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Cell-free DNA methylation as a biomarker for early detection of the three major cancers in males

Vera Salvado Constâncio

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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Cell-free DNA methylation as a biomarker for early detection of the three major cancers in males

Dissertação de Candidatura ao grau de **Mestre em Oncologia** – Especialização em Oncologia Laboratorial submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto

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“We can only see a short distance ahead,

but we can see plenty there that needs to be done.”

Alan Turing

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RESUMO

Introdução: Os cancros do pulmão (CaPl), próstata (CaP) e coloretal (CaCr) são os três mais incidentes no sexo masculino a nível mundial. Apesar dos avanços científicos, a procura de novos métodos ideais para rastreio de cancro para a população em geral continua a ser uma necessidade atual. A metilação aberrante de promotores de genes é um evento precoce no desenvolvimento do cancro, sendo uma alteração específica da tumorigénese passível de ser identificada em fluídos corporais, constituindo, uma potencial ferramenta minimamente invasiva para a deteção precoce de cancro. Assim, o principal objetivo deste trabalho consistiu em desenvolver um teste minimamente invasivo baseado na metilação de DNA circulante extraído de biópsias líquidas para deteção simultânea de CaPl, CaP e CaCr em homens.

Métodos: O DNA circulante foi extraído de amostras de plasmas provenientes de doentes com CaPl, CaP, CaCr, e de dadores assintomáticos. De seguida, o referido DNA foi sujeito a modificação bissulfito e amplificação. Os níveis de metilação dos genes APC_{me}, $FOXA1_{me}$, $GSTP1_{me}$, $HOXD3_{me}$, $RAR\beta2_{me}$, $RASSF1A_{me}$, $SEPT9_{me}$ e $SOX17_{me}$ foram determinados por PCR quantitativo específico de metilação em multiplex.

Resultados: Os genes $SEPT9_{me}$ e $SOX17_{me}$ foram os únicos biomarcadores partilhados pelos três tipos de cancro, embora tenham detetado CRC com baixa sensibilidade. O painel "PanCancer" ($FOXA1_{me}$, $RAR\beta2_{me}$ e $RASSF1A_{me}$) detetou CaPl e CaCr com 64% sensibilidade e 70% especificidade, sendo complementado com o painel "CancerType" ($GSTP1_{me}$ e $SOX17_{me}$) que distinguiu estes dois cancros com alta especificidade (superior a 90%), embora com sensibilidade limitada. Um painel constituído pelo $HOXD3_{me}$ e $RASSF1A_{me}$ discriminou cancro do pulmão de pequenas células de cancro do pulmão de não pequenas células com 75% sensibilidade e 88% especificidade. Além disso, o painel APC_{me} e $RASSF1A_{me}$ demonstrou ser um preditor independente de sobrevivência específica de doença em doentes com CaPl.

Conclusões: Um teste baseado em metilação de DNA em biópsias líquidas pode permitir o rastreio de CaPl e CaP de forma minimamente invasiva, tendo o potencial de aumentar a adesão dos doentes a programas de rastreio e reduzir os custos dos sistemas de saúde. Além disso, pode igualmente permitir a sub-tipagem e avaliação prognóstica em CaPl.

ABSTRACT

Background: Lung (LC), prostate (PCa) and colorectal (CRC) cancers are the most incident in males worldwide. Despite advances, optimal population-based cancer screening methods remains an unmet need. Due to its early onset, cancer specificity and accessibility in body fluids, aberrant DNA promoter methylation might be a valuable minimally invasive tool for early cancer detection. Herein, we aimed to develop a minimally invasive methylation-based test for simultaneous early detection of LC, PCa and CRC in males, using liquid biopsies.

Methods: Circulating cell-free DNA was extracted from 102 LC, 121 PCa and 100 CRC patients and 136 asymptomatic donors' plasma samples. Sodium-bisulfite modification and whole-genome amplification was performed. Promoter methylation levels of APC_{me}, $FOXA1_{me}$, $GSTP1_{me}$, $HOXD3_{me}$, $RAR\beta2_{me}$, $RASSF1A_{me}$, $SEPT9_{me}$ and $SOX17_{me}$ were assessed by multiplex quantitative methylation-specific PCR.

Results: $SEPT9_{me}$ and $SOX17_{me}$ were the only biomarkers shared by all three cancer types, although they detected CRC with limited sensitivity. A "PanCancer" panel ($FOXA1_{me}$, $RAR\beta2_{me}$ and $RASSF1A_{me}$) detected LC and PCa with 64% sensitivity and 70% specificity, complemented with "CancerType" panel ($GSTP1_{me}$ and $SOX17_{me}$) which discriminated between LC and PCa with high specificity (over 90%), but with modest sensitivity. Moreover, a $HOXD3_{me}$ and $RASSF1A_{me}$ panel discriminated small cell lung carcinoma from non-small cell lung carcinoma with 75% sensitivity and 88% specificity. An APC_{me} and $RASSF1A_{me}$ panel independently predicted disease-specific survival in LC patients.

Conclusions: We concluded that DNA methylation-based test in liquid biopsies might enable minimally invasive screening of LC and PCa, improving patient compliance and reducing healthcare costs. Moreover, it might assist in LC subtyping and prognostication.

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LIST OF ABBREVIATIONS

5mC	5-methylcytosine
AC	Asymptomatic control
ADT	Androgen deprivation therapy
AJCC	American Joint Committee on Cancer
ALK	ALK receptor tyrosine kinase
APC	APC regulator of WNT signalling pathway
APC_{me}	Methylated APC regulator of WNT signalling pathway
BPH	Benign prostatic hyperplasia
BRAF	B-Raf proto-oncogene, serine/threonine kinase
BrC	Breast Cancer
ccfDNA	Circulating cell-free DNA
ccfRNA	Circulating cell-free RNA
CE	*Conformité Européenne*
CEA	Carcinoembryonic antigen
CI	Confidence interval
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CpG	Cytosine-phosphate-guanine
CRC	Colorectal Cancer
CRPC	Castration-resistant prostate cancer
CTCs	Circulating tumour cells
ctDNA	Circulating tumour DNA
ddMSP	Droplet digital methylation-specific PCR
DFS	Disease-free survival
DNMTs	DNA methyltransferases
DRE	Digital rectal examination
DSS	Disease-specific survival
EAU	European Association of Urology
EGFR	Epidermal growth factor receptor
FAP	Familial adenomatous polyposis
FDA	United States Food and Drug Administration
FIT	Faecal immunochemical test
FOBT	Faecal-occult blood test
FOXA1	Forkhead box A1

FOXA1$_{me}$	Methylated forkhead box A1
GSTP1	Glutathione-S transferase pi 1
GSTP1$_{me}$	Methylated glutathione-S transferase pi 1
HDAC	Histone deacetylases
HGNC	HUGO Gene Nomenclature Comittee
HNPCC	Hereditary non-polyposis colon cancer
HOXD3	Homeobox D3
HOXD3$_{me}$	Methylated homeobox D3
HR	Hazard ratio
ISUP	International Society of Urological Pathology
IVD	*In Vitro* Diagnostic
LC	Lung Cancer
LCLC	Large-cell lung carcinoma
LD-CT	Low-dose computed tomography
LUAD	Lung adenocarcinoma
LUSC	Lung squamous cell carcinoma
MBP	Methyl-CpG-binding proteins
MDA	Multiple displacement amplification
MMR	Mismatch repair
MSI	Microsatellite instability
NPV	Negative predictive value
NSCLC	Non-small cell lung carcinoma
OS	Overall survival
PCa	Prostate Cancer
PD-L1	Programmed death ligand 1
PD1	Programmed cell death protein 1
PFS	Progression-free survival
PPV	Positive predictive value
PSA	Prostate-specific antigen
qMSP	Quantitative methylation-specific PCR
RARβ2	Retinoic acid receptor beta 2
RARβ2$_{me}$	Methylated retinoic acid receptor beta 2
RASSF1A	Ras association domain family 1 isoform A
RASSF1A$_{me}$	Methylated Ras association domain family 1 isoform A
ROS1	ROS proto-oncogene 1, receptor tyrosine kinase
SAM	S-adenosylmethionine

SCLC	Small cell lung carcinoma
SEPT9	Septin 9
SEPT9$_{me}$	Methylated septin 9
SOX17	SRY-box transcription factor 17
SOX17$_{me}$	Methylated SRY-box transcription factor 17
TKI	Tyrosine kinase inhibitor
TRUS	Transrectal ultrasound
TSG	Tumour suppressor gene
VEGF	Vascular endothelial growth factor
WGA	Whole genome amplification

I. INTRODUCTION

1. CANCER BIOMARKERS

During the last decades efforts have scaled up worldwide to develop more effective biomarkers as an approach to reduce cancer mortality [1]. A cancer biomarker can be defined as an objectively measurable biomolecule, such as a protein, metabolite, RNA, DNA or an epigenetic alteration, found in body fluids or tissues, that indicates the presence of cancer or provides information on cancer's expected future behaviour [2, 3]. Depending on their purpose in the clinical setting, they can be classified as: "Susceptibility/Risk Biomarkers" designed to identify individuals predisposed to develop cancer; "Screening Biomarkers" used to depict early-stage cancer in asymptomatic individuals; "Diagnostic Biomarkers", used to identify and categorize a patient's cancer; "Prognostic Biomarkers" if the aim is to inform the likely patient clinical outcome, including recurrence or disease progression; "Predictive Biomarkers" if predict the likely response to specific therapeutic interventions; or "Disease monitoring Biomarkers" if indicate the development of disease relapse during follow-up [1, 3].

2. LIQUID BIOPSIES

Thus far, tissue biopsy sampling has been the gold-standard approach for patients' diagnosis and prognostication, however several drawbacks have been pointed out over the years to this approach [4, 5]. Firstly, and mainly, tissue samples might not fully represent the tumour heterogeneity, constituting a limitation for accurate outcome prediction and treatment efficacy [5, 6]. Moreover, early-stage tumour, residual disease and early recurrence detection might be difficult, since tissue biopsy sampling requires a highly invasive intervention with possible risk of complications, and, depending on the tumour anatomical location, can be extremely difficult to obtain [4, 7].

Recently, liquid biopsies obtained from easily assessable body fluids, including blood, urine or sputum, have raised up as a viable alternative to overcome these challenges. Liquid biopsies, mainly based on circulating cell-free DNA (ccfDNA), circulating tumour cells (CTCs), circulating cell-free RNA (ccfRNA) and exosomes [8] are a fast, reliable, cost-effective and minimally invasive approach [9] (Figure 1). Hence, owing to these features they may allow for a real time monitorization of the cancer evolution, while better representing the heterogenous genetic profile of all tumour sub clones [8, 10].

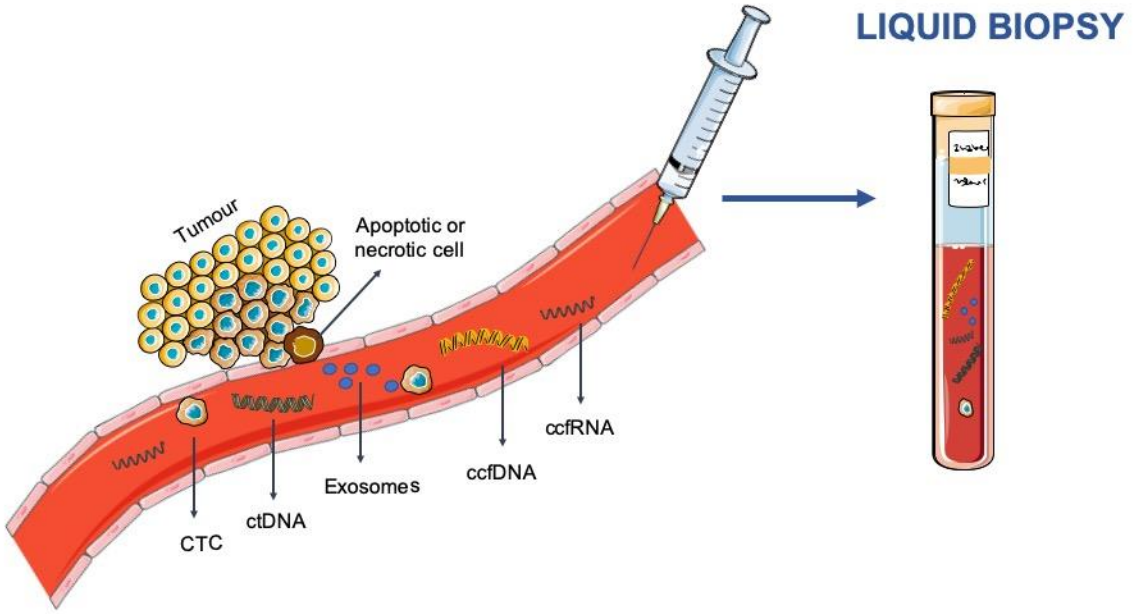

Figure 1. Blood-based liquid biopsy. Circulating tumour cells (CTC), circulating cell-free DNA (ccfDNA) [including circulating tumour DNA (ctDNA)], circulating cell-free RNA (ccfRNA) and exosomes are released from tumour cells to the bloodstream. Hence, blood can be collected and analysed in the context of a liquid biopsy. Constâncio, V. *unpublished.*

2.1. Circulating Cell-free DNA

CcfDNA was firstly described in 1948 when extracellular nucleic acids were found in human blood from healthy individuals by Mandel and Métais [11]. Later on, it was found that, in cancer patients, circulating tumour DNA (ctDNA) fragments between 150 and 1000 base pairs could also be detected due to their release into the bloodstream, either by cell death (apoptosis or necrosis) or active secretion by the release of extracellular vesicles, such as exosomes [10, 12]. Depending on several factors, including, tumour burden, metastatic sites or cellular turnover, ctDNA might account for 0.01 to 90% of the total ccfDNA in the blood of cancer patients [12]. Owing to the fact that ctDNA might represent tumour-specific genetic and epigenetic alterations of all tumour's sub clones present, ccfDNA is an ideal candidate for blood-based liquid biopsies by offering the possibility to test for the presence of cancer and the discrimination of lethally aggressive cancer [13]. Moreover, it gathers also the advantage of representing tumour real-time dynamic changes due to its short half-life (from 15 minutes to few hours), since it is rapidly cleared by the liver and kidney [13].

3. EPIGENETICS

Although the study of tumour mutations were the focus of biomarker research for a long time, their wide diversity has been a challenge for the development of effective diagnostic biomarkers since very large proportions of the genome would need to be examined in order to provide adequate sensitivity [14]. Contrarily, epigenetic alterations seem to be more stable and homogenous in cancer, representing a good alternative for biomarker development [15]. The definition of epigenetics, firstly coined in 1941 by Conrad Waddington, evolved over time and, currently, it is described as the study of gene expression modifications, carried during cell division, that do not change the primary DNA sequence [16, 17]. These alterations are, indeed, an alternative to genetic changes, by mimicking their effect. The main studied epigenetic mechanisms are DNA methylation, histone post-translational modifications, histone variants and chromatin remodelling complexes (Figure 2) [16]. Although these mechanisms are crucial for normal cell development and regulation of specific gene expression patterns, epigenetic processes dysregulation often lead to inappropriate activation or inhibition of several signalling pathways, which can trigger the development of several pathologies, including cancer [18].

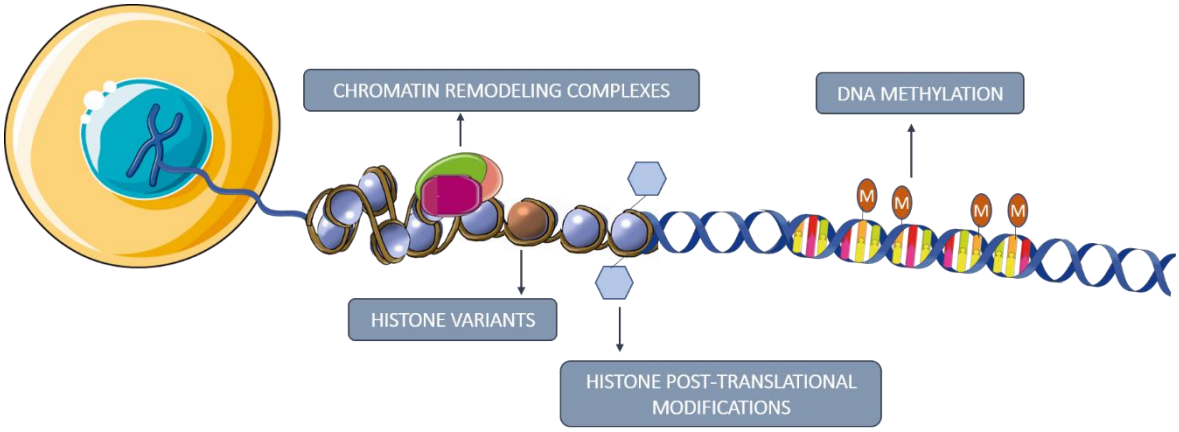

Figure 2. Major studied epigenetic mechanisms involved in gene expression regulation. DNA methylation consists in the addition of a methyl group to a cytosine present in a cytosine-phosphate-guanine (CpG). Histone post-translational modifications refer to the addition of biochemical modifications on histone tails, such as methylation, acetylation, phosphorylation, ubiquitylation and SUMOylation, that regulate gene expression. Histone variants differ a few amino acids from canonical histones and regulate chromatin remodelling and histone post-translational modifications. Chromatin remodelling complexes regulate the nucleosome structure by removing, relocate and shifting histones. Constâncio, V. *unpublished.*

3.1. DNA Methylation

DNA methylation, the most widely studied epigenetic modification in humans, was also the first to be identified in cancer [18, 19]. This epigenetic mechanism consists in a covalent addition of a methyl group, donated by S-adenosylmethionine (SAM), to the 5-position carbon of a cytosine ring to form 5-methylcytosine (5mC) [20, 21]. This modification is catalysed by DNA methyltransferase enzymes (DNMTs), namely, DNMT3a and DNMT3b that catalyse *de novo* DNA methylation during embryonic development, stablishing tissue-specific DNA methylation, and DNMT1 that is often associated with maintenance of methylation patterns during replication [21]. Typically, this process occurs on cytosine residues present at CpG dinucleotides (cytosine followed by a guanine) commonly found in large clusters named CpG islands, which are predominantly located at the 5' end of genes, occupying approximately 60% of human gene promoter regions [18, 21, 22]. Although gene promoter's hypermethylation is associated with transcription repression of the nearby gene (Figure 3), depending on DNA methylation genomic location, it can display different functions [20, 23]. For instance, gene body methylation is correlated with its transcriptional activation [23]. Epigenetic gene silencing by DNA promoter methylation can happen either directly, by blocking transcription factors binding to target sites in or near the promoter, or indirectly, by the binding of methyl-CpG-binding proteins (MBP), which can recruit other enzymes like DNMTs and histone deacetylases (HDAC), leading to chromatin conformation changes that further repress gene transcription [18, 20].

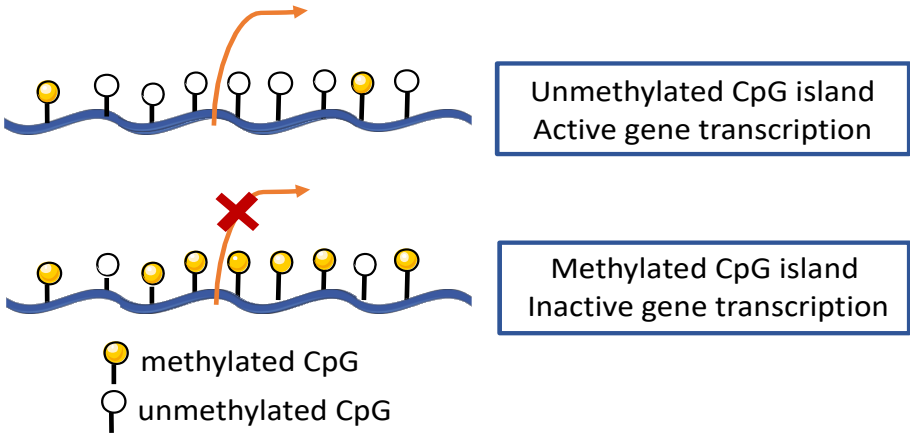

Figure 3. DNA methylation within a gene promoter region. Unmethylated CpG island enable gene transcription. When CpG island is methylated, gene transcription is repressed. Constâncio, V. *unpublished*.

Although, in normal cells, CpG island promoters are usually found unmethylated, physiological hypermethylation associated with promoters is observed on the silenced copy of the X chromosome in females, on imprinted genes, and in a tissue-specific manner [20]. Moreover, CpG dinucleotides within repetitive genomic sequences throughout the genome, retrotransposons and parasite sequences are also highly methylated to prevent their transcription, and to maintain genomic stability [16].

As aforementioned, DNA methylation is crucial for multiple cellular processes, thus it is understandable that its deregulation has been linked to cancer. Indeed, normal and cancer cells display different methylomes. Usually, a global hypomethylation pattern, which contributes to genomic instability and activation of silenced oncogenes, is observed in cancer [21, 24]. Alongside, tumour suppressor genes (TGS) frequently undergo inactivation due to focal promoter's hypermethylation [21, 24]. Currently, the latter process is considered a major contributor of neoplastic transformation [25].

Interestingly, aberrant DNA methylation is thought to occur at very early stages of cancer development and specific genes seem to be methylated at different tumour stages [21]. Moreover, since these alterations can be assessed in several body fluid samples [21], it is widely accepted that DNA methylation-based liquid biopsies are a promising approach, not only for premalignant/early cancer detection but also for prognostic assessment. Furthermore, since some genes seem to acquire tissue-specific DNA methylation, it may be possible to discriminate between different cancer types in the context of metastatic tumours [21] or in liquid biopsies.

4. CELL-FREE DNA METHYLATION BASED BIOMARKERS

According to GLOBOCAN 2018, Lung Cancer (LC), Prostate Cancer (PCa), and Colorectal Cancer (CRC) were estimated to account for 39% of all cancers diagnosed in males worldwide (Figure 4A), or 46% if only European countries were considered [26], representing major public health issues. Therefore, for the purposes of this work, a detailed literature review was conducted to assess the current state of art of the ccfDNA methylation blood-based biomarkers for cancer screening, diagnosis, prognostication, prediction and monitorization of the three most diagnosed cancers in males worldwide. On a PubMed database search, the keywords "Lung Cancer / Prostate Cancer / Colorectal Cancer", "DNA methylation" and "Serum / Plasma" were used.

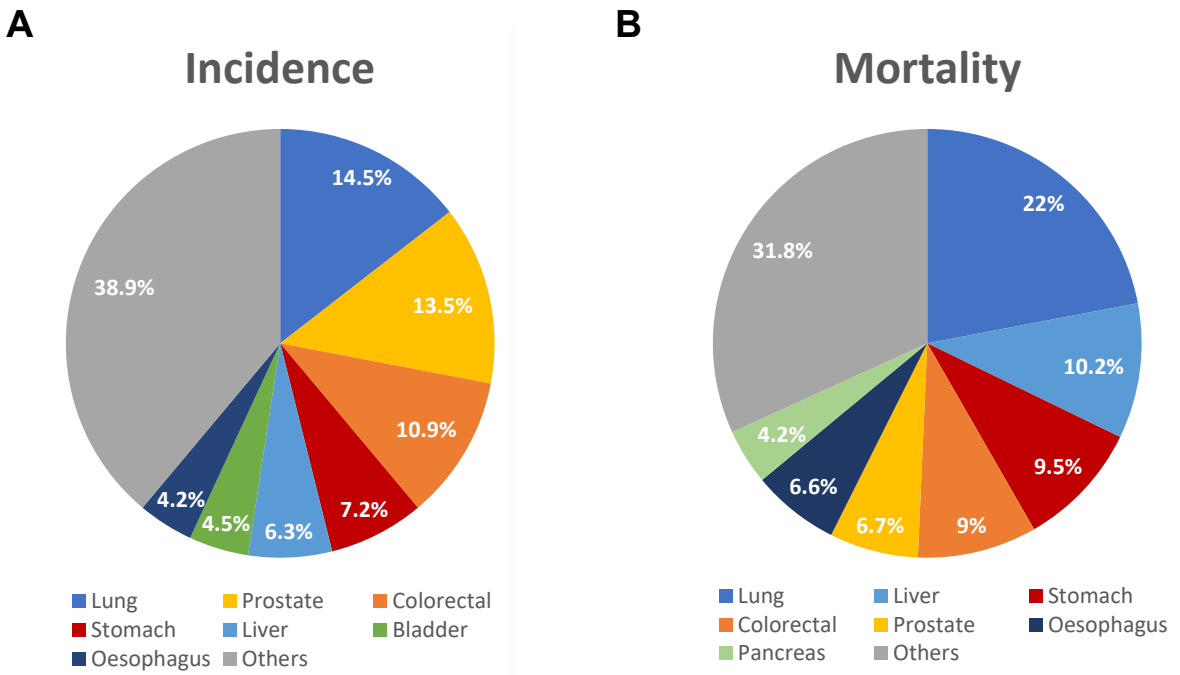

Figure 4. Estimated percentage of cancer-related incidence (A) and mortality (B) in males, worldwide, in 2018. Adapted from [26].

4.1. Lung Cancer

LC is the most incident and lethal cancer worldwide (Figure 4) [26], having a 5-year survival rate of about 15% [27]. The most well-established risk factor for this cancer is tobacco, being estimated that roughly 85% of all LC cases are attributed to cigarette smoking history [28].

LC is a heterogeneous disease, being classified into two major histological subtypes based on the prognostic and therapeutic implications: the small cell lung carcinoma (SCLC) and the non-small cell lung carcinoma (NSCLC) [29, 30].

NSCLC accounts for 85-90% of all cases, and is further classified into adenocarcinoma (LUAD), squamous cell carcinoma (LUSC) and large-cell lung carcinoma (LCLC) [30]. LUAD, the predominant LC subtype, accounts for nearly 40% of all LC, and is an epithelial tumour with glandular differentiation, being mostly located at the peripheral areas of the lung [30]. Although all LC subtypes are strongly associated with tobacco, LUAD is the most common subtype in never-smokers [30]. LUSC accounts for 20% of all LC cases and is usually present in a central location [30]. Accounting for only 3% of all diagnosed LC, LCLC is defined as an undifferentiated NSCLC [30].

SCLC revels neuroendocrine differentiation and arises in a central location [31]. This LC subtype accounts for 10-15% of all cases and is the most associated with smoking (with

95% of all patients having a heavy tobacco exposure history) and the most aggressive LC subtype [32], being characterized by rapid doubling time, early development of widespread metastases and worse survivals [33]. In fact, 60-65% of SCLC patients display metastatic disease at diagnosis, whereas the remaining often present locally advanced disease, which is also not amenable to surgical removal [31, 33].

4.1.1. Screening and Diagnosis

Despite advancements in new treatment options over the years, the high mortality rate observed in LC patients is mainly related with the fact that more than 75% of LC patients are diagnosed with advanced stage disease [34]. Hence, effective screening options aiming to shift LC diagnosis from advanced to curative early stages are crucial to change the fate of this disease [35]. Currently, low-dose computed tomography (LD-CT) is considered the best LC screening method available [36]. Nonetheless, despite The National Lung Screening Trial showed a 20% decrease in LC-related mortality rate among high-risk smokers with LD-CT screening comparing to chest X-ray, which was corroborated by the largest European trial (Dutch-Belgian Lung Cancer Screening Trial), from the 24% positive test results in this trial, 96.4% were classified as false-positive results [37, 38]. Hence, due to the risks related with LD-CT screening, namely, overdiagnosis, radiation exposure, and false positive results leading to unnecessary anxiety and costs [35], the development of specific and accurate screening tools is urgently needed to improve LC survival.

LC diagnosis procedures combine imaging exams and evaluation of histological or cytological specimens, collected either by bronchoscopy, sputum cytology or fine-needle aspiration [29, 30].

In 2002, Usadel *et al.* and Bearzatto *et al.* described for the first time, APC_{me} (Appendix I: Supplementary Table 1) and $p16^{INK4a}{}_{me}$, respectively, in ccfDNA as putative minimally-invasive biomarkers for LC detection [39] [40]. Thenceforth, numerous other methylated gene promoters detected in ccfDNA have been purposed for LC detection either individually or in panel (Table 1). $RASSF1A_{me}$ and $p16^{INK4a}{}_{me}$ represent the two most reported genes in blood-based liquid biopsies, displaying, between 22-66% sensitivity and 57-100% specificity for LC detection, individually [40-42].

After the commercialization in Europe of a test based on $SHOX2_{me}$ assessment in bronchial aspirates, the methylation of this gene was also evaluated as a LC detection biomarker in plasma. Indeed, plasma $SHOX2_{me}$ distinguished LC from control samples with 60% sensitivity and 90% specificity, even though higher sensitivity was found in stages II (72%), III (55%) and IV (83%) compared with stage I patients (27%) [43]. In line with these

results, another study reported that *SHOX2*$_{me}$ discriminated LC in subjects undergoing bronchoscopy with 81% sensitivity and 79% specificity [44]. Later on, Weiss *et al.* reported that *SHOX2*$_{me}$ and *PTGER4*$_{me}$ panel distinguished LC patients with 67% sensitivity for a fixed specificity of 90%, and with 73% specificity for a fixed sensitivity of 90% [45]. Remarkably, in the end of 2017, the "Epi proLung®" assay, developed by Epigenomics AG, based on these two genes received the *Conformité Européenne* (CE) mark for *In Vitro* Diagnostic (IVD) test. According to their validation study comprising 360 patients from the US and Europe, of which 152 were diagnosed with LC, depending on the Epi proLung test score threshold chosen, 85% sensitivity was achieved for 50% specificity, whereas sensitivity decreased to 59% if 95% specificity was considered [46, 47].

Although the majority of these studies have been focused on detection of NSCLC (the most diagnosed LC subtype), different detection frequencies of genes' methylation have been reported among the different subtypes. Indeed, *SHOX2*$_{me}$ detected with higher sensitivity SCLC (80%) and LUSC (63%) than LUAD (39%) [43]. Similarly, *DCLK1*$_{me}$ was more frequent in SCLC than NSCLC [48], and our research team recently reported that higher *APC*$_{me}$ and *RARβ2*$_{me}$ levels were observed in SCLC compared to LUAD in females [42]. Conversely, *SEPT9*$_{me}$ was more frequent in NSCLC (53%) than in SCLC (26%) [49]. A serum-based gene panel (*MARCH11*$_{me}$, *HOXA9*$_{me}$, *CDO1*$_{me}$, *UNCX*$_{me}$, *PTGDR*$_{me}$ and *AJAP1*$_{me}$) detected stage I LUAD and LUSC with 71% specificity and 72% and 60% sensitivity, respectively [50].

Table 1. Circulating cell-free DNA methylation-based biomarkers for lung cancer detection.

Lung Cancer						
Genes	**Number of cases / controls**	**Sensitivity (%)**	**Specificity (%)**	**Sources**	**Methods**	**References**
APC$_{me}$	89 LC / 50 AC	47	100	Serum/Plasma	qMSP	[39]
p16$^{INK4a}{}_{me}$	35 NSCLC / 15 AC	34	100	Plasma	F-MSP	[40]
MGMT$_{me}$/p16$^{INK4a}{}_{me}$/RASSF1A$_{me}$/ DAPK$_{me}$/RARβ_{me}	91 LC / 109 BPD	50	85	Serum	MSP	[51]
p16$^{INK4a}{}_{me}$/CDH13$_{me}$	61 NSCLC / 15 BPD	39	100	Serum	MSP	[52]
RASSF1A$_{me}$	80 LC / 50 AC[a]	34	100	Serum	MSP	[53]
CDH13$_{me}$/p16$^{INK4a}{}_{me}$/FHIT$_{me}$/ RARβ_{me}/RASSF1A$_{me}$/ZMYND10$_{me}$	63 NSCLC / 36 BPD	73	83	Plasma	Two-step MSP	[54]
KLK10$_{me}$	78 NSCLC / 50 AC[a]	38	96	Plasma	MSP	[55]
SFRP1$_{me}$	78 NSCLC / 50 AC[a]	28	96	Plasma	MSP	[56]
DLEC1$_{me}$	78 NSCLC / 50 AC[a]	36	96	Plasma	MSP	[57]
Kif1a$_{me}$/DCC$_{me}$/RARβ2$_{me}$/NISCH$_{me}$	70 LC / 80 BPD	73	71	Plasma	qMSP	[58]
APC$_{me}$/RASSF1A$_{me}$/CDH13$_{me}$/ KLK10$_{me}$/DLEC1$_{me}$	110 NSCLC[b] / 50 AC[a]	84	74	Plasma	MSP	[59]
APC$_{me}$/CDH1$_{me}$/MGMT$_{me}$/DCC$_{me}$ RASSF1A$_{me}$/AIM1$_{me}$	76 LC / 30 AC	84	57	Serum	qMSP	[60]
SHOX2$_{me}$	188 LC / 155 AC[a,c]	60	90	Plasma	qMSP	[43]
TMEFF2$_{me}$	316 NSCLC / 50 AC	9	100	Serum	Two-step MSP	[61]
RARβ2$_{me}$	60 NSCLC / 32 AC	72	62	Plasma	qMSP	[41]
RASSF1A$_{me}$		66	57			
SEPT9$_{me}$	70 LC / 100 AC	44	92	Plasma	qMSP	[49]

p14ARF$_{me}$	107 NSCLC / 20 BPD	25	95	Plasma	Two-step MSP	[62]
DCLK1$_{me}$	65 LC / 95 AC	49	92	Plasma	qMSP	[48]
SOX17$_{me}$	48 Operable NSCLC / 49 AC	56	98	Plasma	qMSP	[63]
	74 Advanced NSCLC / 49 AC	36				
SHOX2$_{me}$	38 LC / 31 BPD	81	79	Plasma	qMSP	[44]
SHOX2$_{me}$/PTGER4$_{me}$	50 LC / 122 AC[a]	67	90	Plasma	Multiplex qMSP	[45]
		90	73			
CDO1$_{me}$/TAC1$_{me}$/SOX17$_{me}$	150 NSCLC[b] / 60 AC	93	62	Plasma	qMSP	[64]
MARCH11$_{me}$/HOXA9$_{me}$/CDO1$_{me}$/ UNCX$_{me}$/PTGDR$_{me}$/AJAP1$_{me}$	43 LUAD[d] / 42 AC	72	71	Plasma	qMSP	[50]
	40 LUSC[d] / 42 AC	60				
NID2$_{me}$	46 NSCLC / 30 BPD	46	80	Plasma	qMSP	[65]
APC$_{me}$	73 LC[e] / 103 AC[e]	36	94	Plasma	Multiplex qMSP	[42]
FOXA1$_{me}$		72	74			
RARβ2$_{me}$		25	95			
RASSF1A$_{me}$		22	98			
SOX17$_{me}$		38	95			
CDKN2A$_{me}$/DLEC1$_{me}$/CDH1$_{me}$/ DAPK$_{me}$/RUNX3$_{me}$	42 NSCLC / 10 AC	95	100	Plasma	Two-step MSP	[66]
RASSF1A$_{me}$/CDKN2A$_{me}$/DLEC1$_{me}$/ CALCA$_{me}$/CDH13$_{me}$/PITX2$_{me}$/ HOXA1$_{me}$/WT1$_{me}$	39 NSCLC[d] / 11 BPD	72	91	Plasma	qMSP	[66]

[a]Included Benign pulmonary Diseases; [b]Only stage I/II; [c]Included other cancer types; [d]Only included stage I; [e]Only included females; Abbreviations: AC – Asymptomatic Controls; BPD – Benign Pulmonary Diseases; F-MSP – Fluorescent methylation-specific PCR; LC – Lung Cancer; LUAD – Lung Adenocarcinoma; LUSC – Lung Squamous Cell Carcinoma; MSP – Methylation-specific PCR; NSCLC – Non-Small Cell Lung Cancer; qMSP – Quantitative methylation-specific PCR

4.1.2. Prognosis, Prediction and Monitorization

Besides LC histological subtype, TNM prognostic stage groups of the American Joint Committee on Cancer (AJCC), based on tumour size and local invasion (T), and presence of nodal (N) and distant (M) metastasis (Appendix II: Supplementary Tables 2-3), remains the most important prognostic feature to predict recurrence and survival, followed by tumour histological grade, gender and age [33, 67]. Recently, molecular subtypes have also emerged in order to provide more personalized genetic information that can improve prognostic estimates and prediction to treatment response [33]. LC treatment options include surgery for early diagnosed cancers, chemotherapy and/or radiotherapy, specific tyrosine kinase inhibitors (TKI) in molecularly-defined NSCLC [presence of epidermal growth factor receptor (*EGFR*) mutation, and ALK receptor tyrosine kinase (*ALK*) or ROS proto-oncogene 1, receptor tyrosine kinase (*ROS1*) rearrangements], as well as, immunotherapy with programmed cell death protein (PD1) and programmed death-ligand 1 (PD-L1) antibodies [29, 68].

Thus far, only few small-scaled studies reported the prognostic, predictive and follow-up potential of ccfDNA methylation for LC, being most of them performed in patients with advanced disease.

DCLK1$_{me}$ and *SOX17*$_{me}$ levels were associated with reduced overall survival (OS) in advanced LC and NSCLC, respectively [48, 63]. Similarly, higher *SHP1P2*$_{me}$ levels observed in advanced NSCLC associated with reduced progression-free survival (PFS) and OS [69], whereas *BRMS1*$_{me}$ with both reduced disease-free survival (DFS) and OS in operable NSCLC, and reduced PFS and OS in advanced NSCLC [70]. Moreover, *RASSF1A*$_{me}$ associated with presence of regional node metastasis [42] and advanced clinical stage [53], whereas *SOX17*$_{me}$ associated with distant metastasis [42].

Interestingly, after neoadjuvant chemotherapy and surgery with intraoperative radiation therapy, NSCLC patients showed decreased *RASSF1A*$_{me}$ and *RARβ2*$_{me}$ levels, similar to levels in healthy subjects. Moreover, methylation levels' increase of at least one of these genes, up to the levels detected before treatment, was observed in all the 5 patients that showed evidence of disease progression [41]. Increased *APC*$_{me}$ and/or *RASSF1A*$_{me}$ levels within 24h after cisplatin-based chemotherapy also associated with increased OS [71].

Remarkably, advanced LC patients who clinically responded to chemo/radiotherapy demonstrated a decrease in plasmatic *SHOX2*$_{me}$ levels, observable at 7-10 days after therapy initiation [72]. Additionally, higher *SHOX2*$_{me}$ levels, both before and 7-10 days after

therapy beginning were indicative of shorter OS [72]. Contrarily, *14-3-3σ$_{me}$* levels in stage IV NSCLC patients before treatment with cisplatin-gemcitabine associated with longer survival [73]. Stage IV NSCLC patients with unmethylated *CHFR* showed longer OS when treated with EGFR-TKI compared to those treated with chemotherapy as second-line therapy [74].

4.2. Prostate Cancer

PCa was estimated to be the second most incident cancer in men in 2018, worldwide (Figure 4A), and the first in Europe [26]. Approximately 95% of all PCa are adenocarcinomas mainly found in the peripheral zone of the prostate [75]. Currently, only nonmodifiable risk factors are well-established for PCa development, namely, advanced age, black race and positive family history [76]. Nonetheless, other factors such as diet, obesity, physical inactivity, smoking and chronic inflammation have also been associated, although with inconsistent results [76].

4.2.1. Screening and Diagnosis

PCa is mostly asymptomatic, therefore, patients might be diagnosed with advanced disease, resulting in worse patient outcomes and limited treatment options [75]. Digital rectal examination (DRE) in combination with serum prostate-specific antigen (PSA) remain the gold standard PCa screening tools [77], however both methods present drawbacks. A DRE positive result is dependent on clinicians' expertise and the majority of cancers detected by this method are at an advanced stage [78], besides compliance is rather low. The widespread adoption of PSA screening since late 1980s has facilitated the shift to detection of PCa at early stages [78]. Nonetheless, despite being highly sensitive, since benign prostatic hyperplasia (BPH) and other benign conditions can also cause PSA elevation, the lack of cancer-specificity of this approach entails a high false-positive rate and overdiagnosis of non-life threatening PCa [77, 79]. Indeed, only less than one third of the patients undergoing transrectal ultrasound-guided (TRUS) biopsy (standard diagnostic approach) due to elevated PSA levels or abnormal DRE are diagnosed with cancer [80]. In parallel, a negative result does not completely rule out the existence of cancer, leading to a large number of unnecessary invasive tissue biopsies that might be repeated due to the uncertainty of diagnosis if elevated PSA levels persist [80]. Thus, the introduction of more specific alternatives is urgently sought.

Besides blood-based liquid biopsies, aberrant DNA methylation in urological cancers can also be detected in urine, which is a non-invasive, easily accessible source of

exfoliated cells and ccfDNA from diverse sites of the urinary system [81]. Hence, several studies have been addressed using this source [81]. Nonetheless, in PCa, higher sensitivities are achieved after manipulation of the prostate, either by massage and DRE, increasing the invasiveness of this procedure [81]. Therefore, blood-based liquid biopsies might represent the most minimally invasive procedure for PCa detection. A summary of the currently reported blood-based DNA methylation PCa detection biomarkers is depicted on Table 2.

GSTP1$_{me}$ is the most described epigenetic alteration in ccfDNA of PCa patients due to its remarkably high specificity for PCa [82-86]. Indeed, a meta-analysis of 10 studies published until 2010, evaluating *GSTP1*$_{me}$ PCa detection performance in plasma/serum, reported a pooled specificity of 90% [non-quantitative methylation specific PCR (non-qMSP)] and 96% (qMSP-based detection), although with a modest sensitivity of 40% (non-qMSP) and 36% (qMSP-based detection) [84].

Since epigenetic alterations are usually multiple and not necessarily overlapped, multigene panels are pivotal to increase the modest sensitivities of individual genes. Indeed, Ellinger *et al.* reported that using a gene panel comprising *GSTP1*$_{me}$, *PTGS2*$_{me}$, *RPRM*$_{me}$ and *TIG1*$_{me}$, PCa diagnostic coverage increased from 42% (*GSPT1*$_{me}$ alone) to 47% (panel), maintaining 93% specificity [82]. Furthermore, Sunami *et al.* reported that *GSTP1*, *RASSF1A* and *RAR*β*2* were hypermethylated in 13%, 24% and 12% of serum samples from PCa patients, respectively, whereas the three gene panel increased the detection rate to 29%, with 100% specificity [83]. More recently, other panels without comprising *GSTP1*$_{me}$ have also been tested. Indeed, *MCAM*$_{me}$, *ER*α_{me} and *ER*β_{me} panel demonstrated 75% sensitivity and 70% specificity for early PCa detection [87]. Likewise, *ZNF660*$_{me}$, *CCDC181*$_{me}$, *ST6GALNAC3*$_{me}$ and *HAPLN3*$_{me}$ in serum displayed 22%, 26%, 31% and 44% sensitivity, respectively, and 100% specificity for PCa. Remarkably, the best gene panel (*ST6GALNAC3*$_{me}$, *CCDC181*$_{me}$ and *HAPLN3*$_{me}$) increased sensitivity to 67%, while keeping 100% specificity [88].

Interestingly, given the modest sensitivities obtained with ccfDNA methylation even in panels, studies have also been addressed to understand if ccfDNA might have a better performance by complementing it with serum PSA levels. Indeed, in a Mexican cohort with biopsy-confirmed PCa, a panel comprising *GSTP1*$_{me}$ and *RASSF1A*$_{me}$ allowed cancer detection with 73% positive predictive value (PPV) and 59.6% negative predictive value (NPV), increasing to 81% and 66%, respectively, when serum PSA was also considered [89]. Similarly, serum *GADD45a*$_{me}$ increased its sensitivity from 38% to 94% when PSA and free circulating DNA levels were also considered, even though the 98% specificity decreased to 88% [90].

Table 2. Circulating cell-free DNA methylation-based biomarkers for prostate cancer detection.

Prostate Cancer						
Genes	**Number of cases / controls**	**Sensitivity (%)**	**Specificity (%)**	**Sources**	**Methods**	**References**
GSTP1$_{me}$/PTGS2$_{me}$/RPRM$_{me}$/TIG1$_{me}$	168 PCa / 42 BPH	47	93	Serum	qMSP	[82]
MDR1$_{me}$	192 PCa / 35 AC[a]	32	100	Serum	qMSP	[91]
GSTP1$_{me}$/RASSF1A$_{me}$/RARβ2$_{me}$	83 PCa / 40 AC	29	100	Serum	MSP	[83]
GSTP1$_{me}$	80 PCa / 51 AC[a]	26	80	Plasma	qMSP	[85]
RASSF2A$_{me}$		28				
HIST1H4K$_{me}$		17				
TFAP2E$_{me}$		12				
GSTP1$_{me}$	Meta-analysis	40	90	Plasma / Serum	Non-qMSP	[84]
		36	96		qMSP	
RARβ2$_{me}$	91 PCa / 94 BPH	93	89	Serum	qMSP	[92]
GSTP1$_{me}$	31 PCa / 44 BPH	93	89	Plasma	MSP	[86]
CDH13$_{me}$	98 PCa / 47 AC[b]	45	100	Serum	MSP	[93]
GADD45a$_{me}$	34 PCa / 48 BPH	38	98	Serum	Pyrosequencing	[90]
MCAM$_{me}$/ERα$_{me}$/ERβ$_{me}$	84 PCa / 30 AC	75	70	Serum	qMSP	[87]
CCDC181$_{me}$/ST6GALNAC3$_{me}$/HAPLN3$_{me}$	27 PCa / 10 BPH	67	100	Serum	ddMSP	[88]
ZNF660$_{me}$		22				

[a]Biopsy negative; [b]Included BPH; Abbreviations: AC – Asymptomatic Controls; BPH – Benign Prostatic Hyperplasia; ddMSP – Digital droplet methylation-specific PCR; MSP – Methylation-specific PCR; PCa – Prostate Cancer; qMSP – Quantitative methylation-specific PCR.

4.2.2. Prognosis, Prediction and Monitorization

PCa is a very heterogenous disease, ranging from small, low grade clinical indolent to large, lethally aggressive tumours [94]. Thus, the main goal after establishing the presence of the disease is to evaluate its extension and aggressiveness through staging, in order to assess prognosis and plan the treatment course. Currently, PCa prognostic stage groups are based on nomograms combining TNM classification, preoperative serum PSA levels, and histological International Society of Urological Pathology (ISUP) Grade Group (Appendix II: Supplementary Tables 4-6) [95, 96], which is based on the evaluation of the two most common differentiation patterns in a tumour [97]. PCa is mostly asymptomatic and organ-confined, amenable to curative intent treatment options, namely, radical prostatectomy and radiotherapy (external beam radiotherapy or brachytherapy) or conservative approaches (monitoring PCa progression while not undergoing definitive therapy) by means of active surveillance and watchful waiting [98]. Moreover, androgen-deprivation therapy (ADT) and chemotherapy are available for locally advanced or metastatic disease [98]. Nonetheless, the median duration of response to ADT is 18-24 months, after which most patients develop a more aggressive form of disease named castration-resistant prostate cancer (CRPC) [99]. Furthermore, it is estimated that 30-50% patients curatively treated may show a rising of serum PSA levels (biochemical recurrence) within 10 years after treatment, with disease progression in up to 40% of these [99, 100]. Thus, considering these uncertainties, there is an urgent need for development and implementation of more trustworthy PCa biomarkers to assist clinicians and patients in decision-making.

Besides the study of its detection value, the potential of methylation-based biomarkers in ccfDNA to predict disease progression and therapy response have also been tackled. Namely, *GSTP1*$_{me}$ [91, 101], *MDR1*$_{me}$, *EDNRB*$_{me}$ and *RARβ2*$_{me}$ [91] were reported to be more frequent in CRPC patients than in early-stage PCa patients. *GSTP1*$_{me}$ levels also associated with Gleason score and presence of metastasis [101] and with reduced disease-specific survival (DSS) along with *APC*$_{me}$ [102] in CRPC patients. Furthermore, *GSTP1*$_{me}$ and *RASSF2A*$_{me}$ were more frequently detected in men with non-organ confined compared to organ-confined disease and both associated with increased Gleason score [85]. Interestingly, preoperative serum *GSTP1*$_{me}$ was also reported as an independent predictor of biochemical recurrence following radical prostatectomy [103]. Sunami *et al.* reported that *GSTP1*$_{me}$, *RASSF1A*$_{me}$ and *RARβ2*$_{me}$ associated with Gleason score and serum PSA levels, whereas *GSTP1*$_{me}$ and *RARβ2*$_{me}$ also associated with advanced stages of disease [83]. Moreover, individually, serum *PCDH17*$_{me}$, *PCDC10*$_{me}$ and *PCDH8*$_{me}$ were

also associated with advanced clinical stage, higher preoperative serum PSA, as well as lymph node metastasis and shorter biochemical recurrence free survival [104-106]. Besides being also associated with Gleason score, advanced tumour stage and high PSA, *CDH13*$_{me}$ was further associated with shorter OS [93].

Remarkably, a recent phase III multicentre trial comprising 600 CRPC patients showed that detectable serum *GSTP1*$_{me}$ levels prior and after two cycles of chemotherapy were both independently associated with decreased OS [107]. In the same study, undetectable serum *GSTP1*$_{me}$ after two cycles of docetaxel further associated with longer time to PSA progression [107].

4.3. Colorectal Cancer

CRC is the third most incident cancer worldwide (Figure 4), being estimate to account for over 1 million new cases detected and almost half a million deaths in males in 2018 [26]. Several risk-factors, such as family history, inflammatory bowel diseases, and lifestyle (western-type diet, sedentarism, smoking and excessive alcohol consumption) are associated with the development of this disease [108, 109].

Thirty five percent of all CRC cases might be attributable to hereditary syndromes including, the autosomal dominant disorders: familial adenomatous polyposis (FAP), characterized by APC regulator of WNT signalling pathway (*APC)* mutation, and Lynch syndrome, the most common hereditary non-polyposis colon cancer (HNPCC) characterized by germline mutations at the mismatch repair (MMR) genes, resulting in microsatellite instability (MSI) [109, 110]. Nevertheless, most CRC cases are sporadic and develop for over 10 years through the accumulation of multiple genetic and epigenetic alterations that lead to loss of genomic stability [109, 111]. Usually, small (<1 cm) polypoid alterations (adenomas) with low-grade dysplasia emerge from normal cells that transform into hyperproliferative cells of the colon epithelium, and develop into high-grade dysplasia and potentially in *in situ* cancers, that can eventually metastasize [111].

The most frequent pathway resulting in genomic instability is chromosomal instability (CIN) which is present in about 85% of sporadic CRC and arises through activating mutations of oncogenes and inactivating mutations in TSG [109, 111]. MSI pathway due to deficiency of DNA MMR genes is characterized by the accumulation of mutations across the whole genome, and occur in 15-20% of sporadic CRC [109, 111]. Alternatively, the CpG island methylator phenotype (CIMP) is characterized by a global hypermethylation of tumour suppressor genes and can be found in 15% of all CRC cases [111].

4.3.1. Screening and Diagnosis

Due to its quite slow progression time and the opportunity to easily remove precancerous and early stage cancerous lesions, if caught in time, CRC entail minimal risk to the patient with about 90% long-term survival [112, 113]. Therefore, CRC screening programs are of great interest. Currently, analysis of trace blood in stool by faecal-occult blood test (FOBT) / faecal immunochemical test (FIT), and internal imaging of the colon by colonoscopy are the principal screening options available for CRC early detection [112, 114]. Additionally, biopsy samples during colonoscopy are mandatory to histological diagnosis [109]. Nevertheless, faecal screening tests have limited sensitivity to detect precancerous lesions, whereas although colonoscopy is very precise and can be used to remove the lesion during the examination, it is a costly and highly invasive procedure with low patients' compliance [115]. Hence, despite being recognized that screening reduces CRC incidence and mortality, the availability and compliance to the current screening tests remain suboptimal [111].

The increasing knowledge of the influence of epigenetic alterations in malignant transformation in the gut, gave rise to an opportunity for development of sensitive and specific minimally invasive epigenetic-based biomarkers for CRC. Hence, plentiful studies have investigated the detection value of these biomarkers (Table 3). *SEPT9$_{me}$* is the main described methylated gene in blood from CRC patients. Remarkably, this marker was the first blood-based IVD assay for detection of occult cancer based on an epigenetic alteration approved by the US Food and Drug Administration (FDA), in 2016, under the name of "Epi ProColon® 2.0" (Epigenomics AG) [46]. Additionally, this CE-IVD marked test is also commercially available in Europe and China [116]. A meta-analysis published in 2017 reported that *SEPT9$_{me}$* sensitivity for CRC detection varies between 73-78% depending on the algorithm used to consider a positive result, while specificity varies between 84-96% [117]. Nevertheless, when the biomarker performance of this gene's methylation was assessed in a multicentre screening setting (PRESEPT clinical trial) with asymptomatic individuals older than 50 years old, the results from 53 CRC cases and 1457 subjects without CRC yielded 48% sensitivity and 92% specificity [118].

Given the importance to detect pre-malignant conditions, several studies have not only studied the CRC detection performance, but also the capability to detect adenomas. Disappointingly, *SEPT9$_{me}$* performance to detect advanced adenomas ranged between 8-31% [119-121], being reported to be 11% in the previously mentioned screening setting study [118]. Thus, the usefulness of this gene for population-based screening is questionable.

As expected, gene panels have improved the performance to detect both adenomas and CRC. Remarkably, APC_{me}, $MGMT_{me}$, $RASSF2A_{me}$ and $WIF1_{me}$ panel discriminated adenomas and early stage CRC with 75% and 87% sensitivity, respectively, and 92% specificity [122], whereas $SFRP1_{me}$, $SFRP2_{me}$, $SDC2_{me}$ and $PRIMA1_{me}$ panel detected adenomas with 89% sensitivity and 87% specificity, and CRC with 92% sensitivity and 97% specificity [123]. Nevertheless, the performance of these panels in a large screening setting remains to be elucidated. Interestingly, $BCAT1_{me}$ and $IKZF1_{me}$ panel performance has been evaluated in large multicentre studies, displaying 62-66% sensitivity and 92-95% specificity for CRC detection, although with a limited 6-9% sensitivity for adenomas detection [124, 125].

Table 3. Circulating cell-free DNA methylation-based biomarkers for colorectal cancer detection.

Colorectal Cancer						
Genes	**Number of cases / controls**	**Sensitivity (%)**	**Specificity (%)**	**Sources**	**Methods**	**References**
p16$^{INK4a}_{me}$	52 CRC / 44 AC[a]	27	100	Serum	MSP	[126]
APC$_{me}$/hMLH1$_{me}$/HLTF$_{me}$	49 CRC / 41 AC	57	90	Serum	qMSP	[127]
ALX4$_{me}$	30 CRC / 30 AC	83	70	Serum	qMSP	[128]
HPP1$_{me}$	38 CRC / 20 AC	49	100	Serum	qMSP	[129]
HLTF$_{me}$		67				
hMLH1$_{me}$		47				
SEPT9$_{me}$	133 CRC / 179 AC	69	86	Plasma	qMSP	[130]
TMEFF2$_{me}$		65	69			
NGFR$_{me}$		51	84			
RASSF1A$_{me}$	45 CRC / 30 AC	29	100	Serum	MSP	[131]
VIM$_{me}$	81 CRC / 110 AC	59	93	Plasma	Methyl BEAMing	[132]
APC$_{me}$/MGMT$_{me}$/RASSF2A$_{me}$/	243 CRC[b] / 276 AC	87	92	Plasma	MSP	[122]
WIF1$_{me}$	64 Adenoma / 276 AC	75				
ALX4$_{me}$/SEPT9$_{me}$/TMEFF2$_{me}$	182 CRC / 170 AC	81	90	Plasma	Multiplex qMSP	[133]
NEUROG1$_{me}$	97 CRC[b] / 45 AC	61	91	Serum	qMSP	[134]
TFPI2$_{me}$	215 CRC / 20 AC	18	100	Serum	qMSP	[135]
DLC1$_{me}$	85 CRC / 45 AC	42	91	Serum	MSP	[136]
CYCD2$_{me}$/HIC1$_{me}$/PAX5$_{me}$/ RASSF1A$_{me}$/RB1$_{me}$/SRBC$_{me}$	30 CRC[b] / 30 AC	84	68	Plasma	Microarray	[137]
HIC1$_{me}$/MDG1$_{me}$/RASSF1A$_{me}$	30 Adenoma / 30 AC	55	65			

SMAD4$_{me}$	60 CRC / 100 AC[a]	52	64	Plasma	MSP-SSCP	[138]
FHIT$_{me}$		50	84			
DAPK1$_{me}$		50	74			
APC$_{me}$		57	86			
CDH1$_{me}$		60	84			
SDC2$_{me}$	131 CRC / 125 AC	87	95	Serum	qMSP	[139]
TAC1$_{me}$/SEPT9$_{me}$	26 CRC[c] / 26 AC	73	92	Serum	qMSP	[140]
NPY$_{me}$/PENK$_{me}$/WIF1$_{me}$	32 CRC / 161 AC	87 59	80 95	Serum	Multiplex qMSP	[141]
CAHM$_{me}$	73 CRC / 74 AC	55	93	Plasma	qMSP	[142]
	73 Adenoma / 74 AC	4				
PPP1R3C$_{me}$/EFHD1$_{me}$	120 CRC / 96 AC	53 (2 genes) 90 (at least 1 gene)	96 (2 genes) 64 (at least 1 gene)	Plasma	MSP	[143]
SYNE1$_{me}$/FOXE1$_{me}$	66 CRC / 140 AC	58	91	Plasma	Multiplex qMSP	[144]
GATA5$_{me}$/SFRP2$_{me}$	57 CRC / 47 AC	43	91	Plasma	MSP	[145]
	30 Adenoma / 47 AC	27				
BCAT1$_{me}$/IKZF1$_{me}$	74 CRC / 144 AC	77	92	Plasma	qMSP	[146]
BCAT1$_{me}$/IKZF1$_{me}$	129 CRC / 450 AC	66	95	Plasma	qMSP	[124]
	338 Advanced Adenoma / 450 AC	6				
	346 Non-Advanced Adenoma / 450 AC	7				
BCAT1$_{me}$/IKZF1$_{me}$	66 CRC / 1315 AC[a]	62	92	Plasma	qMSP	[125]
	170 Advanced Adenoma	9	---			
	278 Non-Advanced Adenoma	9	---			

ALX4$_{me}$	25 CRC / 25 AC	68	88	Serum	MSP	[147]
FGF5$_{me}$	20 CRC / 40 AC	85	82	Plasma	qMSP	[148]
GRASP$_{me}$	44 CRC / 44 AC	55	93			
IRF4$_{me}$	22 CRC / 24 AC	59	96			
PDX1$_{me}$	20 CRC / 20 AC	45	70			
SDC2$_{me}$	44 CRC / 44 AC	59	84			
SEPT9$_{me}$	44 CRC / 44 AC	59	95			
SOX21$_{me}$	20 CRC / 20 AC	85	50			
SPG20$_{me}$	37 CRC / 37 AC	81	97	Plasma	qMSP	[149]
SEPT9$_{me}$	Meta-analysis	78 (1/3) 73 (2/3)	84 (1/3) 96 (2/3)	Plasma/ Serum	---	[117]
ALX4$_{me}$/BMP3$_{me}$/NPTX2$_{me}$/ RARβ_{me}/SDC2$_{me}$/SEPT9$_{me}$/VIM$_{me}$	193 CRC / 102 AC	91	73	Plasma	Two-step qMSP	[150]
SFRP1$_{me}$/SFRP2$_{me}$/SDC2$_{me}$/ PRIMA1$_{me}$	47 CRC / 37 AC	92	97	Plasma	qMSP	[123]
	37 Adenoma / 37 AC	89	87			
BMP3$_{me}$	45 CRC / 50 AC	40	94	Plasma	BS-HRM	[151]
TWIST1$_{me}$	18 CRC / 25 AC	44	92	Serum	Multiplex ddPCR	[152]
	70 Advanced Adenoma / 25 AC	30				
	25 Non-Advanced Adenoma / 25 AC	36				
SEPT9$_{me}$	98 CRC / 253 AC	61	98	Plasma	qMSP	[121]
	101 Adenoma / 253 AC	8				

APC$_{me}$		21	94			
FOXA1$_{me}$		50	88			
RARβ2$_{me}$		17	95			
RASSF1A$_{me}$	72 CRC[d] / 103 AC[d]	14	99	Plasma	Multiplex qMSP	[42]
SCGB3A1$_{me}$		26	90			
SEPT9$_{me}$		11	100			
SOX17$_{me}$		24	90			
SFRP2$_{me}$	62 CRC / 55 AC	69	87	Serum	qMSP	[153]

[a]Included patients with adenomatous polyps; [b]Only included stages I/II; [c]Only included stage I; [d]Only included females; Abbreviations: AC – Asymptomatic Controls; Adenoma – Adenomatous polyps; BS-HRM – Bissulfite specific high-resolution melting analysis; CRC – Colorectal Cancer; ddPCR – Digital droplet PCR; MSP – Methylation-specific PCR; MSP-SSCP – Methylation-specific PCR – single strand conformation polymorphism; qMSP – Quantitative methylation-specific PCR.

4.3.2. Prognosis, Prediction and Monitorization

TMN prognostic stage groups (Appendix II: Supplementary Tables 7-8) in addition to cancer location, patient's age and comorbidities are also the basis for CRC prognosis and patients' management [67, 109]. CRC gold-standard treatment is surgical resection, along with possible neoadjuvant chemoradiotherapy (for patients with rectal cancer), and adjuvant chemotherapy (for patients with stage III/IV and high-risk stage II colon cancer) [109]. Preoperative evaluation of serum carcinoembryonic antigen (CEA) is recommended as a prognostic biomarker and for postoperative follow-up of CRC patients, even though sensitivity for detecting recurrent CRC is limited [154, 155]. For unresectable metastatic CRC, current treatment options include chemotherapy associated with monoclonal antibodies against vascular endothelial growth factor (VEGF), and monoclonal antibodies that inhibit EGFR for patients without *RAS* or B-Raf proto-oncogene, serine/threonine kinase *(BRAF)* mutations [109, 156].

Regarding ccfDNA methylation-based biomarkers, higher $RUNX3_{me}$ levels associated with lymphatic invasion, advanced pathological stage and development of recurrence [157]. $TFPI2_{me}$ and $SFRP2_{me}$ levels associated with poorly differentiated carcinoma, deep invasion, lymph node and distant metastasis [135, 158], being the former also associated with tumour size [135], and the latter with shorter OS [158]. SST_{me} was independently associated with higher recurrence risk and shorter DSS in patients who underwent curative surgical resection [159].

A small-scale study suggested that $p16^{INK4a}{}_{me}$ could reflect the recurrence status during follow-up after surgery [160]. Interestingly, in a prospective cohort study including 150 stage I-III CRC patients from whom serum samples were obtain 1 week before, and 6 months and 1 year after surgery, high levels of $TAC1_{me}$ after 6 months and $SEPT9_{me}$ after 1 year were independent predictors for tumour recurrence and shorter DSS [161]. Additionally, the increment of $TAC1_{me}$ and $SEPT9_{me}$ levels independently predicted disease recurrence, while $NELL1_{me}$ at both 6 months and 1 year associated with DSS [161]. In the same line, $SEPT9_{me}$ was suggested as a follow-up marker for recurrence and metastasis detection, being also associated with tumour size, histological grade and histological type [121]. $GATA5_{me}$, $SFRP2_{me}$, $ITGA4_{me}$, $SHOX2_{me}$ and $SEPT9_{me}$ levels were also associated with tumours' histological grade, TNM stage and lymph node metastasis [145, 162], whereas $GATA5_{me}$ also associated with large tumour size [145]. Furthermore, APC_{me}, $SEPT9_{me}$, $SHOX2_{me}$ and $SOX17_{me}$ levels were also shown to be significantly higher in patients with metastatic disease [42].

Interestingly, various studies have demonstrated the prognostic value of *HLTF$_{me}$* and *HPP1$_{me}$* levels in CRC patients. Indeed, serum *HLTF$_{me}$*, *HPP1$_{me}$* and *hMLH1$_{me}$* were significantly correlated with tumour size, and the two former genes (*HLTF$_{me}$* and *HPP1$_{me}$*) further associated with metastatic disease and tumour stage, as well as with worst outcome [129]. Later, Philipp *et al.*, in a study involving 311 serum samples of CRC patients, reported that *HLTF$_{me}$* and *HPP1$_{me}$* associated with tumour size, stage, grade and metastatic disease, and *HPP1$_{me}$* also associated with nodal status. Moreover, in stage IV patients, high levels of both these genes associated with reduced OS [163]. In patients curatively resected for CRC, pretherapeutic serum *HLTF$_{me}$* levels were associated with increased relative risk of disease recurrence [164]. In a clinical trial including 467 metastatic CRC patients treated with a combination therapy containing a fluoropyrimidine, oxaliplatin and bevacizumab, patients with detectable plasmatic *HPP1$_{me}$* before the start of treatment showed a significantly poorer OS [165]. Moreover, patients that reduced to undetectable levels 2-3 weeks after treatment showed a better OS compared to patients that maintained detected plasmatic *HPP1$_{me}$* levels [165], suggesting the usefulness of *HPP1$_{me}$* as a prognostic and early response biomarker.

Recently, Barault *et al.* also suggested that plasmatic methylation changes of *EYA4$_{me}$, GRIA4$_{me}$, ITGA4$_{me}$, MAP3K14-AS1$_{me}$* and *MSC$_{me}$* panel over time correlate with tumour response in metastatic CRC patients treated with chemo- or targeted therapy [166].

Since 2017, COLVERA™, a Laboratory Developed Test (LDT), based on a two-gene panel (*IKZF1$_{me}$* and *BCAT1$_{me}$*) is being commercialized in the USA, for post-surgery residual disease detection and CRC patients' surveillance [167]. The detection of these two genes in blood associated with stage [124] and showed a rapid reversion after surgical resection (35 of 47 positive patients at diagnosis, became negative after surgery) [168]. Moreover, in patients undergoing surveillance after primary CRC treatment, this panel was positive in 68% plasma samples of the 28 patients with clinically detectable recurrent CRC, whereas CEA was positive in only 32%, although specificity was similar with both tests, 87% and 94%, respectively [169]. Hence, this panel has demonstrated to double the sensitivity of the current gold-standard marker for CRC monitorization. Recently, positivity of this panel after surgery also independently associated with increased recurrence risk [170].

5. CELL-FREE DNA METHYLATION AS A CANDIDATE "PANCANCER" SCREENING BIOMARKER

The screening of these three cancer types faces different challenges. If on the one hand, LC and CRC demand an early cancer detection that is still not fully accomplished with the current screening tools available. On the other hand, PCa diagnosis should not only be focused on early disease detection, but importantly be restrictive to clinically significant disease detection, since a great proportion of PCa currently diagnosed would not cause any harm to the patients during the course of the disease. Hence, new screening biomarkers have been exhaustively searched (Table 1-3). One may wonder if a minimally invasive "PanCancer" detection approach based on liquid biopsies with good sensitivity and specificity for simultaneous cancer detection would increase screening effectiveness. In fact, a study performed in the context of CRC screening revealed that only 37% of the 172 subjects were compliant to screening colonoscopy [171]. Interestingly, 97% of the subjects who refused this modality accepted a non-invasive alternative, of which 83% selected *SEPT9*$_{me}$ blood-test and only 15% the stool test, being the primary reason for this choice the convenience of the procedure [171]. Regardless of being small scaled, this study clearly demonstrates that screening compliance can dramatically increase if a convenient minimally invasive option, such as a liquid biopsy, is offered. Nevertheless, the establishment of a "PanCancer" panel for a simultaneous detection of several cancer types is a challenge given their heterogeneity.

Regarding ccfDNA methylation based biomarkers, although only *RARβ2*$_{me}$ and *RASSF1A*$_{me}$ levels were reported in all three cancer types (LC, PCa and CRC), several other genes have already been reported in at least two of the three different cancer types (Figure 5).

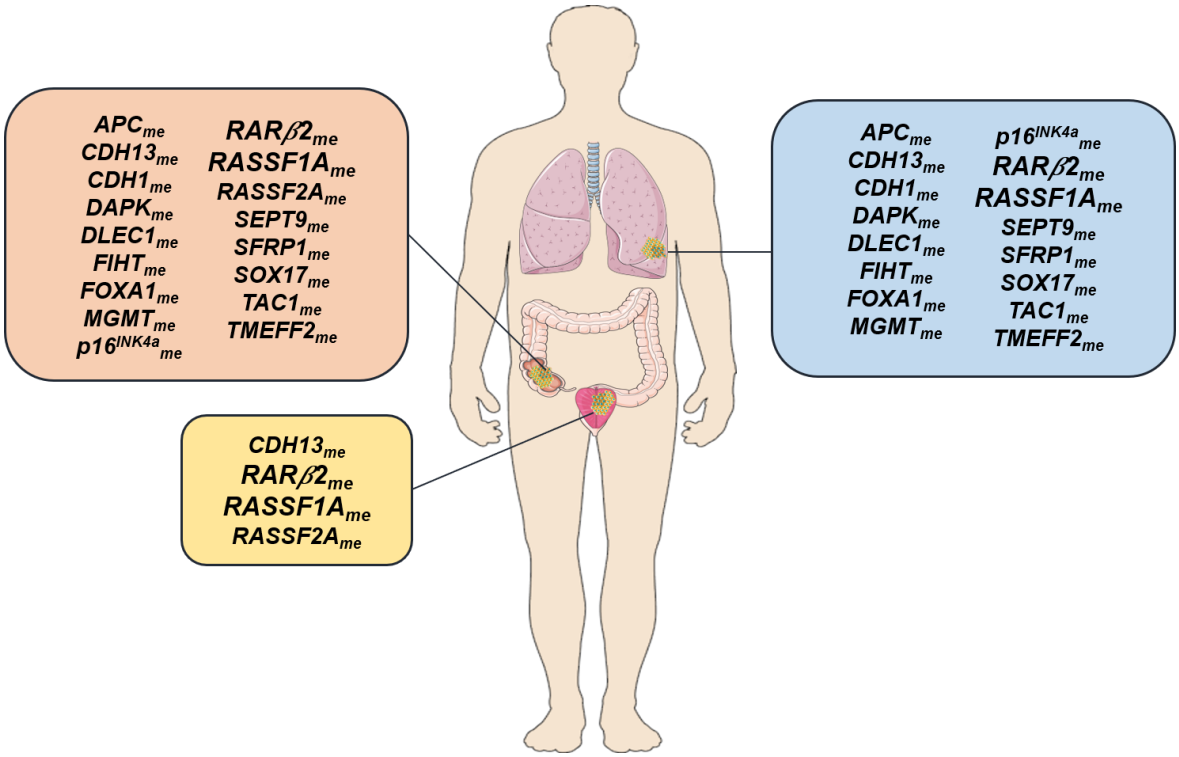

Figure 5. Circulating cell-free DNA methylation-based biomarkers described in literature for cancer detection common to at least two cancer types [Lung Cancer (blue box), Prostate Cancer (yellow box), Colorectal Cancer (orange box)]. Constâncio, V. *unpublished.*

Interestingly, a study published in 2007 that included 70 serum samples from metastatic breast, NSLC, gastric, pancreatic, colorectal and hepatocellular carcinoma and 10 healthy serum controls demonstrated that a gene panel hypermethylation (*RUNX3*$_{me}$*, p16*$_{me}$*, RASSF1A*$_{me}$ *and CDH1*$_{me}$) detected cancer samples with 89% sensitivity and 100% specificity using methylation-specific PCR (MSP) [172], suggesting the putative value of using a single panel to detect several malignancies.

Remarkably, genome-wide DNA methylation studies have also been performed aiming to detect the presence of cancer and underlying cancer type [173-175]. Indeed, Li *et al.* and Kang *et al.* developed "CancerDetector" and "CancerLocator" that can detect cancer using probabilistic approaches based on ccfDNA methylation sequencing [174, 175]. Moreover, Moss *et al.* using Illumina methylation arrays demonstrated that plasma methylation patterns can be used to identify cell type-specific ccfDNA in healthy and pathological conditions, including different types of cancer [176].

Interestingly, in 2018, the Laboratory for Advanced Medicine launched the LDT IvyGene® Test in the USA for detection of breast cancer (BrC), CRC, liver cancer and LC [177]. This test utilizes a multi-target approach derived from sequencing methods to detect ccfDNA methylation profile in 40mL of whole blood samples [177, 178]. Notwithstanding, according to their website, the test detected cancer with 84% sensitivity and 90% specificity,

it was only validated in 197 samples obtained from subjects with either no history of cancer or diagnosis of one of the four cancers [177]. Thus, the performance evaluation of IvyGene® Test in a large screening setting is still warranted.

Although epigenome-wide approaches allow for simultaneous screen of several hundreds of genes and might be advantageous to boost the discovery of new differentially methylated DNA regions, they are still not widely available in clinical laboratories and require high-level bioinformatics expertise, fast data processing and large data storage capabilities [179, 180]. Additionally, depending on the number of samples and genes to be analysed, targeted approaches, such as qMSP or droplet digital MSP (ddMSP), might be more cost-effective [181]. Remarkably, in a recent study performed in our group, in which methylation levels of nine genes were evaluated in plasma samples from female BrC, CRC, LC patients and asymptomatic controls using multiplex qMSP, a "PanCancer" panel (*APC*$_{me}$, *FOXA1*$_{me}$ and *RASSF1A*$_{me}$) was able to detect the three malignancies with 72% sensitivity and 74% specificity, whereas a "CancerType" panel (*SCGB3A1*$_{me}$, *SEPT9*$_{me}$ *and SOX17*$_{me}$) indicated the most likely cancer topography, with over 80% specificity, although with limited sensitivity [42].

Altogether, these data suggest that the hypothetical use of a "PanCancer" panel based on ccfDNA methylation is amenable to increase patient compliance to screening programs and decrease health-systems costs and patient morbidity and mortality.

6. SELECTED GENES UNDER STUDY

For the purpose of this work, eight previously reported methylated genes were selected to be studied in blood-based liquid biopsies of LC, PCa and CRC male patients and asymptomatic controls with the aim to develop a "PanCancer" detection panel.

As previously mentioned, hypermethylation of retinoic acid receptor beta 2 (*RARβ2*) and Ras association domain family 1 isoform A (*RASSF1A*) were already reported for LC, PCa and CRC detection (Table 1-3 and Figure 5), hence, they were selected for our panel as the most promising genes to integrate a "PanCancer" panel in liquid biopsies. *RARβ2* is a TSG that encodes a nuclear receptor, and mediates growth inhibition and differentiation of epithelial cells in the presence of retinoic acid [182]. *RASSF1A*, as a TSG, is thought to regulate multiple biological processes, such as cell-cycle, apoptosis, cell motility and invasion [183].

APC is a well-known TSG involved in the down-regulation of Wnt/β-catenin signalling, cell-adhesion, cytoskeleton stability and apoptosis [184]. Hypermethylation of this gene is described in liquid biopsies for detection of both LC and CRC (Table 1 and

Table 3 and Figure 5), moreover, it is also a widely reported PCa biomarker in tissue and urine samples [185-187].

Forkhead box A1 (*FOXA1*) is a transcription factor that regulates a variety of tissues during embryogenesis and early life. Moreover, its activity is involved in oestrogen and androgen receptor signalling in breast and prostate cells [188]. Interestingly, hypermethylation of this gene was found in plasma samples from BrC, CRC and LC female patients [42] and, so far, it has not been investigated in PCa patients.

Glutathione S-transferase pi 1 (*GSTP1*) is a caretaker gene, family member of the glutathione-S transferases, which are enzymes involved in DNA protection from electrophilic metabolites of carcinogens and reactive oxygen species by catalysing the conjugation of chemically reactive electrophiles with reduced glutathione [189]. The silencing of this gene by methylation is a frequent and early event in prostate carcinogenesis, being largely reported for its cancer-specific nature in prostate tissues [6, 190]. Hence, it is an outstanding putative biomarker for PCa detection in body fluids, although it also reported in other cancer types including BrC, hepatocellular carcinoma and testicular cancer [190].

Septin 9 (*SEPT9*) appears to act as a TSG, being involved in cytokinesis and cell cycle control [191]. As previously mentioned, a test based on $SEPT9_{me}$ in ccfDNA is already commercially available for CRC detection [113], being also described in LC plasma samples [49].

Sex determining region Y box 17 (*SOX17*) transcription factor has been recognized as an antagonist and inhibitor of the Wnt signalling pathway, being involved in the regulation of embryonic development and in determination of the cell fate [63]. Methylation of this gene has been reported in plasma of LC patients (Table 1), being also recently reported in plasma of female CRC patients [42]. As for $FOXA1_{me}$, methylation of this gene was not been accessed in PCa yet.

To the best of our knowledge, homeobox D3 (*HOXD3*) methylation has not been yet investigated in blood-based liquid biopsies. Nevertheless, it was reported to detect PCa in urine with high sensitivity [81], being also detected in LC and hepatocellular carcinoma patients' tissue [192, 193]. *HOXD3* belongs to the HOX family of transcription factors, major regulators of embryonic development, cellular proliferation differentiation and angiogenesis [194].

II. AIM

LC, PCa and CRC cancers remain the three most incident cancers in males worldwide, and the deadliest in Europe and North America, although recent efforts in early cancer detection. Given the shortcomings of current screening methods, the development and implementation of effective screening methods that might allow for detection of these three cancers at curative early stages is crucial. Due to its early onset, cancer specificity and biological stability, DNA methylation has emerged as potential valuable epigenetic-based biomarkers. Additionally, their accessibility in body fluids makes them an attractive target to be studied in liquid biopsies.

Thereby, the aim of this study is to assess the feasibility of a minimally invasive methylation-based test in liquid biopsies for simultaneous detection of LC, PCa and CRC in males. Furthermore, the prognostic value of the selected genes will also be ascertained.

Specifically, we intend to:

1. Assess the methylation levels of eight gene promoters (*APC_{me}*, *$FOXA1_{me}$*, *$GSTP1_{me}$*, *$HOXD3_{me}$*, *$RAR\beta2_{me}$*, *$RASSF1A_{me}$*, *$SEPT9_{me}$* and *$SOX17_{me}$*) by multiplex qMSP in ccfDNA extracted from plasma samples from male cancer patients (LC, PCa and CRC) and asymptomatic controls (AC);
2. Determine the diagnostic performance of genes methylation levels for each cancer type;
3. Establish the most sensitive and specific gene-panel combination “PanCancer” for detection of the three cancers, and a “CancerType” gene-panel suitable to discriminate the three cancer types;
4. Evaluate the association between genes methylation levels and clinicopathological features, also assessing their putative value as prognostic markers.

III. MATERIAL AND METHODS

1. CLINICAL SAMPLES

1.1. Patients and Samples Collection

Pre-treatment blood samples were collected from 323 male patients diagnosed with LC (n=102) or CRC (n=100) between 2015 and 2019, and PCa (n=121) between 2006 and 2010, at Portuguese Oncology Institute of Porto, Portugal. For control purposes, blood samples were donated by 136 male asymptomatic blood donors (AC) older than 48 years old, from 2016 to 2018, at the same institution.

After collection of peripheral blood into EDTA-containing tubes, plasma was separated by centrifuging at 2000 rpm for 10 minutes at 4ºC, and subsequently, stored at -80ºC in the institutional tumour bank until further use. Relevant clinical and pathological data was retrieved from clinical charts and an anonymized database was constructed for analysis purposes.

This study was approved by the institutional review board (Comissão de Ética para a Saúde) of Portuguese Oncology Institute of Porto, Portugal (CES-IPOFG-EPE 120/015). Written informed consent, in accordance with the Declaration of Helsinki ethical principles, were provided by all patients and AC enrolled in this study.

2. CELL-FREE DNA EXTRACTION

CcfDNA was extracted from 2-3 mL of plasma (Figure 6) using QIAmp MinElute ccfDNA (Qiagen, Hilden, Germany), according to manufacturer's instructions. Briefly, Magnetic Bead Suspension, Proteinase K and Bead Binding Buffer were added to the plasma in the appropriate ratio (Table 4) to the sample volume in a 15 mL tube. After a 10 minutes incubation period with shaking at 100 rpm to bind circulating DNA to magnetic beads, the tubes were placed in a magnetic rack and supernatant was discarded when the solution was clear, and magnetic beads with bound DNA were collected in a pellet. To elute the bound DNA from the beads, 200 µL of Bead Elution Buffer were added and the mixture was transferred to a bead elution tube and incubated for 5 minutes with shaking at 300 rpm. Then, the bead elution tube was placed in a 2 mL magnetic rack and the supernatant was transferred to a new tube. 300 µL of buffer ACB were added to the samples to adjust conditions to allow optimal binding of the circulating nucleic acids to the membrane, and the mixture was briefly vortexed and centrifugated. Subsequently, samples were transferred onto a QIAmp UCP MinElute column, and circulating DNA was adsorbed onto the silica membrane while the sample was centrifuged at 6000 x g for 1 minute. The column was washed twice with 500 µL of ACW2 and centrifuged at 20,000 x g for 3 minutes. Then, the

column was transferred to a 1.5 mL elution tube and the samples were incubated at 56ºC for 3 minutes, with the lid open, to dry the membrane to ensure that any residual ethanol is carried over into the next step. Lastly, ccfDNA was eluted in 20 µL of Ultra-clean water, incubated for 2 minutes and centrifuged at 20,000 x g for 1 min. All steps were performed at room temperature. The extracted ccfDNA was stored at -20ºC until further use.

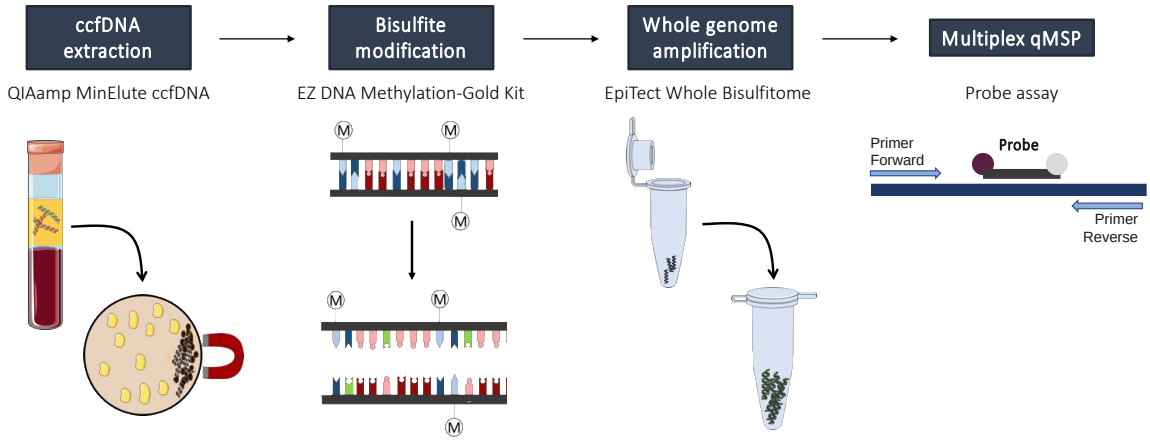

Figure 6. Overview of the techniques performed. Circulating cell-free DNA (ccfDNA) was extracted from plasma samples using a kit based on the concentration of ccfDNA onto magnetic beads. Then, sodium-bisulfite modification was performed to convert unmethylated cytosines into uracil, while maintaining methylated cytosines unchanged, and whole genome amplification (WGA) was performed to increase modified-DNA quantity. Lastly, promoters' methylation levels were assessed by multiplex quantitative methylation specific PCR (qMSP) with TaqMan probes. (Kindly provided by S.P. Nunes *unpublished*)

Table 4. Component mix (QIAmp MinElute ccfDNA Mini Kit).

Plasma (mL)	Magnetic Bead Suspension (µL)	Proteinase K (µL)	Bead Binding Buffer (µL)
2	60	110	300
2.5	75	137.5	375
3	90	165	450

3. SODIUM-BISULFITE MODIFICATION

Sodium-bisulfite modification (Figure 6) is a pivotal gold-standard technique for DNA methylation analyses. This method includes the treatment of DNA with bisulfite, which converts unmethylated cytosines into uracil, while methylated cytosines remain unchanged, based on three consecutive chemical reactions (sulphonation, deamination and desulphonation) [195]. Therefore, after conversion, the DNA methylation profile can be determined by MSP and sequencing, through the design of primers and probes specific for the modified DNA sequence.

All ccfDNA samples were sodium-bisulfite modified using EZ DNA Methylation-Gold™ Kit (Zymo Research, Orange, CA, USA), according to the manufacturer's recommendations. Firstly, 130 µL of CT conversion reagent solution was added to 20 µL of the previously extracted ccfDNA of each sample. Then, samples were incubated at 98ºC for 10 minutes for DNA denaturation, followed by 64ºC for 180 minutes for bisulfite conversion reaction in Applied Biosystems Veriti 96-Well Thermal Cycler (Thermo Fisher Scientific, USA). Next, each sample and 600 µL of M-Binding Buffer were added to Zymo-Spin™ IC column and incubated for 10 minutes. The columns were centrifugated at 10,000 rpm for 30 seconds. Following the addition of 100 µL of M-Wash Buffer and centrifugation, 200 µL of M-Desulphonation Buffer were added to the column, followed by 20 minutes incubation and centrifugation at 10,000 rpm for 30 seconds. After, the column was washed twice with 200 µL of M-Wash Buffer and centrifugated at 10,000 rpm for 30 seconds. Finally, the columns were transferred to 1.5 mL safe-lock tubes and 10 µL of sterile distilled water were added to elute the bisulfite-converted DNA. Following a 5 minutes incubation, the columns were centrifugated at 12,000 rpm for 30 seconds. This process was repeated twice. All steps were performed at room temperature. One µg of CpGenome™ Universal Methylated DNA (Merck Milipore, Burlington, MA, USA) was also modified using the previously mentioned protocol and eluted in 30 µL of sterile distilled water. The bisulfite-converted DNA was stored at -80ºC until further use.

4. WHOLE-GENOME AMPLIFICATION

DNA methylation analysis can be limited by the small amount of sample available. Moreover, DNA quality can further be compromised during sodium bisulfite DNA conversion due to DNA fragmentation. Thus, a whole genome amplification (WGA) step using multiple displacement amplification (MDA) technology was performed in this study to increase DNA

quantity. MDA is based on an isothermal genome amplification using a DNA polymerase with an exonuclease proofreading activity to maintain high fidelity during replication [196].

EpiTect Whole Bisulfitome Kit (Qiagen, Hilden, Germany) was used to perform WGA of sodium-bisulfite modified DNA, according to the manufacturer's instructions. Briefly, 10 µL of modified DNA of each sample were mixed with 30 µL of EpiTect amplification master mix containing 29 µL of EpiTect WBA reaction buffer and 1 µL REPLI-g Midi DNA polymerase. After, samples were incubated in Applied Biosystems Veriti 96-Well Thermal Cycler (Thermo Fisher Scientific, USA) at 28ºC for 8 hours, followed by 95ºC for 5 minutes to inactivate the polymerase. Finally, the amplified DNA was diluted in 25 µL of sterile distilled water, for a final volume of 65 µL, and stored at -20ºC until further use.

5. NUCLEIC ACID QUANTIFICATION

DNA concentration was quantified using Qubit fluorometric method. The Qubit fluorometer measures the fluorescence intensity of a fluorescent dye that bind specifically to DNA (double and single stranded), RNA or protein [197].

The extracted ccfDNA and amplified DNA were quantified using Qubit dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA, USA), whereas the sodium-bisulfite converted DNA was quantified with Qubit ssDNA Assay Kit (Invitrogen, California, CA, USA). A working solution containing 199 µL of Qubit dsDNA HS / ssDNA Buffer and 1 µL of Qubit dsDNA HS / ssDNA Reagent per sample was prepared. Next, 199 µL of working solution were added to 1 µL of DNA sample. The DNA concentration was determined using Qubit 2 Fluorometer (Invitrogen, Carlsbad, CA, USA).

6. MULTIPLEX QUANTITATIVE METHYLATION SPECIFIC PCR

Promoter methylation levels of eight genes (*APC_{me}, $FOXA1_{me}$, $GSTP1_{me}$, $HOXD3_{me}$, $RAR\beta2_{me}$, $RASSF1A_{me}$, $SEPT9_{me}$ and $SOX17_{me}$*) were assessed by multiplex qMSP (Figure 6), which allows the assessment of multiple genes simultaneously. Primers and TaqMan probes designed specifically for the modified gene sequence, plus fluorochromes and quenchers selected for each probe are listed in Table 5. The housekeeping gene β-Actin was used an internal reference gene to normalize the assay.

Table 5. Primers and probes sequences with respective fluorochrome and quencher.

Gene		Sequence (5'–3')	Volume	Vendor
β-Actin	Primers	F – TGGTGATGGAGGAGGTTTAGTAAGT R – ACCAATAAAACCTACTCCTCCCTTAA	0.8 µL	Sigma-Aldrich, Steinheim, Germany
	Probe	Cy5 – ACCACCACCCAACACACAATAACAAACACA – QSY	0.1 µL	Applied Biosystems
APC_{me}	Primers	F – TGTGTTTTATTGCGGAGTGC R – CACATATCGATCACGTACGC	0.8 µL	Sigma-Aldrich, Steinheim, Germany
	Probe	VIC – CAATCGACGAACTCCCGAC – MGB	0.1 µL	Applied Biosystems
$FOXA1_{me}$	Primers	F – CGACGTTAAGACGTTTAAGC R – CGCTCAACGTAAACATCTTAC	0.8 µL	Sigma-Aldrich, Steinheim, Germany
	Probe	FAM – ATATACGAATAAAACGACTTAACG – MGB	0.1 µL	Applied Biosystems
$GSTP1_{me}$	Primers	F – GTCGGCGTCGTATTTAGTATTG R – AAACTACGACGACGAAACTCCAA	0.8 µL	Sigma-Aldrich, Steinheim, Germany
	Probe	FAM – AAACCTCGCACCTCCGAACCTTATAAAA – BHQ1	0.1 µL	NZY Tech, Lisbon, Portugal
$HOXD3_{me}$	Primers	F – TAAAGGTTTATGGTTGCGC R – TTACGAACACTAAACTACACCCG	0.8 µL	Sigma-Aldrich, Steinheim, Germany
	Probe	Cy5 – ACAAAACGTTCCCGACGCTTCTAAAA – BHQ1	0.1 µL	NZY Tech, Lisbon, Portugal
$RARβ2_{me}$	Primers	F – TCGAGAACGCGAGCGATT R – GACCAATCCAACCGAAAC	0.3 µL	BioRad, Hercules, USA
	Probe	HEX – CTTACAAAAAACCTTCCGAATACGTTCCGA – Iowa Black RQ-Sp		
$RASSF1A_{me}$	Primers	F – AGCGAAGTACGGGTTTAATC R – ACACGCTCCAACCGAATA	0.8 µL	Sigma-Aldrich, Steinheim, Germany
	Probe	NED – CGGGAGTTGGTATTCGTTGGGCG – QSY	0.1 µL	Applied Biosystems
$SEPT9_{me}$	Primers	F – TTAGTTAGCGCGTAGGGTTC R – ACCTTCGAAATCCGAAATAA	0.8 µL	Sigma-Aldrich, Steinheim, Germany
	Probe	NED – GCGTTAACCGCGAAATCCGACATAATAACT – QSY	0.1 µL	Applied Biosystems
$SOX17_{me}$	Primers	F – GATCGGTTCGTTTTCGTCG R – GCCCGTATTCTAACCTATCG	0.3 µL	BioRad, Hercules, USA
	Probe	Cy5 – ACCGACCTAATAACACTACGAACGC – Iowa Black RQ-Sp		

Multiplex qMSP assay was carried out in triplicate in 96-well plates using a 7500 Sequence Detector (Applied Biosystems, Perkin Elmer, CA, USA). The multiplex gene combinations used are displayed in Table 6.

Table 6. Gene combinations for multiplex qMSP.

Combination 1	Combination 2	Combination 3
β-Actin	$RAR\beta 2_{me}$	$GSTP1_{me}$
APC_{me}	$SEPT9_{me}$	$HOXD3_{me}$
$FOXA1_{me}$	$SOX17_{me}$	---
$RASSF1A_{me}$	---	---

For each reaction, 6 µL of WGA amplified DNA, 10 µL of Xpert Fast Probe (GRiSP, Porto, Portugal), a mix with forward and reverse primers (10µM) and TaqMan probe (10µM) or Primer PCR Custom Assay (described in Table 5), and sterile distilled water (B. Braun, Melsungen, Germany) were added, to a final volume of 20µL.

The following PCR program was used: 1 cycle at 95 ºC for 3 minutes; 50 cycles at 95ºC for 5 seconds and 60ºC for 30 seconds. All samples were run in triplicate and three wells of sterile distilled water were used as negative control in all plates. WGA amplified CpGenome™ Universal Methylated DNA subjected to six serial dilutions (5x factor dilution) was used to generate a standard curve in each plate, allowing for relative quantification and PCR efficiency evaluation. All plates displayed efficiency values above 90%. Relative methylation levels were calculated as the ratio between the mean methylation levels of each target gene and the respective value for β-Actin, multiplied by 1000.

7. STATISTICAL ANALYSIS

Non-parametric tests were performed to compare methylation levels of each gene's promoter between cases and controls, and to evaluate associations with clinicopathological features. Mann-Whitney U test was used for comparisons between two groups, while Kruskal-Wallis test was used for multiple groups, followed by Mann-Whitney U test with Bonferroni's correction for pairwise comparisons. Spearman non-parametric test was performed to assess correlations between methylation levels and patients' age. A result was considered statistically significant when *p-value*<0.05.

For each gene's promoter, samples were categorized as methylated or non-methylated based on the cut-off values established using Youden's J index (value combining highest sensitivity and specificity), through receiver operator characteristic (ROC) curve analysis [198]. When more than one value fulfilled this condition, the cut-off value allowing for higher sensitivity was chosen. Validity estimates (sensitivity, specificity and accuracy) were determined to assess detection biomarker performance (Table 7). To improve detection performance of the selected genes, panels were constructed considering a positive result whenever at least one gene promoter was plotted as methylated in individual analysis. For "PanCancer" panel, validity estimates were calculated by joining LC and PCa (n=223) vs AC samples (n=136), whereas for "CancerType" panel, these were calculated by comparing one tumour type to the other. Validity estimates for the two panels were assessed by constructing multiple ROC curves via resampling analysis [199]. In short, samples were randomly divided into training (70%) and validation (30%) sets. Then, the cut-off value obtained combining the highest sensitivity and specificity in the training set, was used to calculate validity estimates in the validation set. This procedure was repeated 1000 times, and the mean value of sensitivity, specificity and accuracy was computed.

Table 7. Formulas for biomarker performance calculations.

Tumour *vs.* Control			**Validity Estimates**	
	Tumour	Control		
> Cut-off	A	B	**Sensitivity (%)**	(A/E) x 100
< Cut-off	C	D	**Specificity (%)**	(D/F) x 100
Total (n)	E	F	**Accuracy (%)**	[(A+D) / (E+F)] x 100

Kaplan-Meier curves were constructed, and log-rank test was used to compare disease-specific survival (DSS), progression-free survival (PFS) and disease-free survival (DFS) between groups, considering clinicopathological variables and categorized gene promoter methylation status (Positive: relative methylation levels > 0 and Negative: relative methylation levels = 0). DSS and PFS was calculated as the time between the date of diagnosis and the date of cancer-related death or of the first imaging exam showing disease progression, respectively, whereas DFS as the time between the date of curative intent treatment and the date of the first imaging exam showing disease progression or biochemical recurrence (for PCa). Cox proportional hazards regression was employed to calculate hazard ratios (HR) and 95% confidence intervals (CI). Backwards conditional multivariable Cox-regression model comprising all significant variables on univariable analysis was computed to determine whether genes' promoter methylation status were independently associated with DSS.

Two-tailed *p-values* calculation, ROC curve analysis and survival analysis were performed using SPSS 25.0 for MacOS software (IBM-SPSS Inc., Chicago, IL, USA). Multiple ROC curves via resampling analysis was performed using R v.3.4.4 (Vienna, Austria). All graphics were assembled using GraphPad Prism 7.0a for MacOS Software (GraphPad Software Inc., LA Jolla,CA, USA).

IV. RESULTS

1. CLINICAL AND PATHOLOGICAL DATA

Plasma samples were obtained from 323 male patients diagnosed with LC (n=102), PCa (n=121) and CRC (n=100), and 136 male AC (Table 8). Overall, cancer patients' median age was significantly higher than that of controls (p<0.0001). Nonetheless, except for $SOX17_{me}$ levels that correlated with controls' age (R=0.179; p=0.037), and $FOXA1_{me}$ levels which correlated with cancer patients' age (R=0.144; p=0.010), no other correlations were disclosed.

Table 8. Clinical and pathological features of lung, prostate and colorectal cancer patients and asymptomatic controls included in this study.

Clinicopathological features	Asymptomatic controls	Cancer Patients
Number	136	323
Age median (range)	57 (48-66)	68 (27-93)
		Lung Cancer
Number		102
Age median (range)		66 (45-89)
Histological Type		
Non-small cell lung cancer (NSCLC):		
Adenocarcinoma	n.a	42
Squamous cell carcinoma		43
Large cell carcinoma		1
Small-cell lung carcinoma (SCLC)		16
Primary Tumour (T)[a]		
T1	n.a	12
T2 / T3 / T4		82
Regional Lymph node (N)[b]		
N0	n.a	25
N+		72
Distant Metastasis (M)		
M0	n.a	47
M+		55
Clinical Stage		
I / II	n.a	17
III / IV		85

		Prostate Cancer
Number		121
Age median (range)		71 (52-88)
Histological Type	n.a	
Adenocarcinoma		121
Primary Tumour (T)[c]		
T1 / T2	n.a	104
T3		16
Regional Lymph node (N)		
N0	n.a	119
N+		2
Distant Metastasis (M)		
M0	n.a	116
M+		5
ISUP Grade Group		
1	n.a	59
2		38
3 / 4 / 5		24
Serum PSA levels (ng/mL)		
<10	n.a	71
10-20		27
>20		23
Clinical Stage		
I	n.a	31
II		55
III/IV		35
		Colorectal Cancer
Number		100
Age median (range)		66 (27-93)
Histological Type		
Adenocarcinoma (all subtypes)	n.a	99
Squamous cell carcinoma		1
Tumour location		
Proximal colon	n.a	23
Distal colon		36
Rectum		41
Primary Tumour (T)[d]		
T1/T2	n.a	26
T3/T4		72

Regional lymph node (N)[e]		
N0	n.a	40
N+		57
Distant metastasis (M)		
M0	n.a	82
M+		18
Clinical Stage		
I / II	n.a	39
III / IV		61

[a]No information available in 8 cases; [b]No information available in 5 cases; [c]No information available in 1 case; [d]No information available in 2 cases; [e]No information available in 3 cases; Abbreviations: n.a. – not applicable.

2. GENE PROMOTER METHYLATION LEVELS IN CCFDNA

CcfDNA's APC_{me}, $FOXA1_{me}$, $GSTP1_{me}$, $HOXD3_{me}$, $RAR\beta2_{me}$, $RASSF1A_{me}$, $SEPT9_{me}$ and $SOX17_{me}$ levels were compared between each cancer type and controls. APC_{me} (p=0.033), $FOXA1_{me}$ (p=0.024), $RAR\beta2_{me}$, $RASSF1A_{me}$, $SEPT9_{me}$ and $SOX17_{me}$ (p<0.0001, in all comparisons) levels were significantly higher in LT patients than in AC, whereas no differences were disclosed for $GSTP1_{me}$ and $HOXD3_{me}$ (p=0.718 and p=0.174, respectively) (Figure 7).

In PCa patients, significantly higher levels were observed for $FOXA1_{me}$, $GSTP1_{me}$, $HOXD3_{me}$, $RAR\beta2_{me}$, $SOX17_{me}$ (p<0.0001, in all comparisons), $RASSF1A_{me}$ and $SEPT9_{me}$ (p=0.014 and p=0.0001, respectively), although no differences were apparent for APC_{me} (p=0.443) (Figure 7).

Concerning CRT patients, only $SEPT9_{me}$ and $SOX17_{me}$ (p=0.012 and p=0.014, respectively) displayed significantly higher levels in patients comparing with controls. Moreover, $HOXD3_{me}$ levels (p=0.009) were significantly lower in CRT patients than in controls (Figure 7).

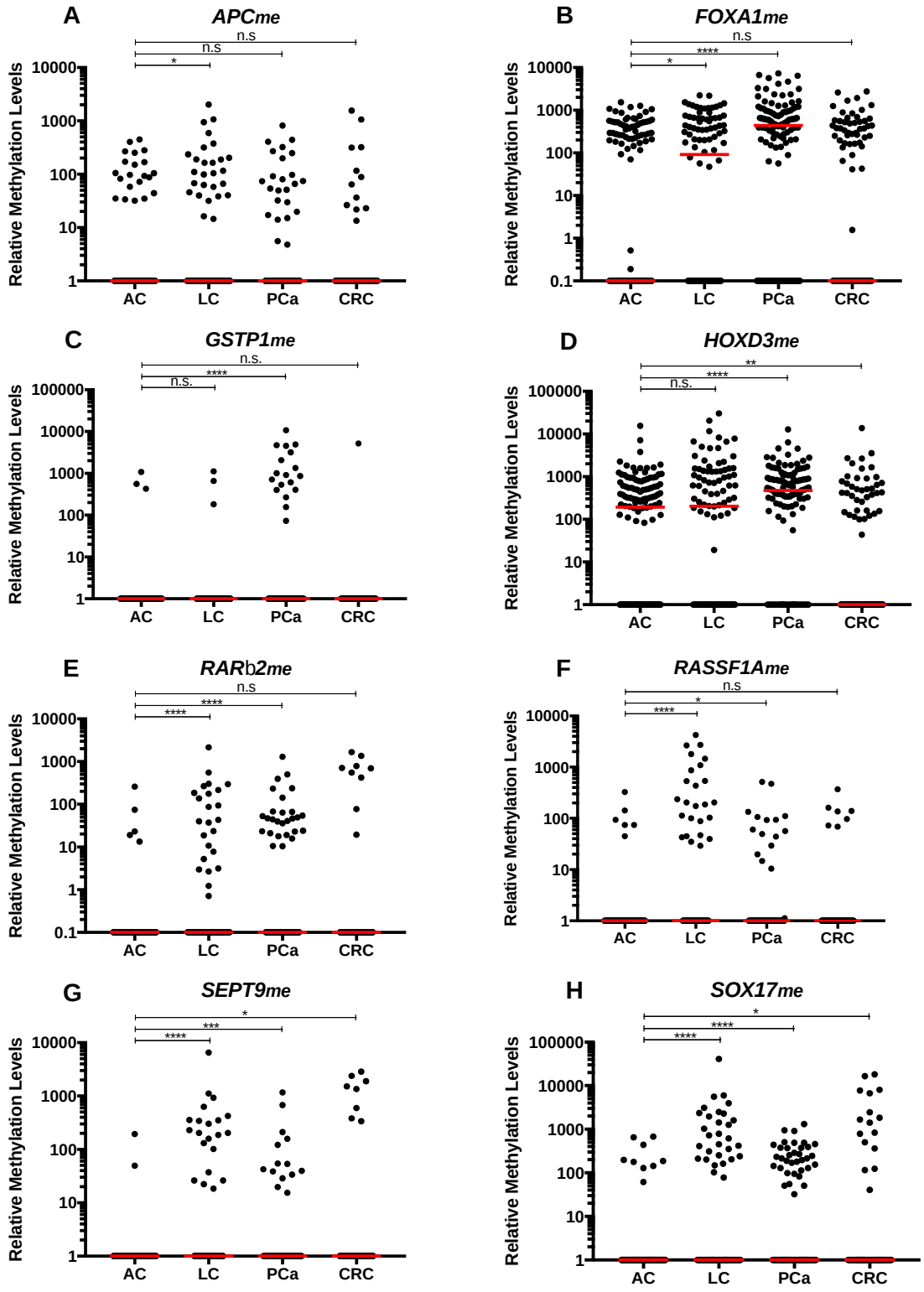

Figure 7. Distribution of (A) *APC*, (B) *FOXA1*, (C) *GSTP1*, (D) *HOXD3*, (E) *RARβ2*, (F) *RASSF1A*, (G) *SEPT9* and (H) *SOX17* relative methylation levels of asymptomatic controls (AC) (n=136), lung cancer (LC) (n=102), prostate cancer (PCa) (n=121) and colorectal cancer (CRC) (n=100) samples. Mann-Whitney U Test between AC and each cancer type, n.s. $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$. Red horizontal lines represent median methylation levels. (Raw data available in Appendix III: Supplementary Table 9)

2.1. Detection Biomarker Performance of ccfDNA

Since our main goal was to test the biomarker performance for LC, PCa and CRC detection of a methylation-based panel in ccfDNA, genes displaying significantly higher methylation levels in cancer patients compared to controls were selected for further analysis.

SEPT9$_{me}$ and *SOX17*$_{me}$ were the only two biomarkers shared by all three cancer types, displaying specificity between 93-100% (Table 9). Nonetheless, these two biomarkers detected CRC with limited sensitivity (8 and 11%, respectively) (Table 9), thus, no further analyses were performed for this tumour.

RARβ2 $_{me}$*, RASSF1A* $_{me}$*, SEPT9* $_{me}$ *and SOX17* $_{me}$, were able to detect both LC and PCa with over 93% specificity (Table 9). Conversely, *FOXA1*$_{me}$ disclosed the highest sensitivity for detecting LC and the second highest for detecting PCa (38% and 61%, respectively), with 77% specificity for both. *SOX17*$_{me}$ detected both LC and PCa, individually, with 29% sensitivity, and *RARβ2*$_{me}$ identified both cancers with 22-24% sensitivity (Table 9).

Gene panels were further constructed to increase detection sensitivity. Hence, the best LC panel (*FOXA1*$_{me}$*, RARβ2*$_{me}$*, RASSF1A*$_{me}$ and *SOX17*$_{me}$) achieved 66% sensitivity and 70% specificity, whereas for PCa, the panel F*OXA1*$_{me}$*, RARβ2*$_{me}$*, RASSF1A*$_{me}$ and *GSTP1*$_{me}$ depicted 72% sensitivity and specificity (Table 9).

Table 9. Biomarker performance of each gene promoter methylation for lung (LC), prostate (PCa) and colorectal (CRC) cancer detection in circulating cell-free DNA.

Genes	Cut-off value	Sensitivity %	Specificity %	Accuracy %
Lung Cancer				
APC$_{me}$	7.2629	26.47	84.56	59.66
FOXA1$_{me}$	301.1682	38.24	77.21	60.50
RARβ2$_{me}$	0.3548	23.53	96.32	65.13
RASSF1A$_{me}$	14.5588	24.51	95.59	65.13
SEPT9$_{me}$	9.1907	20.59	98.53	65.13
SOX17$_{me}$	70.0725	29.41	94.12	66.39
LC panel	---	65.69	69.85	68.07
Prostate Cancer				
FOXA1$_{me}$	295.8133	61.16	77.21	69.65
GSTP1$_{me}$	36.7086	14.88	97.79	58.75
HOXD3$_{me}$	320.9365	80.17	42.65	60.31
RARβ2$_{me}$	5.2251	22.31	96.32	61.48
RASSF1A$_{me}$	0.5627	13.22	95.59	56.81
SEPT9$_{me}$	7.7096	11.57	98.53	57.59
SOX17$_{me}$	16.153	28.93	93.38	63.04
PCa panel	---	71.90	72.06	71.98
Colorectal cancer				
SEPT9$_{me}$	265.2606	8.00	100.00	61.02
SOX17$_{me}$	732.3866	11.00	100.00	62.29
CRC panel	---	12.00	100.00	62.71

LC panel - *FOXA1$_{me}$*, *RARβ2$_{me}$*, *RASSF1A$_{me}$* and *SOX17$_{me}$*; **PCa panel** - F*OXA1$_{me}$*, *RARβ2$_{me}$*, *RASSF1A$_{me}$* and *GSTP1$_{me}$*; **CRC panel** – *SEPT9$_{me}$* and *SOX17$_{me}$*.

Aiming to obtain a gene panel for simultaneous LC and PCa detection (designated as “PanCancer” panel), all genes (*FOXA1$_{me}$*, *RARβ2$_{me}$*, *RASSF1A$_{me}$* and *SEPT9$_{me}$*) that were shared [except for *SOX17$_{me}$* that displayed a relatively different cut-off between these two cancer types (70.0725 for LC and 16.153 for PCa)] (Table 9), were further tested as panel. Additionally, *APC$_{me}$*, *GSTP1$_{me}$*, *HOXD3$_{me}$* and *SOX17$_{me}$* were tested as a suitable panel for tumour’s primary location discrimination (“CancerType” panel). Remarkably, 144 out of 223 patients were correctly identified with “PanCancer” panel (*FOXA1$_{me}$*, *RARβ2$_{me}$* and *RASSF1A$_{me}$*), displaying 64% sensitivity, 70% specificity and 66% accuracy (Table 10 and Figure 8).

Table 10. Biomarker performance of "PanCancer" panel *(FOXA1$_{me}$, RARβ2$_{me}$* and *RASSF1A$_{me}$*) for simultaneous lung and prostate cancer detection in circulating cell-free DNA.

	PanCancer
Sensitivity %	64.3%
Specificity %	69.8%
Accuracy %	66.4%

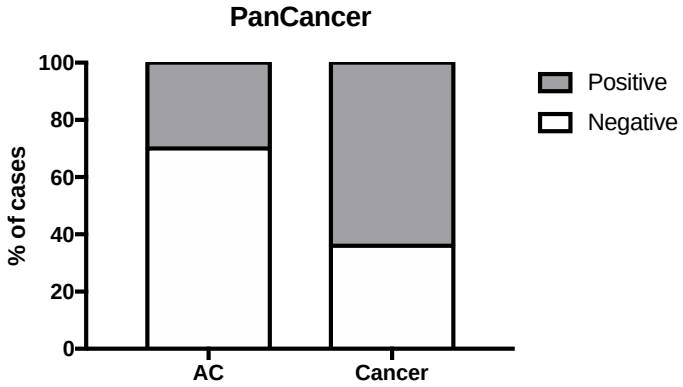

Figure 8. Percentage of cases identified by "PanCancer" panel in cancer samples (64% Positive, 36% Negative) and in asymptomatic controls (AC) (30% Positive, 70% Negative).

As early diagnosis is imperative, and specifically for PCa it is also relevant to detect clinically significant disease, we further tested this panel for those purposes. "PanCancer" panel detected LC stages I and II with 95.0% specificity, however with modest 35% sensitivity, displaying 87.7% accuracy. Importantly, this panel was able to discriminate intermediate and high risk PCa patients (stage II and III-IV) from controls and low-risk (stage I) PCa patients with 71.1% sensitivity, 64.7% specificity and 66.9% accuracy.

Among the four genes tested for "CancerType" panel, *SOX17$_{me}$* and *GSTP1$_{me}$* demonstrated the best performance for discriminating LC from PCa. Indeed, *SOX17$_{me}$* discriminated LC from PCa with 93% specificity, whereas *GSTP1$_{me}$* detected PCa with 97% specificity, although with limited sensitivity (Table 11).

Table 11. Biomarker performance of "CancerType" panel (*GSTP1*$_{me}$ and *SOX17*$_{me}$) for discrimination among lung and prostate cancer in circulating cell-free DNA.

Gene	Sensitivity %	Specificity %	Accuracy %
Lung Cancer			
***SOX17*$_{me}$**	15.2	93.4	57.7
***GSTP1*$_{me}$**	---	---	---
Prostate Cancer			
***SOX17*$_{me}$**	---	---	---
***GSTP1*$_{me}$**	13.6	97.0	51.8

2.2. Association Between Promoters' Methylation Levels and Clinicopathological Features

Concerning associations between promoters' methylation levels and clinicopathological features (Appendix IV: Supplementary Tables 10-12), in LC patients, higher circulating *FOXA1*$_{me}$ and *RARβ2*$_{me}$ levels associated with advanced primary tumour stage (T) (p=0.014 and p=0.044, respectively) (Figure 9), whereas higher levels of those two genes and *APC*$_{me}$ and *HOXD3*$_{me}$ were also depicted in LC patients with regional lymph node (N) involvement (p=0.021, p=0.015, p=0.044 and p=0.022, respectively) (Figure 10).

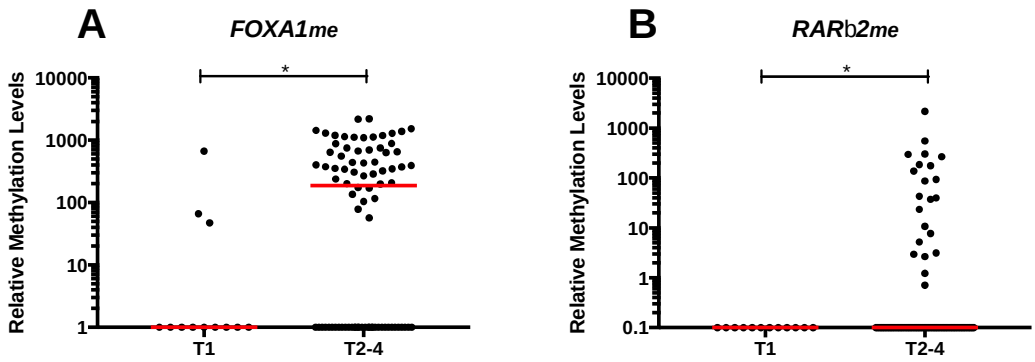

Figure 9. (A) *FOXA1* and (B) *RARβ2* promoter's methylation levels according with T stage [T1 (n=12) and T2-4 (n=82)] in lung cancer patients. Mann-Whitney U Test, *p<0.05. Red horizontal lines represent median methylation levels. (Raw data available in Appendix III: Supplementary Table 9).

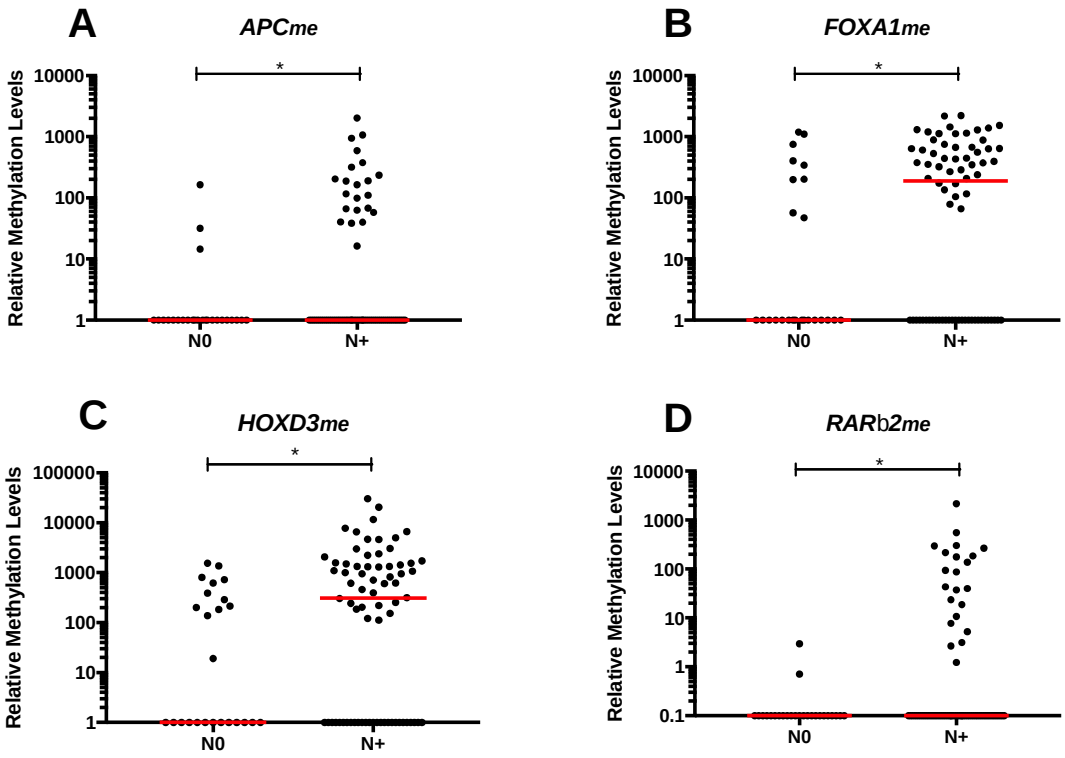

Figure 10. (A) *APC*, (B) *FOXA1*, (C) *HOXD3* and (D) *RARβ2* promoter's methylation levels according with node status, node-negative (N0) (n=25) and node-positive (N+) (n=72) in lung cancer patients. Mann-Whitney U Test, **p*<0.05. Red horizontal lines represent median methylation levels. (Raw data available in Appendix III: Supplementary Table 9)

Furthermore, circulating APC_{me}, $FOXA1_{me}$, $HOXD3_{me}$ and $RASSF1A_{me}$ levels were significantly higher in LC patients with distant metastatic disease (p=0.028, p=0.001, p<0.0001 and p=0.001, respectively) (Figure 11A), whereas $RAR\beta2_{me}$, $SEPT9_{me}$ and $SOX17_{me}$ levels were higher in CRC metastatic patients (p<0.0001, in all comparisons) (Figure 11B). Similarly, significantly higher APC_{me}, $GSTP1_{me}$, $HOXD3_{me}$, $RAR\beta2_{me}$, $RASSF1A_{me}$ and $SEPT9_{me}$ levels were observed in the five PCa patients with metastatic disease at diagnosis (p<0.0001, for all comparisons, except for $HOXD3_{me}$, p=0.003) (Appendix IV: Supplementary Figure 1).

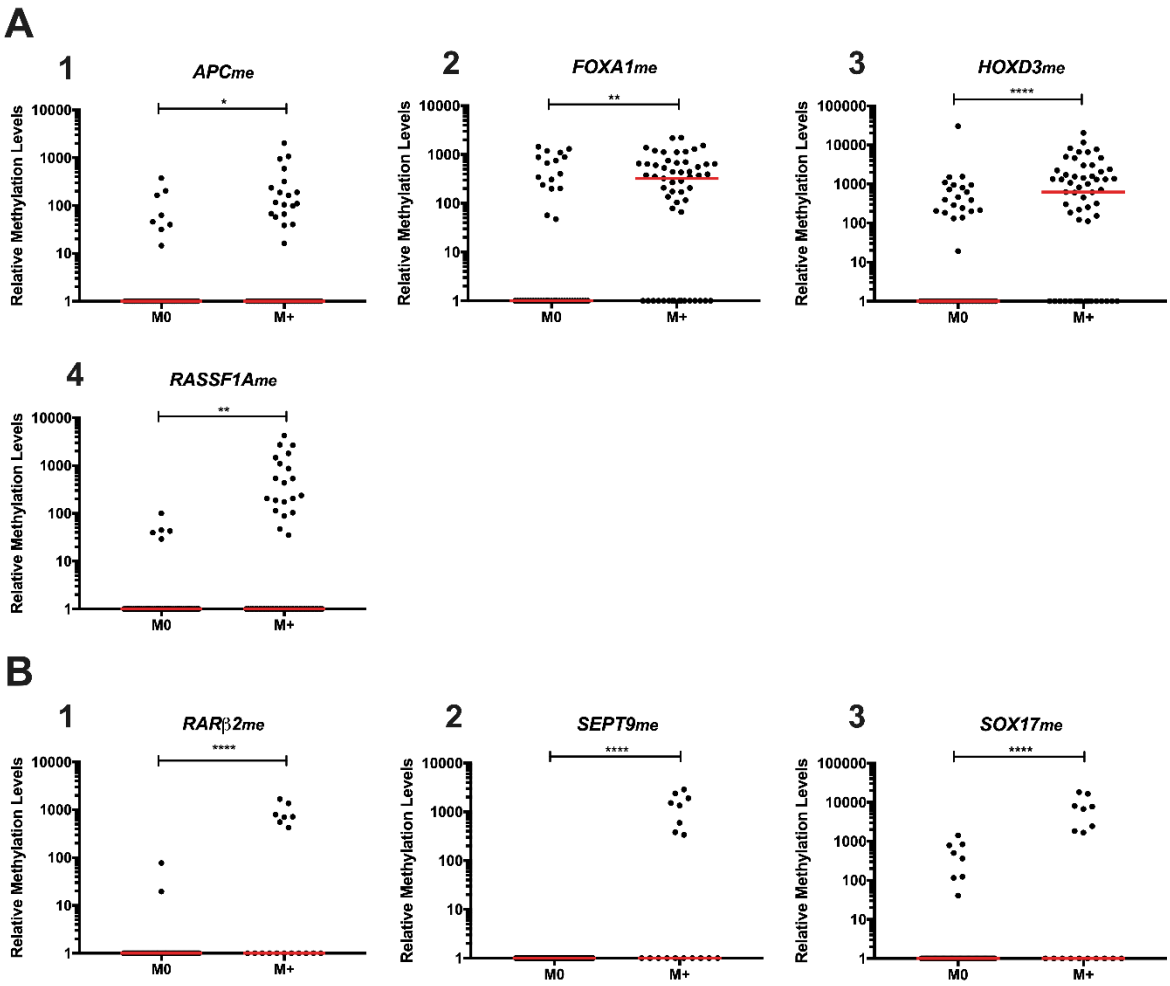

Figure 11. Distribution of methylation levels in lung (LC) (A) and in colorectal (CRC) (B) cancer patients according with metastatic dissemination. (A)-(1) *APC*, (2) *FOXA1*, (3) *HOXD3* and (4) *RASSF1A* promoter's methylation levels in non-metastatic (M0) (n=47) and metastatic (M+) (n=55) LC patients. (B)-(1) *RARβ2*, (2) *SEPT9* and (3) *SOX17* promoter's methylation levels in non-metastatic (M0) (n=82) and metastatic (M+) (n=18) CRC patients. Mann-Whitney U Test, **p*<0.05, ***p*<0.01, *****p*<0.0001. Red horizontal lines represent median methylation levels. (Raw data available in Appendix III: Supplementary Table 9)

Significantly higher circulating $RASSF1A_{me}$ levels were also apparent in LC patients with advanced clinical stage (III and IV) (p=0.043) (Figure 12A), whereas higher $GSTP1_{me}$ levels were observed in PCa patients with advanced clinical stage (III and IV) (p=0.039) comparing to patients with clinical stage I (Figure 12B). Similar results were found for $RARβ2_{me}$ and $SEPT9_{me}$ in CRC patients (p=0.012 and p=0.019, respectively) (Figure 12C).

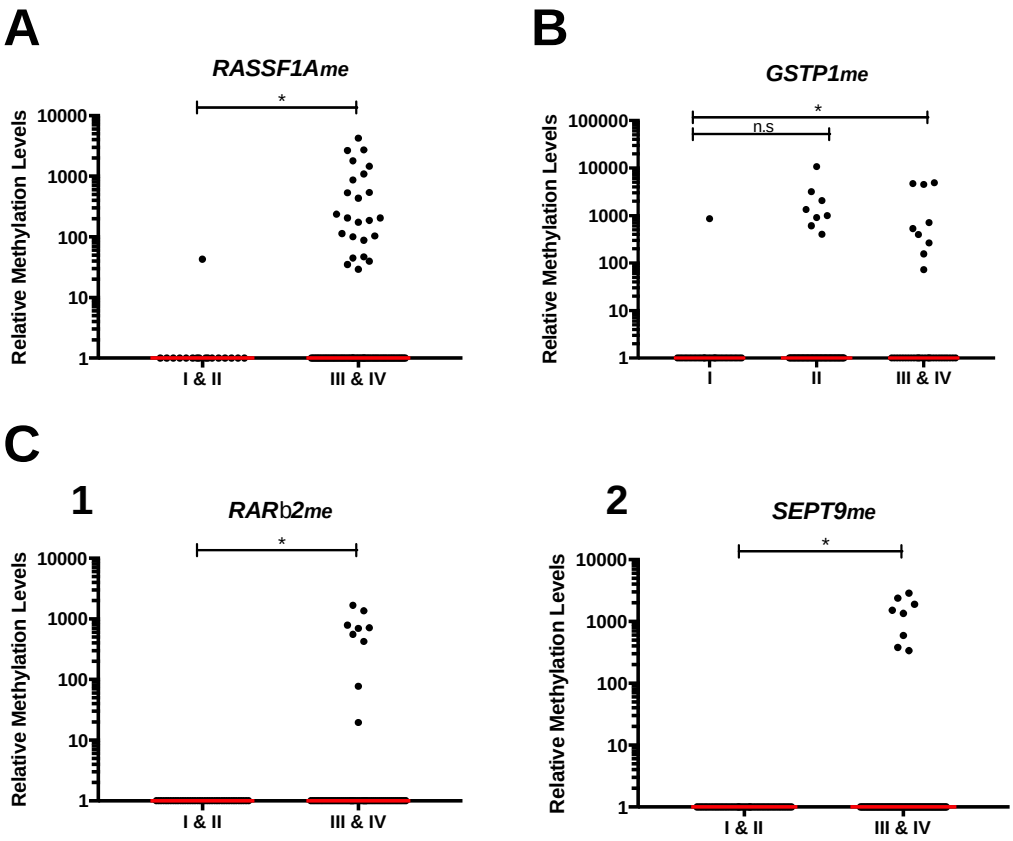

Figure 12. Scatter plot of (A) *RASSF1A* promoter methylation levels between Clinical Stage I & II (n=17) and III & IV (n=85) lung cancer patients. (B) *GSTP1* promoter methylation levels between Clinical stage I (n=31), II (n=55) and III & IV (n=35) prostate cancer patients. (C) (1) *RARβ2* and (2) *SEPT9* promoters' methylation levels between Clinical Stage I & II (n=39) and III & IV (n=61) CRC patients. Mann-Whitney U Test for A and C and Mann-Whitney U test with Bonferroni's correction for B, n.s. $p<0.05$, *$p<0.05$. Red horizontal lines represent median methylation. (Raw data available in Appendix III: Supplementary Table 9)

Concerning LC patients, significantly higher circulating $HOXD3_{me}$, $RASSF1A_{me}$ and $SOX17_{me}$ levels were observed in SCLC *vs* NSCLC patients ($p=0.001$, $p<0.001$ and $p=0.013$, respectively) (Figure 13). Therefore, we further assessed biomarker's performance of these three genes to discriminate between these two major histological subtypes (Table 12). Individually, all three genes identified SCLC with specificities above 75%. $RASSF1A_{me}$ disclosed the highest specificity (97.67%), correctly identifying 10 out of the 16 SCLC patients, misclassifying only 2 out of 86 NSCLC patients. Importantly, the panel including the two genes with the highest specificity ($HOXD3_{me}$ and $RASSF1A_{me}$) discriminated between LC subtypes with 75% sensitivity, 88.37% specificity and 86.27% accuracy (Table 12).

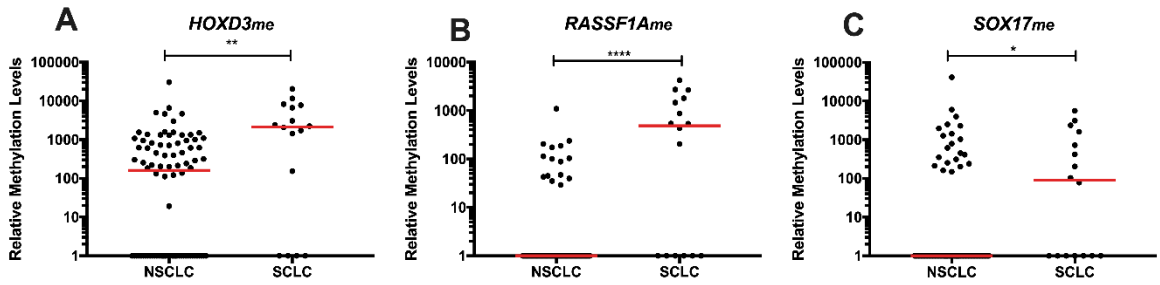

Figure 13. Scatter plot of (A) *HOXD3*, (B) *RASSF1A*, and (C) *SOX17* promoter's methylation levels according with Histological Subtype [Non-Small Cell Lung Carcinoma (NSCLC) (n=86) and Small Cell Lung Carcinoma (SCLC) (n=16)]. Mann-Whitney U Test, *p<0.05, **p<0.01, ****p<0.0001. Red horizontal lines represent median methylation levels. (Raw data available in Appendix III: Supplementary Table 9).

Table 12. Biomarker performance of *HOXD3, RASSF1A* and *SOX17* promoters' methylation levels for discrimination among Small Cell Lung Carcinoma (SCLC) and Non-Small cell Lung Carcinoma (NSCLC) in circulating cell-free DNA.

Genes	Cut-off value	Sensitivity %	Specificity %	Accuracy %
NSCLC *vs* SCLC				
HOXD3$_{me}$	1401.1301	68.75	88.37	85.29
RASSF1A$_{me}$	204.8474	62.50	97.67	92.16
SOX17$_{me}$	30.1876	56.25	75.58	72.55
HOXD3$_{me}$/RASSF1A$_{me}$	---	75.00	88.37	86.27

2.3. Prognostic Biomarker Performance of ccfDNA

The median patient follow-up time for LC patients' cohort was 9 months (range from 0-40 months), during which 62 patients have deceased due to cancer. Since only 15 out of 102 LC patients were submitted to surgery, DFS was not assessed. Moreover, no significant associations were disclosed between circulating methylation levels of the selected genes and PFS (data not shown).

DSS was significantly reduced in LC patients with lymph node involvement or distant metastasis (both p<0.0001), advanced clinical stage (p<0.0001) and SCLC subtype (p=0.011) (Appendix V: Supplementary Table 13 and Supplementary Figure 2), as expected. Interestingly, methylation of *APC* (p<0.0001), *FOXA1* (p=0.033), *RASSF1A* (p<0.0001), *SEPT9* (p=0.013) and *SOX17* (p=0.047) significantly associated with shorter DSS (Figure 14 and Appendix V: Supplementary Table 13).

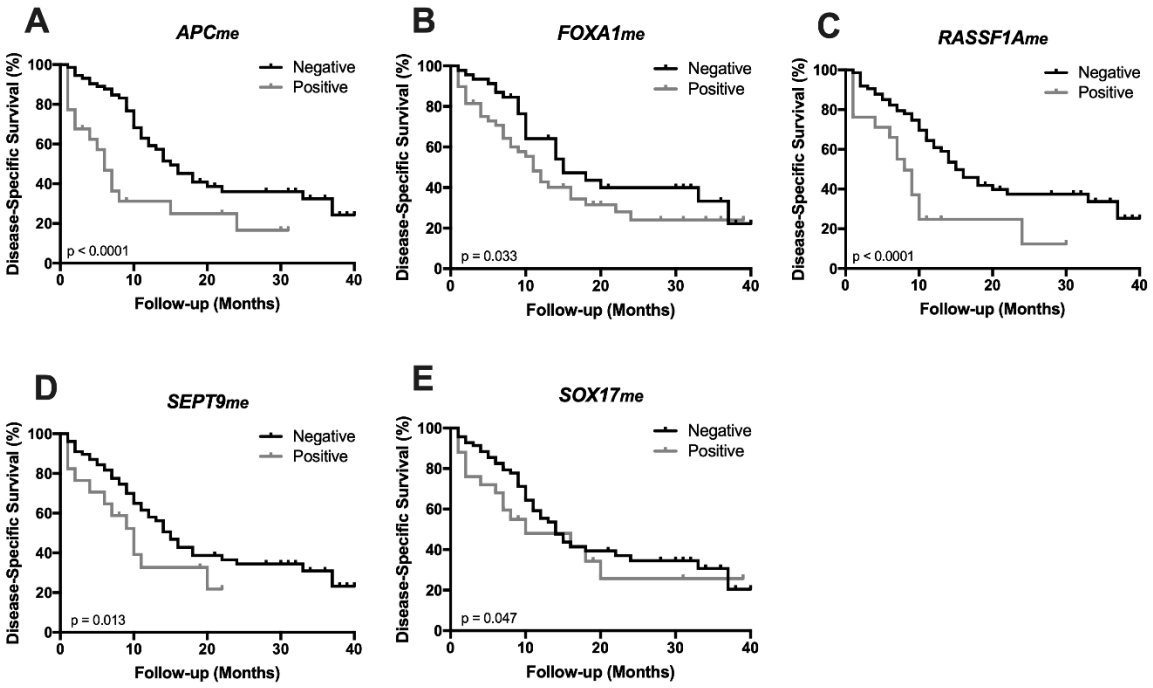

Figure 14. Disease-Specific Survival curves according to (A) *APC*, (B) *FOXA1*, (C) *RASSF1A*, (D) *SEPT9* and (E) *SOX17* promoter methylation status in circulating cell-free DNA of lung cancer patients.

As both *APC* and *RASSF1A* methylation positivity were strongly associated with shorter DSS, we combined them to study the clinical utility as a prognostic panel to better stratify outcome in LC patients. Indeed, this prognostic panel was significantly associated with DSS (p<0.0001) (Figure 15 and Appendix V: Supplementary Table 13). More than half (61.8%) of LC patients were classified as negative for methylation and presented a median survival time of 16 months (95% CI 11.138-20.862), whereas the 25.5% of the patients with one methylated gene promoter endured decreased median survival time to 8 months (95% CI 4.769-11.231). Remarkably, for the 12.7% patients classified as positive for both genes, the median DSS was reduced to 1 month (95% CI 0-3.273) (Figure 15 and Appendix V: Supplementary Table 13).

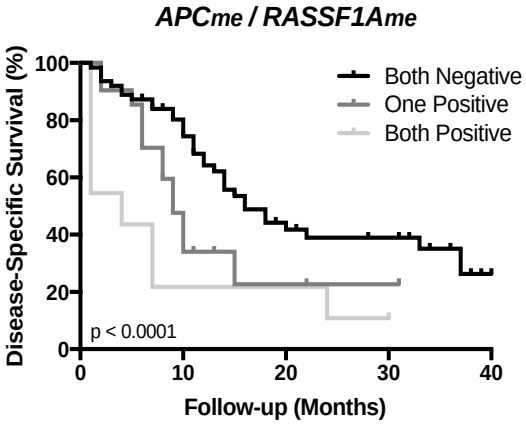

Figure 15. Disease-Specific Survival curves according to panel (*APC$_{me}$* and *RASSF1A$_{me}$*) promoter methylation status in circulating cell-free DNA of lung cancer patients.

On univariable Cox-regression analysis, circulating *APC$_{me}$* (p<0.0001), *FOXA1$_{me}$* (p=0.039), *RASSF1A$_{me}$* (p=0.001), *SEPT9$_{me}$* (p=0.018) and prognostic panel (*APC$_{me}$* and *RASSF1A$_{me}$*) (both negative *vs* one positive and both negative *vs* both positive) (p<0.0001) were predictors of disease-specific survival in LC patients, along with nodal involvement (p=0.001) and distant metastasis (p<0.0001) at diagnosis, clinical stage (p=0.001) and histological subtype (p=0.015) (Table 13). Remarkably, on multivariable Cox-regression analysis comprising the significant variables in univariable analysis [clinical stage, histological subtype, *FOXA1$_{me}$*, *SEPT9$_{me}$* and prognostic panel (*APC$_{me}$* and *RASSF1A$_{me}$*)], both clinical stage (p=0.002) and prognostic panel (*APC$_{me}$* and *RASSF1A$_{me}$*) (p<0.0001) were found to be independent DSS predictors (Table 13). Indeed, LC patients with detectable circulating *APC$_{me}$* and *RASSF1A$_{me}$* displayed a 3.918-fold risk (95%CI for HR 1.935-7.933, p<0.0001) of dying from LC comparing to those lacking methylation (Table 13).

Table 13. Cox-regression models assessing the potential of clinical variables and detection of circulating methylation levels of *APC, FOXA1, GSTP1, HOXD3, RARβ2, RASSF1A, SEPT9* and *SOX17* and prognostic panel (*APC, RASSF1A*) in the prediction of disease-specific survival in lung cancer patients.

Disease-Specific Survival	Variable	HR	95% CI for HR	*p* value
Univariable	**Regional node (N)**			
	N0 vs N+	4.067	1.827-9.051	**0.001**
	Distant Metastasis (M)			
	M0 vs M+	3.320	1.897-5.809	**<0.0001**
	Clinical Stage			
	I&II vs III&IV	5.594	2.012-15.549	**0.001**
	Histological Subtype			
	NSCLC vs SCLC	2.098	1.153-3.819	**0.015**
	APC$_{me}$			
	Negative vs positive	2.800	1.634-4.800	**<0.0001**
	FOXA1$_{me}$			
	Negative vs positive	1.716	1.027-2.867	**0.039**
	RASSF1A$_{me}$			
	Negative vs positive	2.669	1.529-4.660	**0.001**
	SEPT9$_{me}$			
	Negative vs positive	2.011	1.130-3.579	**0.018**
	SOX17$_{me}$			
	Negative vs positive	1.696	0.991-2.902	0.054
	APC$_{me}$ / RASSF1A$_{me}$			**<0.0001**
	Both negative vs One positive	2.582	1.428-4.671	**0.002**
	Both negative vs Both Positive	3.958	1.967-7.965	**<0.0001**
Multivariable	**Clinical Stage**			
	I&II vs III&IV	5.014	1.781-14.114	**0.002**
	APC$_{me}$ / RASSF1A$_{me}$			**<0.0001**
	Both negative vs One positive	1.992	1.100-3.608	**0.023**
	Both negative vs Both positive	3.918	1.935-7.933	**<0.0001**

PCa patient's cohort displayed the longer follow-up time, with a median of 85 months (range: 3 to 155 months). From the 85 patients treated with curative intent (radical prostatectomy, external beam radiotherapy or brachytherapy), 24 developed biochemical relapse and 12 endured disease progression. Moreover, nine out of the remainder 36 patients [subjected to androgen deprivation therapy or active surveillance] presented disease progression. Nonetheless, no relevant significant associations were disclosed between circulating methylation positivity and PFS or DFS (data not shown). Although 38 patients deceased during follow-up, only 11 deaths were attributable to PCa. DSS was significantly reduced in PCa patients with higher serum PSA levels at diagnosis (p<0.0001), higher ISUP Group Grade and advanced clinical stage (p=0.003, for both comparisons), as expected. Interestingly, patients with detectable circulating APC_{me} (p=0.001), $GSTP1_{me}$ (p=0.005), $RAR\beta2_{me}$ (p=0.001), $RASSF1A_{me}$, $SEPT9_{me}$ (both p<0.0001) or $SOX17_{me}$ (p=0.012) also disclosed shorter DSS (Appendix V: Supplementary Table 14 and Supplementary Figure 3). Nevertheless, Cox-regression analysis was not performed due to the reduced number of events in each group.

Regarding CRC patients, a median follow-up time of 13 months (range from 0 to 44 months) was achieved. From the 90 CRC patients submitted to surgery, 10 developed recurrence/progression. Given the difficulty to ascertain whether patients submitted to surgery were indeed free of the disease, DFS was not performed. CRC was the cause of death of nine of the 11 patients that deceased during follow-up. Presence of distant metastasis (p<0.0001) and detection of circulating $RAR\beta2_{me}$ (p<0.0001), $SEPT9_{me}$ (p<0.0001) and $SOX17_{me}$ (p=0.025) significantly associated with shorter DSS (Appendix V: Supplementary Table 15 and Supplementary Figure 4), although due to lack of events, no Cox-regression analysis was performed.

V. DISCUSSION

LC, PCa and CRC are the three most incident cancers in males worldwide, and the most deadly in Europe and North America [26]. Despite efforts to develop more effective screening methods, due to either low sensitivity, high false positive rate leading to overdiagnosis [79, 200, 201], or high invasiveness and cost [115], currently available strategies are suboptimal for population-based screening. Thus, development of new minimally invasive and effective pre-screening methods to triage patients for subsequent screening/detection tests are urgently needed. Aberrant DNA methylation of cancer-related genes occurs at very early stages of tumorigenesis, is cancer-specific, and amenable to be assessed in ccfDNA [3, 21], representing a valuable candidate tool for minimally-invasive early cancer detection. Thus, we assessed the clinical utility of a liquid biopsy-based strategy for simultaneous detection of LC, PCa and CRC in males using multiplex qMSP in ccfDNA.

The eight candidate genes tested were selected based on a critical analysis of published data available in PubMed, including previously published data by our research team [6, 42, 202]. Firstly, the performance of candidate genes was individually analysed for each cancer type. In accordance with previously published studies [39, 41, 42, 49, 51, 54, 60, 63, 64, 203], significantly higher circulating *APC*$_{me}$, *FOXA1*$_{me}$, *RARβ2*$_{me}$, *RASSF1A*$_{me}$, *SEPT9*$_{me}$ and *SOX17*$_{me}$ levels were observed in LC patients. Although the best panel for LC detection - *FOXA1*$_{me}$, *RARβ2*$_{me}$, *RASSF1A*$_{me}$, *SOX17*$_{me}$ - disclosed similar specificity to "Epi proLung®", it showed modest sensitivity [45]. Concerning PCa, except for *APC*$_{me}$ that was only reported in tissue and urine of cancer patients [186, 204], our results are in line with previously published studies showing *GSTP1*$_{me}$, *RARβ2*$_{me}$ and *RASSF1A*$_{me}$ hypermethylation in ccfDNA of PCa patients [82-84, 86]. Furthermore, to the best of our knowledge, we are the first research team reporting the biomarker potential of *FOXA1*$_{me}$, *HOXD3*$_{me}$, *SOX17*$_{me}$ and *SEPT9*$_{me}$ levels for PCa detection in blood-based liquid biopsies. Interestingly, the combination of *FOXA1*$_{me}$, *RARβ2*$_{me}$, *RASSF1A*$_{me}$ and *GSTP1*$_{me}$ identified PCa with 72% sensitivity and specificity. Although a panel comprising *RARβ2*$_{me}$, *RASSF1A*$_{me}$ and *GSTP1*$_{me}$ disclosing 29% sensitivity and 100% specificity to detect PCa was previously shown [83], the addition of *FOXA1*$_{me}$ to this panel in our study increased sensitivity although at expense of lower specificity. Nonetheless, the former panel was assessed using MSP [83], whereas we performed multiplex qMSP, which requires less amounts of DNA, is less time consuming and more sensitive. We also confirmed *SEPT9* and *SOX17* hypermethylation in ccfDNA of CRC patients, although displaying modest sensitivity (12%), in contrast with previous reports [42, 117]. Moreover, the disparate results observed for *APC*$_{me}$, *FOXA1*$_{me}$, *RARβ2*$_{me}$ and *RASSF1A*$_{me}$ [42, 122, 131, 137, 138, 141] might be due to differences in sample collection procedures, methodology, or population

studied, since we have only evaluated these genes in males. Interestingly, *HOXD3* was significantly hypomethylated in CRC samples.

Although the initial goal was to identify a panel suitable for simultaneous detection of LC, PCa and CRC in males, due to the results observed in CRC, we decided to move forward with simultaneous detection of LC and PCa, only, which constitute the two most incident cancers in males. The best combination to this end - "PanCancer" panel (*FOXA1*$_{me}$, *RARβ2*$_{me}$ and *RASSF1A*$_{me}$) – disclosed 70% specificity and 64% sensitivity, which is slightly lower than we have reported for the simultaneous detection of BrC, CRC and LC in women [42]. Nonetheless, compared to LD-CT LC screening [37], our test displayed considerable less false-positive results. Although asymptomatic donors included in our study do not represent a high-risk population, they might better reproduce the "real world" setting, nonetheless. Furthermore, the panel performance for detecting PCa, outperformed serum PSA specificity (which is around 20-45%) [205], although with limited sensitivity, Interestingly, our results are within the range reported for urinary long-non coding RNA Prostate Cancer Antigen 3 (PCA3), which is analysed in urine collected after prostatic massage [205, 206], a more invasive PCa screening approach. Thus, we are tempted to speculate whether our panel might add clinical value to serum PSA, increasing specificity and potentially reducing the number of unnecessary prostate biopsies, a hypothesis that deserves further studies.

Despite the modest sensitivity to detect early stage (I and II) LC, the high specificity of "PanCancer" panel to detect those patients suggests a potential usefulness for early detection of LC, reducing false-positive rates of some previously reported screening strategies. Moreover, our panel also discriminated intermediate and high-risk PCa (stage II-IV), according to the European Association of Urology (EAU) risk groups [96], with 71% sensitivity and 65% specificity, thus increasing the ability to identify clinically significant PCa. Therefore, it has the potential to prevent the excess morbidity associated with unnecessary biopsies and reduce overdiagnosis and consequent overtreatment. Hence, the minimally invasive "PanCancer" panel might be advantageous for population-based screening, limiting drawbacks of currently available methods, with the advantage of potentially detecting two cancer types in the same test.

Following a positive result in "PanCancer" panel, identifying the primary location (i.e., LC or PCa) is of foremost importance. We, thus, propose a second panel ("CancerType") comprising *SOX17*$_{me}$ and *GSTP1*$_{me}$, due to their remarkable specificity to discriminate between LC and PCa. Nonetheless, given the limited sensitivity of these two genes, this strategy must be complemented with other ancillary tests, including imaging. Notwithstanding, a positive "PanCancer" test should always trigger a search for LC or PCa and if any of these is identified, then closer follow-up and repeat testing must be performed.

Correlations between clinicopathological features and circulating methylation levels of candidate genes were also assessed. Interestingly, higher $FOXA1_{me}$ and $RAR\beta2_{me}$ associated with advanced LC tumour stage. Moreover, methylation of those two genes, as well as of APC_{me} and $HOXD3_{me}$ was also increased in node-positive disease. Furthermore, higher APC_{me}, $FOXA1_{me}$, $HOXD3_{me}$ and $RASSF1A_{me}$ associated with metastatic dissemination. To the best of our knowledge, only $RASSF1A_{me}$ and $SOX17_{me}$ in ccfDNA of LC patients have been previously associated with nodal and distant metastasis, and advanced stage [42, 53]. Increased $HOXD3_{me}$ levels were similarly observed in all five PCa patients with distant metastasis at diagnosis, along with higher APC_{me}, $GSTP1_{me}$, $RAR\beta2_{me}$, $RASSF1A_{me}$ and $SEPT9_{me}$ in four out of the same five patients. Likewise, higher $RAR\beta2_{me}$, $SEPT9_{me}$ and $SOX17_{me}$ levels were disclosed in metastatic CRC patients, paralleling recent reports which indicate the same trend for $SEPT9_{me}$ and $SOX17_{me}$ [42, 121, 162]. Thus, despite the early onset of epigenetic alterations in tumorigenesis, higher circulating methylation levels are detectable in cancer progression probably owing to increased tumour burden and metastatic spread.

Remarkably, a panel constituted by $HOXD3_{me}$ and $RASSF1A_{me}$ was able to discriminate SCLC from NSCLC with 75% sensitivity and 88% specificity. Interestingly, $RASSF1A_{me}$ potential for SCLC discrimination from NSCLC in tissue and plasma samples was previously observed in our group (Nunes and Diniz *et al., under review*). This distinction is critical as SCLC and NSCLC require quite different therapeutic strategies and display different prognosis. Although, an IVD approved miRNA-based test (miRview®) identifies the four main LC subtypes, in fine needle aspirates and cytological samples, with 90% sensitivity and specificity [207], our panel can discriminate the two major LC subtypes in liquid biopsies, which has a far less invasive collection method. Moreover, our panel displays similar performance to a three miRNAs panel assessed in plasma [208].

We further evaluated whether gene methylation might also convey independent prognostic information. Indeed, detection of circulating APC_{me}, $FOXA1_{me}$, $RASSF1A_{me}$, $SEPT9_{me}$ and $SOX17_{me}$ associated with reduced DSS in LC patients, alongside with all clinicopathological variables except tumour stage. Remarkably, the prognostic panel (APC_{me} and $RASSF1A_{me}$) is an independent predictor of DSS in LC patients. These results are in line with the prognostic value reported for circulating APC_{me} and $RASSF1A_{me}$ by other research teams, namely as biomarkers for evaluation of treatment efficacy and disease monitoring [41, 71, 209]. Moreover, detectable circulating APC_{me}, $GSTP1_{me}$, $RARB2_{me}$, $RASSF1A_{me}$, $SEPT9_{me}$ and $SOX17_{me}$ methylation, in PCa patients, and $RARB2_{me}$, $SEPT9_{me}$ and $SOX17_{me}$, in CRC patients, associated with reduced DSS, suggesting that methylation of selected genes in plasma might convey prognostic information, at diagnosis. Our observations are limited owing to the short follow-up time and the favourable prognosis of

most PCa and CRC patients, and, thus, additional validation in larger studies with longer follow-up, is warranted.

Overall, and notwithstanding the promising results of “PanCancer” panel for simultaneous detection of LC and PCa in males, it should be acknowledged that the lack of clinical information and long-term follow-up of asymptomatic controls constitutes a limitation. Thus, our results should be validated in future large multicentre prospective studies, including high-risk populations. Moreover, further studies should also be considered to find the best DNA methylation-based test to detect CRC, and ultimately, to be able to detect the four major cancers in both genders (LC, BrC, CRC and PCa).

VI. CONCLUSION & FUTURE PERSPECTIVES

In conclusion, our findings corroborate the hypothesis that a single minimally invasive screening test can be devised to simultaneously detect multiple malignancies, which might improve patient compliance and, thereby, increase tumours' detection rate at earlier stages, reducing cancer morbidity and mortality. Moreover, we also provide a novel tool to aid discrimination between the major LC subtypes and a prognostic panel that carries independent information for those patients.

Considering our findings, we foresee to:

- Test other methylated genes for CRC detection;
- Develop a multiplex ddMSP-based test with selected genes, aiming to overcome qMSP's limited sensitivity;
- Establish the most sensitive and specific gene-panel combination suitable for detection of the four major cancers in both genders (LC, BrC, CRC and PCa);
- Validate the results in large multicentre prospective studies, including high-risk populations.

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VIII. APPENDIX

APPENDIX I

Methylated genes' nomenclature

Supplementary table 1. Methylated genes' nomenclature according to HUGO Gene Nomenclature Committee (HGNC).

Symbol	Name
14-3-3σ$_{me}$ **or** ***SFN$_{me}$***	Methylated stratafin
AIM1$_{me}$ **or** ***CRYBG1$_{me}$***	Methylated crystallin beta-gamma domain containing 1
AJAP1$_{me}$	Methylated adherens junctions associated protein 1
ALX4$_{me}$	Methylated ALC homeobox 4
APC$_{me}$	Methylated APC regulator of WNT signaling pathway
BCAT1$_{me}$	Methylated branched chain amino acid transaminase 1
BMP3$_{me}$	Methylated bone morphogenetic protein 3
BRMS1$_{me}$	Methylated BRMS1 transcriptional repressor and anoikis regulator
CAHM$_{me}$	Methylated colon adenocarcinoma hypermethylated
CALCA$_{me}$	Methylated calcitonin related polypeptide alpha
CCDC181$_{me}$	Methylated coiled-coil domain containing 181
CDH13$_{me}$	Methylated cadherin 13
CDH1$_{me}$	Methylated cadherin 1
CDKN2A$_{me}$ **or** ***p14$^{ARF}{}_{me}$*** **or** ***p16$^{INK4A}{}_{me}$***	Methylated cyclin dependent kinase inhibitor 2A
CDO1$_{me}$	Methylated cysteine dioxygenase type 1
CHFR$_{me}$	Methylated checkpoint with forkhead and ring finger domains
CYCD2$_{me}$ **or** ***CCND2$_{me}$***	Methylated cyclin D2
DAPK1$_{me}$ **or** ***DAPK$_{me}$***	Methylated death associated protein kinase 1
DCC$_{me}$	Methylated DCC netrin 1 receptor
DCLK1$_{me}$	Methylated doublecortin like kinase 1
DLC1$_{me}$	Methylated DLC1 Rho GSTPase activating protein
DLEC1$_{me}$	Methylated DLEC1 cilia and flagella associated protein
EDNRB$_{me}$	Methylated endothelin receptor type B
EFHD1$_{me}$	Methylated EF-hand domain family member D1
ERα$_{me}$ **or** ***ESR1$_{me}$***	Methylated estrogen receptor 1
ERβ$_{me}$ **or** ***ESR2$_{me}$***	Methylated estrogen receptor 2
EYA4$_{me}$	Methylated EYA transcriptional coactivator and phosphatase 4
FGF5$_{me}$	Methylated fibroblast growth factor 5
FHIT$_{me}$	Methylated fragile histidine triad diadenosine triphosphatase
FOXA1$_{me}$	Methylated forkhead box A1
FOXE1$_{me}$	Methylated forkhead box E1
GADD45a$_{me}$	Methylated growth arrest and DNA damage inducible alpha
GATA5$_{me}$	Methylated GATA binding protein 5
GRASP$_{me}$	Methylated general receptor for phosphoinositides 1 associated scaffold protein
GRIA4$_{me}$	Methylated glutamate ionotropic receptor AMPA type subunit 4
GSTP1$_{me}$	Methylated glutathione S-transferase pi 1
HAPLN3$_{me}$	Methylated hyaluronan and proteoglycan link protein 3

HIC1$_{me}$	Methylated HIC ZBTB transcriptional repressor 1
HIST1H4K$_{me}$* or *H4C12$_{me}$	Methylated H4 clustered histone 12
HLTF$_{me}$	Methylated helicase like transcription factor
MLH1$_{me}$	Methylated mutL homolog 1
HOXA1$_{me}$	Methylated homeobox A1
HOXA9$_{me}$	Methylated homeobox A9
HOXD3$_{me}$	Methylated homeobox D3
HPP1$_{me}$	Methylated hyperpigmentation, progressive, 1
IKZF1$_{me}$	Methylated IKAROS family zinc finger 1
IRF4$_{me}$	Methylated interferon regulatory factor 4
ITGA4$_{me}$	Methylated integrin subunit alpha 4
Kif1a$_{me}$	Methylated kinesin family member 1A
KLK10$_{me}$	Methylated kallikrein related peptidase 10
MAP3K14-AS1$_{me}$	Methylated MAP3K14 antisense RNA 1
MARCH11$_{me}$* or *MARCHF11$_{me}$	Methylated membrane associated ring-CH-type finger 11
MCAM$_{me}$	Methylated melanoma cell adhesion molecule
MDR1$_{me}$* or *ABCB1$_{me}$	Methylated ATP binding cassette subfamily B member 1
MGMT$_{me}$	Methylated O-6-methylguanine-DNA methyltranferase
MSC$_{me}$	Methylated musculin
NELL1$_{me}$	Methylated neural EGFL like 1
NEUROG1$_{me}$	Methylated neurogenin 1
NGFR$_{me}$	Methylated nerve growth factor receptor
NID2$_{me}$	Methylated nidogen 2
NISCH$_{me}$	Methylated nischarin
NPTX2$_{me}$	Methylated neuronal pentraxin 2
NPY$_{me}$	Methylated neuropeptide Y
PCDH10$_{me}$	Methylated protocadherin 10
PCDH17$_{me}$	Methylated protocadherin 17
PCDH8$_{me}$	Methylated protocadherin 8
PDX1$_{me}$	Methylated pancreatic and duodenal homeobox 1
PENK$_{me}$	Methylated proenkephalin
PITX2$_{me}$	Methylated paired like homeodomain 2
PPP1R3C$_{me}$	Methylated protein phosphatase 1 regulatory subunit 3C
PRIMA1$_{me}$	Methylated proline rich membrane anchor 1
PTGDR$_{me}$	Methylated prostaglandin D2 receptor
PTGER4$_{me}$	Methylated prostaglandin E receptor 4
PTGS2$_{me}$	Methylated prostaglandin-endoperoxide synthase 2
RARβ2$_{me}$* or *RARβ$_{me}$	Methylated retinoic acid receptor beta
RASSF1A$_{me}$	Methylated Ras associated domain family member 1 isoform A
RASSF2A$_{me}$	Methylated Ras associated domain family member 2 isoform A
RPRM$_{me}$	Methylated reprimo, TP53 dependent G2 arrest mediator homolog
RUNX3$_{me}$	Methylated RUNX family transcription factor 3
SCGB3A1$_{me}$	Methylated secretoglobin family 3A member 1
SDC2$_{me}$	Methylated syndecan 2
SEPT9$_{me}$	Methylated septin 9
SFRP1$_{me}$	Methylated secreted frizzled related protein 1
SFRP2$_{me}$	Methylated secreted frizzled related protein 2

SHOX2$_{me}$	Methylated short stature homeobox 2
SHP1P2$_{me}$ or PTPN6$_{me}$	Methylated protein tyrosine phosphatase non-receptor type 6
SMAD4$_{me}$	Methylated SMAD family member 4
SOX17$_{me}$	Methylated SRY-box transcription factor 17
SOX21$_{me}$	Methylated SRY-box transcription factor 21
SPG20$_{me}$* or *SPART$_{me}$	Methylated spartin
SST$_{me}$	Methylated somatostatin
ST6GALNAC3$_{me}$	Methylated ST6 N-acetylgalactosaminide alpha-2,6-sialytransferase 3
SYNE1$_{me}$	Methylated spectrin repeat containing nuclear envelope protein 1
TAC1$_{me}$	Methylated tachykinin precursor 1
TFAP2E$_{me}$	Methylated transcription factor AP-2 epsilon
TFPI2$_{me}$	Methylated tissue factor pathway inhibitor 2
TIG1$_{me}$* or *RARRES1$_{me}$	Methylated retinoic acid receptor responder 1
TMEFF2$_{me}$	Methylated transmembrane protein with EGF like and two follistatin like domains 2
TWIST1$_{me}$	Methylated twist family bHLH transcription factor 1
UNCX$_{me}$	Methylated UNC homeobox
VIM$_{me}$	Methylated vimentin
WIF1$_{me}$	Methylated WNT inhibitory factor 1
WT1$_{me}$	Methylated WT1 transcription factor
ZMYND10$_{me}$	Methylated zinc finger MYND-type containing 10
ZNF660$_{me}$	Methylated zinc finger protein 600

APPENDIX II

TNM Staging

Supplementary Table 2. TNM classification of lung cancer according to the 8th edition American Joint Committee on Cancer (AJCC) guidelines (adapted from [67]).

Primary Tumour (T)	
Tx	Primary tumour cannot be assessed, or tumour proven by the presence of malignant cells in sputum or bronchial washings but not visualized by imaging or bronchoscopy
T0	No evidence of primary tumour
Tis	Carcinoma *in situ* Squamous cell carcinoma *in situ* (SCIS) Adenocarcinoma *in situ* (AIS): adenocarcinoma with pure lepidic pattern. ≤3 cm in greatest dimension
T1	Tumour ≤3 cm in greatest dimension, surrounded by lung or visceral pleural, without bronchoscopic evidence of invasion more proximal than the lobar bronchus (i.e., not in the main bronchus)
T1mi	Minimally invasive adenocarcinoma: adenocarcinoma (≤3 cm in greatest dimension) with a predominantly lepidic pattern and ≤5 mm invasion in greatest dimension
T1a	Tumour ≤1 cm in greatest dimension. A superficial, spreading tumour of any size whose invasive component is limited to the bronchial wall and may extend proximal to the main bronchus also is classified as T1a, but these tumours are uncommon.
T1b	Tumour >1 cm but ≤2 cm in greatest dimension
T1c	Tumour >2 cm but ≤3 cm in greatest dimension
T2	Tumour >3 cm but ≤5 cm or having any of the following features: • Involves the main bronchus regardless of distance to the carina, but without involvement of the carina • Invades visceral pleural (PL1 or PL2) • Associated with atelectasis or obstructive pneumonitis that extends to the hilar region, involving part or all of the lung
T2a	Tumour >3 cm but ≤4 cm in greatest dimension
T2b	Tumour >4 cm but ≤5 cm in greatest dimension
T3	Tumour >5 cm but ≤7 cm in greatest dimension or directly invading any of the following: parietal pleura (PL3), chest wall (including superior sulcus tumours), phrenic nerve, parietal pericardium; or separate tumour nodule(s) in the same lobe as the primary
T4	Tumour >7 cm or tumour of any size invading one or more of the following: diaphragm, mediastinum, heart, great vessels, trachea, recurrent laryngeal nerve, oesophagus, vertebral body, or carina: separate tumour nodule(s) in an ipsilateral lobe different from that of the primary

Regional Lymph node (N)	
Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in ipsilateral peribronchial and/or ipsilateral hilar lymph nodes and intrapulmonary nodes, including involvement by direct extension
N2	Metastasis in ipsilateral mediastinal and/or subcarinal lymph node(s)
N3	Metastasis in contralateral mediastinal, contralateral hilar, ipsilateral or contralateral scalene, or supraclavicular lymph node(s)
Distant Metastasis (M)	
M0	No distant metastasis
M1	Distant metastasis
M1a	Separate tumour nodule(s) in a contralateral lobe; tumour with pleural or pericardial nodules or malignant pleural or pericardial effusion
M1b	Single extrathoracic metastasis in a single organ (including involvement of a single nonregional node)
M1c	Multiple extrathoracic metastases in a single organ or in multiple organs

Supplementary Table 3. American Joint Committee on Cancer (AJCC) prognostic stage groups of lung cancer according to the 8th edition AJCC guidelines (adapted from [67]).

Primary Tumour (T)	Regional Lymph Node (N)	Distant Metastasis (M)	Stage
Tx	N0	M0	Occult carcinoma
Tis	N0	M0	0
T1mi	N0	M0	IA1
T1a	N0	M0	IA1
T1a	N1	M0	IIB
T1a	N2	M0	IIIA
T1a	N3	M0	IIIB
T1b	N0	M0	IA2
T1b	N1	M0	IIB
T1b	N2	M0	IIIA
T1b	N3	M0	IIIB
T1c	N0	M0	IA3
T1c	N1	M0	IIB
T1c	N2	M0	IIIA
T1c	N3	M0	IIIB
T2a	N0	M0	IB
T2a	N1	M0	IIB
T2a	N2	M0	IIIA
T2a	N3	M0	IIIB
T2b	N0	M0	IIA
T2b	N1	M0	IIB
T2b	N2	M0	IIIA
T2b	N3	M0	IIIB
T3	N0	M0	IIB

T3	N1	M0	IIIA
T3	N2	M0	IIIB
T3	N3	M0	IIIC
T4	N0	M0	IIIA
T4	N1	M0	IIIA
T4	N2	M0	IIIB
T4	N3	M0	IIIC
Any T	Any N	M1a	IVA
Any T	Any N	M1b	IVA
Any T	Any N	M1c	IVB

Supplementary Table 4. TNM classification of prostate cancer according to the 8th edition American Joint Committee on Cancer (AJCC) guidelines (adapted from [67]).

Clinical Primary Tumour (cT)	
Tx	Primary tumour cannot be assessed
T0	No evidence of primary tumour
T1	Clinically inapparent tumour that is not palpable
T1a	Tumour incidental histologic finding in 5% or less of tissue resected
T1b	Tumour incidental histologic finding in more than 5% of tissue resected
T1c	Tumour identified by needle biopsy found in one or both sides, but not palpable
T2	Tumour is palpable and confined within prostate
T2a	Tumour involves one-half of one side or less
T2b	Tumour involves more than one-half of one side but not both sides
T2c	Tumour involves both sides
T3	Extraprostatic tumour that is not fixed or does not invade adjacent structures
T3a	Extraprostatic extension (unilateral or bilateral)
T3b	Tumour invades seminal vesicle(s)
T4	Tumour is fixed or invades adjacent structures other than seminal vesicles such as external sphincter, rectum, bladder, levator muscles, and/or pelvic wall
Pathological Primary Tumour (pT)	
T2	Organ confined
T3	Extraprostatic extension
T3a	Extraprostatic extension (unilateral or bilateral) or microscopic invasion of bladder neck
T3b	Tumour invades seminal vesicle(s)
T4	Tumour is fixed or invades adjacent structures other than seminal vesicles such as external sphincter, rectum, bladder, levator muscles, and/or pelvic wall
Regional Lymph node (N)	
Nx	Regional nodes were not assessed
N0	No positive regional nodes
N1	Metastases in regional node(s)
Distant Metastasis (M)	
M0	No distant metastasis
M1	Distant metastasis
M1a	Nonregional lymph node(s)
M1b	Bone(s)
M1c	Other site(s) with or without bone disease

Supplementary Table 5. Histological ISUP Grade Groups of prostate cancer (adapted from [67]).

ISUP Grade Group	Gleason Score	Gleason Pattern
1	≤6	≤3+3
2	7	3+4
3	7	4+3
4	8	4+4
5	9 or 10	4+5, 5+4, or 5+5

Supplementary Table 6. American Joint Committee on Cancer (AJCC) prognostic stage groups of prostate cancer according to the 8th edition AJCC guidelines (adapted from [67]).

Primary Tumour (T)	Regional Lymph Node (N)	Distant Metastasis (M)	PSA (ng/mL)	ISUP Grade Group	Stage
cT1a-c, cT2a	N0	M0	<10	1	I
pT2	N0	M0	<10	1	I
cT1a-c, cT2a	N0	M0	≥10 <20	1	IIA
cT2b-c	N0	M0	<20	1	IIA
T1-2	N0	M0	<20	2	IIB
T1-2	N0	M0	<20	3	IIC
T1-2	N0	M0	<20	4	IIC
T1-2	N0	M0	≥20	1-4	IIIA
T3-4	N0	M0	Any	1-4	IIIB
Any T	N0	M0	Any	5	IIIC
Any T	N1	M0	Any	Any	IVA
Any T	N0	M1	Any	Any	IVB

Supplementary Table 7. TNM classification of colorectal cancer according to the 8th edition American Joint Committee on Cancer (AJCC) guidelines (adapted from [67]).

Primary Tumour (T)	
Tx	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma *in situ*, intramucosal carcinoma (involvement of lamina propria with no extension through muscularis mucosae)
T1	Tumour invades the submucosa (through the muscularis mucosa but not into the muscularis propria)
T2	Tumour invades the muscularis propria
T3	Tumour invades through the muscularis propria into pericolorectal tissues
T4	Tumour invades the visceral peritoneum or invades or adheres to adjacent organ or structure
T4a	Tumour invades through the visceral peritoneum (including gross perforation of the bowel through tumour and continuous invasion of tumour through areas of inflammation to the surface of the visceral peritoneum)
T4b	Tumour directly invades or adheres to adjacent organs or structures
Regional Lymph node (N)	
Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	One to three regional lymph nodes are positive (tumour in lymph nodes measuring ≥0.2 mm), or any number of tumour deposits are present and all identifiable lymph node are negative
N1a	One regional lymph node is positive
N1b	Two or three regional lymph nodes are positive
N1c	No regional lymph nodes are positive, but there are tumour deposits in the subserosa, mesentery or nonperitonealized pericolic, or perirectal/mesorectal tissues
N2	Four or more regional nodes are positive
N2a	Four to six regional lymph nodes are positive
N2b	Seven or more regional lymph nodes are positive
Distant Metastasis (M)	
M0	No distant metastasis by imaging, etc.; no evidence of tumour in distant sites or organs
M1	Metastasis to one or more distant sites or organs or peritoneal metastasis is identified
M1a	Metastasis to one site or organ is identified without peritoneal metastasis
M1b	Metastasis to two or more sites or organs is identified without peritoneal metastasis
M1c	Metastasis to the peritoneal surface is identified alone or with other site or organ metastasis

Supplementary Table 8. American Joint Committee on Cancer (AJCC) prognostic stage groups of colorectal cancer according to the 8th edition AJCC guidelines (adapted from [67]).

Primary Tumour (T)	Regional Lymph Node (N)	Distant Metastasis (M)	Stage
Tis	N0	M0	0
T1, T2	N0	M0	I
T3	N0	M0	IIA
T4a	N0	M0	IIB
T4b	N0	M0	IIC
T1-T2	N1/N1c	M0	IIIA
T1	N2a	M0	IIIA
T3-T4a	N1/N1c	M0	IIIB
T2-T3	N2a	M0	IIIB
T1-T2	N2b	M0	IIIB
T4a	N2a	M0	IIIC
T3-T4a	N2b	M0	IIIC
T4b	N1-N2	M0	IIIC
Any T	Any N	M1a	IVA
Any T	Any N	M1b	IVB
Any T	Any N	M1c	IVC

APPENDIX III

Distribution of Promoters' Methylation Levels

Supplementary Table 9. Distribution of promoters' methylation levels of the different genes analysed.

		Relative methylation levels	Patients n			
Figure 7			**AC**	**LC**	**PCa**	**CRC**
A (*APC$_{me}$*)		**> 0, = 0**	21, 115	27, 75	24, 97	12, 88
B *(FOXA1$_{me}$)*		**> 0, = 0**	58, 78	55, 47	90, 31	47, 53
C (*GSTP1$_{me}$*)		**> 0, = 0**	3, 133	3, 99	18, 103	1, 99
D *(HOXD3$_{me}$)*		**> 0, = 0**	78, 58	60, 42	97,24	40, 60
E *(RARβ2$_{me}$)*		**> 0, = 0**	5, 131	24, 78	27, 94	9, 91
F *(RASSF1A$_{me}$)*		**> 0, = 0**	6, 130	25, 77	16, 105	7, 93
G *(SEPT9$_{me}$)*		**> 0, = 0**	2, 134	21, 81	14, 107	8, 92
H *(SOX17$_{me}$)*		**> 0, = 0**	9, 127	30, 72	35, 86	16, 84
Figure 9			**T1**	**T2-4**		
A *(FOXA1$_{me}$)*		**> 0, = 0**	3, 9	34, 48		
B *(RARβ2$_{me}$)*		**> 0, = 0**	0, 12	22, 60		
Figure 10			**N0**	**N+**		
A (*APC$_{me}$*)		**> 0, = 0**	3, 22	22, 50		
B *(FOXA1$_{me}$)*		**> 0, = 0**	9, 16	43,29		
C *(HOXD3$_{me}$)*		**> 0, = 0**	12, 13	45, 27		
D *(RARβ2$_{me}$)*		**> 0, = 0**	2, 23	22,50		
Figure 11			**M0**	**M+**		
A	**1** (*APC$_{me}$*)	**> 0, = 0**	8, 39	19, 36		
	2 *(FOXA1$_{me}$)*	**> 0, = 0**	16, 31	39, 16		
	3 *(HOXD3$_{me}$)*	**> 0, = 0**	21, 26	39, 16		
	4 *(RASSF1A$_{me}$)*	**> 0, = 0**	5, 42	20, 35		
B	**1** *(RARβ2$_{me}$)*	**> 0, = 0**	2, 80	7, 11		
	2 *(SEPT9$_{me}$)*	**> 0, = 0**	0, 82	8, 10		
	3 *(SOX17$_{me}$)*	**> 0, = 0**	8, 74	8, 10		

Figure 12		**I & II**	**III & IV**	
A *(RASSF1A$_{me}$)*	**> 0, = 0**	1, 16	61, 24	
		I	**II**	**III & IV**
B (*GSTP1$_{me}$)*	**> 0, = 0**	1, 30	8, 47	9,26
		I & II	**III & IV**	
C **1** *(RARβ2$_{me}$)*	**> 0, = 0**	0, 39	9, 52	
2 *(SEPT9$_{me}$)*	**> 0, = 0**	0, 39	8, 53	
Figure 13		**NSCLC**	**SCLC**	
A *(HOXD3$_{me}$)*	**> 0, = 0**	48, 38	12,4	
B *(RASSF1A$_{me}$)*	**> 0, = 0**	15, 71	10, 6	
C *(SOX17$_{me}$)*	**> 0, = 0**	21, 65	9, 7	
Supplementary Figure 1		**M0**	**M+**	
A (*APC$_{me}$)*	**> 0, = 0**	20, 96	4, 1	
B (*GSTP1$_{me}$)*	**> 0, = 0**	14, 102	4, 1	
C *(HOXD3$_{me}$)*	**> 0, = 0**	92, 24	5, 0	
D *(RARβ2$_{me}$)*	**> 0, = 0**	23, 93	4, 1	
E *(RASSF1A$_{me}$)*	**> 0, = 0**	12, 104	4, 1	
F *(SEPT9$_{me}$)*	**> 0, = 0**	10, 106	4, 1	

APPENDIX IV

Association between Promoters' Methylation Levels and Clinicopathological Features in ccfDNA

Supplementary Table 10. Associations between lung cancer patients' clinicopathological features and *APC*, *FOXA1*, *GSTP1*, *HOXD3*, *RARβ2*, *RASSF1A*, *SEPT9* and *SOX17* promoters' methylation levels. *p*-values obtained by Mann-Whitney U Test.

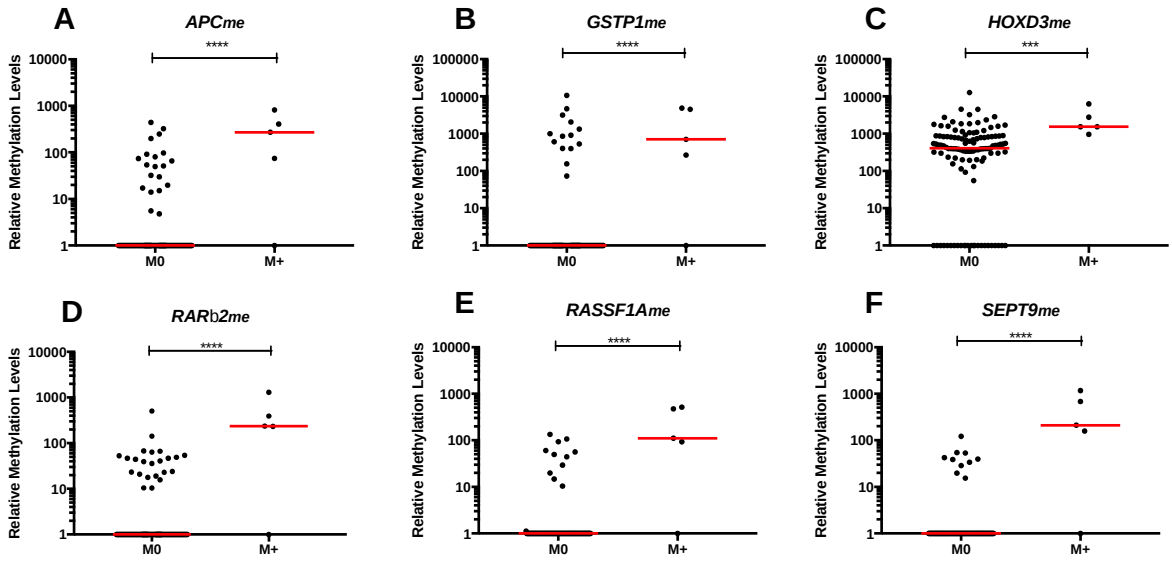
Genes	***APC*$_{me}$**	***FOXA1*$_{me}$**	***GSTP1*$_{me}$**	***HOXD3*$_{me}$**	***RARβ2*$_{me}$**	***RASSF1A*$_{me}$**	***SEPT9*$_{me}$**	***SOX17*$_{me}$**
Clinicopathological features	*p*-value							
Histological type	0.162	0.516	0.379	**0.001**	0.338	**<0.0001**	0.532	**0.013**
Primary Tumour (T)	0.490	**0.014**	0.503	0.622	**0.044**	0.137	0.066	0.091
Regional Node (N)	**0.044**	**0.021**	0.302	**0.022**	**0.015**	0.085	0.381	0.471
Distant Metastasis (M)	**0.028**	**0.001**	0.655	**<0.0001**	0.323	**0.001**	0.179	0.119
Clinical Stage	0.080	0.228	0.434	0.784	0.141	**0.043**	0.277	0.469

Supplementary Table 11. Associations between prostate cancer patients' clinicopathological features and *APC, FOXA1, GSTP1, HOXD3, RARβ2, RASSF1A, SEPT9* and *SOX17* promoters' methylation levels. *p*-values obtained by Mann-Whitney U Test for Primary Tumour (T) and Distant Metastasis (M), and by Kruskal-Wallis Test for ISUP Grade Group, serum PSA levels and Clinical Stage. Regional Node (N) was not evaluated due to the low number of N+ samples.

Genes	***APC*$_{me}$**	***FOXA1*$_{me}$**	***GSTP1*$_{me}$**	***HOXD3*$_{me}$**	***RARβ2*$_{me}$**	***RASSF1A*$_{me}$**	***SEPT9*$_{me}$**	***SOX17*$_{me}$**
Clinicopathological features	*p*-value							
Primary Tumour (T)	0.170	0.861	0.584	0.994	0.357	0.989	0.577	0.865
Distant Metastasis (M)	**<0.0001**	0.979	**<0.0001**	**0.003**	**<0.0001**	**<0.0001**	**<0.0001**	0.111
ISUP Grade Group	0.355	0.091	0.056	0.565	0.269	0.095	0.060	0.852
PSA	0.052	0.413	0.237	0.084	0.396	0.213	**0.038**	0.624
Clinical Stage	**0.001**	0.841	**<0.0001**	0.073	**0.001**	**<0.0001**	**<0.0001**	0.052

Supplementary Table 12. Associations between colorectal cancer patients' clinicopathological features and *APC, FOXA1, GSTP1, HOXD3, RARβ2, RASSF1A, SEPT9* and *SOX17* promoters' methylation levels. *p*-values obtained by Mann-Whitney U Test for Primary Tumour (T), Regional Node (N), Distant Metastasis (M) and Clinical Stage, and by Kruskal-Wallis Test for Tumour Location. Histological type was not evaluated due to the low number of squamous cell carcinoma samples.

Genes	***APC*$_{me}$**	***FOXA1*$_{me}$**	***GSTP1*$_{me}$**	***HOXD3*$_{me}$**	***RARβ2*$_{me}$**	***RASSF1A*$_{me}$**	***SEPT9*$_{me}$**	***SOX17*$_{me}$**
Clinicopathological features	*p*-value							
Tumour Location	0.898	0.884	0.487	0.932	0.068	0.267	0.416	0.249
Primary Tumour (T)	0.941	0.553	0.548	0.524	0.078	0.449	0.101	0.169
Regional Node (N)	0.358	0.534	0.402	0.199	0.083	0.472	0.127	0.460
Distant Metastasis (M)	0.306	0.521	0.639	0.723	**<0.0001**	0.059	**<0.0001**	**<0.0001**
Clinical Stage	0.277	0.638	0.424	0.133	**0.012**	0.549	**0.019**	0.160



Supplementary Figure 1. Distribution of methylation levels in prostate cancer (PCa) patients according with metastatic dissemination. (A) *APC*, (B) *GSTP1*, (C) *HOXD3*, (D) *RARβ2*, (E) *RASSF1A* and (F) *SEPT9* promoter's methylation levels between non-metastatic (M0) (n=116) and metastatic (M+) (n=5) PCa patients. Mann-Whitney U Test, ***p<0.001, ****p<0.0001. Red horizontal lines represent median methylation levels. (Raw data available in Appendix III: Supplementary Table 9)

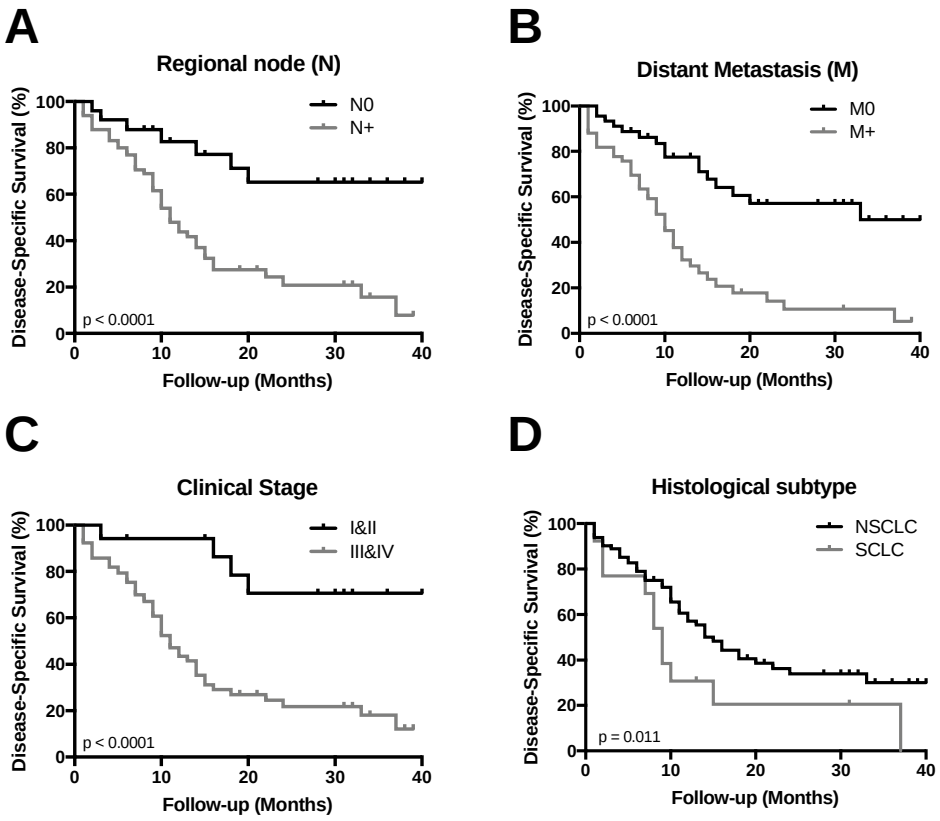
APPENDIX V

Association between Clinicopathological Features and Promoters' Methylation Levels and Disease-Specific Survival in ccfDNA

Supplementary Table 13. Demographics of the clinicopathological features and *APC, FOXA1, GSTP1, HOXD3, RARβ2, RASSF1A, SEPT9* and *SOX17* promoters' methylation levels in lung cancer patients, and their association with disease-specific survival. *p*-values obtained by log-rank test.

	Patients n (%)	**Disease-specific survival *p*-value**
Tumour stage (T)[a] (T1, T2-4)	12 (11.8%), 82 (80.4%)	0.090
Regional node (N)[b] (N0, N+)	25 (24.5%), 72 (70.6%)	**<0.0001**
Distant Metastasis (M) (M0, M+)	47 (46.1%), 55 (53.9%)	**<0.0001**
Clinical Stage (I&II, III&IV)	17 (16.7%), 85 (83.3%)	**<0.0001**
Histological Subtype (NSCLC, SCLC)	86 (84.3%), 16 (15.7%)	**0.011**
APC$_{me}$ (Negative, positive)	75 (73.5%), 27 (26.5%)	**<0.0001**
FOXA1$_{me}$ (Negative, positive)	47 (46.1%), 55 (53.9%)	**0.033**
GSTP1$_{me}$ (Negative, positive)	99 (97.1%), 3 (2.9%)	0.089
HOXD3$_{me}$ (Negative, positive)	42 (41.2%), 60 (58.8%)	0.051
RARβ2$_{me}$ (Negative, positive)	78 (76.5%), 24 (23.5%)	0.229
RASSF1A$_{me}$ (Negative, positive)	77 (75.5%), 25 (24.5%)	**<0.0001**
SEPT9$_{me}$ (Negative, positive)	81 (79.4%), 21 (20.6%)	**0.013**
SOX17$_{me}$ (Negative, positive)	72 (70.6%), 30 (29.4%)	**0.047**
APC$_{me}$* / *RASSF1A$_{me}$ (Both negative, One positive, Both Positive)	63 (61.8%), 26 (25.5%), 13 (12.7%)	**<0.0001**

[a]No information available in 8 cases (7.8%); [b]No information available in 5 cases (4.9%).

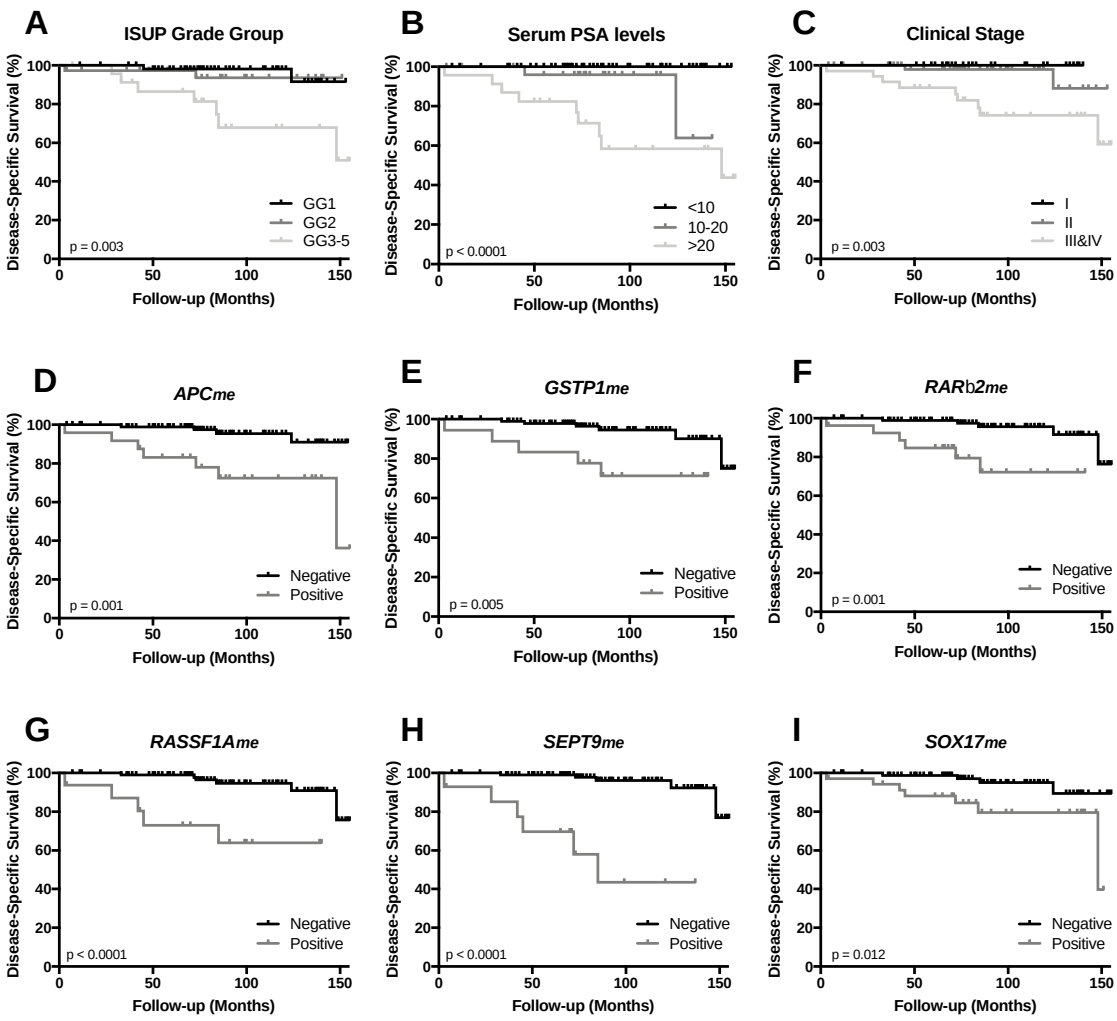


Supplementary Figure 2. Disease-specific survival curves according to clinicopathological variables (A) regional node, (B) distant metastasis, (C) clinical stage and (D) histological subtype in lung cancer patients.

Supplementary Table 14. Demographics of the clinicopathological features and *APC, FOXA1, GSTP1, HOXD3, RARβ2, RASSF1A, SEPT9* and *SOX17* promoters' methylation levels in prostate cancer patients, and their association with disease-specific survival. *p*-values obtain by log-rank test.

	Patients n (%)	**Disease-specific survival *p*-value**
Tumour stage (T)[a] (T1-2, T3)	104 (86%), 16 (13.2)	0.993
ISUP Grade Group (1, 2, 3-5)	59 (48.8%), 38 (31.4%), 24 (19.8%)	**0.003**
PSA levels (<10, 10-20, <20)	71 (58.7%), 27 (22.3%), 23 (19%)	**<0.0001**
Clinical Stage (I, II, III&IV)	31 (25.6%), 55 (45.5%), 35 (28.9%)	**0.003**
APC$_{me}$ (Negative, positive)	97 (80.2%), 24 (19.8%)	**0.001**
FOXA1$_{me}$ (Negative, positive)	31 (25.6%), 90 (74.4%)	0.919
GSTP1$_{me}$ (Negative, positive)	103 (85.1%), 18 (14.9%)	**0.005**
HOXD3$_{me}$ (Negative, positive)	24 (19.8%), 97 (80.2%)	0.111
RARβ2$_{me}$ (Negative, positive)	94 (77.7%), 27 (22.3%)	**0.001**
RASSF1A$_{me}$ (Negative, positive)	105 (86.8%), 16 (13.2%)	**<0.0001**
SEPT9$_{me}$ (Negative, positive)	107 (88.4%), 14 (11.6%)	**<0.0001**
SOX17$_{me}$ (Negative, positive)	86 (71.1%), 35 (28.9%)	**0.012**

[a]No information available in 1 case (0.8%); Regional Node (N) and Distant Metastasis (M) was not evaluated due to the low number of N+ and M+ samples

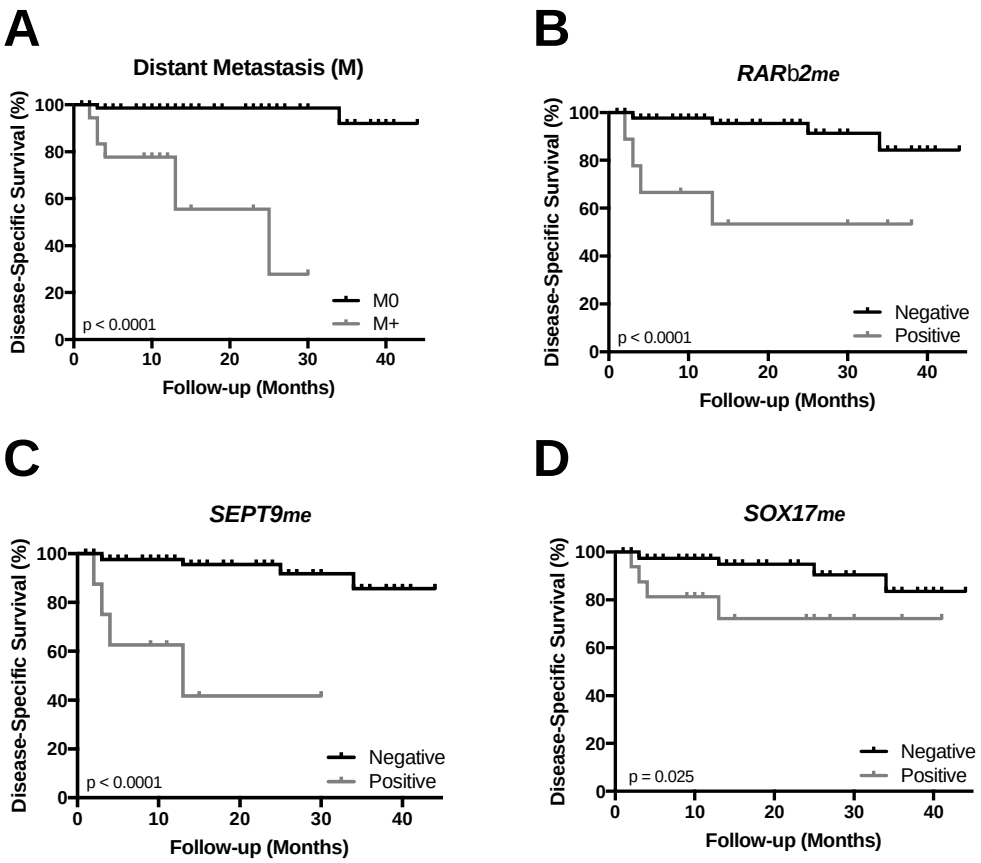


Supplementary Figure 3. Disease-specific survival curves according to clinicopathological variables (A) ISUP Grade Group, (B) serum PSA levels, (C) clinical stage, and (D) *APC*, (E) *GSTP1*, (F) *RARβ2*, (G) *RASSF1A*, (H) *SEPT9*, (I) *SOX17* promoter methylation levels in prostate cancer patients.

Supplementary Table 15. Demographics of the clinicopathological features and *APC, FOXA1, GSTP1, HOXD3, RARβ2, RASSF1A, SEPT9* and *SOX17* promoters' methylation levels in colorectal cancer patients, and their association with disease-specific survival. *p*-values obtained by log-rank test. Histological type was not evaluated due to the low number of squamous cell carcinoma samples.

	Patients n (%)	Disease-specific survival *p*-value
Tumour location (Proximal colon, Distal colon, Rectum)	23 (23%), 36 (36%), 41 (41%)	0.801
Tumour stage (T)[a] (T1/T2, T3/T4)	26 (26%), 72 (72%)	0.179
Regional node (N)[b] (N0, N+)	40 (40%), 57 (57%)	0.776
Distant Metastasis (M) (M0, M+)	82 (82%), 18 (18%)	**<0.0001**
Clinical Stage (I&II, III&IV)	39 (39%), 61 (61%)	0.159
***APC*$_{me}$** (Negative, positive)	88 (88%), 12 (12%)	0.271
***FOXA1*$_{me}$** (Negative, positive)	53 (53%), 47 (47%)	0.129
***GSTP1*$_{me}$** (Negative, positive)	99 (99%), 1 (1%)	0.812
***HOXD3*$_{me}$** (Negative, positive)	60 (60%), 40 (40%)	0.245
***RARβ2*$_{me}$** (Negative, positive)	91 (91%), 9 (9%)	**<0.0001**
***RASSF1A*$_{me}$** (Negative, positive)	93 (93%), 7 (7%)	0.393
***SEPT9*$_{me}$** (Negative, positive)	92 (92%), 8 (8%)	**<0.0001**
***SOX17*$_{me}$** (Negative, positive)	84 (84%), 16 (16%)	**0.025**

[a]No information available in 2 cases (2%); [b]No information in 3 cases (3%).



Supplementary Figure 4. Disease-specific survival curves according to clinicopathological variable (A) distant metastasis, and (B) *RARβ2,* (C) *SEPT9* and (D) *SOX17* promoter methylation levels in colorectal patients.