CHAPTER 5

QUANTIFICATION OF RELATIVE AREA OF pS2 IMMUNOHISTOCHEMICAL STAINING AND EPITHELIAL PERCENTAGE IN BREAST CARCINOMAS. THE EFFECT OF THE LATTER ON THE INTERPRETATION OF A CYTOSOLIC pS2 ASSAY.

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CHAPTER 5

Abstract
In immunochemical assays of specific cell constituents in cytosols from tumors, the relationship between epithelial and stromal fractions is not taken into account. This may influence the outcome of the measurements and result in incorrect categorization as negative or positive. In a setting addressing pS2 (only detectable in the epithelial cells) in breast carcinomas, we investigated three possibilities that may overcome this problem using histologic sections of the breast carcinomas of 50 patients: (a.) visual estimation of area percentage (AREA%) of immunohistochemical staining of the cell constituent of interest performed by three independent individuals, (b.) quantification of AREA% of the IH results by true color image analysis, and (c.) quantification of the epithelial and stromal compartments of the tumors in Heidenhain’s-azan-stained tissue sections, using the true color image analysis system (IAS), to assess the epithelial percentage (EPIT%) in the tumors. This percentage was used as a correction factor for data on pS2 obtained by cytosolic determinations. Visual estimation appeared to be subject to interobserver variation and, subsequently, becomes less applicable in the absence of strict scoring rules. Based on tests for correlation, IAS quantification seemed reproducible in both quantification procedures. However, due to the high magnification necessary to visualize the IH staining product, the effect of field selection caused systematic differences between repeat measurements (Friedman test). As a result of the contrasting colors of the azan staining, the calculation of the EPIT% could be performed at a low magnification. Consequently, here the effect of field selection was not present. Correction of cytosolic values for EPIT% resulted in 8% of the cases changing category. In conclusion, the combined results of the two disciplines (histology and biochemistry) may provide more information than either of them alone.
CHAPTER 5

Introduction
In biochemical assays of cell constituents in breast carcinoma samples, the proportions of epithelial cells and stromal components cannot be taken into account. As a consequence, variation in cellularity in tumors may affect the categorization as negative or positive. With histologic material, it is possible to evaluate the cellularity of a tumor. The introduction of immunohistochemistry allowed performance of quantifications of a specific cell constituent on histologic sections, with a concentration on areas of interest. Several quantification procedures are described, including subjective categorization as positive and negative, estimation of the percentage of positive-staining cells, the histochemical score (HSCORE) and methods using a computerized image analysis system (IAS)\textsuperscript{104,116,117,119,133,134}. However, there is no consensus about scoring or reporting results\textsuperscript{135}. In methods of applying IASs, because of the limited time available for measurements, only a relatively small part of the tumor is generally measured. This may affect the reproducibility of the measurements. As both the biochemical assay and IAS quantification of immunohistochemical (IH) staining have disadvantages, it may be valuable to obtain additional information that allows correction of the values of the biochemical assay. As van Netten et al.\textsuperscript{136} explained over 10 years ago the cellularity of a tumor may be used as correction factor for biochemically obtained values.

In this study we addressed the above mentioned problems in a setting concerning pS2 measurements in 50 cases of breast cancer. We evaluated the reproducibility of quantification of IH pS2 staining by visual estimation and by true color image analysis and compared this to the reproducibility of quantification of the epithelial percentage (EPIT\%\textsuperscript{104}) of the tumors, for which the IAS was also used. Furthermore we evaluated the effect of the EPIT\% as correction factor for immunochemical cytosolic pS2-assay data.

Material and methods
Patient Material
The tumors studied for quantification of IH staining and tissue components were part of the material used before, comprising a group of 205 patients with operable breast cancer who underwent breast conserving surgery or a modified radical mastectomy with axillary lymph node dissection\textsuperscript{109}. From this group we obtained formalin-fixed, paraffin-embedded tissue samples of the breast carcinomas (all of infiltrating duct type) of 50 patients. The material was kindly provided by the Institutes of Pathology of Dordrecht, Middelburg and the Zuiderziekenhuis, Rotterdam, the Netherlands. From five patients we obtained more than one tissue block (two (3x), three (1x), four (1x) blocks). This amounted to a total of 58 tissue
samples.

For IH staining the 58 tissue sections were incubated with a monoclonal antibody directed against pS2 (Mab BC4 anti-pS2, CIS-Bio International, Gif-sur-Yvette, France), an estrogen regulated protein. The IH staining for pS2 was performed on 4 \( \mu \)m thick sections using a streptavidin-biotin peroxidase method and 3-amino-9-ethylcarbazole as described previously.

For quantification of the EPIT% in the tissue samples we used a connective tissue stain, known as Heidenhain’s azan, in which collagen stains blue, and chromatin, muscle tissue and erythrocytes stain red. In the breast carcinoma samples the epithelial parts stained red and the stromal parts, containing merely collagen, blue. Stromal parts containing fat tissue remained unstained. In cases where there was more than one tissue block available only one was used for quantification of the EPIT%. In this way 50 values were available for evaluation of EPIT% as correction factor for the cytosolic pS2 data of the 50 patients. These cytosolic data were obtained with a radiometric immunoassay (ELSA-pS2, CIS-Bio International, Gif-sur-Yvette, France) and were from those reported before. In this ELSA assay, the BC4 monoclonal antibody was coated on the solid phase.

**IAS Quantification**

Image analysis was performed using the VIDAS IAS. The hardware of this system consists of an Axioplan microscope with a halogen illuminator (Carl Zeiss, Oberkochen, Germany) fed by a stabilized power source, a low cost, single chip charge coupled device (CCD) color camera (WV-CD130, Panasonic, Matsushita Communication Co. Ltd., Yokohama, Japan), and a personal computer based on a 286 AT processor equipped with a frame grabber and expanded with a 287 mathematic coprocessor (Kontron Elektronik, Eching, Germany). The software used for quantification was VIDAS 2.0, capable of processing true color images, which are formed by a red, green, and blue (RGB) image partition, respectively. The quantification is performed without shading correction and by application of fixed threshold levels for segmentation, as previously described. Images for quantification of IH staining in the 58 tissue sections were recorded using an oil immersion 16× objective (numerical aperture=0.50). In an area representative for the staining in the tumor, the relative area percentage (AREA%) of positive IH staining of 10 image fields was measured. This correlated to a total measured surface of 1.2 mm\(^2\) on specimen level. In the evaluation of the reproducibility, for which the measurements were repeated, the mean values of the 10 AREA%\(\text{s}\) of IH staining were used. When more than one tissue block was available the AREA%\(\text{s}\) of IH staining of the different tissue sections were used as separate values in this evaluation. The proportion of positive staining cells or the intensity of staining was
not included in the measured values.

The quantification procedure on the 50 tissue sections to obtain the EPIT% was developed in a similar way. In this procedure the red (epithelial), blue and white (stromal) AREA% s were quantified in a maximum of 10 images per specimen, which corresponded to a total measured surface of 47.5 mm² on specimen level, using an objective with a magnification of 2.5× (numerical aperture = 0.075). For segmentation of the EPIT% and stromal AREA% s we used three sets of fixed threshold levels. The measurement of an image field was accepted if the mean sum of the EPIT% and the stromal AREA% s did not exceed 100 ± 10%. As in the quantification of AREA% of IH staining the EPIT% measurements were performed in areas containing tumor. Because it is not possible to differentiate between benign or malignant epithelium in the azan staining measurements were restricted to fields containing merely cancerous epithelium to prevent influence of normal epithelium in the estimation of the EPIT%. As a control for the IAS method, interactive morphometry was performed on 15 specimens by point counting. To evaluate reproducibility the image processing procedure was applied a second time for 12 breast carcinoma samples in which the surface of the tumor exceeded 47.5 mm².

Visual estimation of IH staining.
To evaluate the applicability of visual estimation as compared with IAS quantification we tested for inter- and intraobserver variation. Three investigators (FW, MN, HFE) independently estimated AREA% of IH staining of the positive part of the tumor, evaluating the whole tissue section. Comparison of intraobserver variation was performed by replication of the visual estimation by one of the investigators (FW).

Results
Immunohistochemistry
pS2 Immunoreactivity was localized cytoplasmatic. Both intensity and distribution of positive staining showed some heterogeneity. The most positive part of the tumor was used for measuring. Sometimes there was background staining, mainly restricted to the borders of the specimen. These areas were not used for quantification.

Computerized AREA% measurements of IH staining of the 58 tissue sections ranged between 0.09 and 31.02% (Table 5.I). IAS quantification of one tissue section took 15 minutes. The Spearman rank coefficients of correlation between
Table 5.I. Image analysis system quantifications of area percentage (%) of immunohistochemical staining for pS2

<table>
<thead>
<tr>
<th>Measurement session</th>
<th>1st</th>
<th>2nd</th>
<th>3rd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum</td>
<td>0.11</td>
<td>0.21</td>
<td>0.09</td>
</tr>
<tr>
<td>Maximum</td>
<td>27.78</td>
<td>31.02</td>
<td>24.07</td>
</tr>
<tr>
<td>Mean</td>
<td>7.46</td>
<td>8.42</td>
<td>6.71</td>
</tr>
<tr>
<td>Standard dev.</td>
<td>6.42</td>
<td>7.79</td>
<td>6.73</td>
</tr>
</tbody>
</table>

Table 5.II. Visual estimation of area percentage (%) of immunohistochemical staining for pS2

<table>
<thead>
<tr>
<th>Investigator</th>
<th>FW1a</th>
<th>FW2a</th>
<th>MN</th>
<th>HFE</th>
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</thead>
<tbody>
<tr>
<td>Minimum</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Maximum</td>
<td>90</td>
<td>90</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>Mean</td>
<td>18.14</td>
<td>17.66</td>
<td>21.79</td>
<td>28.32</td>
</tr>
<tr>
<td>Standard dev.</td>
<td>23.49</td>
<td>25.09</td>
<td>26.22</td>
<td>30.12</td>
</tr>
</tbody>
</table>

* FW1 and FW2 represent first and repeat visual estimation by investigator FW
first and repeat AREA% measurements were $r_s=0.84$, $r_s=0.85$ and $r_s=0.86$ (for all $p<0.001$), suggesting acceptable reproducibility. Nevertheless, using analysis of variance, significant systematic differences were found between the successive quantifications (Friedman test, $p<0.0001$).

The values obtained by visual estimation ranged between 0 and 90% (Table 5.II). Visual evaluation of one tissue section never took more than 5 minutes. The Spearman rank coefficients of correlation between the visually obtained AREA% by the three respective investigators were: $r_s=0.90$, $r_s=0.86$ and $r_s=0.85$ (for all $p<0.001$). However, there were significant systematic differences between the three observers (Friedman test, $p=0.012$). There was a high Spearman rank correlation for the intraobserver variation between both estimations performed by FW ($r_s=0.93$, $p<0.001$). The intraobserver results, as compared with the interobserver results, did not show significant systematic differences between the replicate estimations (Friedman test, $p=0.47$).

**IAS Quantification of EPIT%**

The EPIT% in the 50 cases of the set ranged from 18.42 to 85.56% (mean=53.87%, standard deviation=14.47%). The results of the IAS method showed a good correlation with those obtained by point counting ($r_s=0.94$, $p<0.001$). Pairwise comparison of both sets of results did not show significant differences (Wilcoxon signed ranks test, $p=0.40$). Investigation of the influence of field selection showed a Spearman rank coefficient of correlation of 0.91 ($p<0.001$) between the first and second assessment of EPIT%. Analysis of variance did not show significant differences (Friedman test, $p=0.22$) between the repeat measurements. Figure 5.1 shows the effect of the EPIT% on the initial ELSA-pS2 values. Corrections were made according to the equation: \[ \text{corrected ELSA-pS2} = \frac{100}{\text{EPIT%}} \times \text{ELSA-pS2} \]. Correction resulted in one case with low cellularity changing from negative to positive, whereas three tumors with higher cellularity changed from positive to negative when compared with a corrected cutoff level. The initial cutoff level in the set was chosen in such a way that about one-third of the population in this study would be considered positive. The corrected cutoff level was obtained by multiplying the initial cutoff level by the mean overall correction factor.

**Discussion**

Quantification of IH staining in breast carcinomas mostly concerns estrogen receptor content. In studies describing this technique the results are in general compared to biochemical assays. Although these may be
considered as "golden standard" due to their technical and clinical validation there remains a considerable amount of discordance in results. These discrepancies are probably due to the notoriously heterogeneous nature of breast tumor tissues, in fact, this is one of the reasons for which IH quantification was devised. The introduction of true color IASs has made it possible, although with some restrictions, to separate and quantify the relative area of structures in histologic sections based on color information. However, the present study shows that reproducibility of this quantification procedure is limited: although repeat measurements showed good correlations, analysis of variance showed significant systematic differences. This is caused by the effects of field selection. To adequately quantify the AREA% of IH staining a relatively high magnification was needed. For this reason, and because of the limited time available for measurements, this resulted in a small surface covered per tissue section (1.2 mm²). Repeat measurements are therefore likely to be performed in different areas, which may explain the systematic differences. To reduce effects of field selection, measurements should be performed on a larger part of the tumor or a more objective selection method should be used, such as application of motor-controlled object positioning. These alternatives are time consuming and costly, respectively. Another alternative, visual estimation, which may be performed relatively fast and on the entire tissue section, is subject to even more individual variation related to the observer. However, in experimental designs, this method may be sufficiently reliable, provided it is performed under strict, predefined conditions on how and what to quantify.

Using the knowledge obtained with IAS quantification of IH staining, the present study shows that it is possible to quantify the EPIT% reliably and reproducibly. The use of a much lower magnification allowed measurement on a surface of 47.5 mm² within a comparable period of time. This way, no effects of field selection were found. The lower magnification could be used due to the contrasting colors of Heidenhain's azan. The red epithelial compartment could be separated easily from the blue and white stromal compartments by the single-chip CCD color camera. We realize that the azan staining may be suited for breast carcinomas but less so for similar approaches in

Figure 5.1. Effect of the epithelial percentage (X axis) as correction factor for cytosolic pS2 values (Y axis). The initial and corrected values were connected by a line to visualize the effect. When comparing the initial and corrected pS2 values with their respective cutoff levels (initial: solid line; corrected: dotted line), one of the initial values appeared to be false negative (corrected value represented as ▼), whereas three appeared to be false positive (corrected value represented as △).
tissues with more smooth muscle and/or erythrocytes and a heavy lymphocytic infiltration. Because both these components and epithelial cells stain red, this would result in overestimation of the EPIT%. The problem of lymphocytic infiltrations has also been encountered by others in comparable quantification procedures of EPIT% \(^{59}\). In our set of tumors they were not observed. Another reason for misestimation of the EPIT% of breast carcinomas may occur when normal epithelium is included in the measurements. In a method described by others \(^{59}\), as well as in our method, it is not possible to differentiate between normal and cancerous epithelium due to the staining methods used. Therefore, measurements are to be performed within selected areas within the tumors comparable to other quantification procedures in tumor pathology (e.g., approaches for counting mitotic activity).

Evaluation of the effect of EPIT% as correction factor on ELSA-pS2 values of the set of tumors showed that four cases (8%) changed category. In one case, an initially negative cytosol value changed to positive after correction with the EPIT%, whereas in three cases correction resulted in the opposite. Assuming that the pairwise cytosol and histologic samples contained comparable amounts of (cancerous) epithelial and stromal compartments, this may be explained as a result of the fact that in biochemical assays the relation between epithelial and stromal fractions is not taken into account. Hence, tumors with low cellularity containing cells with a high concentration of the cell constituent of interest or, vice versa, highly cellular tumors containing cells with a low concentration are incorrectly classified as negative or positive, respectively. The change of category after correction of the ELSA-pS2 value with the EPIT% of the tumor is an indication of the importance of the idea suggested over 10 years ago by van Netten et al. \(^{136}\). It remains to be determined to what extent an improvement in ELSA-pS2 ranking, or in rankings of other immunochemical parameters by EPIT% as a correction factor, may be reflected by improved patient selection for therapy. It must be noted that all technical aspects of tissue handling should be strictly standardized when the combination of the two techniques is to be performed in routine practice.

In conclusion, the effect of field selection in IAS quantification procedures should not be underestimated. Proper indications and applications of IAS in combination with results of biochemical assays may provide additional information that makes both procedures complementary.