Comparison of Apolipoprotein D Determination Methods in Breast Cancer

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Abstract. *Background: Apolipoprotein D (ApoD) is a promising prognostic and predictive factor in breast cancer, but the analysis methods and results vary. Patients and Methods: Determination of ApoD content by immunoelectrophoresis in tumour cytosol (EPC), immunohistochemistry (IHC) in whole sections (WS) and tissue micro arrays (TMA) were compared in 283 breast carcinomas. Results: With EPC, 45% and with IHC, 71% of the tumours were ApoD-positive. Correlation between the degrees of ApoD positivity by ECP and IHC was poor (R^2=0.04), caused by higher sensitivity of the IHC (resulting in many ECP negative carcinomas being IHC positive) and ApoD positivity of normal tissues and cysts (resulting in ApoD positivity by ECP in up to 33% of the IHC negative cases). Discrepancies between WS and TMA were considerable due to tumour heterogeneity. Conclusion: In breast cancer, IHC ApoD determination is superior to EPC analysis, but intratumor heterogeneity must be carefully considered when using TMA technology.*

Breast cancer is a challenging disease with a complex biology. Many prognostic factors exist (1, 2), while others are still under investigation (3, 4). One of the latter is the lipocalin apolipoprotein D (ApoD), a small glycoprotein of 24 kD. It is expressed in most body tissues and has important roles in normal human cell physiology (5) and also in cancer biology (6) (for review, see (7)). It has been hypothesised that ApoD is a marker for steroid signalling in breast cancer (7, 8), and its high affinity to arachidonic acid (9), progesterone and tamoxifen (10) makes it a very interesting putative prognostic and predictive marker in breast cancer.

In breast cancer research, two different methods have been used for ApoD determination, in tumour cytosol with fresh frozen material by means of a precipitating antibody used in immunoelectrophoresis (EPC) (11) and by an immunohistochemical (IHC) method on paraffin-embedded tumour material (12). The EPC method inevitably analyzes mixtures of epithelial cancer cells and other cells. IHC has the advantage in that epithelial cancer cells can be exclusively analyzed without non-cancer cells blurring the outcome. Another advantage of IHC is the possibility of studying the subcellular localization of ApoD staining, which clearly is impossible by the EPC method. Earlier, different laboratories developed their own antibodies against ApoD, which inevitably varied. In recent years monoclonal antibodies have become commercially available, facilitating standardization of IHC ApoD determination. A comparison of the EPC and IHC ApoD methods is still lacking. Moreover, tissue microarray (TMA) is increasingly used for IHC studies. The advantage of TMA-IHC over conventional whole tissue section (WS) IHC analysis is that much less work is needed. The TMA method is consequently less expensive and allows high throughput evaluation of tumour markers samples from the same area of the tumour. However, it is unclear whether TMA sampling gives the same result as WS-IHC analysis of ApoD. In a recent study on the androgen receptor (AR) (13), TMA sampling required up to 64 cylinders of 0.6 mm in diameter to account for intratumor heterogeneity and obtain a representative reliable result. There are no studies that have fulfilled such a sample size condition that is, of course, impractical. The same may be true for ApoD, and the consequence could be that different IHC studies using

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either WS or TMA technology, would give variable results. We therefore compared ApoD determination in invasive operable breast cancer by EPC, WS-IHC and TMA-IHC.

Patients and Methods

The Regional Ethics Committee (#151.04), the Norwegian Social Science Data Service (#11241), the Norwegian Institute of Public Health, Bio Bank Registry (#1500) and the Norwegian Data Inspectorate (#2004/1432-2) approved all aspects of this study. The study population comprised a population-based cohort of 386 women (median age = 63 years, range 22-89 years) with operable breast cancer (*i.e.* stage I (pT_1, pN_0, M_0) or stage IIa (pT_{0-2}, pN_{0-1}, M_0). The details of these patients have been published previously (14). For the present study both fresh frozen material was needed for EPC and archival paraffin embedded material with confirmed invasive cancer for IHC. As a result, the following cases had to be excluded: ductal carcinoma in situ; n=15, lacking blocks; n=84 and poor quality material; n=4, leaving 283 patients. These patients did not differ in the demographics and tumour characteristics from the original 386 patients (Table I), which indicated that the material in the present study was representative for the whole original material.

The tumour size was measured in fresh surgical breast tumour specimens following excision. Sections (5 mm thick) were fixed in buffered 4% aqueous formaldehyde (=10% Formalin®, CellPath, Newtown Powys, UK) and embedded in paraffin. Next, 4 μm histological sections were cut and stained with haematoxylin-eosin-safran (HES). Then histological type and grade were assessed according to the World Health Organization criteria (15). The tumour stage (pT) and nodal status (pN) were classified according to UICC criteria (16).

For construction of the TMA, one 1.7-mm cylinder sample was taken from the invasive front of each tumour (= donor block), *i.e.* from the area in which the epithelial cancer cells were invading the normal surrounding tissue. This cylinder was re-embedded into a new recipient paraffin block. Each TMA block contained cylinders from 42 patients. New 4-μm-sections were prepared from the recipient TMA blocks and stained for ApoD. In 25 patients (9%), information based on TMA was not available due to technical reasons (either bad quality or not enough tumour tissue left after previous cuts). Both WS and TMA samples were available from the remaining 258 patients for IHC-ApoD quantification.

ApoD determination

EPC determination of ApoD. ApoD was determined in the tumour cytosol by immunoelectrophoresis as described previously (17). Briefly, ApoD was purified from breast cyst fluid by sequential chromatography and used for immunization of rabbits. The isolated ApoD IgG antibody was used in rocket immuno-electrophoresis to quantify the ApoD content in the tumour EPC, from 0.2-0.5 g of tumour tissue chilled and frozen immediately after surgery. The detection limit was 0.05 μg per mg EPC protein (= 4 mm rocket).

IHC determination of ApoD. Antigen retrieval and dilution of the antibodies were optimized before the study started. To guarantee uniform processing for each sample, all the sections were made and immunostained all the same time. The antigen retrieval and immunohistochemical techniques were based on DAKO (Glostrup, Denmark) technology (18). In brief, 4 μm paraffin sections adjacent to the HES sections used for diagnosis were mounted onto silanized slides (#S3002, DAKO), dried overnight at 37˚C, and treated for 1 hour at 60˚C. The sections were deparaffinized in xylene and rehydrated in a graded series of alcohol solutions. The antigen was retrieved with a highly stabilized retrieval system (ImmunoPrep, Instrumec, Oslo, Norway). The retrieval buffer was 10 mM TRIS/1 mM EDTA (pH 9.0). The sections were heated for 3 min at 110˚C followed by 10 min at 95˚C, and cooled to 20˚C. Immunostaining was performed using an autostainer (DAKO). TBS (Tris-buffered saline, DAKO #S1968: 0.05M Tris-HCL, 0.15M NaCl and S-1968) with 0.05% Tween 20 (pH 7.6) was used as the rinse buffer. Endogenous peroxidase activity was blocked by peroxidase blocking reagent (#S2001, DAKO) for 10 minutes and the sections were incubated with the antibody (ApoD clone 36C6, Novocastra Laboratories Ltd., Newcastle upon Tyne, UK, at 1:200 dilution) for 30 min. The immune complex was visualized with the DAKO REAL EnVision Detection System, Peroxidase/DAB, Rabbit/Mouse (K5007; DAKO) using incubation with EnVision / Horseradish Peroxidase, Rabbit/Mouse for 30 min, and DAB+ chromogen for 10 min. The sections were counterstained with haematoxylin, dehydrated, and mounted.

IHC protocol for ApoD in breast cancer cells. IHC ApoD staining was evaluated in whole sections for the different tissue components (*i.e.* cancer cells, normal glands, cystic glands and

Table I. *Study population characteristics (n=283).*

	Number	%
Age group (years)*		
<55	84	30
55-69	122	43
≥70	77	27
Premenopausal**	66	23
Postmenopausal	212	75
Unknown	5	2
Clinical stage I ***	96	34
Clinical stage II	187	66
pT1	125	44
pT2	152	54
Missing	6	2
pN0	141	50
pN+	142	50
Grade 1#	31	11
Grade 2	155	55
Grade 3	97	34
ER +##	227	80
ER –	56	20
PR+##	192	68
PR –	91	32

*Norwegian Breast Cancer Group[25], **clinical status, ***UICC staging: Stage I (pT1, pN_0, M_0), Stage II (pT_{0-2}, pN_{0-1}, M_0)[16], #WHO grading protocol[15], ##positive if ≥10 pmol/g (dextran-coated charcoal method). ER: oestrogen receptor, PR: progesterone receptor.

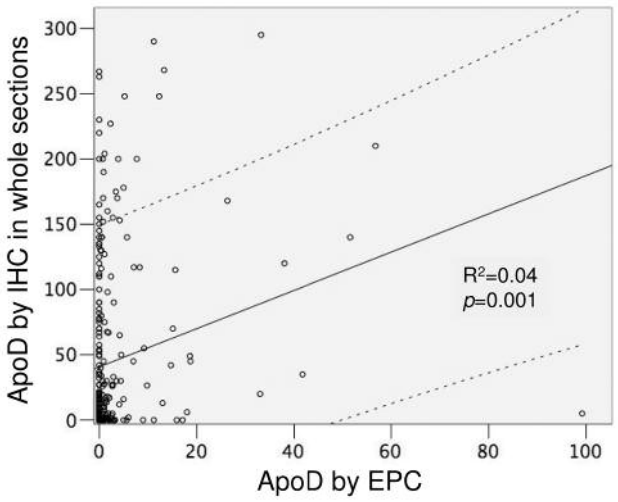

Figure 1. *Correlation between ApoD immunoelectrophoresis in cytosol (EPC) values (μg/mg protein in cytosol) and IHC H-score values in whole sections. Solid line is the regression line; dotted lines are the 95% confidence interval lines.*

fibrous tissue around the tumour, as ApoD positivity in these localisations reflect different physiological functions of ApoD) (19). In the cancer cells, the subcellular localisation of ApoD in the different cell compartments of the tumour cells was evaluated. The quantitative IHC expression pattern was also evaluated by means of the H-score (20), which is the product of staining intensity (0=none, 1+=weak, 2+=moderate, 3+=strong) and the percentage of cells with that staining intensity. The percentage positive cells was calculated by counting 1000 cells at 400x magnification. An example of the H-score calculation is: 20% x (0) + 30% x (1+) + 25% x (2+) + 25% x (3+) = 155; on a scale 0-300. For the tumours with an exclusively granular ApoD expression pattern (see results section for details) staining intensity was slightly modified as follows: 1+ for <1/3 of the cytoplasm filled with granules, 2+ for 1/3-2/3 of the cytoplasm filled with granules and 3+ if >2/3 of the cytoplasm was filled with granules. Validation of the H-score method showed a high intra-observer and inter-observer agreement (R^2=0.98 and R^2=0.91, respectively).

Threshold values for EPC and IHC ApoD. A cut-off value for ApoD in EPC of 0 μg per mg protein, *versus* all other values has often been used, (11, 17, 21) and was also applied in our study. The same threshold method (*i.e.* IHC-ApoD positivity or not), was applied for comparison of the EPC and IHC methods. In addition, whether or not other different thresholds for IHC ApoD expression were better predictors of EPC positivity was analysed by receiver operating curves (ROC) (see below).

Statistics. SPSS statistical software, version 13 (SPSS, Chicago, IL, USA) was used for statistical calculations. The Chi-square test was used for comparisons between groups. Two-tailed *p*-values 0.05 were considered statistically significant. As the biomarkers in the tissue samples were expressed with a continuous spectrum of test results, their diagnostic properties, *i.e.* sensitivity and specificity, depended on the chosen cutoff value (22). Receiver operating characteristics curve analysis (ROC) calculates the sensitivity and specificity of every observed result, and thus identifies the cutoff value associated with the highest sensitivity and specificity. The ROC curves were generated by plotting the false positives (1-specificity, x-axis) against the true positives (sensitivity, y-axis) of every observed test result. The diagnostic accuracy of the test is expressed as the area under the ROC curve (AUC) (23). ROC analysis was used to identify the best cutoff value of the various tissue markers to predict EPC positivity (24). For age comparison, the study population was stratified according to the treatment guidelines of the Norwegian Breast Cancer Group (NBCG) (25) (Table I).

Table II. *ApoD category according to different methods of determination (EPC vs. IHC).*

	ApoD WS-IHC H-score value*		
	=0	>0	Sum
ApoD EPC value (μg/mg)			
=0	53	103	156
>0	26	101	127
Sum	79	204	283

*ApoD in whole sections with threshold H-score =0.

Table III. *ApoD category according to different methods of determination (EPC vs. IHC) using ROC derived threshold.*

	ApoD WS-IHC H-score value*		
	<23	>22	Sum
ApoD EPC value (μg/mg)			
= 0	110	46	156
> 0	63	64	127
Sum	173	110	283

*ApoD in whole sections with threshold H-score =22.

Results

Correlation between EPC and IHC ApoD determination. The correlation between ApoD values by EPC and IHC is shown in Figure 1. There was a wide spread. The H-score increased considerably for a slight rise in the EPC value. With the EPC method, 45% (127/283) of patients had an ApoD positive cancer and the values varied (mean 2.9 μg/mg, median= 0 μg/mg, range 0-99.2 μg/mg protein). WS-IHC showed that 71% (204/283) of the carcinomas were positive for ApoD in the epithelial cancer cells. Agreement between EPC and IHC for ApoD negativity or

positivity was found only in 55% (53+101/283) of the tumours (Table II). Many of the EPC ApoD negative tumours were IHC positive (103/156=66%), but the opposite also occurred (26/127=20% of all tumours) (Table II).

The ROC analyses were performed by defining the EPC method as a categorical variable (=0 μg/mg or >0 μg/mg); the IHC variables percentage positive cells and H-score of the epithelial cancer cells (for both the whole sections and the TMA samples) were defined as the continuous variables. Only the best correlating results are described here as the thresholds of the other IHC features were not informative. The optimal threshold for the H-score in the WS to predict EPC positivity/negativity was 22, with an Area Under the Curve (AUC) of 0.61, p=0.0009, sensitivity=50% and specificity=71%. For the percentage positive cells in the WS the threshold was 20%, (AUC=0.61; p=0.0007; sensitivity=45%, specificity=74%). Using these ROC-derived thresholds, in comparison with the threshold of 0 and 0%, the agreement between the EPC and IHC method was only marginally improved from 55% to (110+64)/283=61% (Table III).

Observations of IHC ApoD staining. There was considerable variation in positivity of different tissue compartments, as staining occurred not only in the epithelial cancer cells, but also frequently in fibrous tissue, normal and cystic dilated glands as well. Figure 2 shows a tumour with a high EPC value of ApoD, while the WS-IHC showed the epithelial cancer cells were ApoD negative (this discrepancy pattern occurred in 10% of the cases). The strong EPC positivity is explained by the fact that the adjacent normal glands and also fibrous tissue outside and within the tumour strongly expressed ApoD.

Figure 3 depicts another malignant breast tumour with a high positive EPC ApoD content, but the IHC pattern differed from the specimen described above. There was strong diffuse staining of the epithelial cancer cells, but also strong staining in the adjacent benign cysts. In 32% of the tumours with IHC positive epithelial cancer cells, the tumour stroma also showed positive ApoD staining, and in 66% the fibrous tissue outside the tumour and adjacent normal tissue expressed ApoD. In ApoD negative carcinomas by IHC, 33 % had positive ApoD staining in the benign tissue adjacent to the cancer.

Special attention was paid to whether the brown stain granules in the ApoD-IHC stained sections in fact represented lipofuscin (which is also brown in standard microscopic sections). However, careful analysis (including polarized light microscopy) of serial HES sections and haematoxylin stained sections not stained for ApoD, did not show any brown staining in those areas that were positive in the parallel ApoD stained sections.

Subcellular expression difference. Four different subcellular localisations of ApoD staining in the epithelial tumour cells occurred: at the cell membrane, in the cytoplasm, at the nuclear membrane and in the nuclei (Figure 4). Often, in one cell all four compartments were ApoD positive, but clear variations (with only one or two of the cell compartments being positive) were commonly observed. Moreover, in the cytoplasm unique granular ApoD staining occurred where the ApoD was typically restricted to granules or vesicles (Figure 5). Both smaller and larger ApoD positive granules were observed. In total, 26% (74/283) of the carcinomas predominantly or exclusively exhibited this granular ApoD expression pattern whereas a predominantly diffuse homogenous ApoD staining was found in 23% (65/283) of the cases (Figures 4A and 4C). There were also carcinomas with a mixed granular-diffuse pattern (51/283; 18%) (Figures 4B and 4D). The intensity of the homogenous staining varied (Figure 4C). In some cells strong nuclear staining was found (Figure 4D), mostly together with diffuse cytoplasmic or mixed expression. In addition, staining of the cell membrane (Figure 4A) and the nuclear membrane (Figure 4C) was observed. Interestingly, ApoD staining in the adjacent tumour stroma or normal pre-existing stroma was predominately found in carcinomas with diffuse or mixed cytoplasmatic, but not the granular ApoD staining pattern.

Correlation between WS-IHC ApoD and TMA- IHC ApoD. There was considerable intratumoral heterogeneity in ApoD expression. Some carcinomas had positive central staining and a negative invasive front (Figure 6, A1-3), and vice versa (Figure 6, B1-3). The ApoD results of the WS-IHC and TMA-IHC methods were strongly correlated (p<0.0001), but with a wide spread (R^2=0.60) (Figure 7), mostly due to strong positivity in the TMA (*i.e.* samples from the thin invasive front line in the periphery of the tumour), and weaker ApoD staining in the rest of the tumour, the net overall result of such a tumour was low ApoD expression in the whole section. Figure 8 shows that a non-linear relationship existed between the *percentage* of ApoD positive cancer cells on the one hand, and the *H-score* on the other. For the carcinomas with <80% positive cells, the correlation was very strict and linear, most (apart from 12) of these cases had an H-score <100. However, when a threshold of 80% positive cells was reached, the ApoD *intensity* per cell greatly increased relative to the *number of cells* becoming positive. More specifically, an increase of 80 to 90% (an absolute increase of 10%, or 10/90=12.5% relative) resulted in nearly 25% stronger expression intensity per cell; and another 10% increase (from 90 to 100%, or 11% relative), resulted in nearly 100% ApoD intensity expression (as can be seen from the increase in H-score from 125 to 250 on average).

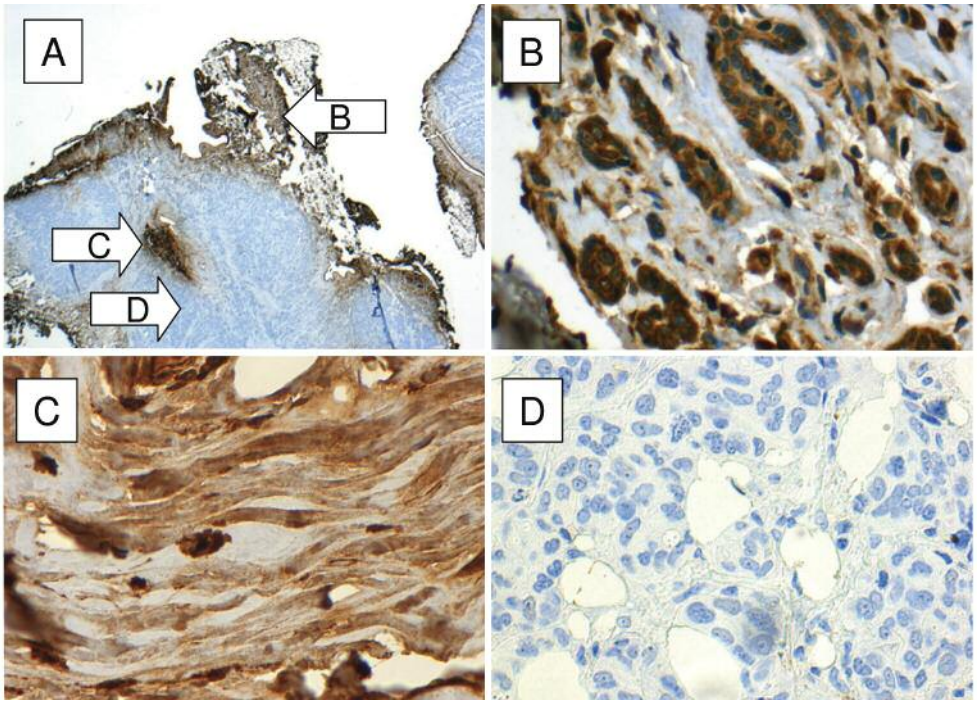

Figure 2. *A: Overview of IHC section at 1.25x magnification showing the different compartments in the section of a tumour with high (99 µg/mg) EPC ApoD value, which was negative in the epithelial cancer cells by IHC ApoD determination. Panels B, C and D are higher magnifications (corresponding to areas indicated by the white arrows in panel A). B: Positive ApoD staining in normal glands adjacent to the tumour tissue at 400x magnification. C: Positive ApoD staining in fibrous tissue at 400x magnification. D: No ApoD staining in the epithelial cancer cells at 400x magnification.*

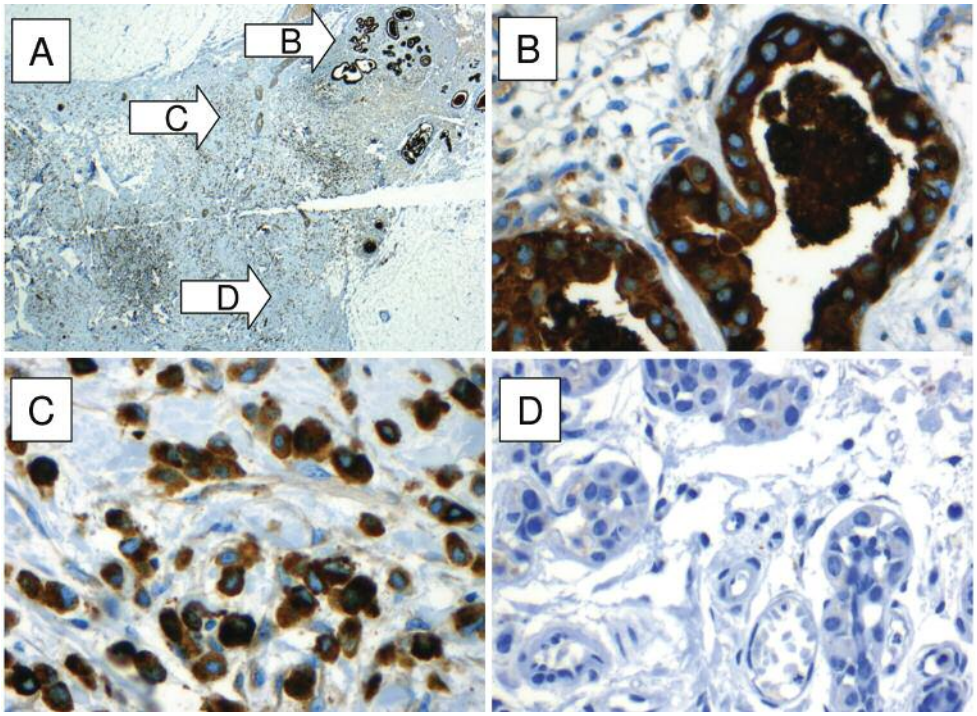

Figure 3. *A: Overview of IHC section at 1.25x magnification of a tumour with a positive (12 µg/mg) EPC ApoD value. Panel B, C and D are higher magnifications (corresponding to areas indicated by the white arrows in panel A). B: Positive ApoD staining in normal glands adjacent to the tumour tissue at 400x magnification. C: Positive ApoD staining in the epithelial cancer cells at 400x magnification. D: No ApoD staining of the normal glands adjacent to the tumour tissue at 400x magnification.*

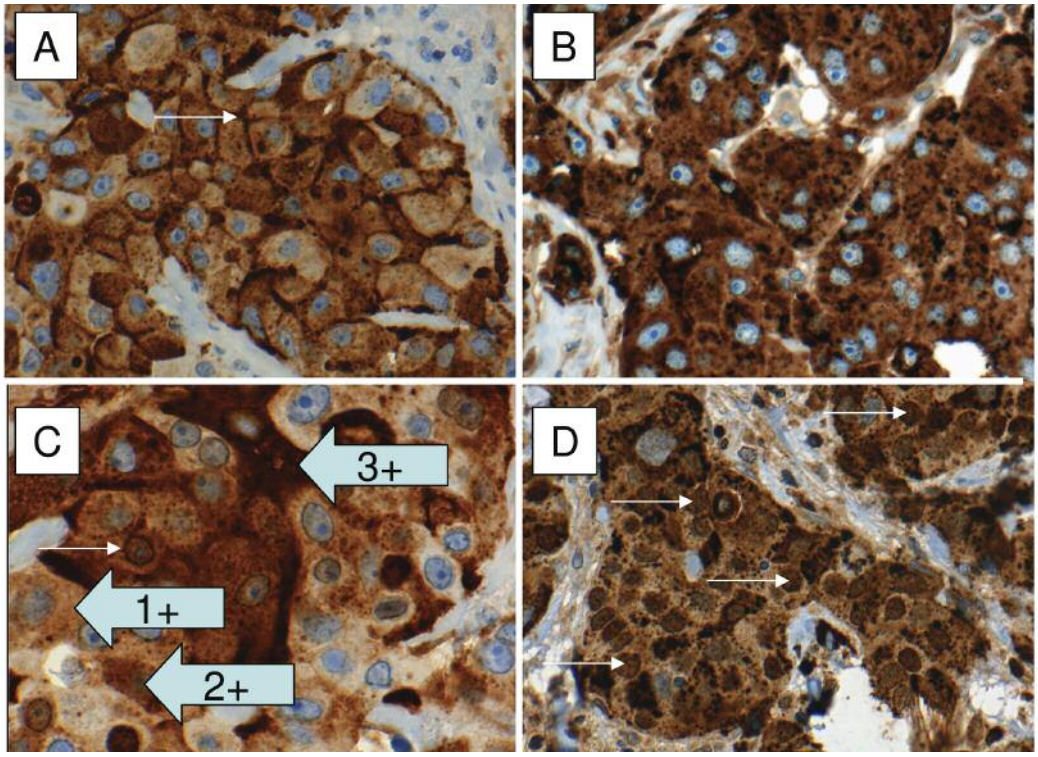

Figure 4. *Sub-cellular localisation of ApoD. A: Diffuse cytoplasmatic staining pattern. White arrow = staining of the outer cell membrane. B: Mixed diffuse and granular cytoplasmatic staining. C: Diffuse cytoplasmatic staining with 1+, 2+ and 3+ intensity (broad light blue arrows). White arrow= staining of the nuclear membrane. D: Nuclear staining, denoted by white arrows.*

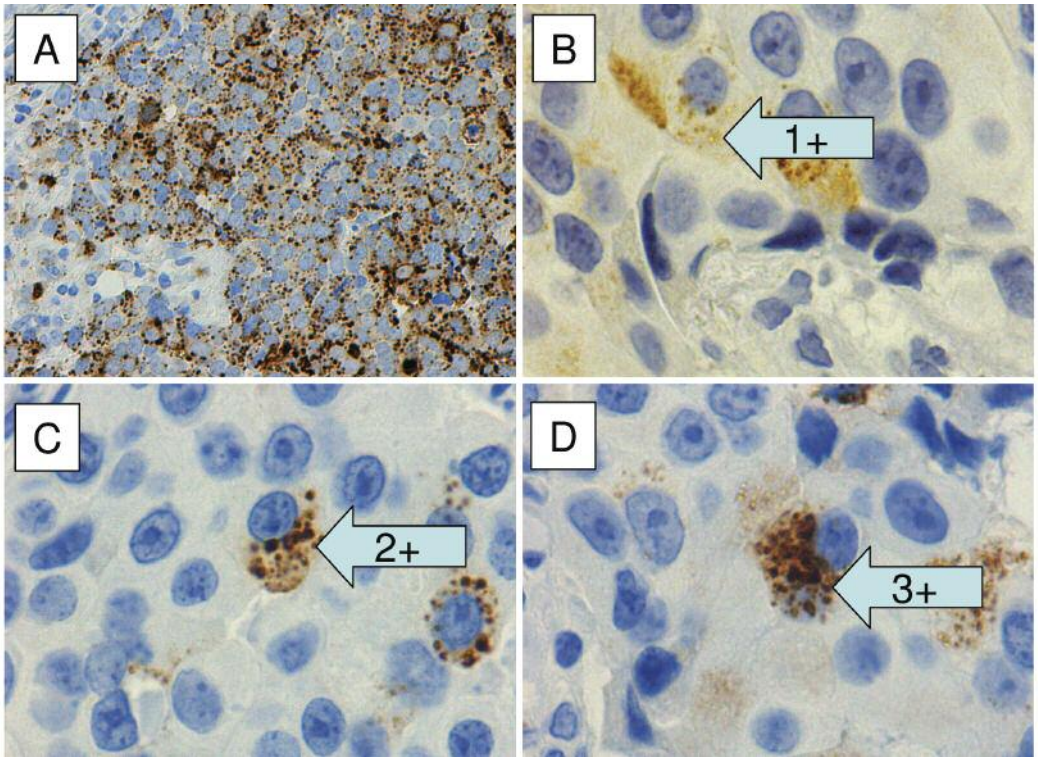

Figure 5. *Example of granular cytoplasmatic staining pattern. A: Overview with intermediate (100x) magnification. B-D, higher magnifications (1000x): Granular 1+ intensity C: Granular 2+ intensity. D: 3+ granular intensity.*

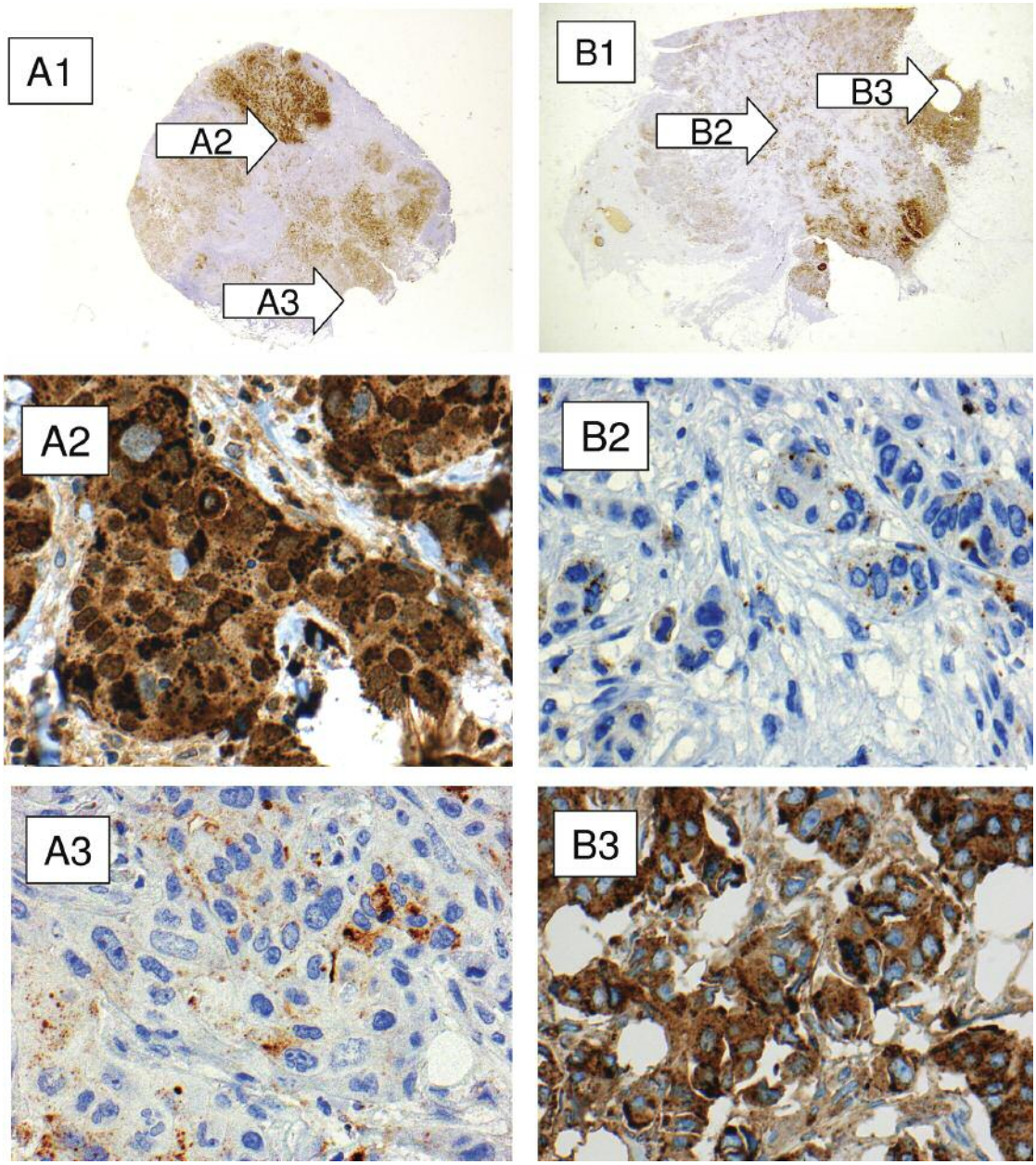

Figure 6. *Examples of intratumoral heterogeneity of ApoD staining in two different patients. Patient A. Panel A1: Overview of IHC ApoD staining of the tumour at 1.25x magnification. A2-arrow = strong IHC staining of ApoD in central part of the tumour shown at high (400x) magnification in panel A2. A3-arrow = weak IHC ApoD staining in the invasive front (= TMA sampling area) shown at high (400x) magnification in panel A3 which is taken from the TMA. Patient B. Panel B1: Overview of IHC ApoD staining of the tumour at 1.25x magnification. B2-arrow = weak ApoD staining of the central part of the tumour shown at high (400x) magnification in panel B2. B3-arrow = strong ApoD staining in the invasive front (=TMA sampling area) shown at high (400x) magnification in panel B3 which is taken from the TMA.*

Discussion

The present study showed that there were major discrepancies between the EPC and the immunohistochemical ApoD results in breast cancer, and also clarified the problems with the EPC determination. The EPC method analysed not only cancer but also non-cancerous tissue compartments, which were often ApoD positive (cystic epithelium, peritumoral fibrous tissue) resulting in false positive results. However, many more carcinomas were ApoD positive by IHC demonstrating that IHC is a much more sensitive method than the EPC method for ApoD

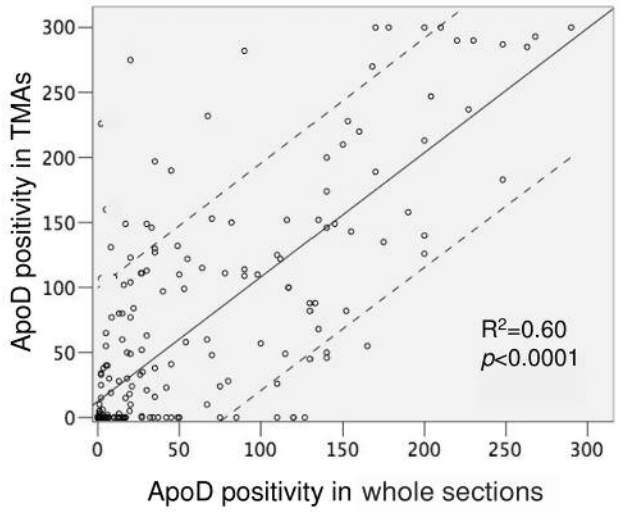

Figure 7. *Correlation between ApoD positivity in IHC whole sections and TMA-IHC (H-score measured in the cytoplasm of the cancer cells). Note the wide spread. Solid line is the regression line; dotted lines are the 95% confidence interval lines.*

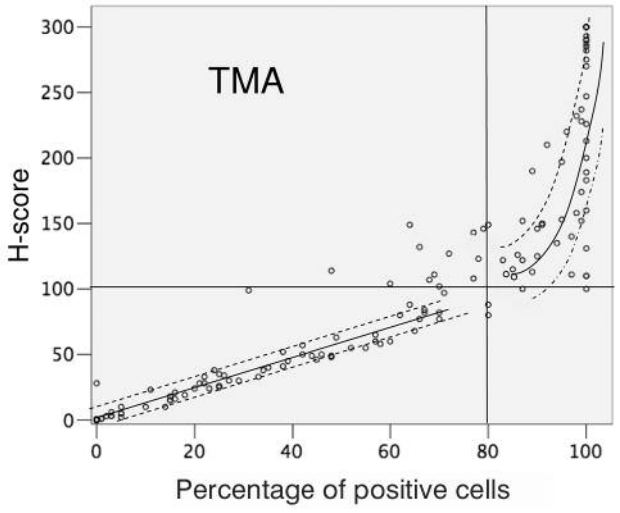

Figure 8. *Relationship between the percentage of positive cells and H-score in TMAs, both measured in the cytoplasm of the cancer cells. Note the steep increase of H-score in the cells that had more than 80% positive cancer cells. Solid lines are the regression lines, dotted lines are the 95% confidence interval lines.*

determination. Clearly, the IHC method allowed a sensitive, accurate and specific tissue compartment determination of ApoD in epithelial cells, thereby making it more accurate. Moreover, the ApoD scoring of the IHC-stained slides was well reproducible between observers although digital image analysis may be required for optimal reproducibility.

The considerable heterogeneity in ApoD expression within the tumour probably reflected differences in the regulation of ApoD transcription and differences in the possible functions of ApoD (8, 26). Until recently, the function of ApoD was not well understood, but recent cell line studies (19) have shown that ApoD is produced, packed and, *via* the Golgi apparatus excreted out of the cell, taken up again and then transported into the nucleus. This new knowledge puts the present staining patterns and locations into an understandable context. The granular pattern probably represented ApoD in granules just secreted by the Golgi apparatus, the stromal staining illustrated the excreted paracrine location, the diffuse cytoplasmatic staining reflected ApoD that was taken up from the stroma again, and the nuclear membrane and nuclear staining, the transportation into the nucleus. The observation of staining of the outer cell membrane and the nuclear membrane was in line with other observations (27, 28). To our knowledge, clear nuclear staining has not been described in breast cancer tissue before. The localisation close to the outer cell membrane could be explained by the strong affinity of ApoD to membrane-bound arachidonic acid (29) and/or cell protection against reactive oxygen species from the microenvironment (19).

Cell senescence due to nutritional starvation has been shown to up-regulate ApoD (30), hence the ApoD expression in parts of the tumour may have been associated with localized marginal nutritional status. The presence of ApoD in stromal fibrous tissue has also been described by others (31). This may indicate that ApoD plays a role in the micro-environmental transportation of ligands. In view of the fact that different cellular staining patterns of ApoD may reflect the different locations and functions, these should be analyzed separately in prognostic studies. The EPC method does not allow such analyses.

Tumour heterogeneity of ApoD staining resulted in many different expression patterns, but two patterns were particularly interesting. Firstly, those which were negative in the invasive front but ApoD positive in other parts of the tumour. Secondly, those with a higher expression in the invasive front than in other parts of the tumour. Whether the latter represented a clonal expansion of cancer cells with amplification of the chromosomal region where the ApoD gene is located (3q26.2q.ter) (32), known to occur in breast cancer (33), remains to be shown. The consequence might be that analysing ApoD in the peripheral growing zone of the tumour may be of importance for analysing its clinical prognostic and predictive value, since prognostication in breast cancer can be dependent of where in the tumour the analyses are done (*i.e.*, the invasive front *versus* the inner, fibrotic centre) (34).

The exact function of ApoD is still not totally clear. Recently, ApoD and its homologs were shown to be part of the cyto-protective system in plant (35) and in animal cells

(36), and in human cancer cell lines (19). Therefore, a random sampling of the TMA cylinders throughout the whole tumour, without considering the biological and prognostic significance of the peripheral growing front of the cancer, could blur the biological and clinical and perhaps prognostic information from ApoD.

However, this is hypothetical at present and should be studied further. Moreover, the relevance of ApoD in breast cancer has been raised significantly by the discovery that it has a tamoxifen-binding effect (10, 14) and, possibly, a relationship with AR and oestrogen receptor alpha (ERα) (7). The current knowledge of ApoD and the present IHC findings, make long-term prognostic studies in breast cancer all the more important.

In conclusion, IHC determination has the advantage of exact intratumoral and subcellular localisation of ApoD in breast cancer and the tumour heterogeneity and the subcellular expression difference of ApoD in breast cancer should be taken into consideration in clinical prognostic studies.

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