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Angiogenesis and invasive recurrence in ductal carcinoma in situ of the breast

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Abstract

The development of an invasive recurrence following treatment for ductal carcinoma *in situ* (DCIS) converts a non-fatal disease to one associated with mortality. To date, no pathological or molecular features have been found to predict for the type of recurrence. Previous studies have suggested that in DCIS angiogenesis may be an important factor in determining the transformation from *in situ* to invasive carcinoma. We looked at 355 cases of DCIS and found that 32 had subsequently developed recurrent disease. In these 32 cases and in matched controls, periductal vascular density was determined using morphometry and anti-endothelial antibodies, von Willebrand factor (vWF) and CD34. Vascular density was related to the risk of both invasive and *in situ* recurrence. Normal lobules at least 2 mm away were used as controls. Differences in the phenotype of individual blood vessels was detected by performing dual staining immunofluorescence on selected cases. The microvessel density (MVD), as detected with the CD34 antibody, was higher around foci of DCIS than around normal breast lobules ($P=0.001$). Furthermore, it was significantly higher in cases of DCIS that recurred ($P<0.0001$). The findings with the vWF antibody were less clear cut and suggested a trend in decreasing MVD with increasingly aggressive disease. Dual immunofluorescence staining shows that the increase in MVD seen around DCIS is due to an increase in CD34+/vWF-blood vessels. An increase in CD34+/vWF–of blood vessels may be able to predict cases of DCIS that are at a high risk of developing a recurrence.

Keywords: Angiogenesis; Ductal carcinoma *in situ*; Breast; Recurrence

1. Introduction

With the advent of screening mammography, the frequency with which ductal carcinoma *in situ* (DCIS) is detected has increased from approximately 1–20% of all breast cancers [1,2]. Although DCIS is a non-fatal disease, in cases that recur, approximately 50% of recurrences are as invasive carcinoma [3–5]. However, to date no pathological or molecular features have been found to predict for the development of invasive disease.

A number of studies have shown that the degree of vascularity within an invasive breast carcinoma may be of prognostic value [6–11]. Several studies have also shown that various premalignant lesions of the breast can induce angiogenesis in animal experimental systems and in the human breast [12–20]. In DCIS, two different patterns of angiogenesis have been described: a diffuse stromal vascularity and a vascular rim around the involved ducts [12]. The former is thought to be mediated via the recruitment of accessory cells which release proangiogenic factors, whilst the latter is thought to result from angiogenic factors being secreted by the tumour cells [13]. It thus seems possible that the pattern or extent of vascularisation around DCIS may be an important factor in determining the transformation from *in situ* to invasive carcinoma. It is likely that the periductal vessels are most important in this respect as incipient invasion is most likely to be associated with changes in vessels in the immediate vicinity of the tumour cells.

The aim of this study was to determine if the vascularity of DCIS was different in those who subsequently developed recurrent cancer compared with those who did not. This was tested by comparing a group of

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women with pure DCIS (no known development of recurrent disease) with a group that subsequently developed recurrence either as *in situ* or invasive carcinoma. As studies of breast cancer angiogenesis in the past have been associated with inconsistent findings, we have used precise morphometric methodology to compare vascularity in DCIS with normal breast and have employed a panel of anti-endothelial antibodies to take account of phenotypic as well as numerical changes.

2. Patients and methods

2.1. Patients and tumours

Out of 355 cases of pure DCIS without microinvasion identified in Merseyside, 32 cases of recurrence were found (20 cases as *in situ* and 12 cases as invasive carcinoma). The cases spanned a period of 10 years from 1989 to 1999. Formalin-fixed paraffin-embedded breast samples from 52 patients with ductal carcinoma *in situ* were collected from the archives of five hospital pathology departments; 20 with no history of recurrence, 20 that subsequently recurred as DCIS and 12 that subsequently recurred as invasive carcinoma. The original haematoxylin and eosin (H&E) stained sections for the primary DCIS were reviewed by two pathologists for classification according to the guidelines of the European Commission and UK National Breast Screening Programme [21,22]. A representative block for each patient was selected for subsequent immunostaining.

Initially, a pilot study on 20 cases was performed in which four different anti-endothelial antibodies were used. When DCIS was compared with normal breast lobules, all showed a change in microvessel density (MVD): increases in CD31, CD34 and CD141 and a decrease in von Willebrand factor (vWF). CD34 was chosen for the main study as it gave the highest values for MVD and has been shown in the past to be the most sensitive method for detecting small blood vessels [23], vWF was included as it appeared to be identifying a different sub-population of vessels.

2.2. Immunohistochemistry

Sections were stained for endothelial cells using monoclonal anti-CD34 (Qbend/10, DAKO, Denmark) and polyclonal anti-human vWF (DAKO, Denmark). Sections were dewaxed through two changes of xylene and industrial methylated spirits (IMS). Endogenous peroxidase activity was blocked with a mixture of H_2O_2/methanol (12 ml H_2O_2 in 400 ml methanol) for 12 min. Antigen retrieval was performed by treating the sections with 0.2 g of trypsin and 0.4 g of calcium chloride in 440 ml Tris Buffered Saline, TBS (50 mM Tris–HCL, 150 mM NaCl, pH 7.4) at 37 °C for 20 min. Prior to staining with the polyclonal antiserum, sections were treated with a mixture of 5% BSA/TBS (1 g Bovine Serum Albumin in 20 ml TBS) for 10 min.

The antibodies were diluted 1:20 for anti-CD34 and 1:1000 for anti-human vWF in 5% BSA/TBS. The sections were incubated with primary antibodies at room temperature for 40 min. Secondary antibodies were incubated for 40 min using EnVision Labelled Polymer (mouse or rabbit as appropriate). Sections were washed with TBS between incubation steps. 33′ diaminobenzidine (DAB) was used as a chromogen. The last two steps were carried out using a commercial kit (DAKO EnVisionTM + System, Peroxidase (DAB), USA).

The cell nuclei were counter-stained blue with Haematoxylin solution. The sections were dehydrated through four changes of IMS and three changes of xylene before being mounted in resinous mountant (DPX, BDH Laboratory Supplies, UK). Omission of the primary antibody was used as a negative control and the microvessels of the normal adjacent breast tissue served as internal positive controls.

2.3. Assessment of tumour vascularity

Vascular density was determined without knowledge of the clinical outcome or the pathological classification of the cases. No scanning of stained microvessels to identify 'hot-spots' at low magnification was undertaken. The assessment of completely transected involved ducts started from the upper right of all stained sections, moving downwards and to the left. All or the first 50 foci of transected DCIS encountered were assessed on each section, thus eliminating selection bias.

2.4. Evaluation of DCIS area

For each focus of DCIS evaluated, the area of each DCIS focus (core area) and the area with a circumference that was 100 μm from the edge of the individual focus (total area) were calculated. The areas were measured at ×200 magnification using an image analysis system (Zeis Axiohome with software version 3.0, Germany). The appropriate area in which MVD was then evaluated was calculated by subtracting the core area from the total area for each focus.

2.5. Counting microvessels

The number of microvessels within 100 μm of a focus of DCIS was manually counted at high magnification (×400). Eligible microvessels included any immunostained endothelial cell or cluster of cells around a visible lumen clearly separated from adjacent microvessels, tumour cells and other connective tissue components. The presence of red blood cells was not required. It was not possible to distinguish blood and lymphatic vessels. Where vessels were in clusters, each was counted as

separate if it met the above criteria. The MVD for each individual focus was obtained by dividing the number of microvessels counted by the appropriate area.

2.6. Controls

For each case of DCIS evaluated, adjacent normal lobules from the same section were used as controls. Up to ten normal lobules were assessed for MVD in the same way as that described for DCIS. The normal lobules were assessed only if they were situated more than 2 mm from the nearest DCIS focus.

2.7. Dual labelling immunofluorescence

To determine whether a specific type of blood vessel accounted for any differences in MVD, 12 cases of pure DCIS were assessed using dual labelling immunofluorescence for CD34 and vWF; three lesions were low, three intermediate and six high nuclear grade. The method used was as follows.

Sections were dewaxed through two changes of xylene and IMS, respectively. The sections were microwaved on full power in 500 ml of Ethylenediamine-Tetraacetic Acid (ED2SS; Sigma Chemical, St Louis, USA) for 20 min. Prior to incubation with primary antibodies, sections were treated with a mixture of 5% BSA/TBS for 10 min. The antibodies were diluted 1:10 for anti-CD34 and 1:1000 for anti-human vWF in 5% BSA/TBS. The sections were incubated with primary antibodies at room temperature for 40 min. Secondary antibodies were applied for 30 min using a biotinylated sheep anti-mouse antibody (Amersham Pharmacia Biotech, Essex, UK) diluted 1:100 and a swine anti-rabbit TRITC Conjugated antibody (DAKO, Cambridge, UK) diluted 1:50. Next, sections were incubated for 30 min using Flourescein Avidin DCS (A-2011; Vector Labs, Peterborough, UK) diluted 1:100 and Swine α Anti-Rabbit Immunoglobulin TRITC diluted 1:50. Sections were washed with TBS between incubation steps.

DNA was stained by immersion in a solution of 4′,6-diamidino-2-phenylindole (Sigma) at a concentration of 250 ng/ml in phosphate-buffered saline for 10 min and coverslips were mounted onto the tissue sections in an antifading medium (Vectashield, Vector Laboratories, Peterborough, UK). Control slides were included in each analysis by performing the same procedures and substituting non-immune serum for primary antibodies and secondary antibodies individually.

2.8. Assessment of slides

Quantification of the fluorochrome-labelled vessels was performed as above. Each vessel was examined under a high-power lens for the red (TRITC), green (fluorescein) and blue (4′,6-diamidino-2-phenylindole) fluorochromes using the appropriate filters in succession to assess the presence or absence of double-labelled vessels. A triple band filter in which all three fluorochromes could be seen simultaneously was used for confirmation of dual staining.

The data were analysed by using the non-parametric Mann–Whitney and Kruskal–Wallis tests using the Statistical Package for the Social Sciences (SPSS) Version 10.0 software for Windows 97/NT.

3. Results

The clinical data for the three groups of DCIS are summarised in Table 1. It was not possible to retrospectively

Table 1
Clinical and pathological data from the three groups of DCIS patients

	DCIS with no recurrence	DCIS with *in situ* recurrence	DCIS with invasive recurrence
Number of cases	20	20	12
Age (years)			
Range	49–87	45–78	43–82
Mean	66.2	65.6	63.3
Follow-up (months)			
Range	29–138	9–98	7–52
Mean	94	43	48
Death	2 (both not related to breast cancer)	0	1 (died of breast cancer)
Operation			
Wide local excision	18 (90%)	20 (100%)	10 (83%)
Mastectomy	2 (10%)	0 (0%)	2 (17%)
Adjuvant therapy			
None	9 (45%)	9 (45%)	5 (42%)
Hormonal	11 (55%)	11 (55%)	7 (58.3%)
Radiotherapy	0 (0%)	0 (0%)	0 (0%)
Others	0 (0%)	0 (0%)	0 (0%)
Nuclear grade			
Low	4 (20%)	3 (15%)	4 (33%)
Intermediate	4 (20%)	6 (30%)	2 (17%)
High	12 (60%)	11 (55%)	6 (50%)
Van Nuys pathological grade			
1	2 (10%)	3 (15%)	3 (25%)
2	6 (30%)	6 (30%)	3 (25%)
3	12 (60%)	11 (55%)	6 (50%)
Size (mm)			
Range	1–65	5–20	1–22
Mean	16.6	13.5	11.7
No of cases not assessable	0	9	3
Excision margin (mm)			
<1	7 (44%)	4 (33%)	3 (50%)
$1-\leqslant 10$	4 (25%)	4 (33%)	0 (0%)
>10	5 (31%)	4 (33%)	3 (50%)
No. of cases not assessable	4	8	6

DCIS, ductal carcinoma *in situ*.

assess the size of the DCIS and the excision margins in a number of cases.

3.1. Normal breast

The mean MVD around normal lobules was 79 vessels/mm^2 for CD 34 and 91 vessels/mm^2 for vWF. There was no significant difference in MVD for CD34 when normal lobules were compared in cases of DCIS with and without recurrence (Mann–Whitney, $P=0.5$). In contrast, staining with vWF was more variable, the normal lobules of patients with DCIS that subsequently developed recurrent disease had a lower MVD than those that did not (Mann–Whitney, $P=0.024$). In addition, MVD in cases with invasive recurrence was significantly lower than those that developed recurrence as *in situ* carcinoma (Mann–Whitney, $P<0.001$).

3.2. DCIS (Fig. 1)

The MVD for the three groups of DCIS are summarised in Table 2.

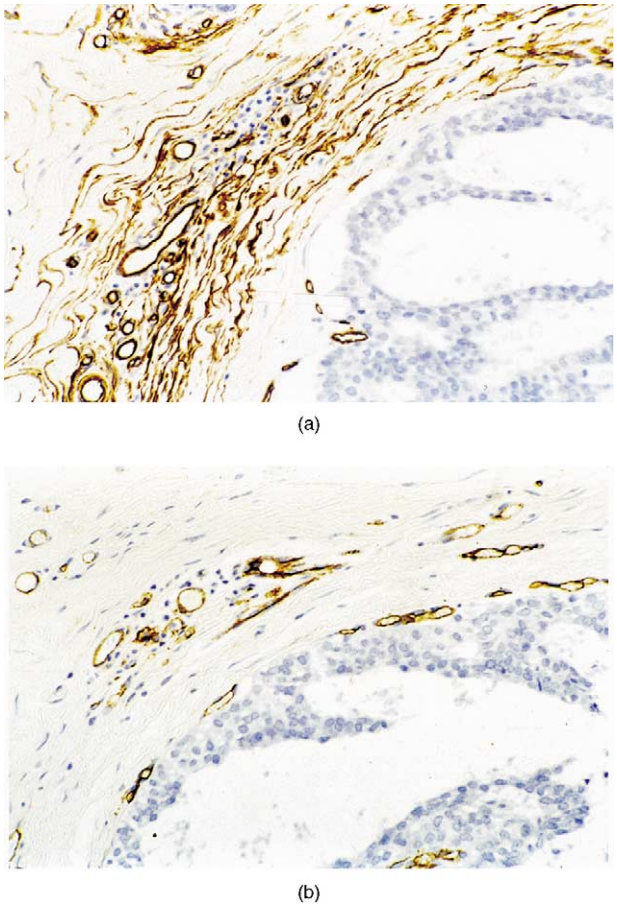

Fig. 1. Periductal microvessels stained with (a) anti-CD34 and (b) anti-vWF antibodies in pure DCIS with no known history of recurrence (high-power field).

Table 2
Microvessel density (MVD) for CD34 and vWF for normal lobules and DCIS

	CD34 Mean MVD (vessels/mm^2)	vWF Mean MVD (vessels/mm^2)
Normal lobules	79 (S.D. = 60)	91 (S.D. = 72)
DCIS (Non-recurrence)	95 (S.D. = 49)	80 (S.D. = 65)
DCIS (*In situ* recurrence)	119 (S.D. = 66)	89 (S.D. = 65)
DCIS (Invasive recurrence)	126 (S.D. = 59)	77 (S.D. = 53)

vWF, von Willebrand factor; S.D., standard deviation.

3.3. CD34

The highest MVD for CD34 was seen in cases of DCIS that subsequently developed an invasive recurrence and the lowest in cases of DCIS that were not known to have developed recurrent disease (Fig. 2). The mean MVD was significantly higher around cases of DCIS that subsequently developed recurrent disease than those that did not (Mann–Whitney, $P<0.001$). The MVD was higher in cases that developed an invasive recurrence than those that developed recurrent DCIS. However, the confidence interval between these two groups largely overlaps. In addition, all three groups of DCIS had a higher MVD than that seen in normal breast (highest $P=0.001$).

3.4. vWF

The data for vWF-positive vessels was less clear (Fig. 3). Normal lobules had the highest value of MVD, and DCIS with subsequent invasive recurrence the lowest.

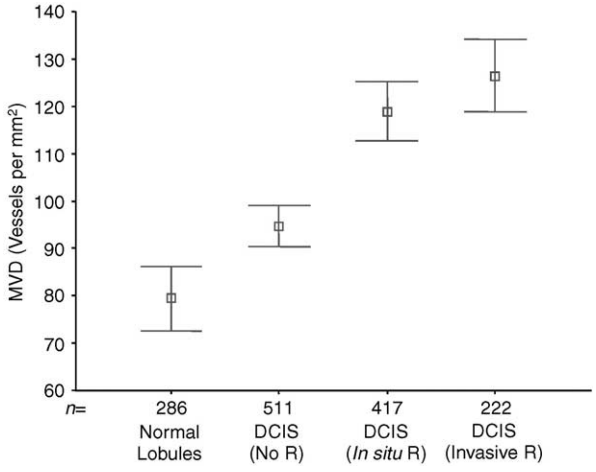

Fig. 2. Comparison of CD34 microvessel density (MVD) among normal lobules and three groups of ductal carcinoma *in situ* (DCIS) (no recurrence (No R), recurrence with ductal carcinoma *in situ* (*In situ* R), recurrence with invasive carcinoma (Invasive R)). The squares represent the mean values and the bars represent the 95% Confidence Intervals of the mean.

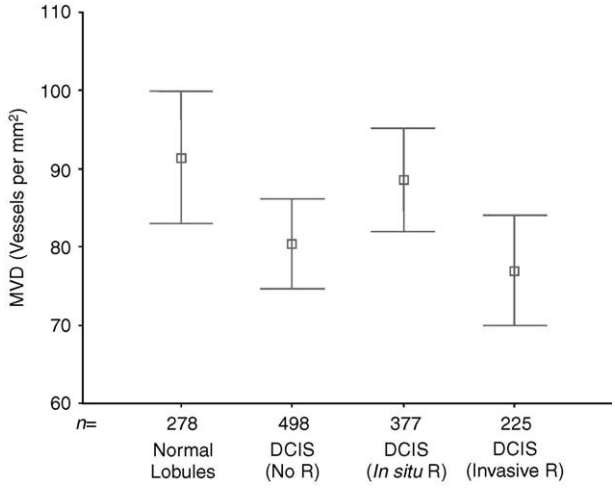

Fig. 3. Comparison of vWF microvessel density (MVD) among normal lobules and three groups of ductal carcinoma *in situ* (DCIS) (no recurrence (No R), recurrence with ductal carcinoma *in situ* (*In situ* R), recurrence with invasive carcinoma (Invasive R)). The squares represent the mean values and the bars represent the 95% Confidence Intervals of the mean.

3.5. Dual immunofluorescence for CD34 and vWF (Fig. 4)

On the 12 cases of DCIS on which dual immunofluorescence was performed, three different immunophenotypes of blood vessels were identified around normal breast and DCIS: CD34+/vWF−, CD34+/vWF+ and CD34−/vWF+.

3.5.1. Normal lobules

The mean MVD for CD34-positive vessels was 83 vessels/mm^2; of these 45% coexpressed vWF. The mean MVD for vWF-positive vessels was 56 vessels/mm^2 and of these 68% coexpressed CD34.

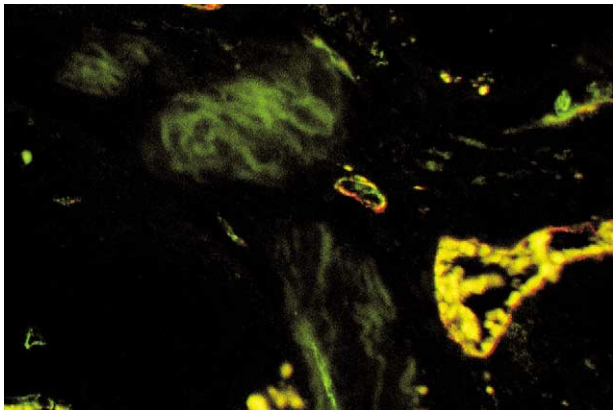

Fig. 4. High-power field: CD34+ (green), vWF+ (red), and dual positive (in the middle) microvessels around pure DCIS with no known history of recurrence.

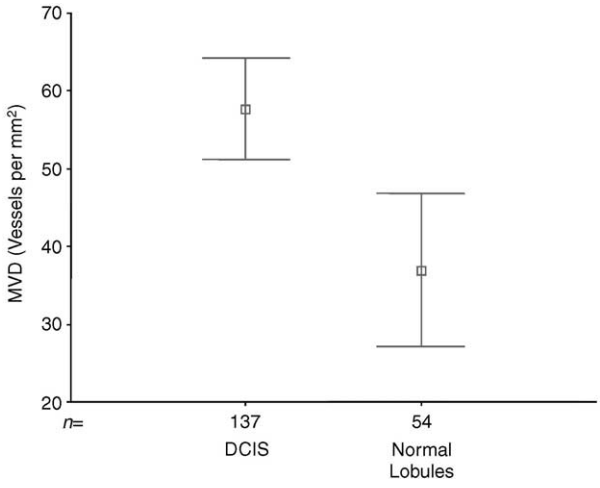

Fig. 5. Comparison of microvessel density (MVD) for CD34+/vWF– vessels around ductal carcinoma *in situ* (DCIS) and normal lobules. The squares represent the mean values and the bars represent the 95% Confidence Intervals of the mean.

3.6. DCIS

The mean MVD for CD34-positive vessels around DCIS was 87 vessels/mm^2, and of these 34% coexpressed vWF. The mean MVD for vWF-positive vessels around DCIS was 42 vessels/mm^2 and of these 72% coexpressed CD34. The MVD of CD34+/vWF–vessels was significantly higher around DCIS than normal lobules (Mann–Whitney, $P<0.001$) (Fig. 5). In contrast, there was no difference between CD34+/vWF+ or CD34−/vWF+ vessels between normal breast and DCIS (Mann–Whitney, lowest $P=0.4$).

4. Discussion

In this study, we have identified different populations of microvessels in DCIS and normal lobules. In DCIS, there was a significant increase above normal in CD34+ vessels; however, for vWF the changes were less clear cut and overall were more in keeping with a drop in MVD rather than an increase. This suggests that there is a change in phenotype, as well as vascular density, from the transition from normal breast to DCIS, although significant vascular proliferation must have taken place in order to maintain roughly normal density around greatly enlarged structures.

The groups of DCIS that subsequently recurred had higher mean values for CD34+ MVD than those that did not. Increases in CD34 MVD thus not only correlated with the progression from normal to *in situ* neoplasia, but also appeared to be able to detect cases that are likely to recur. Furthermore, MVD appeared to be independent from the size, and excision margin status as these were appropriately matched between the groups.

However, the small numbers in each of these groups precludes further comment. In contrast, the findings for vWF were more difficult to interpret. Differences were seen between normal lobules from the three different DCIS groups. It is not certain whether the changes in vWF MVD is due to the proximity of the DCIS. However, we attempted to minimise this by evaluating normal lobules at least 2 mm from the nearest focus of DCIS. Furthermore, no such change was seen for CD34. Alternatively, staining with vWF may be more sensitive to the effects of tissue fixation and processing, the differences in staining reflecting these rather than definite changes in the microvessel phenotype. DCIS also showed differences in MVD for vWF between the three groups. However, unlike that seen for CD34 a clear step-wise increase was not seen and instead there was a trend towards a decrease in MVD. When dual immunofluorescence was used, the values for CD34 were comparable to those obtained by standard immunohistochemistry. However, far fewer vWF-positive vessels were detected, again suggesting that this antibody may be affected by the methodology.

The dual immunofluorescence staining did, however, give us further insight into the change in MVD. There was no difference between normal lobules and DCIS when vessels showing a CD34+/vWF+ or a CD34-/vWF+ phenotype were compared. In contrast, vessels with a CD34+/vWF−phenotype showed a significant increase between normal lobules and DCIS. This suggests that the increased vasculature around DCIS is primarily due to an increase in CD34+/vWF−vessels. This is in keeping with observations on colorectal carcinoma where vWF immunostaining has been found to be absent from some of the capillaries in the tumour [24]. It has been shown that anti-vWF stains large vessels more strongly than small ones [25]. In contrast, anti-CD34 antibodies are sensitive endothelial markers that stain small and large vessels in normal and tumour tissue equally [25]. Perivascular stromal cells and a proportion of lymphatic vessels are also stained [25], the former were excluded from our study by using a rigid definition for a vessel. The role of the latter in neoangiogenesis is unknown. Consequently, our findings could reflect the immaturity of newly formed tumour-associated vessels. The density of CD34+/vWF−vessels could thus be related to the rate of tumour angiogenesis and the consequences it has for the biological behaviour of DCIS. It would therefore be interesting to know the phenotype of the blood vessels in the subsequent recurrences.

A number of other studies have also looked at angiogenesis around pre-invasive disease. Ottinette and colleagues [26] examined vascular number and size within a 100 μm perimeter of proliferative breast disease and DCIS with a morphometric method. On each section, 3–5 fields were examined and larger vessels with a layer of smooth muscle were excluded. They found an increased mean vascular size in proliferative breast disease ($n=13$) and DCIS ($n=10$) relative to normal epithelium ($n=11$), but unlike the present study no increase in vessel numbers. Guidi and colleagues [12] used both a 1–3+ estimate of vascularity and quantitative counts of microvessels within 500 μm of DCIS. For each section, microvessel counts from five most vascular fields were recorded and averaged. Vessels with muscular walls; within the fibrovascular cores of neoplasms; and in immediate apposition to the basement membrane, were not counted. The vascularity by semiquantitative assessment was greater in comedo than non-comedo DCIS ($P=0.004$), and was proportional to nuclear grade ($P=0.05$). Both semiquantitative and quantitative vascular scores correlated with Her2/neu ($P=0.03$ and $P=0.0002$, respectively).

Heffelfinger and colleagues [27] estimated angiogenic grade (0–4) according to the proportion of basement membrane of involved ducts touched by vessels. Up to 18 affected ducts on each section from 90 patients were examined. 58 (64%) patients had at least one form of proliferative breast disease, 61 (68%) patients had some form of carcinoma in situ, and 56 (62%) individuals had invasive carcinoma. The study showed that the vascularity of normal epithelium was higher in breasts containing invasive disease than in breasts lacking invasive disease ($P=0.006$); simple proliferative breast disease had a higher count than normal breast epithelium ($P<0.0001$) and, vascularity increased in proportion to epithelial lesion progression and relative risk of invasion. Interestingly, Guinebretiere and colleagues [28] showed that periductal vascularity in fibrocystic disease was a predictor of progression to invasive disease. 10 most hyperplastic lesions and the surrounding 50 μm tissue were assessed on each section in 24 cases and 24 control subjects. In our study, we concentrated our investigations on periductal vascularity as we hypothesised that risk of recurrence is likely to be associated with vascular changes in the immediate vicinity of the tumour foci.

In conclusion, this study shows that the blood vessels surrounding DCIS appear to have a different immunophenotype when compared with blood vessels surrounding normal breast lobules. Furthermore, increases in vascular density, as detected with the CD34 antibody, correlates with the risk of recurrence.

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