An appraisal of 11 ~~epithelial and myoepithelial~~ immunnohistochemical markers in adenoid cystic carcinoma of the breast: A unique reverse staining pattern of cytokeratin 5/6 excludes its mimickers

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ABSTRACT

Adenoid cystic carcinoma (AdCC) of the breast is an uncommon but distinct neoplasm composed of ~~epithelial and myoepithelial cells~~ a dual cell population polarized around true glandular (luminal) spaces and pseudolumina, respectively. The aim of this study was to clarify whether various ~~epithelial and myoepithelial~~ immunohistochemical markers (CK7, EMA, CD117, p63, calponin, CD10, S100, CK5/6, CK14, vimentin, and type IV collagen) can distinguish the dual cell population in classical AdCC (n=14) and collagenous spherulosis (n=5). The sensitivity and specificity of these 11 markers for distinguishing ~~epithelial~~ luminal from ~~myoepithelial~~ abluminal cells were evaluated using a curve created by plotting the true-positive rate (sensitivity) against the false-positive rate (1 − specificity) at four threshold settings of 0%, 10%, 50%, and 70%. The most sensitive and specific markers for ~~epithelial~~ luminal cells in AdCC were CK7 and EMA; those for ~~myoepithelial~~ abluminal cells were type IV collagen, p63, and vimentin. CD10 and S100 did not act as ~~myoepithelial~~ abluminal markers of AdCC. ~~Although~~ CK5/6, ~~is believed to~~

~~be~~ one of the basal/myoepithelial markers, ~~our data indicated that CK5/6~~ was expressed more frequently in ~~epithelial~~ luminal than in ~~myoepithelial~~ abluminal cells of AdCC. Thus, CK5/6 immunostaining resulted in a reverse expression pattern analogous to that we recently documented in clear cell lesions of mammary adenomyoepithelioma (Virchows Arch 2015;466;191-198.). In conclusion, compared with myoepithelial/abluminal cells of normal breast or collagenous spherulosis, the neoplastic ~~myoepithelial~~ abluminal cells of classical AdCC are characterized by enhanced vimentin and attenuated CD10. Furthermore, the ~~epithelial~~ luminal cells of AdCC show a unique aberrant staining of CK5/6 that may aid in excluding its mimickers.

Keywords: Adenoid cystic carcinoma, myoepithelial cells, cytokeratin 5/6, vimentin, collagenous spherulosis

Introduction

Adenoid cystic carcinoma (AdCC) of the breast is a rare but distinct neoplasm. It has attracted researchers because of its favorable outcome, even better than its salivary gland counterpart, despite negative hormone receptors and its basal-like phenotype [1-4]. Morphologically, it is composed of ~~basaloid/myoepithelial-like (abluminal) cells and ductal epithelial (luminal)~~ luminal and abluminal cells arranged into classical tubular or cribriform architecture. Differentiating classical AdCC from its malignant mimickers, cribriform carcinoma, is usually straightforward because the neoplastic cells in the latter are monotonous, positive for hormone receptors and negative for high molecular weight cytokeratins. However, it is occasionally challenging to distinguish AdCC from its benign mimicker, collagenous spherulosis, especially in needle core biopsies, because both lesions show not only the cribriform architecture but also immunophenotypic overlap [2, 5-7].

In order to uncover the dual cell population of AdCC, various epithelial and myoepithelial/basal cell markers have been used. Previous studies have suggested that some of the myoepithelial markers such as calponin [5, 8], smooth muscle myosin heavy chain [1, 5], CD10 [6, 9], S100 [10-13], and muscle-specific actin [6] that should be expressed in normal myoepithelial cells of the breast are not useful for identifying ~~basaloid/myoepithelial-like~~ abluminal cells in AdCC of the breast. It has been suggested that epithelial and myoepithelial marker expression may be modified or altered in AdCC [14, 15]. Recently, our group has found that high molecular weight keratins, CK5/6 and CK14,

show a unique paradoxical or reverse staining pattern in clear cell lesions of adenomyoepithelioma of the breast, with diffusely positive inner epithelial cells and completely negative outer myoepithelial cells [16]. This prompted us to formally explore the expressions of various epithelial and myoepithelial/basal cell markers in AdCC, another mammary neoplasm with a dual cell population. This study differs from the previous ones on a similar topic in that the ability of each marker to discriminate between epithelial/luminal and myoepithelial/abluminal cells was assessed using the sensitivity vs (1 − specificity) plot, a graphical representation of the relationship between sensitivity and specificity over four threshold settings of 0%, 10%, 50%, and 70%.

Materials and methods

Fourteen cases of mammary AdCC were retrieved from the pathology archives of Nagoya Medical Center (n=2), Nara City Hospital (n=2), Iwate Medical University Hospital (n=2), Kochi Health Sciences Center (n=2), Tokushima University Hospital (n=1), Toyota Kousei Hospital (n=1), and Okayama University Hospital (n=1). Three cases were retrieved from the breast pathology consultation file of SI. All samples were anonymized prior to the analysis. The clinicopathological features of AdCC are shown in Table 1. The age of the patients ranged from 49 to 87 years (mean 65 years). All cases were women. Tumor diameter (the greatest dimension) was 5-43 mm (mean 16.1 mm). As to estrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor receptor type 2 (HER2), the

majority (70%) were so called triple-negative (data not shown). The remaining, approximately 30%, were luminal A (ER-positive HER2-negative). The range of the MIB-1 index was broad, but there was no difference between solid and classical types.

We reviewed hematoxylin and eosin-stained sections of each case to assess the proportions of morphologic components (cribriform, tubular, and solid patterns). AdCC with a solid pattern in more than 90% of the tumor (n=7) was excluded from this study. Therefore, the present study included 14 cases of AdCC with classical cribriform or tubular patterns. Five cases of collagenous spherulosis were retrieved from the pathology archives of Nagoya Medical Center. They were non-complicated microscopic foci of collagenous spherulosis incidentally found in the background of malignant or benign breast lesions. The underlying pathology with collagenous spherulosis is shown in Table 2.

As ~~ductal/epithelial or basaloid/myoepithelial~~ immunohistochemical markers, CK7, EMA, CD117 (c-KIT), CK5/6, CK14, S100, vimentin, calponin, CD10, p63, and Type IV collagen, which are commonly used in many institutions for routine practice, were used. The antibodies, manufacturers, and dilutions of immunohistochemistry are shown in Table 3. Representative 4-μm-thick sections of AdCC, normal TDLU (terminal duct lobular unit), normal ducts, and collagenous spherulosis were cut and subjected to immunohistochemical analysis. Signals were detected using a Leica Bond-Max automated immunostainer (Leica Biosystems, Tokyo, Japan).

Immunohistochemical expression in AdCC was evaluated by focusing on the two landmark

structures of the tumor, namely a true lumen and a false lumen. The former is small and contains neutral periodic acid-Schiff-positive mucin. The latter is of varying shape, mostly round, and contains a myxoid acidic stromal substance that stains with Alcian blue or straps of collagen with small capillaries [7]. The definitions of epithelial and myoepithelial cells in adenoid cystic carcinoma (AdCC) are not as clear as in normal TDLU and ducts. In this study, therefore, topographical terms, luminal and abluminal, rather than epithelial and myoepithelial cells, were adopted in AdCC. ~~epithelial~~ Luminal cells in AdCC were defined as the cells facing the true lumina, and ~~myoepithelial~~ abluminal cells were defined as the cells facing the false lumina in cribriform structures. Similarly, ~~epithelial~~ luminal cells in collagenous spherulosis were defined as the cells facing the true lumina, and the ~~myoepithelial~~ abluminal cells were defined as the cells rimming the round spaces containing eosinophilic, hyaline, acellular spherules.

The proportion of epithelial/luminal or myoepithelial/abluminal cells that were positive for a marker was scored into five categories as follows: completely negative (0), less than 10% (1+), 10–49% (2+), 50–69% (3+), and 70% or more (4+), as previously described [16].

Expressions of the 11 markers for epithelial/luminal and myoepithelial/abluminal cells were evaluated in 12 TDLUs and 14 ducts observed in the background of AdCC (Tables 4 and 5), 5 collagenous spherulosis (Table 6), and 14 AdCC cases (Table 7). The sensitivity and specificity for detecting epithelial/luminal or myoepithelial/abluminal cells were calculated for each marker at four cut-off values, 0%, 10%, 50%, and 70%. Expression was assessed using a curve created by plotting the

true positive rate (sensitivity) against the false positive rate (1 − specificity) at four threshold settings of 0%, 10%, 50%, and 70%. Online Resource 1 shows how the curves were generated based on the sensitivity and the specificity of each marker expression in normal TDLU/duct, collagenous spherulosis, and AdCC. This curve is a comparison of the true positive rate and the false positive rate as the criterion for epithelial/luminal marker changes. It depicts relative trade-offs between true and false positives. The best possible prediction method for epithelial/luminal markers would yield a point in the upper left corner or coordinate (0,1) of the sensitivity vs (1 − specificity) space, representing 100% sensitivity and 100% specificity (called a perfect classification). Absolutely no classification ability would give a point along a diagonal line from the left bottom to the top right corners. It is important to note that the result of a consistently poor predictor for epithelial/luminal markers could simply be inverted to obtain a good predictor for myoepithelial/abluminal ones. In this study, therefore, the best myoepithelial/abluminal markers would yield a point in the lower right corner or coordinate (1,0) of the sensitivity vs (1 − specificity) space, representing 100% sensitivity and 100% specificity for myoepithelial/abluminal cells.

Results

Normal breast

Expressions of 11 markers were assessed in normal TDLUs (n=12) and ducts (n=14) observed in 14 cases of AdCC (Table 4, 5). Between TDLU and ducts, there was no significant difference in

sensitivity and specificity of each marker for detecting epithelial or myoepithelial cells. The most sensitive and specific epithelial markers for normal TDLU/ducts were CK7 and EMA, with perfect sensitivity and specificity (Tables 4, 5, Figures 1, 2). CD117 (c-KIT) also showed excellent, albeit not perfect, specificity and sensitivity for detecting epithelial cells of normal TDLU/ducts. The most sensitive and specific myoepithelial markers for detecting normal TDLU/duct were p63 and CD10 (Tables 4, 5, Figures 1, 2). Type IV collagen, vimentin, CK14, calponin, and S100 were also useful markers, although they were less sensitive or less specific than p63 and CD10. CK5/6 was unsatisfactory in both specificity and sensitivity to differentiate between epithelial and myoepithelial cells of normal TDLU/ducts.

Collagenous spherulosis (Figure 3a)

Expressions of the 11 markers in 5 cases of collagenous spherulosis are shown in Table 6; Figure 4 shows that the best ~~epithelial~~ luminal marker of collagenous spherulosis was CK7, with perfect sensitivity and specificity (Figure 3b). Although EMA and CD117 showed 100% specificity for ~~epithelial~~ luminal cells of collagenous spherulosis, their sensitivities were less than CK7. The best myoepithelial abluminal cell markers for collagenous spherulosis were p63 (Figure 3c), CD10, and type IV collagen (Figure 3d). Calponin and vimentin were less sensitive than p63, CD10, and type IV collagen, but they showed perfect specificity for ~~myoepithelial~~ abluminal cells of collagenous spherulosis. CK14 and CK5/6 were also informative but suboptimal in their sensitivity and specificity.

Adenoid cystic carcinoma (Figure 5a)

Expressions of the 11 markers in ~~true and false lumina~~ luminal and abluminal cells of 14 AdCC cases are shown in Table 7. Figure 6 indicates that EMA (Figure 5b) and CK7 (Figure 5c) were the most sensitive and specific markers for confirming ~~epithelial~~ luminal cells in AdCC. CD117 was also a highly sensitive marker for ~~the true lumen~~ luminal cells, but its specificity was less than that of EMA and CK7. The most sensitive and specific markers for ~~myoepithelial~~ abluminal cells of AdCC were type IV collagen (Figure 5d), p63 (Figure 5e), and vimentin. The present data also indicated that CK5/6 (Figure 5f) was a marker for ~~epithelial~~ luminal rather than abluminal ~~myoepithelial~~ cells of AdCC. Expressions of CD10 and S100 were, unlike those in normal TDLU/ducts and collagenous spherulosis, approaching a diagonal line from the left bottom to the top right corners, indicating almost no classification ability.

Discussion

Recognizing two types of spaces within the tumor is a key for the correct diagnosis of AdCC[1]. One, called a true lumen containing neutral PAS-positive mucin, is composed of ~~ductal epithelial~~ luminal cells. The other is called a false lumen and contains amorphous glycosaminoglycans, believed to be surrounded by myoepithelial/basaloid cells. In order to identify these two types of lumina, various antibodies have been used. To the best of our knowledge, systematic appraisals of ~~epithelial and myoepithelial~~ various immunohistochemical markers for AdCC using the sensitivity vs (1 − specificity) plot have not been performed. The results of the present study are summarized in Table 8.

To detect myoepithelial/basaloid cells, a panel approach including antibodies directed against

basal cytokeratins and myofilaments has been recommended.[7] The recent reviews on mammary AdCC, as well as the latest version of the WHO Blue Book, stated that the myoepithelial/basal cells of AdCC are immunoreactive for basal cytokeratins (CK5, CK5/6, CK14, CK17), myoepithelial markers (p63, actin, calponin, S-100 protein), vimentin, and epidermal growth factor (EGFR).[4, 7, 17] This study, however, indicated that S100 is not useful as a myoepithelial marker for AdCC and that CK5/6-positive cells in AdCC are more frequently around the true lumen of AdCC, indicating that CK5/6 is a marker for ~~epithelial~~ luminal rather than ~~myoepithelial~~ abluminal cells in AdCC (Figures 5f, 6).

A meticulous bibliographic survey identified sporadic descriptions of CK5/6 expression in AdCC, although they rarely specified whether the expression was around the pseudolumen or the true lumen.[3, 11, 18, 19] Azoulay et al. assessed immunohistochemical expressions of CK5/6, CK8/18, and p63 in 18 cases of AdCC of the breast. In cribriform and tubular areas of AdCC, the cells around glandular lumina are CK8/18 and CK5/6-positive. Their observation is consistent with ours. However, there has been no discussion as to the significance of this phenomenon. To the best of our knowledge, the present study is the first formal report on the reverse staining pattern of CK5/6 in AdCC of the breast. We have recently reported on a similar paradoxical phenomenon in clear cell lesions of adenomyoepithelioma of the breast.[16] It is important to note that there is a difference in the paradoxical expression of high molecular weight keratins between adenomyoepithelioma and AdCC; the reverse staining pattern was observed for both CK5/6 and CK14 in adenomyoepithelioma, whereas it was observed only for CK5/6 in

AdCC. Recognition of this difference would be useful for distinction of AdCC cases from adenomyoepithelioma.

With regard to the origin of salivary gland-like breast tumors including AdCC and adenomyoepithelioma, Boecker et al. speculated that CK5/CK14-positive progenitor cells have a potential to differentiate to glandular and myoepithelial lineages and also generate heterogeneous cell differentiations such as squamous and mesenchymal progenies[20]. It would be interesting to know the molecular mechanisms for the reverse expression of CK5/6 in AdCC and both CK5/6 and CK14 in adenomyoepithelioma, since this may reflect the difference in tumorigenesis of these closely related mammary neoplasms.

CK7, EMA, and CD117 have been used as luminal epithelial markers for AdCC [4, 7, 21]. The present results showed that CK7 and EMA are stable, sensitive, and specific luminal epithelial markers for AdCC, as well as normal TDLU/ducts. Basically, the present data on epithelial markers of AdCC are consistent with those of other researchers. Nikitakis et al. noted that AdCCs were diffusely CK7-positive in 14 of 25 cases and focally positive in 11 of 25 cases.[22] In focally positive AdCCs, the immunoreactivity of CK7 was limited to the luminal cells, while expression in ~~myoepithelial~~ abluminal cells was very weak or negative. CD117 (C-kit) has also been proven to be an excellent luminal epithelial marker in normal breast, which is consistent with other reports on CD117 expression in normal tissues.[23] However, the specificity of CD117 for detecting ~~epithelial~~ luminal cells decreased in AdCC

because ~~myoepithelial~~ abluminal cells express this antigen in half of the cases (Table 7). This is consistent with other studies on CD117 expression in most AdCCs [3, 19, 24-26]. All AdCCs (16/16, 100%) examined by Azoulay et al. expressed KIT protein.[3] Five of six (83%) AdCCs of the breast expressed CD117 in more than 50% of tumor cells [25]. In AdCC with a classic or solid cystic pattern, C-kit expression was localized to the inner cell layer. In the solid basaloid pattern AdCC, C-kit expression was seen in all cell layers [25].

The present data showed that p63 is a stable, sensitive, and specific myoepithelial/abluminal marker in normal breast, collagenous spherulosis, and AdCC. In addition, the present data suggest that some myoepithelial markers' expressions are modified or altered according to the myoepithelial lesions. Both CD10 and S100 are useful myoepithelial markers in normal breast and collagenous spherulosis, but their sensitivities and specificities for detecting abluminal cells decreased in AdCC. These results are consistent with those reported by Neves et al., who examined CD10 expression in 20 cases of AdCC of the salivary glands.[9] According to their report, CD10 was not expressed in neoplastic cells or only in less than 10% of them. The authors suggest that CD10 immunohistochemistry could be a useful adjunct to separate epithelial myoepithelial carcinoma from AdCC, because the former showed significantly higher CD10 expression (positive in 83% of the 12 cases examined). With regard to S100, Morice et al. reported that 12 (80%) of the AdCCs reacted with anti-S100, but the strength of reaction was 0-5% of cells in 6 cases and 5-50% in 6 cases [27].

Although unexpected to us, vimentin is a very sensitive and specific abluminal/myoepithelial marker for AdCC and normal breast, but not in collagenous spherulosis. These results have been pointed out by Morinaga et al., who observed that the myoepithelial cells of AdCC were always positive for vimentin; the epithelial cells of AdCC were negative for vimentin and strongly positive for keratin [15].

Collagenous spherulosis is an incidentally discovered benign myoepithelial lesion, often observed in intraductal papillomas, as well as usual ductal hyperplasia, adenosis, and other breast conditions. It features intraluminal eosinophilic, hyaline, acellular spherules rimmed by myoepithelial cells, histologically mimicking cribriform ductal carcinoma in situ or AdCC [7]. According to Rabban et al., AdCCs are CD117(+), calponin(-), whereas collagenous spherulosis lesions are CD117(-), calponin(+). This statement appears to be an oversimplification, because the present results indicate that 64% of AdCCs expressed calponin in ~~myoepithelial~~ abluminal cells (Table 7), and 80% of collagenous spherulosis cases expressed CD117 in ~~myoepithelial~~ abluminal cells (Table 6), although their scores were generally small. The present data suggest that aberrant expression of myoepithelial markers including reverse CK5/6, enhanced vimentin, and attenuated S100 and CD 10 favors AdCC over collagenous spherulosis.

In conclusion, based on systematic evaluation of 11 ~~epithelial and myoepithelial~~ markers using sensitivity and (1 − specificity) plots, we recommend CK7 and EMA as ~~epithelial~~ luminal markers and type IV collagen, p63, and vimentin as ~~myoepithelial~~ abluminal cell markers of AdCC. S100 and CD10

are not appropriate as ~~myoepithelial~~ abluminal cell markers of AdCC. Although CK5/6 and CK14 are generally believed to be myoepithelial/basal markers, the present data indicate that CK5/6 is a luminal epithelial marker of AdCC, which may aid in excluding its mimickers, including collagenous spherulosis, adenomyoepithelioma, and cribriform carcinoma.

Conflict of interest statement

The authors have no conflict of interest to declare.

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Figure legends:

Fig. 1 Sensitivity vs (1－specificity) plot of 11 markers in normal terminal duct lobular units (TDLUs) of the breast

The Y axis is sensitivity, and the X axis is 1 − specificity for luminal epithelial cells. CK7 and EMA are located in the upper left corner, signifying 100% sensitivity and specificity for luminal epithelial cells. P63 and CD10 are located around the lower right corner, indicating excellent classification ability for myoepithelial cells. Vimentin is also a highly sensitive myoepithelial marker but less specific than p63 and CD10. In contrast, calponin and Type IV collagen are highly specific myoepithelial markers but less sensitive than p63 and CD10. S100 and CK14 are informative myoepithelial markers with similar sensitivity and specificity. CK5/6 approaches the diagonal line from the left lower corner to the upper right corner, suggesting a suboptimal epithelial or myoepithelial marker in normal TDLUs.

Fig. 2 Sensitivity vs (1 − specificity) plot of 11 markers in normal ducts of the breast

The Y axis is sensitivity, and the X axis is 1 − specificity for epithelial cells. CK7 and EMA are located in the upper left corner, signifying 100% sensitivity and specificity as luminal epithelial markers. P63, calponin, and CD10 are located at the lower right corner, indicating perfect classification as myoepithelial markers. Vimentin and Type IV collagen show excellent specificity but less sensitivity for myoepithelial cells than p63, calponin, and CD10. The status of the other markers is similar to those in normal TDLUs.

Fig. 3 Hematoxylin and eosin (HE)-stained section of collagenous spherulosis (a). Luminal cells around the true glandular spaces are positive for CK7 (b), while the abluminal cells rimming the round spaces containing acellular spherules are positive for p63 (c) and collagen type IV (d).

Fig. 4 Sensitivity vs (1 − specificity) plot of 11 markers in collagenous spherulosis of the breast

The Y axis is sensitivity, and the X axis is 1 − specificity for epithelial cells. CK7 is located in the upper left corner, indicating perfect classification for luminal epithelial cells. EMA and CD117 are less sensitive than CK7 as luminal epithelial markers of collagenous spherulosis. P63 and CD10 are located at the lower right corner, signifying perfect sensitivity and specificity for ~~myoepithelial~~ abluminal cells. Type IV collagen, calponin, vimentin, S100, CK14, and CK5/6 are also informative ~~myoepithelial~~ abluminal cell markers of collagenous spherulosis.

Fig. 5 Hematoxylin and eosin (HE)-stained section of adenoid cystic carcinoma with a cribriform pattern (a). There are two types of structures: true glandular spaces and pseudolumina. Luminal cells ~~Cells~~ around the true glandular spaces are positive for EMA (b), CK7 (c), and CK5/6 (f), while the abluminal cells around the pseudolumen are positive for p63 (e) and collagen type IV (d). Note that CK5/6 is a marker for ~~epithelial~~ luminal rather than ~~myoepithelial~~ abluminal cells in adenoid cystic carcinoma.

Fig. 6 The sensitivity vs (1 − specificity) plot of 11 markers in Adenoid cystic carcinoma (AdCC) of the breast

The Y axis is sensitivity, and the X axis is 1 − specificity for ~~epithelial~~ luminal cells. EMA and CK7 are located around the upper left corner, implying that they are excellent ~~epithelial~~ luminal cell markers in AdCC. P63, Type IV collagen, and vimentin are located around the lower right corner, indicating that these are excellent ~~myoepithelial~~ abluminal cell markers in AdCC. Note that CK5/6 acts as an abluminal cell ~~myoepithelial~~ marker in AdCC. CD10 and S100 are almost along the diagonal line from the left lower corner to the upper right corner, indicating they do not act as ~~epithelial or myoepithelial~~ luminal or abluminal cell markers in AdCC.

Prof. Fred T. Bosman
Editor-in-Chief
Virchows Archiv
February 24, 2016

Dear Prof. Bosman,

Re: Manuscript reference No. VIAR-D-15-00461R1
Please find attached a revised version of our manuscript "An appraisal of 11 immunohistochemical markers in adenoid cystic carcinoma of the breast: A unique reverse staining pattern of cytokeratin 5/6 excludes its mimickers", which we would like to resubmit for publication as an original article in Virchows Archiv.

The comments and suggestions of the reviewers were highly insightful and enabled us to greatly improve the quality of our manuscript. In the following pages are our point-by-point responses to each of the comments of the reviewers.

Revisions in the text are shown using yellow highlight for additions and strikethrough font for deletions. We hope that the revisions in the manuscript and our accompanying responses will be sufficient to make our manuscript suitable for publication in Virchows Archiv.

We look forward to hearing from you at your earliest convenience.

Yours sincerely,

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Responses to the comments of Reviewer#1

Reviewer #1: This is a well written article and its content is correct. Nevertheless no new information is provided. Most of what is presented is found in textbooks and articles.

Response: Many previous studies dealt with immunohistochemical marker expressions of adenoid cystic carcinoma (AdCC). However, most studies involved only a few markers; a systematic appraisal of various immunohistochemical markers for AdCC using the sensitivity vs (1 − specificity) plot has not been performed. Based on the analysis of 11 markers, this study recommends CK7 and EMA as luminal markers and type IV collagen, p63, and vimentin as abluminal markers of AdCC. S100 and CD10 are not appropriate as abluminal cell markers of AdCC. Furthermore, this is the first formal study indicating that CK5/6 is a luminal cell marker of AdCC rather than an abluminal one, which may provide valuable information in the differential diagnosis between AdCC and its mimickers, including collagenous spherulosis, adenomyoepithelioma, and cribriform carcinoma.

Reviewer #1: It is not clear why the Authors have excluded the solid type of ACC from their cases.

Given the suggestions of Reviewer #2, we adopted topographical terms, luminal and abluminal cells, instead of epithelial and myoepithelial cells for the dual cell populations of AdCC. Since the two types of lumina are obscure in cases of the solid type of AdCC, it is difficult to differentiate between the luminal and abluminal cells in the solid type AdCC. This is the reason why we excluded the solid type of AdCC from the present study. We would like to explore whether immunostaining with the sensitive and specific immunohistochemical markers for classical AdCC provide useful information for the diagnosis and classification of the solid variant of AdCC in our next study.

Responses to the comments of Reviewer#2

Reviewer #2: This immunohistochemical study compares collagenous spherulosis, adenoid-cystic carcinoma of the breast, and normal breast epithelium using 11 epithelial biomarkers such as K7, EMA, CD117, Calponin, p63, CK5/6, CK14, vimentin and collagen IV. The aim of the study was to determine whether these markers could

distinguish the dual differentiation of adenoid-cystic carcinomas. Each marker was scored into five categories. Specificity and sensitivity of each marker are determined and normal breast tissue is used as reference. As expected the most sensitive and specific epithelial marker for TDLUs/ducts were K7, EMA, and CD117, for myoepithelial cells p63, CD10, collagen IV, K14 and calponin. In adenoid-cystic carcinomas the markers were used to classify true glandular lumina and pseudolumina. For luminal cells the most specific and sensitive markers were CK7, EMA and to a lesser degree CD117, for cells lining pseudolumina p63, S100 and collagen IV. Unexpectedly, the CK5/6 was found to be a marker of epithelial rather than myoepithelial cells and was not consistently found in cells of the lining the pseudolumina. The authors interpreted the CK5/6 expression in the epithelial lining of true lumina as aberrant expression of a myoepithelial marker in epithelial cells of adenoid-cystic carcinoma. In conclusion the authors suggested that these markers may help to distinguish these lesions from its mimickers.
This is a well done study with important results with regard to the use of such markers in the differential diagnostic setting.
There are some minor drawbacks that should be considered by the authors:

1. The authors use the two terms epithelial and myoepithelial to describe the two differentiation states of breast epithelium. However the term epithelial in breast includes glandular/luminal and myoepithelial/basal as the two main layers. Thus myoepithelial cell layer is part of the epithelial tissue. Therefore the reviewer would suggest to use luminal or glandular instead of epithelial to define the K7-positive cells. In this context it is important what the authors mean with epithelial and myoepithelial in tables 6 and 7. Is it the immunophenotype (and what type?), is it the cellullar appearance?, is it the lining of lumina?. This should be defined in more detail.

Response: As pointed out by Reviewer #1, the definitions of epithelial and myoepithelial cells in collagenous spherulosis and adenoid cystic carcinoma (AdCC) are not as clear as in normal TDLU and ducts. With regard to the definitions of the dual cell populations in collagenous spherulosis and AdCC, we stated the following in the Methods of the initial manuscript. “In this study, epithelial cells in AdCC were defined as the cells facing the true lumina, and myoepithelial abluminal cells were defined as the cells facing the false lumina in cribriform structures. Similarly, epithelial cells in collagenous spherulosis were defined as the cells facing the true lumina, and the myoepithelial cells were defined as the cells rimming the round spaces containing eosinophilic, hyaline, acellular

spherules." (Text p8).
Given the suggestions of Reviewer #2, we added the following sentences in the Materials and methods (p7):
"The definitions of epithelial and myoepithelial cells in adenoid cystic carcinoma (AdCC) is not as clear as those in normal TDLU and ducts. In this study, therefore, topographical terms, luminal and abluminar, rather than epithelial and myoepithelial cells, were adopted in AdCC."
We replaced "epithelial" with "luminal" and "myoepithelial" with "abluminal" throughout the text as shown in the revised text with yellow highlight.

2. The authors regard collagen IV as myoepithelial markers. However collagen IV is found in the extracellular matrix of the basement membrane and in eosinophilic globules within the tumor. It is not observed in the cytoplasm of cells. Furthermore collagen IV has been reported in tumors that do not show a myoepithelial differentiation potential (for example trichoblasboma or hidradenoma of the skin etc). That means collagen IV is not a specific cellular marker and far more important it is not necessarily a myoepithelial marker.

Response: We agree with the comment by Reviewer #2 that collagen IV is not a myoepithelial marker. We changed the term "epithelial and myoepithelial markers" to "immunohistochemical markers" in the text.

4. Furthermore the authors regard p63 as a myoepithelial marker. Again, p63 has also been shown to be a reliable marker of stem cells, both in squamous epithelium and in different other epithelial tissues. So it may be misleading to interpret the occurrence of p63 in tumor cells as indicating myoepithelial differentiation. This view is supported by the fact that the classical myoepithelial markers SMA etc. are often negative or at least reduced in adenoid-cystic carcinomas.

Response: We agree with the comment by Reviewer #2 that p63 is not a myoepithelial marker. We changed the term "epithelial and myoepithelial markers" to "immunohistochemical markers" in the text.

5. In tables 6 and 7 the authors use the terms epithelial and myoepithelial. The reviewer would like to suggest to specify how they define the different types of cells within these lesions.

Response: This question is equivalent to the first question raised by Reviewer #2. Please refer to the response to the first question.

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Figure1

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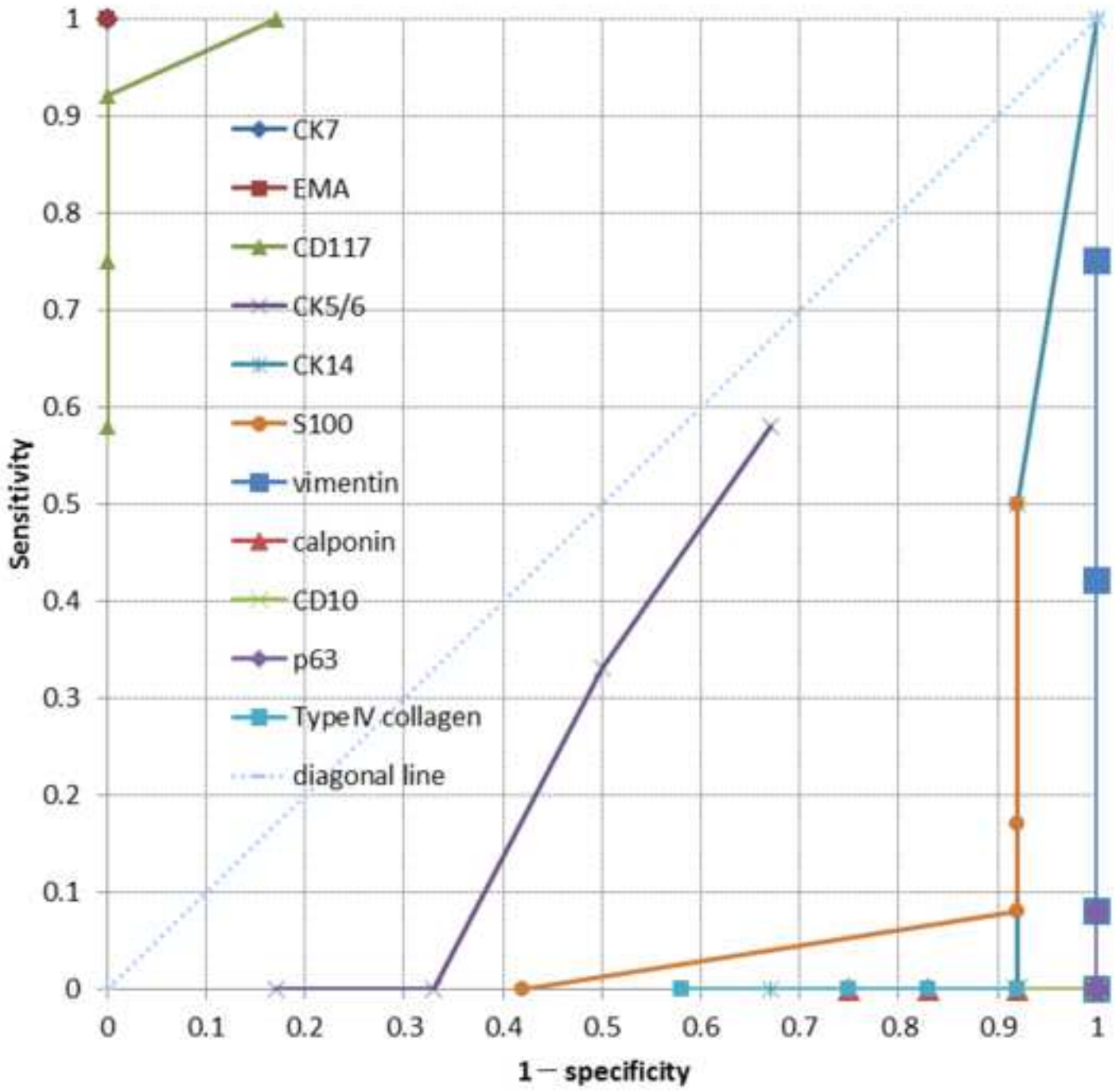

Figure2

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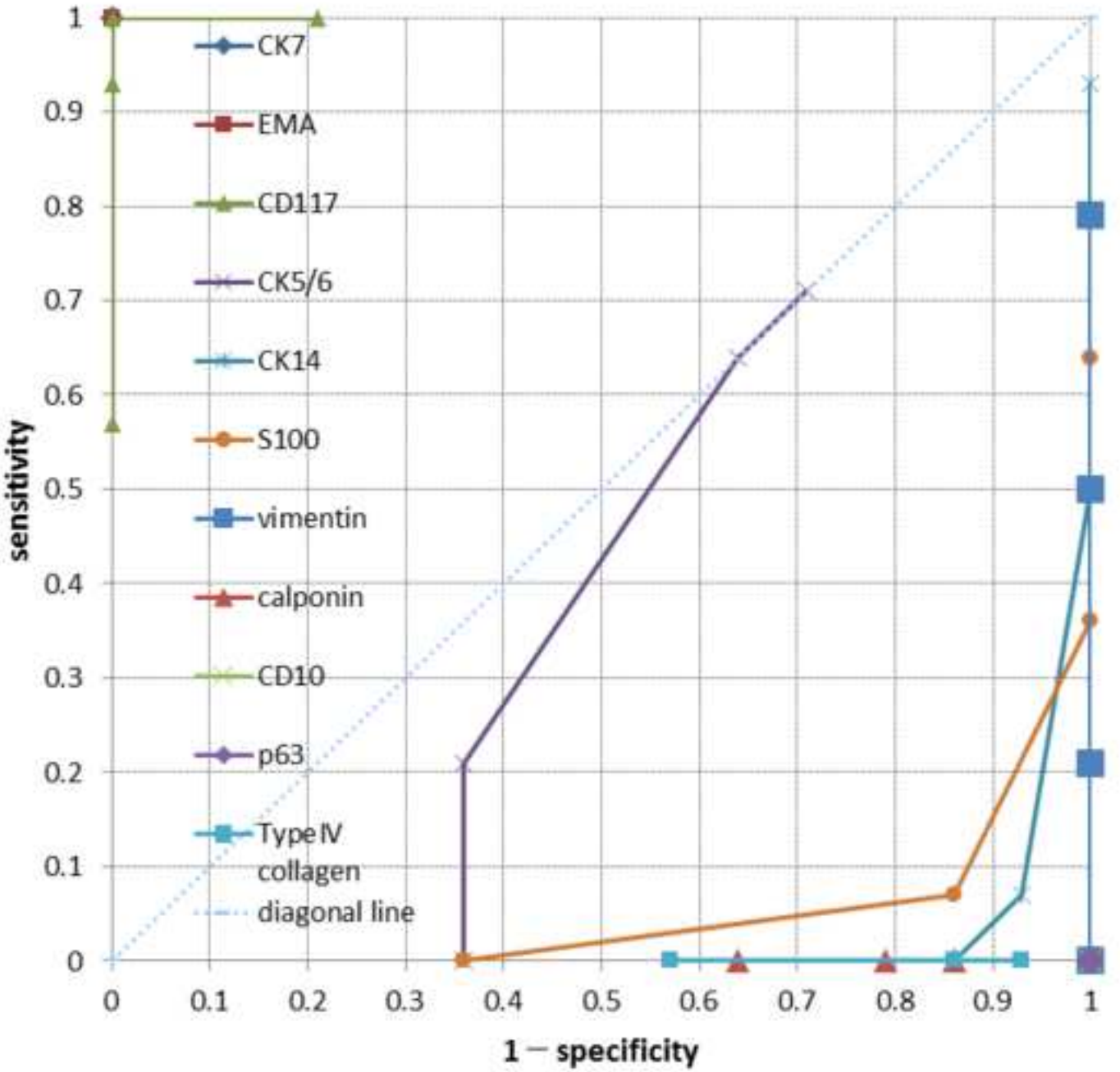

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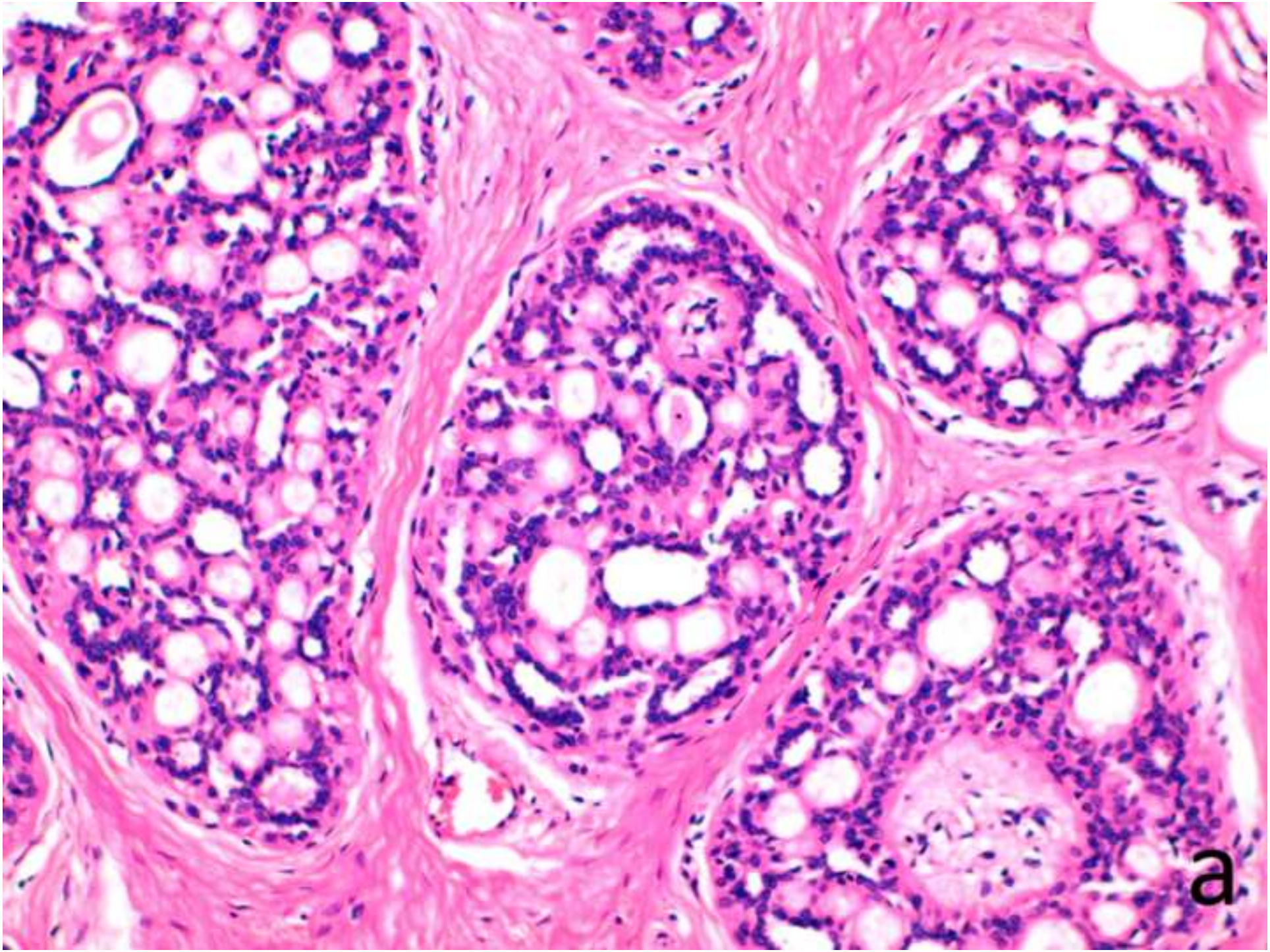

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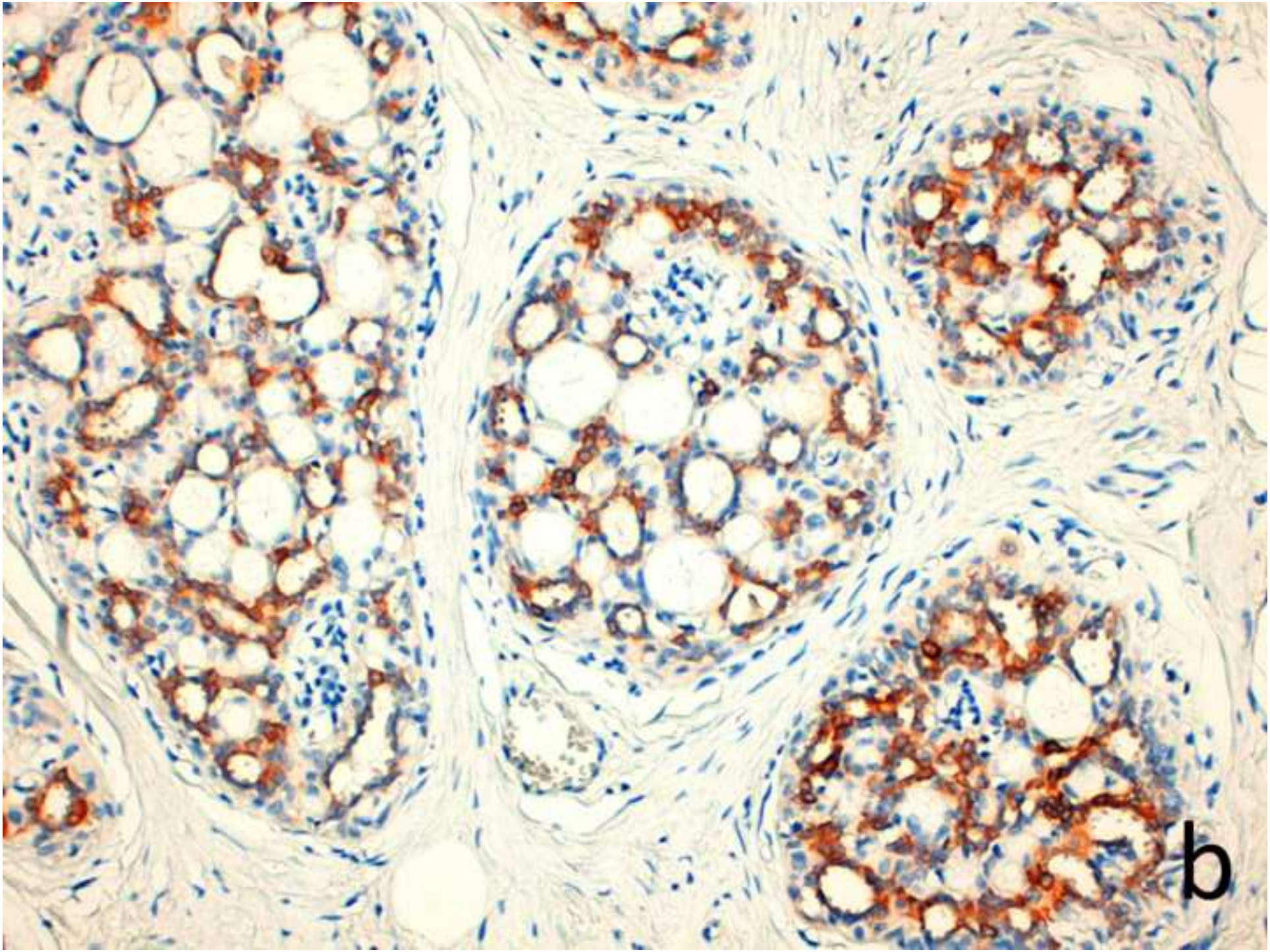

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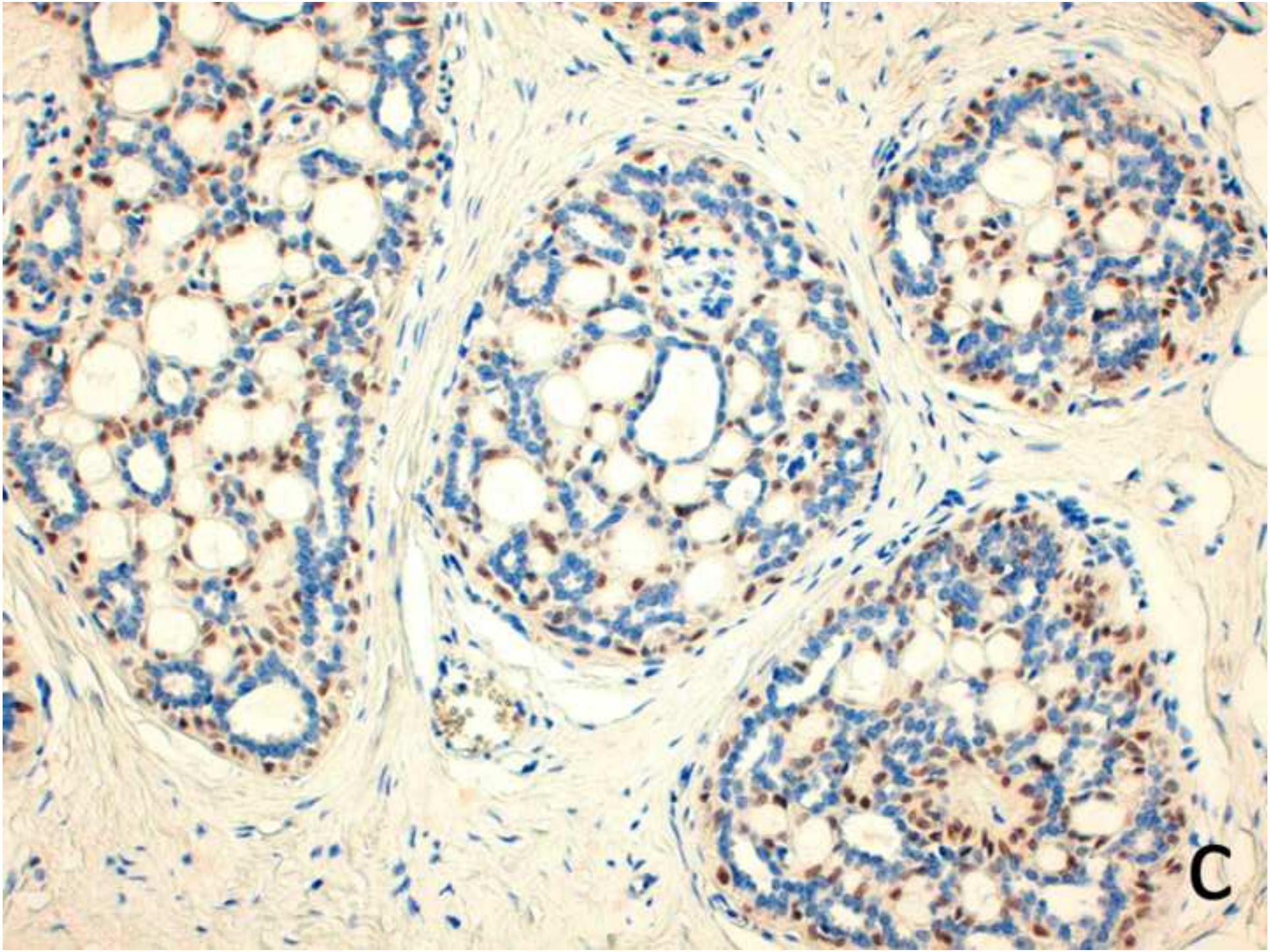

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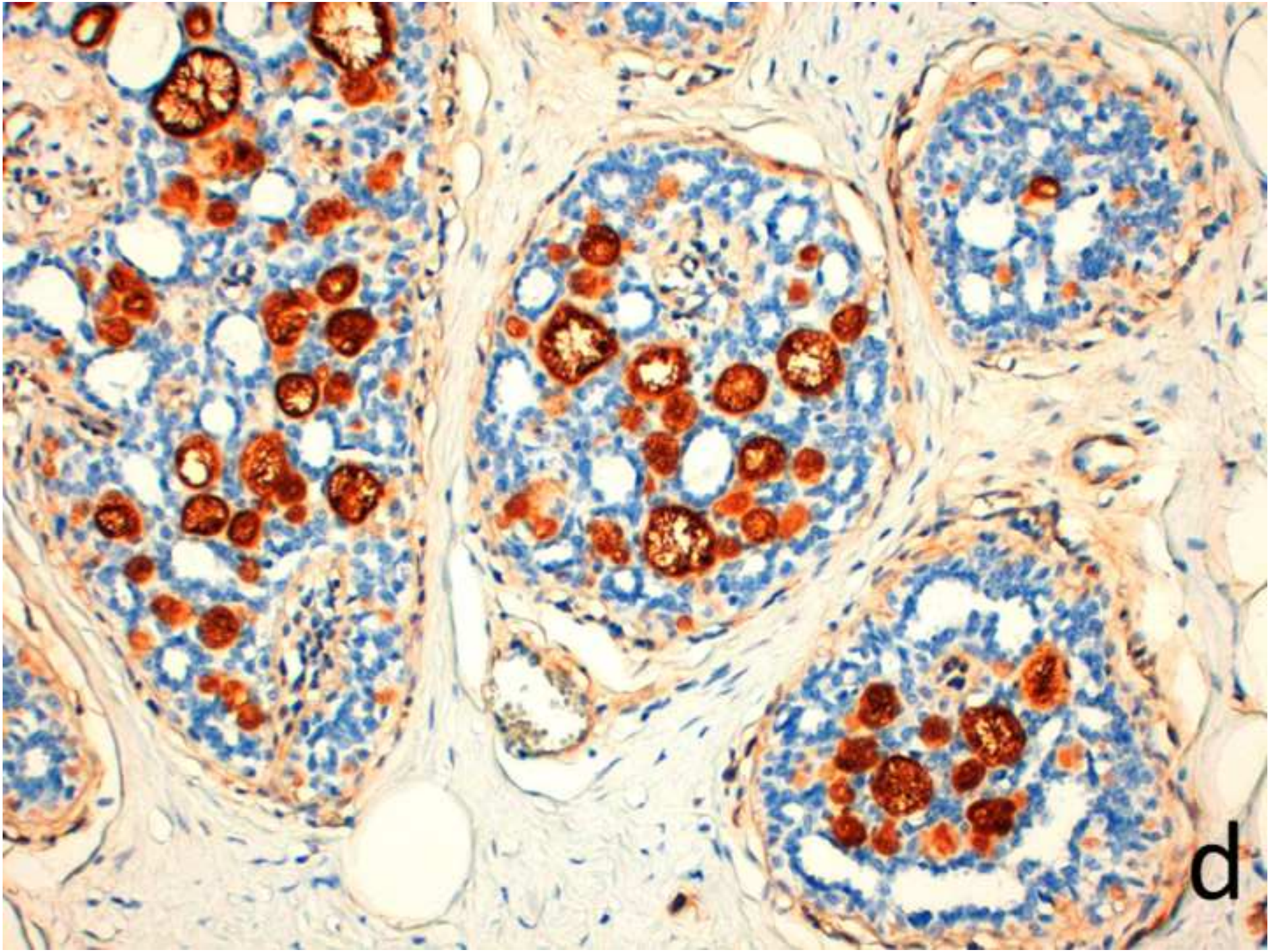

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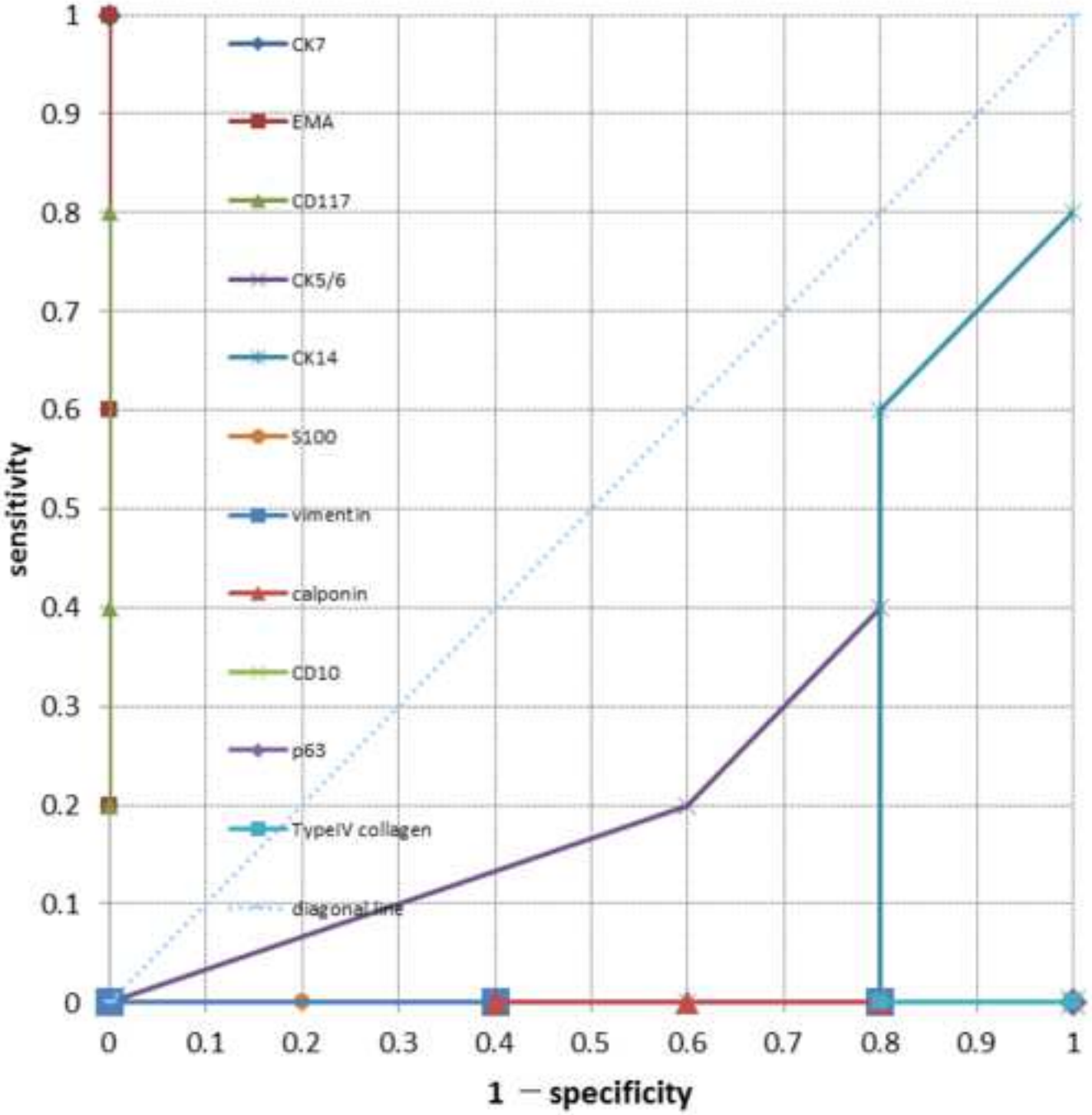

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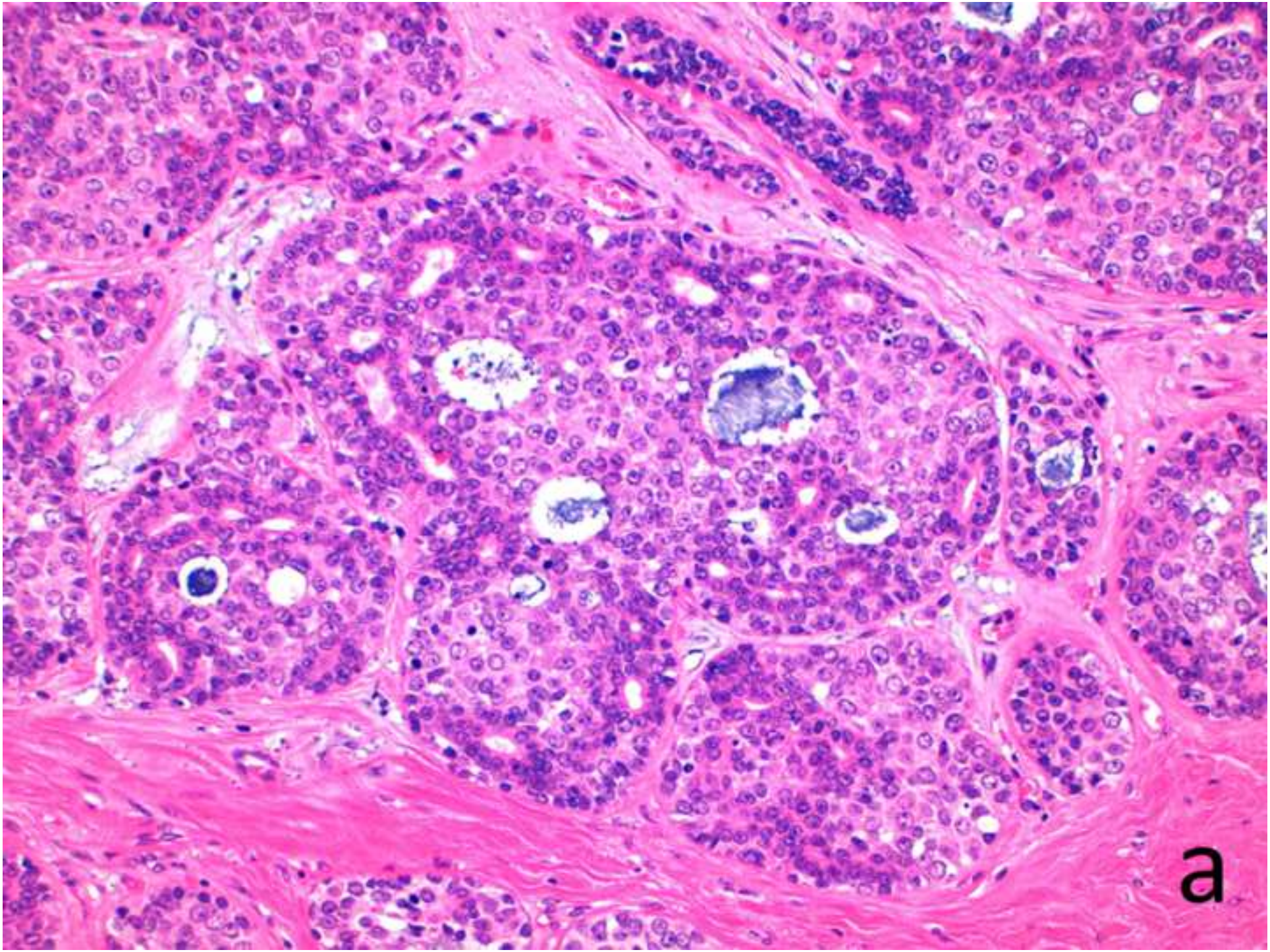

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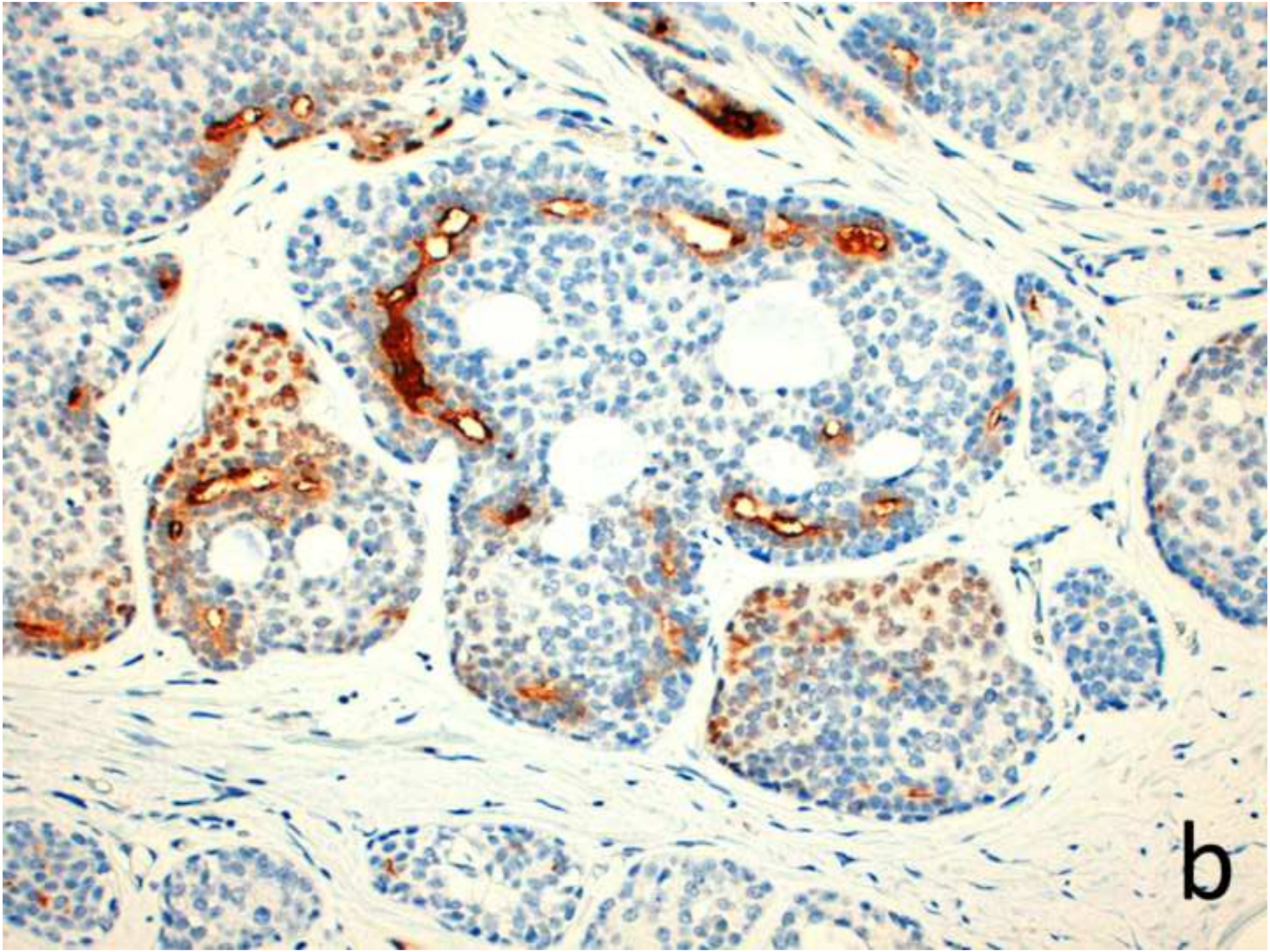

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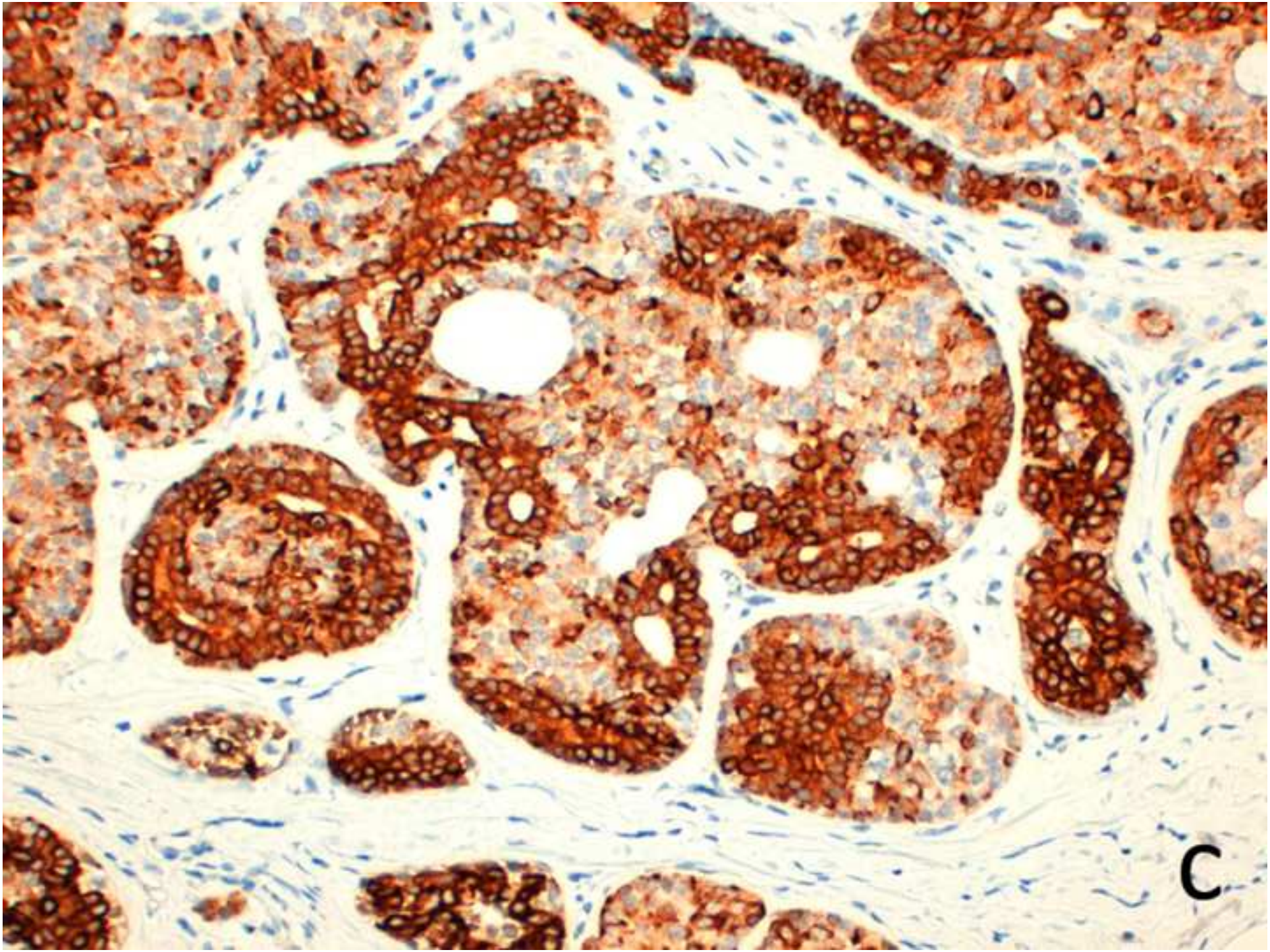

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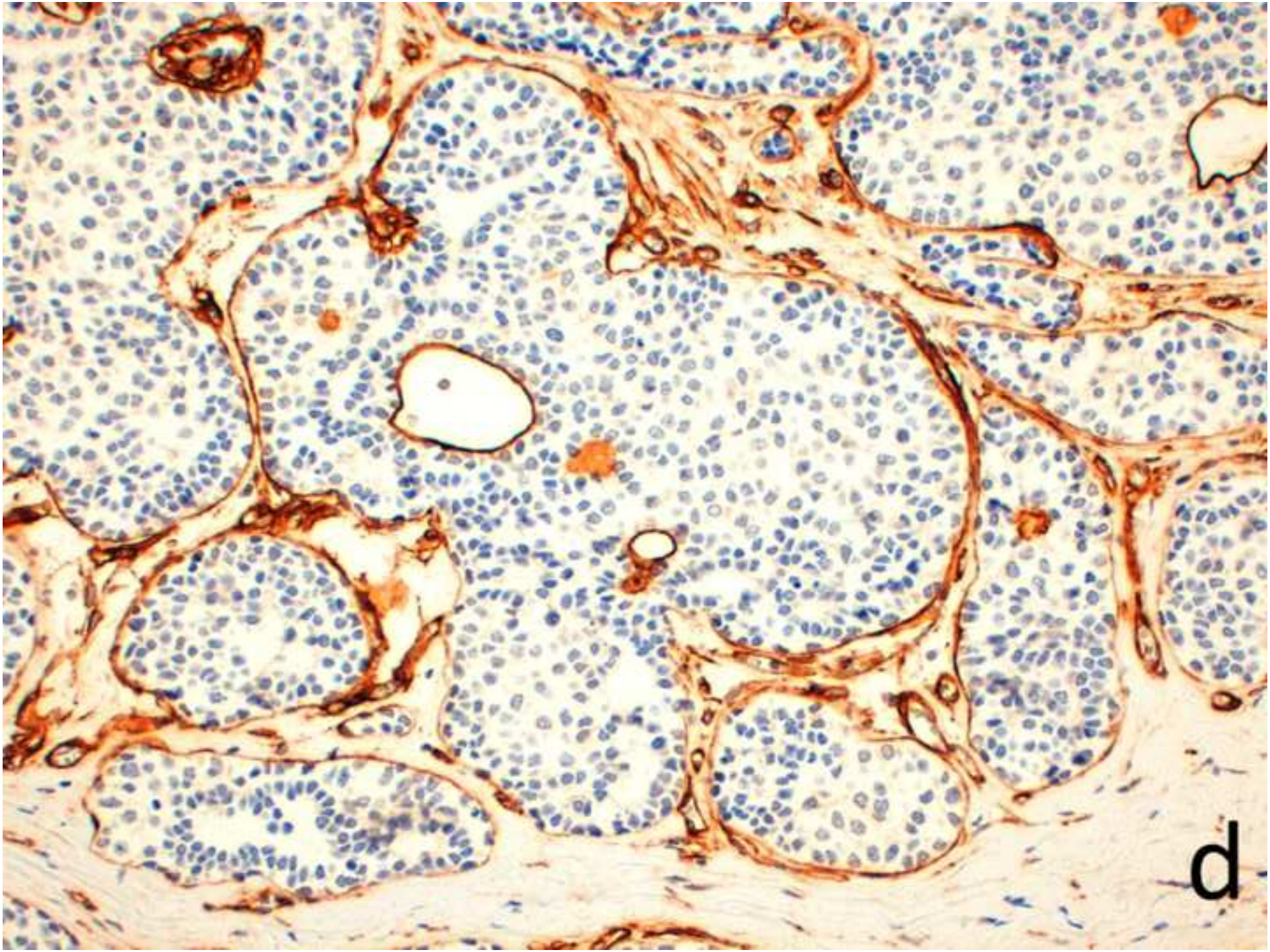

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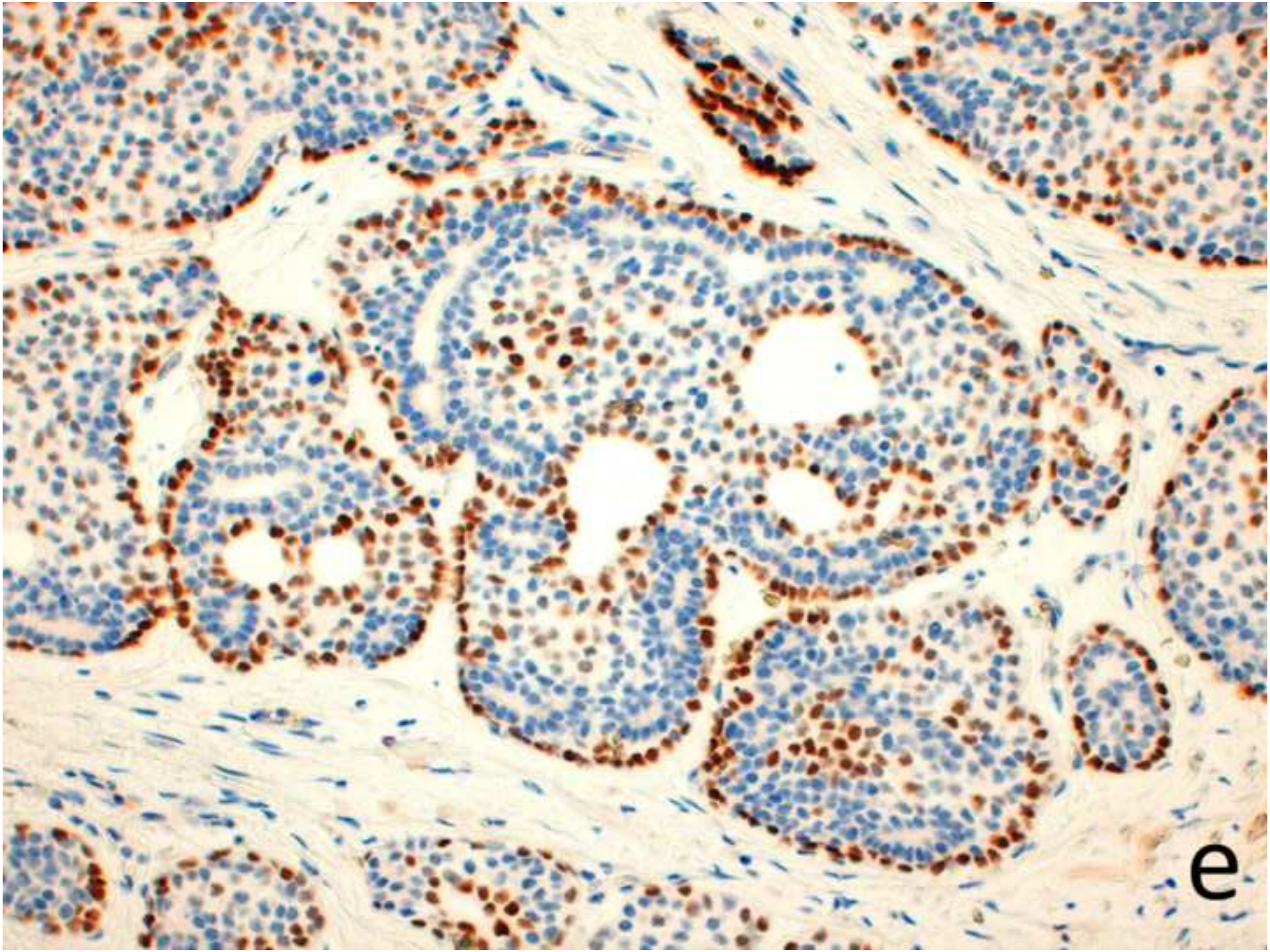

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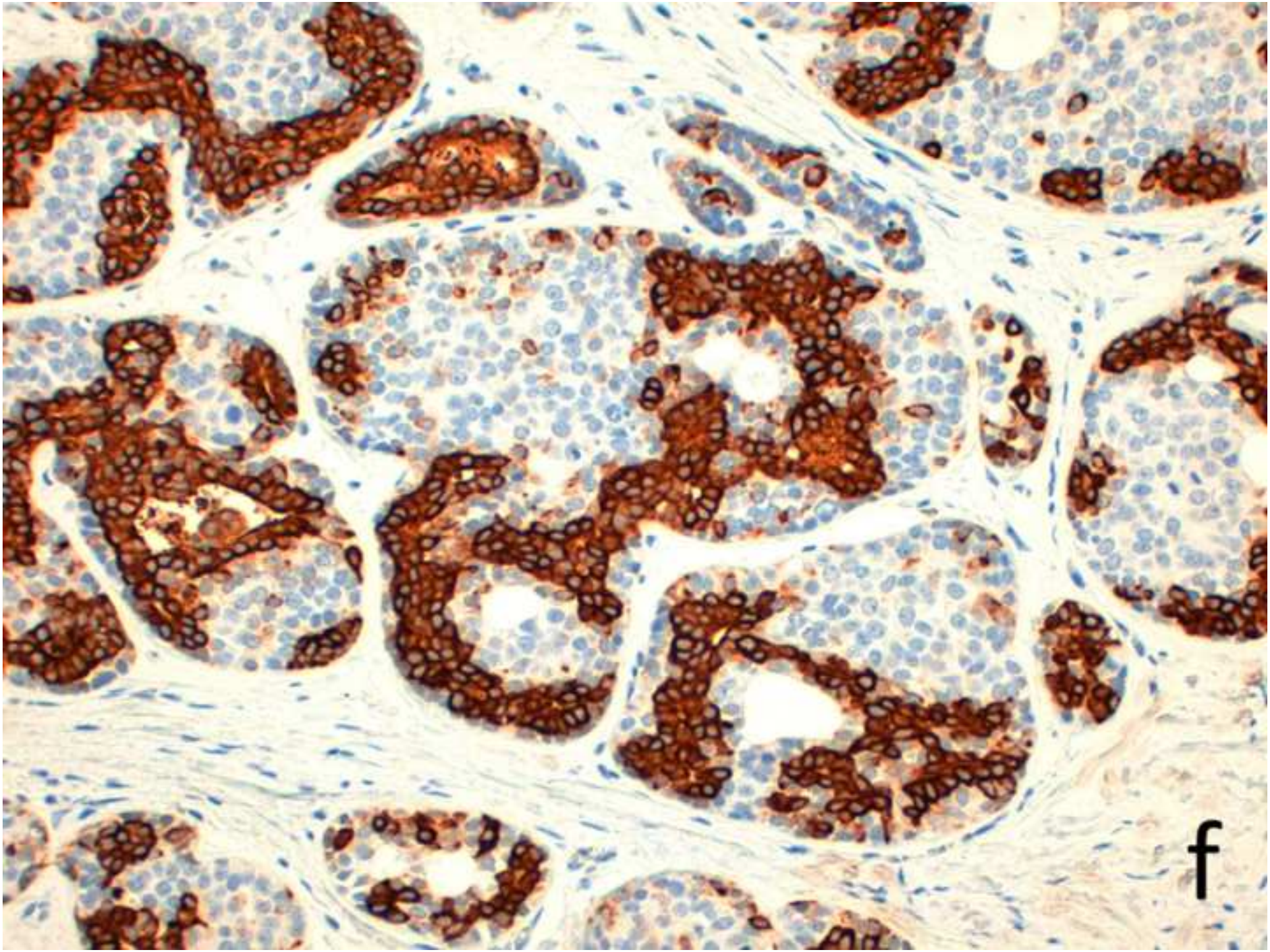

Figure6

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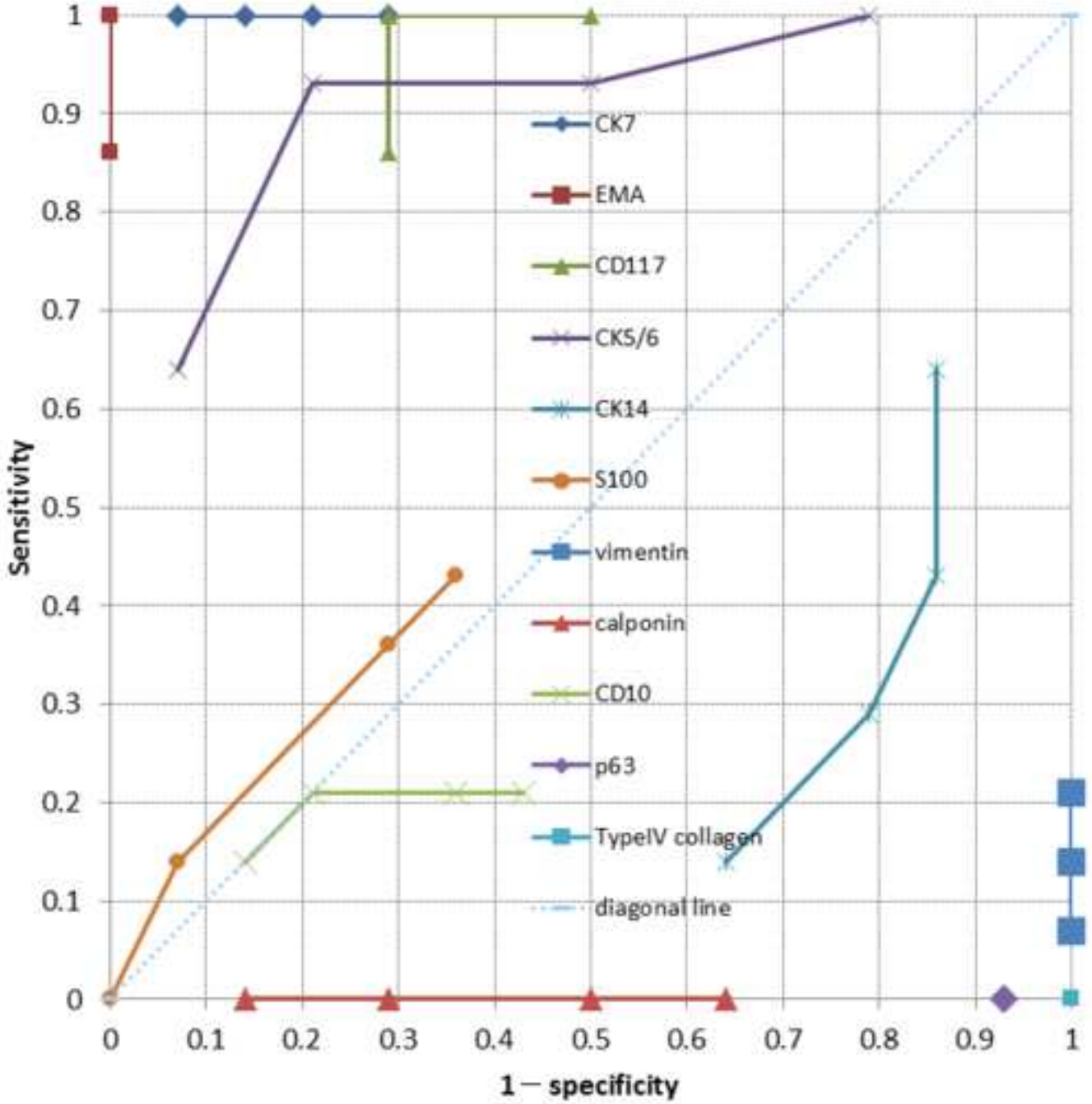

Table 1. Clinicopathological features of adenoid cystic carcinoma of the breast				
Case	Age(years)	Laterality	Size (mm)	Pattern
1	61	Right	15	TUB
2	68	Right	8	TUB
3	87	Left	35	CR>TUB
4	69	Left	18	CR
5	65	Left	43	CR
6	59	Left	20	CR>TUB
7	52	Right	5	TUB
8	69	Left	10	CR>SOL
9	62	Right	10	CR
10	74	Right	8	TUB
11	49	Right	−(CNB)	CR
12	72	Left	10	TUB>CR
13	66	Right	7	CR
14	56	Left	20	CR

CR, cribriform: TUB, tubular: SOL, solid: CNB, core needle biopsy

Table 2. Underlying pathology in collagenous spherulosis			
Case	Age (years)	Specimen type	Primary Diagnosis
1	54	Mastectomy	Invasive ductal carcinoma, NST
2	46	Wide local excision	Intraductal papilloma
3	52	Mastectomy	Invasive ductal carcinoma, NST
4	53	Mastectomy	Ductal carcinoma in situ, high grade
5	44	Mastectomy	Ductal carcinoma in situ, high grade

NST no special type

Table 3. Antibodies used in this study				
Antibody	**Clone**	**Dilution**	**Antigen retrieval**	**Source**
CK5/6	D5/16 B4	x150	Heat	DC
CK7	OV-TL 12/30	x200	Heat	Novo
CK14	LL002	x40	Heat	Novo
CD10	56C6	x50	Heat	Novo
CD117	Polyclonal	x200	Heat	DC
Calponin	26A11	x10	Heat	Novo
EMA	E29	x1200	Heat	Novo
P63	7JUL	x100	Heat	Novo
S-100	Polyclonal	x2	Heat	DC
Vimentin	V9	x4000	Heat	DC
Type4 collagen	PHM-12	x1600	Enzyme	Novo

DC Dako Cytomation, Carpinteria, CA, USA, *Novo* Novacastra Laboratories Ltd, Newcastle upon Tyne, UK,

Table 4. Expression of 11 markers in normal TDLUs (N=12)						
Marker	Topology	Score				
		0	1+	2+	3+	4+
CK7	epithelial	0	0	0	0	12
	myoepithelial	12	0	0	0	0
EMA	epithelial	0	0	0	0	12
	myoepithelial	12	0	0	0	0
CD117	epithelial	0	1	2	2	7
	myoepithelial	10	2	0	0	0
CK5/6	epithelial	5	3	4	0	0
	myoepithelial	4	2	2	2	2
CK14	epithelial	0	6	6	0	0
	myoepithelial	0	1	0	3	8
S100	epithelial	6	4	1	1	0
	myoepithelial	1	0	0	6	5
vimentin	epithelial	3	4	4	1	0
	myoepithelial	0	0	0	0	12
calponin	epithelial	12	0	0	0	0
	myoepithelial	1	0	1	1	9
CD10	epithelial	12	0	0	0	0
	myoepithelial	0	0	0	1	11
p63	epithelial	12	0	0	0	0
	myoepithelial	0	0	0	0	12
TypeIV collagen	epithelial	12	0	0	0	0
	myoepithelial	1	1	1	2	7

The proportion of epithelial or myoepithelial cells that were positive for a marker was scored into five categories as follows: completely negative (0), less than 10 % (1+), 10–49 % (2+), 50–69 % (3+) and 70% or more (4+) as previously described.

Table 5. Expression of 11 markers in normal ducts (N=14)						
Markers	**Topology**	**Score**				
		0	1+	2+	3+	4+
CK7	epithelial	0	0	0	0	14
	myoepithelial	14	0	0	0	0
EMA	epithelial	0	0	0	0	14
	myoepithelial	14	0	0	0	0
CD117	epithelial	0	0	1	5	8
	myoepithelial	11	3	0	0	0
CK5/6	epithelial	4	1	6	3	0
	myoepithelial	4	1	4	0	5
CK14	epithelial	1	6	6	1	0
	myoepithelial	0	0	1	1	12
S100	epithelial	5	4	4	1	0
	myoepithelial	0	0	2	7	5
vimentin	epithelial	3	4	4	3	0
	myoepithelial	0	0	0	0	14
calponin	epithelial	14	0	0	0	0
	myoepithelial	2	0	1	2	9
CD10	epithelial	14	0	0	0	0
	myoepithelial	0	0	0	0	14
p63	epithelial	14	0	0	0	0
	myoepithelial	0	0	0	0	14
TypeIV collagen	epithelial	14	0	0	0	0
	myoepithelial	1	0	1	4	8

The proportion of epithelial or myoepithelial cells that were positive for a marker was scored into five categories as follows: completely negative (0), less than 10 % (1+), 10–49 % (2+), 50–69 % (3+) and 70% or more (4+) as previously described.

Table 6. Expression of 11 markers in collagenous spherulosis (N=5)						
Markers	**Topology**	**Score**				
		0	1+	2+	3+	4+
CK7	luminal	0	0	0	0	5
	abluminal	5	0	0	0	0
EMA	luminal	0	2	0	2	1
	abluminal	5	0	0	0	0
CD117	luminal	1	2	1	1	0
	abluminal	5	0	0	0	0
CK5/6	luminal	3	1	1	0	0
	abluminal	1	1	3	0	0
CK14	luminal	1	1	3	0	0
	abluminal	0	1	0	1	3
S100	luminal	5	0	0	0	0
	abluminal	0	0	0	4	1
vimentin	luminal	5	0	0	0	0
	abluminal	1	2	2	0	0
calponin	luminal	5	0	0	0	0
	abluminal	1	1	1	0	2
CD10	luminal	5	0	0	0	0
	abluminal	0	0	0	0	5
p63	luminal	5	0	0	0	0
	abluminal	0	0	0	0	5
TypeIV collagen	luminal	5	0	0	0	0
	abluminal	0	0	0	1	4

The proportion of luminal or abluminal cells that were positive for a marker was scored into five categories as follows: completely negative (0), less than 10 % (1+), 10–49 % (2+), 50–69 % (3+) and 70% or more (4+) as previously described.

Table 7. Expression of 11 markers in adenoid cystic carcinoma (N=14)						
Marker	**Topology**	**Score**				
		0	1+	2+	3+	4+
CK7	luminal	0	0	0	0	14
	abluminal	10	1	1	1	1
EMA	luminal	0	0	0	2	12
	abluminal	14	0	0	0	0
CD117	luminal	0	0	0	2	12
	abluminal	7	0	3	0	4
CK5/6*	luminal	0	1	0	4	9
	abluminal	3	4	4	2	1
CK14	luminal	5	3	2	2	2
	abluminal	2	0	1	2	9
S100	luminal	8	1	3	2	0
	abluminal	9	1	3	1	0
vimentin	luminal	11	1	1	0	1
	abluminal	0	0	0	0	14
calponin	luminal	14	0	0	0	0
	abluminal	5	2	3	2	2
CD10	luminal	11	0	0	1	2
	abluminal	8	1	2	1	2
p63	luminal	14	0	0	0	0
	abluminal	1	0	0	0	13
TypeIV collagen	luminal	14	0	0	0	0
	abluminal	0	0	0	0	14

The proportion of luminal or abluminal cells that were positive for a marker was scored into five categories as follows: completely negative (0), less than 10 % (1+), 10–49 % (2+), 50–69 % (3+) and 70% or more(4+) as previously described.

*Although CK5/6 was used as an abluminal markers, it was turned out to be a luminal marker in AdCC.

Table 8. Summary of immunohistochemical markers that distinguish between luminal and abluminal cells in collagenous spherulosis and adenoid cystic carcinoma				
	Topology	**Highly recommended**	**Informative**	**Not recommended**
Collagenous spherulosis	luminar	CK7	EMA CD117	
	abluminar	P63 CD10 TypeIV collagen	S100 Calponin CK14 Vimentin CK5/6	
Adenoid cystic carcinoma	luminar	CK7 EMA	CD117 CK5/6	
	abluminar	p63 Vimentin TypeIV collagen	CK14 Calponin	CD10 S100