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The Multi-Center Morphometric Mammary Carcinoma Project (MMMCP) in The Netherlands: Value of Morphometrically Assessed Proliferation and Differentiation

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Abstract The Multi-Center Morphometric Mammary Carcinoma Project (MMMCP) was set up to investigate the reproducibility and prognostic value of routine assessments of morphometric parameters [mean nuclear area (MNA), mitotic activity index (MAI), and multivariate prognostic index (MPI)] and cytometric features (DNA ploidy and index, % S-phase cells, as well as other cell cycle data) in comparison with classical prognostic parameters and steroid receptors. Thirty-four hospitals in six geographic regions participated. In 1988–1989, 3427 patients entered the study and morphometric assessments were made. An interim (1993) survival analysis indicated that MAI, MNA, and MPI are the strongest predictors of outcome. A Phase III randomized prospective multi-center trial [Premenopausal Morphometric Intervention Study (PREMIS)] using these endpoints was initiated in Europe to evaluate adjuvant [cyclophosphamide, methotrexate and 5-fluorouracil (CMF)] chemotherapy versus observation in morphometrically high risk (*i.e.*, MAI > 10), premenopausal, lymph node negative (LN–) breast cancer patients.

Key words: Breast carcinomas, mitotic activity index, morphometry, prognostic value, reproducibility

Breast cancer patient mortality is high, and the incidence of the disease is increasing [1]. The purpose of adjuvant chemotherapy (ACT) of surgically treated breast cancer is to eliminate *clinically* occult metastases and thus improve survival. The presence of axillary lymph node metastases is usually the selection criterion for ACT [2,3]. However, there is no generally accepted treatment for lymph node negative (LN–) premenopausal patients, despite the relatively high risk of distant metastases (15–20% of patients eventually die from metastatic disease). As a result, research into prognostic factors for these patients is intense. Such factors should be accurate, reproducible, and simple to use. The results of trials using qualitative histomorphological criteria are controversial [4,5], probably due to the lack of marker reproducibility. Estrogen receptor (ER) negativity and tumor size larger than 3 cm have been used as selection criteria for ACT, but the recurrence-free survival advantage at 4 years is only 10% [6,7]. This is understandable and in fact predictable, as the survival difference between ER-positive and -negative patients (both biochemically and histochemically evaluated) vanishes 4–6 years after diagnosis [8–10]. Overexpression of c-*erb*B2 (*neu*) seemed promising at first, but recent long-term follow-up studies are disappointing [11].

In contrast, a number of studies have shown that patients with LN– breast cancers with high proliferation rates do worse than those with low proliferation rates [12,13]. Retrospective as well

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TABLE I. Relapse and Death Risks in Premenopausal Lymph Node-Negative Patients [10]

Threshold[1]	Rel. Inc.	%Relapse[2]	%Deaths[2,3]
MAI < 10	0.54	6	3
MAI ≥ 10	0.46	37	27

[1] MAI = Mitotic Activity Index
[2] at 5 years follow-up
[3] due to lethal, distant metastases

as prospective studies by different groups have shown that the morphometric proliferation feature mitotic activity index (MAI) is the strongest single prognostic factor in clinically and population-detected small and large, LN– and positive (LN+), pre- and postmenopausal breast cancers [9–11,14–16]. The value of MAI was further enhanced by the finding that geographic survival differences were explained by differences in proliferation [17]. In premenopausal patients, a threshold of MAI = 10 gave the best results for the prediction of prognosis (Table I).

It is conceivable that ACT will be especially effective, and thus improve survival in premenopausal, LN–, morphometrically high-risk (MAI > 10) breast cancer patients. Before the morphometric features can be generally introduced, it is necessary to investigate whether such assessments routinely performed in several laboratories at different locations can provide reproducible results. The MMMCP was set up to evaluate the reproducibility of morphometric features, identify and control possible influencing factors, and prospectively assess the prognostic value.

HISTORY, SAMPLES, METHODS

In total, 34 hospitals and 15 pathology laboratories in six different regions of The Netherlands participated in the MMMCP. The last three months of 1987 were used as a test period, and after satisfying preliminary results, all primary invasive breast cancer patients were enrolled in 1988–1989. Clinical data were gathered in the hospitals and both standard pathological investigations and morphometric assessments were carried out in selected laboratories. Patient data, measurement results, and tumor material were transferred to the Free University for quality control. Data were stored in a central database, and a simple but safe procedure was constructed to ensure the privacy of patient data [18]. Tissue processing was carefully standardized for all laboratories. The tissue sample had to be taken from the periphery of the tumor, maximally 5 mm thick, and fixed at 18°C in 4% neutral (buffered, as pH of the fixative may influence nuclear area) formaldehyde, pH = 7.0 [19]. Fixation time was no shorter than 6 hours and not to exceed 72 hours. Standard 4 μm tissue sections were cut and stained with hematoxylin and eosin (H&E). Two fresh tissue samples were frozen for cytometric as well as other studies. The measuring procedure for morphometry was carefully outlined. The responsible pathologist marked a 5 × 5 mm area in the most representative H&E slide at low magnification using strict criteria. Detailed protocols for counting mitotic figures and nuclear measurements have been previously described [20] and were carefully followed.

REPRODUCIBILITY OF MITOSIS COUNTS IN 2469 BREAST CANCER SPECIMENS

Analysis of the reproducibility of the assessments in 2469 patients showed correlation coefficients between 0.81 and 0.96 (mean = 0.91) for the MAI, between 0.78 and 0.96 (mean = 0.87) for the mean nuclear area (MNA), and between 0.91 and 0.97 (mean = 0.96) for the multivariate prognostic index (MPI) [21]. The reproducibility was constant over time, although it showed a slight drop in the middle of the two-year intake period. A prognostically relevant discrepancy in MAI between the original and quality control assessments was found in only 7.2% of the cases. When analyzing the reasons for these discrepancies, a plausible explanation could be found in all cases (Table II). Since these error sources are now identified and controlled, reproducibility has been achieved.

With digital image processing (DIP), this can be further guaranteed [22].

STABILITY OF MITOSES

In spite of the reproducibility of the mitotic counts, stability of mitoses is a matter of debate. It has been argued that mitoses may complete their cycle due to the absence of blood supply and thus disappear. However, the mitotic rate of Bullough's and Graem's [23,24] samples was extremely high (comparable with a MAI range of 35–100; compared with a median MAI = 8 in breast cancers). Donhuijsen *et al.* [25] found that the mitoses counted by two observers decreased by 46% and 39% on average after a 12-hour fixation delay. However, the mitoses "decrease" was related to the degree of mitotic activity of individual tumors, and was minimal in sarcomas with the lowest rate, which was 35 (still very high!).

TABLE II. Factors Causing Mitotic Activity Index Discrepancies [21]

Factors	%	Solution
Poor section quality	10	Reject
Overstaining	15	DIP[1]
Wrong area selected	23	VEPI[2]
Wrong starting point within selected area	27	VEPI
Doubtful mitosis-like structures counted	25	DIP

[1] DIP = by means of digital image processing
[2] VEPI = assess volume percentage epithelium to select most epithelium-rich area

Most importantly, they concluded that the decrease in counts is largely due to the reduced identification of mitotic figures and only partly attributable to completion of the cell cycle. This is further supported by two facts. First, well-preserved mitotic figures, demonstrable after 12 hours, indicate that proliferative activity only gradually decreases in unfixed biopsy specimens. Second, the flow cytometric data did not change substantially; only a slight increase in the G_2+M-phase fraction was observed. Moreover, Start *et al.* [26] found that a fixation delay of 6–24 hours resulted in little change in mitotic rates. This is the usual delay in routine breast cancer specimens.

We therefore conclude it is unlikely that a fixation delay of up to 24 hours has any decisive influence on the MAI as a prognostic factor in breast cancers, or on the decision threshold (MAI = 10) used in the Premenopausal Morphometric Intervention Study (PREMIS) study (see below).

INTERIM PROGNOSTIC EVALUATION (1993)

Recently, an interim survival analysis was performed on patients with at least 12 months of follow-up (range = 12–56 months; median = 35 months). Table III shows the descriptive statistics, and Table IV the survival analysis, using the Mantel-Cox statistic. In both the total group and the LN– group (48% of the total group), the morphometric features (MNA, MAI, MPI) were among the strongest prognosticators.

THE PREMIS STUDY

The above mentioned results imply that in LN– patients the MAI is a practical, generally applicable, stable, reproducible, powerful, and inexpen-

TABLE III. MMMCP—Interim, Descriptive Evaluation 1993

Features	Mean	Min.	Max.
Age (years)	58.8	22.4	90.8
Tumor diameter (μm)	2.5	0.2	25.0
Mean Nuclear Area (μm^2)	61.4	22.7	163.5
Mitotic Activity Index	10.3	0.0	135.0

TABLE IV. MMMCP—1993 Interim Evaluation Survival Analysis

	Total		LN– Only	
	p[1]	MC[2]	p	MC
Lymph node status				
neg vs. pos	<0.0001	21.5	—	
neg vs. <4 pos vs. >3 pos	<0.0001	38.2	—	
Tumor diameter				
<2 vs. 2–3 vs. >3	0.0003	16.1	0.21	3.1
Estrogen Receptor				
pos vs. neg	0.0001	14.5	too few negatives	
Progesterone Receptor				
pos vs. neg	0.003	8.9	0.22	1.5
DNA				
diploid vs. non diploid	0.41	0.68	0.27	1.2
diploid vs. tetraploid vs. aneuploid	0.70	0.70	0.43	1.7
Mean Nuclear Area				
<41.7 vs. 41.7–53.0 vs. >53.0	0.0001	25.0	0.009	9.5
MAI				
<9 vs. >9	<0.0001	25.0	<0.0001	27.5
<9 vs. 9–19 vs. >19	<0.0001	34.3	<0.0001	39.5
MPI				
<0.6 vs. >0.6	<0.0001	50.7	<0.0001	32.2
<0.0 vs. 0.0–0.6 vs. >0.6	<0.0001	58.2	<0.0001	38.4
<0.6 vs. 0.6–1.0 vs. >1.0	<0.0001	53.4	<0.0001	31.2

[1] p = Probability of No Difference
[2] MC = Mantel-Cox Value

sive prognostic factor. This has led to a Phase III, two-armed randomized trial to compare adjuvant CMF chemotherapy (six classical courses each month) with no treatment in premenopausal, LN–, morphometrically unfavorable (MAI > 10) invasive breast cancer patients. Treatment must be started within four weeks of surgery, or within four weeks after the end of radiotherapy, if applicable. Patients with high medical risks or other malignancies are not eligible for the trial, except for those with adequately treated *in situ* carcinoma of the uterine cervix or basal cell carcinoma of the skin. Informed consent and cooperation of the patients for treatment and follow-up are required. To attain significance (alpha: 0.05 one-sided, power = 0.90) the required sample size is 166 patients per arm. Currently, 110 patients are enrolled.

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REFERENCES

1. Nemoto R, Vana J, Bedwani RH, Baker HW, McGregor FH, Murphy GP. (1980) Management and survival of female breast cancer: Results of a national survey by the American College of Surgeons. Cancer 45:2917–2924.
2. Early Breast Cancer Trials Collaborative Group. (1988) Effects of adjuvant tamoxifen and cytotoxic therapy on mortality in early breast cancer. New Eng J Med 319:1680–1692.
3. Bonadonna G, Valagussa P, Rossi A, Tancini G, Brambilla C, Zambetti M, Veronesi U. (1985) Ten-year experience with CMF based adjuvant chemotherapy in resectable breast cancer. Breast Cancer Res Treat 5:95–115.
4. Rosen PP, Saigo PE, Braun DW Jr, Weathers E, DePalo A. (1981) Predictors in recurrence in stage I ($T_1N_0M_0$) breast carcinoma. Ann Surg 193:15–25.
5. Bilik R, Mor C, Wolloch Y, Rintsman M. (1986) Histopathology risk factors influencing the prognosis for patients with early breast cancer ($T_1N_0M_0$). Am J Surg 151:460–464.
6. Fisher B, Redmond C, Dimitrov NV, Bowman D, Legault-Poisson S, Wickerham DL, Wolmark N, Fisher ER, Margolese R, Sutherland C. (1989) A randomised clinical trial evaluating sequential methotrexate and fluorouracil in the treatment of patients with node-negative breast cancer who have estrogen-receptor-negative tumors. New Engl J Med 320:473–478.
7. Mansour EG, Gray R, Shatila AH, Osborne CK, Tormey DC, Gilchrist KW, Cooper MR, Falkson G. (1989) Efficiency of adjuvant chemotherapy in high risk node negative breast cancer. New Engl J Med 320:485–490.
8. Raemakers JMM, Beex LVAM, Koenders AJM, Pieters GFFM, Small AGH, Benraadt THJ, Kloppenberg PWC & the Breast Cancer Group. (1985) Disease-free interval and estrogen receptor activity in tumor tissue of patients with primary breast cancer: Analysis after long-term follow-up. Breast Cancer Res Treat 6:123–130.
9. Linden JC van der, Baak JPA, Lindeman J, Hermans J, Meijer CJLM. (1987) Prospective evaluation of prognostic value of morphometry in patients with primary breast cancer. J Clin Pathol 40:302–306.
10. Uyterlinde AM, Baak JPA, Schipper NW, Peterse H, Matze E, Meijer CJLM. (1990) Further evaluation of the prognostic value of morphometric and flow cytometric parameters in breast-cancer patients with long follow-up. Int J Cancer 45:1–7.
11. Baak JPA, Chin D, van Diest PJ, Ortiz R, Matze-Cok P, Bacus SS. (1993) Comparative long-term prognostic value of quantitative HER-2/*neu* protein expression, DNA ploidy, and morphometric and clinical features in paraffin-embedded invasive breast cancer. Lab Invest 64:215–223.
12. Silvestrini R, Daidone MG, Gasparini G. (1985) Cell kinetics as a prognostic marker in node-negative breast cancer. Cancer 56:1982–1987.
13. Meyer JS, Province M. (1988) Proliferative index of breast carcinoma by thymidine labelling: Prognostic power independent of stage, estrogen and progesteron receptor. Breast Cancer Res Treat 12:191–204.
14. Baak JPA, Dop H van, Kurver PHJ, Hermans J. (1985) The value of morphometry to classic prognosticators in breast cancer. Cancer 56:374–382.
15. Uyterlinde AM, Baak JPA, Schipper NW, Peterse HJ, Meijer JWR, Vooijs PG, Matze E. (1991) Prognostic value of morphometry and DNA flow-cytometry features of invasive breast cancers detected by population screening: Comparison with a control group of hospital patients. Int J Cancer 48:173–181.
16. van Diest PJ, Baak JPA. (1991) The morphometric prognostic index is the strongest prognosticator in premenopausal lymph node-negative and lymph node-positive breast cancer patients. Hum Pathol 22:326–330.
17. Baak JPA, Wisse-Brekelmans ECM, Kurver PHJ, van Gorp LH, Voorhorst FJ, Miettinen OS. (1993) Regional differences in breast cancer survival are correlated with differences in differentiation and rate of proliferation. Hum Pathol 23:989–992.
18. Baak JPA, van Diest PJ, Stroet-van Galen CM, Wisse-Brekelmans ECM, Matze-Cok P, Littooy JG, Kurver PH. (1989) Data processing and analysis in the multicenter morphometric mammary carcinoma project (MMMCP). Pathol Res Pract 185:657–663.
19. Baak JPA, Noteboom E, Koevoets JJM. (1989) The influence of fixatives and other variations in tissue processing on nuclear morphometric features. Anal Quant Cytol Histol 11:219–224.
20. Fleege JC, van Diest PJ, Baak jPA. (1991) Reliability of quantitative pathological assessments: Standards and quality control. In Baak JPA (ed): "Manual of quantitative pathology in cancer diagnosis and prognosis." Chapter 12. New York: Springer-Verlag, pp 151–181.
21. van Diest PJ, Baak JPA, Matze-Cok P, Wisse-Brekelmans ECM, other MMMCP collaborators. (1992) Reproducibility of mitosis counting in 2,469 breast cancer specimens: results from the Multicenter Morphometric Mammary Carcinoma Project. Hum Pathol 23:603–607.
22. ten Kate TK, Belien JAM, Smeulders AWM, Baak JPA. (1993) Method for counting mitoses by image processing in Feulgen stained breast cancer sections. Cytometry 14:241–250.
23. Bullough WS. (1950) Mitotic activity in the tissue of dead mice, and in tissues kept in physiological salt solution. Exp Cell Res 1:410–420.

24. Graem H, Helweg-Larsen K. (1979) The influence of delay in fixation on mitotic activity of a human osteogenic sarcoma grown in athymic nude mice. Acta Pathol Microbiol Scand 87:375–378.
25. Donhuijsen K, Schmidt U, Hirc H, Beuningen D van, Budach V. (1990) Changes in mitotic rate and cell cycle fractions caused by delayed fixation. Hum Pathol 21:709–714
26. Start RD, Flynn MS, Cross SS, Rogers K, Smith JHF. (1991) Is the grading of breast carcinomas affected by a delay in fixation? Virch Arch A419:475–477.