Abstract. Background: So far discordant results regarding the significance of tumour cells circulating in peripheral blood (CTCs) of breast cancer (BC) patients have been reported. Our aim was to evaluate the association of indirect CTC detection by amplification of human mammaglobin (hMAM) gene expression with traditional prognostic markers of clinical outcome in BC at the time of diagnosis. Patients and Methods: Peripheral blood samples from 190 patients with invasive and 12 patients with in situ BC, before therapy and/or surgery, from 184 patients with benign breast disease and from 146 healthy volunteers were tested for hMAM expression by a nested reverse transcriptase-polymerase chain reaction (RT-PCR) assay. Correlations between CTCs and age at diagnosis, tumour type and size, grading, lymph node involvement, oestrogen and progesterone receptor status, HER-2/neu expression and Ki-67/MIB-1 labelling index were assessed through the odds ratio (OR) point estimates, considering OR >2.0 or <0.5 as being clinically relevant. ORs and their corresponding 95% confidence limits (95% CL) were obtained by logistic regression analysis. Results: Expression of hMAM was found only in peripheral blood of patients with invasive BC (9.5%) and multivariate logistic regression analysis indicated its association with lymph node involvement (pN1-pN3 vs. pN0, OR=5.6, 95% CL=1.4-22.6; p=0.009), tumour size (pT2-pT4 vs. pT1, OR=2.3, 95% CL=0.6-9.0; p=0.207) and negative ER status (OR=2.5, 95% CL=0.6-10.0; p=0.227). Conclusion: Our data show that CTC detection in invasive BC at the time of diagnosis is associated with poor prognosis and may also be used as an additional prognostic indicator.

Breast cancer (BC) has been shown to shed malignant cells into peripheral blood (PB) at the earliest stages of primary development (1). Circulating tumour cells (CTCs) are potentially able to form metastases (2) and may be regarded as valuable predictors of poor clinical outcome, being associated with shorter disease-free and overall survival (3-6). In addition, CTC levels may be useful for the follow-up evaluation and monitoring of the therapeutic efficacy (3-6). However, although numerous studies suggest the promising role of CTCs in routine management of BC patients, their clinical implication is still under investigation.

Among the numerous molecular markers being currently investigated for the indirect detection of occult micrometastasis in BC patients, mRNA expression of the human mammaglobin (hMAM) gene is considered one of the most promising due to its high sensitivity and specificity (7). The hMAM gene, a member of the uteroglobin gene family mapping to human chromosome 11q13, was cloned by Watson and Fleming in 1996 (8). hMAM has been recognized as a breast epithelial cell-associated glycoprotein, expressed in 93% of BC cases (9, 10) and overexpressed in up to 23% (11). hMAM has been proposed as a marker for CTC detection by several authors (11-25). Many studies have reported the association between indirect detection of CTCs using hMAM expression in PB and traditional clinicopathological prognostic factors in BC before therapy and/or surgery (16-19, 25). However, we still do not have a clear view of this issue because the results are often contradictory. Therefore, other studies are still required to enable final conclusion to be drawn.

We previously developed a nested RT-PCR assay for amplifying the hMAM mRNA transcript as an indirect index
of the presence of CTCs in BC patients (12). This methodology proved to be highly sensitive and capable of detecting as few as one BC cell per million normal haematopoietic cells from healthy donors (12). With this level of sensitivity, transcripts of hMAM were not detected in PB collected from 58 patients affected by benign breast diseases, nor in PB from 65 healthy volunteers (12).

In the present study, on the basis of the discordant results reported by other authors, we investigated the association of the presence of CTCs with well established prognostic factors of BC at the time of diagnosis. In particular, we focused the statistical analysis on the odds ratio (OR) point estimates as, representing the more relevant index of association between each prognostic factor and the study outcome, they can better help to identify the prognostic implications of CTC detection in PB.

**Patients and Methods**

**Patients.** PB from BC patients was collected at the time of diagnosis, before neoadjuvant therapy and/or surgery, at the Division of Surgery of ASL5 La Spezia (Italy) between February 2002 and October 2004. Blood samples (5 ml) were drawn in ethylenediaminetetraacetic acid (EDTA) vacutainer after informed consent and approval of the study design by the Institutional Ethics Committee (protocol n. 53 15/11/2001). The study enrolled 190 patients with invasive BC (median age of 63.2, range 33-93 years), of whom 155 had ductal BC and 35 had other types of BC.

The study also included 12 patients with *in situ* BC (10 ductal and 2 lobular) and 330 controls consisting of 184 patients with benign breast disease and 146 with pathologies different from breast disease, or who were healthy blood donor volunteers.

**Enrichment of PB tumour cells and molecular analysis.** PB mononuclear cells were obtained by Ficoll-Hipaque (Biochrome AG, Berlin, Germany) density gradient centrifugation, followed by blockage of the interface cells. The collected cells, containing the CTCs, were washed twice in phosphate-buffered saline, dissolved in RLT solution (RNeasy Mini Kit, QIAGEN GmbH, Hilden, Germany) and snap-frozen at −30°C, ready for RNA extraction (12, 26).

Total RNA was extracted using RNeasy Mini Kit (QIAGEN GmbH) following the manufacturer’s instructions and 1 μg of total RNA was reverse transcribed into cDNA as previously described (12, 26).

A 10 μl sample of cDNA was amplified by nested PCR described elsewhere (12, 26) using primers that amplify *hMAM* gene mRNA (GenBank accession number U33147). Briefly, the first PCR reaction (total volume 50 μl) contained 1.5 mM MgCl₂, 0.2 mM dNTPs, 1.5 U Platinum Taq DNA Polymerase (Invitrogen, Milano, Italy) with its buffer and 0.4 μM primers. The cycling conditions were 2 min at 94°C followed by 40 cycles of 94°C for 30 s, 64°C for 20 s, 72°C for 5 s and a final incubation at 72°C for 2 min. The nested PCR reaction (total volume 50 μl) was carried out using 5 μl of the first PCR product with conditions identical to those of the first PCR but using the appropriate primers and 30 cycles of amplification. As internal control, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene amplification was carried out for each cDNA sample under conditions identical to those for *hMAM* first amplification. PCR products were separated by standard electrophoresis on 1.5% agarose gel containing ethidium bromide. The MDA-MB415 BC cell line (ICLC, National Institute for Cancer Research, Genova, Italy) was used as an *hMAM* positive control as previously described (26). The *hMAM*-positive samples were confirmed in a second RT-PCR experiment.

**Histopathology and immunohistochemistry.** The antigens were localized by means of a Ventana Medical Systems/view DAB detection kit and the reactions were performed by an automated immunostainer NEX ES (Ventana Medical Systems, S.A. Strasbourg, France) programmed for antigen retrieval. Finally, the sections were counterstained with haematoxylin.

The following monoclonal antibodies were used: clone 6F11 for oestrogen receptor (ER) (Ventana Medical Systems), clone 16 for progesterone receptor (PGR) (Ventana Medical Systems), and clone MIB-1 for Ki-67 (Dakocytomation Denmark A/S, Glostrup, Denmark). The cells were considered positive for ER, PGR and Ki-67 only when distinct nuclear staining was identified and the percentage of obtained cells was higher than 10. For HER-2 detection, the semi-quantitative detection kit PATHWAY® anti-HER-2/neu (4B5) Rabbit Monoclonal Primary Antibody (Ventana Medical Systems) was used. The histopathological grading for invasive BC was evaluated according to Elston and Ellis (27).

The percentage of immunoreactive tumour cells and tumour grading were evaluated independently by two observers (PD and BB or FF) who re-evaluated the conflicting results together.

**Statistical analyses.** Logistic regression analysis (28) was applied to assess the univariate and multivariate association between CTC status (positive vs. negative), indirectly detected by *hMAM* gene expression, and the following selected prognostic factors: age at diagnosis, tumour type and size, grading, lymph node involvement, ER and PGR receptor status, HER-2/neu expression and Ki-67/MIB-1 labelling index. The odds ratio (OR) was used as an index of strength of association between each prognostic characteristic and study outcome, considering OR >2.0 or <0.5 as being clinically relevant. For each OR, 95% confidence limits (95% CL) were also computed (28).

In order to evaluate the degree of dependence of each factor on the others in predicting the CTC status, ORs were estimated through both univariate (*i.e.* including only one factor) and multivariate (*i.e.* including all factors) logistic modelling. For each selected factor, the discrepancy between the two ORs was used as a tool for disclosing the degree of correlation with the other prognostic characteristics. Finally, statistical significance of each OR was assessed through the likelihood-ratio chi-square test (28) with *p*-values less than <0.05 being regarded as significant.

All statistical analyses were performed using Stata V10.1 (StataCorp, Stata Statistical Software, College Station, TX, USA).

**Results**

**Detection of CTCs in peripheral blood of BC patients and controls.** We investigated the presence of CTCs in PB of BC patients and controls through the *hMAM* transcript amplification by a previously described RT-PCR approach (12, 26).

*hMAM* expression was analysed in the mononuclear cell fraction of 532 PB samples derived from 190 patients with
invasive BC at primary diagnosis, 12 patients with in situ BC and 330 controls without BC including 184 patients with benign breast disease and 146 patients without breast disease or healthy blood donors.

HMAM transcript was detected in 18/190 (9.5%) PB samples from invasive BC patients but in none of those with in situ BC, nor in those without BC, as shown in Table I.

Detailed hMAM expression results, according to selected prognostic factors for patients with invasive BC are summarized in Table II.

Figure 1. The Figure shows a representative ethidium bromide-stained 1.5% agarose gel of RT-PCR amplified hMAM and internal control GAPDH. The hMAM band (334 bp) is evident in PB from a patient with breast cancer (lane 3), in the breast cancer cell line MDA-MB415 used as positive control (lane 5), but not in PB from a patient with benign breast disease (lane 1). The internal control GAPDH band (295 bp) is shown for each sample (lanes 2, 4, 6). hMAM, human mammaglobin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PB, peripheral blood; MW, molecular weight marker.

Univariate and multivariate analyses. In univariate analysis, clinically remarkable direct correlations between CTC-positive status and selected prognostic factors (i.e., OR >2.0 or OR <0.5) were found for nodal status (positive vs. negative, OR=8.2, p<0.001), tumour size (>2 cm vs. ≤2 cm, OR=5.9, p<0.001), histological grading (G3 vs. G1+G2, OR=5.6, p=0.058) and Ki-67/MIB-1 labelling index (>10% vs. ≤10%, OR=2.3, p=0.098), as shown in Table III.

A noteworthy association with the ER status (negative vs. positive, OR=3.3, p=0.055) was also observed, whereas no association with the other prognostic factors was found (Table III).

By comparing univariate to multivariate logistic regression analysis, it is possible to detect changes in OR point estimates basically due to the degree of correlation among the prognostic factors. In particular, a strong attenuation effect (i.e. OR towards the value of 1) was seen for some prognostic markers including tumour size (OR=2.3, p=0.207), histological grading (OR=1.8, p=0.379) and Ki-67/MIB-1 labelling index (OR=1.1, p=0.849). Although the OR for lymph node involvement exhibited a discernible reduction on multivariate analysis (OR=5.6), it was the only factor which maintained the highest and statistically significant correlation (p<0.009). The multivariate modelling confirmed the association of CTC detection with a negative ER status (OR=2.5, p=0.227) whereas it increased the
overexpression) were detected. Characteristics (age at diagnosis, PGR status and HER-2
expression) have been regarded as breast tissue-associated markers and a promising molecular indicator of
micrometastatic disease in BC (8). hMAM for the indirect detection of CTCs in BC has been investigated by many
authors who provided clear evidence for its high specificity (11-24). In this study, we evaluated the presence of CTCs
through the analysis of hMAM gene expression by RT-PCR in PB samples from BC patients at the time of diagnosis and
from controls.

Our results, obtained in a larger number of patients and controls, confirm those reported supporting the crucial
importance of hMAM as a marker for CTC detection, specificity being an absolute requirement in this field. In fact,
misleading identification of tumour cells may lead to wrong choices in clinical decisions and may represent a source of
dilemma and anxiety for the patients.

The frequency of hMAM expression in PB from invasive BC varies among the studies. Our finding of 9.5% positive
samples at primary diagnosis is in line with the frequencies reported by some authors: 8% by Grünewald et al. (16) and
Gargano et al. (15), 13% by Mikhitarian et al. (17), but in contrast to the higher frequencies reported by others: 18%
by Marques et al. (21), 20% by Benoy et al. (25), 38% by Lin et al. (18), 41% by Cerveira et al. (13) and 61% by
Zehentner et al. (19).

In addition, in the multivariate analysis, we found that the following four factors, nodal status, tumour size, tumour
type, and negative ER status, played a role in predicting hMAM-positivity in our series of patients. A significant
association of hMAM expression in PB with nodal status was reported by Grünewald et al. in patients with invasive BC
at the time of diagnosis or during the follow-up (16).

A trend towards a positive correlation of hMAM expression with lymph node involvement and with increased
tumour size was reported by Suchy et al. in PB of patients with invasive BC (20), whereas a significant association with
increased tumour size was reported by Ignatiadis et al. in early invasive BC (14).

Concerning the association of hMAM expression with high histological grading, we confirmed the data reported by
Mikhitarian et al. (17), as we found significant association of hMAM expression in PB samples obtained before surgery
with G3 grading in univariate analysis, although this finding was not confirmed by multivariate analysis. Moreover, at
variance with Mikhitarian et al. (17), who reported only a trend towards an association of hMAM expression with ER-
negative tumours, we found this association to be statistically significant.

On the other hand, our findings are in contrast with those of Zehentner et al. (19), Lin et al. (18), and Benoy et al.
(25), who did not find any association between poor prognostic factors and hMAM expression in PB samples from
BC patients collected before surgery.

The discrepancies in CTC detection through the use of hMAM marker may be partially linked to the number of
patients studied, age or disease stage, amount of cellular hMAM mRNA, or to other variables, including the amount
of PB analysed, blood collected and its storage, PCR methodology and RT-PCR conditions (primers, markers, reagents,
etc.).

In general, further conflicting findings regarding CTC detection in BC may arise from the non-uniformity of the

### Table III. Correlation between CTC detection in PB of patients with invasive BC and selected prognostic factors.

<table>
<thead>
<tr>
<th>Prognostic factor</th>
<th>Univariate</th>
<th>Multivariate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR</td>
<td>95% CL</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤50</td>
<td>1.0</td>
<td>0.547</td>
</tr>
<tr>
<td>&gt;50</td>
<td>1.5</td>
<td>0.4-5.3</td>
</tr>
<tr>
<td>Tumour type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Invasive ductal</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>Other*</td>
<td>1.8</td>
<td>0.6-5.5</td>
</tr>
<tr>
<td>Tumour size (cm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤2 (pT1)</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>&gt;2 (pT2-pT4)</td>
<td>5.9</td>
<td>1.9-18.7</td>
</tr>
<tr>
<td>Nodal status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative*</td>
<td>0.55</td>
<td>0.1-2.6</td>
</tr>
<tr>
<td>Positive</td>
<td>3.3</td>
<td>1.0-10.0</td>
</tr>
<tr>
<td>HER-2/Neu</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>Negative</td>
<td>1.25</td>
<td>0.5-3.3</td>
</tr>
<tr>
<td>Ki-67/MIB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤10%</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>&gt;10%</td>
<td>2.3</td>
<td>0.9-6.2</td>
</tr>
</tbody>
</table>

CTC, Circulating tumour cells; PB, peripheral blood; BC, breast cancer; ER, oestrogen receptor; PGR, progesterone receptor; OR, odds ratio; CL, confidence limits. *Other (n): lobular (23), papillary (3), mioepithelial-rich (1), apocrine (1), sarcomatoid (1), squamous (1), tubular (4), undifferentiated (1).

prognostic role of tumour type (OR=2.8, p=0.135). Finally, no substantial changes in the remaining prognostic
characteristics (age at diagnosis, PGR status and HER-2 overexpression) were detected.

### Discussion

hMAM gene expression has been regarded as a breast tissue-associated marker and a promising molecular indicator of
micrometastatic disease in BC (8). hMAM for the indirect detection of CTCs in BC has been investigated by many
authors who provided clear evidence for its high specificity (11-24). In this study, we evaluated the presence of CTCs
through the analysis of hMAM gene expression by RT-PCR in PB samples from BC patients at the time of diagnosis and
from controls.
methodologies applied \( (i.e. \) direct versus indirect methods and markers used). Finally, the results are difficult to interpret and compare owing to the scarce knowledge of the functional biology of CTCs \( (4,5) \). In particular, the time and number of CTC shed in the systemic circulation, besides their half-life in PB, not yet completely known, may contribute to the variability of results.

Therefore, in spite of the large number of studies, we still do not have a clear view of the clinical impact of CTCs. The concordance of results represents the basis conferring a clinical significance on CTCs and supports the application of CTC detection in routine clinical decisions.

In this regard, important progress has arisen from the use of an automated enrichment and immunocytochemical detection system (CellSearch system, Veridex, Warren, NJ, USA) reported by Riethdorf \textit{et al.} providing evidence for a robust tool allowing standardized detection and quantification of CTCs in PB of patients with metastatic BC \( (29) \). In addition, the American Society of Clinical Oncology (ASCO), for the first time in 2007, included CTC detection among the specific markers recommended in the guidelines for the use of tumour markers in BC. However, ASCO advises that CTCs “should not be used to make the diagnosis or to influence any treatment decisions until further validation confirms the clinical value of this test” \( (30) \). On the basis of our experience, we are in line with ASCO guidelines and we believe that further research is necessary to standardize methodologies and to fill the gap in the incomplete understanding of the functional biology of CTCs in BC.

Acknowledgements

This work was supported by grants from Ricerca Sanitaria Regionale Liguria, from Comitato Assistenza Malati e Lotta Contro i Tumori (Sarzana, Italy) and Associazione Italiana Contro le Leucemie/Linfoma e Mieloma (AIL, Sezione Francesca Lanzone, La Spezia, Italy). The Authors thank the staff of the Laboratory of Histopathology and Cytopathology of Sant’Andrea Hospital (La Spezia, Italy), Dr. Gaetano Leto for critically reading the manuscript, and Mrs. Anna Morabito for her valuable technical assistance.

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