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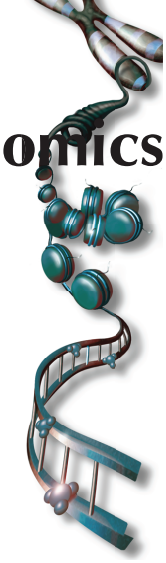
Research Article

Epigenomics



cfDNA methylation in liquid biopsies as potential testicular seminoma biomarker

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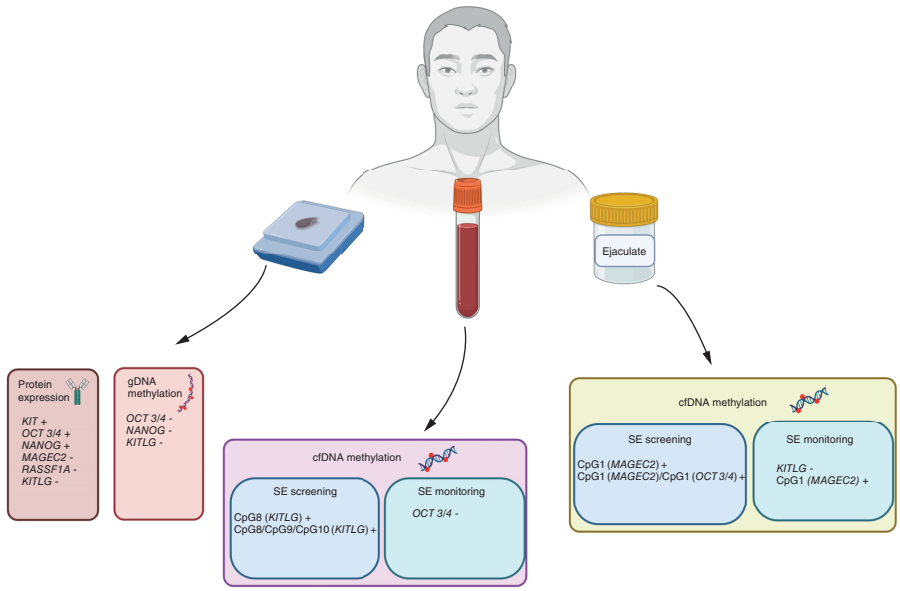
Background: Seminoma is a testicular tumor type, routinely diagnosed after orchidectomy. As cfDNA represents a source of minimally invasive seminoma patient management, this study aimed to investigate whether cfDNA methylation of six genes from liquid biopsies, have potential as novel seminoma biomarkers. **Materials & methods:** cfDNA methylation from liquid biopsies was assessed by pyrosequencing and compared with healthy volunteers' samples. **Results:** Detailed analysis revealed specific CpGs as possible seminoma biomarkers, but receiver operating characteristic curve analysis showed modest diagnostic performance. In an analysis of panels of statistically significant CpGs, two DNA methylation panels emerged as potential seminoma screening panels, one in blood CpG8/CpG9/CpG10 (*KITLG*) and the other in seminal plasma CpG1(*MAGEC2*)/CpG1(*OCT3/4*). **Conclusion:** The presented data promote the development of liquid biopsy epigenetic biomarkers in the screening of seminoma patients.

Plain language summary: Seminoma belongs to testicular cancer, which represents a common malignancy among men of reproductive age. Diagnosis of seminoma is a multistep process that also includes checking tumor biomarkers from blood. However, these biomarkers are not specific for seminoma and to conclude a definite diagnosis of seminoma immunohistochemical analysis is needed, which requires the removal of a whole or partial testicle. Therefore, there is a need for novel, noninvasive biomarkers. cfDNA is the most extensively investigated source of minimally invasive tumor markers. Therefore, this study investigated cfDNA methylation of six genes as potential noninvasive biomarkers for the management of seminoma patients. By examining CpG sites of selected genes by pyrosequencing, the authors detected significant differences. However, receiver operating characteristic curve analysis showed modest results. Therefore, the authors tested possible panels of significantly different CpGs and detected two possible DNA methylation panels for seminoma screening. These findings suggest the further investigation of possible epigenetic biomarkers for seminoma patient management from liquid biopsies.

Tweetable abstract: Novel research of potential epigenetic biomarkers for the management of seminoma patients from liquid biopsies.

Future Medicine

Graphical abstract:



DNA methylation was analyzed in gDNA from tissue and cfDNA from liquid biopsies of seminoma patients. Possible panels for seminoma screening emerged. Created with BioRender.com.

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Keywords: biomarkers • cfDNA • DNA methylation • liquid biopsy • seminoma

Testicular germ cell tumors (TGCTs) represent the most common malignancy among men of reproductive age [1]. TGCTs are a very heterogenous group divided into seminoma (SE) as the most frequent type and nonseminoma [2]. Along with classical histopathology, immunohistochemistry is the main diagnostic tool in SE patient management [3]. OCT3/4 and KIT are routine biomarkers for identification of the cancer stem cell population [4], with NANOG as an alternative [5]. Normally, *OCT3/4* and *NANOG* are expressed during embryonal development, after which their expression decreases and disappears [6]. *KIT* interacts with *KITLG* and they are very important for the cell migration process [7]. *MAGEC2* is a part of the MAGEC family and it enables good discrimination between SE and embryonal carcinoma [8]. *RASSF1A* is a tumor suppressor gene, which aberrant DNA methylation is associated with various tumors [9], as well as with TGCTs, and whose high expression was detected in a premalignant lesion [10]. Aberrant DNA methylation is recognized as a mechanism underlying disrupted gene expression in many tumor tissues, including SE [11]. Indeed, *OCT3/4, NANOG, KIT*, *KITLG, RASSF1A* and *MAGEC2* are reported as hypomethylated in SE tissue [12]. The connectivity network of selected genes is shown in Figure 1.

The majority of reported DNA methylation data were obtained on gDNA from SE tissue, requiring orchidectomy as an invasive procedure. However, analysis of circulating cfDNA from liquid biopsies represents a minimally invasive approach that enables a real-time assessment of a molecular tumor landscape [13]. Blood (plasma and serum) is the most investigated body fluid. It can be used to detect a broad range of molecular changes reflecting tumor presence [14]. Serum markers α-fetoprotein, β-human chorionic gonadotropin and lactate dehydrogenase represent important tools in TGCT patient management. However, in patients with pure SE, these are either slightly elevated or not elevated at all [15,16], making them less valuable in managing SE compared with nonseminoma. Therefore, this investigation was focused on SE with the aim to identify better distinguishing biomarkers with a less invasive liquid biopsy approach. Seminal plasma should, for purely anatomical reasons, reveal information related exclusively to the testis and prostate. Indeed, cfDNA has been demonstrated to be helpful for prostate cancer diagnosis [17]. Accordingly, seminal plasma could represent a valuable source of readily available cfDNA for early screening and monitoring of SE [18].

It has already been reported that gDNA methylation of genes of interest discriminates healthy from SE tissue [19]. This study aimed to investigate whether detected aberrant DNA methylation in SE tissue is reflected in cfDNA

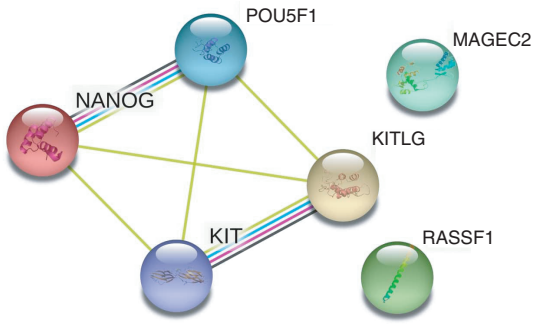

Figure 1. Predicted network of interactions between proteins encoded by selected genes, by the Search Tool for Retrieval of Interacting Gene/Proteins database (version 11.5) [52]. *NANOG* is a transcription regulator involved in the self-renewal and proliferation of inner cell mass and embryonic stem cells. *OCT3/4* encodes for the POUF1 transcription factor, crucial for embryonic stem cell pluripotency and early embryogenesis. Disrupted expression of these two genes is used in the identification of the cancer stem cell population in TGCTs. *KIT* and *KITLG* code for the cell-surface receptor and its ligand, respectively. This association is essential for the regulation of cell survival and proliferation, stem cell maintenance, gametogenesis, cell migration and cell function. In response to the binding of KITLG, KIT activates several signaling pathways. These four genes present significant functional connections, unlike *MAGEC2* and *RASSF1A*. Still, *MAGEC2* is a cancer/testis antigen (i.e., expression is restricted to male germ cells in the healthy testis and is highly expressed during the early phases of spermatogenesis). Finally, tumor suppressor gene *RASSF1A* is related to seminoma as well, with disrupted DNA methylation serving as a trigger/driver for tumor development. Each node represents proteins produced by a single protein-coding gene locus. Different-colored lines represent the types of evidence used to identify protein–protein interactions. Yellow: text mining; purple: experimentally determined; black: coexpression; turquoise: from curated databases.

methylation in blood and seminal plasma, and if it could be translated to a liquid biopsy SE patient management approach.

Materials & methods

Study population

24 SE patients were recruited at two urological centers in Zagreb, the University Hospital Centre Sestre Milosrdnice and the University Hospital Centre Zagreb, after a surgical pathologist concluded the diagnosis of a pure SE. 37 healthy volunteers (HVs) represented the control group for the cfDNA analysis, while for gDNA analysis, nonmalignant (nonmalignant testicular tissue [NTT]) tissue samples from 12 patients were collected from the University Hospital Centre Sestre Milosrdnice paraffin tissue archive.

Ethical statement

Before patients' admission into the study, information about the study was given to all participants and they signed written consent. The study was conducted according to the Declaration of Helsinki. The Ethics Committee of the School of Medicine University of Zagreb (protocol code 380-59-10106-17-100/187, 641-01/17-02/01), University Hospital Centre Sestre Milosrdnice (protocol code, number: EP-7259/17-9) and University Hospital Centre Zagreb (protocol code 8.1-17/107-2, number: 02/21 AG) approved the collection and manipulation of all tissue samples.

Sample collection

SE tissue samples were collected after radical orchidectomy, performed as a part of routine patient management. Histopathological review of paraffin-embedded archive NTT and SE tissue samples was done by two pathologists. Defined areas were determined for subsequent immunohistochemical analysis and gDNA isolation.

Liquid biopsy samples were organized as shown in Figure 2. Preoperative samples were taken up to 2 days before the orchidectomy and any chemo- or radio-therapy, while postoperative samples were collected up to 8 days after surgical excision.

12 ml of peripheral venous blood was collected in two 6 ml tubes containing ethylenediaminetetraacetic acid (Greiner Bio-One, NC, USA). To prevent contamination of plasma with other cells, peripheral venous blood was processed by dual centrifugation (1400× g for 10 min and then 4500× g for 10 min). Obtained blood plasma was stored in a vacutainer (Greiner Bio-One, NC, USA) at -80°C.

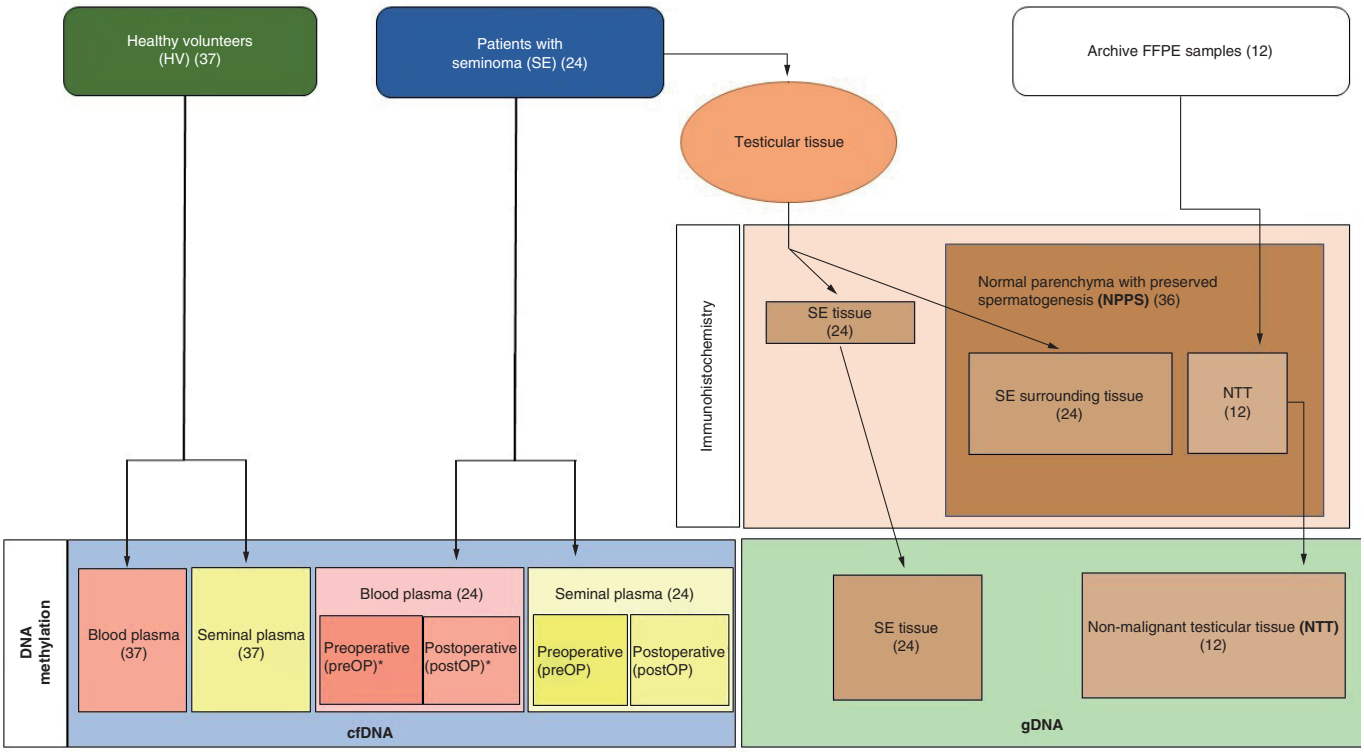

Figure 2. Flowchart of study samples: the number and origin of collected samples. DNA methylation of the selected genes was analyzed for cfDNA and gDNA. The expression of the selected genes at the protein level was analyzed on tissue samples. The number of analyzed samples of blood plasma for each gene marked with * is represented in Supplementary Table 1.

Ejaculate samples were collected by masturbation after sexual abstinence of 3–5 days. The ejaculate was liquified at room temperature for 30 min. The protocol for ejaculate processing to seminal plasma was obtained from Li *et al.* [20] with slight modifications: the ejaculate was centrifuged in three steps (400× g for 10 min, 12,000× g for 10 min and 20,000× g for 10 min. Obtained seminal plasma was stored in 1.5 m tubes (Eppendorf, Hamburg, Germany) at -80°C.

Tissue biomarker analysis

Tissue biomarker analysis was performed by immunohistochemistry followed by morphometric analysis. Additional information is shown in Supplementary Table 2. Both were performed as previously reported [10]. All slides were analyzed by two pathologists and any doubt was resolved with the joint committee. During further statistical analysis, normal cells from testicular parenchyma and spermatogenesis were joined into one group called normal parenchyma with preserved spermatogenesis (NPPS).

Isolation of gDNA & cfDNA

After isolating gDNA from SE tissue [21], the authors measured the concentration with a NanoDrop 2000c spectrophotometer. NucleoSnap cfDNA kit for cfDNA from plasma (Machery-Nagel, Düren, Germany) was used for the isolation of cfDNA from liquid biopsies, as well as a vacuum pump (Qiagen, Hilden, Germany) [17]. Elution of cfDNA was done by 100 µl of elution buffer into 1.5 m DNA low bind tubes (Eppendorf, Hamburg, Germany). Quant-iT™ PicoGreen® dsDNA detection kit (Thermo Fisher Scientific, MA, USA) was used to quantify cfDNA. The intensity of fluorescence was measured on a spectrofluorometer (Tecan, Männedorf, Switzerland).

DNA methylation analysis

DNA was subjected to bisulfite conversion by EpiTect Bisulfite Kit (Qiagen, Hilden,Germany). Bisulfite-converted DNA was further amplified by PyroMark PCR Kit (200; Qiagen, Hilden, Germany) using previously reported or newly designed primers (PyroMark assay design software; Supplementary Table 3). Sequencing was performed by the PyroMark Q24 system using PyroMark Q24 advanced CpG reagents (4 × 24; Qiagen, Hilden, Germany).

Statistical analysis

Immunohistochemistry results were analyzed using the Mann–Whitney test with Bonferroni correction and Kruska–Wallis with Dunn's multiple comparisons statistical tests with two-tailed p-value reported. Raw CpG methylation levels were normalized by performing log2 transformation of the ratio of methylated and unmethylated probes [17]. Before assessing differences in DNA methylation between CpG groups of six preselected genes, the Fligner–Killeen test was used to test the homogeneity of variances between the samples as one of the assumptions of nonparametric tests. Comparison of DNA methylation levels between unpaired and paired data was performed by Wilcoxon rank-sum (Mann–Whitney U) test and Wilcoxon signed-rank test. Receiver operating characteristic (ROC) curves on raw data for individual genes and combinations of multiple genes were evaluated. The optimal balance of sensitivity and specificity for each ROC curve per gene or gene panel was obtained by calculating Youden index (J). Logistic regression was performed to investigate the impact of combinations of individual CpGs on the discriminatory power of methylation levels between control/preOP and preOP/postOP samples. Evaluation measurements, such as the area under the curve (AUC), sensitivity and specificity, were computed for each significant CpG site. Spearman's rank correlation coefficient was used to evaluate the association between DNA methylation levels in SE tissue, preoperative and postoperative blood and ejaculative samples with a two-tailed p-value reported. All statistical analyses were performed in R, except the Kruskal–Wallis test, which was performed in GraphPad Prism 9 (GraphPad Software). All p-values were considered significant if <0.05. To analyze the difference between healthy volunteers' and patients' ages, the Mann–Whitney test was used. The stratification method was used to analyze confounding effects.

Results

Clinicopathological data

Clinicopathological and demographic data are shown in Supplementary Table 4. The cohort considered 24 SE patients, the majority being stage I (71%). Between SE patients (median age: 35) and healthy volunteers (median age: 26), a significant age difference was detected ($p < 0.001$). Furthermore, the correlation detected between the patient's age and methylation level was low (Supplementary Table 5). Regarding demographic data, associations between smoking and alcohol consumption were found for certain CpGs of selected genes (Supplementary Table 5).

Tissue biomarker expression

KIT, NANOG and *OCT3/4* were highly expressed in SE, while *MAGEC2, RASSF1A* and *KITLG* showed higher expression in NPPS (Figure 3).

DNA methylation analysis in tissue & liquid biopsies

Considering gDNA methylation data, *OCT3/4* and *NANOG* were significantly hypermethylated in NTT and *KITLG* in SE. Other genes showed similar gDNA methylation levels in NTT and SE tissue (Figure 4). Regarding blood, cfDNA methylation of *OCT3/4* differed significantly between preoperative and postoperative samples. cfDNA methylation of other selected genes did not differ significantly (Figure 4). In seminal plasma, cfDNA methylation of *OCT3/4* and *KITLG* significantly differed between HV and preoperative samples. cfDNA methylation of *OCT3/4* was statistically different between HV and postoperative samples. The cfDNA methylation level of *KITLG* significantly differed between preoperative and postoperative samples (Figure 4).

DNA methylation level was considered for each CpG site. In blood, *KITLG* CpG8 significantly differed between HV and preoperative samples, while for *OCT3/4* CpG1 significally differed between preoperative and postoperative samples (Figure 5). In seminal plasma, a significant difference was found between HV and preoperative samples in *KITLG* CpG1, CpG2, CpG3 and CpG6, as well as in *OCT3/4* CpG1 and *MAGEC2* CpG1. *MAGEC2* CpG1 methylation frequency differed between preoperative and postoperative samples (Figure 5).

Relation of gDNA & cfDNA methylation

A significant ($p = 0.036$) positive correlation was detected for *NANOG* (Figure 6) in preoperative versus postoperative blood samples. No significant correlations were detected in the seminal plasma. However, a strong positive correlation trend for *KIT* and *MAGEC2*, while negative for *OCT3/4* and *KITLG*, was observed in preoperative versus postoperative seminal plasma samples (Figure 6). Analyzing the correlation between blood and seminal plasma samples, the authors found a significant ($p = 0.015$) negative correlation for *MAGEC2*, while just a trend for *KIT* and *NANOG*, all in preoperative blood versus postoperative seminal plasma (Figure 6).

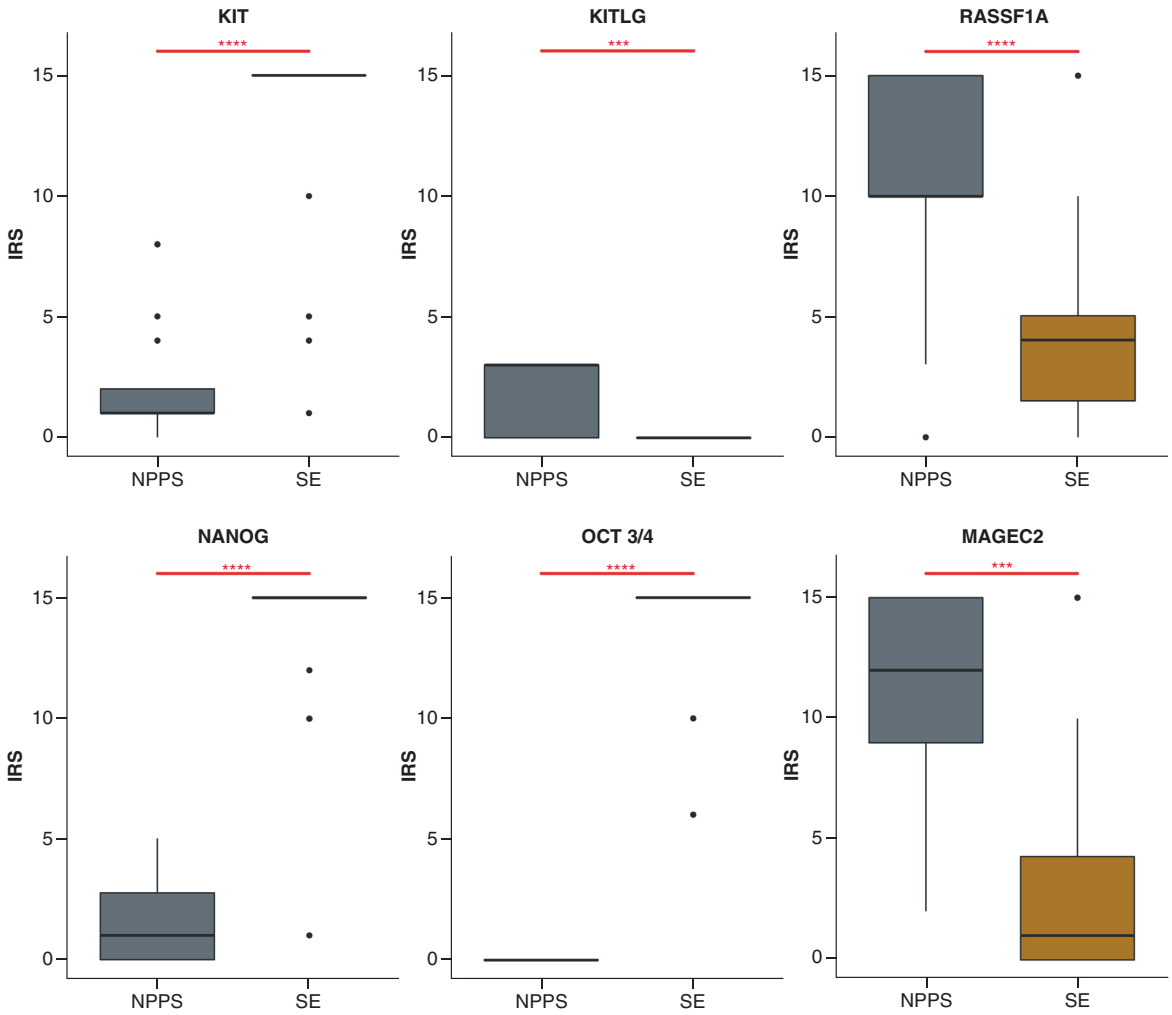

Figure 3. Protein expression in normal parenchyma with preserved spermatogenesis and seminoma. Statistically significant differences between groups are indicated as *p < 0.05; **p < 0.01; ***p < 0.001 and ****p < 0.0001.

ROC curve analysis of cfDNA data

Three *KITLG* CpG sites significantly or indicatively distinguished HV from preoperative samples in blood: CpG8 (75% sensitivity; 79% specificity; AUC: 0.72), CpG9 (62% sensitivity; 79% specificity; AUC: 0.71) and CpG10 (75% sensitivity; 76% specificity; AUC: 0.75; Figure 7). In seminal plasma, CpG sites of selected genes showed significance in discriminating HV from preoperative samples. ROC curve analysis of *MAGEC2* CpG1 methylation level showed 73% sensitivity and 63% specificity (AUC: 0.7), while for *OCT3/4* CpG1 sensitivity was 100% and specificity 55% (AUC: 0.7; Figure 6). ROC curve analysis revealed that not all significant CpG sites have good biomarker potential; for example, CpG1–CpG4 and CpG6 of *KITLG* had either high sensitivity and low specificity or *vice versa* (AUC < 0.41; data not shown).

Possible screening & monitoring cfDNA methylation panels

In blood, a combination of all three significant *KITLG* CpGs emerged as a putative panel for SE screening, displaying 71% sensitivity with 79% specificity (AUC: 0.72; Figure 8). In seminal plasma, a panel comprising *MAGEC2* CpG1 and *OCT3/4* CpG1 displayed significant sensitivity (87%) with notable specificity (60%; AUC: 0.65), marking them a possible panel for SE screening (Figure 8).

Discussion

In current TGCT patient management, a highly invasive orchidectomy is required promptly after the detection of most testicular pathology [22,23]. The cancer-related serum biomarkers are valuable but limited in their sensitivity

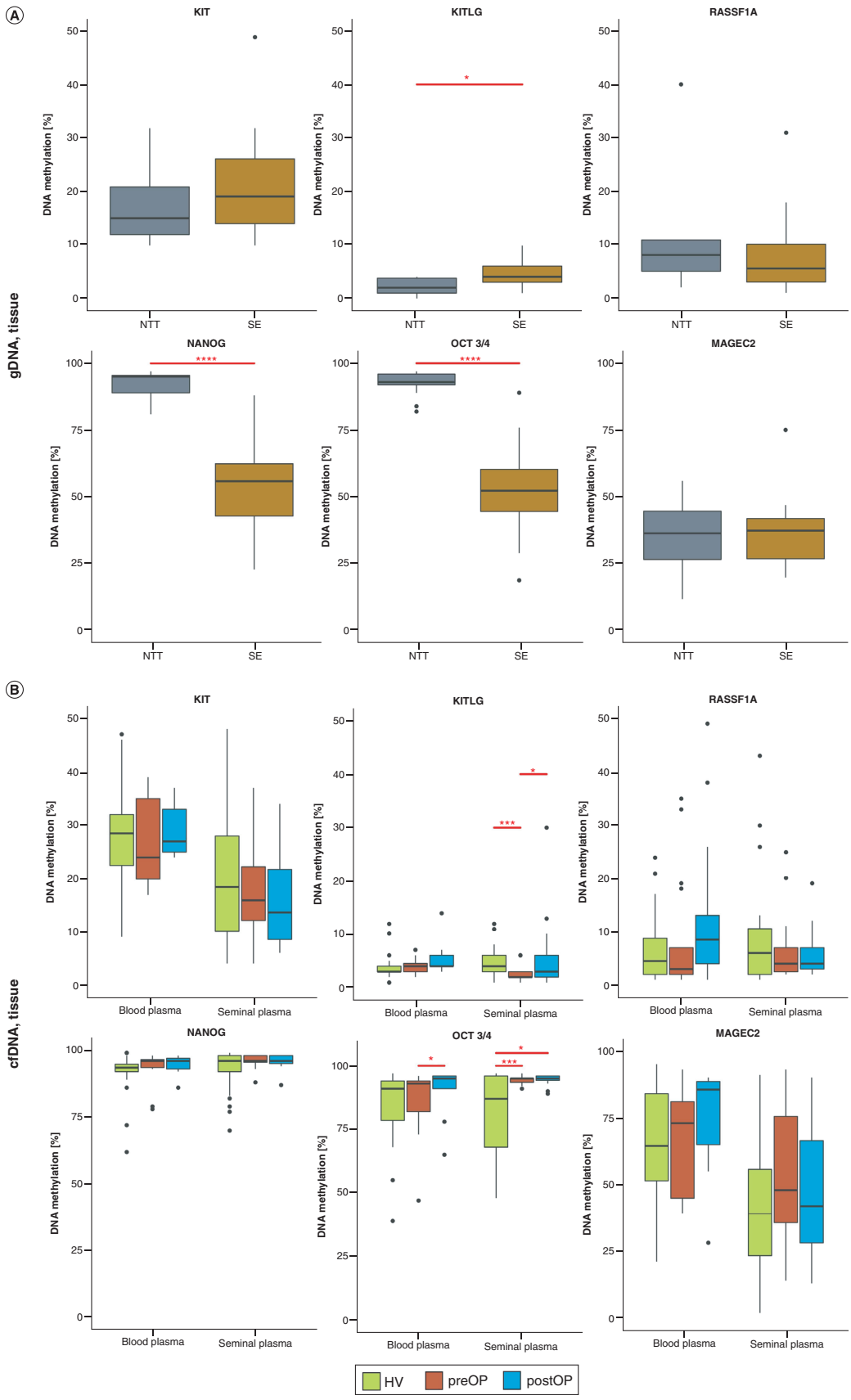

Figure 4. Methylation analysis of gDNA from tissue and of cfDNA from liquid biopsies. (A) gDNA methylation analysis. **(B)** Methylation analysis of cfDNA from blood and seminal plasma. Black horizontal lines represent the median of a group. A statistically significant difference is indicated as *$p < 0.05$; **$p < 0.0$; ***$p < 0.001$ and ****$p < 0.0001$.
SE: Seminoma.

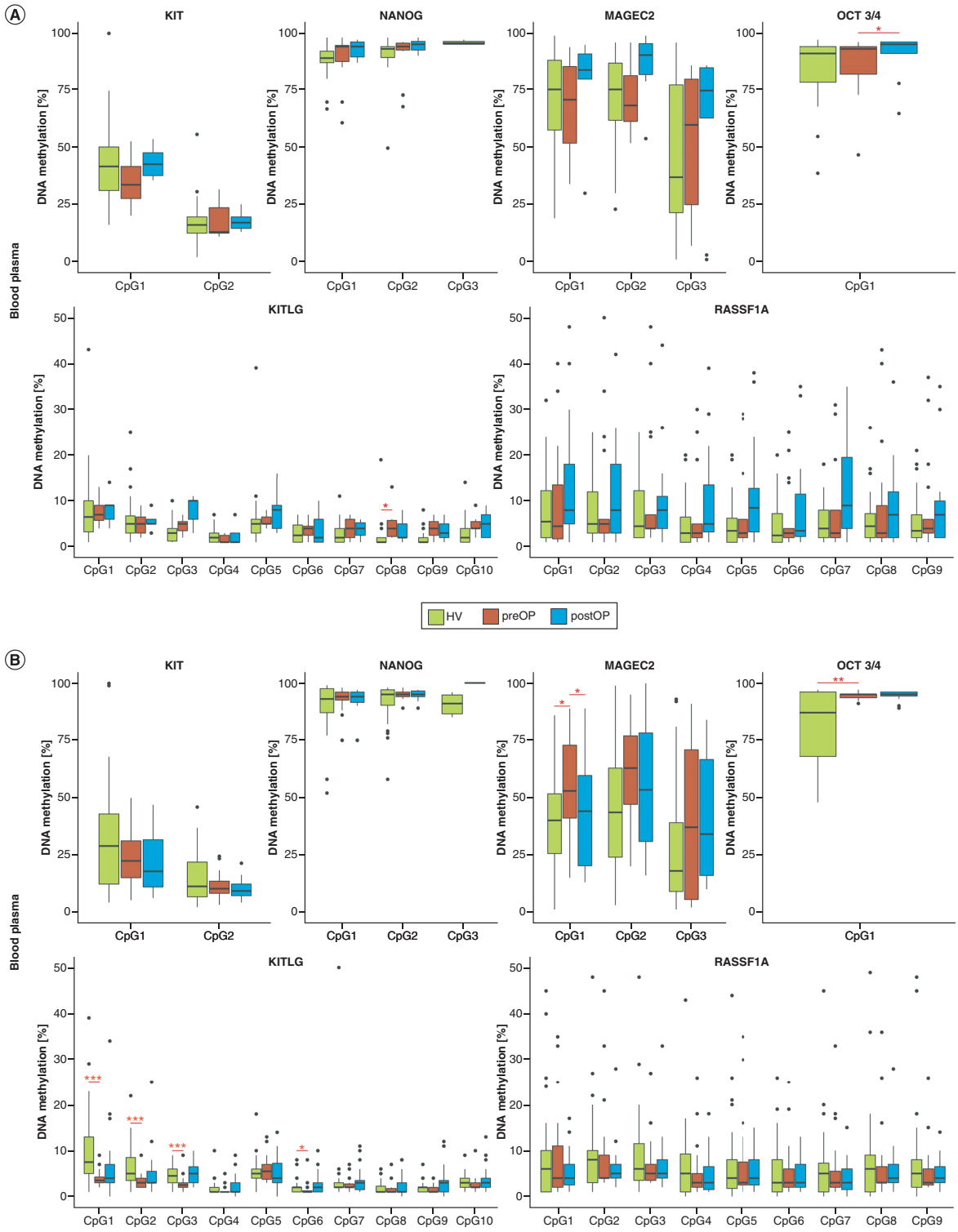

Figure 5. DNA methylation analysis of each CpG from selected genes in liquid biopsies. The methylation level of selected genes in **(A)** blood and **(B)** seminal plasma are shown. Statistically significant differences between groups are indicated as *p < 0.05; **p < 0.01; ***p < 0.001 and ****p < 0.0001.

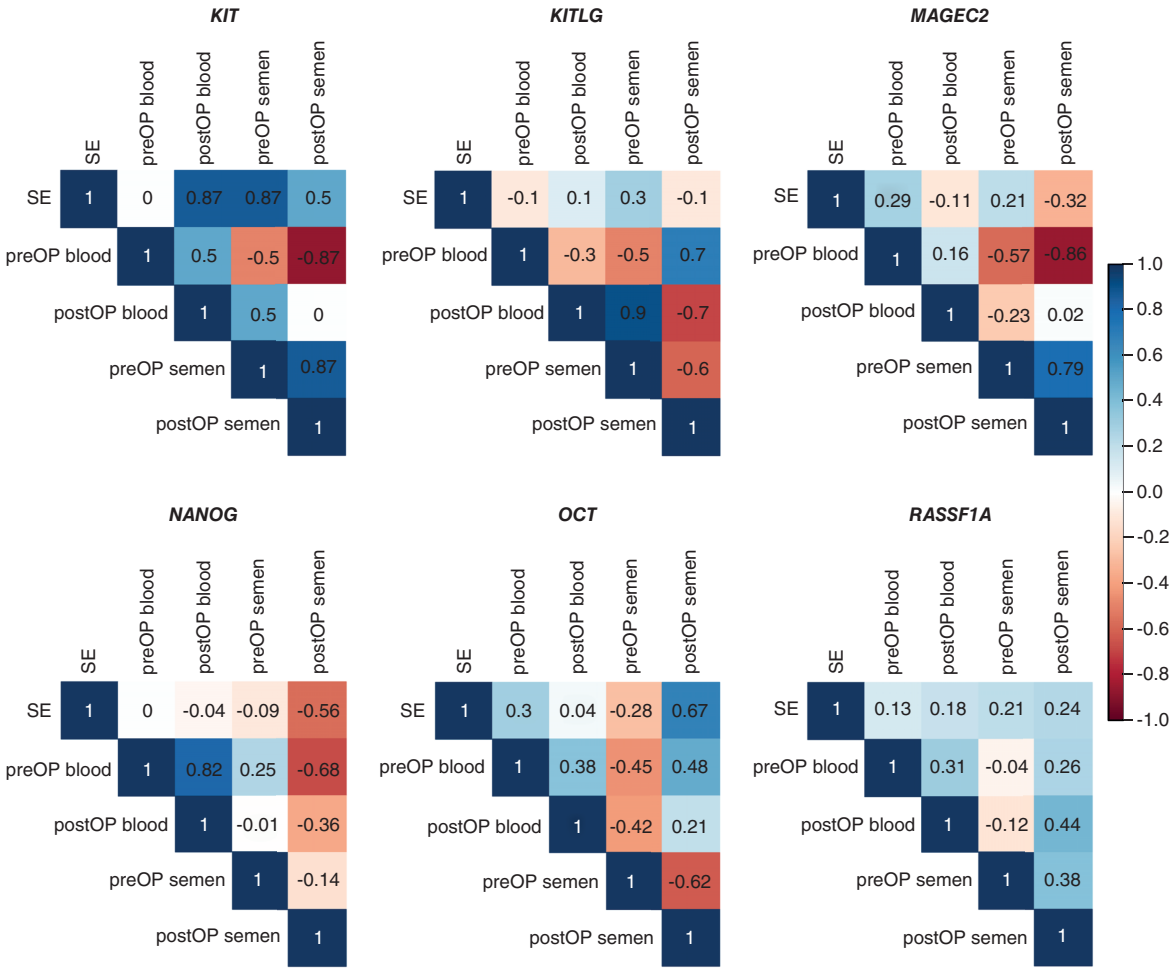

Figure 6. Correlation of DNA methylation level in seminoma tissue and preoperative and postoperative liquid biopsy samples.

and specificity, especially for SE [24], arguing for the development of high-quality, noninvasive SE biomarkers. Cancer cell DNA fragments are present in cfDNA fraction of a liquid biopsy, retaining all features specific for SE. Still, only a few studies are focused on investigating the potential clinical value of liquid biopsy in SE, especially those related to cfDNA methylation [25,26]. Therefore, this study aimed to determine the potential of cfDNA methylation in seminal and blood plasma as a SE liquid biopsy biomarker.

Clinicopathological data

The number of SE samples analyzed in this study could cause possible concern (i.e., could be viewed as low), especially after Medvedev *et al.* [27] detected the two subtypes of SE. However, the mentioned research did not find any statistically significant difference in DNA methylation on the level of methylation of specific probes (despite the overall, genomewide trend). Furthermore, the Medvedev *et al.* study was bioinformatic research that performed a statistical analysis of the data from different databases. While presented results are valuable for tumor biology, differences in clinical representations were beyond the scope of the Medvedev *et al.* study. Also, SE is well known to be a rare tumor, and the included samples represented almost all cases collected over the span of 2 years, in the two largest Croatian urological centers. Similar investigations of novel SE biomarkers have a comparable or even lower number of SE samples [28–30], with only a few containing a greater number of samples collected during a greater period of time or samples collected in several institutions in at least two countries [31,32]. Therefore, the authors are sure that this sample size is both comparable to current, state-of-the-art publications and sufficiently broad-based to include sample profiles with respect to subtypes. The statistically significant difference found between the age of healthy controls and patients could present a possible limitation of the study, since DNA methylation changes are

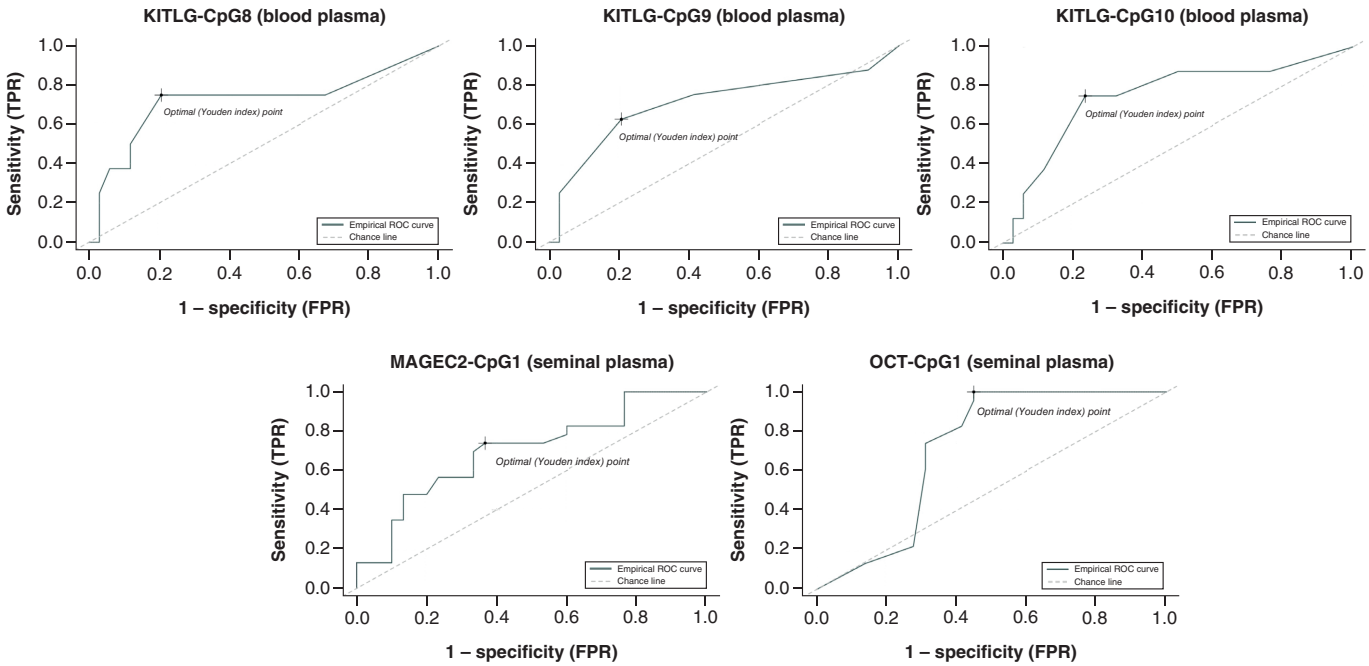

Figure 7. Receiver operating characteristic curve analysis of each significant CpG in liquid biopsies. Receiver operating characteristic curve with corresponding area under the curve, sensitivity and specificity. In blood plasma, *KITLG* CpG8, CpG9 and CpG10 discriminate between healthy volunteer and preoperative samples. J(CpG8) = 0.54, J(CpG9) = 0.42 and J(Cpg10) = 0.541. In seminal plasma, *MAGEC2* CpG1 and *OCT3/4* CpG1 discriminate between healthy volunteer and preoperative samples. For *MAGEC2* J(CpG1) = 0.37 and for *OCT3/4* J(CpG1) = 0.5.

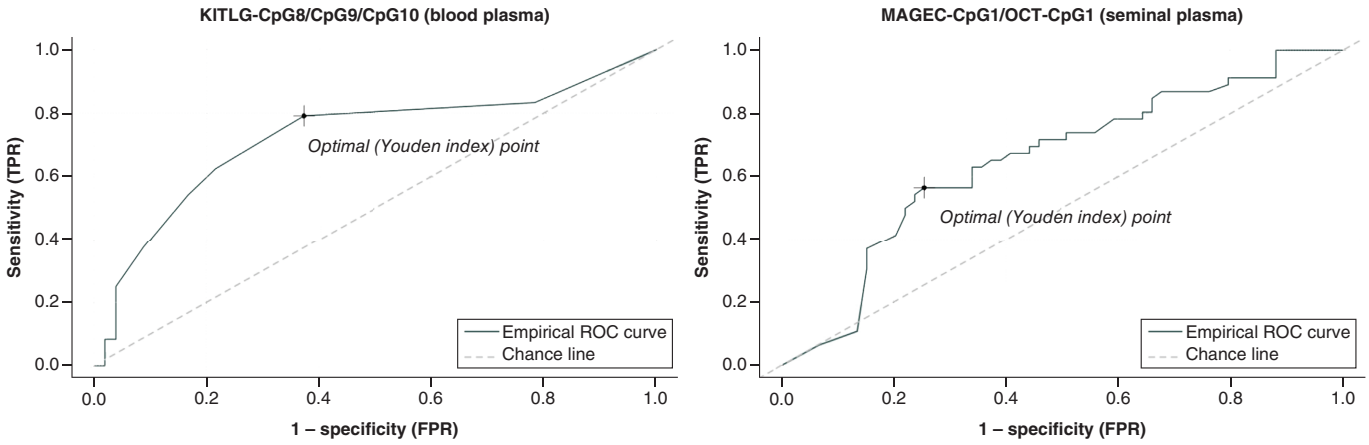

Figure 8. Receiver operating characteristic curve. (A) ROC curve for CpG8/CpG9/CpG10 of *KITLG* panel across healthy volunteer and preoperative blood samples. **(B)** ROC curve for CpG1 *MAGEC2*/CpG1 *OCT3/4* panel for discriminating between healthy volunteer and preoperative seminal plasma samples (dark line: ROC curve for CpG8/CpG9/ CpG10 *KITLG* and CpG1 *MAGEC2*/CpG1 *OCT3/4* panel; light grey dashed line: reference line).
AUC: Area under the curve; ROC: Receiver operating characteristic.

age-related. There was an age-related confounding effect for CpG1 in the *MAGEC2* gene in the ejaculate, as well as two other seemingly high effects for CpG8 and CpG4 in the *KITLG* gene in blood and ejaculate tissues, respectively. However, keep in mind that the authors were unable to compute the risk ratio for each selected stratum of data due to a lack of representative samples (Supplementary File 5). Therefore, there is a reasonable level of certainty that the detected differences in DNA methylation between the two observed groups are SE-specific [33,34]. Furthermore, associations between smoking and alcohol consumption with significant aberrant methylation of *KITLG* CpGs and *MAGEC2* CpGs in liquid biopsies were found, which is in line with already reported investigations [35,36].

Relation of protein expression & gDNA methylation levels in SE

OCT3/4 and *NANOG* showed high protein expression in combination with gDNA hypomethylation in SE, which was expected, according to previous reports [37,38]. Detection of hypomethylated *RASSF1A* in SE is in line with other reports as well [39]. The *KIT-KITLG* pathway is central to TGCT molecular pathology, and *KIT* is an SE biomarker on the protein level [1]. *KITLG* was found to be hypermethylated in SE, which is congruent to the absence of its expression in SE. However, no difference in *KIT* gDNA methylation was found in NTT and SE, despite the significantly higher protein expression in SE. It suggests that the activation of the *KIT-KITLG* pathway is more influenced by *KIT* mutations [40] and *KITLG* SNPs [6]. *MAGEC2* was found to be highly expressed in NPPS, again in line with other reports [8]. Still, differential DNA methylation was not detected. This suggests the involvement of other molecular mechanisms in the regulation of *MAGEC2* expression.

Reflection of SE gDNA methylation in cfDNA as a potential screening biomarker

Hypomethylated *OCT3/4* and *NANOG* detected in SE tissue were not observed in blood cfDNA. This could be explained by the fact that gDNA methylation data were obtained exclusively from SE tissue, while cfDNA data were obtained by analyzing cfDNA in blood plasma, encompassing cfDNA from healthy cells and not only from SE [23]. *OCT3/4* and *NANOG* are strongly hypermethylated in differentiated cells [41], being embryonal biomarkers; it is plausible that cfDNA from healthy tissue completely overshadowed cfDNA from SE [42]. *KITLG* gDNA methylation in SE tissue was reflected in blood cfDNA. Still, a statistically significant difference was not detected between HV and preoperative samples. The reduced reflection of *KITLG* gDNA methylation in cfDNA could be caused by the blood–testis barrier [43], which limits the release of SE cfDNA into the blood. Indeed, the smaller amount of tumor cfDNA released into the bloodstream was detected for various cancers associated with the presence of either a blood–tissue barrier or an organ capsule [42]. Seminal cfDNA methylation of *OCT3/4* and *KITLG* was similar to that found in NTT, which could be due to the larger release of cfDNA from healthy testicular tissue into ejaculate, rather than from SE. However, they could be considered possible SE biomarkers, since they discriminate HV from preoperative samples. Regarding *RASSF1A*, the obtained results are in accordance with a previous study [26], but a statistically significant difference was not detected, possibly owing to the small sample size here. *MAGEC2* and *KIT* cfDNA methylation did not differ between HV and preoperative blood or ejaculate, meaning they seem not to have strong potential as SE epigenetic biomarkers. DNA methylation analysis of each CpG site revealed three differentially methylated *KITLG* CpGs in blood as potential SE screening biomarkers. Their characteristic hypermethylation in SE tissue [44] seems to be detectably retained in cfDNA. Analysis of seminal plasma revealed CpG1 in *MAGEC2* and four CpGs in *KITLG* as possible screening biomarkers. These findings confirm that cancer-specific gDNA methylation could remain, at least partially, detectably reflected in cfDNA and consequently studied in epigenetic noninvasive biomarker development.

cfDNA methylation as a surgery follow-up biomarker

For all selected genes, the cfDNA methylation in postoperative samples reached the cfDNA methylation of HV samples. Indeed, after orchidectomy, *OCT3/4* in postoperative blood reached the DNA methylation of HV samples. The same was observed for *KITLG* and *MAGEC2* (CpG1) in the postoperative seminal plasma. Based on correlation analysis, after orchidectomy, their methylation pattern was completely contrary to that detected before. This is because cfDNA originates from only healthy cells after orchidectomy, confirming treatment success [44]. Therefore, these genes could be considered potential biomarkers for SE monitoring.

Evaluation of possible screening & monitoring panels

ROC curve analysis of statistically significant or indicative CpGs disclosed varied AUC values (0.4–0.75), limiting their potential as SE biomarkers. To improve diagnostic performance, a panel of CpGs for SE screening and monitoring was designed. The panel of three CpGs, CpG8/CpG9/CpG10 in *KITLG*, detected in blood showed 79% specificity and 71% sensitivity, while the panel of CpG1 (*MAGEC2*)/CpG1 (*OCT3/4*) detected in seminal plasma showed 87% sensitivity and 60% specificity. Due to higher specificity, the panel from blood was considered superior for SE screening. Furthermore, a recent study reported a TGCT diagnostic panel from the blood that displayed a sensitivity of 100% [30]. However, even though in this study the sensitivity was 100%, the usage of serum for cfDNA analyses represents a limitation. During the clotting process of white blood cells in the collection tube, a high concentration of nonspecific gDNA is released and it could camouflage the tumor-specific cfDNA [45]. In the present study, the authors overcame this limitation by using plasma instead. The reported panel combined

hypermethylated *RASSF1A* with the quantification of miR-371a-3p. Indeed, miR-371a-3p alone represents a novel TGCT biomarker that is on the way to being translated into clinical practice. However, it is important to emphasize that miRNA is very sensitive to preanalytical variables [46], which influence the analysis results of the miRNA amount [47]. The levels of miRNAs could be influenced by blood sampling and preparation [48], hemolysis [49], the time in which the samples are processed [31], the number of freeze–thaw cycles [48], the type of sample (serum or plasma) and the isolation technique [50]. Even though the cfDNA also requires careful sampling, processing and analysis [17], DNA methylation represents the most stable and easily detectable chemical change on cfDNA, which is not so sensitive to preanalytical variables [51]. The authors of the present study believe that the detection of aberrant DNA methylation of genes playing a crucial role in TGCT could complement the detection of miR-372-3p and improve their diagnostic performance, as Lobo *et al.* [30] already implicated.

Study limitations

The results presented in this study should be further tested on a larger cohort. Still, even with a larger sample size, cancer-specific cfDNA methylation could remain camouflaged by cfDNA methylation from healthy cells, as indicated in this study.

Conclusion

The obtained data promote the cfDNA methylation of *OCT3/4*, *KITLG* and *MAGEC2* as potential noninvasive epigenetic biomarkers in liquid biopsy valuable for SE patient management. Newly designed CpG panels are presented as promising in SE patient screening. Furthermore, blood seems to be a better source of SE biomarkers than ejaculate, based on epigenetic changes detected in cfDNA. The presented data strongly promote the further development of liquid biopsy epigenetic biomarkers in SE patient management. However, TGCTs are a heterogenous group, divided into SE and nonseminoma, the latter consisting of four different histological subtypes. Therefore, to draw conclusions about potential epigenetic biomarkers for SE, cfDNA methylation in nonseminoma patients has to be investigated and compared with the presented data. Regarding confounding variables, despite the fact that some CpG loci showed changes in methylation confounded with the effects of alcohol consumption, smoking and age, we detected a few CpGs (in bld_*KITLG* and ej_*OCT3/4*) with high clinical biomarker potential, irrespective of standard confounding variables. This trend is yet to be confirmed in a larger cohort.

Summary points

- Testicular germ cell tumor (TGCT) represents the most prevalent malignancy among the young population of men and seminoma accounts for 60% of TGCTs.
- Routine serum biomarkers are used as a help in TGCT diagnosis but they are limited in their sensitivity and specificity, especially for seminoma.
- cfDNA represents a molecular biology platform for minimally invasive seminoma diagnosis and monitoring.
- This study aimed to investigate the cfDNA methylation of six genes from liquid biopses of seminoma patients and healthy volunteers as potential seminoma biomarkers.
- Different DNA methylation was detected in blood as well as in seminal plasma for *OCT3/4, KITLG* and *MAGEC2*.
- After receiver operating characteristic curve analysis, two potential screening panels emerged, in blood and seminal plasma.
- The diagnostic performance of potential panels was better than serum biomarkers.
- There is a need to determine clinical value, but the presented data promote the development of liquid biopsy epigenetic biomarkers in the screening of seminoma patients.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/epi-2022-0331

Author contributions

N Sincic and AK Bojanac contributed to the conception and design of the study. D Oršolić and A Barešić organized the database and performed the statistical analysis. M Tomić, NN Gabaj, N Gelo and T Kuliš contributed to the methodology. D Raos performed the investigation and formal analysis. D Oršolić, M Ulamec and S Mašić participated in the visualization of the results. M Ulamec, N Sincic, I Tomašković and Ž Kaštelan supervised and administrated the project. D Ježek and N Sincic provided funds and resources for the investigation. D Raos prepared the first draft. D Raos, AK Bojanac, J Krasić, N Sincic and A Barešić reviewed and edited the manuscript. All authors contributed to the manuscript revision and read and approved the submitted version.

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Ethical conduct of research

The authors state that they have obtained institutional approval from the Ethics Committee of the School of Medicine, University of Zagreb (protocol code 380-59-10106-17-100/187, 641-01/17-02/01), University Hospital Centre Sestre Milosrdnice (protocol code, number: EP-7259/17-9) and University Hospital Centre Zagreb (protocol code 8.1-17/107-2, number: 02/21 AG). Also, the authors followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent was obtained from the participants involved.

Data sharing statement

The raw data supporting the conclusions of this manuscript will be made available by the corresponding author, N Sincic (nino.sincic@mef.hr), without undue reservation, to any qualified researcher.

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