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# Novel methods to diagnose leptomeningeal metastases in breast cancer

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**Abstract**

Leptomeningeal metastases (LM) in breast cancer patients are rare but often accompanied by devastating neurological symptoms and carry a very poor prognosis, even if treated. To date, two diagnostic methods are clinically used to diagnose LM: gadolinium MRI of the brain and/or spinal cord and cytological examination of cerebrospinal fluid (CSF). Both techniques are, however, hampered by limited sensitivities, often leading to a long diagnostic process requiring repeated lumbar punctures and MRI examinations. To improve the detection rate of LM, numerous studies have assessed new techniques. In this review, we present the current workup to diagnose LM, set out an overview of novel techniques to diagnose LM, and give recommendations for future research.

**Keywords**

biomarkers | breast cancer | cerebrospinal fluid | leptomeningeal metastases

Leptomeningeal metastases (LM) are caused by tumor involvement of the leptomeninges, and its occurrence is often accompanied by devastating symptomatology. Malignant cells can invade the leptomeninges through different ways, including hematogenous spread or direct infiltration from (para)vertebral metastases or any other metastasis in close contact with cerebrospinal fluid (CSF). Once shed into the CSF, cancer cells may float along CSF pathways to other areas of the nervous system where they may settle and grow.[1] Both solid as well as hematological malignancies can give rise to LM.

Breast cancer (BC) is one of the most common solid malignancies that metastasize to the leptomeninges, accounting for 19–36% of all LM cases.[2–5] BC is a heterogeneous disease comprising several molecular subtypes, differing between each other in natural course, molecular background, and sensitivity to antitumor treatments. Among BC patients with LM, 23–40% have a triple negative subtype, 35–46% are estrogen receptor positive/human epidermal growth factor receptor 2 (HER2) negative, and 22–28% have HER2-positive disease.[6–8] In addition, in postmortem case series, LM were found in 12–16% of the patients with lobular carcinoma compared with only 0.3–5% of patients with ductal carcinoma.[9,10] During the past decades the incidence of LM has seemed to increase, probably as a result of higher success rates of systemic treatments, resulting in more patients achieving long-term survival, allowing LM to develop.

The prognosis of BC patients with symptomatic untreated LM is dismal, with a median survival time of 4–8 weeks.[5] Currently, there is no consensus for choice of treatment for these patients. Treatment options consist of radiotherapy of clinically symptomatic areas and systemic and/or intrathecal delivered chemotherapy, improving the median overall survival to 3–8 months.[6,8,11,12] An explanation for the dismal prognosis of BC patients with LM could be the delay in diagnosing LM. This is due both to the frequently discrete symptoms at presentation and to the limited sensitivity of the currently available diagnostic techniques, especially early on in the development of LM. Consequently, once diagnosed, patients often have a poor clinical condition, resulting in an impaired tolerance of systemic treatment or even worse, the inability of starting treatment at all.

Currently, cytological identification of malignant cells in CSF is the gold standard for diagnosing LM.[3] Although this technique has a high specificity (>95%), the sensitivity is only 45–75% at initial CSF examination and increases to 64–84% after a second CSF examination.[2,3,13,14] Clearly, improvement of the diagnostic workup for suspicion of LM is needed. In this review we summarize the current diagnostic workup to diagnose LM, and different methods that have been investigated over the years to detect LM with a particular focus on patients with BC. Of note, these data are to a great extent generalizable and relevant for LM from other solid malignancies.

## Clinically Available Diagnostic Techniques

### CSF: General Laboratory Assessments

Nearly all patients with LM have some kind of abnormality in their CSF, including elevated opening pressure (30–57%), elevated leukocyte counts (44–57%), increased protein concentration (74–86%), and decreased glucose concentration (31–56%).[2,13,14] Nevertheless, none of these are pathognomonic for LM.

### CSF: Pathology

After obtaining CSF by lumbar puncture (LP), a cytospin is made and stained with May-Grünwald Giemsa. A positive cytology result is defined as the presence of tumor cells in CSF. For BC, stainings as pan-cytokeratine, estrogen receptor and progesterone receptor can be helpful to confirm LM.

The diagnostic value of a positive CSF cytology has been evaluated by comparing premortem CSF examinations of patients diagnosed with cancer with autopsy results. The presence of malignant cells as diagnosed by cytology in the CSF was in 96% confirmed by leptomeningeal involvement at autopsy. In this study false positives, defined as no pathological evidence of LM at autopsy, were rare: only 5 of 117 CSF examinations concerning 4 patients with a hematological malignancy and 1 with medulloblastoma.[3] Detection of malignant cells in CSF by cytopathological analysis therefore has a specificity of >95%. In patients with solid tumors, CSF examination showed no false-positive results, indicating an even higher specificity of nearly 100%. In addition, in 42 patients with only parenchymal brain metastases found at autopsy, no tumor cells were found in CSF. However, only 30 of 51 autopsy-proven LM patients had a premortem positive cytology, resulting in a relatively low sensitivity of 59%.[3] The number of analyzed CSF samples per patient was not indicated.

To improve the sensitivity of CSF cytology, several recommendations have been made, including[15]: analyzing a large volume (ideally >10.5 mL) of CSF; sampling from a clinically or radiographically suspicious location (ie, LP in case of spinal signs or symptoms and ventricular fluid in case of cranial signs or symptoms); processing CSF immediately after collection; and performing a second or even third CSF sample if the first examination remains negative. Repeated LPs have been shown to increase the sensitivity of CSF cytology by 30%.[2,13,14]

### Gadolinium-Enhanced Magnetic Resonance Imaging

In addition to CSF cytology, gadolinium-enhanced magnetic resonance imaging (Gd-MRI) of the neuraxis is used to detect LM. Until now, no MRI studies for LM have been compared with autopsy studies and due to a limited number of studies it remains difficult to appreciate the diagnostic accuracy of Gd-MRI. Reported sensitivities and specificities range 53–80% and 77–93%, respectively.[4,6,11,12,16]

Comparison of T1-weighted Gd-MRI with contrast enhanced computed tomography (CE-CT) in patients with cytologically confirmed LM showed a favorable detection rate for Gd-MRI: 70% versus 36%, and all abnormalities detected by CE-CT were also detected by Gd-MRI.[17] As a consequence, CE-CT should only be considered as a diagnostic tool when an MRI is contraindicated.

## Current Diagnostic Workup for Clinical Suspicion of LM

As recommended in the first edition of the European Association for Neuro-Oncology (EANO)–European Society for Medical Oncology (ESMO) clinical practice guideline for LM, cancer patients with suspicion of LM should undergo Gd-MRI assessment as first-choice evaluation.[18] In patients presenting with typical clinical signs and symptoms of LM, corresponding abnormalities on Gd-MRI are sufficient to diagnose LM without cytological confirmation. Whenever the Gd-MRI results are inconclusive, an LP for CSF cytology is recommended. In case the first CSF examination is negative, a second LP is advised.[14]

### The Role of Biomarkers in CSF

Due to the limited sensitivities of Gd-MRI and cytopathology, together with the urge for earlier diagnosis of LM, there is an unmet need for novel diagnostic tests to improve the detection rate of LM. To achieve that, numerous potential biomarkers have been studied in CSF of diverse tumor types, including BC. To compare and interpret the value of these diagnostic tests, test characteristics such as sensitivity and specificity are necessary, requiring the presence of a gold standard. These comparisons are challenging because diagnostic criteria are not standardized, resulting in different definitions for LM in various publications. Until now, the majority of clinical trials have evaluated diagnostic tests for detection of LM using either positive CSF cytology or a combination of positive CSF analysis, MRI findings, and clinical presentation consistent with LM as reference standard. Definitions used for the diverse studies for LM-positive cases and control groups are among others presented in Table 1; an overview of normal values for these biomarkers is shown in Table 2.

**Table 1** Test characteristics for the detection of leptomeningeal metastases in breast cancer patients by using CSF biomarkers

| Ref. | Method | Number of Patients with LM from BC versus LM from all tumor types | Biomarker | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) | Definition of LM | Definition Control Group (no. of patients) |
|---|---|---|---|---|---|---|---|---|---|
| **Pro-angiogenic proteins** | | | | | | | | | |
| Stockhammer et al[19] | ELISA VEGF | 4 out of 11 | VEGF | NA | NA | NA | NA | Clinical signs, MRI findings, and positive CSF cytology | I: Pts with solid malignancies without LM (16)<br>II: Pts with infectious meningitis (35)<br>III: Pts with nonmalignant and noninfectious diseases (100) |
| Corsini et al[20] | ELISA VEGF | 11 out of 18 | VEGF index (cutoff 10) | 83.3 | 88.4 | NA | NA | Positive CSF cytology | Non-malignant neurological diseases: inflammatory (33), neurodegenerative (17) |
| Herrlinger et al[21] | ELISA VEGF | 5 out of 37 | VEGF (cutoff 100 pg/mL)<br>VEGF (cutoff 250 pg/mL) | 73<br>51.4 | 93<br>98.3 | 77.1<br>90.5 | 91.5<br>86.3 | Positive CSF cytology, or contrast-enhancing subarachnoid tumor cell deposits on MRI or both | Non-malignant neurological disease (50), multiple sclerosis (28), presumed CNS infectious disease (37) |
| Reijneveld et al[22] | ELISA VEGF | 31 out of 53 | VEGF | NA | NA | NA | NA | Clinical features compatible with LM and positive CSF cytology | I:Pts with malignancies without LM (negative CSF and no signs of LM or brain metastases on imaging) (18)<br>II: No malignancy (no systemic malignancy, no CNS infection, no signs of LM or brain metastases on imaging) (25) |
| Langerijt, van de et al[23] | ELISA VEGF, tPA | 9 out of 19 | VEGF index<br>tPA index<br>VEGF + tPA | 54[a]/*62*[b]<br>85[a]/*85*[b]<br>100 | 72[a]/*71*[b]<br>79[a]/*96*[b]<br>73 | 54 | 100 | Positive CSF cytology and/or enhancement of leptomeninges on MRI | I: Pts with solid malignancy (negative cytology and MRI) (54) = [a]<br>II: Infectious meningitis: viral (16), bacterial (16), nonmalignant and noninfectious neurologic disorders (27) = [b] |

**Table 1** Continued

| Ref. | Method | Number of Patients with LM from BC versus LM from all tumor types | Biomarker | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) | Definition of LM | Definition Control Group (no. of patients) |
|---|---|---|---|---|---|---|---|---|---|
| Groves et al[24] | ELISA VEGF | 8 out of 22 | VEGF (all tumor types)<br>VEFG (BC) | 71<br>75 | 97<br>97 | 88<br>86 | 91<br>94 | Positive CSF cytology | I: BC (33), lung (25), melanoma (9) pts suspected for LM with negative CSF cytology.<br>II: No disease at all (1) |
| **CK-BB, LDH, β2-microglobulin** | | | | | | | | | |
| Bach et al[25] | CK-BB bio-luminescence assay | 12 out of 12 | CK-BB (cutoff 0.2 U/l)<br>LDH (cutoff 80 U/l) | 83<br>50 | 87<br>77 | 60<br>38 | 96<br>92 | Positive CSF cytology or LM at autopsy less than 1 month after assayed CSF | I: BC pts with brain metastases (18).<br>II: BC pts without CNS metastases (34) |
| Twijnstra et al[26] | LDH with ACA method | 15 out of 15 | β2-microglobulin (cutoff >2.2 mg/l)<br>LDH (cutoff >26 U/l) | 60<br>60 | 88<br>93 | NA<br>NA | NA<br>NA | Positive CSF cytology (13) or LM at autopsy (2) | Neurological disorders without CNS metastases (16) |
| Twijnstra et al[27] | LDH with ACA method | 15 out of 34 | LDH (cutoff >26 U/l) | 79 | 83 | 33 | 83 | Positive CSF cytology (32) or LM at autopsy (2) | I: Pts with solid tumors (66), hematological tumors (10), benign primary CNS tumors (7), malignant primary CNS tumors (10), others (112)<br>II: Controls (110) |
| **CA15.3, CEA** | | | | | | | | | |
| Le Rhun et al[28] | CSF CA15.3 Automatized immuno-enzymatic technology | 20 out of 20 | CA 15.3 (cutoff 3 UI/mL) | 80 | 70 | NA | NA | Positive CSF cytology or clinical signs in combination with positive MRI | I: LM from other cancers than BC (20) (group 2).<br>II: BC pts with brain metastases only (20) (group 3).<br>III: No malignancy (20) (group 4) |
| Yap et al[29] | Triple-isotope double antibody method | 23 out of 23 | CEA (cutoff 1.5 ng/mL) | 70 | 100 | NA | NA | Positive CSF cytology and absence of parenchymal brain metastases on CT | Stage IV BC pts and no evidence of metastatic CNS disease (10) |
| Corsini et al[20] | CEA, CA15.3, CA125, CA19.9 (Modular Analytics SWA) | 11 out of 18 | Intrathecal synthesis of CEA/CA 15.3/CA125/ CA19.9 | 100 | 100 | NA | NA | Positive CSF cytology | Nonmalignant neurological diseases: inflammatory (33), neurodegenerative (17) |
| Twijnstra et al[26] | CEA | 15 out of 15 | CEA (cutoff >4 ng/mL) | 60 | 93 | - | - | Positive CSF cytology or LM at autopsy | Neurological disorders without CNS metastases (16) |

**Table 1** Continued

| Ref. | Method | Number of Patients with LM from BC versus LM from all tumor types | Biomarker | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) | Definition of LM | Definition Control Group (no. of patients) |
|---|---|---|---|---|---|---|---|---|---|
| **Proteomics** | | | | | | | | | |
| Dekker et al[30] | MALDI-TOF MS | 54 out of 54 | | 79 | 76 | NA | NA | Positive CSF cytology or a compatible neurological syndrome and diagnostic MRI | I: BC pts with negative cytology and follow-up incompatible with LM (54).<br>II: No cancer and no neurological disease |
| **EpCAM-based assays** | | | | | | | | | |
| Nayak et al[31] | CellSearch | 7 out of 15 | Cutoff: presence of CSFTC | 100 | 97.2 | 93.8 | 100 | Positive CSF cytology or positive MRI findings observed within 1 month of the initial evaluation | I: Pts with clinical suspicion of LM (36).<br>II: Nonmalignant neurologic disease (9) |
| Lee et al[32] | CellSearch | 18 out of 18 | Cutoff >1.9 cells/mL | 81 | 85 | NA | NA | Positive CSF cytology | I: Non-malignant neurologic disease (14).<br>II: BC pts with negative cytology (20) |
| Lin et al[33] | CellSearch | 14 out of 30 | Cutoff ≥1 cell/mL | 93 | 95 | 90 | 97 | Positive CSF cytology or unequivocal MRI findings performed within a month of the CSF analysis. Unequivocal MRI findings defined as: LM enhancement associated with subarchnoid nodules, basal cistern enhancement, or nerve root enhancement/ clumping. | Cancer pts with clinical suspicion of LM without LM confirmation (65) |
| Kerklaan et al[34] | EpCAM FCI | 7 out of 13 | Cutoff ≥2 CSFTC/ 5 mL CSF | 100 | 100 | NA | NA | Positive CSF cytology or a MRI with positive MRI or progressive neurological symptoms compatible with LM and exclusion of other causes | Cancer pts with clinical suspicion of LM without LM confirmation (16) |
| Subirá et al[35] | EpCAM FCI | 32 out of 49 | Cutoff 10 clustered events | 75.5 | 96.1 | 97.4 | 67.6 | Positive CSF cytology or the combination of clinical signs and either MRI or biochemical CSF findings | Cancer pts with clinical suspicion of LM in whom LM was excluded (26) |
| Subirá et al[36] | EpCAM FCI | 39 out of 94 | Cutoff 16 clustered events | 79.8 | 84 | 90.36 | 68.85 | Positive CSF cytology and/ or compatible clinical signs plus MRI findings with biochemical CSF abnormalities | Cancer pts with clinical suspicion of LM in whom LM was excluded (50) |

**Table 1** Continued

| Ref. | Method | Number of Patients with LM from BC versus LM from all tumor types | Biomarker | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) | Definition of LM | Definition Control Group (no. of patients) |
|---|---|---|---|---|---|---|---|---|---|
| **ctDNA** | | | | | | | | | |
| Bougel et al[37] | Methylation of *hTERT* with MS-HRM | 8 out of 9 | Methylation *hTERT* | 92 | 100 | 100 | 95 | Positive CSF cytology | No malignancy (21) |

**Abbreviations:** MS-HRM = methylation-sensitive high resolution melting; NA = not available; pts = patients.
[a]Sensitivity and specificity to discriminate LM from LM negatives.
[b]Sensitivity and specificity to discriminate LM from control patients.

## Pro-angiogenic Proteins: VEGF, uPA, tPA, TGFβ

Angiogenesis, the process leading to the formation of new blood vessels from preexisting vasculature, plays an important role in tumor growth, invasion, and metastasis formation.[41] Vascular endothelial growth factor (VEGF), urokinase-type plasminogen activator (uPA), tissue-type plasminogen activator (tPA), and transforming growth factor–beta (TGFβ) are involved in angiogenesis and have been evaluated as biomarkers for detection of LM.[23]

Stockhammer et al[19] measured VEGF levels using enzyme-linked immunosorbent assay (ELISA) in CSF and matched serum from 11 patients with solid malignant tumors, including 4 patients with BC and cytology- or MRI-proven LM. In this small study, all 11 patients with LM showed high VEGF levels in CSF (median 6795 pg/mL, range 745–18 791 pg/mL), compared with matched serum (median 438 pg/mL, range 47–580 pg/mL). Patients with LM had significantly higher CSF VEGF concentrations than patients with bacterial meningitis (median 38 pg/mL, range <25–633 pg/mL; $P < 0.001$). In patients with brain metastasis without LM, VEGF levels were undetectable. To discriminate between intrathecal VEGF production and passive influx of VEGF from blood, a VEGF index was calculated. Higher VEGF indices were found in LM patients, suggesting local VEGF production or (less likely) active import. These data were supported by the study by Corsini et al[20] in which 15/18 (83%) patients with cytology-proven LM had an increased VEGF index compared with only 3/26 patients with nonmalignant neurologic diseases resulting in a specificity of 88%.

Thereafter, 4 studies confirmed increased CSF VEGF levels or VEGF indices in patients with LM, with 3 studies reporting sensitivities of 51–75%.[21–24] Reijneveld et al[22] found besides increased VEGF CSF levels also higher uPA CSF levels in patients with LM. In another prospective study, paired serum and CSF from patients with metastatic disease with and without LM were collected, from patients with bacterial and viral meningitis and a control group of patients with nonmalignant neurologic diseases.[23] VEGF, uPA, tPA, and TGFβ1 indices were calculated. Although the VEGF concentration was significantly higher in CSF of patients with LM than in all other groups, the VEGF index was not significantly different between groups. In contrast, the tPA index was significantly decreased in LM compared with other groups ($P < 0.01$), whereas uPA and TGFβ1 CSF indices showed no differences between groups.

Based on these studies, we can conclude that VEGF CSF levels are increased in patients with LM; however, the threshold for increased VEGF differs between the various studies. In addition, the sensitivity of VEGF in CSF does not improve the sensitivity of cytology and therefore is not promising enough to replace cytology.

## Enzymes: CK-BB and LDH

Creatine kinase BB (CK-BB) is one of 3 isoenzymes of creatine kinase that reversibly catalyzes the conversion of creatine in phosphocreatine, consuming ATP. Since tumor cells have increased cellular activities to meet the demand for their high energy consumption, high cytosol

**Table 2** Normal values of biomarkers in CSF

| Biomarker | Normal Range | Unit | Ref |
|---|---|---|---|
| VEGF | 0–633 | pg/mL | 19,22,23 |
| VEGF index | 12–41 | | 23 |
| TGF-beta | 65–115 | pg/mL | 23 |
| uPA | 41–282 | pg/mL | 22,23 |
| tPA | 122–222 | pg/mL | 23 |
| tPA index | 13–42 | | 23 |
| CK-BB | 0.04–0.19 | U/L | 25 |
| LDH | 0–26 | U/L | 26 |
| β2-microglobulin | 0.65–2.2 | mg/L | 26 |
| CA15.3 | 0–0.3 | IU/mL | 38 |
| CEA | 0.8–4.0 | ng/mL | 26 |
| CSFTC (CellSearch) | Range varies among studies, <1–2 | cells/mL | 32–34 |
| CSFTC (FCI) | Range varies among studies, <10–16 | clustered events | 39,40 |
| ctDNA | No established cutoff. Since ctDNA is tumor specific, it should be undetectable in healthy controls. | | |

concentrations of CK-BB have been measured.[42] These increased CK-BB levels in tumor cells lead to the hypothesis that CK-BB levels in CSF of patients with LM may also be elevated. Bach et al[25] measured CK-BB in CSF of BC patients suspected of having central nervous system (CNS) metastases. Elevated CK-BB levels (cutoff 0.20 U/L) in CSF were reported in 83% of BC patients with LM; however, levels were also elevated in 39% of BC patients with parenchymal brain metastases but no LM. In a companion study, CK-BB levels in CSF of BC patients without LM were significantly lower compared with patients with CNS metastases (median 0.12 U/L vs 0.42 U/L, $P < 0.001$).[43] From these studies, it can be concluded that elevated CK-BB could be an indicator of CNS metastases; however, it seems unsuitable to distinguish between LM from parenchymal metastases.

Lactate dehydrogenase (LDH) is an enzyme reversibly catalyzing lactate into pyruvate by converting NAD+ into NADH. The normal range of total CSF LDH level is 0–26 U/L.[44] Increased LDH levels in CSF have been reported in numerous conditions, including cerebrovascular accidents, infectious meningitis, acute brain injury, primary CNS tumors, CNS metastases, and LM of solid and hematological tumors.[14,26,27,44] LDH consists of 5 isoenzymes, expressed at different levels in various regions of the brain. In normal brain tissue, particularly aerobically active isoenzymes such as LDH-1 and LDH-2 are expressed.[45] Malignant cells, which are more dependent on anaerobic glycolysis, have a preponderance for anaerobically active LDH-4 and LDH-5 enzymes.[46] However, quantification of total LDH levels in CSF did not distinguish patients with LM from patients with bacterial meningitis.[44] In this latter study, determination of isoenzymes was not performed, because increased CSF LDH-4 and LDH-5 levels were already reported in infectious meningitis and therefore are not specific for LM.[45,47] Thus, LDH levels in CSF, even when considering the isoenzymes LDH-4 and LDH-5, are not specific enough to detect LM.

## β2-Microglobulin

β2-microglobulin ($B_2$-m) is a protein, a small subunit of the human leucocyte antigens, present on the surface of all nucleated cells, but particularly expressed on lymphocytes and macrophages. $B_2$-m is shed from cellular membranes, and various (non)malignant conditions lead to detectable $B_2$-m levels in plasma, serum, urine, saliva, amniotic fluid, and CSF.[48,49] Theoretically, high cell membrane turnover rates, as is the case in malignancies, would lead to increased $B_2$-m levels in surrounding fluids. However, only 60% of advanced BC patients with LM defined by either positive CSF cytology or autopsy had increased $B_2$-m CSF levels, which is disappointing.[26]

## CA15.3 and CEA

Cancer antigen 15.3 (CA15.3) is a large transmembrane glycoprotein, produced by normal glandular breast epithelial cells. CA15.3 is often increased in BC but sometimes also in other malignancies, such as lung, pancreatic, colon, ovarian, and prostate cancers, and benign conditions such as liver cirrhosis.[50] Nevertheless, elevated serum levels of CA15.3 are quite specific for BC and could serve as a useful marker for the diagnosis of LM.[51]

Interestingly, Le Rhun et al[28] compared CA15.3 levels in serum and CSF in 4 patient groups: (i) BC patients with LM, (ii) patients with LM from other primary solid malignancies, (iii) BC patients with parenchymal brain metastases without LM, and (iv) women undergoing a diagnostic LP for various nonmalignant neurological indications. LM was defined as positive cytology and/or clinical signs and imaging. Significantly elevated CA15.3 levels in CSF were observed in BC patients with LM (median 8.7 IU/mL, range 0.1–251.0 IU/mL) compared with the other groups (median of patients with brain metastases 0.5 IU/mL, range 0.1–18.5

IU/mL). A cutoff CA 15.3 level of 3.0 IU/mL in CSF resulted in a sensitivity of 80% and a specificity of 70% for detecting LM in BC.

Carcinoembryonic antigen (CEA) is a cell surface glycoprotein involved in cell adhesion. CEA is elevated in colon cancer but also in BC and some benign diseases of the gastrointestinal tract and the liver.[38] Yap et al[29] studied CEA levels in CSF of 23 BC patients with cytologically proven LM who were treated with whole brain irradiation and intrathecal methotrexate. In 16 patients (70%) the CEA level before treatment was above the limit of detection of 1.5 ng/mL and decreased in patients with response and remained elevated in 2 patients without response. No correlation was found between CEA levels in CSF and in serum, suggesting local synthesis of CEA within the CSF of patients with LM.[29] Corsini et al[20] measured the well-known tumor markers CEA, CA15.3, CA125, and CA19.9 in serum and CSF of 18 patients with LM of solid tumors (11 BC patients) and 50 patients with other neurological diseases. Based on Reiber's formula, intrathecal synthesis of the tumor markers was calculated.[20] All patients with LM had intrathecal synthesis for at least one tumor marker, while none of the controls had tumor marker production in CSF. Interestingly, intrathecal synthesis of CEA was observed in 17 of 18 (in 10/11 BC) patients. CA125 and CA19.9 were elevated and intrathecally synthesized in 6 (55%) of the BC patients. For now, limited data are available regarding the sensitivity and specificity of these markers and therefore short-term implementation into the clinic is not expected.

## Proteomics

Multiplex immune assays and mass spectrometry can obtain information on intra- and extracellular protein expression that could be relevant to the biology of LM. Dekker et al[30] developed a matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) mass spectrometry assay, requiring only 20 μL of CSF, to investigate protein expression profiles in CSF of patients with advanced BC with and without LM. Patients were classified into 3 groups: BC patients with (group I, $n = 54$) and without LM (group II, $n = 52$) and control patients without any neurological disease (group III, $n = 52$). The 3 patient groups were discriminated by 164 peptide peaks ($P < 0.1$). After bootstrap validation, a sensitivity of 79% and specificity of 76% to distinguish patients with LM from patients without LM were found. Using this method, it is not possible to identify specific peptides.

To detect the exact masses of the peptides, electrospray Fourier transform ion cyclotron resonance mass spectrometry was performed on a subset of samples of the study by Dekker et al.[52] Using this method, 17 peptides corresponding to 9 proteins were identified. Proteins detected in the samples of patients with LM were mainly related to host–disease interaction, inflammation, and immune defense (serotransferrin, alpha 1-antichymotrypsin, hemopexin, haptoglobin, and transthyretin).

Based on previously obtained in vivo evidence that tumor cell adhesion is crucial for LM progression in mice and that leptomeningeal tumor growth elicits an intrathecal inflammatory response in the CSF, Brandsma et al[35,39] measured a profile of 9 proteins, including adhesion molecules, cytokines, and chemokines, by using a multiplex immunoassay. CSF of patients with cytologically proven LM ($n = 57$), patients with systemic malignancy without LM ($n = 20$), patients with aseptic or viral meningitis ($n = 11$), and patients with (non)neurological diseases ($n = 19$) were analyzed. Significantly higher in patients with LM compared with the control groups were median CSF levels of soluble vascular cell adhesion molecule 1 (sVCAM-1), soluble intercellular adhesion molecule 1 (sICAM-1), interleukin (IL)-8, pulmonary and activation regulated chemokine (PARC), IL-18, and interferon-γ inducible protein (IP-10). Sensitivity and specificity for these markers were not calculated, which makes it hard to appreciate the diagnostic value of these proteins.

In summary, proteomic studies give more insight into the biology of LM—for example, showing that inflammatory proteins do play a role in LM. Despite the fact that unbiased mass spectrometry does elucidate more of the biology of LM, a more well-defined and LM-specific set of proteins is needed before clinical implementation can be considered. Nevertheless, if such a subset of proteins could be identified and determined by techniques that could be swiftly implemented in routine diagnostic workup, protein CSF analyses could be a promising diagnostic tool.

## EpCAM-Based Circulating Tumor Cell Detection

Solid tumors of epithelial origin like BC frequently express epithelial cell adhesion molecule (EpCAM) on the cell surface, allowing for detection and enumeration of these cells using anti-EpCAM antibodies. The most important methods for circulating tumor cell detection and enumeration in peripheral blood are the FDA-approved CellSearch method and EpCAM-based flow cytometry immunophenotyping (FCI) assays. In short, the CellSearch assay uses immunomagnetic enrichment of circulating tumor cells after adding anti-EpCAM ferrofluid to 7.5 mL of peripheral blood. Subsequently, stainings for the nucleus (4′,6′-diamidino-2-phenylindole), cytokeratin (8, 18, and 19) and pan-leukocyte marker CD45 are added, the latter to stain leukocytes which need to be distinguished. Finally, a reviewer counts all cells meeting the criteria for circulating tumor cells.[53]

The other frequently used technique, EpCAM-based fluorescent activated cell sorting (FACS), uses antibodies for anti-EpCAM, anti-CD45 for discrimination of leukocytes, and markers for detection of nucleated cells (Hoechst or DRAQ5).[35]

Until now, 4 studies have used the CellSearch method to detect tumor cells in CSF (CSFTC) of BC patients. Two small pilot studies with patients with BC and LM showed that detection and enumeration of CSFTC are feasible.[39,54]

Nayak et al[31] detected CSFTC in 15/15 patients (8/8 BC patients) with LM defined as positive cytology or clear MRI findings and in 1 patient without LM, resulting in a sensitivity of 100% and specificity of 97.2%. One patient had a false-positive result, developed 6 months after the initial LP evidence of LM on MRI, suggesting that CSFTC detection may have preceded findings on MRI being a very sensitive tool for LM detection.

In a prospective study, Lee et al[32] showed a high correlation (Pearson's $r = 0.94$) between the CellSearch technique

and EpCAM-based FCI in CSF of 38 advanced BC patients suspected or known to have LM. To define the specificity of the CellSearch assay, 14 patients with hematological malignancies were included as controls. Patients with either positive cytology in one of their CSF samples or unequivocal MRI signs were considered having LM. With a cutoff of ≥1.9 cell/mL CSF, a sensitivity of 81% and specificity of 85% were reached. Explorative analysis of serial CSFTC levels in 7 patients receiving treatment for LM showed that patients with a decrease and at least one negative CSFTC measurement had longer survival times than patients who did not clear CSFTCs. Recently, the largest CellSearch-based study so far involving 95 patients with solid tumors and clinical suspicion of LM, of whom 36 had BC, showed a high sensitivity of 93% and a high specificity of 95% using a cutoff of ≥1 CSFTC/mL CSF.[33]

Using a cutoff of ≥2 CSFTC/5 mL CSF measured with EpCAM-based FCI, Kerklaan et al[34] demonstrated a sensitivity and specificity of 100% in 13 patients with LM from solid tumors.

Another EpCAM-based FCI study, in 78 patients with carcinomas (44 BC), of whom 49 ultimately had diagnoses of LM based on positive cytology or the combination of clinical signs and either MRI or biochemical CSF findings, found a sensitivity of 75.5% and a specificity of 96.1%, using a cutoff of 10 clustered events.[35]

In a subsequent study of 144 patients with carcinomas, in 94 of whom LM was diagnosed (now using a higher cutoff of 16 events), an even higher sensitivity of 79.8% was found, but with a lower specificity of 84%.[36]

In conclusion, EpCAM-based assays show promising sensitivities in the range of 76–100% and specificities ranging 85–100% and allow for the absolute quantification of cells present in a certain volume of CSF.[31–36] Hence, quantification of CSFTC could be used for disease monitoring and response assessments in addition to the new RANO response criteria for LM.[40,55] Moreover, the lower leukocyte background compared with peripheral blood also allows for more sensitive molecular characterization of the enriched CSFTC. In addition, recent whole exome sequencing efforts have revealed that the molecular profile of brain metastases differs from matched primary tumors.[56] Next, these brain metastases harbored clinically informative alterations, as a homozygous missense mutation in *BRCA2* and an activating *EGFR* (L858R) mutation in 2 patients with BC, which were not detected in their primary tumors.[56] Hence, isolation and molecular characterization of CSFTCs could potentially reveal why these cells give rise to LM and hopefully could lead to targets for therapy.[57]

However, important to stress is that neither technique, CellSearch or FCI, provides cytogenetic proof that EpCAM-positive cells are truly malignant. Another disadvantage of EpCAM-based assays is that not all tumor cells express EpCAM on their cell surface, and subsets of EpCAM-negative tumor cells could be missed.[58] Future studies should focus on the ideal cutoff for CSFTC positivity.

## CSF Circulating Tumor DNA

Another potential diagnostic method for early diagnosis of LM is the analysis of circulating tumor DNA (ctDNA) in CSF. Solid malignant tumors, like BC, shed significant amounts of tumor-specific DNA into the systemic circulation mainly through cellular necrosis or apoptosis. Circulating tumor DNA contains tumor-specific DNA alterations such as somatic mutations, copy number alterations, and epigenetic modifications as methylation, but is present in a background of cell-free DNA derived from normal cells.[59] The challenge for sensitive variant detection in plasma ctDNA is the relative abundance of wild-type DNA derived from normal tissue and leukocytes. Although cell counts in CSF of LM patients are raised in 44–57% of the patients, the amount of leukocytes is still much lower compared with blood.[60] Therefore, the background of contaminating DNA derived from healthy cells may probably be less important, allowing for more sensitive detection of tumor derived alterations in CSF.

To date, only small studies have been performed focusing on detection of cell-free DNA free DNA (cfDNA) in CSF of patients with LM of BC. In CSF, human telomerase reverse transcriptase (*hTERT*) methylation status has been studied in various cancer types, including BC.[37] CSF *hTERT* methylation was detected in 11/12 samples from 9 patients (8 with BC) with a positive CSF according to cytopathology and *hTERT* methylation in the primary tumor, resulting in a sensitivity of 92%. Human *TERT* methylation was not detected in control samples, consisting of inflammatory conditions or viral syndromes. In CSF samples of patients with a suspicious cytological result (insufficient intensity of cell atypia and/or insufficient number of atypical cells), with a corresponding *hTERT* methylated primary tumor, *hTERT* methylation was detected in 17/26 samples. In 10 patients without *hTERT* methylation in the primary tumor, no *hTERT* methylation could be detected in suspicious CSF samples, underscoring that it is essential to know the *hTERT* methylation status of primary tumor in order to report results of CSF analysis based on *hTERT* methylation. A panel targeting the most frequently mutated genes or epigenetic aberrations could overcome the problem of an unknown molecular status of the primary tumor.

In a cohort of metastatic patients, including 6 BC patients with CNS metastases, targeted capture massive parallel sequencing was performed on CNS metastases, CSF, and plasma ctDNA. Genomic alterations were detected in all CSF samples and confirmed in a matching CNS metastasis. In "warm" autopsy materials from a patient with HER2-positive metastatic BC, 3 mutations (*PIK3CB* M819L, *PIK3CB* Q818H, and *AHNAK2* L5292V) were exclusively present in a meningeal lesion and CSF but not in the extracranial metastases or plasma. This may indicate that some CNS derived genomic alterations are exclusively present in CSF and that the genetic landscape of CNS metastases should preferentially be examined in CSF.[61] CSF cytology from an advanced BC patient with clinical suspicion of LM was 3 times negative; however, mutations in *ESR1*, *PTEN*, and *MRPS33* were detected at mutant allelic frequencies ranging 20–50% in CSF. LM was confirmed at autopsy, suggesting that CSF ctDNA assessed with next-generation sequencing techniques could detect LM in a more sensitive way than CSF cytology.

Even though the number of currently available papers on CSF cfDNA is limited, CSF cfDNA seems a promising tool to diagnose CNS metastases. Recently, in patients

with LM from non–small cell lung cancer, driver genes were detected in all 26 CSF samples.[62] Future CSF cfDNA analyses should, based on the research question, either use whole exome sequencing, targeted sequencing panels, or digital PCR assays with or without including analyses of tumor tissue and germline DNA. For the investigation of the presence or absence of tumor DNA in CSF, tumor tissue containing a sufficient percentage of tumor cells should ideally be sequenced for detection of patient-specific genomic alterations. These patient-specific alterations then could be analyzed using a targeted sequencing approach or digital PCR in the suspected CSF sample. If the primary tumor or metastasis is not amenable for analysis or if unbiased genotyping of CSF is preferred, one could apply targeted sequencing panels covering the most frequently mutated genes in BC or less sensitive assays as whole exome sequencing and whole genome copy number analyses. With the advent of unique molecular identifiers in targeted sequencing panels, it is possible to quantify the number of mutated molecules in a certain volume of CSF. Nevertheless, it is important to emphasize that CSF ctDNA in patients with concurrent brain metastases could be derived from both brain metastases and LM, which diminishes the chance that ctDNA can be used to distinguish these conditions but can be of important value to determine actionable targets for therapy and could be used in response assessments.[61,63]

## Conclusions and Recommendations

Many biomarkers have been evaluated in an attempt to improve the diagnostic sensitivity and accuracy for detection of LM. However, the majority of studied biomarkers have not reached a wide clinical use, due to limited specificity, sensitivity, and/or lack of validation. A major problem of the investigation of new biomarkers for the detection of LM is the use of a suboptimal "gold standard" reference such as cytopathology and MRI, as theoretically, autopsy is the ultimate proof of absence or presence of LM. In order to ease the comparison of different biomarker studies, a uniform definition of LM should be used across studies. The EANO-ESMO guideline[18] suggests to use the following definitions: **confirmed** for patients with cytologically or histologically proven LM; **probable** for patients with a history of cancer, without cytological proof but with typical clinical findings and neuroimaging findings as linear contrast enhancement, nodules, or a combination of both MRI findings; **possible** for patients with a history of cancer, without cytological proof, without typical clinical findings but with linear and/or nodular MRI findings or typical clinical findings only; **lack of evidence** for patients without cytological proof, without MRI findings, and without typical clinical signs. Although these criteria and the recently proposed RANO criteria have to be validated in prospective clinical trials, we believe that the use of uniform definitions will allow for better comparability of results from new trials.[18]

In addition to optimizing the LM definition, trials should include control groups to determine the specificity of their assays. Selection of the appropriate control group could be done based on the clinical differential diagnosis. For example, patients with a history of cancer who are suspected of having LM are often not suspected of having bacterial meningitis because the latter can be easily distinguished from LM based on CSF protein, white blood cells, and culture together with clinical signs and symptoms. In patients with an oncological history there is a need for markers which could distinguish patients with brain metastases only, brain metastases in combination with LM, LM only, and no malignant CNS pathology at all. This discrimination is important because this will have clinical implications resulting in different treatment strategies, and these 4 groups should therefore be included in future clinical trials.

New markers will only reach clinical use if they (i) are more sensitive than the currently established diagnostic methods: MRI and cytology; or (ii) do add significantly to the sensitivity of established methods; or (iii) can be used in a quantitative manner instead of being only qualitative, as cytology and MRI, enabling for response evaluation; or (iv) could predict outcome of therapies for LM; and (v) are cost-effective. Applying these criteria to the markers described in this paper—identification and enumeration of CSFTC by EpCAM-based assays and detection of CSF cfDNA—both seem the most promising tumor-specific candidates to detect LM at an earlier stage.

To determine the value of CSFTC and CSF cfDNA in the diagnosis and management of patients with LM of BC, studies with larger numbers of patients have to be performed to validate and standardize these methods. As a consequence of the increasing use of DNA sequencing in the diagnostic field, the implementation of CSF cfDNA analyses will become feasible soon if standard operating procedures (SOPs) become available. To achieve this, we have to determine the optimal way of CSF collection and subsequent DNA isolation before sequencing and to subsequently evaluate the cost-effectiveness of these new tests in diagnostics. These SOPs will be even more important for CSFTC detection, since only some centers have a CellSearch system available and shipment of samples will therefore be required.

In addition, since brain metastases can harbor other genetic alterations than matched primary tumors,[56] studying the genomic profile of CSFTC and ctDNA could lead to better insight into why these cells metastasize to the leptomeninges and could potentially reveal actionable targets for therapy.[62] For now, cytopathology remains the "gold standard," but it is important to gain additional proof for the value of new tumor-specific markers in CSF.

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