × 250 µm i.d., 0.25-µm film thickness)	PCA, OPLS-DA
Silva et al., 2011 [32]	· 12 CRC · 21 HC	HS-SPME with CAR/PDMS (75 µm) + GC-MS	SVM: hydrocarbons; aldehydes; sulfur compounds	30 m × 0.25 mm ID × 0.25 µm film thickness BP-20	one-way ANOVA, LSD, PCA
Cheng et al., 2012 [33]	· 103 CRC:	solvent extraction with methanol and derivatization	SNM: amino acids; organic	DB-5MS capillary column (30 m × 250	PCA, OPLS-DA, ROC curve, Student's *t*-test,

	Ø stage I: 24 Ø stage II: 45 Ø stage III: 27 Ø stage IV: 5 . 101 HC	with methoxyamine (in pyridine) and BSTFA (1% TMCS) + GC-TOFMS	acids; saccharides	µm I.D., 0.25-µm film thickness; (5%-phenyl) methyl-polysiloxane bonded and cross-linked	Wilcoxon−Mann−Whitney test
Arasaradnam et al., 2014 [34]	. 83 CRC . 50 HC	ITEX + GC-MS	SVM: ketones; aldehydes; nitrogen compounds	Rxi-624Sil column (20 m length, 0.18 mm ID, 1.0 µm df)	FDA, KNN method
Liesenfeld et al., 2015 [35]	Total for GC-MS and ^{1}H-NMR is 199 CRC: . CRC pre-surgery: Ø s0: 5; sI: 12; sII: 40; sIII: 22; sIV: 18 . CRC post-surgery:	solvent extraction with methanol and derivatization with methoxyamine (in pyridine) and BSTFA (1% TMCS) + GC-MS	SNM: alcohols; amino acids; organic acids; saccharides	HP-5 MS fused silica column (30 m × 0.25 mm; 0.25 µm film thickness of the 5% phenyl 95% dimethylpolysiloxane stationary phase	Wilcoxon–Mann–Whitney tests, PLS-DA, one-way ANOVA, ROC curve

	Ø sI: 4; sII: 4; sIII: 2; sIV: 2 . CRC 6 months follow-up: Ø sI: 12; sII: 17; sIII: 15; sIV: 8 . CRC 12 months follow-up: Ø sI: 7; sII: 13; sIII: 14; sIV: 4				
Delphan et al., 2018 [36]	. 163 CRC pre-surgery: Ø stage I/II: 76; stage III/IV: 87 . 83 with 6 months follow-up: Ø stage I/II: 36; stage III/IV: 47	solvent extraction with methanol and derivatization with methoxyamine (in pyridine) and BSTFA (1% TMCS) + GC-MS	SNM: amino acids	HP-5 MS fused silica column (30 m × 0.25 mm; 0.25 µm film thickness of the 5% phenyl 95% dimethylpolysiloxane stationary phase	one-way ANOVA, Pearson Chi-squared test, Pearson's partial correlation coefficients, Cox proportional hazard models

	. 54 with 12 months follow-up: Ø stage I/II: 32; stage III/IV: 25				
Mozdiak et al., 2019 [37]	. 12 CRC . 80 adenoma . 14 diverticular disease · 5 haemorrhoids . 14 inflammatory bowel disease · 1 excluded . 37 HC	not specified + GC-IMS	undetermined	not specified	ROC curve, Sparse logistic regression, Random Forest, Gaussian process classifier, Support vector machine, Neural network
FECAL SAMPLES					
Weir et al., 2013 [38]	· 10 CRC	solvent extraction with isopropanol:acetonitrile:water	SNM: amino acids; organic	TG-5MS column (30 m, 0.25 mm i.d., 0.25	AMOVA, Student' *t* test, ANOVA, Pearson

	· 11 HC	and derivatization with methoxyamine (in pyridine) and MSTFA (1% TMCS) + GC-MS	acids; lipids; steroids	µm film thickness), SCFA determination: TG-WAX-A column (30 m, 0.25 mm ID, 0.25 µm film thickness)	correlation, PLS-DA
Phua et al., 2014 [39]	· 11 CRC: Ø sB: 6; sC: 5 · 10 HC	solvent extraction with methanol:water and derivatization with methoxyamine (in pyridine) and MSTFA (1% TMCS) + GC-TOFMS	SNM: lipids; saccharides	DB-1 (30 min × 250 µm i.d.) fused silica capillary column with 0.25 µm film thickness	PCA, OPLS-DA, ROC curve, Welch *t* test
Bond et al., 2016 [40]	· 21 CRC · 56 with adenomatous polyp/s · 60 HC	HS-SPME + GC-MS	SVM	not specified	Student's *t* test, Fisher's exact test, ANOVA, false discovery rate correction, PLS-DA, factor analysis, ROC curve
Wang et al., 2017 [41]	· 15 CRC: Ø sII: 4; sIII: 6; sIV: 5 · 12 HC	solvent extraction with isopropanol:acetonitrile:water and derivatization with pyridine-methoxy amino acid salt solution, SCFA determination: solvent extraction and derivatization with sulfuric	SNM: amino acids; organic acids; lipids; steroids	30-m TG-5MS column	Student's *t*-test, Pearson correlation

		acid solution (50%) and diethyl ether + GC-MS			
Song et al., 2018 [42]	· 26 CRC: Ø sI: 3; sIIa: 5; sIIc: 1; sIIIb: 11; sIIIc: 3; sIVa: 3 · 28 HC	Analysis of Long-Chain Fatty Acids: solvent extraction with chloroform:methanol (Folch method) and derivatization with BCl_3–MeOH Analysis of Short-Chain Fatty Acids: solvent extraction with HCl and diethyl ether and derivatization with PFBB in acetonitrile and EDIPA + GC-MS	lipids	HP-5 MS 30 m × 250 µm × 0.25 µm column	Chi-square test, Fisher's exact test, Mann–Whitney *U* test
Bond et al., 2019 [43]	· 21 CRC · 56 with adenomatous polyp/s · 60 HC	HS-SPME with CAR/PDMS + GC-MS	SVM: esters; alcohols	60 m long Zebron ZB-624 capillary column with an inner diameter of 0.25 mm. The column was lined with a 1.4 µm film of 94% dimethyl polysiloxane and 6% cyanopropylphenyl	Student's *t* test, Mann-Whitey tests, Fisher's exact test, ANOVA, false discovery rate correction, PLS-DA, factor analysis, ROC curve
BREATH SAMPLES					
Haines et al. 1977 [44]	· 30 CRC	direct gas sampling by means of:	gases	not specified	*p* value

	· 64 with non-malignant large-bowel disorders · 208 without known large-bowel disorders	either a modified Haldane–Priestley tube' or a 3-bag collecting system in which one bag contains sample which can then be transferred to a syringe or evacuated aerosol can for later analysis + GC			
Piqué et al. 1984 [45]	· 47 CRC · 156 HC	direct gas sampling by means of a 3-bag collecting system in which one bag contains sample which can then be transferred to a syringe or evacuated aerosol can for later analysis + GC-FID	gases	not specified	*p* value
Peng et al., 2010 [46]	· 26 CRC: Ø sI: 3; sII: 7; sIII: 7; sIV: 7 · 22 HC	HS-SPME with PDMS/DVB + GC-MS	SVM: hydrocarbons	H5-5MS 5% phenyl methyl siloxane (30 m length, 0.25 mm i.d., 0.25 µm thickness)	PCA
Altomare et al., 2013 [47]	· 37 CRC · 41 HC	adsorption of VOCs on to sorbent cartridges and thermal desorption + GC-MS	SVM: hydrocarbons	SUPELCOWAX, polyethylene glycol 30 m × 0.25 mm ID. × 0.25 µm stationary phase thickness	PNN, ROC curve

	· 15 CRC · 20 with colonoscopic diagnosis of colonic polyps · 15 HC	adsorption of VOCs on to sorbent cartridges and thermal desorption + GC-MS	undetermined	not specified	LDA
Wang et al., 2014 [49]	· 20 CRC · 20 HC	HS-SPME with CAR/PDMS (75 µm) + GC-MS	SVM: alcohols; hydrocarbons	DB-5MS (length 30 m × inner diameter (ID) 0.250 mm × film thickness 0.25 µm)	PCA, PLS-DA, Kruskal–Wallis rank sum test
Altomare et al., 2015 [50]	· 48 CRC · 55 HC	adsorption of VOCs on to sorbent cartridges and thermal desorption + GC-MS	SVM: hydrocarbons	HP-5MS, 95% polydimethylsiloxane, 5% polydiphenylsiloxane, 30 m × 0.25 mm ID, 0.25 µm stationary phase thickness	Mann–Whitney *U* test, chi-square test, Student's *t* test, PNN, ROC curve
Amal et al., 2016 [51]	· 65 CRC · 22 with advanced or nonadvanced adenomas · 122 HC	adsorption of VOCs on to sorbent cartridges and thermal desorption + GC-MS	SVM: hydrocarbons; ketones; esters; alcohols	SLB-5ms capillary column (with 5% phenyl methyl siloxane; 30 m length; 0.25 mm internal diameter; 0.5 µm thicknesses)	Student's *t* test, DFA, ROC curve

VOC—volatile organic compound; CRC—colorectal cancer; HC—healthy controls; s—stage of cancer; ITEX—in-tube extraction; ^{1}H-NMR—proton nuclear magnetic resonance; GC-MS—gas chromatography-mass spectrometry; HS-SPME—headspace-solid-phase microextraction; CAR/PDMS—Carboxen/Polydimethylsiloxane; PDMS/DVB—Polydimethylsiloxane/Divinylbenzene; GC-FID—gas chromatography with flame ionization detection; GC-IMS—gas chromatography coupled with ion mobility spectrometry; GC-TOFMS—gas chromatography/time-of-flight mass spectrometry; PCA—principal component analysis; OPLS-DA—orthogonal partial least squares discriminant analysis; ANOVA—analysis of variance; AMOVA—analysis of molecular variance; LSD—least significant difference; ROC—receiver operating characteristic; FDA—Fisher discriminant analysis; KNN—k-nearest neighbors algorithm; PLS-DA—partial least squares discriminant analysis; PNN—probabilistic neural network; LDA—linear discriminant analysis; DFA—discriminant function analysis; MSTFA—*N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide; SCFA—short-chain fatty acid; PFBB—pentafluorobenzyl bromide; BSTFA—*N*,*O*-bis(trimethylsilyl)trifluoroacetamide; TMCS—trimethylsilyl chloride; ECF—ethyl chloroformate; EDIPA—3'-*O*-ethyl-*N*,*N*-diisopropylphosphoramidite; SNM—screening of nonvolatile metabolites; SVM—screening of volatile metabolites.

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