Highly-specific early detection of colorectal cancer by novel non-invasive serum methylation biomarkers

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ABSTRACT

Early detection has proven to be the most effective strategy to reduce the incidence and mortality of colorectal cancer (CRC). Nevertheless, most current screening programs suffer from low participation rates. A blood test may improve both the adherence to screening and the selection to colonoscopy. In this study, we conducted a serum-based discovery and validation of cfDNA methylation biomarkers for CRC screening in a multicentre cohort of 453 serum samples including healthy controls, benign pathologies, advanced adenomas (AA), and CRC. First, we performed an epigenome-wide methylation analysis with the MethylationEPIC array using a sample pooling approach, followed by a robust prioritization of candidate biomarkers for the detection of advanced neoplasia (AN: AA and CRC). Then, candidate biomarkers were validated by pyrosequencing in independent individual cfDNA samples. We report GALNT9, UPF3A, WARS, and LDB2 as new non-invasive biomarkers for the early detection of AN. The combination of GALNT9/UPF3A by logistic regression discriminated AN with 78.8% sensitivity and 100% specificity, outperforming the commonly used fecal immunochemical test and the methylated SEPT9 blood test. Overall, our results suggest that the combination methylated GALNT9/UPF3A has the potential to serve as a highly specific and sensitive blood-based test for screening and early detection of CRC.

Keywords: Advanced adenomas, Circulating cell-free DNA, Colorectal cancer, DNA methylation, Non-invasive biomarkers, Liquid biopsy, Screening, Serum.
INTRODUCTION

Colorectal cancer (CRC) is the third cancer with the highest incidence worldwide and the second leading cause of cancer death in both sexes (1). Diagnosis at advanced symptomatic stages is responsible for low survival (14% for stage IV) compared to 90% five-year survival for stage I and II (2). A recent decrease in CRC-related mortality and incidence has been reported, mainly due to the implementation of screening programs that enable early detection of preclinical CRC and the removal of precancerous colorectal adenomas (3–5). Despite strong evidence supports the reduction of both CRC incidence and mortality related to screening (6, 7), the overall participation rate in stool-based screening programs using the fecal immunochemical test (FIT) followed by a confirmatory colonoscopy remains modest (49.5% in Europe and 43.8% worldwide) (4, 8). Since the effectiveness of a screening test relies not only on the diagnostic performance of the test but also on its acceptance by the target population, test preference for CRC screening has been evaluated. A survey-based study reported as first choice a blood test over a stool one (9); similarly, among screening-enrolled individuals who refused colonoscopy, 83% preferred a non-invasive blood-based test, 15% chose a fecal test and 3% refused any test (10). Therefore, participation in screening programs could significantly improve by offering a non-invasive blood-based test.

Liquid biopsy has emerged as a non-invasive alternative to traditional procedures for sampling. Blood-based screening has the advantage of being easily available, repeatable, and minimally invasive for the patient (11). Circulating cell-free DNA (cfDNA) can be detected in body fluids and has been proposed as a source of liquid biopsy biomarkers as it reflects alterations occurring during neoplastic transformation, such as aberrant methylation in colorectal carcinogenesis (12, 13). Recent epigenome-wide methylation analyses have reported that alterations in DNA methylation arise during the early stages of tumor progression and that the heterogeneity of the different pathways to CRC is already detectable in colorectal adenomas.
The Illumina MethylationEPIC BeadChip array has proven to be reliable and consistent for DNA methylation analysis (16), and its combination with sample pooling represents a particularly suitable strategy for the cost-effective analysis of large sample sets aiming to discover differentially methylated signatures (17, 18). In this study, following a cfDNA pooling strategy, we aimed to identify non-invasive methylation biomarkers for the early detection of both colorectal cancer and advanced precancerous lesions. Here we report the discovery and independent validation of novel serum-based methylation biomarkers that could be implemented in CRC screening programs.
RESULTS

The study was conducted in three phases: (i) a high-throughput discovery analysis, paired with a statistically robust biomarker prioritization, was performed to identify candidate non-invasive methylation biomarkers for CRC screening, using a sample pooling strategy. Next, targeted assays were designed and optimized for the quantification of the candidate biomarkers in an independent cohort of patients (individual serum samples). The targeted analysis was divided into (ii) a preliminary evaluation and feature selection of the candidate biomarkers, and (iii) a subsequent validation and final statistical model construction based on the selected biomarkers. An overview of the study design is shown in Figure 1. A description of the independent cohorts is presented in Table 1.
Figure 1. Study workflow. The study was divided into a biomarker discovery phase, a candidate biomarker evaluation phase, and a selected biomarker validation. cfDNA: cell-free DNA; CRC: colorectal cancer (AJCC staging system); IBD: inflammatory bowel disease; NCF: no colorectal findings; BEN: benign pathology; NAA: non-advanced adenomas; D-AA: distal advanced adenomas; P-AA: proximal advanced adenomas; NN: no neoplasia; AN: advanced neoplasia; DMP: differentially methylated position; RRBS: reduced representation bisulfite sequencing.
|                          | Discovery cohort n=280 | Biomarker evaluation cohort n=48 | Biomarker validation cohort n=105 |
|--------------------------|------------------------|---------------------------------|----------------------------------|
| **Total (n)**            | AN:140 NN:140          | AN:27 NN:21                     | AN:66 NN:39                      |
| **Age median (range)**   | 62.0 (51-72)           | 62.5 (50-75)                    | 63.5 (50-72)                     |
| **Sex**                  |                        |                                 |                                  |
| Male                     | 70                     | 15                               | 36                                |
| Female                   | 70                     | 13                               | 30                                |
| **NCF**                  | -                      | -7                               | -15                               |
| **BEN**                  | -                      | -7                               | -13                               |
| Hemorrhoids              | -                      | -                                | -                                 |
| Diverticula              | -                      | -                                | -                                 |
| **NAA**                  | -                      | -7                               | -11                               |
| **AA**                   | 100                    | 16                               | 42                                |
| **AA histology**         | -                      | -                                | -                                 |
| T (size>10mm)            | 52                     | 11                               | 24                                |
| TV                       | 36                     | 3                                | 15                                |
| V                        | 12                     | 2                                | 3                                 |
| **AA dysplasia**         |                        |                                  |                                   |
| Without dysplasia        | 7                      | -                                | 5                                 |
| LGD                      | 89                     | 13                               | 31                                |
| HGD                      | 4                      | 3                                | 7                                 |
| **AA localization**      |                        |                                  |                                   |
| Distal                   | 50                     | 8                                | 23                                |
| Proximal                 | 50                     | 8                                | 19                                |
| **CRC**                  | 50                     | 16                               | 24                                |
| **CRC AJCC stage**       |                        |                                  |                                   |
| Stage I                  | 16                     | 3                                | 8                                 |
| Stage II                 | 14                     | 3                                | 8                                 |
| Stage III                | 14                     | 4                                | 2                                 |
| Stage IV                 | 6                      | 1                                | 6                                 |
| **CRC localization**     |                        |                                  |                                   |
| Distal                   | 39                     | 9                                | 12                                |
| Proximal                 | 11                     | 2                                | 12                                |

Table 1. Independent cohorts used for discovery, evaluation, and validation of biomarkers. The number of patients, age median and range, sex, and colorectal findings is provided for each sub-cohort. AN: advanced neoplasia; NN: no neoplasia; NCF: no colorectal findings; BEN: benign pathology; NAA: non-advanced adenomas; D-AA: distal advanced adenomas; P-AA: proximal advanced adenomas; T: tubular; TV: tubulovillous; V: villous; LGD: low-grade dysplasia; HGD: high-grade dysplasia; CRC: colorectal cancer; AJCC: American Joint Committee on Cancer staging system.
Sample pooling

A total of 28 cfDNA pooled samples were used in the epigenome-wide methylation analysis for biomarker discovery. As described in (18), 10 individuals with the same colorectal pathology were included in each pool and were matched by recruitment hospital, sex, and age (median 62, range 51-72). There was no statistically significant difference in the mean age between pools (ANOVA, p-value < 0.05). The age range matches the USPSTF guideline recommendation for CRC screening (50-75 years) (19). Pools were divided into no neoplasia and advanced colorectal neoplasia. The no neoplasia (NN) group comprised 13 cfDNA pooled samples: 3 pools of individuals with no colorectal findings (NCF), 5 pools of individuals with benign pathologies (BEN; hemorrhoids and diverticula), and 5 pools of individuals with non-advanced adenomas (NAA). On the other hand, the 15 cfDNA pooled samples of advanced neoplasia (AN) included 5 pools of individuals with advanced adenomas located only in the proximal colon (P-AA), 5 pools of individuals with advanced adenomas located in the distal colon or in both proximal and distal locations (D-AA), 3 pools of CRC patients stages I and II, and 2 pools of CRC stages III and IV. The final quantity of cfDNA in the pools ranged from 62 to 403 ng (Supplementary Table 1).

Epigenome-wide biomarker discovery

MethylationEPIC BeadChip was used for quantitative DNA methylation profiling in the 28 cfDNA pooled samples. We correctly detected 97.78% of the total probes present on the array. All failed positions (1,795 probes with a detection p-value > 0.01 and 17,452 probes with a bead count < 3) were removed before analysis. After quality control and data preprocessing, 18,035 probes were discarded as they did not hold the assumptions for linear regression model fitting (i.e. linearity, homoscedasticity, uncorrelatedness, and normality of the standardized residuals). After quality control and preprocessing, a total of 741,310 CpG sites...
mapped to the human genome assembly GRCh37/hg19 were left for differential methylation testing. No samples were removed due to quality issues.

The purpose of screening programs includes the early detection of preclinical CRC and the detection and removal of AA (3, 20); therefore, differential methylation was assessed between NN and AN groups. The analysis between these cfDNA pools revealed 376 differentially methylated positions (10% FDR, BH-adjusted \( p \)-value) (Fig. 2A), annotated to a total of 290 gene regions and 183 CpG islands. Chromosomes 1 and 2 hold the majority of DMPs (10.4% and 7.7%, respectively) possibly due to their larger length. Most CpG sites (326 DMPs, 86.7%) were found to be hypermethylated in AN (Fig. 2B). Concerning the distribution across functional elements, DMPs were mainly located in open sea regions (51.59%) and CpG islands (23.67%) (Fig. 2C). Differentially hypermethylated positions were significantly enriched in CpG islands, shelves, and gene body regions (Fig. 2D). Clustering analyses of all pooled samples based on the methylation values of the 376 DMPs (Fig. 3A) suggest the capacity of this differentially methylated signature to discriminate advanced neoplasia from no neoplasia controls.
Figure 2. Distribution and annotation of differentially methylated positions (DMPs) between advanced neoplasia and no neoplasia pooled samples. A. Manhattan plot for differential methylation. The \(-\log_{10}(p\text{-value})\) for the 741,310 probes analyzed are sorted by chromosome location. Significant DMPs (376) appear above the red dashed line (FDR 10%). B. Volcano plot showing the \(-\log_{10}(p\text{-value})\) versus differences in methylation levels (\(\Delta\beta\): obtained by subtracting the DNA methylation levels (beta-values) of NN from AN). Significant hypermethylated (\(\Delta\beta > 0\)) and hypomethylated (\(\Delta\beta < 0\)) positions appear highlighted in color and above the red dashed line (FDR 10%). C. Distribution of the DMPs relative to CpG islands and functional genomic locations. D. Enrichment of DMPs in relation to CpG island annotation and functional genomic regions. The color scale indicates the fold enrichment of all DMPs (grey), hypermethylated (red), and hypomethylated (blue) positions. The bolded numbers indicate annotations that are enriched with respect to the distribution of probes on the MethylationEPIC array (one-sided Fisher’s exact test p-value < 0.05). Functional characterization of probes according to the Methylation EPIC Manifest (R package version 0.6.0) (21): CpG island: region of at least 200bp with a CG content > 50% and an observed-to-expected CpG ratio ≥ 0.6; CpG island-shore: sequences 2 kb flanking the CpG island; CpG island-shelf: sequences 2 kb flanking shore regions; opensea: sequences located outside these regions, promoter regions (5’UTR, TSS200, TSS1500, and first exons), intragenic regions (gene body and 3’UTR), and intergenic regions. TSS200, TSS1500: 200 and 1500 bp upstream the transcription start site, respectively.
Figure 3. Unsupervised clustering analyses of the 28 cfDNA pooled samples. A. Hierarchical clustering and heatmap showing the methylation levels across all samples for the 376 DMPs. B. Hierarchical clustering and heatmap showing the methylation levels across all samples for the 26 candidate biomarker. Each column represents one pool while rows correspond to CpG sites. Dendrograms were computed and reordered using Euclidean distance and a complete clustering agglomeration. Methylation levels are expressed as beta-values ranging from 0 (blue, unmethylated) to 1 (red, fully methylated).

In order to identify the most relevant features we followed a robust strategy of selection. We first applied the constraint-based SES feature selection algorithm (22) on the 376 DMPs to identify combinations of CpGs whose performances for the NN vs AN classification were statistically equivalent. A total of 3,256 combinations of CpG pairs were obtained. Secondly, we used two strategies for filtering: (a) the CpG pairs were used to build classification models (support vector machine, logit regression, and random forest) that were cross-validated in the
cfDNA pools by the leave-one-out strategy, due to limited sample size. CpG sites present in models with more than 20% prediction error were discarded. And then (b) the remaining CpG sites were ranked according to the mean difference in the methylation levels. From the ranked list we selected the top 15 CpG sites with greater methylation differences and present in models with minimum classification error. Finally, due to the limited sensitivity of FIT for the detection of advanced adenomas, especially those of proximal location, we also selected 3 additional CpG sites that presented 0% prediction error for the detection of advanced adenomas (1 CpG site derived from the NN vs D-AA comparison; 2 CpG sites derived from the NN vs P-AA comparison).

In parallel, we carried out a comparison between an external reduced representation bisulfite sequencing (RRBS) dataset and our methylation microarray data. The RRBS dataset comprised serum and tissue samples from patients with CRC, AA, and healthy controls. We combined both datasets to select a subset of 8 CpG sites that reported more than 30% differences in the methylation levels by bisulfite sequencing, and whose methylation differences showed the same direction in our methylation microarray data (i.e. hyper/hypomethylated in AN in both datasets).

Altogether, a total of 26 CpG positions were selected as candidate biomarkers (Supplementary Table 2) whose methylation levels and their ability to discriminate colorectal advanced neoplasia are shown in Figure 3B.

**Evaluation of candidate methylation biomarkers and further selection in individual samples**

To evaluate the performance of the 26 candidate biomarkers in individual serum samples, methylation of the CpG positions of interest was quantified by bisulfite pyrosequencing in an independent cohort of 153 individuals (Table 1), which was randomly split between a biomarker evaluation cohort (30%, n=48) and a biomarker validation cohort (70%, n=105). Methylation levels of the v2 promoter region of the SEPT9 gene were also quantified in all
samples. A detailed description of the pyrosequenced regions and the number of CpG positions analyzed within each biomarker can be found in Supplementary Table 3. The construction of methylation models for CRC screening was conducted in two phases.

First, the 26 candidate biomarkers were evaluated in a subset of 48 serum samples (30%). The methylation levels of the 26 candidate biomarkers in both pooled (MethylationEPIC-derived) and individual cfDNA samples (bisulfite pyrosequencing) are shown in Figures 4A and B. There was a significant positive correlation (Pearson’s r > 0.6, p-value < 0.001) between cfDNA methylation in pooled and serum samples (Fig 4C, D). To further reduce the number of amplicons for validation, penalized logistic regressions were fitted to the complete list of 26 candidate biomarkers. Classification performance parameters were derived by leave-one-out cross-validation. We produced sparse models containing two (GALNT9 and UPF3A; AUC: 0.905, 95% CI 0.801-1) and four (GALNT9, UPF3A, WARS, and LDB2; AUC: 0.827, 95% CI 0.651-1) methylation biomarkers, derived by LASSO and Elastic net regularization, respectively. Also, the application of Elastic net to the 26 biomarkers, adding the clinical variables age and sex, generated a model containing 20 biomarkers and sex (AUC: 0.820, 95% CI 0.675-0.966). The four biomarkers selected by the sparse models are included in the 20-biomarker signature. Hence, this 20 biomarker set was selected to proceed with the validation phase.
Figure 4. Methylation levels of the 26 candidate biomarkers. A. Methylation levels of the 26 CpG sites selected as candidate biomarkers in the 28 pooled cfDNA samples, with the corresponding MethylationEPIC CpG probe ID. Average methylation of the probes targeting the v2 promoter region of the SEPT9 gene (cg02884239, cg20275528, and cg12783819) is also shown. Methylation is shown as beta-values ranging from 0 (unmethylated) to 1 (fully methylated). B. Evaluation of the methylation levels of the 26 candidate CpG sites and SEPT9 promoter in the biomarker selection cohort (n=48) of individual serum cfDNA samples. Methylation percentage was obtained through bisulfite pyrosequencing. C. Strip-plot showing the concordance of methylation levels between pooled and individual samples. Each dot represents the methylation level of one sample. D. Scatterplot showing the positive significant correlation between methylation in pooled and individual cfDNA samples for the 26 candidate CpG sites.
Validation of the selected biomarkers and final model construction

The final 20 selected methylation biomarkers were then quantified in the remaining 105 serum samples (70%) (Fig. 5A) to validate their use as non-invasive biomarkers in individual samples. A detailed description of the biomarkers selected for validation is shown in Table 2, including the genomic region and the number of CpG sites analyzed by pyrosequencing. Multivariate logistic regressions were fitted to the selected biomarker subsets obtained from the three best performing models from the previous step, to derive three new models containing 2 (GALNT9 and UPF3A), 4 (GALNT9, UPF3A, WARS, and LDB2), and 20 biomarkers. Performances of the diagnostic prediction models in the biomarker validation cohort are summarized in Table 3, while ROC curves are provided in Figure 5B. ROC curves and performance parameters were obtained by leave-one-out cross-validation.
| MethylationEPIC probe ID | Gene symbol | Pyrosequenced region (GRCh37/hg19) | CpG sites analysed | Relation to CpG island | Regulatory feature |
|-------------------------|-------------|------------------------------------|-------------------|------------------------|-------------------|
| CG2 cg05445162          | GCKR        | chr2:27730152-27730225             | 1                 | Opensea                | Body              |
| CG3 cg11113216          | GALNT9      | chr12:132847616-132847751          | 3                 | Island                 | Body              |
| CG4 cg06522913          | IDH2        | chr15:90630673-90630817            | 1                 | Opensea                | Body              |
| CG5 cg14838992          | WARS        | chr14:100814702-100814791          | 3                 | Opensea                | Body              |
| CG6 cg06148974          |             | chr4:1864164-186424               | 3                 | Island                 |                   |
| CG8 cg03111938          |             | chr6:169289158-16928923           | 5                 | Island-shelf           |                   |
| CG9 cg07253636          | NOSIP       | chr19:50077734-50077874            | 2                 | Opensea                | 5'UTR             |
| CG11 cg25942688         | CENPA       | chr2:27016702-27016821             | 1                 | Opensea                | 3'UTR             |
| CG12 cg01987330         |             | chr3:142797286-142797375          | 1                 | Opensea                |                   |
| CG14 cg10641001         | RDX         | chr11:110104045-110104122          | 2                 | Opensea                | Body              |
| CG15 cg01550272         | UPF3A       | chr13:115050863-115050975          | 1                 | Island-shelf           | Body              |
| CG16 cg03640756         | PCDHG gene | chr5:140864546-140864667          | 11                | Island                 | Body              |
| CG17 cg26024530         |             | chr10:130281707-130281864         | 3                 | Opensea                |                   |
| CG19 cg15442105         | ZNF498      | chr7:99227410-99227486             | 4                 | Opensea                | Body              |
| CG20 cg04544475         | RNF43       | chr17:56435427-56435508            | 3                 | Opensea                | Body              |
| CG21 cg16639692         | IncRNA LOC648987 | chr5:43037639-43037813     | 1                 | Island-shore           |                   |
| CG23 cg04600077         | IncRNA ENSG0000024996 | chr5:1852792-1852864     | 1                 | Island-shore           |                   |
| CG24 cg14503564         | LDB2        | chr4:16723341-16723457             | 6                 | Opensea                | Body              |
| CG25 cg18044585         | EIF2C2      | chr8:141619395-141619523           | 6                 | Opensea                | Body              |
| CG26 cg23653187         | PNPLA3      | chr22:44319222-44319299            | 2                 | Island-shore           | TSS1500           |

Table 2. 20 biomarkers selected for the validation phase. Regulatory features and relation to CpG island of biomarkers annotated according to the Methylation EPIC Manifest: CpG island: region of at least 200 bp with a CG content > 50% and an observed-to-expected CpG ratio ≥ 0.6; Island-shore: sequences 2 kb flanking the CpG island; Island-shelf: sequences 2 kb flanking shore regions; Opensea: sequences located outside these regions; Body: gene body (intragenic region); TSS1500: 1500 bp upstream the transcription start site.
The model composed of \textit{GALNT9} and \textit{UPF3A} showed the best diagnostic performance for CRC screening, yielding an AUC of 0.896 (95\% CI 0.835-0.958), discriminating advanced neoplasia from no neoplasia controls with 78.8\% sensitivity and 100\% specificity. That model identified 33 out of 42 AA cases (78.6\%) and 19 out of 24 CRC patients (79.2\%), with great detection of CRC early stages (87.5\% and 100\% for stages I and II, respectively). This 2-biomarker panel detected 87\% of distal AA and 68.4\% proximal AA. On the other hand, the resulting AUC for the model containing \textit{GALNT9}, \textit{UPF3A}, \textit{WARS}, and \textit{LDB2} was 0.864 (95\% CI 0.798-0.931), with a sensitivity of 62.1\% and a specificity of 97.4\%, detecting 28 out of 42 AA (66.7\%; 78.3\% distal and 52.6\% proximal), and 13 out of 24 CRC cases (54.2\%; 37.5 stage I and 75\% stage II). These two models demonstrated no significant differences in the detection of distal AA compared to proximal ones (Fisher’s exact test \textit{p}-value > 0.05). The model containing 20 biomarkers and sex, reported the highest sensitivity (92.4\%) and the highest detection rates for AA and CRC (92.9\% and 91.7\%, respectively), but with considerably less specificity (17.9\%) when compared to the previous models. The logistic classification rules of the model containing \textit{GALNT9}, \textit{UPF3A}, \textit{WARS}, and \textit{LDB2} and the \textit{GANL9/UPF3A} model are detailed in Supplementary information. \textit{GALNT9} (CG3) was hypermethylated in AN with a methylation difference of 0.36\%, whilst \textit{UPF3A} (CG15), \textit{WARS} (CG5), and \textit{LDB2} (CG24) showed hypomethylation in AN with methylation differences of 1.25\%, 0.22\%, and 0.96\%, respectively (Fig. 5A and C).

Since plasma samples are most commonly used as a source of cfDNA, methylation of \textit{GALNT9}, \textit{UPF3A}, \textit{WARS}, and \textit{LDB2} was also evaluated in 8 pairs of matched serum and plasma samples. No statistical differences were found between serum and plasma methylation levels (Wilcoxon signed-rank test \textit{p}-value > 0.01) (Fig. 5E).
|                | AUC (95% CI) | Advanced Neoplasia classification | AA detection % | CRC detection % |
|----------------|-------------|-----------------------------------|----------------|-----------------|
|                |             | Specificity % (95% CI) | Sensitivity % (95% CI) | NPV % (95% CI) | PPV % (95% CI) | Distal | Proximal | All | Stage I | Stage II | Stage III | Stage IV | All |
| **GALNT9, UPF3A** | 0.896 (0.835–0.958) | 100 (91–100) | 78.8 (67–88) | 73.6 (60–85) | 100 (93–100) | 87 | 68.4 | 78.6 | 87.5 | 100 | 100 | 33.3 | 79.2 |
| **GALNT9, UPF3A, WARS, LDB2** | 0.864 (0.798–0.931) | 97.4 (87–100) | 62.1 (49–74) | 60.3 (47–72) | 97.6 (87–100) | 78.3 | 52.6 | 66.7 | 37.5 | 75 | 100 | 33.3 | 54.2 |
| **20 methylation biomarkers, sex** | 0.553 (0.407–0.639) | 17.9 (8–34) | 92.4 (83–97) | 58.3 (28–85) | 65.6 (55–75) | 91.3 | 94.7 | 92.9 | 87.5 | 100 | 100 | 83.3 | 91.7 |
| **SEPT9** | 0.504 (0.389–0.618) | 100 (91–100) | 15.1 (8–26) | 41.1 (31–52) | 100 (69–100) | 4.3 | 10.5 | 7.1 | 0 | 37.5 | 50 | 50 | 29.2 |

Table 3. Diagnostic performance of the models and SEPT9 for the detection of advanced neoplasia in the biomarker validation cohort (n=105), with their corresponding AA and CRC detection rates. No significant differences were found between the detection of distal versus proximal AA (Fisher’s exact test p-value>0.05). ROC curves and performance parameters were derived by the leave-one-out cross-validation approach. AUC: area under the curve; AA: advanced adenoma; CRC: colorectal cancer; NPV: negative predictive value; PPV: positive predictive value.
Analysis and performance of Septin9 methylation

The methylation EPIC array contains three probes targeting the region of the v2 promoter of the SEPT9 gene commonly evaluated for CRC screening (23, 24) (cg02884239, cg20275528, cg12783819). None of them reported significance for the detection of AN in the epigenome-wide differential methylation analysis performed on the 28 cfDNA pooled samples, and showed an average of 0.14% methylation difference between NN and AN (Fig. 4A). We reported a hypomethylation of SEPT9 in AN, with methylation differences between NN and AN of 2.92% in the biomarker evaluation cohort (n=48) (Fig. 4B), and 0.44% in the biomarker validation cohort (n=105) (Fig. 5A). The diagnostic performance of the SEPT9 promoter methylation was also evaluated in the biomarker validation cohort, with an AUC for AN detection of 0.504 (95% CI 0.389-0.618) and values of sensitivity and specificity of 15.2% and 100%, respectively (Table 3, Figure 5D). Only 7.1% of AA and 29.2% of CRC were detected.

Evaluation of the classification models in non-colorectal tumors

To assess the ability of the models for the specific detection of colorectal advanced adenomas and cancer, methylation of GALNT9, UPF3A, WARS, and LDB2 was quantified in serum samples from patients with lung, breast, kidney, prostate, and ovarian cancer (n=16) (Fig. 5C, Supplementary Table 4). When applied to different cancer types, the GALNT9/UPF3A model classified as advanced colorectal neoplasia 3 out of 16 cancer cases (18.75%: prostate, kidney, and breast cancer). On the other hand, the model composed of GALNT9, UPF3A, WARS, and LDB2 identified 1 out of 16 cancer cases (6.25%: ovarian cancer) as advanced neoplasia.
Figure 5. Diagnostic performance of the models and methylation levels in the biomarker validation cohort (n=105). A. Methylation levels of the final 20 selected biomarkers in the validation cohort. B. ROC curve analysis and corresponding AUCs for the three models evaluated for CRC screening, derived by leave-one-out cross-validation. The red dots indicate the sensitivity and specificity values for the best cut-offs based on the Youden Index method. C. Serum methylation levels of GALNT9, UPF3A, WARS, and LDB2 in the biomarker validation cohort (n=105) and in lung, breast, kidney, prostate, and ovarian cancer cases (n=16). D. Methylation levels and classification performance (ROC curve) of the SEPT9 promoter. The red dot indicates the best sensitivity and specificity values (Youden Index). E. Comparison of methylation levels between matched serum and plasma samples. AUC: area under the curve; NN: no neoplasia; AA: advanced adenomas; CRC: colorectal cancer.
DISCUSSION

Early detection has proven to be the most effective strategy to reduce both the incidence and mortality of CRC (6, 7); nevertheless, most current screening programs suffer from low participation rates. The FIT is the most widely implemented and recommended non-invasive test for CRC screening despite having a modest sensitivity for the detection of premalignant AA (22-56%, 90-94% specificity) (25–28) and also improbable compliance (4, 8). Currently, there is no non-invasive biomarker for the early detection of CRC and AA that meets all the characteristics of an ideal screening test: easy to perform, safe, highly specific and sensitive, cost-effective, and well accepted by patients. To this end, the development of liquid biopsy technology has shown to be a promising approach for CRC screening, diagnosis, follow-up, and treatment guidance (11), with the potential to be integrated into the clinic.

In this study, we first conducted an epigenome-wide analysis with the MethylationEPIC array using a cfDNA pooling approach to discover potential blood-based biomarkers for CRC. After prioritization of candidate biomarkers and evaluating their methylation levels in individual samples from an independent cohort, we developed and cross-validated three prediction models for the detection of AN. The first one, the 20 methylation biomarkers with sex, yielded a sensitivity of 92.4% for AN, at 18% specificity. Despite its high sensitivity and highest detection rate for AA (92.9%) and CRC (91.7%), such low specificity is not cost-effective for screening programs. Secondly, the model composed of GALNT9, UPF3A, WARS, and LDB2 reported 62.1% sensitivity and 97.4% specificity, whilst the GALNT9/UPF3A model discriminated AN with 78.8% sensitivity and 100% specificity, showing the best prediction performance for CRC screening. The sensitivities reported for our biomarkers are comparable to that of FIT for CRC detection (73-88%) and higher than its sensitivity for AA (22-56%) (28). The combination GALNT9/UPF3A also fulfills the main objective of CRC screening, that is the detection of preclinical CRC and premalignant AA, as reported great detection rate for AA (78.6%) and early CRC stages I and II (87.5% and 100%, respectively). Also, no statistically significant differences were reported between the detection of distal (87%) and proximal (68.4%) AA, in contrast with the FIT that performs better for distal lesions than proximal ones (29). The decreased
detection of stage IV tumors (33.3%) would not affect the effectiveness of CRC screening, since individuals with late-stage cancers are commonly symptomatic and therefore will not be enrolled in average-risk programs.

Only UPF3A has been previously related to CRC. Located in a CpG island shelf, we reported the hypomethylation of UPF3A in AN; and high expression levels of this gene associate with TNM stage, liver metastasis, and recurrence in CRC (30). GALNT9 is also located in a CpG island and showed hypermethylation in AN, which was also reported in brain metastasis from breast cancer (31). On the other hand, WARS and LDB2 also show hypomethylation in AN but are located within opensea regions. The methylation of regions outside GpG islands does not always directly relate to gene silencing. High expression levels of WARS were found in MSI-H gastrointestinal adenocarcinomas, associated with poor prognosis (32), whilst a decreased expression of LDB2 was associated with a more favorable outcome in lung adenocarcinoma patients (33).

Nowadays, the only non-invasive methylation biomarker approved by the FDA for the detection of CRC in blood is based on the methylation of the SEPT9 promoter (34). Nevertheless, the results on the diagnostic performance of this plasma biomarker are variable and inconsistent, with sensitivities ranging from 48.2-95.6% for CRC and 11.2-35% for AA, with 79.1-99.1% specificity. In an asymptomatic average-risk cohort, SEPT9 showed lower screening performance than FIT (sensitivity: 68% vs. 79%; specificity: 80% vs. 94%, respectively), reviewed in (35–38). In our study, we evaluated the performance of SEPT9 in both our discovery and validation cohorts. The 3 CpG sites targeting SEPT9 interrogated in the MethylationEPIC BeadChip, showed an average of 0.14% methylation difference between NN and AN; while in the final validation cohort, the sensitivity for AN and AA resulted in 15.1% and 7%, respectively, with 100% specificity. Unlike the commercial test, based on qPCR positive reactions (39), we quantified the methylation of the SEPT9 promoter by pyrosequencing and thus the results may not be fully comparable.

Several blood-based methylation biomarkers have recently emerged for the early detection of CRC. Plasma BCAT1 and IKZF1 methylation reported sensitivities of 66% and 5% for CRC and AA, respectively, at 95% specificity (40). Methylation markers C9orf50, KCNQ5, and CLIP4 reported 85% sensitivity and 99% specificity.
for CRC detection (41). Methylated SFRP2 and SDC2 detected CRC and AA with sensitivities of 76.2% and 58.3%, and 87.9% specificity (42). A single plasma methylation biomarker, cg10673833, demonstrated 89.7% sensitivity and 86.8% specificity for CRC, but the sensitivity dropped up to 33.3% against precancerous lesions (including AA) (43). In contrast with the aforementioned available studies, which are based on results reported in cell lines, tissue, or stool, the design of our study targets the final sample format for both the discovery and validation phases, enhancing this way the possibility to discover and translate a robust non-invasive biomarker. From our proposed models, the combination GALNT9/UPF3A showed an acceptable 78.8% sensitivity for the joint detection of AA and CRC, and superior specificity of 100% than other reported potential biomarkers. A cost-effective biomarker for CRC screening should present a low false-positive rate to minimize the number of unnecessary, expensive, and invasive colonoscopies. Thus, the highest specificity is sought for CRC screening.

In the ongoing validation of CRC screening biomarkers, it is important to consider data on protocol acceptability. A blood-based test has the potential to improve compliance and participation in CRC screening, as reported by a randomized controlled trial where 99.5% of subjects who were offered a SEPT9 as the first option for CRC screening completed the test within six weeks, compared to 88.1% of participants in the FIT arm (44). It was also reported that 83% of individuals enrolled for screening who refused colonoscopy, preferred a blood-based test (10). To optimize the participation in CRC screening, perhaps both a fecal and a blood-based test should be offered to target different preferences. A blood test could be proposed as a screening option to invitees refusing FIT and other guidelines recommended tests. This is indeed the indication for the SEPT9 test in average-risk individuals older than 50 (38, 45). Another option for implementing a blood test in screening programs is triaging FIT-positive individuals for improved selection to colonoscopy (46). Figure 6 shows a schematic representation of the possible implementation of a blood test in CRC screening, both as an alternative to FIT aiming to increase participation rates, and as a triage approach to optimize selection to colonoscopy.
Figure 6. Schematic representation of the proposed approach for the implementation of a blood-based test in CRC screening. To target different sample preferences and improve participation rates, a blood test could be offered to individuals refusing the FIT (left arm). In combination with the FIT, the blood test may improve selection to follow-up colonoscopy after a positive FIT (right arm). A FIT test would be offered every two years to individuals rejecting both the FIT and the blood test, and to individuals with a previous negative result in either the FIT or blood-based test.

To the best of our knowledge, this is the first study conducting a serum-based discovery and validation of cfDNA methylation biomarkers for CRC screening. Our multicentre cohort includes not only CRC, AA, and healthy controls, but also benign pathologies typically found during screening programs that influence the specificity, such as non-advanced adenomas, hemorrhoids, and diverticula. It is worth mentioning that our healthy individuals are not self-declared but colonoscopically confirmed and that the criteria of inclusion and age range of the patients follow the USPSTF guidelines for CRC screening (19). Also, we have checked that there is no difference in the methylation levels of our biomarkers between serum and plasma cfDNA, and the low volume of serum used (up to 2 mL) is feasible in clinical practice. The ability of our methylation biomarkers to specifically detect CRC was also assessed, identifying 18.75% cancer samples as advanced neoplasia by the GALNT9/UPF3A model, and 6.25% by the GALNT9, UPF3A, WARS, and LDB2 model.
Nevertheless, our study has some limitations. Firstly, CRC cases were mostly diagnosed having symptoms, and secondly, the proportions of CRC cases, tumor stages, and the rest of pathologies are not fully representative of a screening population. A large prospective validation in an asymptomatic average-risk screening population will be necessary before the implementation of any of the proposed methylation models.

In conclusion, we have discovered and reported *GALNT9*, *UPF3A*, *WARS*, and *LDB2* as new potential non-invasive biomarkers for the early detection of colorectal neoplasia. Overall, our results suggest that the combination *GALNT9/UPF3A* is the most promising to serve as a highly specific blood-based test for screening and detection of CRC at an early and curable stage, even at the premalignant lesion phase, which needs further validation in a real screening setting.
METHODS

Patients and samples

Individuals were recruited from the following Spanish Hospitals: Complexo Hospitalario Universitario de Ourense (Ourense), Hospital Clínico de Barcelona (Barcelona), Hospital Donostia (San Sebastián), and Hospital General Universitario de Alicante (Alicante). A total of 435 individuals between 50-75 years old were selected. Exclusion criteria included a personal history of CRC, digestive cancer or inflammatory bowel disease, a severe synchronic illness, and a previous colectomy. All individuals included in the study underwent a colonoscopy which was performed by experienced endoscopists following the recommendations from the Spanish guidelines on quality of colonoscopy (47). Blood samples were obtained immediately before the colonoscopy procedure. Blood samples were coagulated and subsequently centrifuged according to the manufacturer’s instructions for serum collection. Serum samples were stored at −20 °C until cfDNA extraction. Circulating cell-free DNA (cfDNA) was extracted from 0.5-2 mL according to availability.

Individuals were classified according to the most advanced colorectal finding. Lesions were considered ‘proximal’ when located only proximal to the splenic flexure of the colon and ‘distal’ when found only in the distal colon or in both distal and proximal colon. Advanced adenomas (AA) are defined as adenomas ≥ 1 cm, with villous components or high-grade dysplasia.

We performed a stratified random sampling using colorectal findings and sex as stratifying variables. Strata were matched by age and recruitment hospital. This multicenter cohort was separated into two independent subsets: Biomarker discovery sample set (n=280; 140 female and 140 male) and Biomarker validation sample set (n=153; 72 female and 83 male).

Biomarker discovery sample set included 30 individuals with no colorectal findings (NCF), 50 with benign pathologies (BEN: hemorrhoids and diverticula), 50 with non-advanced adenomas (NAA), 50 with proximal AA (P-AA), 50 with distal AA (D-AA), and 50 CRC cases (17 stage I, 13 stage II, 13 stage III and 7 stage IV,
according to the AJCC staging system (48). On the other hand, the Biomarker validation sample set comprised 22 NCF individuals, 20 benign pathologies, 18 NAA, 31 D-AA, 27 P-AA, and 35 CRC cases (11 stage I, 11 stage II, 6 stage III, and 7 stage IV). ‘Advanced colorectal neoplasia’ (AN) was defined as AA or CRC. In contrast, NCF, BEN, and NAA were considered together as ‘no neoplasia’ (NN) (Table 1).

The specificity of the biomarkers for the detection of colorectal cancer and advanced adenomas was evaluated in an independent cohort of 16 patients with different cancer types, including breast (n=4), kidney (n=2), lung (n=5), ovary (n=1), and prostate (n=4) cancer (Supplementary Table 4). Additionally, 8 pairs of matched serum and plasma samples from the same individual were used to account for differences in the methylation levels between serum and plasma (3NCF, 2 BEN, and 3 NAA).

**DNA extraction and sample pooling**

cfDNA from samples used for biomarker discovery was extracted with a phenol-chloroform protocol as described by Hufnagl et al. (49), with minor modifications. DNA was quantified in each sample using Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). For methylation biomarker discovery we followed a DNA pooling strategy. A detailed description of the pooling protocol can be found in (18). 28 independent cfDNA pooled samples were constructed using equal amounts of cfDNA from 5 men and 5 women from the same pathological group, recruitment hospital- and age-matched. The cfDNA pools were stored at ~ 20 °C and were sent to the Cancer Epigenetics and Biology Program (PEBC) facilities at the Bellvitge Biomedical Research Institute (IDIBELL, Barcelona, Spain) for processing and methylation quantification.

The QIAmp circulating nucleic acids kit (Qiagen, Hilden, Germany) was used for cfDNA extraction from serum and plasma samples in the validation phase. Individual cfDNA samples were bisulfite-converted using EZ DNA Methylation-Direct Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer’s protocol. Bisulfite treated cfDNA samples were stored at -80°C.

**Genome-wide DNA methylation measurements**
DNA methylation of pooled samples was measured with the Infinium MethylationEPIC BeadChip array (Illumina, San Diego, CA, USA). Pooled samples were bisulfite-treated and arrays were hybridized following the manufacturer’s instructions. A total of 865,859 CpG sites were quantified throughout the genome, covering promoter CpG islands, RefSeq genes, open chromatin, and enhancer intergenic regions identified by FANTOM5 and ENCODE projects (50–52). Importantly, to minimize the potential impact of batch effects and confounder variability, samples of each pathological group were randomly allocated to the slides.

**Methylation biomarker discovery**

Illumina methylation data were preprocessed and analyzed using the R environment (versions 3.3.3 and 3.4.0) (53) with R and Bioconductor (54) packages (see supplementary information for details on quality control and preprocessing). Methylation levels were expressed as beta-values for visualization and intuitive interpretation of the results. Methylation expressed as M-values (logit transformation of the beta-values) were used for differential methylation analysis and biomarker selection, as recommended by Du et al. (55).

To test for differentially methylated positions (DMPs) between AN (AA or CRC) and NN (NCF, BEN, or NAA) we used the *limma* package [8]: linear models were fitted for each CpG site across all samples by generalized least squares, and an empirical Bayes method was used to compute the p-values. As recommended by Mansell et al., (56) linear regression assumptions were checked for each model using the *gvlma* package (57).

To select and prioritize the DMPs as candidate biomarkers we first applied the constraint-based Statistically Equivalent Signature (SES) algorithm for feature selection, contained in the *MXM* package (22): multiple CpG sets with minimal size and maximal predictive power for the binary classification problem NN vs AN were obtained by iteratively comparing logistic regression models through a chi-square test. Then, the different CpG subsets were used to build classification models based on support vector machine (*e1071* package) (58), random forest (*randomForest* package) (59), and logistic regression. Models were cross-validated to select candidate CpG biomarkers with minimum prediction error for NN vs AN classification.
Methylation microarray data were compared with an external RRBS (Reduced Representation Bisulfite Sequencing) dataset targeting CpG-rich regions of 20 AA and 27 CRC cases, with matched adenoma/tumor, healthy mucosa, and serum samples of each patient; and 24 serum samples from healthy controls. A subset of CpG sites that reported concordance in methylation differences between NN and AN in both datasets were added to the biomarker list.

**Targeted methylation quantification by bisulfite pyrosequencing**

The methylation levels of the candidate biomarkers, together with the v2 promoter region of the SEPT9 gene, were evaluated by bisulfite pyrosequencing in 153 individual serum samples. PCR and sequencing primers were designed with the PyroMark Assay Design software (version 2.0.1.15, Qiagen, Hilden, Germany). Bisulfite-converted cfDNA (2 μl) was subjected to PCR amplification using primers flanking the CpG candidate biomarkers. Multiplex reactions including 3-6 candidate markers were performed, followed by nested singleplex PCR reactions using a biotin-labeled primer. Primers and PCR conditions for multiplex and singleplex PCR are provided in Supplementary Information and Supplementary Table 3. Biotin-labeled amplicons were further resolved and visualized on 3% agarose gels. Pyrosequencing was performed using a PyroMark Q96 ID pyrosequencer (Qiagen, Hilden, Germany) according to the manufacturer’s protocol for CpG methylation quantification. Data acquisition and methylation measurements were conducted at the Biomedical Research Institute of Malaga facilities (IBIMA, Málaga, Spain) using PyroMark Q96 ID software, CpG analysis mode (version 1.0.11).

Fully methylated and unmethylated controls were included in each run and were used to check bisulfite conversion, the performance of the assay, and to account for batch-to-batch variation. DNA extracted from peripheral blood from a donor was used to prepare controls. For the fully methylated control DNA was treated with CpG methyltransferase (M.SssI; New England Biolabs, Ipswich, MA, USA), while for the unmethylated control whole genome amplification was performed to eliminate any methylation marks (illustra GenomiPhi V2 DNA Amplification Kit, GE Healthcare; Chicago, IL, USA).

**Biomarker selection and classification model development**
Both raw and log10-transformed methylation percentages were subjected to multivariate analyses for the development and validation of methylation-based classification models. The validation set of 153 individual samples was randomly divided for the multi-step process of methylation panel development (Table 1).

First, a preliminary evaluation of the methylation levels of the candidate biomarkers was carried out in a 30% sample subset (n=48, 21 NN, 8 D-AA, 8 P-AA, and 11 CRC cases). Penalized logistic regressions Least Absolute Shrinkage and Selection Operator (LASSO) and Elastic net were applied to the candidate biomarkers, age, and sex for feature selection, with the \texttt{glmnet} R package (60). The minimum mean cross-validation error was used to define the penalty factor for LASSO and Elastic Net models. Biomarkers present in the LASSO and Elastic net-derived models were selected for validation. Then, multivariate logistic regressions were fitted in the remaining 70% samples (n=105, 39 NN, 23 D-AA, 19 P-AA, and 24 CRC cases) to derive models based on the selected biomarkers.

**Data availability**

The Infinium MethylationEPIC data from all the pooled samples generated and analyzed during this study has been deposited in the NCBI Gene Expression Omnibus (GEO) (www.ncbi.nlm.nih.gov/geo) and are accessible through GEO Series accession number GSE186381.

**Statistics**

All statistical analyses were performed with the R environment (version 3.4.0). In the epigenome-wide methylation analyses, \( p \)-values were adjusted for multiple testing with the Benjamini-Hochberg procedure to control the false discovery rate (FDR). One-sided Fisher’s exact tests were used to assess the significance of the enrichment of the DMPs to functionally annotated elements using the annotation of the complete array as background. To assess the performance of the classification models, receiver-operating characteristic (ROC) curves were elaborated, derived by the leave-one-out cross-validation approach, and AUC, sensitivity, and specificity values, were estimated with their corresponding 95% confidence intervals. The best cut-off values were determined by the Youden Index method (61). Negative and positive predictive values (NPV,
PPV) were also estimated for the best cut-off values. Fisher’s exact tests were employed to compare the proportion of distal and proximal lesions detected. Non-parametrical Wilcoxon signed-rank test was used to compare methylation levels between matched serum and plasma samples.

**Study approval**

The study was conducted according to the clinical and ethical principles of the Spanish Government and the Declaration of Helsinki and was approved by the Ethics Committee for Clinical Research of Galicia (2018/008).
Author’ contributions

LD conceived and designed the study. LD, DGC, JC, and ME supervised the study. MGG, LD, and SM contributed to the sample preparation and data acquisition. MGG, NP, and MRG performed the analysis and interpretation of data. MGG, MRG, NP, DGC, and LD contributed to the experimental design. JC, LB, AE, AC, FB, and RJ provided clinical advice for the study design, collection, and management of clinical data. MGG and LD prepared the manuscript. All authors critically reviewed and approved the final manuscript.

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Abbreviations

AA: Advanced adenoma; AN: Advanced neoplasia; BEN: benign pathologies, cfDNA: Circulating cell-free DNA; CRC: Colorectal cancer; D-AA: distal advanced adenomas; DMP: Differentially methylated position; FDR:
False discovery rate; NAA: non-advanced adenomas; NCF: No colorectal findings; P-AA: proximal advanced adenomas.
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