ORIGINAL PAPER

DOI: http://dx.doi.org/10.15403/jgld-4710

Factors Affecting Performance of DNA Methylation as a Potential Biomarker in Ascites for Peritonitis and Peritoneal Carcinomatosis

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Received: 06.11.2022
Accepted: 16.03.2023

ABSTRACT

Background & Aims: Despite limited sensitivity, the gold standard for the diagnosis of malignant cells in ascites is still cytology. The aim of this prospective proof-of-principle study was to evaluate DNA methylation as a molecular tool for the differential diagnosis of benign and malignant ascites.
Methods: A cohort of 79 patients with malignant and non-malignant ascites was prospectively enrolled. Ascites was assessed by cytopathological and laboratory examination. Cell pellets obtained by centrifugation were analyzed for differences in DNA methylation of of long interspersed nuclear element-1 (LINE-1) and microRNA-137. Quantitative determination of methylation in bisulfite-converted DNA was performed by pyrosequencing. In a subsequent stage, we compared our data to previously published data in the field following systematic review of the literature.
Results: Methylation status of studied LINE-1 and microRNA-137 could be reliably detected in all samples. Systematic evaluation revealed reliable reproducibility with satisfactory short- and long-term stability against degradation. Ascites from patients with a malignancy had a significantly higher methylation level of microRNA-137 compared with patients without tumor disease, whereas patients with peritonitis had significantly decreased methylation of microRNA-137. In contrast, differences in the measurement of the methylation status of LINE-1 could only be detected between patients with portal hypertension and a combination of malignant and infectious ascites. Inflammatory cells reflecting peritonitis correlated to DNA methylation changes.
Conclusions: Analysis of DNA methylation in ascites is technically feasible, well reproducible and may lead to identification of potential biomarkers for peritoneal carcinomatosis and other conditions. Inflammatory cells due to peritonitis may also be associated with DNA methylation changes and need to be considered in future studies. Profiling studied under standardized conditions will be needed to identify the appropriate biomarkers for differential diagnosis of ascites.

Key words: DNA methylation – LINE-1 – miR-137 – microRNA-137 - ascites – peritoneal carcinomatosis – peritonitis.

Abbreviations: CA: carcinoma; LINE-1: long interspersed nuclear element-1; miR: microRNA; non-LTR: non-long terminal repeat transposable elements; PHT: portal hypertension; PMN: polymorphonuclear neutrophil granulocytes; PCA: peritoneal carcinomatosis; SBP: spontaneous bacterial peritonitis.

INTRODUCTION

Ascites is a symptom which may be caused by benign and malignant conditions and has substantial impact on health and disease prognosis. In Western countries, ascites is mainly caused by liver cirrhosis or by portal hypertension for other reasons in up to 75% of cases. However, the occurrence of ascites in the context of peritonitis and peritoneal carcinomatosis should be seen as a condition with enormous prognostic relevance [1]. Patients with spontaneous bacterial peritonitis (SBP), which is one of the most common complications of ascites virtually always occurs in patients suffering from liver cirrhosis, and therefore, have substantially reduced overall survival [2]. Patients with peritoneal carcinomatosis (PCA) have a devastating prognosis, and if not treated with tumor-specific therapy, have a median survival of approximately 3.1 months [3].

Diagnostic procedures include diagnostic paracentesis, in addition to obtaining a general history and physical status, standard laboratory tests, and urine analysis. Apart from

the macroscopic aspect, various investigations with different specificity and sensitivity can provide clues about the genesis of ascites.

Spontaneous bacterial peritonitis along with urinary tract infection, are the most common causes of infection in patients with liver cirrhosis. Bacterial infections are associated with a 4-fold increase in mortality in patients with liver cirrhosis [4]. Microbiological examination by inoculation of culture bottles shows positive culture results in 36-59% of cases in patients with SBP [5]. However, the gold standard for the diagnosis of SBP is the determination of cell count and cell differentiation with an increase of polymorphonuclear neutrophil granulocytes (PMN) to > 250/µl. Even so, cell count and percentage of PMN may also be increased in malignant ascites, making the diagnosis challenging in patients with cancer.

Peritoneal carcinomatosis occurs frequently in patients with gastrointestinal tumors and ovarian cancer and is defined by tumor dissemination in the abdominal cavity. The frequency of PCA varies depending on the specific entity involved, for example in about 60-80% of cases of ovarian carcinoma. However, PCA is also present in up to 50% of malignancies of the gastrointestinal tract, depending on the entity and tumor stage [6]. In approximately 35% of non-gynecological tumors, patients present with ascites at the timepoint of diagnosis [3]. When ascites first appears, diagnostic and often therapeutic ascites puncture should be performed. Here, detection of malignant cells and immunotyping may provide very valuable knowledge on the primary origin of the tumor. Although cytologic detection of malignant cells in ascites is quite specific, the sensitivity of a positive cytology, as described in the literature with conventional effusion cytology, reaches only up to 60%, though this sensitivity can be further increased with multiple examinations and the use of increased ascites volume [7-9].

In recent years, the analysis of epigenetic modifications has gained increasing attention in translational research [10]. Epigenetic modifications include DNA methylation, histone modification, and microRNA (miR) analyses. During DNA methylation, postreplicative methyl groups are transferred by methyltransferases to the 5th carbon atom of the base cytosine located within a CpG dinucleotide. This often occurs in promoter or enhancer regions of DNA, reducing or preventing transcription of downstream genes. Alterations in DNA methylation is a common event in carcinogenesis, which can be considered in diagnosis [11] or cancer prevention [12].

Long interspersed nuclear element-1 (LINE-1), a surrogate marker for global methylation status, belongs to the non-long terminal repeat transposable elements (non-LTR), a subset of the repetitive elements of DNA, which account for approximately 50% of the human genome. LINE-1 accounts for a significant proportion of this, approximately 17% [13, 14]. Since 1993, when a study on the methylation status of LINE-1 in tumor tissues was first published, numerous papers have appeared showing global hypomethylation and subsequent DNA instability in various tumor tissues compared with non-tumor tissues [15, 16]. In addition to global methylation analysis, local hypo- or hypermethylation of promoter regions, e.g. of protooncogenes or tumor suppressor genes, has also been investigated in numerous studies [17]. For instance, this is attributed to miR-137, which is embedded in CpG islands and has therefore often been linked to carcinogenesis. Several studies reported on hypermethylation of miR-137 in tumor tissues [18], and miR-137 promoter hypermethylation has been linked to colon and gastric carcinogenesis [19, 20].

Although DNA methylation has been considered as a biomarker for various cancers in feces, blood, and tissues, only limited knowledge has been obtained on DNA methylation in ascites. Several studies reported on the feasibility of DNA methylation mostly using peritoneal lavage and only partially ascites. However, systematic analysis in particular on potential factors including abdominal inflammation are still required.

In our prospective proof-of-principle study we investigated the feasibility of evaluation of DNA methylation in ascites as a potential biomarker using LINE-1 and miR-137 DNA and explored whether specific epigenetic alterations could be associated with an etiology of ascites. Most importantly, we evaluate the role of various factors on performance of DNA methylation analysis.

METHODS

Study Population and Ethical Approval

The study protocol was conducted in accordance with the declarations of Helsinki and approved by the Ethics Committee of the Otto-von-Guericke-University Magdeburg (No 85/10). All patients provided their written informed consent prior to the study. Between 2010 and 2013, a total of 79 patients who underwent diagnostic or therapeutic ascites puncture/drainage were included. Patients were divided into 4 main groups based on confirmed diagnosis as follows: 30 patients with portal hypertension (PHT) (no SBP/PCA, negative cytology, PMN < 250 cells/mm^3, negative bacterial culture); 20 patients with peritonitis (PMN > 250 /µl and/or positive bacterial culture); 4 patients with evidence of carcinoma (CA) (without evidence of PCA, infection or PHT); 25 patients with PCA (detection of neoplastic cells in cytology or histology, primary peritoneal carcinoma). Detailed characteristics of the patients are provided in Table I.

Routine Workup and Cytology

After informed consent and preparation, diagnostic and/or therapeutic ascites drainage/puncture was performed as part of regular ward or outpatient work. In addition, the material was sent for examination to clinical chemistry, microbiology and pathology and examined using established techniques as previously described [21].

DNA Isolation and Measurement

The samples were obtained in a standard technique and, following collection, were utilized according to the protocol as partially described elsewhere [21]. The ascites samples were either stored as they were or in centrifuge at 3600 rpm for 10 minutes from either 1 or 8 ml of ascites to create a pellet for the subsequent analysis. The samples were stored at -80°C until further analysis. Measurements were performed in batches in a central laboratory after thawing of the samples. DNA isolation was performed using the RNeasy Plus Universal Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer‘s

Table I. Characteristics of the patient population.

	Total	PHT	Peritonitis	CA	PCA
Number of patients, n (%)	79 (100)	30 (38.0)	20 (25.3)	4 (5.1)	25 (31.6)
Age (years)	62.8±11.1	59.2±10.3	61.9±12.5	66.8±15.3	67.0±8.8
Gender, n (%)					
Male	58 (73.4)	27 (46.6)	16 (27.6)	1 (1.7)	14 (24.1)
Female	21 (26.6)	3 (14.3)	4 (19.0)	3 (14.3)	11 (52.4)
Liver cirrhosis, n (%)	51 (64.6)	30 (58.8)	13 (25.5)	1 (2.0)	7 (13.7)
ASH/NASH	2	2	0	0	0
Hemochromatosis	2	2	0	0	0
Cardiogenic	2	2	0	0	0
Cryptogenic	9	1	4	1	3
Toxic	34	21	9	0	4
Viral	2	2	0	0	0
Carcinoma, n (%)	48 (60.8)	10 (20.8)	9 (18.8)	4 (8.3)	25 (52.1
Bronchial carcinoma	2	0	1	0	1
B-cell lymphoma	1	1	0	0	0
CCC/Klatskin tumor	9	2	2	3	1
CUP	3	0	0	0	3
Gallbladder carcinoma	2	0	1	1	0
HCC	12	6	4	0	2
Hypopharyngeal carcinoma	1	1	0	0	0
Colorectal carcinoma	2	0	0	0	2
GC/AEG	6	0	0	0	6
Renal cell carcinoma	1	0	1	0	0
Ovarian carcinoma	1	0	0	0	1
Pancreatic carcinoma	7	0	0	0	7
Peritoneal carcinoma	1	0	0	0	1
Other. n (%)	2 (2.5)	0 (0)	2 (100)	0 (0)	0 (0)
HIV	1	0	1	0	0
Pancreatitis	1	0	1	0	0
Blood results					
Albumin (g/dl)	28.8 ± 6.3	29.5±6.1	25.0±6.1	25.6*	31.4 ± 5.4
White blood cells (Gpt/l)	10.4 ± 6.3	8.2 ± 4.1	13.0 ± 8.6	14.6±4.6	10.2 ± 5.4
CRP (mg/l)	75.4 ± 62.2	37.1 ± 38.6	100.1 ± 60.9	124.9±132.1	98.1 ± 53.8
Ascites results					
Albumin (g/dl)	12.3 ± 9.2	9.6 ± 8.2	9.1 ± 4.2	6.1 ± 3.8	22.9 ± 9.2
Cell count (Mpt/l)	3,674.4 ± 14,496.4	150.0 ± 101.5	13,084.8 ± 27,118.5	102.0±54.4	929.7 ± 1,492.7
PMN (Mpt/l)	3,506.2 ± 14,221.6	42.0 ± 50.0	12,760.4 ± 25,966.2	15.4±11.5	448.1 ±757.1
PMN (% of cell count)	38.3 ± 31.4	25.2±24.2	67.7±30.1	19.5±7.8	29.4 ± 24.2
Methylation (%)					
LINE-1	71.3±3.6	70.2±3.7	72.1 ± 3.7	72.3±3.4	71.5 ± 3.8
miR-137	18.4±14.8	18.9±14.2	10.3 ± 8.2	20.8±23.0	23.1 ± 16.0

Mean ± SD: mean ± standard deviation; PHT: portal hypertension; CA: carcinoma; PCA: peritoneal carcinomatosis; ASH/NASH: alcoholic steatohepatitis/non-alcoholic steatohepatitis; CCC: cholangiocarcinoma; CUP: cancer of unknown primary; HCC: hepatocellular carcinoma; GC/AEG: gastric carcinoma/adenocarcinoma of esophagogastric junction; HIV: human immunodeficiency virus; g/dl: grams/deciliter; CRP: C-reactive protein; mg/l: milligrams per liter; Gpt/l: gigaparticles per liter; Mpt/l: megaparticles per liter; PMN: polymorphonuclear neutrophil granulocytes; LINE-1: long interspersed nuclear element-1; miR-137 microRNA; * ± SD not determinable, only 1 value available.

protocol with minor modifications. Ascites (100 µl) and cell pellet were mixed with QIAzol Lysis Reagent, homogenized, incubated at room temperature, then mixed after addition of chloroform, and the phases were separated by centrifugation. The upper RNA-containing aqueous phase was removed and further processed; the DNA-containing interphase was

added with 100% ethanol. After homogenization, incubation on ice and under room air, centrifugation, and addition of 75% ethanol and sodium hydroxide solution, a supernatant was obtained, which was mixed with 0.1 M HEPES and 0.1 M EDTA solution and stored at -20°C, if not immediately further processed. The quantification and quality of the DNA were assessed by measuring the absorbance with the spectrophotometer BioPhotometer (Eppendorf, Hamburg, Germany) at the ratio 260/280 nm.

Quantitative Measurement of DNA Methylation

Bisulfite modification of the purified DNA was performed using the Cells-to-CpG Bisulfite Conversion Kit (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's protocol with minor modification as previously described [20]. Briefly, 500 ng of the purified DNA was mixed with RNase-free water and treated with the various reagents and buffer solutions included in the kit. The eluate obtained at the end contained the bisulfite-modified DNA. PCR was performed using biotinylated primers for miR-137 and LINE-120, to which water and HotStarTaq Plus Master Mix were added. 27 µl of this mix and 3 µl of the recovered DNA were then incubated in the CFX96 cycler at various temperatures, and the modified DNA was stored at 4 °C until further processing. Agarose gel electrophoresis was performed to monitor success. Quantitative determination of methylation was performed by pyrosequencing with PyroMark Q96 ID and the use of PyroMark Gold Q96 reagents according to the manufacturer's protocol. Beads coated with streptavidin bind to the biotin of the reverse primer. The PCR products are denatured prior to pyrosequencing. The bound DNA strand serves as a template strand to which the sequencing primer attaches. A mix of enzymes and substrates and, individually, the nucleotides needed to extend the sequencing primer are then added. When the nucleotide is incorporated, a flash of light is generated. The mean values of the methylation status of the DNA regions to be determined were calculated by integrated software using the pyrogram. The percentages of DNA methylation were calculated from the measured difference of T and C (because methylated C is not converted in the sodium bisulfite treatment). Samples with insufficient DNA quality and/or for which the determination of the methylation status was not reliably successful were excluded from the analyses.

Statistical Analysis

Methylation results were obtained using the PyroMark Q96 ID integrated easy-to-use PyroMark Q96 ID Software 2.5. Further statistical analysis of all data was performed using GraphPad Prism 6.0 (GraphPad Software Inc, La Jolla, California). The Shapiro-Wilk test was used to test for normal distribution. Quantitative evaluation of nonparametric analyses was performed using the Wilcoxon test for 2 paired samples and the Mann-Whitney test for 2 unpaired samples. For 3 or more samples, the Friedman test was used for paired and the Kruskal-Wallis test with Dunn's multiple comparison post-hoc test (Dunn's post-test) for unpaired. Correlation analyses were performed with Spearman's test. P values of ≤0.05 were considered statistically significant.

RESULTS

In this study we completed the preanalytical analysis to assess first the feasibility and potential influence of sample handling or storage, reproducibility, and the impact of different aliquoting volumes. Subsequently, in a second step, the methylation status in disease groups was compared, and the correlation to various clinical parameters was assessed.

Extraction, Reproducibility, Stability, and Aliquoting Volumes

Multiple studies have investigated epigenetic alterations not only in tissues but also in body fluids. However, only limited data are available related to DNA methylation in a cell pellet obtained from ascites; therefore, we performed the first basic feasibility analysis. DNA was successfully isolated and DNA methylation status determined for both LINE-1 and miR-137, with average values for LINE-1 of 71.29±3.59% and for miR-137 of 18.47±14.72% in the entire patient population (Fig. 1A) with good reproducibility (Fig. 1B). In addition, comparable methylation results were shown for LINE-1 and miR-137 following original sample incubation under room temperature

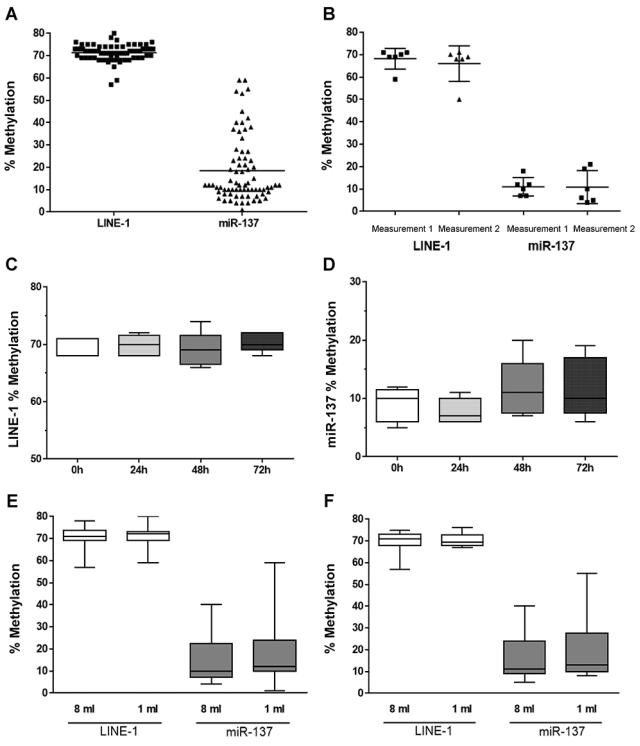

Fig. 1. Feasibility and preanalytical analysis of DNA methylation in ascites. (A) Feasibility and overview of methylation analysis for LINE-1 and miR-137. (n = 79). (B) Detection of DNA methylation in ascites and reproducibility of results using 6 pairs of data each for LINE-1 and miR-137. Analysis was performed using the Wilcoxon assay. (C, D) Stability of DNA methylation. DNA methylation of (C) LINE-1 and (D) miR-137 of 5 samples at 4 different incubation times, each time point 0 h, 24 h, 48 h, 72 h. Analysis was performed using Friedman's test. (E, F) Influence of different aliquoting volumes (1 ml and 8 ml) on the methylation status of LINE-1 and miR-137. Samples from (E) all groups (n=24 for 8 ml, n=51 for 1 ml) and (F) PHT group (n=15 for 8 ml, n=16 for 1 ml) were examined. Analysis was performed using the Mann-Whitney test. PHT: portal hypertension.

at different time points, mimicking the clinical settings (Figs. 1C and D). Furthermore, no significant differences were seen in the comparison of 1 ml with 8 ml, both in the whole patient collective and in the PHT group (Figs. 1E and F).

DNA Methylation in Comparison to Different Groups

Comparison of ascites samples from the PHT group with those from other subgroups combined (peritonitis, CA, and PCA) revealed that there was a significant difference for LINE-1 (70.12±3.73% vs. 71.94±3.37%, p=0.0183), but not for miR-137 (Fig. 2A).

Comparison of the methylation status of the 4 main groups (PHT, peritonitis, CA, and PCA) with each other failed to present significant values for LINE-1 but showed a trend toward lower methylation of the PHT vs. peritonitis group (70.12±3.73 vs. 72.30±2.96%, p=0.0280; adjusted significance level due to multiple testing: ≤ 0.0142). There were significant differences in methylation status between PHT vs. peritonitis in miR-137 (19.04±14.16% vs. 10.47±8.08%, p=0.0035) (Figs. 2B and C).

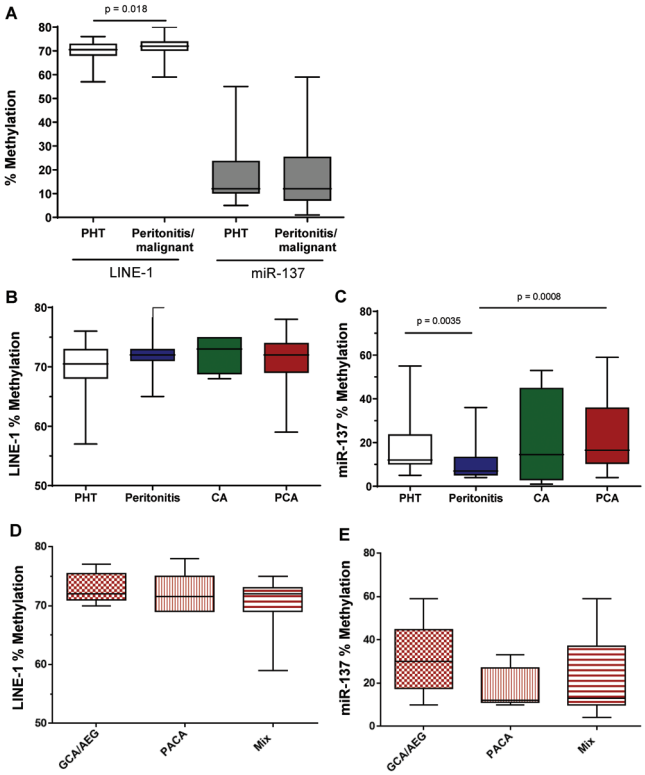

Fig. 2. Methylation status of LINE-1 and miR-137 of PHT group and the other groups. (A) Methylation status of PHT group (n=30) compared to all other groups (n=49). Peritonitis (n=20), CA (n=4), PCA (n=25). Analyses were performed using the Mann-Whitney test. (B, C) Methylation status of LINE-1 and miR-137 of the different major groups according to the genesis of ascites. Methylation status of LINE (B) and of miR-137 (C) comparing the 4 main groups PHT (n=30), peritonitis (n=20), CA (n=4), PCA (n=25). A preselection with the Kruskal-Wallis test was performed, followed by individual testing using the Mann-Whitney test at adjusted significance level (αadjusted: 0.0142). (D, E) Methylation status of LINE-1 and miR-137 in different cancer entities of peritoneal carcinomatosis group. Comparison of methylation status of LINE-1 (D) and of miR-137 (E) between GCA/AEG (n=6), pancreatic cancer (n=7), Mix (n=12). A preselection with the Kruskal-Wallis test was performed, followed by individual testing with the Mann-Whitney test at adjusted significance level (αadjusted: 0.025). Portal hypertension (PHT), carcinoma (CA), peritoneal carcinomatosis (PCA), gastric carcinoma/adenocarcinoma of the esophagogastric junction (GCA/AEG), pancreatic carcinoma (PACA), association of various less common cancer entities (Mix).

Compared to the peritonitis group, the samples from patients with malignancies or from patients with proven PCA showed higher miR-137 with greater variation, with the most significant difference occurring between peritonitis and PCA (10.47 ± 8.08 % vs. 23.98 ± 16.05 %, p=0.0008) (Figs. 2B and C). The PCA group was further divided based on the tumor of primary origin into GCA/AEG, pancreatic cancer, and a mix of other cancer entities (Table I). Despite interindividual differences, for both LINE-1 and miR-137 methylation analysis, no significant results were detected (Figs. 2D and E).

In the following section we evaluated DNA methylation changes related to the presence or absence of malignancies, to the presence or absence of peritonitis, and to possible differences in patients with and without cirrhosis. As shown in Figs 3A and B, we divided all patients into non-malignant (PHT, peritonitis) and malignant (PCA, CA) groups. The non-malignant group showed lower methylation level compared to the malignant group (15.80±12.82% vs. 22.75±16.70%, p=0.0398). Further subgroup analysis of the malignant group into CA and PCA as well as a miR-137 comparison of all 3 groups with each other did not yield significant results for either LINE-1 or miR-137.

While no differences were observed for LINE-1 DNA methylation levels, the miR-137 analysis revealed lower DNA methylation in the peritonitis subgroup of patients compared to other patients (10.47±8.08% vs. 20.89±15.46%, p=0.0007) (Figs. 3C and D). The peritonitis group was furthermore subdivided into SBP and secondary peritonitis; however, there were no differences between these groups.

Comparison of the SBP group vs. the group without peritonitis revealed no relevant differences for LINE-1. For miR-137, there was again a trend toward lower methylation in the SBP group (12.25±9.01% vs. 20.89±15.46%, p=0.0273; adjusted significance level: ≤ 0.025). Similarly, for the secondary peritonitis group vs. the group without peritonitis, we were able to show a significant difference, but only for miR-137 (6.20±2.39% vs. 20.89±15.46%, p=0.0007) (Figs. 3E and F), suggesting that level of inflammation may be associated with level of miR-137 methylation. In the peritonitis group, neither positive nor negative bacterial detection was associated with changes in LINE-1 or miR-137 DNA methylation; likewise, no association was found with either the presence or absence of liver cirrhosis.

Comparison of Laboratory Chemical Parameters in the Groups

Before performing analysis on potential correlation with objective clinical parameters, we evaluated the differences of routine parameters in the subgroups. As expected, CRP values were higher in ascites of patients with peritonitis vs. without peritonitis (100.1±60.9 mg/l vs. 66.2±60.8 mg/l, p=0.0352), peritonitis vs. PHT (100.1±60.9 mg/l vs. 37.1±38.6 mg/l, p=0.0003), and PCA vs. PHT (98.1±53.8 mg/l vs. 37.1±38.6

mg/l, p<0.0001). In contrast, no significant differences could be observed between peritonitis vs. PCA for both CRP and leukocyte count in the blood (Figs 4A and B). In similar peritonitis; however, count was higher in patients with peritonitis vs. without peritonitis (13.0±8.6 Gpt/l vs. 9.5±4.9 Gpt/l, p=0.0297) and in patients with peritonitis vs. PHT (13.0±8.6 Gpt/l vs. 8.2±4.1 Gpt/l, p=0.0044) (Figs. 4C and D). Comparison with the cancer group was not performed due to partially limited values and relatively small cohort size.

Correlation Analyses

Analysis between miR-137 DNA methylation in ascites and cell count and/or PMN revealed a negative association, while no differences were observed for LINE-1 methylation (r=-0,35, p=0.0061), suggesting that higher cell count may be associated with an increased number of lymphocytes with lower miR-137 DNA methylation as compared to peritoneal cells or even tumor cells (Figs. 5B and D). Correlation analysis in subgroups of patients with proven PCA as well as in patients with peritonitis revealed no association between methylation and the cell count.

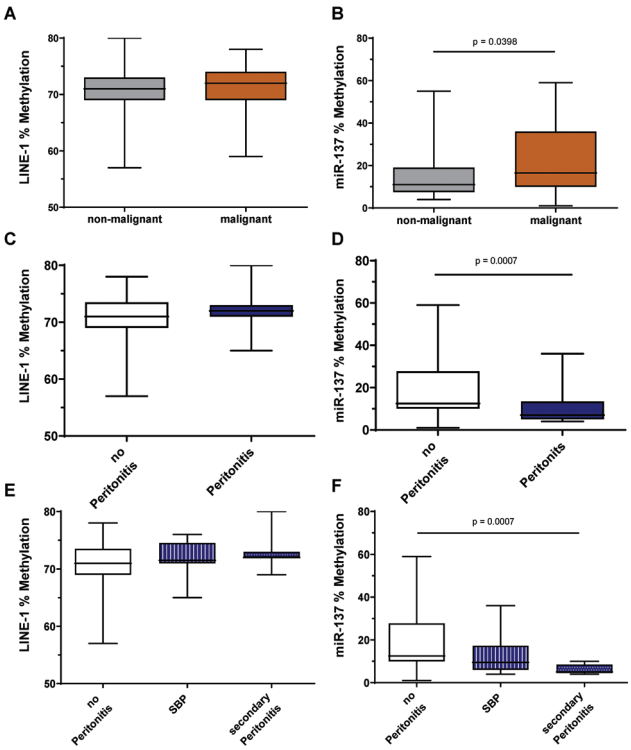

Fig. 3. Comparison of DNA methylation based on etiology of ascites. Methylation status of LINE-1 (A) and of miR-137 (B) comparing non-malignant vs. malignant (non-malignant: n=50; malignant: n=29). Analysis was performed using the Mann-Whitney test. Comparison of the methylation status of LINE-1 (C) and of miR-137 (D) between no Peritonitis (n=59) vs. Peritonitis (n=20). Analysis was performed using the Mann-Whitney test. Comparison of methylation status of LINE-1 (E) and of miR-137 (F) between no Peritonitis (n=59) vs. SBP (SBP and bacterascites, n=12) vs. secondary peritonitis (n=8). A preselection with the Kruskal-Wallis test was performed, followed by individual testing with the Mann-Whitney test at adjusted significance level (αadjusted: 0.025). SBP: spontaneous bacterial peritonitis.

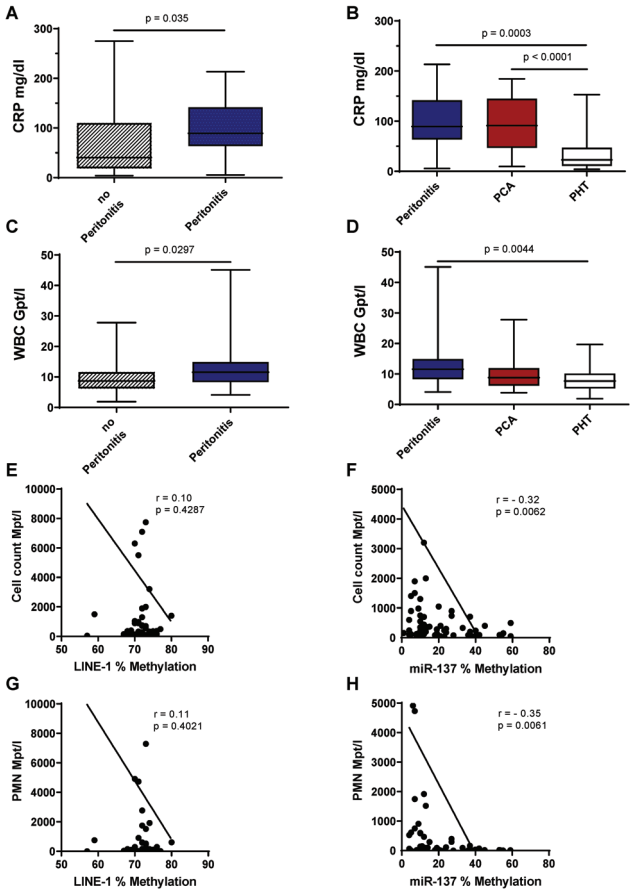

Fig. 4. Association of inflammation markers and DNA methylation status. Comparison of CRP and white blood cells (WBC) in blood between different groups. (A) CRP in the no peritonitis (n=59) and peritonitis (n=20) group and (C) WBC in the no peritonitis (n=59) and peritonitis (n=20) groups. Analysis was performed using the Mann-Whitney test. (B) CRP values in the groups with peritonitis (n=20), PCA (n=25), PHT (n=30), and (D) WBC in the groups with peritonitis (n=20), PCA (n=25), PHT (n=30). A preselection with the Kruskal-Wallis test was performed, followed by individual testing with the Mann-Whitney test at adjusted significance level (αadjusted: 0.025). C-reactive protein (CRP), portal hypertension (PHT), peritoneal carcinomatosis (PCA). Methylation status of LINE-1 and miR-137 in correlation with cell number and PMN (n=79). Correlation of LINE-1 (E) and miR-137 (F) with cell number. Correlation of LINE-1 (G) and miR-137 (H) with PMN. Analysis was performed using the Spearman test. PMN: polymorphonuclear neutrophil granulocytes.

DISCUSSION

In this proof-of-principle study we investigated the methylation behavior of LINE-1 and miR-137 in an ascites-derived cell pellet and their use as potential biomarkers. As previously reported for the DNA methylation analysis in our specimens, our data suggest that DNA methylation evaluation of targets in ascites can be easily performed with a good reproducibility of results with commercially available and easy-to-use kits. Consistently, the quality at different aliquoting volumes and stability over 72 h are comparable to the recent

study with a focus on the miR performance as a biomarker in ascites [21]. Although the implemented DNA methylation targets did not show significant differences between specific disease subgroups, several key messages can be drawn from our work. Firstly, the inflammatory cells may impact the proportion of malignant to inflammatory cells and thus shift the miR-137 or LINE-1 DNA methylation. Furthermore, the selection of appropriate targets may be crucial, as certain cancer types may be associated with different methylation patterns. Interestingly, the miR-137 methylation was higher in cell pellets of patients with malignant ascites compared to the non-malignant group, suggesting that other targets may substantially improve the performance of DNA methylation as a biomarker in ascites. Methylation analyses is highly robust and reproducible and can be successfully performed both in tissues and as so-called liquid biopsies, or even in cell pellets [22-26].

As reported above, several studies related to LINE-1 and miR-137 have been published; however, only limited knowledge exists for the evaluation in ascites. We selected LINE-1 as one of the frequently used surrogate markers for global DNA methylation. Lower methylation of LINE-1 was detectable in tumor tissues of gastric carcinomas and CRC compared to their adjacent non-malignant tissues [15]. Therefore, the lower level of LINE-1 or higher levels of miR-137 in ascites might be an indirect marker for PCA, respectively. However, it is effectively impossible to define a control cohort, as ascites is essentially always a pathological state. Therefore, to overcome this limitation, we included the PHT group as potentially the most "normal" state possible.

In our patient population, there was no significant difference in LINE-1 when comparing the PHT group with the PCA group, which may be explained by several factors. Firstly, the cohort of subjects with PCA may be too small to identify subtle differences. Furthermore, as touched on above, LINE-1 methylation may differ between tumors of different entities, resulting in a higher variability of the results. Overall, the cytology and histology were considered to be a gold standard in defining PCA; however, the number of cells may also differ, and cytology could potentially miss some patients with false negative cytology results. Most importantly, assuming that tumor cells have lower LINE-1 methylation, the values could be masked by the number of lymphocytes in ascites. Zhu et al. [27] examined the LINE-1 methylation of leukocytes and showed that LINE-1 in blood may be rather higher than expected in peritoneal or tumor cells, which, if compared to our values, was significantly higher than the LINE-1 in the cohort of PHT in our study. Moreover, in ascites from patients with peritonitis, there are per definition more cells present, including inflammatory cells, which may lead to differences in DNA quantity and proportion, which again could explain the trend towards lower LINE-1 methylation in the PHT group compared to the peritonitis group. In addition, it is also conceivable that infection itself could lead to DNA alteration or that alteration of DNA methylation could lead to infection, as suggested previously [28].

Methylation analysis of miR-137 revealed a significantly lower methylation level in patients with peritonitis (SBP, secondary peritonitis) compared to those without evidence for peritonitis. This lower miR-137 methylation was present not only when compared between patients with peritonitis and PHT, but most importantly, when compared to PCA. Those differences may be explained by the number of neoplastic cells that can be associated with increased methylation, as previously well reported for gastrointestinal neoplasia [29, 30], but also by the low methylation level in inflammatory cells. Therefore, one may speculate that those changes in DNA methylation in ascites need to be considered while screening for biomarkers and interpreting the data. Recently, a few studies have reported on the association between miR-137 hypomethylation and the progression of inflammation of chondrocytes in osteoarthritis [31, 32].

Peritonitis in patients with PHT is defined by the number of inflammatory cells in ascites, which was validated for our cohort and consistent with previous publications [33-35]. It has been previously suggested that LINE-1 methylation in blood may be associated with CRP and leukocytes count. However, a small number of studies failed to confirm those results [36, 37]. Correlation analyses revealed no differences between LINE-1 methylation and inflammatory markers, while miR-137 was negatively associated with leukocytes count, PMN and CRP, suggesting an indirect impact of inflammation on the DNA methylation level.

To provide the detail overview of our data in view of the existing studies, we performed systematic analysis of the published data related to DNA methylation analysis in ascites (Table II). Overall, using predefined criteria which included studies with focus on ascites/peritoneal lavage and involved cancer patients, we identified 18 studies. Majority of those studies deal with gastrointestinal or gynecologic tumors with a particular focus on peritoneal lavage during surgery. Therefore, the strength of our work is the methylation analysis using ascites samples only. While peritoneal lavage is important for various types of cancer, ascites is a consequence of malignant disease and accordingly differs in clinical situation and management. Furthermore, most of the trials looked at single cancer entity, which substantially limit the power of the analyses.

The data from our study represent the real-life clinical setting, reflecting the everyday clinical practice, in which other differential diagnoses of ascites, including liver pathology, play an important role in addition to various cancer entities.

Furthermore, the clinical and laboratory parameters were available to us to correlate and evaluate the significance of the methylation status of LINE-1 and miR-137 in a clinical context. In addition, in this work were obtained systematic insights on the impact of inflammation on the power of DNA methylation, which has been poorly assessed to date.

There are still limited data on the feasibility of DNA methylation analysis for the differential diagnosis of ascites. As this study was designed as a proof-of-principle work, there are several limitations that need to be mentioned. In this work, we utilized previously well-established, validated DNA methylation targets known to be deregulated in gastrointestinal tumors, but it is obvious that the selection may not be sensitive enough, and other biomarkers need to be evaluated. Based on the assumption that tumor cells may likely be the main source of DNA methylation alterations, we analyzed cell pellets. However, for the ascites with a high number of inflammatory cells in particular, it is likely that those cells may substantially

Table II. Recent studies analyzing the methylation status of peritoneal fluid

Reference	Year	Country	Profiling	Target gene(s)	Patients	Tumor entity	Method	Source
Ibanez de Caceres et al. [38]	2004	USA	no	BRCA1, RASSF1A	42	Ovarian cancer	MSP	Peritonael fluid (lavage and ascites)
Müller et al. [39]	2004	Austria	no	TIMP3, CDH1, CDH13, APC, PPP1R13B, HSPA2, HSD17B4, ESR1, GSTP1, CYP1B1, BRCA1, MYOD1, SOCS1, TITF1, GSTM3	61	Ovarian cancer	qMSP	Peritoneal fluid (3 lavage and 58 ascites)
Chen et al. [40]	2007	Taiwan	no	P15, P16, APC, E-cadherin	70	Metastatic adenocarcinoma	MSP	Ascites, pleural effusion
Kamiyama et al. [41]	2009	Japan	no	CDH1, CDKN2A (p16), MGMT, APC	51	Colorectal cancer	qMSP	Peritoneal lavage
Hiraki et al. [42]	2010	Japan	no	CHFR, p16, RUNX3, E-cadherin, hMLH1, ABCG2, BNIP3	80	Gastric cancer	qMSP	Peritoneal lavage
Du et al. [43]	2010	China	no	RECK	40	Gastric cancer	MSP	Peritoneal lavage
Hiraki et al. [44]	2011	Japan	no	BNIP3, CHFR, CYP1B1, MINT25, SFRP2, RASSF2	107	Gastric cancer	qMSP	Peritoneal lavage
Han et al. [45]	2014	China	no	MINT2	92	Gastric cancer	qMSP	Peritoneal lavage
Yu et al. [46]	2014	China	no	TIMP 3	92	Gastric cancer	qMSP	Peritoneal lavage
Jung et al. [47]	2016	Germany	no	SHOX2, SEPT9	283	mix	qMSP	Ascites
Choi et al. [48]	2017	Korea	yes	profiling	4	Ovarian cancer	microarray	Ascites
Ushiku et al. [49]	2017	Japan	no	CDO1	102	Gastric cancer	qMSP	Peritoneal fluid (lavage and ascites)
Giri et al. [50]	2019	Finland	yes	profiling	6	Ovarian cancer	microarray	Ascites
Valle et al. [51]	2020	USA	no	HIST1H2BB, MAGI2	non specified	Ovarian cancer	qMSP	Ascites
Harada et al. [52]	2021	Japan	no	CDO1	400	Gastric cancer	qMSP	Peritoneal lavage
Hu et al. [53]	2021	China	no	THBS1	92	Gastric cancer	qMSP	Peritoneal lavage
Werner et al. [54]	2021	Australia	no	HOXA9, IFFO1	18	Ovarian cancer	qMSP targeted sequencing	Ascites
Current work: Bose et al.	2023	Germany	no	LINE-1, miR-137	79	mix	Pyro-sequencing	Ascites

qMSP: quantitative Methylation-specific PCR

change the value of screened targets. Further studies are required to explore cell-free ascites with a focus on circulating DNA targets.

CONCLUSIONS

In this proof of principle study, we provide systematic level evidence for the feasibility of DNA methylation analyses in ascites as a potential diagnostic tool in inflammation and cancer research. The DNA for the methylation analysis is well preserved in unprocessed samples with a good short-term and long-term stability, even at room temperature. Multiple cytopathological abdominal conditions, including portal hypertension, peritonitis, and peritoneal carcinomatosis were associated with DNA methylation alterations. However, further large-scale profiling studies with respect to identified factors will be necessary to determine cancer- and disease-specific biomarkers in ascites.

Conflicts of interest: None to declare.

Author' contributions: K.B. performed the formal analysis, investigation, validation, visualization, wrote the original draft, and revised the paper. F.G.S. performed the formal analysis, visualization, wrote the original draft, and revised the manuscript. C.T. performed the investigations and revised the manuscript. S.F., C.S., P.M. found the resources and revised the manuscript. A.L. conceive the study, performed data curation, formal analysis, methodology, validation, visualization, wrote the original draft and revised the manuscript. A.L was responsible for the project administration and supervision.

Acknowledgements: Grant support: A.L. is supported by the funds of the European Commission through the "European funds for regional development" (EFRE) as well as by the regional Ministry of Economy, Science and Digitalization of Saxony-Anhalt as part of the „Autonomy in old Age" (AiA) research group for "LiLife" Project (Project ID: ZS/2018/11/95324). We would like to thank all colleagues who supported the sample collection. We thank Ursula Stolz, Marion Holley und Simone Philipsen (Gastrointestinal Research Laboratory of the Department of Gastroenterology, Hepatology and Infectious Diseases, Otto-von-Guericke University, Magdeburg) for their support

in processing the ascites samples during the experimental part of the work. Furthermore, the authors would like to thank Erin Calabria for the language editing.

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