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Research Paper

Combination of calretinin, MALAT1, and GAS5 as a potential prognostic biomarker to predict disease progression in surgically treated mesothelioma patients

Laura V. Klotz [a,c,e,1,*], Swaantje Casjens [b,1], Georg Johnen [b], Dirk Taeger [b], Alexander Brik [b], Florian Eichhorn [a,c], Laura Förster [d], Nina Kaiser [b], Thomas Muley [c,d], Christa Stolp [d], Marc Schneider [c,d], Jan Gleichenhagen [b], Thomas Brüning [b], Hauke Winter [a,c], Martin Eichhorn [a,c,1], Daniel G. Weber [b,1]

[a] Department of Thoracic Surgery, Thoraxklinik, University of Heidelberg, Roentgenstraße 1, 69126 Heidelberg, Germany
[b] Institute for Prevention and Occupational Medicine of the German Social Accident Insurance, Institute of the Ruhr University Bochum (IPA), Bürkle-de-la-Camp Platz 1, 44789 Bochum, Germany
[c] German Center for Lung Research (TLRC), Germany
[d] Section for Translational Research, Thoraxklinik, University of Heidelberg, Roentgenstraße 1, 69126 Heidelberg, Germany
[e] Division of Molecular Thoracic Oncology, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280, 69120 Heidelberg, Germany

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ABSTRACT

Background: The role of cytoreductive surgery for epithelioid pleural mesothelioma within a multimodal treatment approach remains controversial. Carefully selected patients benefit from cytoreductive surgery and adjuvant chemotherapy, but there is no established biomarker to predict tumor recurrence or progression during the course of the disease. The aim of this study was to identify potential biomarkers to predict therapeutic response in terms of progression-free survival.

Methods: Between 03/2014 and 08/2022, preoperative blood samples were collected from 76 patients with epithelioid pleural mesothelioma who underwent cytoreductive surgery as part of a multimodal treatment approach. Identification of potential biomarkers was performed by determination of mesothelin and calretinin, as well as specific long non-coding RNAs and microRNAs. Receiver operating characteristic analysis, Kaplan-Meier survival analysis, and Cox regression were used to assess the association between biomarker concentrations and patient recurrence status and survival.

Results: MALAT1, GAS5, and calretinin showed statistically significant increased biomarker levels in patients with recurrence in contrast to recurrence-free patients after surgical treatment ($p < 0.0001$, $p = 0.0190$, and $p = 0.0068$, respectively). The combination of the three biomarkers resulted in a sensitivity of 68 % and a specificity of 89 %.

Conclusion: MALAT1, GAS5, and calretinin could be potential biomarkers for the prediction of tumor recurrence, improving the benefit from multimodal treatment including cytoreductive surgery.

1. Introduction

Pleural mesothelioma is a fatal cancer of the pleural cavity, primarily caused by asbestos. Overall survival (OS) after diagnosis is poor and the long latency period of the disease after asbestos exposure is responsible for the still increasing incidence of mesothelioma worldwide [1,2]. Several options are available for the treatment of pleural mesothelioma to improve OS, ranging from multimodal therapy including surgery and chemotherapy to chemotherapy or immunotherapy [3,4]. Monitoring the disease progression after treatment is essential, but imaging techniques are of limited value in mesothelioma, in contrast to several other tumors. Therefore, circulating biomarkers reflecting disease progression

* Corresponding author at: Thoraxklinik, University Hospital Heidelberg, Roentgenstraße 1, 69126 Heidelberg, Germany.
E-mail address: Laura.Klotz@med.uni-heidelberg.de (L.V. Klotz).
[1] Authors contributed equally.

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or recurrence may be a complement or alternative to imaging [5,6]. Potential prognostic biomarkers provide information about patient's likely health outcome, such as recurrence, progression-free survival, and overall survival, regardless of the treatment used [7]. Currently, there is no reliable prognostic biomarker that provides information on disease progression and that allows estimation of response to multimodal therapeutic options for locoregional disease [8].

Liquid biopsies facilitate the detection of circulating biomarkers such as proteins, DNA, and RNA – including microRNA (miRNA) and long non-coding RNA (lncRNA) – in body fluids such as urine and plasma [9]. They represent a powerful tool, not only for non– or minimally-invasive diagnosis and screening, but also for prognosis and prediction in cancer patients [10].

Circulating mesothelin, calretinin, miR-132-3p, miR-126-3p, and growth arrest-specific 5 (GAS5) have already been shown to be suitable for differentiating mesothelioma patients from (asbestos-exposed) controls [11–15]. Additionally, metastatic-associated lung adenocarcinoma transcript 1 (MALAT1) and survivin [16,17] are overexpressed in mesothelioma and play a role in its development, representing additional promising candidate biomarkers. However, less is known about the prognostic potential of these biomarkers in mesothelioma patients. Thus, in the current study, the plasma levels of candidate biomarkers were determined and analyzed regarding their association with patient outcome. The combination of different molecular classes was a special focus of this study, as the expected complementary effect might lead to an improved performance of the biomarkers [18].

2. Methods

2.1. Study population

Mesothelioma patients were retrospectively selected from a prospectively maintained database of patients at the Thoraxklinik Heidelberg who underwent cytoreductive surgery for mesothelioma at the clinic between 03/2014 and 08/2022. Patients with biphasic or sarcomatoid mesothelioma and patients without cytoreductive surgery were excluded from further analysis. Neoadjuvant treatment was defined as an exclusion criterion due to possible influence on biomarker measurement. Patients with an OS $>$ 3 months and $<$ 60 months were included in the subsequent analyses. Patients with OS $<$ 3 months were excluded due to death without tumor recurrence. Patients with OS $>$ 60 months were excluded due to potentially relevant differences concerning tumor biology which might influence the definition of biomarkers on tumor progression. Criteria for surgery were defined as no relevant shrinkage of the affected hemithorax, good cardiopulmonary function test according to the guidelines of the European Respiratory Society for mesothelioma, and potentially resectable tumor mass on contrast-enhanced computed tomography [3,19]. Contraindications were relevant cardiopulmonary comorbidities or impaired performance status. A flowchart of the identification of eligible patients for the study is shown in Fig. 1. Blood samples were prospectively collected prior to surgery and stored in a standardized manner in our biobank at the Department of Translational Research as described below. Cytoreductive surgery was performed as previously described [3]. Multimodal treatment included cytoreductive surgery and four cycles of adjuvant chemotherapy with pemetrexed and cisplatin or carboplatin, based on the adjuvant chemotherapy paradigm for non-small cell lung cancer. The initial multimodal treatment approach did not include radiation or immunotherapy.

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). All participants provided written informed consent. The institutional ethics committee of Heidelberg University Hospital approved the collection and analysis of blood samples and patient data (No. S-174/2019).

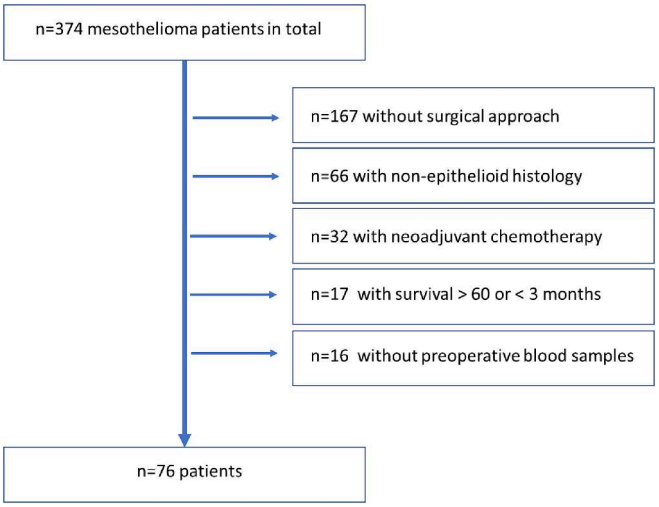

Fig. 1. Flowchart of the identification and selection of eligible mesothelioma patients at Thoraxklinik Heidelberg.

2.2. Blood collection and storage

On the day of admission, additional blood samples were collected during routine clinical peripheral blood draws using EDTA and serum tubes (Sarstedt AG & Co., Nümbrecht, Germany). The tubes were stored upright at room temperature for 15 min and then centrifuged at 2.000 x g for 10 min at 10 °C. Plasma, serum, and buffy coat were then pipetted into 2D cryotubes (LVL Technologies, Crailsheim, Germany) according to a standardized scheme. Samples were registered immediately after processing using the laboratory management software STARLIMS (STARLIMS, Hollywood, FL, United States) and frozen at −80 °C until use.

2.3. RNA isolation

Plasma samples were assayed for free hemoglobin (Hb) prior to RNA isolation by measuring the absorbance at 415 nm (total Hb), 450 nm (bilirubin), and 700 nm (sample turbidity) using a NanoDrop ND-100 spectrophotometer (Thermo Fisher Scientific, Waltham MS, USA). Hb concentrations were quantified using the formula Hb (mg/dl) = 154.7 x A415–130.7 x A450–123.9 x A700 [20,21]. Hemolysis is defined for Hb concentrations $>$ 0.3 ng/ml [22]. Only samples with Hb concentrations below this threshold were used for analysis. RNA was isolated from 0.5 ml plasma samples using the miRVana PARIS kit (Thermo Fisher Scientific) according to the manufacturer's instructions, modified by the addition of 5 µl carrier RNA MS2 (Roche, Mannheim, Germany). Isolated RNA was eluted in 100 µl elution solution and separated into two aliquots. For lncRNA analysis, 50 µl of RNA was treated with DNase using the Turbo DNA free kit (Thermo Fisher Scientific).

2.4. Determination of lncRNAs and microRNAs

Expression analysis of lncRNAs was performed on a Veriti 96-well thermal cycler (Thermo Fisher Scientific) for reverse transcription (RT) and preamplification, and a 7900 HT Fast Real-Time PCR System (Thermo Fisher Scientific) for quantitative real-time PCR (qPCR) using commercial assays (Integrated DNA Technologies, Leuven, Belgium) according to the manufacturer's instructions. In brief, RT was performed in a 20 µl reaction volume with 5 µl RNA as template at 37 °C for 60 min and 95 °C for 5 min. Intermediate preamplification with 14 cycles was performed in 10 µl reaction volume with 2.5 µl cDNA as template at 95 °C for 10 min, followed by 14 cycles of 95 °C for 15 s and 60 °C for 4 min. Finally, qPCR was performed in 20 µl reaction volume with 5 µl DNA at a 1:20 dilution as template at 95 °C for 10 min, followed by 40

cycles of 95 °C for 15 s and 60 °C for 1 min. Reactions were performed in duplicate and non-template controls were included.

Expression analysis of miRNAs was performed using commercial TaqMan microRNA assays (Thermo Fisher Scientific) according to the manufacturer's instruction. Briefly, 5 µl of RNA was used as a template for RT and 5 µl of cDNA as template for subsequent qPCR. RT was performed at 16 °C for 30 min, 42 °C for 30 min, and 85 °C for 5 min and PCR was performed at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All reactions were performed in duplicate using a 7900 HT Real-Time PCR System (Thermo Fisher Scientific) and included non-template controls.

The IDs of the commercially available assays are listed in Supplementary Table S1.

Normalization was performed as described elsewhere [23] using RPLP0 as reference for MALAT1 and GAS5, and miR-146b-5p and U6 snRNA as reference for miR-132-3p and miR-126-3p, respectively.

2.5. Determination of calretinin and mesothelin

Enzyme-linked immunosorbent assays (ELISAs) were used for the determination of calretinin and mesothelin in plasma. For calretinin, the Calretinin ELISA kit (DLD Diagnostika GmbH, Hamburg, Germany) was used according to the manufacturer's instructions with 15 µl plasma. All incubation steps were performed at 22 °C on a temperature-controlled shaker. During the substrate reaction, the temperature was increased to 24 °C. For mesothelin the Human Mesothelin Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA) was used according to the manufacturer's instructions with 15 µl plasma diluted 10-fold with calibrator diluent. All samples were assayed in duplicate. Optical densities were measured using a SpectraMax M3 plate reader (Molecular Devices, Sunnyvale, CA, USA) and standard curves were generated by four-parameter curve fitting using SoftMax Pro 7.0.3 software (Molecular Devices).

2.6. Determination of survivin

Measurement of survivin was performed according to Gleichenhagen et al. [24] with minor modifications. In brief, the proximity probes were prepared according to the manufacturer's instructions. Streptavidin-coated magnetic beads (Dynabeads MyOne Streptavidin T1, Thermo Fisher Scientific) were functionalized by binding of biotinylated anti-survivin antibody, and 5 µl was mixed with 175 µl binding buffer (PBS-BSA 0.05 %) and 20 µl plasma. The mixture was incubated overnight at 4 °C on a rotator with 15 rpm and magnetic beads were isolated using a magnetic stand. The beads were resuspended in 10 µl binding buffer containing 0.1 µl of proximity probes. After incubation, the beads were washed and resuspended in ligation mix according to the manufacturer's instructions. After ligation, the beads were separated and resuspended in 20 µl of qPCR mix from the TaqMan Protein Assay Kit (Thermo Fisher Scientific), and qPCR was performed using a 7900 HT Fast Real-Time PCR System (Thermo Fisher Scientific). A 4-parameter curve fitting was performed using GraphPad Prism, version 9 (GraphPad Software, Inc., San Diego, CA, USA).

2.7. Statistical analyses

Biomarker concentrations were presented as box plots with median and interquartile range (IQR), overlaid with dot plots. Whiskers represent minimum and maximum values. The two-sample Wilcoxon rank-sum test was applied to compare the biomarker distributions according to recurrence status. Sensitivity and specificity were determined using receiver operating characteristic (ROC) curves, which illustrate the performance of biomarkers in discriminating recurrence status. Areas under the curves (AUCs) were calculated with the corresponding 95 % Wald confidence intervals (CI). For the combination of the three biomarkers MALAT1, GAS5, and calretinin, the discriminatory power was calculated iteratively for all possible biomarker combinations with corresponding cutoffs. The sensitivities and specificities thus obtained resulted in a point cloud instead of a conventional ROC curve. Furthermore, it is possible that for one specificity-sensitivity combination, there are several cutoff opinions for the three biomarkers, but only one cutoff option is presented here. The AUC for the biomarker combination was calculated as a descriptive measure for the "best-case" scenario, so that the highest sensitivity per observed specificity was considered in each case. The "worst-case" scenario is given by the lowest sensitivity per observed specificity. The corresponding interval shows the minimum and maximum achievable AUCs. Differences in OS between patients with and without recurrence were evaluated with a logrank test using Kaplan-Meier survival analysis. Kaplan-Meier curves for recurrence-free survival of patients with high and low levels of selected pretherapeutic biomarkers were also evaluated. Cox regression was used to assess the influence of biomarkers on recurrence-free survival. Hazard ratios (HR) and 95 % CIs are given. Because of the exploratory nature of the study, no adjustments were made for multiple comparisons [25]. Statistical analyses were performed using SAS software, version 9.4 (SAS Institute Inc., Cary, NC, USA). Graphs were generated using GraphPad Prism, version 9 (GraphPad Software).

Biomarker values and characteristics of the study population are presented in Supplementary Table S2.

3. Results

The study population comprised 62 male and 14 female patients with epithelioid pleural mesothelioma. The median age at diagnosis was 67 years. All patients underwent cytoreductive surgery as part of a multimodal treatment concept at the Thoraxklinik Heidelberg. The median OS was 27.2 months and at the end of the study, 33 patients were alive (43.3 %) and 27 patients were free of recurrence (35.5 %). Further details on patient characteristics, recurrence, and overall survival are shown in Table 1.

Recurrence of mesothelioma is associated with reduced OS of the patients after surgical treatment (Fig. 2). After 60 months, 96 % of recurrence-free patients (n = 27) are still alive in contrast to 14 % of the

Table 1
Description of the study population.

Characteristics		n (%)
Sex	Men	62 (81.6)
	Women	14 (18.4)
Age [years]	Median (IQR)	67 (62–72.8)
TNM Classification (pT)	1	3 (3.9)
	2	14 (18.4)
	3	54 (71.1)
	4	5 (6.6)
TNM Classification (pN)	0	56 (73.7)
	1	19 (25.0)
	2	1 (1.3)
Tumor stage	IA	2 (2.6)
	IB	50 (65.8)
	II	2 (2.6)
	IIIA	15 (19.7)
	IIIB	6 (7.9)
	IV	1 (1.3)
Survival Status	Alive	33 (43.4)
	Dead	43 (56.6)
Overall survival [months]	Median (IQR)	27.2 (14.4–40.3)
Recurrence-free	Yes	27 (35.5)
	No	49 (64.5)
Local recurrence		41 (83.7)
Distant recurrence		2 (4.1)
Local + distant recurrence		6 (12.2)
Recurrence-free survival [months] (n = 48)*	Median (IQR)	16.1 (10.2–24.3)

*Date of recurrence is missing for one person. TNM: tumor, node, metastasis; p: stage given by histopathologic examination of a surgical specimen. IQR: interquartile range.

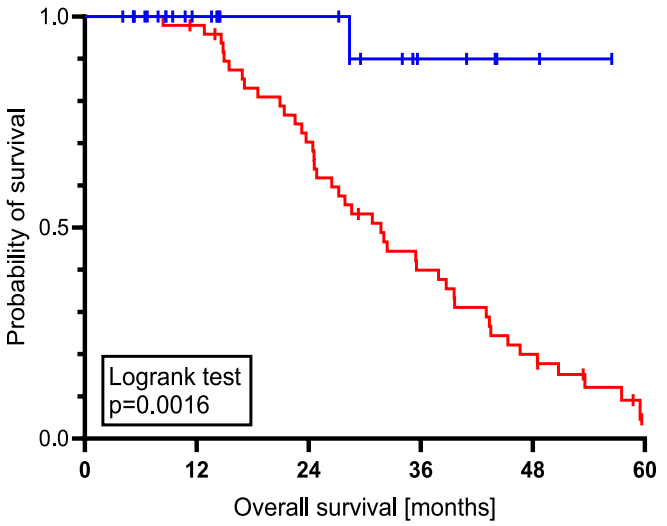

Fig. 2. Overall survival according to recurrence status. Blue: Recurrence-free patients (n = 27), red: patients with recurrence (n = 49). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

patients with recurrence of pleural mesothelioma (n = 49).

MALAT1, GAS5, and calretinin were determined in the plasma of mesothelioma patients and showed statistically significant increased biomarker levels of MALAT1, GAS5, and calretinin ($p < 0.0001$, $p = 0.0190$, and $p = 0.0068$, respectively) in patients with recurrence in contrast to recurrence-free patients (Fig. 3). MALAT1, GAS5, and calretinin did not differ between tumor stages (I and II vs. III and IV), pT stage (1 and 2 vs. 3 and 4), and the presence or absence of metastases (pN = 0 vs. pN = 1 and 2); (data not shown).

ROC analyses were conducted to evaluate the performance of the biomarkers, revealing AUCs of 0.801 (95 % CI 0.702–0.900), 0.664 (95 % CI 0.526–0.802), and 0.689 (95 % CI 0.565–0.812), respectively (Fig. 3). In contrast, mesothelin, survivin, miR-132-3p, and miR-126-3p did not show altered biomarker levels between patients with and without recurrence (Supplementary Table S2). Therefore, these biomarkers were not further analyzed.

When determining recurrence status based on biomarker levels, MALAT1 showed the highest sensitivity of 57 % compared to calretinin (43 %) and GAS5 (15 %) with a pre-specified high specificity of 89 %. The combination of the three biomarkers led to an increased sensitivity of 67 % at a specificity of 89 % (Table 2).

Compared to the single biomarkers, the combination of MALAT1, GAS5, and calretinin resulted in an enhanced AUC of 0.859 in the "best-case" scenario (Fig. 4). The AUC of the "worst-case" scenario was 0.675. For selected specificities (lower x-axis labels), the corresponding sensitivities are given in Fig. 4, e.g., at 70 % specificity a sensitivity of 82 % was observed.

Although the results were not statistically significant, univariate Cox regression models showed that the risk of recurrence increased by 6 % for MALAT1 per ΔCt unit (HR 1.06, 95 % CI 0.89–1.26), by 29 % for GAS5 per ΔCt unit (HR 1.29, 95 % CI 0.98–1.69), and by 10 % for calretinin per 1 ng/mL (HR 1.10, 95 % 0.91–1.33). The Kaplan-Meier curve showed that patients with GAS5 values below the median had a better recurrence-free survival than patients with GAS5 values above the median (Fig. 5 B), but this was statistically not significant due to the crossing survival curves before twelve months. The recurrence-free survival curves showed no differences for MALAT1 (Fig. 5 A) and calretinin (Fig. 5 C) stratified by median biomarker values.

However, the survival for patients with MALAT1 values above the median was better in the first 45 months. Regarding overall survival, on the other hand, MALAT1 and calretinin showed better survival ($p = 0.0503$ and $p = 0.1152$, respectively) for patients with values above the median or below the median, respectively, with crossing survival curves until 18 months (Supplementary Figure S1). Additionally, Kaplan-Meier curves for overall and recurrence-free survival did not differ according to tumor stage, pT, and pN (data not shown).

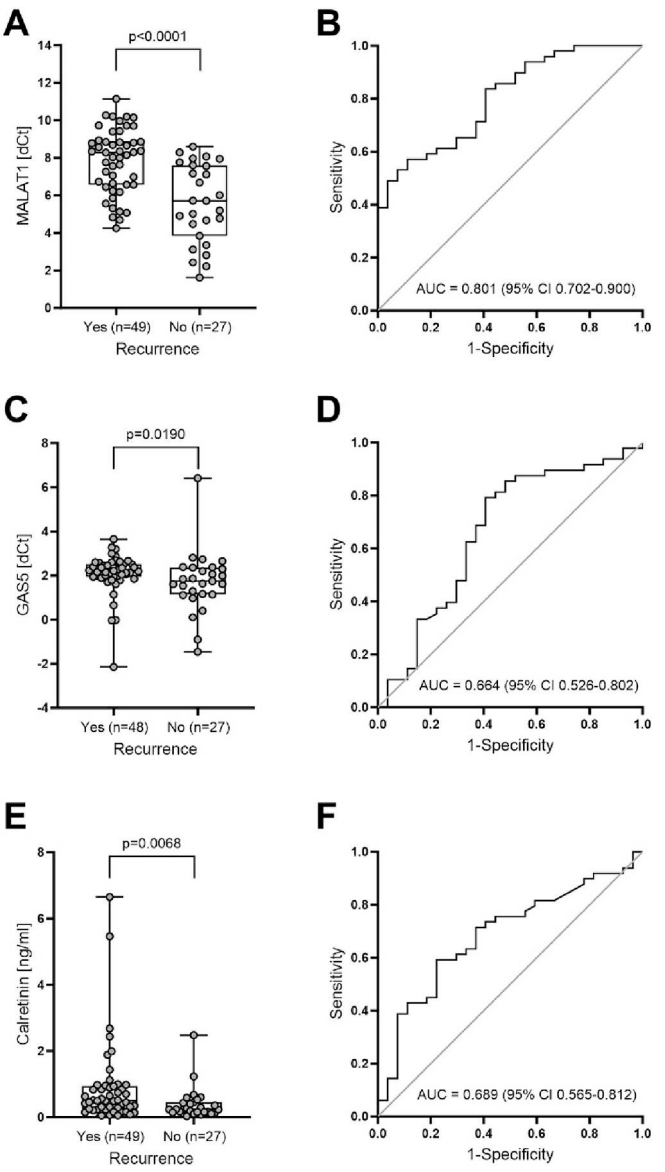

Fig. 3. Boxplots and Receiver Operating Characteristics (ROC) curves of MALAT1 (A and B), GAS5 (C and D), and calretinin (E and F) according to recurrence status. Boxes represent 25th percentile, median, and 75th percentile and whiskers represent the minimum and maximum. P-values (p) of two-sampled Wilcoxon rank-sum tests comparing biomarker distributions according to recurrence status are shown. Area under curve (AUC) values are based on 49 (MALAT1 and calretinin) and 48 (GAS5) patients with recurrence and 27 recurrence-free patients.

4. Discussion

Due to the late onset of symptoms in most patients, the diagnosis of mesothelioma is usually made late. However, mesothelin and calretinin have recently been shown to be useful for early detection of mesothelioma in high-risk individuals [26,27]. In case of early-stage disease, but also in general, an appropriate multimodal treatment concept for the individual patient is required. Mesothelioma requires an individual treatment concept for each patient. Recently, multimodal treatment

Table 2
Biomarker cutoffs to determine recurrence status (specificity 89%).

Biomarker	Cutoff	TP	TN	FP	FN	Sensitivity [%]	Specificity [%]
MALAT1 [ΔCt]	> 7.997	28	24	3	21	57.1	88.9
GAS5 [ΔCt]	> 2.670	7	24	3	41	14.6	88.9
Calretinin [ng/ml]	> 0.630	21	24	3	28	42.9	88.9
Combination*							
MALAT1 [ΔCt]	> 8.627	33	24	3	16	67.3	88.9
GAS5 [ΔCt]	> 1.879						
Calretinin [ng/ml]	> 0.411						

*Detection of recurrence is assumed if at least two of three biomarkers are positive. TP: True positive values, TN: True negative values, FP: False positive values, FN: False negative values.

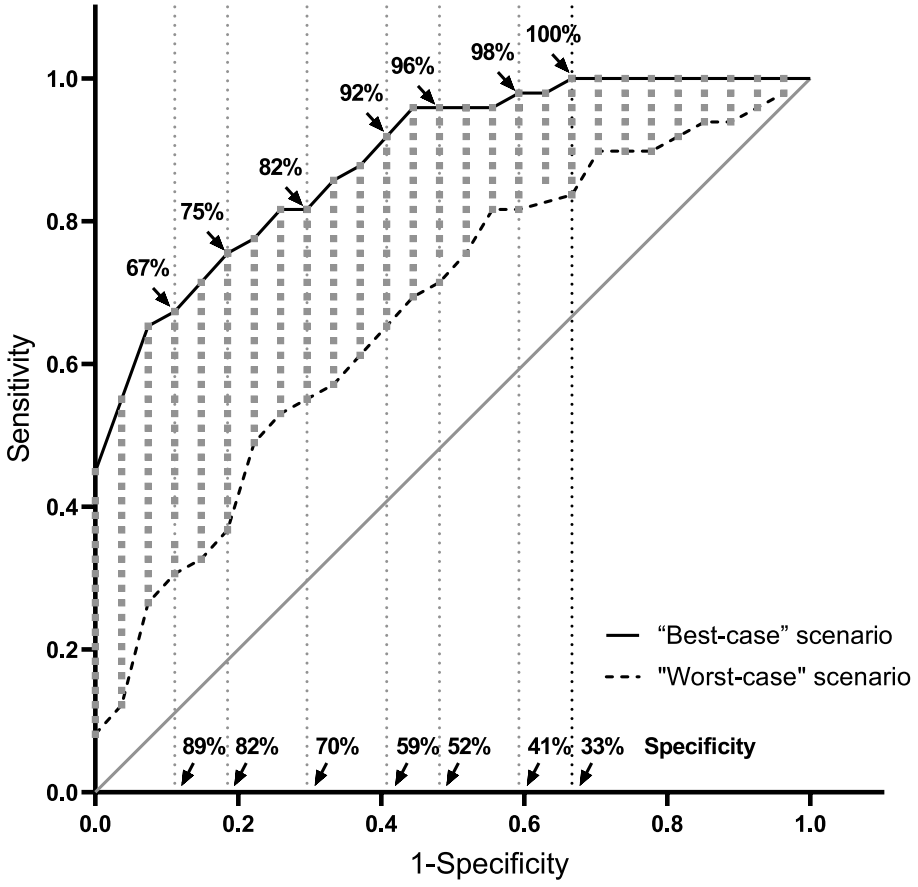

Fig. 4. ROC curve of the biomarker combination including MALAT1, GAS5, and calretinin presenting "best-case" and "worst-case" scenarios to determine recurrence status. The percentages shown on the "best-case" ROC curve are sensitivities associated with the corresponding specificities as given on the dashed vertical lines.

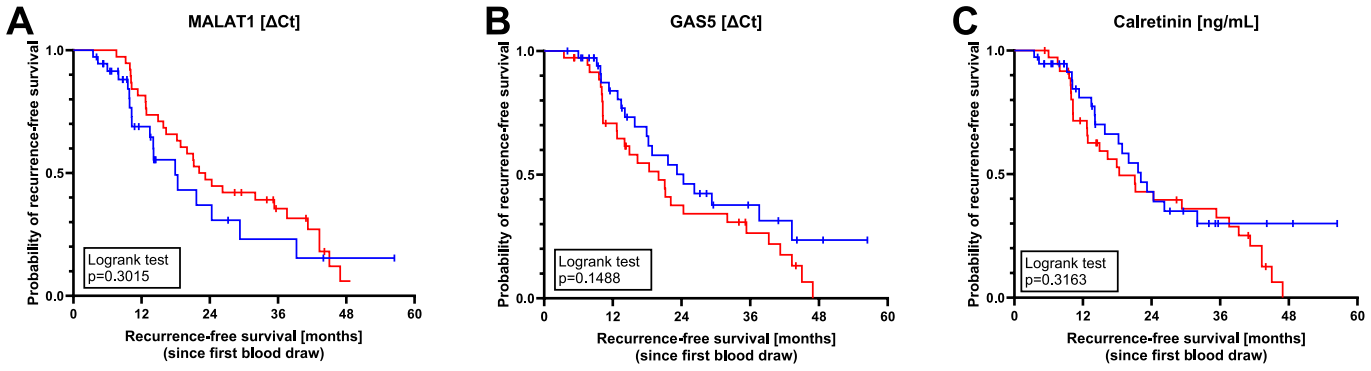

Fig. 5. Recurrence-free survival as a function of (A) MALAT1, (B) GAS5, and (C) calretinin values with values ≤ median (blue) or values > median (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

concepts that also include surgery have been critically discussed [28]. The MARS2 trial compared patients receiving chemotherapy with those receiving chemotherapy plus cytoreductive surgery. However, there are some relevant criticisms of this randomized, multicenter study. In addition to the administration of various systemic therapies and different number of cycles, including immunotherapy, patients were not critically selected for cytoreductive surgery. In addition to the inclusion of all histological subtypes, patients with an advanced tumor stage were also randomized to surgery. As a result, the study does not represent the real-life outcomes of a multimodally treated cohort of selected patients with epithelioid pleural mesothelioma.

Decisive and critical patient selection is of central importance to improve overall survival after multimodal treatment [29,30,3]. In addition, a clinical trial is investigating the ideal constellation of multimodal treatment with regard to the ideal treatment sequence of neoadjuvant or adjuvant chemotherapy in combination with cytoreductive surgery [31].

The recurrence of mesothelioma affects most patients, even after multimodal treatment [32,33]. However, in this work, 96 % of recurrence-free patients remained alive up to 60 months after surgical treatment. The improved survival was due to the multimodal treatment as described by Klotz et al. [3]. Nevertheless, the follow-up of mesothelioma patients after treatment remains a challenge as the assessment of tumor recurrence based on imaging techniques is complicated by unique growth patterns and postoperative changes [32]. Therefore, the evaluation of circulating biomarkers could be a useful alternative to assess disease progression after treatment and provide insight into appropriate patient follow-up for tumor recurrence [6,32].

In this study, we analyzed plasma samples and the corresponding clinical data of patients with cytoreductive, lung-sparing surgery and adjuvant chemotherapy for epithelioid pleural mesothelioma for relevant biomarkers of disease progression after multimodality treatment.

For use in clinical practice, biomarkers must be sufficiently robust. Several factors such as gender and sample matrix can impact marker concentrations. Calretinin is widely used in antibody panels for clinical diagnosis by immunohistochemistry and is one of the best immunohistochemical markers for the diagnosis of mesothelioma [26]. The combination of the biomarkers MALAT1, GAS5, and calretinin showed promising results with high sensitivity and specificity for predicting disease progression of epithelioid pleural mesothelioma after surgical treatment, taking into account that biomarkers for mesothelioma should have either sensitivity or specificity > 80 % [34]. Elevated levels of these biomarkers were associated with an increased incidence of disease recurrence. Comparable results were observed by Bogar et al. analyzing mesothelin, midkine, and sestrin 1 in patients after chemotherapy, showing increased biomarker levels in patients with disease recurrence [35]. In addition, Nakamura et al. observed that positive biomarker levels of mesothelin, CYFRA21-1, and TPA were associated with a high likelihood of disease recurrence in patients who underwent surgery for mesothelioma after induction chemotherapy [32]. In contrast to these two studies mentioned above, mesothelin could not be confirmed as a prognostic biomarker in this study. However, the results on mesothelin remain inconsistent and criteria for increasing or decreasing mesothelin levels have not yet been developed [35]. A detailed comparison between the studies is also difficult due to heterogeneous and relatively small study groups as well as different therapeutic treatment regimens. In addition to the best-known biomarker mesothelin, calretinin is also an established biomarker for mesothelioma [14,36]. Recently, Zupanc et al. evaluated serum calretinin as a predictive biomarker for mesothelioma, showing that calretinin is associated with OS and chemotherapy treatment outcome [37]. Similarly, the combination of the immunohistochemical biomarkers calretinin, BAP1, and WT1 is appropriate to predict OS and response to chemotherapy [38]. In addition, Link et al. indicated that elevated calretinin levels were associated with worse survival and increased risk of tumor recurrence in ovarian cancer after chemotherapy or surgery [39]. Thus, in addition to its usefulness as a biomarker for the diagnosis [14,36] and early detection of mesothelioma [26,27], calretinin could play an additional role as a prognostic and predictive biomarker.

In addition to these proteins, the inclusion of other molecular classes in a biomarker panel could be useful, as they serve as an additional source of information about the patient's health status. In particular, lncRNAs could serve as biomarkers for diagnosis and prognosis because they can be accurately detected [40]. Currently, the expression level of lncRNAs is used for clinical diagnosis of various tumor entities [41]. MALAT1 and GAS5 are well-described lncRNAs that are dysregulated in a variety of cancer types and could be promising candidates for diagnosis [42,43]. In contrast, less is known about the potential role of lncRNAs to predict pleural mesothelioma growth. We have previously shown that GAS5 may have an impact as a complementary biomarker for the detection of mesothelioma [11]. In this study, an association between increased GAS5 levels and decreased recurrence-free survival was observed, but the results were not statistically significant. However, Kresoja-Rakic et al. indicated that decreased GAS5 levels were associated with shorter survival and shorter progression-free intervals in patients treated with neoadjuvant chemotherapy and surgery [44]. Again, the differences could be due to heterogenous and relatively small study groups as well as different therapeutic treatments. As far as we are aware, MALAT1 was analyzed for the first time as a prognostic biomarker in mesothelioma. This showed a correlation between elevated levels with reduced recurrence-free survival, although the observation was not statistically significant. In other tumors, MALAT1 was shown to have no significant association with recurrence of non-small cell lung cancer or bladder cancer [45,46].

The role and evidence of miRNA and lncRNAs as potential biomarkers and therapeutic targets for mesothelioma have been repeatedly discussed in the literature [47]. In recent years, miRNAs have shown their potential to serve as biomarkers in mesothelioma diagnostics [48]. Circulating miRNAs from blood samples of mesothelioma patients, asbestos workers, and healthy individuals have been analyzed to identify relevant miRNAs upregulated in mesothelioma patients [49]. Due to their role in a variety of cellular processes, they appear to have promising potential for diagnostic and therapeutic implications [11,44]. For example, Kresoja-Rakic et al. analyzed the role of miR-625–3 and GAS5 as prognostic biomarkers for mesothelioma patients treated as part of a multimodal treatment concept including extrapleural pneumonectomy. While lncRNAs appeared to have prognostic value in our study the included miRNA candidates did not reach statistical significance.

Our results underline the usefulness of the investigated proteins and lncRNAs as circulating prognostic biomarkers. However, the results are based on small numbers and should be confirmed in larger study groups. The sensitivity of 68 % with a specificity of 89 % shows the benefit of the biomarker combination, especially the combination of different classes of molecules, but leaves room for improvement. Therefore, it may be useful to combine the newly identified biomarkers with other biomarkers, e.g., midkine, sestrin 1, CYFRA21-1, and TPA, to determine whether any synergistic effects could lead to an improved biomarker panel. The performance of the current biomarker panel needs to be optimized before it can be considered for routine clinical use.

In summary, altered biomarker levels in preoperative plasma samples may be a useful tool to identify patients at increased risk of tumor recurrence or progression. Consequently, these patients may not benefit from a multimodal treatment approach including cytoreductive surgery or the treatment needs to be intensified in randomized studies. The combination of MALAT1, GAS5, and calretinin seems to be a promising approach to overcome the limitations of current imaging modalities with a minimally invasive blood test.

CRediT authorship contribution statement

Laura V. Klotz: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Data curation,

Conceptualization. **Swaantje Casjens:** Writing – review & editing, Visualization, Validation, Formal analysis, Data curation. **Georg Johnen:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Dirk Taeger:** Writing – review & editing, Writing – original draft, Visualization, Formal analysis. **Alexander Brik:** Writing – review & editing, Methodology, Formal analysis. **Florian Eichhorn:** Writing – review & editing, Data curation. **Laura Förster:** Data curation. **Nina Kaiser:** Methodology, Data curation. **Thomas Muley:** Writing – review & editing, Supervision, Resources, Methodology. **Christa Stolp:** Methodology, Data curation. **Marc Schneider:** Methodology, Data curation. **Jan Gleichenhagen:** Writing – review & editing, Validation, Methodology. **Thomas Brüning:** Writing – review & editing, Supervision, Resources, Methodology. **Hauke Winter:** Writing – review & editing, Supervision, Resources. **Martin Eichhorn:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Daniel G. Weber:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The IPA has supplied DLD Diagnostika GmbH with the antibodies for the production of the Calretinin ELISA kits. In return, the IPA has received Calretinin ELISA kits at a reduced price and may benefit from future sales of the kits. Otherwise, the individual authors declare any competing interests.

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Appendix A. Supplementary data

Appendix 1, Table S1**:** IDs of the commercially available assays. **Appendix 1,** Table S2**:** Distribution of biomarker values stratified by recurrence status. **Appendix 1,** Figure S1**:** Kaplan-Meier curves for overall survival regarding MALAT1, GAS5, and calretinin with biomarker values $\leq$ median or values $>$ median. **Appendix 2:** Data values and patients' characteristics. Supplementary data to this article can be found online at https://doi.org/10.1016/j.lungcan.2024.107802.

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