Mitotic figure counts are overrated in resection specimen of invasive breast carcinoma.

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Abstract

In order for some patients to benefit from aggressive chemotherapy for invasive breast carcinoma, many patients are currently being treated without little or no benefit. Enormous effort is hence being directed towards the identification of those patients who will need chemotherapy and those who will not. Since chemotherapy targets proliferating cells pathologists focus on the proliferative activity of tumors, as assessed by mitotic figure counts or by cell cycle specific immunohistochemical markers, such as Ki-67 and H3 histone. As far as the tumor grade is concerned, many of these studies have reported a tendency to up-grade carcinomas in resection specimen when compared to the initial diagnosis on the biopsy material, and most studies have noted that the upgrade in resection specimen is due solely or to a large extent to an increase in the mitotic figure count. In the present study, we propose a different explanation for the divergence in mitotic figure counts between biopsy and resection material. We assessed the proliferative activity of 52 invasive ductal carcinomas and confirm that the number of mitotic figures significantly increased by a factor of more than 3 in resection specimen over the biopsy material, while at the same time the pan-cell cycle specific marker MIB-1 yieldes comparable results. we propose that the delayed formalin fixation of resection specimen allows cell cycle activities to continue for a long time, up to many hours, and

that this leads to an arrest of mitoses in metaphase where they are readily identified by the pathologist. We propose that the mitotic figure count in the rapidly fixed biopsy cores better represent the tumor biology and should be used as a basis for chemotherapy therapeutic decisions.

Key words:
Breast cancer – mitotic figure counts – MIB-1 – biopsy – resection specimen

Introduction

In order for some patients to benefit from aggressive chemotherapy for invasive breast carcinoma, many patients are currently being treated without little or no benefit. Enormous effort is hence being directed towards the identification of those patients who will need chemotherapy and those who will not. Since chemotherapy targets proliferating cells (Amadori et al., 2000) pathologists focus on the proliferative activity of tumors, as assessed by mitotic figure counts or by cell cycle specific immunohistochemical markers, such as Ki-67 (Cattoretti et al., 1992) and H3 histone (Tapia et al., 2006). In fact, the mitotic figure count is a key element of the time-honored gradeing of invasive breast carcinomas and has been routinely assessed in breast carcinomas for more than five decades (Elston and Ellis, 1991). Since the advent of core needle biopsies to diagnose breast lesions, many authors have systematically compared the results obtained in biopsy material with the results obtained in resection specimen. As far as the tumor grade is concerned, many of these studies have reported a tendency to up-grade carcinomas in resection specimen when compared to the initial diagnosis on the biopsy material, and most studies have noted that the upgrade in resection specimen is due solely or to a large extent to an increase in the mitotic figure count (Di Loreto et al., 1996; Dahlstrom et al., 1996; Sharifi et al., 1999; Denley et al., 1998; Harris et al., 2003; Badoual et al., 2005 Burge et al., 2006). Explanations for this phenomenon have been sought in intratumoral heterogeneity and in sampling artefacts, proposing that the biopsy material is either too small or taken at random from anywhere within the tumor and not necessarily from the proliferatively most active tumor periphery (Sharifi et al., 1999; Denley et al., 2000; Harris et al., 2003; Badoual et al., 2005 Burge et al., 2006). It has even been suggested that discrepancies in tumor grades could be avoided by increasing the number and the size of the biopsy cores, but no hard data have ever been provided to underscore that idea (Harris et al.,2003; Badoual et al., 2005). Finally, several authors have put forward that in case of grade discrepancies, therapeutic decisions should be based on the grade obtained in the resection specimen (Denley et al, 2000; Lorgis et al., 2011).

In the present study, we propose a different explanation for the divergence in mitotic figure counts between biopsy and resection material. We assessed the proliferative activity of 52 invasive ductal carcinomas and confirm that the number of mitotic figures significantly increased by a factor of more than 3 in resection specimen over the biopsy material, while at the same time the pan-cell cycle specific marker MIB-1 yieldes comparable results. In contrast, we find that the proportion of post-metaphase figures (anaphase and telophase) among the counted mitoses in resection specimen is less than half of the number found in biopsies. Based on these findings, we propose that the delayed formalin fixation of resection specimen allows cell cycle activities to continue for a long time, up to many hours, and that this leads to an arrest of mitoses in metaphase where they are readily identified by the pathologist. We propose that the mitotic figure count in the rapidly fixed biopsy cores better represent the tumor biology and should be used as a basis for chemotherapy therapeutic decisions.

Methods

Patients: We selected 52 consecutive cases of invasive ductal carcinomas from the archives of the institute of pathology. Only those cases were chosen for which we disposed of paraffin blocks of true-cut biopsy material as well as resection specimen (tumorectomies or mastectomies). The slides were assigned random codes from 1 to 104 which were used for the entire series of experiments. No patient identifiers were used for any of the different part of the study. The study protocol was approved by the local ethical committee (CEV-VD BB09-15).

Mitotic figure counts: Hematoxylin and eosin stains were reformed according to standard protocols on 4µm section and used for mitotic figure counts. Mitoses were counted in 10 high power fields (Olympus BX-45 diagnostic microscope, 0.52mm diameter of the high power field), corresponding to grade cut-offs of 0-6 (grade 1), 7-14 (grade 2) and >15 (grade 3) (quote EUSOMA guidelines). As in our daily diagnostic routine, the guidelines of van Diest and coworkers (1992) were applied for the identification of mitotic figures. In biopsy material, areas were selected at random for mitotic figure counting typically where the high power field was filled with invasive carcinoma as much as possible. Ten complete high power fields could be counted in 47 or the 52 carcinomas, and in the remaining 5 cases, between 5 and 9 high power fields could be quantified. We had excluded from the study another 8 cases because we considered that insufficient material was contained in the biopsy core to permit a reliable comparison. In resection specimen, we selected the proliferatively most active tumor periphery, but avoiding hot-spots. Mitotic figures were counted jointly by two observers (HAL and CR)

under a discussion microscope. Each mitotic figure was pointed out with an arrow in the microscope tube and only those mitoses were counted for which consensus was obtained by the both observers. We not only counted the total number of mitotic figures, but noted their phase of the cell cycle (prophase, prometaphase, metaphase, anaphase, and telophase, see figure 1). In addition, we estimated for each HPF the percentage of the field covered by tumor cells (vs. stroma and preexisting glands/lobules). In the same way, we counted the number of apoptotic figures per 10 HPF, again under a discussion microscope and by consensus between two observers (AN and CR).

Immunohistochemistry: Immunohistochemical assays were performed using an automated system from Ventana (Benchmal XL, Ventana, Tucson, AZ) on 4µm sections on super frost slides, after heat induced epitope retrieval (30min, EDTA buffer, proprietary information retained by Ventana). We used antibodies to Ki-67 (MIB-1, clone M7240, DAKO, Glostrup, Denmark, dilution 1:40) and the phosphorylated histone H3 (Ser28, clone 117C826, IMGENEX, San Diego, CA, dilution 1:1000).

Quantification of MIB-1 immunostained slides was performed in three different ways: (i) rough estimation of the percentage of MIB-1 immunostained tumor cells, usually done at a 20x magnification and assessing about 3-5 fields in the biopsy at random or in the proliferatively active tumor periphery in resection specimen, avoiding hotspots. In analogy to mitotic figure counting, MIB-1 estimation was performed by two observers (HAL and CR) using a discussion microscope and consensus was obtained for all cases. In practice, both observers jointly scanned about five to ten 20x fields for about 30seconds, each, made mental notes of our MIB-1 based proliferative index, and then compared our results. In case of discrepancy, the case was discussed and a consensus index could be obtained in every case. (ii) The percentage of MIB-1 positive tumor cells was counted by hand (CR) on high quality color print-outs of five circular high power fields that were cropped from screen-shots of virtual microscope slides (Hamamatsu NanoZoomer, Hamamatsu City, Japan). (iii) Finally, we used the publicly available internet-based analysis software IMMUNORATIO to quantify the percentage of MIB-1 immunostained tumor nuclei (http:// imtmicroscope.uta.fi/ immunoratio/), uploading jpg files of the circular high power fields cropped from the virtual slides. This software identifies the surface covered by hematoxcylin counterstained (blue) cells and the surface covered by immunoreactive (brown) cells and calculates an index of immunostained cells over the total cell number (Tuominen et al., 2010). Quantification of H3 immunohistochemistry was performed by estimating the number of immunoreactive (brown) cells among all tumor cells in five 20x fields.

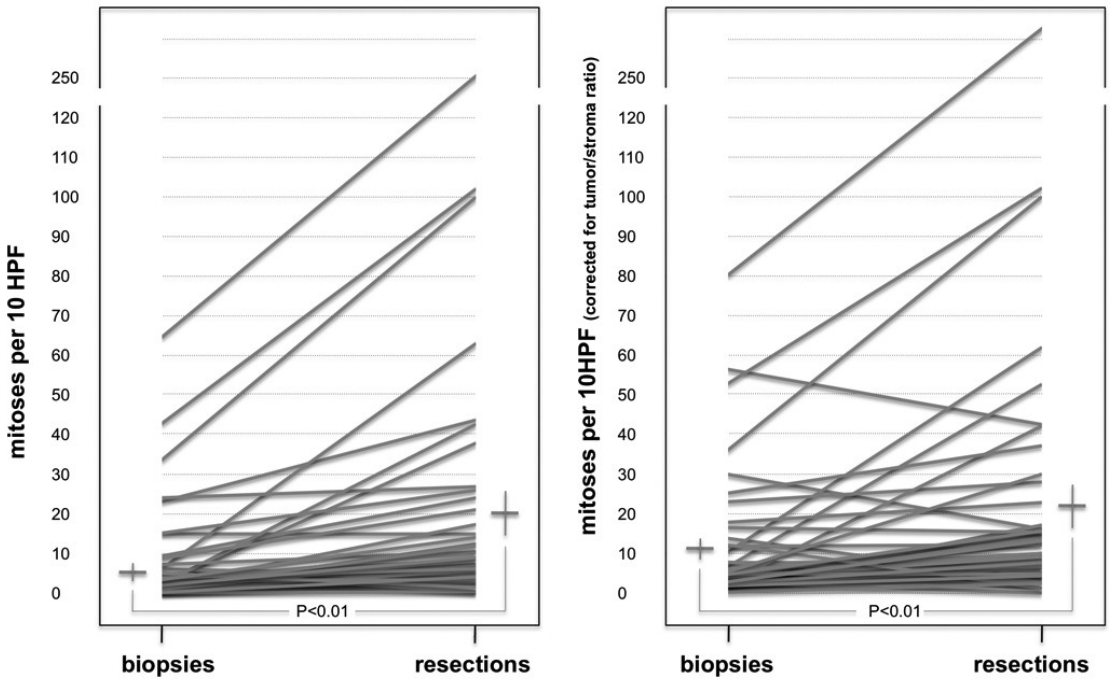
Photoshop-based image analysis: We used photoshop-based image analysis to quantify the size of the biospy cylinders. For that purpose, the 52 biopsy slides were scanned using a commercially available flatbed scanner (EPSON perfection photo 3200) and the image was opened in Photoshop (version CS3, Adobe Systemc Inc, San José) on a MacPro (Apple computers, Cupertino, CA). In analogy to previously published procedures (Lehr et al., 1999), the biopsy cores were individually selected using the magic wand tool and the number of pixels (indicated in the histogram window) was then used to calculate the surface of the cores (in mm2).

Statistical analysis: The data obtained in the various analyses were manually entered in an excel file and statistical analyses were calculated using a publicly available internet-based program (http://www.wessa.net/stat.wasp). We performed simple regression and Spearman analyses for the correlations between mitotic figure counts and immunohistochemical read-outs, and bivariate paired t-tests to compare the results obtained in biopsies with those obtained in resection specimen. P-values of <0.01 were considered statistically significant.

Results

52 breast carcinomas were analysed, from which we had slides and blocks of both true cut biopsies and resection specimen in our archive. The cases were from the years 2008 to 2011.

The **number of mitoses per 10 high power fields** ranged from 0 to 64 for biopsies and from 0 to 250 for tumorectomies. Mean values ± SD were 6.23 ± 11.70 for biopsies and 19.99 ± 39.36 (P=0.00800 paired t-test, A vs. I). The increase in the number of mitotic figures from the biopsy to the resection specimen was 3.43-fold (SD 3.71), with a range of 0.25 to 12.30. The results are shown in graphic form in Figure 1A. When the numbers of **mitotic figures were normalized to the surface of the HPFs taken up by tumor cells (**vs. stroma and preexisting glands/lobules), the numbers ranged from 0 tp 80 for biopsies and 0-263 for tumorectomies. Mean values ± SD were 10.11 ± 15.53 for biopsies and 21.12 ± 40.58 for tumorectomies (P=0.00737 paired t-test, Q vs. R). The increase in the number of mitotic figures from the biopsy to the resection specimen was 2.96-fold (SD 3.18), with a range of 0.08 to 18.90. The results are shown in graphic form in Figure 1B.



Motivated by the proposal by Harris (2003) and others that the discrepancy of mitotic figure counts between biopsies and resection specimen should be reduced by increasing the size of the biopsies, we correlated the increase of mitotic figure counts for each case pair with the size of the biopsy fragments, as assessed by Photoshop-based image analysis, which ranged from 2.5 to 104mm^2 (mean 9,6+/-14.3), but could not identify such a correlation (rho=0.01859, P=0.8952, Spearman I/A vs. CD)

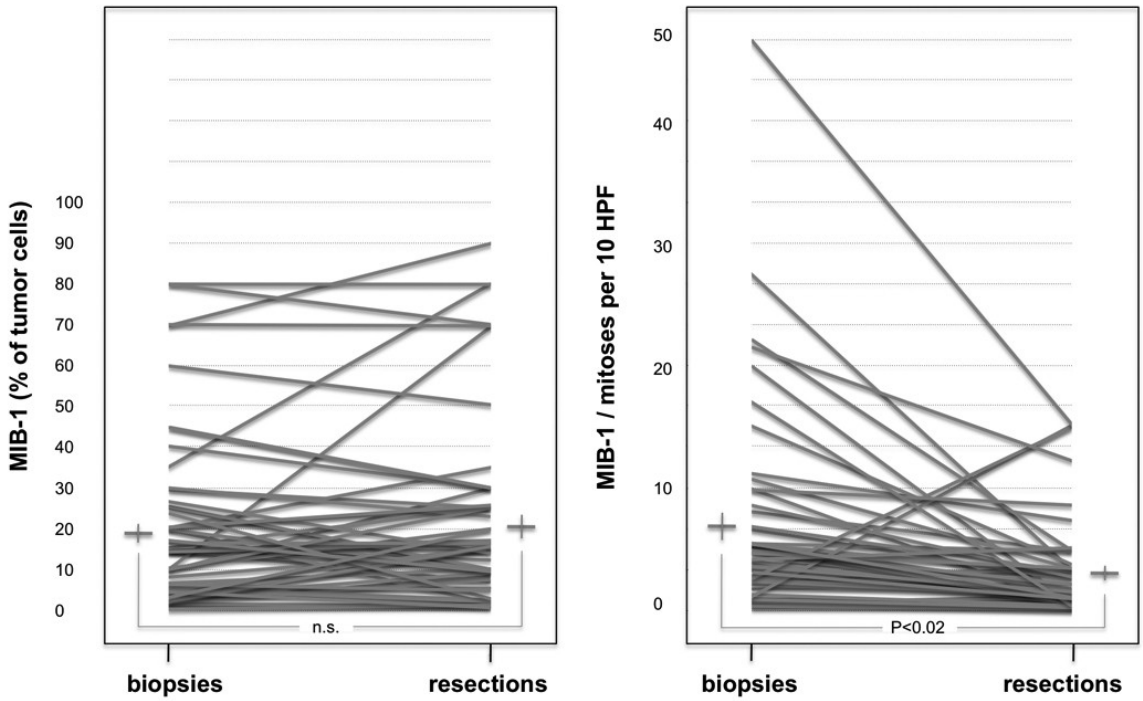
We next analysed whether this increase in **mitotic figures affect the overall grades** of the respective carcinomas. We found that based on our mitotic figure counts (per 10HPF), 14 of the 52 carcinomas were upgraded in the tumorectomies (26.9%; 6 from grade 1 to grade 2 and 8 from grade 2 to grade 3) and 5 were downgraded (9.6%; 4 from grade 2 to grade 1 and 1 from grade 3 to grade 2). The concordance rate between grades in biopsies and resection specimen was 63.4%. Similar numbers were obtained when the mitotic figures are corrected for the tumor/stroma ratio: 15 carcinomas were upgraded (28.8%; 6 from grade 1 to grade 2 and 9 from grade 2 to grade 3) and 6 were downgraded (11.5%; 4 from grade 2 to grade 1). Concordance rate: 59.6%. If we compare this to the grades that were recorded in the original pathology reports of the respective patients, the observations were virtually superposable: 15 cases experienced an upgrade (28.8%; 5 from grade 1 to grade 2 and 10 from grade 2 to grade 3) and 5 a downgrade (9.6%; 3 from grade 2 to grade 1 and 2 from grade 3 to grade 2). The concordance rate was 61.5%. These figures are at the lower end of the spectrum that has been reported by others (concordance rates between 62% and 81%; Di Loreto et al., 1996; Dahlstrom et al., 1996; Sharifi et al., 1999; Denley et al., 1998; Harris et al., 2003; Badoual et al., 2005 Burge et al., 2006).

Next, we sub-classified the phases of the cell cycle of all the mitotic figures that we counted and found that there was a significant reduction in the percentage of **post-metaphase figures** (anaphase and telophase) from 6.93% of all mitotic figures in biopsies to 3.09% of all mitotic figures in resection specimen ($P<0.005$ paired Wilcoxon rank sum test with continuity correction AX vs AK).

Along with the increase in mitotic figures in resection specimen, we observed an increase - albeit somewhat less pronounced when compared to mitotic figures - in the count of **apoptotic figures** per 10 high power fields from 10.4 ± 11.44 (mean +/- SD) to 15.4 ± 12.4 (P=0.00261029 paired t-test H vs P).

We obtained **MIB-1 immunohistochemical stains** of all biopsies and tumorectomies and analyzed the percentage of MIB-1 stained tumor cells among all tumor cells by three different means: (i) rough estimation („eyeballing“) by two observers (HAL and CR), (ii) counting (on high quality color print-outs of circular areas, 0.74mm in diameters, cropped from digitized slides), and (iii) image analysis using a IMMUNORATIO. We found that for all three modes of MIB-1 quantification, there was no difference between biopsies and tumorectomies: 19.8% vs. 21.6% for eyeballed data (Fig. 2A), 21.7% vs. 22.4% for counted data and 14.2% vs. 13.6% for image

analysis, biopsies versus tumorectomies, respectively (n.s. paired t-test). At the same time, the ratzion of MIB-1 over mitoses was significantly reduced in resection specimen (Fig. 2B).

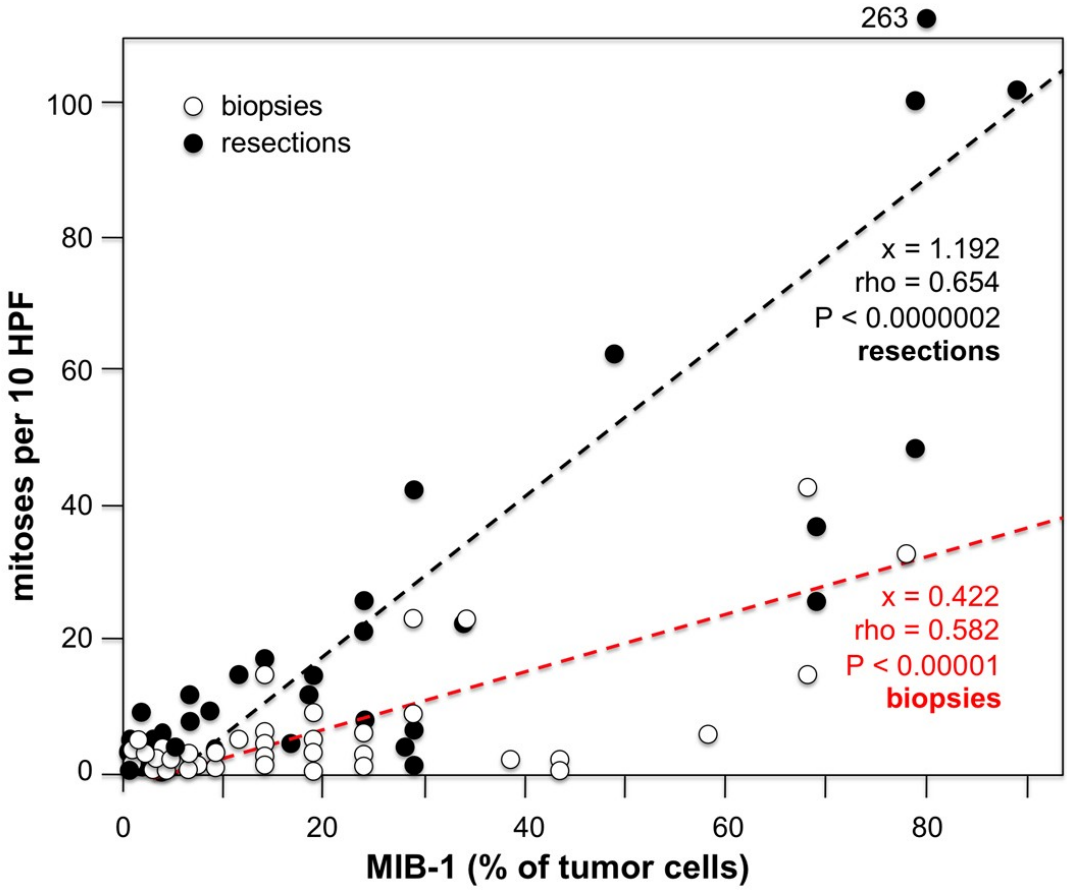


We next calculated simple linear regressions between mitotic figure counts (with and without correction for tumor/stroma ratio) and the percentage of MIB-1 positive nuclei (using the three distinct modes of assessment). The results are shown in table 1.

Table 1: **Linear regression analysis mitoses vs. MIB-1 immunohistochemistry in biopsies and tumorectomies/mastectomies**

	biopsies		**tumorectomies / mastectomies**	
	mitoses/10HPF	mitoses (corr)	mitoses/10HPF	mitoses (corr)
MIB-1 eyeballed	slope x=0.423 rho=0.583 P<0.00000582	slope x=0.523 rho=0.472 P<0.00040464	slope x=1.204 rho=0.654 P<0.0000001	slope x=1.192 rho=0.514 P<0.000098
MIB-1 counted	slope x=0.407 rho=0.546 P<0.00002808	slope x=0.514 rho=0.458 P<0.00064135	slope x=1.235 rho=0.644 P<0.00000025	slope x=1.260 rho=0.518 P<0.0000830
MIB-1 Immunor.	slope x=0.559 rho=0.461 P<0.00058774	slope x=0.707 rho=0.396 P<0.0036890	slope x=1.668 rho=0.592 P<0.0000038	slope x=1.69 rho=0.496 P<0.0001847

We noted a strong, highly significant correlation between the number of mitotic figures and the percentage of MIB-1 positive tumor cells. The correlation was slightly stronger for uncorrected data (i.e. for mitotic figure counts than for mitotic figure counts that were corrected for the tumor/stroma ratio). The rho values for the correlation between MIB-1 and mitotic figure counts correspond well to data published previously by ourselves (Lehr et al., 1999) and others (Weidner et al., 1994). It also became apparent – in agreement with the significantly higher numbers of mitoses in tumorectomies versus biopsies - that the slopes of the regression curves differ markedly between biopsies and resection specimen, so that for a comparable MIB-1 index, the number of mitotic figures was consequently higher in the resection specimen than in the biopsies. These data are shown in graphic form in figure 3 for uncorrected mitoses and estimated MIB-1 values.



Using the paired t-test, we found highly significant differences concerning the ratio of MIB-1 values over mitotic figures counts in biopsies and their corresponding resection specimen (P values were 0.00169, 0.000184, and 0.0000951 for estimated, counted, and computer-based MIB values over mitoses/10HPF and 0.0196, 0.00995, and 0.00517 over mitotic counts normalized to the tumor/stroma ratio, respectively, paired t-test BH/BI, BD/BE, AZ/BA and BJ/BK, BF/BG, and BB/BC).

The percentage of H3-immunostained cells per total number of tumor cells showed a high variation between cases and was comparable in biopsies and resection specimen (3.27+/- 3.21 vs. 3.65+/- 3.67, n.s. paired Student t-test).

Discussion

This paper confirms observations made by other authors in the past, namely that the number of mitotic figures is higher in resection specimen of breast carcinomas than in core needle biopsies and that this results in an increase in tumor grades in around 25-35% of carcinomas (Di Loreto et al., 1996; Dahlstrom et al., 1996; Sharifi et al., 1999; Denley et al., 1998; Harris et al., 2003; Badoual et al., 2005 Burge et al., 2006). However, our interpretation of these data is fundamentally different from the one advanced in these previous reports. We do not believe that the discrepancy in mitotic figure counts is due to a sampling artefact, where biopsies are taken at random from anywhere within the tumor, and not necessarily the proliferatively active tumor periphery. If this was the case, the percentage of MIB-1 positive tumor cells should also be higher in resection specimen than in biopsies. However, neither our study (Fig 2A) nor results published by other authors suggest that this is the case (Cavaliere et al, 2005). Also, we have no reason to believe that the discrepancy in mitotic figure counts could be ascribed to the limited size of the biopsies (which could potentially preclude proper mitotic figure counting, Harris et al. 2003), because there appeared to exist no correlation between the size of the biopsies and the increase in mitotic figure counts between biopsies and resection specimen (rho=0.01859, P=0.8952).

Rather, we propose that the difference in mitotic figure counts between biopsies and resection specimen reflects a biological phenomenon, presumably secondary to intraoperative stress and specimen handling after removal from the breast. While biopsies are taken from the intact tumor and immediately immersed in formalin, resection specimen are exposed to intraoperative trauma and warm ischemia (i.e. due to ligation of feeding arteries to avoid bleeding), postoperative ischemia (i.e. during transport to radiology for specimen radiography and then on to pathology), and a poorly defined fixation delay in the formaldehyde container due to slow penetration of the fatty tissue by the fixative. If one assumes that formaldehyde penetrates tissues at a speed of 1mm/hour (Start et al., 1992), then the biopsy is entirely fixed within 30min, arresting all biological processes. In contrast, tumor cells in the resection specimen may benefit from many additional hours of more or less unimpaired biological activity before eventually being stopped by dwindling energy sources or by being fixed by the slowly advancing formaldehyde front. With this consideration in mind, we assume that tumor cells, once having

trespassed the G0/G1 checkpoint, are fatefully bound towards mitotic cell division. The cell cycle machinery will continue to work towards that aim even after removal of the tumor from the breast and metabolic activities are maintained by intracellular glucose through the Warburg effect. However, in the absence of sustained energy supply, the gradually declining energy reserves may eventually arrest the cell cycle at the G2/M checkpoint: recent studies have shown that the mitotic machinery involves a metabolic sensor, the AMP-activated protein kinase (AMPK, Koh & Chung, 2007) , which binds to various structures of the mitotic apparatus, including centrosomes, spindle poles, and the spindle midzone (Vazquez-Martin et al., 2009A). This mechanism is understood as en energy gauge that assures that the cell disposes of sufficient energy to complete faithful chromosome separation and thus exerts an important cytokinetic suppressor function (Vazquez-Martin et al., 2009B). It is conceivable that mitotic activity continues in resection specimen up to the G2/M checkpoint, and that in the absence of sufficient energy reserves, tumor cells arrest in metaphase, where they are easily identified and counted by the diagnosing pathologist. The fact that we found significantly fewer post-metaphase figures (anaphase, telophase) in the resection specimen of our study (3% versus almost 7% in biopsies), would be consistent with such a concept. Also, this idea would reconcile the apparent dilemma that despite markedly increased mitotic figure counts, MIB-1 immunoreaction seems not to go up in resection specimen (fig.2A and Cavaliere et al., 2005). MIB-1 labels all cells in the cell cycle (Cattoretti et al., 1992), including cells in S and in G2 phase (which are not identifiable as proliferating cells in standard H&E sections), and would hence not be altered by a gradual (and artefactual) progress of these cells into mitose.

Finally, evidence for ongoing biological activity in resection specimen may also be drawn from the observation of significantly increased numbers of apoptotic figures in resection specimen when compared to biopsies, albeit to a smaller extent and likely involving other biological mechanisms then the ones considered to apply to mitoses (Klein et al., 2005).

In conclusion, we propose that the increased mitotic figure counts in resection specimen are secondary to poorly defined artefacts, which are probably quite variable from specimen to specimen and likely difficult to control in the clinical setting. Putative mechanisms include warm ischemia and/or protracted formalin fixation. Based in the consideration raised above, we propose that in the clinical routine, chemotherapy treatment decisions should be based on the assessment of mitotic figures in the biopsies, and not - as proposed by most authors - in the resection specimen (Harris et al., 2003; Badoual et al., 2005). This also suggest that in case of discrepancies, particular attention should be payed to the MIB-1 based proliferative activity, which appears not to be affected by the described problems. In fact, similar recommendations have been advanced also for other biomarkers, including hormone receptors and the Her2/*neu*

status and should raise the awareness that good biopsy material of sufficient quantity and quality is an essential basis for (neo-) adjuvant treatment decisions, and that the examination of the resection specimen should be limited to additional informations concerning tumor extension and spread (size, lymphovascular invasion, resection margins, presence of associated in situ lesions).

H3: sensitive of delayed fixation, as suggested by Tapia et al., 2006. This has also been described by Hirata and coworkers (2004). May hence not work in resection specimen. It this due to a similar mechanism, where metaphase-arrested cells do not react with the antibody because the antigen degrades/desintegrates?

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