

# Prognostic comparative study of S-phase fraction and Ki-67 index in breast carcinoma

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## Abstract

**Aims**—To investigate the prognostic value of recently proposed flow cytometric S-phase fraction (SPF) variables (average SPF and SPF tertiles) compared with conventional SPF, and to compare the one with the best predictive value with the immunohistochemical Ki-67 index in breast carcinoma.

**Methods**—A short term follow up study (median, 39.6 months) of a large series of patients (n = 306) was conducted. DNA ploidy was analysed on fresh/frozen tumour samples by flow cytometry, and the SPF was calculated from the DNA histogram using an algorithm. The Ki-67 index was assessed on paraffin wax embedded material by immunohistochemistry (cut off point, 10%). The two methods were compared by means of  $\kappa$  statistics, and the prognostic significance of both in relation to disease free survival (DFS) and overall survival (OS) was determined.

**Results**—SPF and Ki-67 analysis was performed on 234 (76.5%) and 295 (96.4%) tumours, respectively. The two assessments were simultaneously available in 230 cases. All SPF variables analysed in the whole series significantly correlated with disease evolution, with the conventional median SPF (cut off point, 6.1%) showing the highest predictive value in relation to both DFS ( $p = 0.0001$ ) and OS ( $p = 0.0003$ ). SPF tertiles and median SPF evaluated according to DNA ploidy status had no prognostic significance. The Ki-67 index showed a trend in relation to DFS ( $p = 0.086$ ) that did not reach significance, and no correlation with OS was found ( $p = 0.264$ ). The comparative analysis of SPF and Ki-67 revealed some agreement between the two methods (agreement, 69.13%;  $\kappa$  statistic, 0.3844;  $p < 0.001$ ), especially in the subgroup of diploid tumours.

**Conclusions**—Flow cytometric SPF is a better prognosticator than the Ki-67 index, but only SPF variables applied in the whole series show potential clinical usefulness.

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**Keywords:** breast carcinoma; DNA flow cytometry; immunohistochemistry; S-phase fraction; Ki-67; prognosis

choice of the best method for the assessment of proliferation and the standardisation of criteria for interpretation of results, have limited its clinical application.<sup>4,5</sup>

In a previous study,<sup>6</sup> we showed that the S-phase fraction (SPF) is an independent marker of disease outcome in breast carcinoma. For prognostic purposes, some investigators adopt the median value as the cut off point, whereas others use two thresholds for defining a three group classification system.<sup>2,7,8</sup> Therefore, we investigated which SPF variable has the greatest predictive value and would provide useful prognostic information for the clinic. In addition, we sought to determine the best cell proliferation method for predicting disease outcome, comparing distinct markers in the same series of patients.<sup>9-15</sup> We tested Ki-67, a nuclear antigen present in all active phases of the cell cycle (G1, S, G2, and mitosis (M)),<sup>16</sup> which is a valuable indicator of tumour proliferation and prognosis in patients with breast cancer.<sup>17-20</sup> The immunohistochemical Ki-67 index has the technical advantage, in relation to flow cytometry, of allowing the morphological evaluation of proliferating cell populations.

Our study was designed to investigate the following three areas in a series of 306 patients with breast cancer, namely: (1) to elicit the SPF category with the best prognostic strength, by applying SPF variables with distinct cut off points; (2) to compare SPF with immunohistochemical Ki-67 results; and (3) to correlate both indices with disease outcome (disease free survival (DFS) and overall survival (OS)).

## Materials and methods

The study group consisted of 306 women with primary operable invasive breast cancer (stage I/II of the disease), diagnosed and treated at the Instituto Português de Oncologia, Lisbon between October 1990 and December 1996. The eligibility criteria for patients included the lack of treatment before surgery, the availability of frozen samples for flow cytometry, and accurate follow up information. The mean age of the patients was 58.5 years (range, 23-88). The histological type and tumour staging of breast carcinomas were evaluated according to the TNM-UICC system.<sup>21</sup> The series comprised 273 invasive ductal carcinomas (89.2%) and 33 carcinomas of other histological types (10.8%). One hundred and twenty tumours (39.2%) were classified as pT1 (< 2 cm), 163 (53.3%) as pT2 (2-5 cm), and 23 (7.5%) as pT3 (> 5 cm). One hundred and seventy three patients (56.5%) had no axillary lymph node positivity (pN0), whereas 133 (43.5%) had

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It is well recognised that the proliferative activity of neoplastic cells influences the clinical course of certain types of human malignancy.<sup>1-3</sup> However, methodological issues, such as the

nodal involvement (pN1). Two hundred and seventy eight patients (90.9%) underwent modified radical mastectomy and axillary lymphadenectomy, and 28 (9.1%) were submitted to conservative surgery (tumorectomy or quadrantectomy) and axillary lymph node dissection as primary surgical treatment. Heterogeneous adjuvant therapeutic regimens were given to the patients: 84 received chemotherapy; 46 hormonotherapy; 33 chemotherapy, hormonotherapy, and radiotherapy; 43 chemotherapy and hormonotherapy; 84 chemotherapy and radiotherapy; and nine hormonotherapy and radiotherapy, whereas seven patients received no adjuvant treatment. Information on DFS and OS was obtained from clinical chart review or consultation of the epidemiological registry service at our institution (ROR-Sul). DFS and follow up period were defined as the time that elapsed between primary surgical resection and the first recurrence, locally or at a distance, and the last clinical observation or death, respectively. The median follow up was 39.6 months (range, 3–84). At the end of follow up time, 254 patients (83%) were alive without evidence of disease, 17 (5.6%) were alive with disease, and 34 (11.1%) had died of their disease. One patient who died from an unrelated cause was censored from the survival analysis study.

#### DNA FLOW CYTOMETRY STUDY

Flow cytometric analysis was performed on representative fresh/frozen samples obtained at the time of surgery, as described previously.<sup>6</sup> Briefly, the tissue samples were mechanically disaggregated in cold phosphate buffered saline (PBS) using scalpel blades, and the cell suspension obtained was rinsed twice in PBS and checked by counting in a Bürker haemocytometer. For DNA staining, the cells were treated with 1 mg/ml ribonuclease in PBS and 0.05% Nonidet P40 non-ionic detergent, and incubated with 50 µg/ml propidium iodide (PI), in Tris/MgCl<sub>2</sub> buffer, for one hour in the dark at 4°C. Immediately before the flow cytometric analysis, the specimens were passed through a 27 gauge needle and then filtered through a 55 µm nylon mesh. The samples were analysed on an Epics Profile II flow cytometer (Coulter Electronics, Hialeah, Florida, USA) equipped with a 488 nm, 15 mW argon ion laser as light source and a 575 nm bandpass filter for PI detection. Fluorescent beads (DNA-Check; Coulter) were used daily for instrument alignment. Chicken red blood cells were added to each sample as an internal control to define the G0/G1 diploid population. At least 20 000 nuclei at a rate of 100–150/second were acquired in each run, and recorded on a single parameter 256 channel integrated fluorescence histogram.

#### DNA histogram interpretation

Flow cytometric data analysis was performed using the MultiCycle Software Program (Phoenix Flow Systems, San Diego, California, USA) developed by PS Rabinovitch (University of Washington, Seattle, Washington, USA), which is based on the mathematical method

described by Dean and Jett.<sup>22</sup> The coefficient of variation (CV) of tumour G0/G1 peaks, estimated as half peak width, ranged from 2.4 to 7.8 (mean, 4.2). Histograms with CVs over 8% were not included in our study. DNA ploidy status was defined according to guidelines proposed at the DNA cytometry consensus conference.<sup>5</sup> To standardise SPF evaluation more accurately, we have followed the cell cycle analysis criteria used by Bergers *et al*,<sup>23</sup> which provided the best prognostic results in breast cancer flow cytometric studies, namely the zero order S-phase calculation and “sliced nuclei” debris option with aggregates correction. Therefore, SPF was calculated in 234 cases (76.5%) of our series, according to this polynomial model. In the remaining 72 tumours (23.5%), all but four being DNA aneuploid, SPF determination could not be reliably assessed because of: (1) samples with a high amount of background debris (critical percentage > 20%); (2) a small (< 15%) but definitely non-diploid population; and (3) overlapping of two or more populations (near diploidy or multiploidy).

For prognostic purposes, three SPF variables were assessed: (1) the “average SPF” (total number of cells in all S-phases/total number of cells × 100), using the median 5.9% value as the cut off point for discriminating low (< 5.9%) and high (≥ 5.9%) proliferative tumours; (2) the “conventional SPF” of only DNA diploid or DNA aneuploid cell cycles, depending on the ploidy status, also using the median (whole series, 6.1%; DNA diploid tumours, 3.8%; DNA aneuploid tumours, 12%) as cut off values; and (3) the “SPF tertiles”—the low, intermediate, and high proliferative tumours subgrouping, using two thresholds (whole series, 4.5% and 9.2%; DNA diploid tumours, 3% and 4.7%; DNA aneuploid tumours, 10% and 13%) for classification.

Using average SPF instead of conventional SPF, a higher number of cases (five more in our series, which totals 239 cases) could be estimated, the difference being related to multiploid tumours (fig 1).

#### IMMUNOHISTOCHEMICAL STAINING

Ki-67 immunostaining was performed on 2–3 µm thick sections cut from formalin fixed, paraffin wax embedded tissue using the streptavidin–biotin complex peroxidase technique.<sup>24</sup> First, the sections were attached to gelatine coated slides and dried overnight at 37°C. Dewaxing in xylene and washes in 100% ethanol were followed by two pretreatment procedures: endogenous peroxidase was blocked by 0.6% hydrogen peroxide in methanol for 10 minutes, and antigen retrieval was carried out using a pressure cooker and citrate buffer, pH 6.0 for one minute.<sup>25</sup> After washing in water, the sections were rinsed in Tris buffered saline (TBS), pH 7.4–7.6, and incubated for 30 minutes at room temperature with primary monoclonal anti-Ki-67 antibody (anti-Ki-67/7B11 clone; Zymed Laboratories, San Francisco, California, USA) at a 1/50 dilution. The sections were then washed in TBS

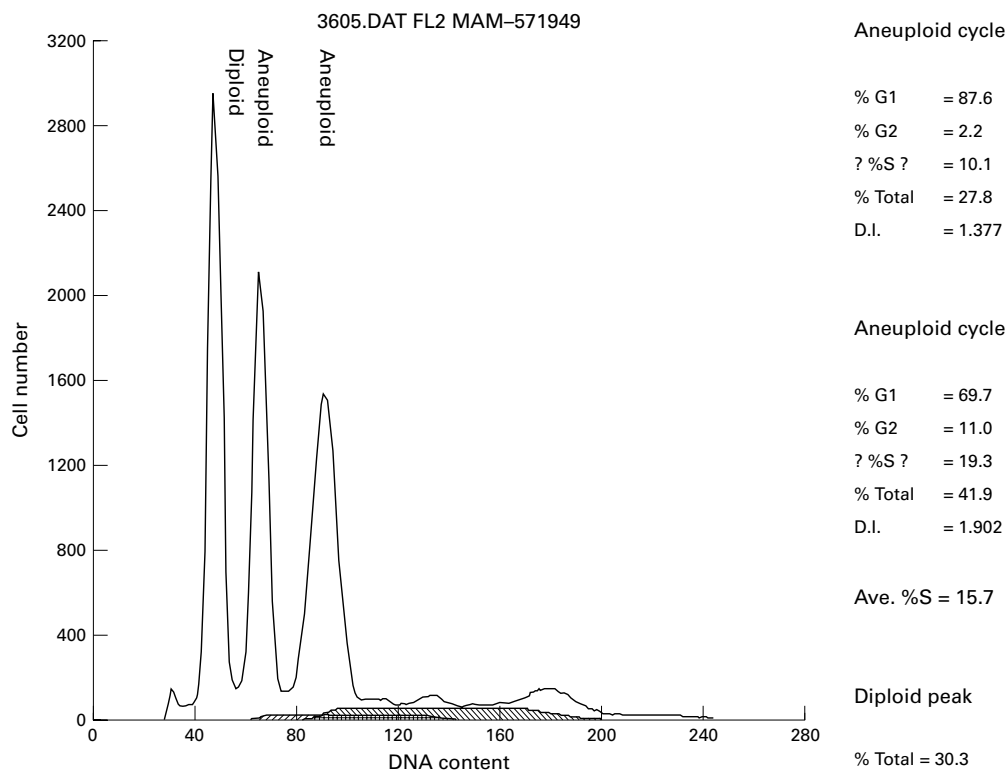


Figure 1 Flow cytometric DNA histogram showing a multiploid tumour with overlapping cell populations. Only “average S-phase fraction (SPF)” was determined (15.7%), and the tumour was classified as highly proliferative by flow cytometry and histochemistry methods.

and incubated with a secondary biotinylated goat antimouse/antirabbit serum (K492; Dako) at a 1/100 dilution for 30 minutes. The sections were rinsed again in TBS, and the StreptABC complex (K492; Dako) at a 1/100 dilution was applied for 30 minutes. After washing in TBS, diaminobenzidine tetrahydrochloride (D-5637; Sigma, St Louis, Missouri, USA) was used as chromogen for eight minutes. The sections were then washed in water and finally counterstained with Mayer’s haematoxylin. As negative control, staining was performed without primary antibody, and human normal appendix tissue was used as positive control.

**Staining assessment**

The entire slide was scanned for immunostaining evaluation by two observers using a two headed light microscope. All malignant cells with nuclear staining were considered to be

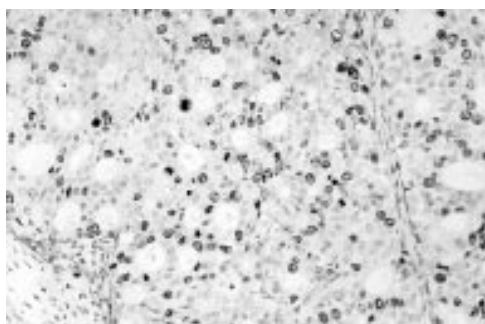


Figure 2 Immunohistochemical staining (anti-Ki-67 antibody) of the same ductal invasive carcinoma shown in fig 1.

positive (fig 2). When Ki-67 immunoreactivity was distributed diffusely, randomly chosen tumour cells were assessed in several high power fields; whenever there was focal/heterogeneous staining, the scoring was carried out in the area with the highest number of positive nuclei.<sup>26</sup> The Ki-67 index was expressed semiquantitatively only in the invasive component of the tumour in at least 200 neoplastic cell nuclei. A cut off point of 10% was used to distinguish between the categories of low and high proliferative tumours.<sup>19 27 28</sup>

**STATISTICAL ANALYSIS**

The κ statistic was used to compare flow cytometric SPF and immunohistochemical Ki-67 results.<sup>29 30</sup> κ Values between 0.21 and 0.40 suggested a reasonably better agreement and values between 0.00 and 0.20 suggested a slightly better agreement than would be expected by chance alone.<sup>30</sup> Analysis of survival data was performed using the Kaplan-Meier method,<sup>31</sup> with differences between survival curves being evaluated by the log rank test.<sup>32</sup> Probabilities of p < 0.05 were regarded as significant.

**Results**

Table 1 illustrates the correlation between the SPF categories and the disease outcome as assessed by DFS and OS. Only the SPF variables analysed in the whole series showed a significant correlation with the evolution of the disease, with the “conventional median SPF” having the greatest predictive strength in relation to both DFS (p = 0.0001) and OS

Table 1 Correlation between SPF variables and disease outcome in breast carcinoma

Variables	Disease free survival			Overall survival	
	n	No. recurrences	p Value	No. deaths	p Value
% Average SPF (whole series)					
<5.9	119	8	0.0003	4	0.0010
≥5.9	120	29		21	
% SPF tertiles (whole series)					
<4.5	78	4	0.0034	1	0.0054
4.5–9.2	78	12		8	
≥9.2	78	19		14	
% SPF tertiles (diploid tumours)					
<3	37	2	0.4625	0	0.1600
3–4.7	50	3		2	
≥4.7	42	5		4	
% SPF tertiles (aneuploid tumours)					
<10	34	7	0.9935	5	0.6834
10–13	35	8		4	
≥13	36	9		8	
% Median SPF (whole series)					
<6.1	117	7	0.0001	3	0.0003
≥6.1	117	28		20	
% Median SPF (diploid tumours)					
<3.8	66	4	0.4468	1	0.1070
≥3.8	63	6		5	
% Median SPF (aneuploid tumours)					
<12	52	14	0.1776	8	0.3887
≥12	53	10		9	

SPF, S-phase fraction.

(p = 0.0003). Therefore this SPF category was used for comparison with Ki-67 results. Neither SPF tertiles nor median SPF evaluated by DNA ploidy status (DNA diploid v DNA aneuploid) showed significance in relation to disease outcome.

SPF analysis was feasible in 234 cases (76.5%), half of which were considered as slowly proliferative tumours and the other half as highly proliferative. The Ki-67 index was obtained in 295 cases (96.4%), including 159 low and 136 high proliferative tumours. In the remaining 11 cases, Ki-67 was not determined because of the lack of representative pathological material in the paraffin wax blocks.

Table 2 Comparison between flow cytometric SPF and immunohistochemical Ki-67 index methods

Variable	SPF			p Value
	n	Low	High	
Ki-67 index				<0.001
Low	134	88	46	
High	96	25	71	

Agreement: 69.13%  
κ: 0.3844

SPF cut off point, 6.1%; Ki-67 cut off point, 10%.  
SPF, S-phase fraction.

Table 3 Relation between SPF and Ki-67 proliferation indices in DNA diploid breast carcinomas

Variable	SPF			p Value
	n	Low	High	
Ki-67 index				0.005
Low	93	84	9	
High	32	23	9	

Agreement: 74.40%  
κ: 0.2154

SPF cut off point, 6.1%; Ki-67 cut off point, 10%.  
SPF, S-phase fraction.

Table 4 Relation between SPF and Ki-67 proliferation indices in DNA aneuploid breast carcinomas

Variable	SPF			p Value
	n	Low	High	
Ki-67 index				0.077
Low	41	4	37	
High	64	2	62	

Agreement: 62.86%  
κ: 0.0783

SPF cut off point, 6.1%; Ki-67 cut off point, 10%.  
SPF, S-phase fraction.

The concomitant assessment of both cell proliferation parameters was available in 230 cases.

Table 2 shows the statistical agreement between flow cytometric SPF and immunohistochemical Ki-67 methods. Their relation, according to DNA ploidy status, is illustrated in tables 3 and 4 for DNA diploid and DNA aneuploid tumours, respectively. Overall, a reasonable agreement was verified between the two techniques (agreement, 69.13%; κ statistic, 0.3844; p < 0.001), especially in the subgroup of DNA diploid tumours (agreement, 74.40%; κ statistic, 0.2154; p = 0.005). In the DNA aneuploid group only a slight agreement was observed (agreement, 62.86%; κ statistic, 0.0783; p = 0.077).

Univariate survival analysis in this series of patients with breast cancer revealed a significant correlation between SPF and either DFS (p < 0.001) or OS (p < 0.001) (fig 3), whereas the Ki-67 index showed a trend in relation to DFS, which did not reach significance (p = 0.086), and no correlation with OS (p = 0.264) (fig 4).

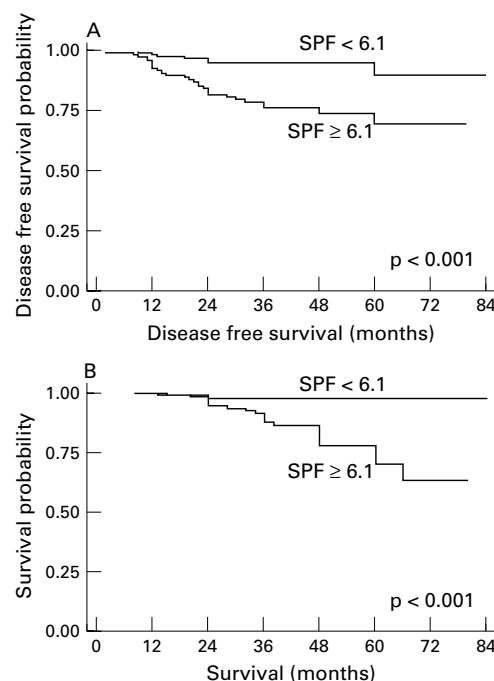


Figure 3 (A) Probability of disease free survival and (B) overall survival according to S-phase fraction (SPF) (cut off point, 6.1%) in breast carcinoma (n = 234). Low SPF groups have a more favourable outcome (p < 0.001).

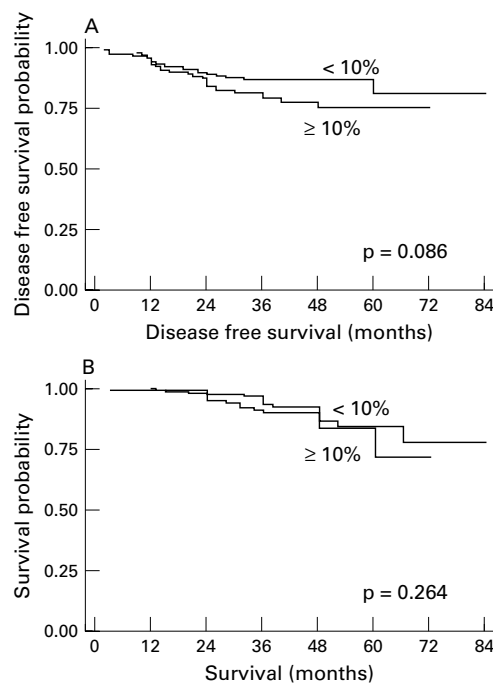


Figure 4 (A) Probability of disease free survival (DFS) and (B) overall survival (OS) according to Ki-67 index (cut off point, 10%) in breast carcinoma ( $n = 294$ ). Weak differences between survival curves in relation to DFS (not significant;  $p = 0.086$ ) were verified. No differences in relation to OS were found ( $p = 0.264$ ).

### Discussion

The technical issues of validity and reproducibility related to prognostically useful methods to measure tumour cell proliferation are a matter of controversy. Although some authors<sup>33</sup> have demonstrated a highly reproducible way of estimating the mitotic index in breast carcinoma, it has been difficult to reach general consensus on standardised conditions for SPF assessment, as well as on the cut off values to be used for prediction purposes.<sup>7,8</sup> In our study, we evaluated the prognostic value of three SPF variables (average SPF, SPF tertiles, and conventional SPF) through their correlation with disease outcome. The use of the SPF tertiles classification, which applies two thresholds in an attempt to optimise the definition of prognostic risk groups, was strongly recommended by a consensus review on the subject.<sup>2,5</sup> The biological rationale underlying the use of average SPF determination is related to the fact that proliferating cells from all DNA diploid and DNA aneuploid populations influence the way that tumours grow, and therefore have prognostic implications.<sup>23</sup> Moreover, some studies have shown that the average SPF is the most reproducible method for estimating S-phase cells in breast cancer.<sup>23,34</sup> Our data showed that all SPF variables applied in the whole series are significantly correlated with both DFS and OS, with the conventional median SPF being the best indicator in terms of prognostic strength (table 1). In contrast, the SPF variables evaluated according to DNA ploidy status showed no predictive significance, which does not support the view that the use of separate cut points improves the prognostic

impact of SPF in DNA ploidy subgroups (DNA diploid *v* DNA aneuploid).<sup>5</sup>

We also investigated the prognostic value of the immunohistochemical Ki-67 index in the same series of breast carcinomas and, although a trend was found in relation to DFS ( $p = 0.086$ ), no significant correlation between this marker and OS was verified. The study profile characteristics, with a relatively short follow up period, could explain this, but other investigators, using longer follow up studies, have reported identical findings.<sup>9,10,27,28</sup> In contrast to our results, some authors have shown a positive correlation between Ki-67 index and OS.<sup>17-19,35</sup> Conflicting results might be caused by difficulties in counting and interpreting the positivity of Ki-67 stained cells, particularly when tumour staining is heterogeneous. Another reason relates to the well known intra-tumour heterogeneity of neoplastic cell populations.<sup>36</sup> In an attempt to overcome this fact, we assessed Ki-67 in the areas with the highest number of positive nuclei, taken as those with greater proliferation rates (so called "hot spots"). The determination of a cut off point to discriminate low from high Ki-67 proliferative tumours is crucial. Median values ranging from 0.6% to 25% have been used by some authors, and arbitrary values between 10% and 20% were adopted by others.<sup>16,17,26</sup> It is also known that fixation conditions as well as the fixative used may affect Ki-67 determination, because the antigen is very sensitive to chemical denaturation and can even be destroyed in very dilute formalin solutions.<sup>37,38</sup>

In our study, the comparative analysis of the SPF and Ki-67 index showed reasonable agreement (agreement, 69.1%;  $\kappa$  statistic, 0.38) between the two methods (table 2), similar to the results of Brown *et al* (agreement, 67%;  $\kappa$  statistic, 0.22).<sup>10</sup> When analysed according to DNA ploidy status, the relation was more evident among DNA diploid tumours, with the DNA aneuploid group showing only slight agreement. Some studies<sup>10-12</sup> suggested that the overall correlation is mostly dependent on the aneuploid group, whereas others<sup>13,14</sup> found a significant correlation between both methods, irrespective of the DNA ploidy status. In contrast, Jansen *et al* failed to demonstrate such a correlation.<sup>9</sup>

The comparative study revealed the existence of two groups of tumours exhibiting apparently contradictory results (table 2): one group comprised tumours with a low Ki-67 index and high SPF ( $n = 46$ ), and the other comprised tumours with a high Ki-67 index and low SPF ( $n = 25$ ). The discordant data found in this last group could be explained by the fact that the two methods evaluate different cell cycle compartments of proliferating cell populations: Ki-67 stained cells in the G1 phase being responsible for the higher percentage of cycling elements in these tumours.<sup>16,20</sup> The other group included a few tumour samples that contained numerous mitotic figures but lacked Ki-67 immunostaining, a surprising finding because Ki-67 staining commonly identifies G2/M phases.<sup>20</sup> Several

reasons have been advocated for the discrepancy; namely, a very low amount of Ki-67 antigen undetectable by the antibody used, or the occurrence of a mutated protein.<sup>12</sup> The alteration of protein expression in nutritionally deprived cells has also been suggested,<sup>39</sup> together with the inability of the Ki-67 antibody to identify S-phase arrested tumour cells.<sup>38</sup>

The main finding of our study is that flow cytometric SPF is the most useful cell proliferation method in predicting the short term prognosis of patients with breast cancer, with the conventional median SPF category being the best indicator of disease outcome compared with other SPF variables and the Ki-67 index. Gasparini *et al* compared SPF with other immunohistochemical indicators of cell proliferation, such as Ki-67 and proliferating cell nuclear antigen (PCNA), in a consecutive series of 195 patients with breast cancer, and also concluded that SPF is the best cell kinetics marker to assess disease prognosis.<sup>15</sup> Similarly, Dettmar *et al* determined SPF and MIB-1 indices in their retrospective study of 90 node negative breast carcinomas, and showed by multivariate analysis that SPF has the highest prognostic value.<sup>12</sup> However, it has to be taken into account that, despite promising results, SPF could not be assessed in 23.5% of our cases, owing to technical drawbacks. Furthermore, the high intratumour heterogeneity of breast carcinoma might affect SPF determination, which is a crucial problem when applying this parameter to the individual patient. To improve the accuracy of the method, some authors<sup>36</sup> have recommended the separate analysis of multiple samples from the same specimen.

In conclusion, the comparative study of SPF and the Ki-67 index in breast carcinoma showed that: (1) the two methods show reasonable agreement; (2) Ki-67 appears to have limited prognostic usefulness; (3) flow cytometric SPF is a better prognosticator than the Ki-67 index; and (4) only the SPF variables assessed in the whole series constitute reliable proliferative indicators for estimating the disease outcome.

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# MP

## MOLECULAR PATHOLOGY

### Contents

### June 2001 Vol 54 No 3

#### Review

- 121 The IGF system in thyroid cancer: new concepts *V Vella, L Sciacca, G Pandini, R Mineo, S Squatrito, R Vigneri, A Belfiore*
- 125 The three dimensional structure of the type I insulin-like growth factor receptor *C W Ward, T P J Garrett, N M McKern, M Lou, L J Cosgrove, L G Sparrow, M J Frenkel, P A Hoyne, T C Elleman, T E Adams, G O Lovrecz, L J Lawrence, P A Tulloch*
- 133 IGF-I receptor signalling in transformation and differentiation *B Valentinis, R Baserga*
- 138 The IGF axis and hepatocarcinogenesis *J-G Scharf, F Dombrowski, G Ramadori*
- 145 Signalling pathways involved in antiproliferative effects of IGFBP-3: a review *R C Baxter*
- 149 Insulin-like growth factor 1 and oestradiol promote cell proliferation of MCF-7 breast cancer cells: new insights into their synergistic effects *J Dupont, D Le Roith*

#### Papers

- 155 The molecular basis of allergenicity: comparative analysis of the three dimensional structures of diverse allergens reveals a common structural motif *R Furmonaviciene, F Shakib*
- 160 Loss of sequences on the short arm of chromosome 17 is a late event in squamous carcinoma of the cervix *C S Herrington, M Worsham, S A Southern, P Mackowiak, S R Wolman*
- 165 A high degree of chromosomal instability at 13q14 in cutaneous squamous cell carcinomas: indication for a role of a tumour suppressor gene other than Rb *D P O'Connor, E W Kay, M Leader, G M Murphy, G J Atkins, M J E M F Mabruk*
- 170 5' flanking sequence of the human immediate early responsive gene *ccn1* (*cyr61*) and mapping of polymorphic CA repeat sequence motifs in the human *ccn1* (*cyr61*) locus *N Schütze, N Rücker, J Müller, J Adamski, F Jakob*
- 176 Regulation of connective tissue growth factor (*ccn2*; *ctgf*) gene expression in human mesangial cells: modulation by HMG CoA reductase inhibitors (statins) *M Goppelt-Strube, A Hahn, D Iwanciw, M Rehm, B Banas*
- 180 The control of *ccn2* (*ctgf*) gene expression in normal and scleroderma fibroblasts *A Leask, S Sa, A Holmes, X Shirven, C M Black, D J Abraham*
- 184 The expression of *ccn3* (*nov*) RNA and protein in the rat central nervous system is developmentally regulated *B-Y Su, W-Q Cai, C-G Zhang, V Martinez, A Lombet, B Perbal*
- 192 Identification of human *ccn2* (connective tissue growth factor) promoter polymorphisms *I E Blom, A J van Dijk, R A de Weger, M G J Tilanus, R Goldschmeding*

#### Short report

- 197 In situ amplification of oestrogen receptor  $\alpha$  mRNA in breast cancer cell lines and tumours *L Lespagnard, D Gancberg, Y Maaroufi, G Leclercq, D Larsimont, A Verhest*

#### Miscellanea

- 200 Correspondence • Correction • Notice