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Estrogen receptor and Signal Transducer and Activator of Transcription 3 expression in equine mammary tumors

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Brief Communication

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Domestic herbivores develop mammary tumors at a much lower frequency than domestic carnivores. Mammary tumors in mares are rare, in spite of the fact that horses in the United Kingdom generally represent a companion animal group rather than a production animal species, and as such often reach an advanced age. In general, equine mammary tumors are carcinomas. They tend to be locally aggressive and metastasize to the regional lymph nodes and other organs [2 3 8 9 11]. The scarcity of equine mammary tumors is exemplified by the fact that these tumors have mainly been documented by means of case reports [3 8 2] and small case series [9 11]. Molecular characterisation, which is gaining increasing prominence in small animal mammary oncology [13], has so far been limited, although staining for estrogen receptor and progesterone receptor has been attempted [11] and successfully demonstrated in one mare [2].

The purpose of the current study was to characterise the morphology of equine mammary tumors in conjunction with expression of selected intermediate filaments and the adhesion molecule E-cadherin. Estrogen receptor α (ERα) status was assessed in view of the previously reported estrogen receptor positive case [2] and the possible prognostic implications. Finally, nuclear expression of STAT3 in equine mammary tumors was interrogated as a potential future prognostic marker. The signal transducers and activators of transcription (STATs) are transcription factors that influence cellular differentiation, proliferation, survival and death. STAT3 is constitutively activated in approximately 50% of primary human breast tumors [1], and may predict a poor prognosis in ERα positive cases [7]. STATs are generally activated by transient phosphorylation, prior to dimerization and translocation to the nucleus to control transcription and thus nuclear localization may indicate transcriptional activity.

Methods

A search of the histopathology and necropsy databases at Beaufort Cottage Laboratories from 2006 to 2013 (totalling 15,789 equine submissions) identified six cases of equine mammary carcinoma and no cases of equine mammary hyperplasia or adenomas. A further equine mammary carcinoma was identified from the histopathology submission records of Bridge Pathology Ltd, which comprised 470 equine histopathology submissions received between 2008 and 2013. Representative histological sections were examined and the molecular characteristics of the tumors were investigated.

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Clinical and histopathological findings

Limited signalment and clinical data was available. For five of the seven mares, the age range was 10 years to 20 years (median 11 years). Two were cobs, one was a Thoroughbred and for the others breed was not known. Information on parity and lactation status was not available. In some cases the mass had been present for some time (horse Nos. 1 and 2). In two cases (horse Nos. 1 and 5) the lesion was originally diagnosed and treated as mastitis. In one instance (horse No. 3) unilateral mastectomy was performed, but generally treatment was limited or not recorded. Euthanasia was frequently undertaken following the results of biopsy. In one case (horse No. 2), necropsy was carried out, and metastatic spread to the sublumbar, prefemoral and caudal cervical lymph nodes, lung, spleen and liver was identified and confirmed histologically.

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Three out of seven cases (horse Nos. 1, 4 and 5) exhibited strong nuclear staining for ERα (Figs. 7 and 8), whilst three of the seven equine mammary carcinomas (horse Nos. 4, 5 and 7) exhibited strong nuclear expression of STAT3 (Fig. 9 and 10). Expression of the intercellular adhesion molecule E-cadherin was strong in all seven cases (Figs. 7 and 10).

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ERα expression status did not correlate with a particular histopathological pattern in the present case series. However, although it is not possible to draw firm conclusions from analysis of seven cases, it is interesting that ERα positive staining tended to be observed in tumors with weak or absent vimentin staining. This correlates with recent evidence suggesting that in humans, vimentin expression, which tends to be associated with epithelial-mesenchymal transition (EMT), is higher in triple-negative breast cancers than in other subtypes such as those expressing estrogen receptor [12].

Pan-cytokeratin staining in these tumors was generally mild to moderate in intensity, which is in contrast to a previous report where equine mammary ductal carcinoma cells were strongly positive for pan-cytokeratin (clone Lu-5) [3]. Notably even in Horse No. 6, in which approximately 60% of the neoplastic cells exhibited mild to moderate cytoplasmic expression of cytokeratin, staining was considerably weaker in the neoplastic cells than in adjacent non-neoplastic mammary tissue. In non-neoplastic tissue, luminal epithelia exhibited intense staining with the pan-cytokeratin antibody, whilst weaker staining was noted in cells with location and morphology consistent with basal epithelia. Consequently, a specific basal

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Membranous expression of E-cadherin was strong in all seven cases; in the one carcinoma that was known to have metastasised (Horse No. 2) expression remained strong in both the primary tumor and pulmonary metastases. This is consistent with the human breast cancer literature, where it has been demonstrated that E-cadherin expression in metastatic ductal carcinomas may be the same intensity or stronger than in the equivalent primary masses [6]. It is traditionally considered most likely that E-cadherin expression is down-regulated during the process of epithelial-mesenchymal transition (EMT) during metastasis, with subsequent re-expression in metastatic foci. However, it has recently been suggested that in some instances of human inflammatory breast carcinoma E-cadherin may also be up-regulated in tumor microemboli, favouring intravasation [10]. The immunofluorescence findings in the present study could be compatible with either hypothesis.

Interestingly, strong nuclear localisation of STAT3 was detected in three of the seven equine mammary carcinomas (horse Nos. 4, 5 and 7), suggesting a potential role for STAT3 activity in a subset of equine mammary carcinomas. These cases exhibited a tubulo-papillary to solid growth pattern and in two cases exhibited convincing ERα expression, tentatively suggesting that a more luminal phenotype might be associated with nuclear STAT3

expression. The three cases which exhibited moderate to intense positive staining for cytokeratin 14 (horse Nos. 1, 3 and 6), indicating a more basal phenotype, exhibited rare to absent nuclear STAT3 expression, potentially further supporting this interpretation. In human breast cancer, some authors have suggested a correlation between STAT3 expression and the luminal subtype [7], although this is not consistently demonstrable [1].

A major limitation of this study is that the small number of available cases precludes any meaningful correlations or prognostic assertions. Given the rarity of equine mammary tumors, wide-ranging multi-centre collaborations are required to build up adequate case material for any such prognostic or molecular relationships to be adequately interrogated. Such investigations would also require comprehensive clinical follow-up. Although all of the cases for which outcome data was available were euthanized following diagnosis, the records available do not indicate whether this decision was based on clinical condition of the mare, or prognostic advice from the attendant veterinary surgeon.

Taken together, our histopathological data confirms previous reports that equine mammary tumors have an invasive and malignant phenotype. Importantly, the histological phenotype, and molecular characteristics appear to be heterogeneous, with variable expression of intermediate filaments, ERα, and nuclear STAT3. The role of STAT3 activity in equine mammary tumors merits further investigation, particularly to confirm or refute the potential inverse correlation with cytokeratin 14 expression. Our findings also confirm that a subset of equine mammary tumors exhibit ERα expression, which suggests that these tumors may have similar molecular characteristics to their feline and canine counterparts.

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References

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4 Hughes K, Wickenden JA, Allen JE, Watson CJ. Conditional deletion of Stat3 in mammary epithelium impairs the acute phase response and modulates immune cell numbers during post-lactational regression. *The Journal of pathology* 2012;227(1):106-117.

5 Itoh M, Iwamoto T, Matsuoka J, et al. Estrogen receptor (ER) mRNA expression and molecular subtype distribution in ER-negative/progesterone receptor-positive breast cancers. *Breast cancer research and treatment* 2014;143(2):403-409.

6 Kowalski PJ, Rubin MA, Kleer CG. E-cadherin expression in primary carcinomas of the breast and its distant metastases. *Breast cancer research : BCR* 2003;5(6):R217-222.

7 Liu LY, Chang LY, Kuo WH, et al. Prognostic features of signal transducer and activator of transcription 3 in an ER(+) breast cancer model system. *Cancer informatics* 2014;13:21-45.

8 Munson L. Carcinoma of the mammary gland in a mare. *J Am Vet Med Assoc* 1987;191(1):71-72.

9 Prendergast M, Bassett H, Larkin HA. Mammary carcinoma in three mares. *Vet Rec* 1999;144(26):731-732.

10 Rodriguez FJ, Lewis-Tuffin LJ, Anastasiadis PZ. E-cadherin's dark side: possible role in tumor progression. *Biochimica et biophysica acta* 2012;1826(1):23-31.

11 Seahorn TL, Hall G, Brumbaugh GW, Honnas CM, Lovering SL, Snyder JR. Mammary adenocarcinoma in four mares. *J Am Vet Med Assoc* 1992;200(11):1675-1677.

12 Yamashita N, Tokunaga E, Kitao H, et al. Vimentin as a poor prognostic factor for triple-negative breast cancer. *Journal of cancer research and clinical oncology* 2013;139(5):739-746.

13 Zappulli V, Rasotto R, Caliari D, et al. Prognostic Evaluation of Feline Mammary Carcinomas: A Review of the Literature. *Veterinary pathology* 2014. Online first. doi:10.1177/0300985814528221.

Figure legends

Figures 1-10. Mammary carcinoma, horse, mammary gland. **Figures 1-3** HE. **Figure 1.** Tubulopapillary pattern; horse No. 5. **Figure 2.** Solid with comedone pattern of necrosis; horse No.1. **Figure 3.** Tumor cells present within a lymphatic vessel (black arrow); horse No. 3. **Figure 4.** IHC for vimentin shows that the majority of the neoplastic cells in this tubulopapillary to solid carcinoma exhibit strong cytoplasmic expression (black arrowhead); horse No. 2. **Figure 5.** IHC for pan-cytokeratin shows that approximately 60% of the neoplastic cells in this tubulopapillary to solid carcinoma exhibit mild to moderate cytoplasmic expression; horse No. 6. Intense cytoplasmic expression is observed in adjacent non-neoplastic mammary tissue (red arrow; positive internal control tissue). **Figure 6.** IHC for cytokeratin 14 shows that the vast majority of the neoplastic cells exhibit intense cytoplasmic expression; horse No. 1. **Figure 7.** Nuclear expression of estrogen receptor alpha (ERα) and membranous expression of E-cadherin in a solid carcinoma; horse No. 1. Immunofluorescence staining for ERα (red), E-cadherin (E-cad) (green) and DNA (Hoechst; blue). **Figure 8.** Nuclear expression of estrogen receptor alpha (ERα) in a solid carcinoma; horse No. 4. Immunofluorescence staining for ERα (red), and DNA (Hoechst; blue). **Figure 9.** IHC for STAT3 shows both cytoplasmic and nuclear localization (arrowhead); horse No. 4. **Figure 10.** Nuclear localization of STAT3 (white arrow) in an equine mammary tumor. Not all neoplastic cells exhibit nuclear STAT3 (white arrowhead) corroborating specificity of staining; horse No. 7. Immunofluorescence staining for STAT3 (red), E-cadherin (E-cad) (green) and DNA (Hoechst; blue).

Supplemental Figures S1 and S2. Quiescent mammary tissue, horse, mammary gland.

Supplemental Figure S1. IHC for pan-cytokeratin demonstrates that pan-cytokeratin preferentially stains luminal epithelial cells compared to basal epithelial cells (arrow).

Supplemental Figure S2. IHC for cytokeratin 14 stains basal epithelial cells.

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3 Hirayama K, Honda Y, Sako T, et al. Invasive ductal carcinoma of the mammary gland in a mare. *Vet Pathol* 2003;40(1):86-91.

4 Hughes K, Wickenden JA, Allen JE, Watson CJ. Conditional deletion of Stat3 in mammary epithelium impairs the acute phase response and modulates immune cell numbers during post-lactational regression. *The Journal of pathology* 2012;227(1):106-117.

5 Itoh M, Iwamoto T, Matsuoka J, et al. Estrogen receptor (ER) mRNA expression and molecular subtype distribution in ER-negative/progesterone receptor-positive breast cancers. *Breast cancer research and treatment* 2014;143(2):403-409.

6 Kowalski PJ, Rubin MA, Kleer CG. E-cadherin expression in primary carcinomas of the breast and its distant metastases. *Breast cancer research : BCR* 2003;5(6):R217-222.

7 Liu LY, Chang LY, Kuo WH, et al. Prognostic features of signal transducer and activator of transcription 3 in an ER(+) breast cancer model system. *Cancer informatics* 2014;13:21-45.

8 Munson L. Carcinoma of the mammary gland in a mare. *J Am Vet Med Assoc* 1987;191(1):71-72.

9 Prendergast M, Bassett H, Larkin HA. Mammary carcinoma in three mares. *Vet Rec* 1999;144(26):731-732.

10 Rodriguez FJ, Lewis-Tuffin LJ, Anastasiadis PZ. E-cadherin's dark side: possible role in tumor progression. *Biochimica et biophysica acta* 2012;1826(1):23-31.

11 Seahorn TL, Hall G, Brumbaugh GW, Honnas CM, Lovering SL, Snyder JR. Mammary adenocarcinoma in four mares. *J Am Vet Med Assoc* 1992;200(11):1675-1677.

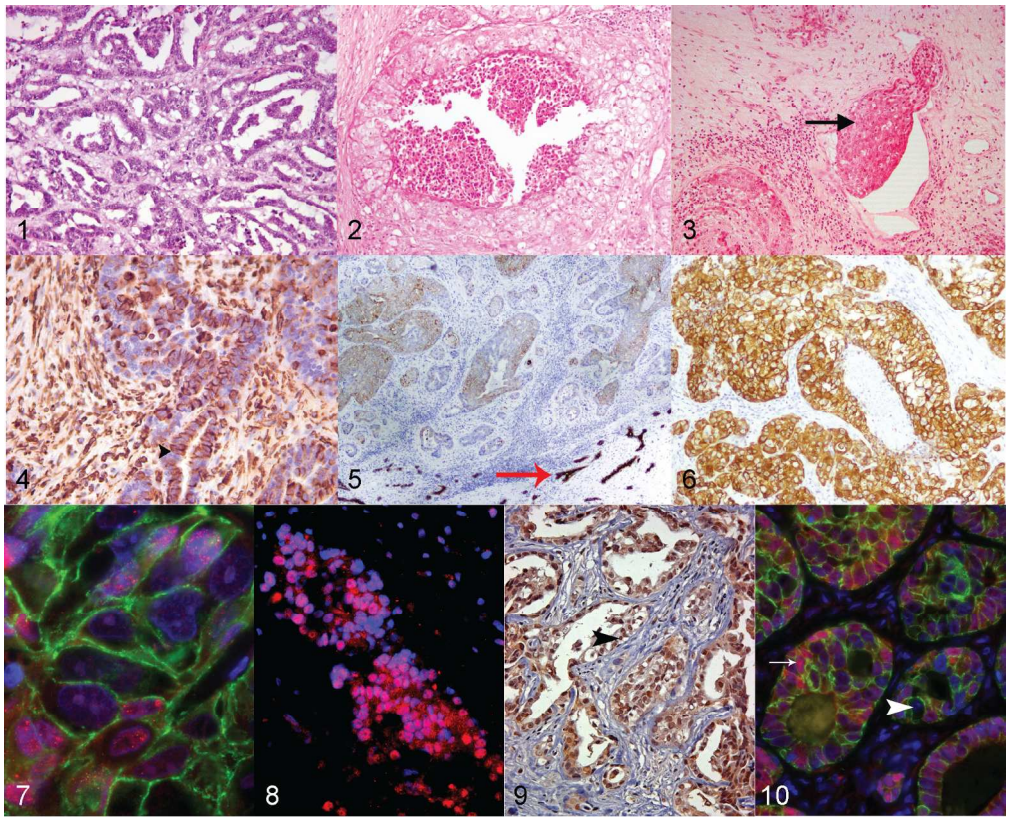
12 Yamashita N, Tokunaga E, Kitao H, et al. Vimentin as a poor prognostic factor for triple-negative breast cancer. *Journal of cancer research and clinical oncology* 2013;139(5):739-746.

13 Zappulli V, Rasotto R, Caliari D, et al. Prognostic Evaluation of Feline Mammary Carcinomas: A Review of the Literature. *Veterinary pathology* 2014. Online first. doi:10.1177/0300985814528221.

Figure legends

Figures 1-10. Mammary carcinoma, horse, mammary gland. **Figures 1-3** HE. **Figure 1.** Tubulopapillary pattern; horse No. 5. **Figure 2.** Solid with comedone pattern of necrosis; horse No.1. **Figure 3.** Tumor cells present within a lymphatic vessel (black arrow); horse No. 3. **Figure 4.** IHC for vimentin shows that the majority of the neoplastic cells in this tubulopapillary to solid carcinoma exhibit strong cytoplasmic expression (black arrowhead); horse No. 2. **Figure 5.** IHC for pan-cytokeratin shows that approximately 60% of the neoplastic cells in this tubulopapillary to solid carcinoma exhibit mild to moderate cytoplasmic expression; horse No. 6. Intense cytoplasmic expression is observed in adjacent non-neoplastic mammary tissue (red arrow; positive internal control tissue). **Figure 6.** IHC for cytokeratin 14 shows that the vast majority of the neoplastic cells exhibit intense cytoplasmic expression; horse No. 1. **Figure 7.** Nuclear expression of estrogen receptor alpha (ERα) and membranous expression of E-cadherin in a solid carcinoma; horse No. 1. Immunofluorescence staining for ERα (red), E-cadherin (E-cad) (green) and DNA (Hoechst; blue). **Figure 8.** Nuclear expression of estrogen receptor alpha (ERα) in a solid carcinoma; horse No. 4. Immunofluorescence staining for ERα (red), and DNA (Hoechst; blue). **Figure 9.** IHC for STAT3 shows both cytoplasmic and nuclear localization (arrowhead); horse No. 4. **Figure 10.** Nuclear localization of STAT3 (white arrow) in an equine mammary tumor. Not all neoplastic cells exhibit nuclear STAT3 (white arrowhead) corroborating specificity of staining; horse No. 7. Immunofluorescence staining for STAT3 (red), E-cadherin (E-cad) (green) and DNA (Hoechst; blue).

Supplemental Figures S1 and S2. Quiescent mammary tissue, horse, mammary gland. **Supplemental Figure S1.** IHC for pan-cytokeratin demonstrates that pan-cytokeratin preferentially stains luminal epithelial cells compared to basal epithelial cells (arrow). **Supplemental Figure S2.** IHC for cytokeratin 14 stains basal epithelial cells.



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Supplemental table 1

Antibodies employed for immunohistochemistry and immunofluorescence.

Antibody and species	Dilution	Manufacturer	Catalogue number
Mouse anti-human vimentin	1:500	Dako	M0725
Mouse anti-human cytokeratin. Clone MNF116. Reacts with cytokeratins 5, 6, 8, 17 and probably also 19.	1:100	Dako	M0821
Mouse anti-cytokeratin 14 [LL002]	1:200	Abcam	ab7800
Rabbit anti-mouse STAT3	1:50	Cell Signaling Technology	#9132
Rabbit anti-mouse estrogen receptor α[a]	1:50	Santa Cruz Biotechnology	sc-542
Mouse anti-bovine estrogen receptor α[b]	1:50	Santa Cruz Biotechnology	sc-787
Mouse anti-human E-cadherin	1:200	BD Biosciences	610182

[a] Cases 1-4 only.

[b] Cases 5-7 only.

Supplemental table 2

Histopathological data for seven equine mammary tumors.

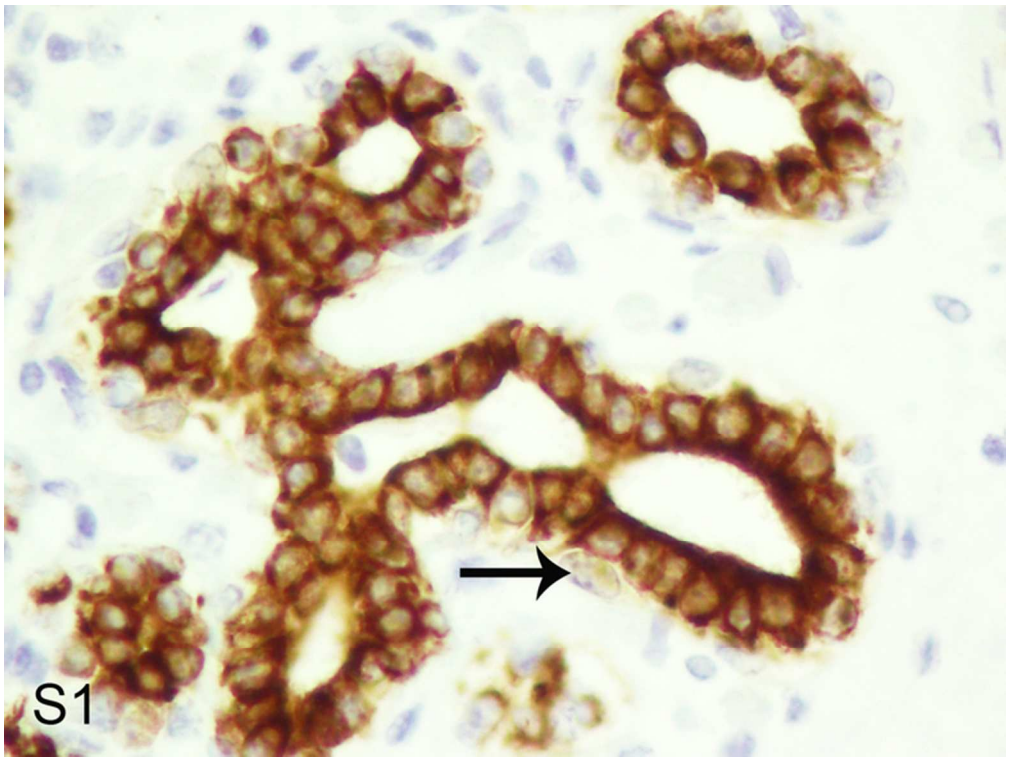
Case	Histological classification	Mitotic rate primary tumor per 10 hpf	Pan-cytokeratin	Cytokeratin 14	Vimentin	E-Cadherin	Estrogen receptor	Nuclear Stat3
1	Solid carcinoma with comedone pattern of necrosis	8	-	++	+/-	++	++	+/- Rare individual cells
2	Tubulo-papillary to solid carcinoma	23	+/-	-	++	++	+/- Rare individual cells	- Occasional positive cells in pulmonary metastases
3	Solid carcinoma with extensive necrosis	17	+	+	+	++	-	-
4	Tubulo-papillary to solid carcinoma	~10*	+	NP	-	++	++	++
5	Tubulo-papillary carcinoma	38	++	-	-	++	++	++
6	Tubulo-papillary to solid carcinoma with multifocal comedone pattern of necrosis	9	++	++	++	++	-	-
7	Tubulo-papillary to solid carcinoma with intraductular growth pattern	10	+/-	+	+	++	-	++

Hpf = high power fields (400x magnification).

* Only small biopsy pieces available.

NP = not performed; insufficient tissue.

– indicates no positive staining in tissue planes examined. +/- indicates rare positive staining individual cells or multifocal areas of very weak staining. + indicates less than 20% of neoplastic cells exhibit moderate levels of staining. ++ indicates that 20% or more of neoplastic cells exhibit moderate levels of staining.



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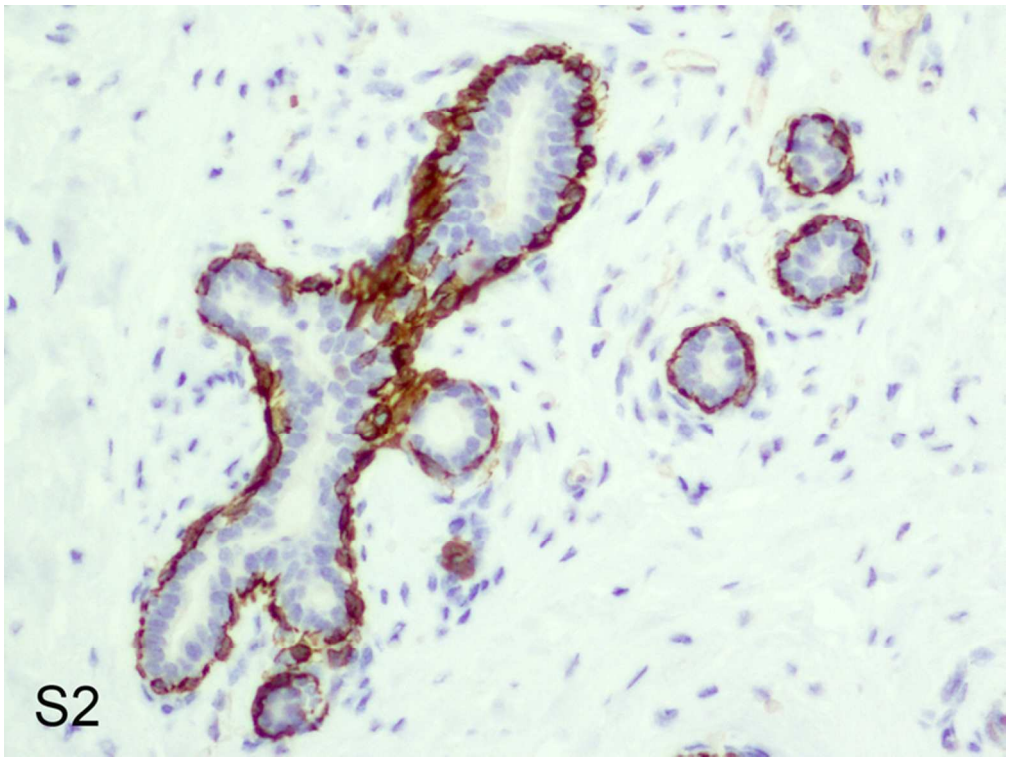

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