UNICA IRIS Institutional Research Information System

This is the Author's accepted manuscript version of the following contribution:

Giulia Casula, Stefano Lai, Eleonora Loi, Loredana Moi, Patrizia Zavattari, Annalisa Bonfiglio,

An innovative PCR-free approach for DNA methylation measure: An application for early colorectal cancer detection by means of an organic biosensor,

Sensors and Actuators B: Chemical,

Volume 398,

2024,

134698,

ISSN 0925-4005,

The publisher's version is available at:
https://doi.org/10.1016/j.snb.2023.134698.

When citing, please refer to the published version.

This full text was downloaded from UNICA IRIS https://iris.unica.it/

Title: An innovative PCR-free approach for DNA methylation measure: an application for early colorectal cancer detection by means of an organic biosensor

Authors: *Giulia Casula[a,]*, Stefano Lai[a], Eleonora Loi[b], Loredana Moi[b], Patrizia Zavattari[b], and Annalisa Bonfiglio[a,c,]**

*Corresponding authors

E-mail: giulia.casula@unica.it, annalisa.bonfiglio@unica.it

Affiliations:

[a] *Department of Electrical and Electronic Engineering, University of Cagliari, Piazza D'Armi, 09123, Cagliari, Italy*

[b] *Department of Biomedical Sciences, Unit of Biology and Genetics, University of Cagliari, 09042, Cagliari, Italy*

[c] *Scuola Universitaria Superiore IUSS di Pavia , Palazzo del Broletto, Piazza della Vittoria 15 – 27100, Pavia, Italy*

Abstract. The colorectal cancer (CRC) is one of the most treatable cancers, but high mortality levels have been reported as a result of its asymptomatic nature in the very early stages of the disease. This justifies the importance of mass screening programs to fight colorectal cancer, and thus the interest in the development of minimally invasive, cost-effective tests. In this paper, a novel approach for the detection of CRC biomarkers in DNA stool samples is reported. The strategy is based on the detection of DNA methylation alterations as highly sensitive and selective epigenetic biomarkers by means of an organic transistor-based sensor, particularly suited for the integration in portable, low-cost and easy-to-use sensing system. A clear demonstration of the detection strategy effectiveness is provided using a standard CRC biomarker, namely *SEPT9* (encoding for septin9), and further deepened using an innovative biomarker, *GRIA4* (encoding for glutamate ionotropic receptor AMPA type subunit 4), in several samples coming from CRC patients. The correct detection of biomarkers, as confirmed by independent optical analysis, is demonstrated both on amplicons and unamplified genomic DNA

derived from stool samples, thus making the proposed approach particularly attractive for the future development of non-invasive, PCR-free and low-cost mass screening tests.

Keywords: Epigenetic biomarker, DNA methylation alteration, non-invasive cancer test, methylight, quantitative-PCR method

1. Introduction

Colorectal cancer (CRC) is the third most common cancer diagnosed worldwide, the second in terms of mortality [1], although it is one of the most preventable ones. Indeed, patients receiving early diagnosis experience successful treatment, while most cases are diagnosed in advanced and incurable stages. Therefore, early diagnosis and treatment are fundamental for reducing the burden of disease, and this justifies the interest of national healthcare systems in the promotion of screening programs [2, 3]. Currently, CRC diagnosis mainly relies on invasive methods such as colonoscopy, which enables highly sensitive detection and removal of precancerous lesions [4]. Nevertheless, invasive methods are expensive, require thorough bowel preparations and cause discomfort, leading to poor compliance among patients and to a questionable effectiveness in mass screening programs. Non-invasive approaches, such as faecal occult blood test (FOBT), guaiac-FOBT, faecal immunochemical test (FIT), have a much broader acceptance among patients [5, 6], but their restrained sensitivity and specificity are unfortunately an issue [7, 8]. For this reason, the current trend in research is focused on the development of non-invasive screening procedures and tools endowed with high sensitivity and specificity at very early stage of the disease. For clinical applications, a great attention has been paid to markers detectable in body fluids or in biospecimens non-invasively collected (stool, urine, saliva). As regards CRC, different biomarkers that can be identified in blood or stool samples have been proposed in literature in the last years [9, 10, 11]. In particular, the role of epigenetic factors in cancer onset, such as DNA methylation, has been thoroughly investigated. DNA methylation is a covalent modification of cytosines consisting in the addition of a methyl group leading to 5-methylcytosine (5mC). Changes in DNA methylation present several features making them promising cancer biomarkers. Firstly, these alterations are frequent events in cancer genesis, and detectable even

in precancerous lesions, thus being potential biomarkers for early cancer diagnosis [12-22]. Moreover, they can also be found in cell-free circulating tumour DNA (ctDNA) in various body matrices, making their detection possible through non-invasive methods. Finally, DNA methylation is a stable epigenetic marker, and a multitude of well-established techniques can be used for its detection [23]. Although an immense number of studies about DNA methylation-based biomarkers have been published, a few of them are commercially available and have been included in clinical guidelines, and even less have been approved by the Food and Drug Administration (FDA) to be introduced in clinical practice for CRC screening [24]. So far, three DNA-methylation-based blood tests are commercially available for CRC detection: Epi proColon (Epigenomics), ColoVantage (Quest Diagnostics) and RealTime mS9 (Abbott) [25]. All these three tests are based on a well-known CRC methylation biomarker, namely *SEPT9.* Although *SEPT9* exhibited overall high sensitivity and specificity in detecting CRC and some *SEPT9*-based diagnostic tests have been developed [26-30], it has been found that detection performance increases with CRC stage [31, 32].To date, the power of *SEPT9* methylation-based test on detecting adenomas and early stage CRC is limited [28]. A DNA methylation-based stool test commercially available is ColoSure (LabCorp), evaluating *VIM* (encoding for vimentin) methylation, but still with a high risk of false negative results [33, 34]. The proposed kits are believed to significantly impact the CRC diagnosis practice, introducing non- (or minimally-) invasive approaches with high sensitivity and specificity. Nonetheless, relatively high costs (a few-several hundreds of Euros) and time-to-result (ranging from a few days to several weeks) strongly mitigate the potential advantages in their employment in mass screening programs. At the state-of-the-art laboratory-scale approaches, such as quantitative Polymerase Chain Reaction (qPCR), are employed to detect epigenetic biomarkers in samples collected in these kits, requiring several preparation passages and the involvement of highly-skilled personnel. Therefore, there is an increasing effort in the development of new diagnostic devices with higher efficacy, possibly avoiding PCR amplification or the employment of sophisticated laboratory instrumentation. Cancer biosensors attracted a significant interest in the scientific community for their potential superior

analytical performance, real-time and parallel detection [35]. Among possible approaches, Field-Effect Transistor (FET)-based biosensors, namely bioFETs, are attracting a huge attention. Differently from most electrochemical approaches, bioFETs ensure direct, often label-free, detection of several biochemical species [36]. The electronic transduction provided by bioFETs significantly reduces the readout complexity, as the biochemical reaction under analysis is directly converted into an electronic signal. Moreover, sensor fabrication can benefit of the low-cost fabrication processes of electronic technologies. The combination of these advantages makes bioFETs ideal for the development of low cost, portable instrumentation for mass screening. Interestingly enough, bioFETs have been already employed for the detection of cancer biomarkers [37]. Protein detection by means of ImmunoFETs has been demonstrated, among others, for breast cancer in saliva [38], for bladder cancer in urine [39], and for liver cancer in serum [40]. Recently, multibiomarker detection for early cancer biomarkers using organic FET-based immunosensor was also reported [41]. Molecular detection of microRNA by GenFETs, which is by far the most sensitive and selective, has been demonstrated for breast cancer [42, 43]. Although some preliminary results for biomarker detection by means of bioFET are thus available, even without the need for PCR amplification [43], a huge effort must be paid in an effective transfer of this approach to the real application scenario. Moreover, the promising epigenetic biomarker approach is so far unexplored, due to the fact that classic bioFETs perform genetic detection by exploiting the complementarity of DNA molecules. In this paper, a bioFET conceived for PCR-free detection of epigenetic cancer biomarkers is reported, exploiting for the first time ever, to our knowledge, in this field the peculiar advantages of flexible organic electronics as cost-effective, large-area technology suitable for the development of low cost, easy-to-handle mass screening kits. The detection of CRC DNA methylation-based biomarkers will occur in samples extracted from stool specimens by exploiting the working principle of the Organic Charge-Modulated Field-Effect Transistor (OCMFET), which has been thoroughly investigated as DNA hybridization sensor [44-46]. Here, the hybridization detection is employed to recognize different methylation patterns by exploiting the conversion of non-methylated regions in the molecule, i.e. by

encoding the epigenetic information into a modification in the base sequence. The methylation profile of two genomic regions altered in CRC has been tested: the first is the *SEPT9*, and the second one, recently demonstrated as a CRC biomarker with superior specificity and informativity, is *GRIA4* [17, 47], identified by our group with Illumina genome-wide microarray technology, allowing the interrogation of more than 450K CpG loci. In this work, different tests will be presented, demonstrating that the device is capable of discriminating positive and negative samples, in agreement with a classic fluorescence-based method for DNA methylation analysis (MethyLight) carried out as reference standard. Interestingly, the device functionality has been demonstrated on both PCR-amplified and unamplified DNA extracted from stool samples. Genomic DNA detection with bioFETs was previously demonstrated on bacterial, highly-purified samples [48]; here, the possibility of recognizing human genomic DNA is demonstrated for the first time. The proposed approach will combine several features, such as low-cost fabrication over large areas enabled by organic electronic processes, direct and label-free electronic detection enabling quasi-real-time result, PCR-free and easy sample collection and preparation. Consequently, it will be particularly suited for the development of an innovative class of user-friendly, portable CRC diagnosis kit for mass screening, ensuring high sensitivity and specificity at affordable costs.

2. Experimental section

2.1. Device structure

Fig. 1 shows a cross-section of the OCMFET structure, which is based on a modified bottom-gate, bottom-contact transistor in order to keep the gate electrically floating. The operating point of the device is fixed by means of a control gate, capacitively coupled with the floating gate. The latest is electrically connected to the sensing area of the device, where the DNA probes are anchored. The detailed fabrication of the device has been reported elsewhere [45]: all metal layers were obtained by thermal evaporation and patterned by means of photolithography. Aluminum was employed for the floating gate, in order to have native aluminium oxide in combination with a thin Parylene C

(Specialty Coating Systems) layer, deposited by Chemical Vapor Deposition, to obtain a sufficiently high gate capacitance (15 nF/cm^2) for low voltage operation. Gold was used as second metal layer for source, drain, control gate and sensing area. This material allows easy functionalization chemistry and optimal biocompatibility of the sensing area. Moreover, gold is a standard choice for source and drain electrodes when 6,13-Bis(triisopropylsilylethynyl)-pentacene (TIPS-pentacene, Sigma-Aldrich) is employed as semiconductor. TIPS pentacene ensures high stability and easy processing, as it can be deposited from liquid phase (1wt% solution in anisole anhydrous). Source and drain were patterned following a self-alignment process with the aim of enhancing sensitivity, as described in [49]. In order to perform biochemical measurements, 3D printer incubation chambers (custom fabrication using a Makerbot Replicator 2x, Makerbot) were fixed on the sensing area by means of polydimethylsiloxane (PDMS, Sylgard 186®, Dow Inc.). Finally, the device was encapsulated by means of a 2 μm-thick Parylene C layer to further improve the stability of the electrical performances in time.

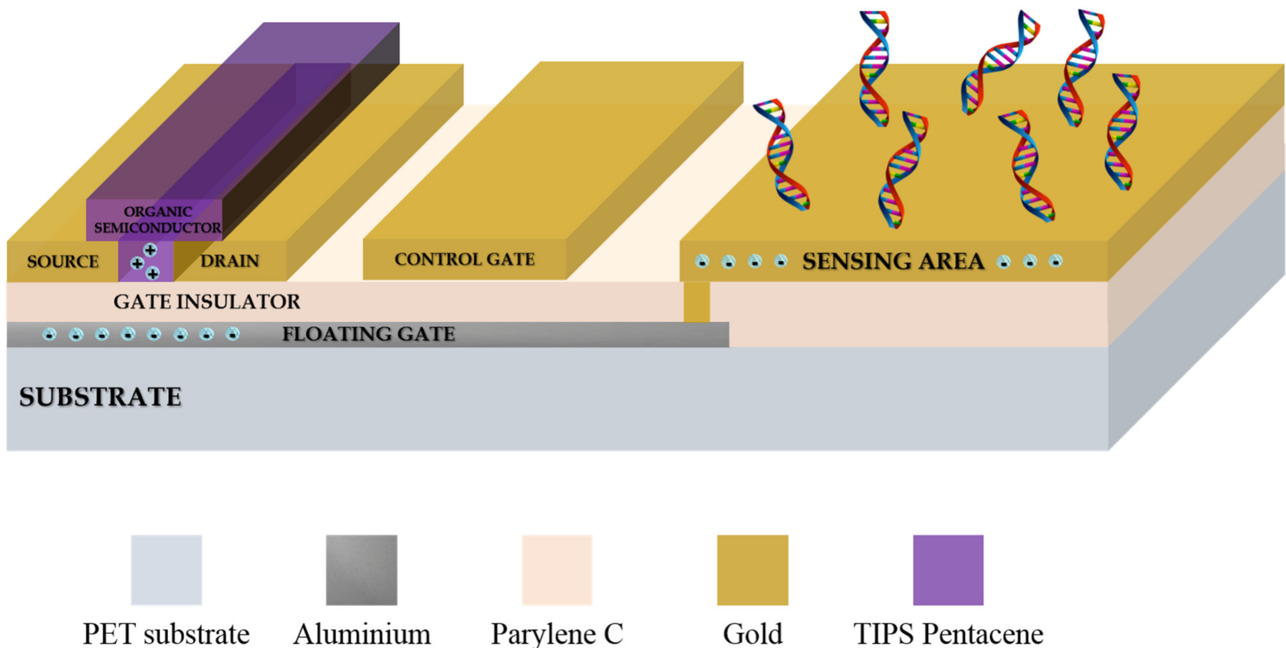

Fig. 1: *Cross-section of OCMFET structure based on a bottom-gate bottom-contact OFET.*

2.2. Sensor functionalization

The specificity of the OCMFET to the selected biomarkers was obtained by the immobilization of appropriate DNA probes onto the gold sensing area. To this aim, thiol-modified DNA sequences have been designed. For *SEPT9* experiments, the employed probe was (HS)-5'-$(T)_5$-CAAAATCCTC TCCAACACGTCCGCGACCGC-3'. As it can be deduced by the sequences of the probes, they are interrogating 4 CpGs for *SEPT9* and 6 CpGs for *GRIA4*. For *GRIA4*, the employed probe was (HS)-5'-$(T)_5$-ACACTAACGCCGCGACCGCCACACGCGCTA–3'. These probes (Sigma-Aldrich) were designed to be complementary to the methylated specific genomic regions to be interrogated. Here, the first five thymines act as spacer between the probe and the sensing surface. In order to promote probe anchoring on gold, the sensing area was preliminary cleaned with a 1:10 aqueous solution of hydrochloric acid (HCl, Sigma Aldrich). Then, a solution containing a 100 nM dispersion of the probe in a functionalization buffer (1M KH_2PO_4, 1M H_3PO_4, Sigma Aldrich, pH 4.7, I = 1.04 M) was spotted on the sensing area, followed by 1 μM solution of 6-mercapto-1-hexanol (MCH, Sigma Aldrich). MCH acts as a spacer between probes to enhance the ordering of the self-assembled monolayer. Devices have been stored at room temperature overnight in this functionalization solution. Before hybridization tests, the sensing areas were thoroughly rinsed with a 50 mM phosphate buffered saline solution (PBS, Sigma-Aldrich), containing sodium chloride (NaCl, Sigma Aldrich) at a concentration of 50 mM, in order to remove any a-specific absorbed molecule.

2.3. Electrical measurement setup

Electrical measurements were performed and recorded in liquid environment (phosphate buffered saline solution 50 mM, 50 mM NaCl, pH = 7.04, I = 50 mM), in ambient conditions and at room temperature, employing a Keithley® 2636 SourceMeter and custom-made Matlab® scripts. In particular, output and transfer characteristic curves have been acquired in order to characterise device electrical performances and extrapolate basic parameters, such as threshold voltage and mobility (Fig.

S1 in Supplementary Material). Sensor characterization was performed by the real-time acquisition of the output current of the OCMFETs.

2.4. Samples collection and DNA samples preparation

Stool samples of eight CRC patients were available at the Laboratory of Molecular Biology, Genomics and Epigenomics, Department of Biomedical Sciences, collected from the Hospital of the University of Cagliari (Italy). Clinical characteristics of the tumours are described in Table 1. Samples were taken intraoperatively from the bowel resection specimen and immediately frozen after collection and stored at -80 °C until being processed. For patient number 3, normal tissue sample was taken at a distance >10 cm from the neoplastic tissue during surgical resection. All the biological samples analysed were obtained with written informed consent signed from patients and ethical approval granted by the relative Ethics Committee.

DNA was extracted from stool samples by using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Genomic DNA from the normal tissue sample was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). DNA samples were subjected to sodium bisulfite conversion using EZ DNA Methylation Gold Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions.

SAMPLE ID	TUMOUR LOCATION	STAGE AT DIAGNOSIS	GRADE	BIOLOGICAL MATRIX
2	Left colon	I	G2	Stool
3	Right colon	III	G2	Stool / Normal tissue
14	Rectum	III	G3	Stool
19	Right colon	II	G2	Stool
21	Transversal colon	II	G2	Stool
29	Right colon	II	G2	Stool
33	Right colon	IV	G2	Stool
34	Rectum	III	G2	Stool

Table 1:Clinical characteristics of CRC patients.

2.5 Methylation Analysis by MethyLight

Validation of *SEPT9* and *GRIA4* methylation was evaluated in MethyLight qPCR by using primers and probes designed with Beacon Designer™ (Premier Biosoft, San Francisco, CA, USA) (Table 2). As it can be deduced by the sequences of the probes, they are interrogating 3 CpGs for *SEPT9* and 4 CpGs for *GRIA4*. The probes were labelled with the 6-Carboxyfluorescein (6-FAM) fluorophore at the 5′ end. A primer-probe mix containing 300 nM of each primer and 100 nM of the probe was prepared. Each assay was performed in triplicate using: 15 µl of TaqMan Genotyping Master mix 2X (Applied Biosystems, Foster City, CA, USA), 4.5 µL of primer-probe mix, 5 µL of bisulfite-converted DNA (10 ng/µL) and 5.5 µL of RNase-free water. The experiments were conducted on a DNA Engine Opticon 2 Real-Time Cycler (Bio-Rad, Hercules, CA, USA) using the following thermal conditions: initial PCR activation step at 95 °C for 10 min, followed by 50 cycles of denaturation step at 95 °C for 15 s and annealing/extension step at 60 °C for 60 s. The results are reported as Ct (threshold cycle), that is the intersection between an amplification curve and a threshold line. It represents a relative measure of the target concentration in the PCR reaction.

Target	*Forward primer (5′–3′)*	*Reverse primer (5′–3′)*	*Probe (5′–3′)*	*Sequence between primers (5′-3′)*
SEPT9	GGATTTAGAAGGTGGG TGTTGG	CCAAACCCACCCCCA AAATCCTCTC	CCGCGACCGCAACAA CC	GTTGGTTGTTGYGGTY GYGGAYGTGTTG
GRIA4	GGGTTGGTGTAGGTTT GTT	CTCCCCCCTTACTTTC TCACATACACACAA	AACGCCGCGACCGCC ACAC	GGGGGATGTYGGTTG ATGYGAGTTGGAGAG YGYGTGTGGYGGTYGY GGYGTTAGTGT

Table 2:Primers and probe sequences for MethyLight assay.

Notes: Y indicates C/T of CpG sites

3. **Results and discussion**

3.1. Epigenetic biomarkers detection strategy

The detection of epigenetic biomarkers by means of the OCMFET requires a modification of the detection strategy previously employed for genetic applications. In that case, the OCMFET

transduced the intrinsic negative charge of target, single-stranded DNA (ssDNA) molecules captured by ssDNA probes anchored on the surface of the sensing area. Indeed, when hybridization occurs, double-stranded DNAs (dsDNAs) are formed on the sensing surface: as the total negative charge increases, the floating gate charge distribution is modulated and a variation of the threshold voltage of the transistor is obtained. A complete description of this working principle is thoroughly discussed elsewhere [50]: briefly, the threshold voltage shift ΔV_{TH} can be related to the additional charge of target molecules ΔQ according to the equation:

$$\Delta V_{TH} = -\frac{\Delta Q}{C_{TOT}}$$

where C_{TOT} is a capacitive term related to the sensor layout. This detection strategy relies on the complementarity between the target molecules to be detected and a self-assembled monolayer of probes acting as bioreceptors. This strategy can be easily adapted for the detection of the epigenetic DNA methylation alteration as biomarker, by exploiting the sodium bisulfite ($NaHSO_3$) conversion of target molecules. Indeed, alterations in the methylation pattern predominantly occurs in the context of CpG dinucleotides defined as CpG sites, often grouped into high CpG density regions, termed as CpG islands (CGIs), often overlapping with gene regulatory regions. In fact, the region interrogated in the present study is included within a CGI located in the promoter of *GRIA4*. As schematically shown in Fig.2a, bisulfite conversion is a process in which genomic DNA is denatured and treated with sodium bisulfite, leading to deamination of unmethylated cytosines in uracils, while 5mC remained unchanged. This allows to differentiate between methylated and unmethylated cytosines, potentially offering a single-nucleotide resolution information about the methylated loci of DNA, converted into different sequences of bases that can be thus inferred by exploiting the hybridization reaction. Indeed, if DNA probes are designed to be complementary for a specific sequence, obtained by the conversion of a certain methylation pattern, only target molecules showing the expected pattern are captured and can determine a charge variation on the sensing area, which remains unvaried otherwise (Fig. 2b). If hybridization occurs, the threshold voltage shift determines the modification

of the working point of the transistor: as a consequence, a variation in the output current of the device is obtained (Fig. 2c) for the perfect matching, while no specific current variation is expected otherwise.

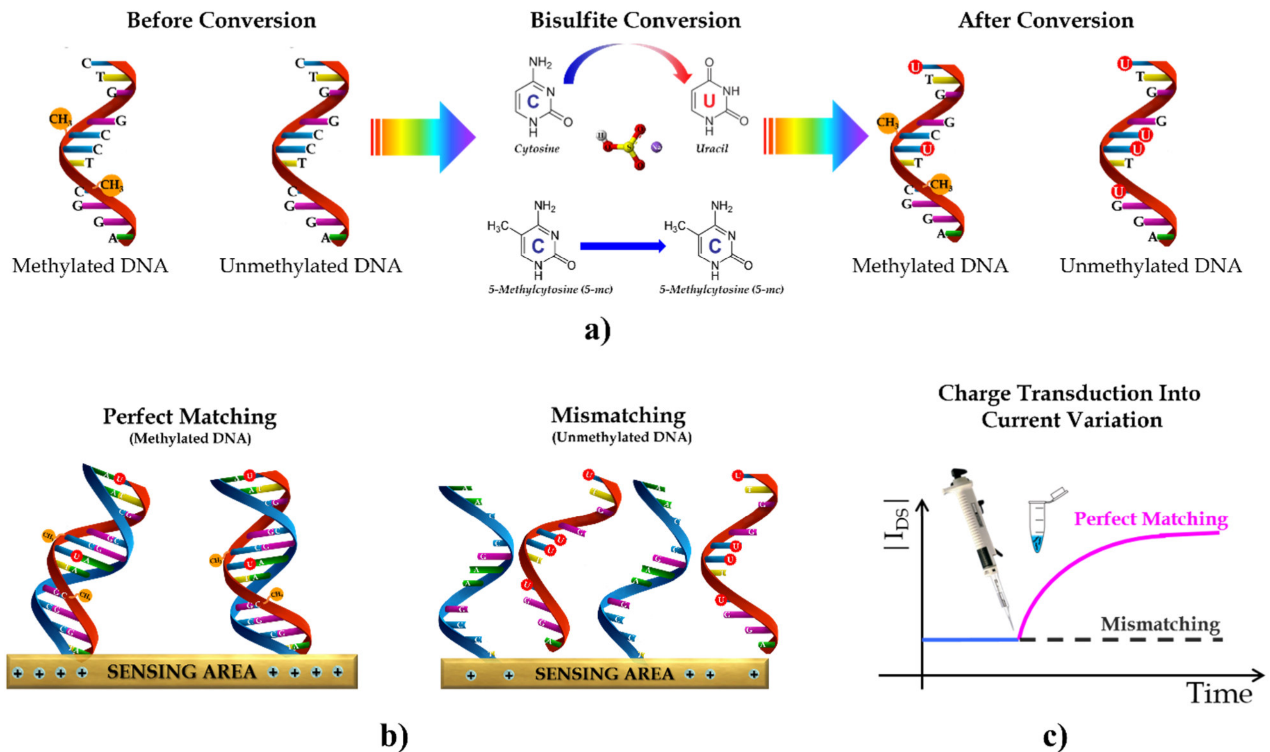

Fig. 2. *(a) Sodium bisulfite conversion of DNA molecules (target): unmethylated cytosines are converted to uracils, while methylated molecules remain unaffected. (b)Probes designed for the detection of a specific biomarker allow hybridization only with perfect matching target molecules. (c) In an OCMFET, when a biomarker is detected (perfect matching), a variation in the output current (I_{DS}) occurs, while it remains unvaried otherwise.*

3.2. Electronic detection of SEPT9

At first, the OCMFET structure was evaluated as sensor for the detection of epigenetic biomarkers by employing *SEPT9*. OCMFET sensors were functionalized with specific probes designed for the detection of *SEPT9*, as described in Experimental Section. Electronic detection was investigated by recording in real time the output current of the devices during hybridization process. A constant source to drain voltage V_{DS} = -5 V was applied, while the control gate was biased with a squared voltage V_{GS} in order to reduce as much as possible the bias stress of the OFET. This effect leads to a

continuous charge trapping at the semiconductor/insulator interface, thus determining an unspecific reduction of the current in time. The amplitude and the frequency of the voltage were chosen in order to make the device working in over-threshold conditions. Hybridization was performed at room temperature by spotting a solution of amplicons diluted in a 50 mM phosphate buffered saline (PBS) solution, with a NaCl concentration of 50 mM. This is a standard measurement environment for OCMFET testing with ssDNA molecules, ensuring an optimal trade-off between hybridization efficiency (which requires significant salt concentration) and sensitivity, which may be influenced by Debye screening length [51]. Initially, tests were performed employing amplicons, *i.e.* oligonucleotides obtained from the amplification through PCR of the target region of *SEPT9*. Specifically, the final concentration of the amplicons was around 900 ng/µl starting from 100 ng of stool DNA subjected to bisulfite conversion (45 cycles of PCR). Representative results are reported in Fig. 3a in terms of percentage current variation with respect to the baseline current, *i.e*. the current recorded before hybridization. It is possible to observe that an increase of the current is obtained when amplicons from DNA stool sample is employed: according to the OCMFET working principle, this effect is related to an increase of the negative charge on the sensing area, which is coherent with the detection of the *SEPT9* biomarker. On the other hand, when a target from non-tumoral DNA is employed, a reduction of the output current was recorded, meaning that *SEPT9* was correctly not identified: indeed, since no charge is anchored on the sensing surface, current should remain stable but the residual bias stress effect leads to a (even minimum) current decrease. The different behaviours obtained in the two cases can be interpreted with a positive detection *of SEPT9* when a current increase is recorded, as predicted by the OCMFET model. Fig. 3b shows the average data on four technical replicates for stool sample DNA, and three for non-tumoral tissue DNA sample. Devices show a reproducible response for both kinds of samples: in particular, low standard deviation is observed for stool DNA samples, while larger error bars characterize the result obtained for non-tumoral tissue DNA sample. This is due to the effect of bias stress, which can significantly vary among devices. Nonetheless, as average and standard deviation values of the two categories of

samples are not overlapped, these results demonstrate that the OCMFET can be employed for specific biomarkers detection.

Subsequently, the possibility of recognizing cancer specific biomarkers directly from human genomic DNA was investigated and demonstrated. Indeed, the same experiments just described for the detection of *SEPT9* from amplicons, were performed on unamplified stool DNA samples coming from the same patient. In particular, as well as amplicons, genomic sequences are diluted in a 50 mM phosphate buffered saline (PBS) solution, with a NaCl concentration of 50 mM. Fig. 3c reports representative results for *SEPT9* detection in genomic (unamplified) stool DNA samples, which are perfectly in line with those related to amplicons: indeed, a significant increase (of about 6%) of the OCMFET current is recorded, while no increase can be observed when the target comes from non-tumoral DNA sample. It is noteworthy that a higher response was always recorded from unamplified targets, as clearly observable also from the average data reported in Fig. 3d. This could be justified by the fact that the captured genomic fragments are surely longer than amplicons, as they comprehend additional nucleotides to the *SEPT9* target sequence. Although these additional nucleotides are not involved in the hybridization with the probe, they can act as a "label", bringing an additional charge on the surface. Since the OCMFET has been already demonstrated to be capable to operate at low Debye length [51], it is reasonable that this additional charge is also sensed by the device, contributing to the variation of its output current. It is noteworthy that the specificity is unaltered, as probes are designed to interact specifically with the *SEPT9* target sequence in the fragment. As for Fig. 3b, larger error bars in the negative control are related to the different extent of bias stress drift in different devices; however, the distinction with positive response is unambiguous.

The same samples were analysed in parallel with a classic optical method, *i.e.* MethyLight, a quantitative PCR-based method to measure DNA methylation levels in real time using fluorescent probes. As shown in Fig. 3e and f, we observed an earlier Ct curve for the two technical replicates using stool DNA (corresponding to the higher number of copies of the target of interest and to a lower Ct) followed by the Ct curve for the two technical replicates using DNA extracted from non-tumoral

tissue (with a lower number of copies of the target of interest corresponding to a higher Ct). Therefore, MethyLight confirmed that *SEPT9* biomarker was strongly detectable in stool DNA samples and as expected at a lower level in a non-tumoral tissue DNA sample, in accordance with what obtained with OCMFET analysis.

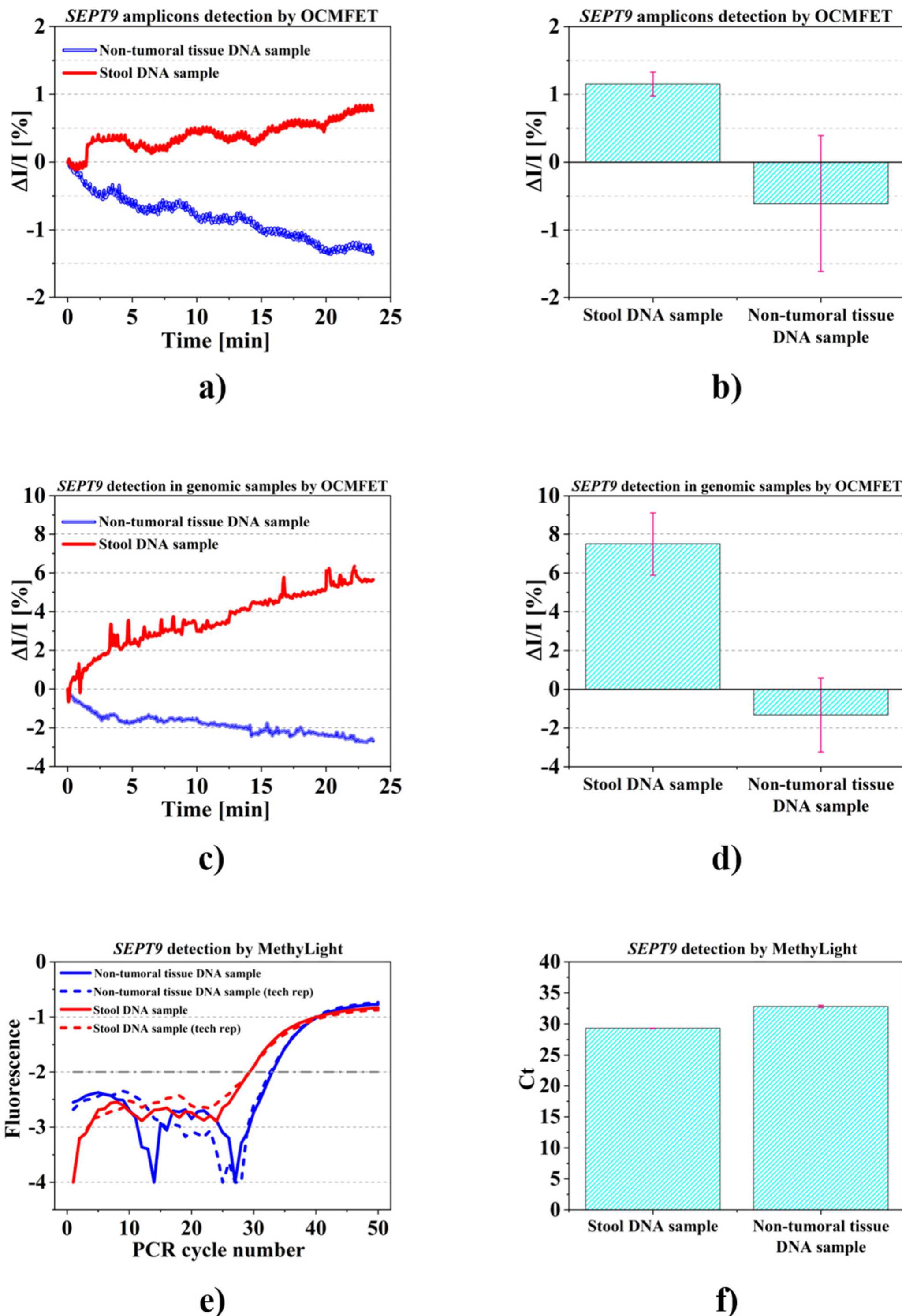

Fig. 3. *(a, c). Representative real time percentage current variation IΔ of OCMFET sensors during hybridization tests, normalized to the baseline current I, recorded before the spotting of target sequences. (a) SEPT9 amplicons detection test in non-tumoral tissue DNA sample (decrease of device current means no response) and stool DNA sample (increase of device current means biomarker identified) coming from the same CRC patient. (c) SEPT9 detection test in genomic non-tumoral tissue DNA sample and stool DNA sample coming from the same CRC patient. (b, d). Average data of experiments, carried out on at least three different devices for both SEPT9 amplicons (error bars are 1-σ tolerance bands) (b) and genomic samples (d). (e, f). SEPT9 Validation results obtained by*

MethyLight, a quantitative PCR-based optical method to measure DNA methylation levels in real time using fluorescent probes. (e) Plot showing the measured fluorescence, in logarithmic scale, at increasing PCR cycles for each replica (plots in exponential scale are available in Supplementary Information, Fig. S3). (f) Bar plots showing average Ct and standard deviation for the two technical replicates of each tested sample (error bars are 1-σ tolerance bands).

3.3. Electronic detection of an innovative CRC biomarker

Proved that OCMFET sensor is able to detect epigenetic biomarkers, the focus was set on the detection of the new promising CRC biomarker, *GRIA4*, directly on DNA extracted from stool samples of eight CRC patients. This biomarker, thoroughly examined elsewhere [21], shows superior informativity at the very early stages of the disease, representing a valid alternative to SEPT9 for early diagnosis applications.

DNA stool samples from the same eight CRC patients were analysed by MethyLight. A broad range in DNA methylation was observed, from very high levels to undetectable, with a non-existing curve or a curve remaining below the threshold (Fig. 4a, b). Sample 19 appears as an outlier with the highest methylation levels (earliest Ct curve for the two technical replicates, lowest Ct). A group of samples showed from low (sample 34 and sample 3) to intermediate methylation levels (samples 29, 2, 33). For two samples (sample 14 and sample 21), it was not possible to estimate Ct (under threshold) and thus the methylation was not detected.

The same samples have been then employed for electronic detection using the OCMFET in blind mode, *i.e.* without any information about the methylation level and kind of sample provided to the operators that carried out electrical measurements. Fig. 4c, d show typical real-time responses and average data (obtained from at least three technical replicates of the same sample, with the sole exception of sample 34), respectively. It is possible to observe that the data are coherent with those obtained by MethyLight: sample 19 gave rise to the higher signal amplitude (among 4% of average current variation) and the lowest Ct, while the lower sensor's response was obtained for sample 34 with the highest Ct among the detectable samples. Sample 29 and 33 are consistent with the intermediate methylation level recorded by MethyLight. The only remarkable difference among the

two techniques was observed for sample 2 and sample 3: this may be due to possible heterogeneity of samples, which will present variable methylation levels in the various molecules extracted (especially due to the fact that these are somatic, not germline, epimutations), thus bringing to different measurements on different replicas. Nonetheless, a positive methylation pattern was in any case detected, coherently with what obtained by MethyLight. Interestingly, a low methylation level was observed for sample 14: this is consistent with previous analysis on that sample carried out by droplet digital PCR (ddPCR, [21]), which is another quantitative PCR-based method characterized by a higher sensitivity. Sample 21 resulted in an undetectable methylation level, coherently with what obtained by MethyLight and ddPCR. Therefore, the proposed approach is capable to correctly discriminate among different methylation levels in genomic samples, *i.e.* without the need for biomarker amplification, with a performance comparable to the one of quantitative PCR methods. Similar results have been obtained by the capturing of *GRIA4* amplicons obtained by PCR (Fig. S2 in Supplementary Material).

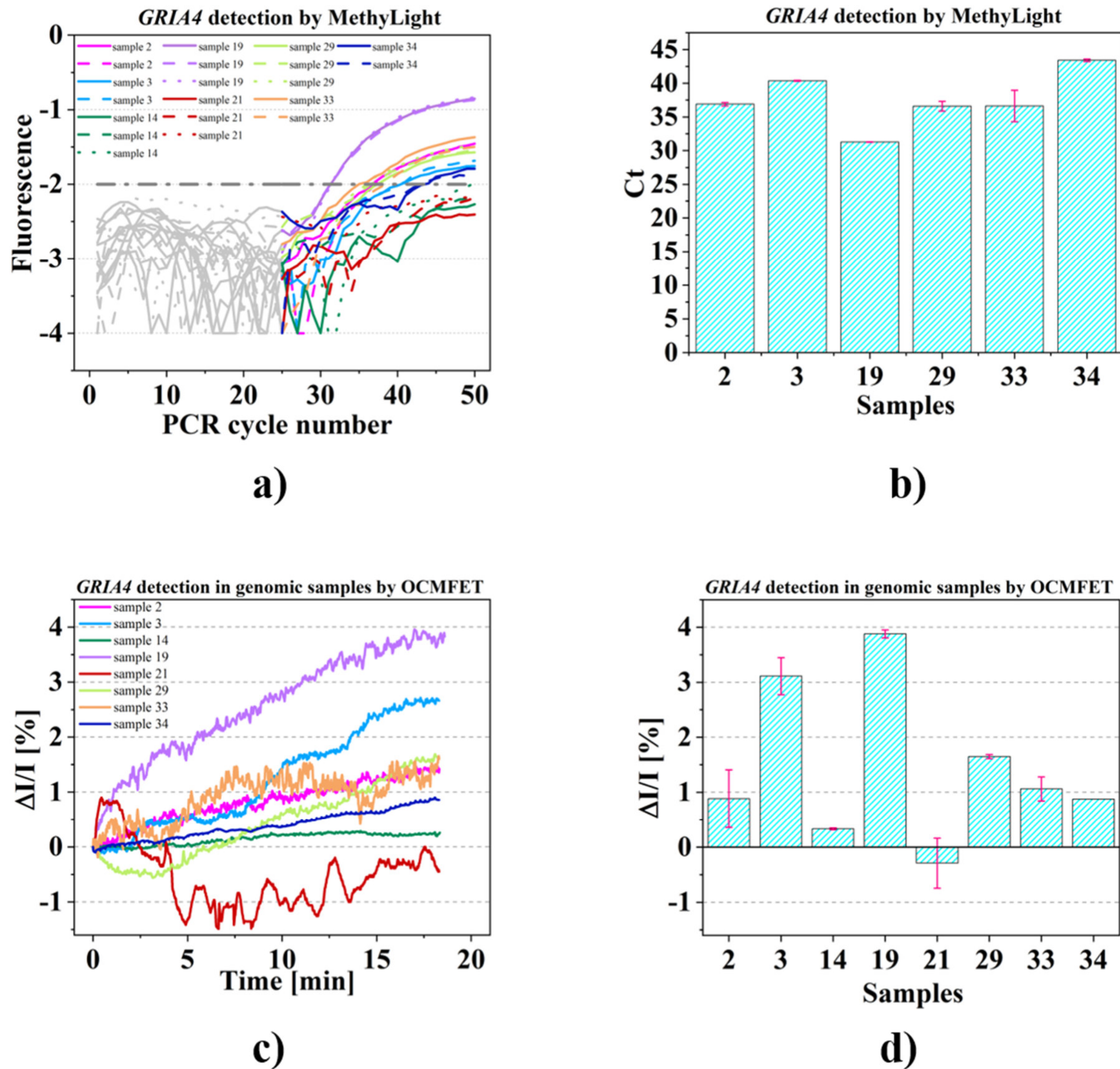

Fig. 4. *(a, b). GRIA4 methylation analysis results obtained by MethyLight, a quantitative PCR-based optical method to measure DNA methylation levels in real time using fluorescent probes. (a) Plot showing the measured fluorescence, in logarithmic scale, at increasing PCR cycles for each replicates of the analyzed sample (plots in exponential scale are available in Supplementary Information, Fig. S4). (b) Bar plots showing average Ct and standard deviation for the two technical replicates of each tested sample (1-σ tolerance band). (c, d). GRIA4 methylation analysis results obtained by electronic detection with OCMFET. (c) Plot showing the real time current relative variation for a representative replicate of each tested sample. (d) Average current variation for at least three technical replicates of each tested sample (with the sole exception of sample 34, 1-σ tolerance band).*

4. **Conclusions**

In this paper, we proposed an innovative approach for the direct detection of epigenetic biomarkers of colorectal cancer by means of an organic transistor-based biosensor. The considered biomarkers are alterations of the methylation pattern, and the proposed strategy is based on the conversion of such epigenetic factor into a genetic information (*i.e.*, a modification in the base sequence of the target molecule). For the first time ever, this strategy is combined with the electronic detection of DNA hybridization, which ensures direct, highly sensitive, real-time transduction of the biochemical phenomena with minimum sample preparation. This working principle has been validated using a recognized, standard optical approach, namely MethyLight, and a well-known epigenetic biomarker for CRC, namely *SEPT9*. The obtained results demonstrated the correct discrimination between non-tumoral tissue and tumoral stool DNA samples coming from CRC patients in both amplicons and genomic (unamplified) material. This last result was thoroughly deepened by employing the OCMFET for the detection of an innovative epigenetic CRC biomarker, namely *GRIA4*, characterized by superior specificity and informativity. Indeed, different methylation levels has been characterized in a blind test carried out on genomic (unamplified) stool samples coming from eight CRC patients, in accordance with what obtained by MethyLight analyses. The proposed results represent a first important demonstration of the possibility of an electronic detection of epigenetic cancer biomarkers; further tests on a statistically relevant set of samples and technical replicates are obviously requested to upscale this proof-of-concept validation to a more representative application scenario. Similarly, OCMFET figures of merits needs to be further investigated and characterized in order to fit with the performances required in real application scenario, and several optimizations in the measurement setup can be also implemented. In particular, the optimization of target capture and signal transduction play a fundamental role for an adequate sensitivity: this will require the definition of optimal (minimal) ionic strength in the measurement solution, and the reduction of electrostatic repulsion between probe and target molecules by means of PNA probe employment. Nonetheless, the described activity could pave the way for the development of an innovative approach for cancer

biomarker detection by means of low-cost, portable devices, suitable for the integration of easy-to-use, cost-effective and PCR-free kits for mass screening.

CRediT authorship contribution statement

Giulia Casula: Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing Original Draft, Writing Review & Editing, Visualization. **Stefano Lai:** Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing Original Draft, Writing Review & Editing. **Eleonora Loi:** Methodology, Softwares, Validation, Formal analysis, Investigation, Data curation, Writing original draft, Writing review and editing. **Loredana Moi:** Methodology, Validation, Investigation, Data curation. **Patrizia Zavattari**: Conceptualization, Methodology, Softwares, Formal analysis, Investigation, Data curation, Writing original draft, Writing review and editing, Supervision, Project administration, Funding acquisition. **Annalisa Bonfiglio:** Conceptualization, Writing Original Draft, Writing Review & Editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

This project has received funding from Fondazione di Sardegna, Italy (Prot. 738/2012-0246 in 2012 to P.Z.; F72F16003120002 to P.Z. in 2016; U278.2021/AI.259.RP to P.Z. in 2021), Regione

Autonoma della Sardegna, Italy (F72I15000920002 to P.Z. in 2015), POR FESR Sardegna, Italy (F31G17000050002 to P.Z. and A.B. in 2018).

References

[1] H. Sung, J. Ferlay, R. L. Siegel, M. Laversanne, I. Soerjomataram, A. Jemal, F. Bray, Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries, CA-Cancer J. Clin. 71(3) (2021) 209-249, https://doi.org/10.3322/caac.21660

[2] R. H. Henderson, D. French, T. Maughan, R. Adams, C. Allemani, P. Minicozzi, M. P. Coleman, E. McFerran, R. Sullivan, M. Lawler, The economic burden of colorectal cancer across Europe: A population-based cost-of-illness study, Lancet Gastroenterol. Hepatol. The 6(9) (2021) 709-722, https://doi.org/10.1016/S2468-1253(21)00147-3

[3] R. Sharma, M. Abbasi-Kangevari, R. Abd-Rabu, H. Abidi, E. Abu-Gharbieh, J. M. Acuna, M. S. Hosseini, Global, regional, and national burden of colorectal cancer and its risk factors, 1990–2019: a systematic analysis for the Global Burden of Disease Study 2019, Lancet Gastroenterol. Hepatol. The 7(7) (2022) 627-647, https://doi.org/10.1016/S2468-1253(22)00044-9

[4] R. Hultcrantz, Aspects of colorectal cancer screening, methods, age and gender, J. Intern. Med. 289(4) (2021) 493-507, https://doi.org/10.1111/joim.13171

[5] L. L. Song, Y. M. Li, Current noninvasive tests for colorectal cancer screening: An overview of colorectal cancer screening tests, World J. Gastrointest. Oncol. 8(11) (2016) 793, https://doi.org/10.4251%2Fwjgo.v8.i11.793

[6] I. A. Issa, M. Noureddine, Colorectal cancer screening: An updated review of the available options, World J. Gastroenterol. 23(28) (2017) 5086, https://doi.org/10.3748%2Fwjg.v23.i28.5086

[7] G. P. Young, E. L. Symonds, J. E. Allison, S. R. Cole, C. G. Fraser, S. P. Halloran, E. J. Kuipers, H. E. Seaman, Advances in fecal occult blood tests: the FIT revolution, Dig. Dis. Sci. 60(3) (2015) 609-622, https://doi.org/10.1007/s10620-014-3445-3

[8] J. Meklin, K. Syrjänen, M. Eskelinen, Colorectal cancer screening with traditional and new-generation fecal immunochemical tests: a critical review of fecal occult blood tests, Anticancer Res. 40(2) (2020) 575-581, https://doi.org/10.21873/anticanres.13987

[9] O.O. Ogunwobi, F. Mahmood, A. Akingboye, Biomarkers in colorectal cancer: current research and future prospects, Int. J. Mol. Sci. 11 (2020), 5311. https://doi.org/10.3390/ijms21155311.

[10] E. Koncina, S. Haan, S. Rauh, E. Letellier, Prognostic and predictive molecular biomarkers for colorectal cancer: updates and challenges, Cancers 12(2) (2020) 319, https://doi.org/10.3390/cancers12020319

[11] G. Jung, E. Hernández-Illán, L. Moreira, F. Balaguer, A. Goel, Epigenetics of colorectal cancer: biomarker and therapeutic potential, Nat. Rev. Gastroenterol. Hepatol. 17(2) (2020) 111-130, https://doi.org/10.1038/s41575-019-0230-y

[12] T.R. Church, M. Wandell, C. Lofton-Day, S.J. Mongin, M. Burger, S.R. Payne, E. Castaños-Vélez, B. A. Blumenstein, T. Rösch, N. Osborn, N. Snover, R.W. Day, D.F. Ransohoff, Prospective evaluation of methylated SEPT9 in plasma for detection of asymptomatic colorectal cancer, Gut (2014) 63(2) 317-325, http://dx.doi.org/10.1136/gutjnl-2012-304149

[13] Y. Luo, C. J. Wong, A. M. Kaz, S. Dzieciatkowski, K. T. Carter, S. M. Morris, J. Wang, J.E. Willis, K. W. Makar, C. M. Ulrich, J. D. Lutterbaugh, M. J. Shrubsole, W. Zheng, S. D. Markowitz, W. M. Grady, Differences in DNA methylation signatures reveal multiple pathways of progression from adenoma to colorectal cancer, Gastroenterology 147(2) (2020) 418-429, https://doi.org/10.1053/j.gastro.2014.04.039

[14] Y. Toiyama, Y. Okugawa, A. Goel, DNA methylation and microRNA biomarkers for noninvasive detection of gastric and colorectal cancer, Biochem. Biophys. Res. Commun. 455(1-2) (2014) 43-57, https://doi.org/10.1016/j.bbrc.2014.08.001

[15] S. Alonso, B. González, T. Ruiz-Larroya, M. Durán Domínguez, T. Kato, A. Matsunaga, K. Suzuki, A. Y. Strongin, P. Gimènez-Bonafé, M. Perucho, Epigenetic inactivation of the extracellular matrix metallopeptidase ADAMTS19 gene and the metastatic spread in colorectal cancer, Clin. Epigenetics 7(1) (2015) 1-15, https://doi.org/10.1186/s13148-015-0158-1

[16] M. Antonelli, A. Fadda, E. Loi, L. Moi, C. Zavattari, P. Sulas, D. Gentilini, C. Cameli, E. Bacchelli, M. Badiali, A. Arcella, I. Morra, F. Giangaspero, P. Zavattari, Integrated DNA methylation analysis identifies topographical and tumoral biomarkers in pilocytic astrocytomas, Oncotarget 9(17) (2018) 13807, https://doi.org/10.18632%2Foncotarget.24480

[17] A. Fadda, D. Gentilini, L. Moi, L. Barault, V. P. Leoni, P. Sulas, L. Zorcolo, A. Restivo, F. Cabras, F. Fortunato, C. Zavattari, L. Varesco, V. Gismondi, M. R. De Miglio, A. M. Scanu, F. Colombi, P. Lombardi, I. Sarotto, E. Loi, F. Leone, S. Giordano, F. Di Nicolantonio, A. Columbano, P. Zavattari, Colorectal cancer early methylation alterations affect the crosstalk between cell and surrounding environment, tracing a biomarker signature specific for this tumor, Int. J. Cancer 143(4) (2018) 907-920, https://doi.org/10.1002/ijc.31380

[18] E. Loi, P. Zavattari, CpG Islands Methylation Alterations in Cancer: Functionally Intriguing Security Locks, Useful Early Tumor Biomarkers, The DNA, RNA, and Histone Methylomes (2019) 53-62, Springer, Cham, https://doi.org/10.1007/978-3-030-14792-1_3

[19] E. Loi, L. Moi, A. Fadda, G. Satta, M. Zucca, S. Sanna, S. A. Nia, G. Cabras, M. Padoan, C. Magnani, L. Miligi, S. Piro, D. Gentilini, M. G. Ennas, M. C. Southey, G. G. Giles, N. W. Doo, P. Cocco, P. Zavattari, Methylation alteration of SHANK1 as a predictive, diagnostic and prognostic biomarker for chronic lymphocytic leukemia., Oncotarget 10(48) (2019) 4987, https://doi.org/10.18632%2Foncotarget.27080

[20] A. F. Vega-Benedetti, E. Loi, L. Moi, S. Blois, A. Fadda, M. Antonelli, A. Arcella, M. Badiali, F. Giangaspero, I, Morra, A. Columbano, A. Restivo, L. Zorcolo, V. Giusmondi, L. Varesco, S. E.

Bellomo, S. Giordano, M. Canale, A. Casadei-Giardini, L. Faloppi, M. Puzzoni, M. Scartozzi, P. Ziranu, G. Cabras, P. Cocco, M. G. Ennas, G. Satta, M. Zucca, D. Canzio, P. Zavattari, Clustered protocadherins methylation alterations in cancer, Clin. Epigenetics 11(1) (2019) 1-20, https://doi.org/10.1186/s13148-019-0695-0

[21] A. F. Vega-Benedetti, E. Loi, L. Moi, S. Orrù, P. Ziranu, A. Pretta, E. Lai, M. Puzzoni, L. Ciccone, A. Casadei-Giardini, F. Cabras, F. Fortunato, A. Restivo, L. Zorcolo, M. Scartozzi, P. Zavattari, Colorectal cancer early detection in stool samples tracing CpG islands methylation alterations affecting gene expression, Int. J. Mol. Sci. 21(12) (2020) 4494, https://doi.org/10.3390/ijms21124494

[22] A. F. Vega-Benedetti, E. Loi, L. Moi, A. Restivo, F. Cabras, S. Deidda, S. Pretta, P. Ziranu, S. Orrù, M. Scartozzi, L. Zorcolo, P. Zavattari, Colorectal cancer promoter methylation alteration affects the expression of glutamate ionotropic receptor AMPA type subunit 4 alternative isoforms potentially relevant in colon tissue, Hum. Cell. 35(1) (2022) 310-319, https://doi.org/10.1007/s13577-021-00640-x

[23] T. Mikeska, J. M. Craig, DNA methylation biomarkers: cancer and beyond, Genes 5(3) (2014) 821-864, https://doi.org/10.3390/genes5030821

[24] A. Koch, S. C. Joosten, Z. Feng, T. C. de Ruijter, M. X. Draht, V. Melotte, K. M. Smits, J. Veeck, J. G. Herman, L. Van Neste, W. Van Criekinge, T. De Meyer, M. van Engeland, Analysis of DNA methylation in cancer: location revisited, Nat. Rev. Clin. Oncol. 15(7) (2018) 459-466, https://doi.org/10.1038/s41571-018-0004-4

[25] S. R. Payne, From discovery to the clinic: the novel DNA methylation biomarker m SEPT9 for the detection of colorectal cancer in blood, Epigenomics 2(4) (2010) 575-585, https://doi.org/10.2217/epi.10.35

[26] M. T. Gyparaki, E. K. Basdra, A. G. Papavassiliou, DNA methylation biomarkers as diagnostic and prognostic tools in colorectal cancer, J. Mol. Med. 91(11) (2013) 1249-1256, https://doi.org/10.1007/s00109-013-1088-z

[27] Y. N. Lamb, S. Dhillon, Epi proColon® 2.0 CE: a blood-based screening test for colorectal cancer, Mol. Diagn. Ther. 21(2) (2017) 225-232, https://doi.org/10.1007/s40291-017-0259-y

[28] Y. Wang, P. M. Chen, R. B. Liu, Advance in plasma SEPT9 gene methylation assay for colorectal cancer early detection, World J. Gastrointest. Oncol. 10(1) (2018) 15, https://doi.org/10.4251%2Fwjgo.v10.i1.15

[29] Y. Liu, G. Zhao, J. Miao, H. Li, Y. Ma, X. Liu, S. Li, Y. Zhu, S. Xiong, M. Zheng, S. Fei, Performance comparison between plasma and stool methylated SEPT9 tests for detecting colorectal cancer, Front. Genet. 11 (2020) 324, https://doi.org/10.3389/fgene.2020.00324

[30] S. Bach, I. Paulis, N. R. Sluiter, M. Tibbesma, I. Martin, M. A. van de Wiel, J. B. Tuynman, I. Bahce, G. Kazemier, R. D. M. Steenbergen, Detection of colorectal cancer in urine using DNA methylation analysis, Sci. Rep. 11(1) (2021) 1-11, https://doi.org/10.1038/s41598-021-81900-6

[31] L. Song, J. Jia, X. Peng, W. Xiao, Y. Li, The performance of the SEPT9 gene methylation assay and a comparison with other CRC screening tests: A meta-analysis, Sci. Rep. 7(1) (2017)1-12, https://doi.org/10.1038/s41598-017-03321-8

[32] R. Hariharan, M. Jenkins, Utility of the methylated SEPT9 test for the early detection of colorectal cancer: a systematic review and meta-analysis of diagnostic test accuracy, BMJ Open Gastroenterol. 7(1) (2020) e000355, https://10.1136/bmjgast-2019-000355

[33] R. M. Ned, S. Melillo, M. Marrone, Fecal DNA testing for colorectal cancer screening: the ColoSure™ test, PLoS Curr. 3 (2011), https://doi.org/10.1371%2Fcurrents.RRN1220

[34] A. Amiot, H. Mansour, I. Baumgaertner, J. C. Delchier, C. Tournigand, J. P. Furet, J.-P. Carrau, F. Canoui-Poitrine, I. Sobhani, CRC group of Val De Marne, The detection of the methylated Wif-1

gene is more accurate than a fecal occult blood test for colorectal cancer screening, PLoS One 9(7) (2014) e99233, https://doi.org/10.1371/journal.pone.0099233

[35] Q. Zhang, Y. Wu, Q. Xu, F. Ma, C. Y. Zhang, Recent advances in biosensors for in vitro detection and in vivo imaging of DNA methylation, Biosens. Bioelectron. 171 (2021) 112712, https://doi.org/10.1016/j.bios.2020.112712

[36] D. Sung, J. Koo, A review of BioFET's basic principles and materials for biomedical applications, Biomed. Eng. Lett. 11 (2021) 85–96, https://doi.org/10.1007/s13534-021-00187-8

[37] A. Chakraborty, P. Dutta, A. Wakankar, C. RoyChaudhuri, Recent field effect transistors and electrical impedance spectroscopy based biosensing strategies for cancer biomarker screening: A mini review, Biosens. Bioelectron.: X 2022 100253, https://doi.org/10.1016/j.biosx.2022.100253

[38] Z. Hao, Y. Pan, W. Shao, Q. Lin, X. Zhao, Graphene-based fully integrated portable nanosensing system for on-line detection of cytokine biomarkers in saliva, Biosens. Bioelectron. 134 (2019) 16-23, https://doi.org/10.1016/j.bios.2019.03.053

[39] Y. Yang, B. Zeng, Y. Li, H. Liang, Y. Yang, Q. Yuan, Construction of MoS_2 field effect transistor sensor array for the detection of bladder cancer biomarkers, Sci. China Chem. 63 (2020) 997–1003, https://doi.org/10.1007/s11426-020-9743-2

[40] C. Sun, R. Li, Y. Song, X. Jiang, C. Zhang, S. Cheng, W. Hu, Ultrasensitive and reliable organic field-effect transistor-based biosensors in early liver cancer diagnosis, Anal. Chem. 93(15) (2021) 6188-6194, https://doi.org/10.1021/acs.analchem.1c00372

[41] C. Sun, G. Feng, Y. Song, S. Cheng, S. Lei, W. Hu, Single Molecule Level and Label-Free Determination of Multibiomarkers with an Organic Field-Effect Transistor Platform in Early Cancer Diagnosis, Anal. Chem. 94 (2022) 6615–6620, https://doi.org/10.1021/acs.analchem.2c00897

[42] C.- C. Huang, Y.- H. Kuo, Y.- S. Chen, P. -C. Huang, G. -B. Lee, A miniaturized, DNA-FET biosensor-based microfluidic system for quantification of two breast cancer biomarkers, Microfluid Nanofluid 25 (2021) 33, https://doi.org/10.1007/s10404-021-02437-8

[43] T. Li, Y. Liang, J. Li, Y. Yu, M. M. Xiao, W. Ni, Z. Zhang, G. J. Zhang, Carbon nanotube field-effect transistor biosensor for ultrasensitive and label-free detection of breast cancer exosomal miRNA21, Anal. Chem. 93(46) (2021) 15501-15507, https://doi.org/10.1021/acs.analchem.1c03573

[44] S. Lai, M. Demelas, G. Casula, P. Cosseddu, M. Barbaro, A. Bonfiglio, Ultralow voltage, OTFT-based sensor for label-free DNA detection, Adv. Mater. 25 (2013) 103-107, https://doi.org/10.1002/adma.201202996

[45] S. Lai, M. Barbaro, A. Bonfiglio, Tailoring the sensing performances of an OFET-based biosensor, Sens. Actuat. B. Chem. 233 (2016) 314-319, https://doi.org/10.1016/j.snb.2016.04.095

[46] C. Napoli, S. Lai, A. Giannetti, S. Tombelli, F. Baldini, M. Barbaro, A. Bonfiglio, Electronic detection of DNA hybridization by coupling organic field-effect transistor-based sensors and hairpin-shaped probes, Sensors 18 (2018) 990, https://doi.org/10.3390/s18040990

[47] L. Barault, A. Amatu, G. Siravegna, A. Ponzetti, S. Moran, A. Cassingena, B. Mussolin, C. Falcomatà, A. M. Binder, C. Cristiano, D. Oddo, S. Guarrera, S. Bustreo, K. Bencardino, S. Maden, A. Vanzati, P. Zavattari, G. Matullo, M. Truini, W. M. Grady, P. Racca, K. B. Michels, S. Siena, M. Esteller, A. Bardelli, A. Sartore-Bianchi, F. Di Nicolantonio, Discovery of methylated circulating DNA biomarkers for comprehensive non-invasive monitoring of treatment response in metastatic colorectal cancer, Gut 67(11) (2018) 1995-2005, http://dx.doi.org/10.1136/gutjnl-2016-313372

[48] S. Subramanian, K. H. Aschenbach, J. P. Evangelista, M. B. Najjar, W. Song, R. D. Gomez, Rapid, sensitive and label-free detection of Shiga-toxin producing Escherichia coli O157 using carbon nanotube biosensors, Biosens. Bioelectron. 32 (2012) 69-75, https://doi.org/10.1016/j.bios.2011.11.040

[49] S. Lai, P. Cosseddu, G.C. Gazzadi, M. Barbaro, A. Bonfiglio, Towards high frequency performances of ultra-low voltage OTFTs: Combining self-alignment and hybrid, nanosized dielectrics, Org. Electron. 14 (2013) 754-761, https://doi.org/10.1016/j.orgel.2012.11.032

[50] M., Demelas, S. Lai, G. Casula, E. Scavetta, M. Barbaro, A. Bonfiglio, An organic, charge-modulated field effect transistor for DNA detection, Sens. Actuators B Chem. 171 (2012) 198-203, https://doi.org/10.1016/j.snb.2012.03.007

[51] S. Lai, M. Barbaro, A. Bonfiglio, The role of polarization-induced reorientation of DNA strands on organic field-effect transistor-based biosensors sensitivity at high ionic strength, Appl. Phys. Lett. 107 (2015) 86, https://doi.org/10.1063/1.4930303