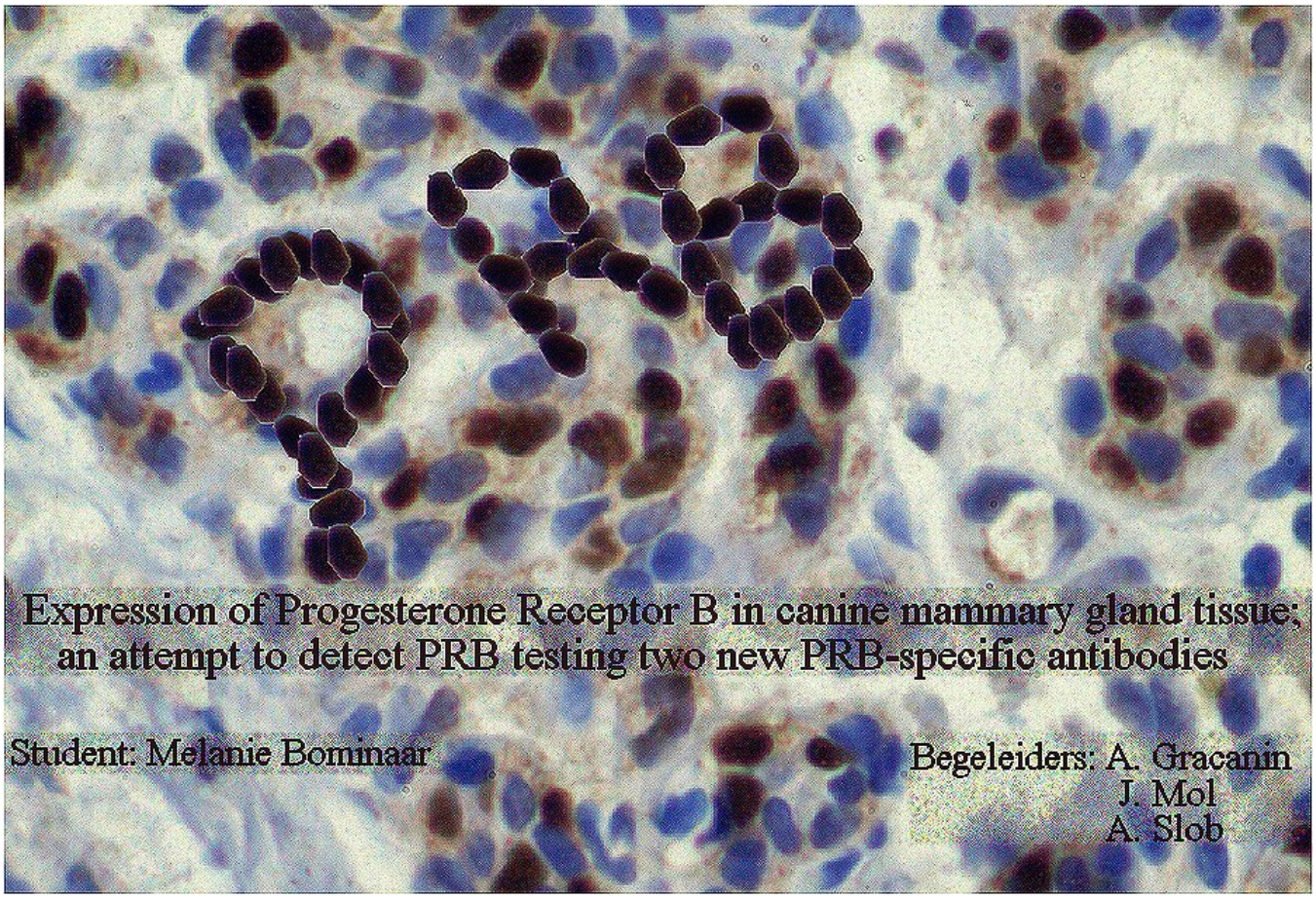

Abstract

In the female dog, the mammary gland tumor is the most prevalent tumor. The Progesterone Receptor exists in two independently regulated functional isoforms, Progesterone Receptor A (PRA) and Progesterone Receptor B (PRB). Dogs are known to have a different sequence and function of an important regulatory function element of PRB, compared to other species. This may be a causative factor in the very high prevalence of canine mammary gland tumors. So far, PRB expression has never been identified in dogs. The main goal of this 3 month research was thus to detect the PRB isoform in canine mammary gland tissue using immunohistochemistry and Western Blot. For PRB detection, 2 new PRB-specific antibodies were tested, the results of which were compared to an antibody that is known to detect both PRA and PRB. Using the two new antibodies did result in staining of the slides, and we continued our research with the one that we thought was best. For this antibody we found mild to moderate staining of the nucleus as well as the cytoplasm of epithelial cells. Staining of the stromal compartment was also found, but very infrequent. Testing of the specificity of this antibody for Progesterone Receptor B then followed. At present, we are unable to prove that the results obtained in immunohistochemistry were the result of specific binding of the investigated primary antibody to Progesterone Receptor B, but do have some reasons not to conclude it's definitely not caused by specific binding either. The advice would be to continue on after this project with the antibody used, with some adjustments in order to be able to test our hypothesis.

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Background

The objective of this report is to describe the results that were obtained during a student research project of 3 months duration in the canine mammary gland tumor department. During this research the expression of Progesterone Receptor isoform B (PRB) was investigated using immunohistochemistry and Western Blotting techniques, testing 2 new PRB specific antibodies.

Progesterone

Progesterone is an ovarian hormone that has important regulatory effects on the reproductive system. Progesterone's effects include a role in ovulation, the development of the uterus and mammary gland, sexual behavior and the establishment and maintenance of pregnancy. (Conneely 2000) In the normal mammary gland, progesterone regulates the proliferation and differentiation of this tissue. (Aupperlee 2007) Progesterone or progestins (progesterone's synthetic equivalents) exert its effect through binding to the progesterone receptor (PR) (Scarpin 2009)

Progesterone receptor

Isoforms PRA and PRB

PR is a nuclear ligand-activated transcription factor and acts on promoters that contain Progesterone Response Elements (PRE). (Scarpin 2009, Daniel 2009). It belongs to a large family of steroid hormone receptors including the estrogen receptor (ER), androgen receptor (AR), mineralocorticoid receptor and glucocorticoid receptor. (Lange 2008) PR exists in two independently regulated functional isoforms, PRA and PRB, that are expressed from a single gene but different promoters. (Aupperlee 2005, Conneely 2000)

PR consists of two main domains; a DNA binding domain (DBD) located at the central part and a ligand-binding domain (LBD) located at the carboxyl end of PR. PR also consists of different elements divided into (trans)activation function (AF) elements and inhibitory function (IF) elements. (Scarpin 2009)

PRA and PRB only differ in their amino(N)-terminus, where PRB contains an additional sequence of amino acids, 128 to 165 amino acids long, depending on the species. (Conneely 2000, Scarpin 2009) These extra amino acids form a region that is also being called the PRB upstream sequence (BUS) region. The BUS region contains a transactivation function that is specific for PRB: AF3. (Conneely 2000, Scarpin 2009) AF1 (PRB as well as PRA) and AF3 (PRB only) play a role in recruiting co-activator proteins thus regulating the level of the activation of target genes, as well as the specificity of the promoter. The amino-terminal also contains an inhibitory domain (ID), which recruits co-repressor proteins that inhibit transcription. Of these elements AF3 in particular may play a key role in explaining functional differences between PRA and PRB, since it is located in the BUS region, and is thus lacking in PRA. (Conneely 2000) (Scarpin 2009) The amino-terminal region is poorly conserved among species. (Conneely 2000, Conneely 2003, Daniel 2010, Mulac-Jericevic 2004) In contrast, the centrally located DNA binding domain (DBD) is highly conserved. It contributes to receptor dimerization and specificity of DNA binding.

Daniel 2010 describes the hinge region, which is located between the DBD and LBD. PR transcriptional activation is regulated by acetylation, phosphorylation and nuclear

retention of the receptor. All of these events are regulated by the hinge region of PR which thus regulates PR transcriptional responses to hormone. (Daniel 2010)
The ligand-binding domain (LBD) is located downstream of this small region and contains AF2. The LBD has multiple functions; it binds progesterone, interacts with heat shock proteins in inactive receptors, contains sequences that are required for dimerization, and through AF2 recruits co-activators (which is hormone-dependent). (Conneely 2000, Conneely 2003, Daniel 2010, Mulac-Jericevic 2004)

Figure 1 Overall structure of PRB. BUS; PRB upstream sequence, DBD; DNA binding domain, H;hinge region, HBD;hormone-binding domain. In PRA, BUS is lacking. In between BUS and DBD, the amino-terminal region is located. (Daniel 2010)

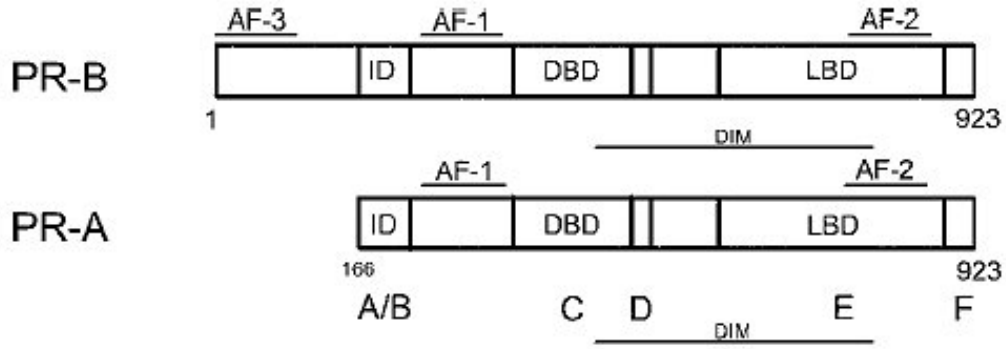


Figure 2 Structure of the human PRA and PRB isoforms. AF-1; activation function 1, AF-2; activation function 2; AF-3; activation function 3, DBD; DNA binding domain, LBD; ligand binding domain, ID; inhibitory domain, DIM; sequences that are important for receptor dimerzation (Mulac-Jericevic 2004)

Genomic actions of PR

Chaperone proteins make sure the unliganded PR is properly folded and capable of binding its ligand. Binding of ligand causes a conformational change of PR, an increase of receptor phosphorylation and dimerization of the ligand-receptor complexes.

Dimerization of the PR isoforms results in 3 species: PRA-PRA and PRB-PRB homodimers and the PRA-PRB heterodimer. (Conneely 2000) Since each of the 3 dimers regulates a different set of genes, which one of these 3 dimers predominates within a cell has an important influence on the transcription. The ratio of PRA to PRB is species-, cell type-, and tissue-specific, and in the reproductive tissues the ratios also vary as a consequence of developmental and hormonal status. In different diseased mammary gland or breast tissue different abnormal ratios can be found, and ratios may also change during treatment of disease. (Scarpin 2009)
In human cells the PRA to PRB ratio is 1:1 in the majority of PR^+ cells, and the heterodimer most likely plays an important role in progesterone action. However, in mouse and rat tissues for example the homodimer is frequently observed. It is important to take this difference between species into account.

After dimerization, binding of the receptor dimers to PREs that are located in the promoters of target genes plus interaction with co-regulators and general transcription

factors occurs and this eventually leads to the regulation of the transcription of target genes. (Conneely 2000, Lange 2008, Scarpin 2009) Binding of a PR agonist promotes the interaction of co-activator proteins with the AFs. Co-activators stimulate remodeling of the chromatin and bridging with general transcription factors, eventually forming a 'transcription initiation complex' located at the promoter that is regulated by PR. Binding of PR antagonists however, promotes a conformational change of PR making it impossible for AFs to bind co-activators. Instead, binding with co-repressor proteins is stimulated and transcriptional activity of PR is inhibited. (Conneely 2003 (2))

Gene regulation is however more complex since the majority of genes that are regulated in response to progesterone do not contain PREs and in genes that do contain PREs it proves to be hard to predict regulation. Also, some genes are downregulated by progesterone or PR. (Lange 2008)

Non-genomic actions of PR

Besides the classic PR action as nuclear ligand-activated transcription factor, PR can also affect cytoplasmic/membrane signal transduction pathways (extranuclear or non-genomic effects). (Daniel 2007, Lange 2008, Boonyaratanakornkit 2001) These effects occur rapidly compared to the more slowly occurring genomic actions of PR and are caused by the interaction between progesterone and cytoplasmic PR localized at the cell membrane. (Daniel, 2007, Lange 2008, Camacho-Arroyo 2007) Membrane-localized and nuclear PRs each have a different structure and functional activity. (Camacho-Arroyo 2007)

The membrane-localized PR activates cytoplasmic signaling molecules and proteins involved in the Mitogen-Activated Protein Kinases (MAPK)-pathway. This interaction is hormone dependent. (Boonyaratanakornkit 2001, Daniel 2007, Lange 2008)). After activation of the cytoplasmic signaling molecules by liganded membrane-localized PR, these molecules in turn phosphorylate nuclear PR, which may potentiate the nuclear actions (transcription) of these PRs (which are ligand-dependent), but also stimulate ligand-independent actions of PR. Through activating the MAPK-pathway, PR can also regulate genes that do not contain a PRE within their promoter. (Lange 2008, Daniel 2007) A different subset of genes may then be regulated since activated MAPK interacts with different transcription factors that are independent of nuclear receptors. In the normal breast Epidermal Growth Factor (EGF) acts through Epidermal Growth Factor Receptor (EGFR) and enhances the proliferative effects of progesterone and estrogen on mammary gland epithelium. (Daniel 2007, Lange 2008)

The role of this non-genomic PR action is not entirely clear yet, but seems to also regulate gene transcription. (Scarpin 2009) So, the non-genomic pathway of PR may also be able to regulate gene transcription, in a way that direct interaction of PR and DNA in the nucleus is unnecessary. (Boonyaratanakornit 2001) (figure 3)

PRB is known to be present in the nucleus but a significant part can be found in the cytoplasm, while PRA is localized mainly inside the nucleus. The activation of the MAPK-pathway thus seems to be predominantly the result of PRB action because of its localization. Indeed, the potential of PRB to activate the Src protein kinase has been established in experiments. (Daniel 2007, Lange 2008, Narayanan 2005) Lim concludes that the amino-terminal extension of PRB significantly affects the transport

of PR between the cytoplasm and nucleus (called shuttling) resulting in the different distribution of both isoforms in the cell. (Lim 1999)

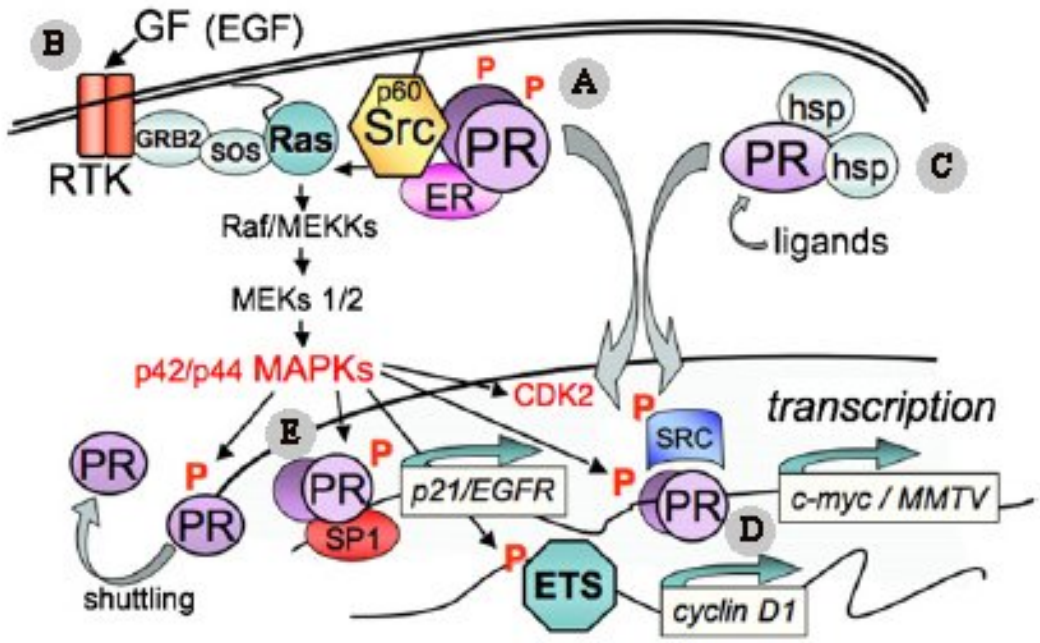


Figure 3 overview of genomic and non-genomic pathways of PR action Growth factors (GF) as well as liganded membrane-associated PR are able to activate protein kinases of the MAPK-pathway. As can be seen membrane-associated PR binds to and activates a signaling molecule (Src) which in turn is connected to RAS (part of the MAPK-pathway) (A; non-genomic pathway). GF binds to its receptor which is located in the cell membrane, thus activating the MAPK-pathway. (B) Upon binding of its ligand, PR loses the heat shock proteins (hsps; part of the chaperone molecules that keep PR properly folded and capable of binding ligand) and is being translocated into the nucleus (C; genomic pathway). Once inside the nucleus PR performs its nuclear (genomic) tasks; regulation of gene expression. (D; genomic pathway). Activation of the MAPK-pathway, either by GF or membrane-associated PR, may result in the phosphorylation of nuclear PR (E; non-genomic pathway). This phosphorylation enhances nuclear PR function. (Adapted from Lange 2008)

Function of PR

PRA and PRB are both regulators of gene transcription but they differ in function and generated response to progesterone; both isoforms regulate a different (largely non-overlapping) subset of genes expressed by a cell. (Aupperlee 2005, Conneely 2000) The difference in action between PRA and PRB is related to their structure; PRB possesses AF3 that is absent in PRA. The presence of AF3 allows binding of certain co-activators that cannot efficiently be recruited by PRA. Because of this function certain genes can be activated by PRB and not PRA. (Camacho-Arroyo 2007, Conneely 2000, Scarpin 2009) Meanwhile, an inhibitory function (IF) that is located next to AF1 is only active in PRA. PRB contains more phosphorylation sites compared to PRA. Both isoforms also have different affinity to the co-regulators. Different conformations of PRA and PRB possibly leads to interaction with different co-regulators. In humans PRA and PRB are expressed in equal amounts in most tissues. Differing levels of PRA- and PRB specific co-regulators in the different tissues may explain why the effects of progesterone are tissue-specific even here. (Camacho-Arroyo 2007, Scarpin 2009)

In general, PRB is the stronger transcription activator. Researchers found that PRA may even repress transcriptional activity mediated by PRB and thus decrease overall responsiveness to progesterone of specific target genes. (Camacho-Arroyo 2007, Conneely 2000) However, this inhibitory effect may only be observed when PRA is in very significant excess over PRB, levels that are never observed endogenously. (Conneely 2000, Scarpin 2009)

Function in the development of the mammary gland

Both estrogen and progesterone are the principle steroid hormones that are involved in the normal development of the mammary gland. Estrogen is held responsible for the development of the primary ductal system (acting through ERα). Progesterone controls proliferation and differentiation; it is necessary for the development of the terminal end buds (TEBs; the tips of elongating mammary ducts) and alveoli (lobuloalveolar development). IGF-1 and EGF enhance the effects of progesterone and estrogen on the mammary tissue. (Lange 2008, Robinson 2000)

PRB

PRBs most dominant role is that in the lobuloalveolar differentiation of the mammary gland. The specific effects of PRB have been investigated in Progesterone Receptor B Knock-Out (PRBKO) mice. In these mice pregnancy-associated side branching of the ducts and lobuloalveolar development are significantly decreased because of a decrease in proliferation of ductal and alveolar epithelial cells as well as a decrease in the survival of alveolar epithelial cells. PRB seems to be sufficient to result in normal proliferation and differentiation in response to progesterone in the mammary glands from Progesterone Receptor A Knock-Out (PRAKO) mice. (Conneely 2000)

PRA

The role of PRA in the normal development of the mammary gland seems less definite. It's been linked to the development and sidebranching of the ductal structure. (Aupperlee 2007, Lange 2008) Overexpression of PRA leads to abnormal morphology of the ducts, enhanced lateral branching and hyperplasia. (Robinson 2000) Whether PRA function is an absolute requirement or just a contributing factor.

So PR is thought to have a role in proliferation of cells of the mammary gland. PR^+ cells usually lie closely to the proliferating (steroid hormone receptor negative) epithelial cells but in general are not dividing themselves. It is thought that PR^+ cells are capable of proliferation but are inhibited to do so, while stimulating the proliferation of PR^- cells through a paracrine signal. (Lange 2008, Robinson 2000) Possibilities for this paracrine signal are: Wnts (Wnt-4 proved to be required for ductal side branching), Insulin-like Growth Factor II (IGF-II), hepatocyte growth-factor (HGF) (Conneely 2001, Conneely 2003 (2), Lange 2008, Robinson 2000) and receptor activator of NF-κB-ligand (RANKL; essential in alveolar proliferation). Progesterone alone can cause sidebranching and the start of alveologenesis but the combination of estrogen and progesterone enhances and accelerates overall proliferation. (Aupperlee 2007)

Expression

In the normal adult mammary gland tissue ER^+ /PR^+ cells constitute 7-10% of the total amount of luminal epithelial cells. (Lange 2008) Beleut 2010 mention a percentage of 30% present in the luminal cells. (Beleut 2010)
In mice PRB is predominantly expressed during pregnancy and after involution. There

is no expression of either isoform during lactation. However, until the first pregnancy, in the virgin mammary gland, PRA is predominantly expressed in the mammary tissue, which at the time is composed mostly of ducts. In rats, pregnancy is required for complete lobuloalveolar differentiation. In the human adult, non-pregnant breast however, the amount of lobules is higher than the amount of ducts. (Aupperlee 2005, Aupperlee 2007) The canine mammary gland completely differentiates into lobuloalveolar tissue during each luteal phase, whether the dog is pregnant or not. (Chandra 2010) Since PRB expression is thought to be required in order for alveologenesis to occur (and remain), a physiologic difference in mammary gland morphology may explain the difference in PRB expression between different species.

Regulation of expression

The responsiveness to progesterone is largely dependent on the level of expression of receptors in a cell. (Bagamasbad 2011, Cui 2005)
Progesterone itself regulates the expression of its own receptor (autoregulation) but so does estrogen; PR is an estrogen-regulated gene. (Bagamasbad 2011, Cui 2005)
Estrogen acts through the Estrogen Receptor (ER), which like PR, is a hormone-regulated nuclear transcription factor that induces the expression of a number of genes including the gene encoding PR. (Cui 2005)

It seems that, in general progesterone has a down-regulatory effect on the expression of its receptor but, has a different effect on both isoforms. Progesterone down-regulates PRA, weakens the up-regulation by estrogen, but up-regulates PRB. Progesterone alone is enough to induce PRB expression (and proliferation). However, the addition of estrogen accelerates and enhances the up-regulation of PRB by progesterone. (Aupperlee 2007)

Estrogen has an antagonistic effect; it upregulates PR expression. (Bagamasbad 2011) The up-regulatory effect of estrogen liganded to ER is most likely caused by estrogen response elements that are located in the promoter of the PR gene. Bagamasbad 2011, McGowan 2007) As was the case for progesterone, the hormonal regulation of the expression of PRA and PRB differs between the two isoforms; Estrogen up-regulates PRA, most likely through a direct mechanism. For PRB, estrogen enhances the increase in expression that is induced by progesterone, but estrogen treatment alone is not sufficient to up-regulate PRB expression; an indirect mechanism thus seems to be the case. (Aupperlee 2007)

Since PR expression is regulated by estrogen and progesterone, a physiological fluctuation in expression during the estrous cycle can thus be expected. Chandra 2010 and Rehm 2007 investigated the expression of PR during the estrous cycle. The results have been summarized in appendix 2.

Localization of PR

Previous researchers have focused on the expression of PR and its localization. So far, the precise localization of PR in the mammary gland cells remains controversial. Regarding its localization in the cytoplasm different opinions can be found. The same is true for the localization in the different cell types. Whether PR is only localized in glandular luminal epithelail cells (Aupperlee 2005, Chang 2009) or possibly also in myoepithelial cells and/or stromal fibroblasts (de Las Mulas 2002, de Las Mulas

2005). Also PRs distribution between alveolar structures and ductal structures is a source of disagreement.

Progesterone/PR and mammary gland tumorigenesis

In the normal human breast, proliferation is regulated by estrogen and progesterone, acting through ER and PR. Disorders in the function and expression of these receptors are linked to breast cancer. (McGowan 2007) The role of estrogen and ER in mammary gland tumorigenesis is very well studied and estrogen is considered a potent mammary gland mitogen. The role of progesterone and the PR to date is less clear and has been studied significantly less. (Lange 2008) When studied it proves to be difficult to separate the effects of progesterone in tumorigenesis from those of other hormones and growth factors such as Epidermal Growth Factor (EGF), Insulin-Like Growth Factor (IGF-1), prolactin and also estrogen. So far, research found enough reason to believe that PR indeed does have an important tumorigenic potential in the mammary gland. Some of these findings have been summarized in table 1.

Ovariohysterectomy significantly reduces the risk of developing mammary gland tumors, as long as it is performed at a young age.	The ratio of PRA:PRB is often altered in pathological conditions of the breast, but may also change during treatment, for example with the development of drug resistance (Cui 2005, Scarpin 2009)
The intake of progestins in addition to estrogen results in an increase of breast cancer risk as well as the invasiveness of breast cancers diagnosed. Progestin agonists have been shown to be able to reverse the antitumor effects of tamoxifen, an antiestrogen. (Conneely 2003 (2), Cui 2005, Daniel 2010, Lange 2008)	The loss of expression of PR in tumors has been linked to an increased risk of high histologic grade, high clinical stage, involvement of the lymph nodes, metastases, invasion of the tumor, disease progression, recurrence and death. (Chang 2009, Cui 2005)
Progesterone regulates many genes that control cell adhesion to the extracellular matrix. Progesterone may therefore contribute to the process of epithelial to mesenchymal transition (EMT) which is known to precede invasion and metastasis. (Lange 2008)	There also appeared to be a difference in expression between different diseased tissues; In canine mammary gland tumors PR expression was found to be higher in small malignant tumors and benign mammary lesions have a significantly higher PR expression compared to malignant tumors in the dog and cat. (Chang 2009, Geraldes 2000, de las Mulas 2002) In malignant tumors, the expression of PR was higher in complex carcinomas compared to simple carcinomas and sarcomas. (Chang 2009)
Previous studies have shown that progesterone can induce the expression of growth factors and transcription factors (including EGFR, STAT5a and STAT5b) as well as cyclin D1 in breast cancer cells. One possible explanation of PRs contribution to tumorigenesis is an abnormal regulation of cyclin D1 in breast cancer cell lines. (Conneely 2000, Conneely 2003 (2))	It is believed that PR expression can be used as a prognostic factor both in human breast cancer as in canine mammary gland tumors. Expression of ERα and PR may be of help in predicting the level of differentiation of neoplastic cells. In breast cancer, ER and PR expression is a marker of responsiveness to therapy and patient outcome. (Chang 2009, McGowan 2007)
The proliferative effects of PR have been linked to the early progression of breast malignancies. (Daniel 2010)	An increase in PRB expression (through polymorphism of the promoter) is linked to an increased breast cancer risk. When highly expressed, PRB was able to overcome the therapeutic effects of antiestrogens. (McGowan 2007)
The majority of breast cancers express PR at the time of diagnosis and in at least 60% of advanced cases PR expression remains high. (Lange 2008) One of the earliest responses to experimental exposure to carcinogens is the emergence of ER^+ and PR^+ proliferating mammary epithelial cells. In many human breast tumors the majority of ER^+ and PR^+ cells are proliferating. (Conneely 2003 (2))	Disorders in expression or activity of PR coregulators are linked to cancer. (Scarpin 2009)
The elimination of PR in Progesterone Receptor Knock-Out mice resulted in a reduction of tumor growth and loss of preneoplastic changes in response to carcinogens (Conneely 2003, Conneely 2003 (2))	Studies of RANKL (a possible paracrine signal used by PR^+ cells to stimulate proliferation of PR^- cells) and its receptor showed that signaling through RANK is strongly mitogenic. (Beleut 2010, Conneely 2003 (2))
A strong connection between PR action and mechanisms of cell cycle control is suspected. This suggests a role for the Cyclin A/CDK2 complex in linking the cell cycle to the regulation of PR activity (transcription); PR activity is stimulated by cyclin A/Cdk2.(Pierson-Mullany 2003, Lange 2008, Narayanan 2005)	Levels of Src and MAPK activity are often enhanced in breast cancer, possibly because of the membrane-associated PR induced activation of this pathway. (Lange 2008)

Table 1: Summary of articles that investigated progesterone's/progestins and/or PRs possible role in tumorigenesis in the mammary gland/breast.

The proliferating PR^+ cells that are found in breast cancer research (table 1) could be explained by a shift from paracrine signaling to autocrine signaling.

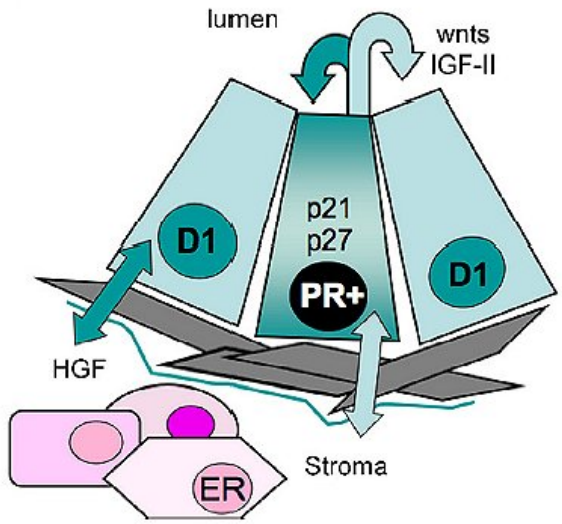


Figure 4 Paracrine signaling mechanism versus autocrine signaling mechanism. A shift from paracrine signaling to autocrine signaling could explain why PR^+ and ER^+ cells seem to be able to proliferate in breast cancers, while in normal breast tissue these cells are in general non-proliferating. (Adapted from Lange 2008)

Canines

In all canines, interesting differences exist compared to other species investigated that make canines even more interesting for the research of PR. Since most research is done with domestic dogs, from now on we will refer to the special characteristics of canines by using the term “dog”.

Canine PR

1. The amino acid sequence of the BUS region strongly differs from the sequence in humans.
2. Also, specific sequences in the AF3 region (called “motifs”), which are known to be essential for normal AF3 function, differ in the dog.
3. The potential to activate transcription proved to be reduced in the dog compared to human PRB.
4. Lower expression of PRB, compared to PRA is found in the dog. (Gracanin 2012)

These findings strongly raise the question if canine PRB is even fully functional.

Other characteristics

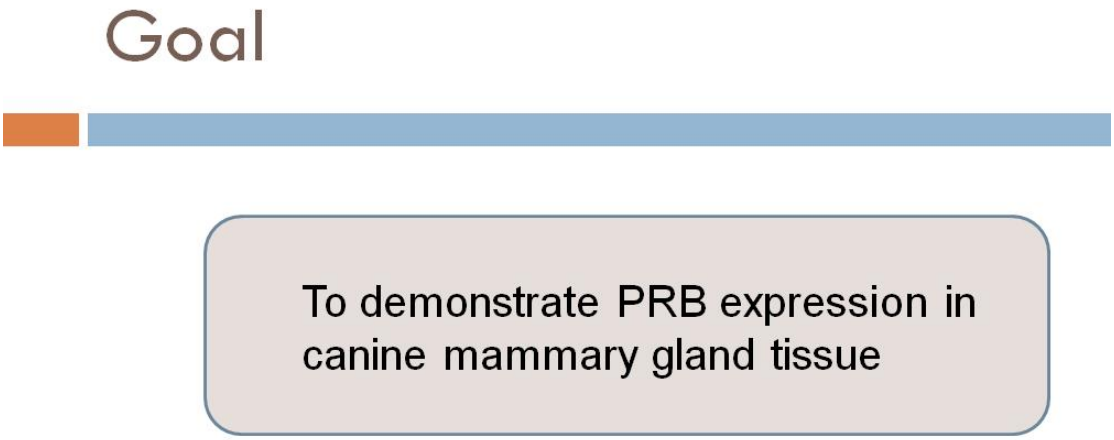
1. Remarkable however is that dogs, despite uncertainty with respect to PRBs functionality in the dog, do develop normal mammary gland tissue. Even more extraordinary is that during each luteal phase, whether the dog is pregnant or not, the mammary gland tissue fully differentiates into lobuloalveolar tissue, requiring PRB. Of all species investigated, this differentiation into lubuloalveolar tissue is most extreme in the dog. In Appendix 4 and Appendix 5 the morphology of the canine mammary gland, including its characteristic extreme lobuloalveolar differentiation has been depicted.
2. The incidence of mammary gland tumors in dogs is extremely high. Of all neoplasms found in the domestic dog, the mammary tumor is the most prevalent one. Just to give some insight; A cancer registry for cats and dogs in

California, USA, found that 41% of all cancers, of a total of 971 investigated female dogs, were of mammary gland origin. In a study where beagles were followed during their whole lives, 70,8% developed at least 1 mammary tumor.

3. The dog has an unique estrous cycle among mammals. Characteristic of the canine estrous cycle is the very long, progesterone dominated, luteal phase in non-pregnant bitches as well. (Chandra 2010, Kooistra 2002, Rehm 2007) Estrous cycles of the dog, mouse/rat and human are summarized in appendix 3.
4. The expression of ER and PR in canine mammary gland carcinomas is comparable to what is seen in human hormone-dependent breast carcinoma studies; The dog proves to be a good animal model for breast cancer studies. (de Las Mulas 2002)

To date, not that many research has focused on dogs however, much of it focusing on human breast cancer and rats or mice. The points mentioned above should have clarified why the dog is so interesting in PR research. The questionable functionality of canine AF3 combined with the high prevalence of mammary gland tumors in the dog raises the suggestion of an involvement of PRB in mammary gland tumorigenesis. But also the role of canine PRB in reproductive functions in the dog is an interesting topic for investigation.

The first step in the further investigation of canine PRB, was to prove that PRB is indeed expressed in the mammary gland of the dog. This proof of PRB expression thus was the most important goal of the present research project. In order to prove PRB expression in canine mammary gland tissue, two new antibodies, supposedly PRB-specific were tested in immunohistochemistry and Western Blotting techniques.



Methods

For the detection of PRB immunohistochemical staining and Western Blotting were performed.

Immunohistochemistry

Histologic sections were cut from paraffin-embedded canine mammary tissues (tissues were called “Zagreb xx” and “M3”) and mounted on slides, approximately 2 weeks before the start of this 3 month research (Zagreb). The tissues were deparaffinized and rehydrated in xylene and different concentrations of ethanol as follows:
Xylene I: 5 minutes
Xylene II: 5 minutes
Ethanol 96% I: 3 minutes
Ethanol 96% II: 3 minutes
Ethanol 80%: 3 minutes
Ethanol 70%: 3 minutes
Ethanol 60%: 2 minutes

After this the slides were washed in Tris Buffered Saline (TBS; 50mM Tris and150mM NaCl); 2x 5 minutes. Then, blocking of the endogenous peroxidase followed, through putting the slides in 3% hydrogen peroxide (H_2O_2 diluted in TBS) for 15 minutes. Blocking of the endogenous peroxidase is necessary because a horseradish peroxidase (HRP) labeled polymer is conjugated to the goat anti-rabbit secondary antibody. This peroxidase cleaves a substrate (the chromogen) which results in a brown staining at the location of the protein. Blocking of endogenous peroxidase activity thus decreases background staining. (Abcam IHC)

After this blocking step the slides were washed for 2x 5 minutes in TBS, followed by the antigen retrieval step. Antigen retrieval exposes the antigenic sites, that are masked due to the formation of methylene bridges during fixation leading to cross-linking of proteins. Antigen retrieval breaks the methylene bridges so that the antibodies are able to bind to the antigenic sites. (Abcam IHC) Antigen retrieval methods that were tested during this research were the heat induced epitope retrieval (using sodium citrate) and an enzymatic epitope retrieval method (using protein kinase). Based on the results, we decided to work with sodium citrate from that moment on; Antigens were retrieved by putting the slides in a holder filled with sodium citrate (10 mM, pH 6,0, 0.05% of Tween 20) in a water bath at 98˚C for 15-20 minutes. After 15-20 minutes the holder containing the slides in the sodium citrate solution was removed from the water bath and left to cool down on the lab table for 20 minutes. Two wash steps followed; 2x 5 minutes in TBS. Slides were dried, taking off excessive fluid, making sure however that the tissues were never entirely dry. Drying of the tissues leads to non-specific binding and ultimately high background staining. (Abcam IHC) Circles were then drawn around the tissues with ImmEdge pen.

The tissues were then blocked in 10% normal goat serum (NGS), 1% bovine serum albumin (BSA) (both diluted in TBS) for 30 minutes. Normal goat serum is used because the secondary antibody is raised in a goat and may cross react with endogenous immunoglobins in the tissue. Pre-treating the tissue with normal goat serum reduces this cross reaction of the secondary antibody with endogenous

immunoglobins (non-specific binding) to a minimum. It also eliminates Fc receptor binding of both the primary and secondary antibody when used before the primary antibody is applied. (Abcam IHC) Fc receptors are located on the surface of certain cells and bind the Fc region of antibodies. It is thought that Fc receptors bind the Fc region of antibodies not only in vivo, but also during immunohistochemical procedures. (Buchwalow 2011). BSA reduces the non-specific binding caused by hydrophobic interactions. (Abcam IHC) The slides were kept inside a plastic box on top of cut pipettes with wet tissue paper on the bottom, to make sure the tissues remained humid.

After 30 minutes the blocking solution was carefully removed from the slides using tissue paper (making sure the tissue on the slides never dried out) and primary antibody was applied. The primary antibody must have been raised in a species that is different from the species of the tissue that is being stained. Using a primary antibody from the same species as the tissue that is being stained, leads to very high background, since the secondary antibody will not only bind the primary antibody, but also to all the endogenous IgG in the tissue. (Abcam IHC) After determining the optimal antigen retrieval method, the optimal antibody concentration was established. We mainly worked with two primary antibodies during this research. The first one was called "SC539" (Santa Cruz), an affinity purified rabbit polyclonal antibody raised against a peptide mapping within an internal region of PR of human origin (so; rabbit anti-human) (datasheet sc539), that recognized PR in general (i.e. this antibody did not distinguish PRA from PRB), at 1:1000 dilution. The second antibody was called KAQD, at dilution 1:100 and this antibody supposedly specifically recognized PRB, not PRA. A third antibody called AAEP was tested in the beginning of this research at dilution 1:200 and 1:400, however later on the decision was made to work with KAQD (for explanation of this decision see results/discussion). KAQD and AAEP antibodies were polyclonal antibodies raised in rabbit that were especially made through immunization with specific peptides. (Davids Biotechnologie) The serum was then purified using affinity purification in order to increase specificity.

Primary antibodies were diluted in TBS with 1% BSA. Negative control slides were treated with no primary antibody, the rest of the protocol was the same for all slides. Negative control slides are incorporated to test the specificity of the antibody. Ideally, no staining should be shown when omitting the primary antibody.
The tissues were incubated overnight at 4˚C in the plastic box to avoid drying of the tissues.

The second day started with washing the slides; 2x 5 minutes in TBS. The slides were then dried to remove excessive fluid and the secondary antibody was applied. For the secondary antibody a goat anti-rabbit antibody (Envision, Dako) was used. After an incubation period of 30 minutes, two wash steps followed; TBS 2x 5 minutes.

Slides were dried again to remove excessive fluid before applying the chromogen 3,3'-diaminobenzidine (DAB) (Dako). DAB is the substrate for the enzyme horseradish peroxidase (HRP) that is conjugated to the secondary antibody, and the result is a brown staining. DAB was incubated on the slides for exactly 5 minutes. The concentration used was 1 drop/ml substrate. After 5 minutes the slides were put in a container with demineralised water. Slides were dried again to remove excessive fluid before covering the tissues with hematoxylin (Vector Laboratories) for a few seconds,

to counterstain the sections. After approximately 5-10 seconds the hematoxylin was drained from the slide and the slides were put in a glass holder filled with tap water until all slides were ready. Next the glass holder with the slides was put in the sink under running tap water for approximately 10 minutes. Sections were dehydrated as follows:
Ethanol 60%: 2 minutes
Ethanol 70%: 2 minutes
Ethanol 80%: 3 minutes
Ethanol 96% I: 3 minutes
Ethanol 96% II: 3 minutes
Xylene I: 5 minutes
Xylene II: 5 minutes
Slides were dried and mounted with VectaMount (Vector Laboratories), a permanent mounting medium.

Negative control slides were treated exactly as the rest of the slides, except for the primary antibody; Negative control slides were incubated overnight in TBS with 1% BSA without the primary antibody.

The positive controls, in the beginning consisted of sections from the uterus, a tissue that is known to express (high) PR. Later on M3 (canine mammary tissue) was used because this tissue proved to be strongly positive for PR. A positive control is added to an immunohistochemical procedure to evaluate if the procedure is optimized and working correctly; when the positive control turns out positive and the samples are negative, the positive control can be used to support the validity of the negative results. (abcam)

The next day the slides were ready to be viewed. Staining with KAQD antibody was compared to the slides that were stained with SC539 because SC539 has been tested before while KAQD was not. So therefore SC539 was considered to be the reliable antibody to which KAQD and AAEP would be compared.

In order to determine if the staining observed in the sections was the result of specific binding of the antibody to PRB, blocking peptide (BP) was added in later experiments. When using blocking peptide, an excess of peptide corresponding to the epitope that is recognized by the antibody is added. Blocking peptide consists of the protein or peptide that is used to generate the antibody (Burry). The blocking peptide is meant to compete with the protein of interest that is present in the tissue mounted to the slides. For this reason, the blocking peptide has to be added in excess, so the binding of antibody with the blocking peptide instead of with the protein of interest is favored. (Stratech) Antibody bound to the blocking peptide is no longer available to bind to the epitope present in the protein of interest. The antibody is thus being neutralized. By comparing the results of the combination of blocking peptide and the antibody with the results when antibody alone is added, it’s possible to say which staining is specific. Any binding of the antibody to the tissue of interest in the presence of the peptide is nonspecific. (stratech) The specific staining is absent from the immunostaining in which the antibody was neutralized by blocking peptide. (Abcam BP, Stratech)

Western Blot

On day 1 a piece of tissue of approximately 0,5cm^2 was cut off of the frozen mammary gland tissue. This piece of tissue was put in an eppendorf and 400µl of Radio Immuno Precipitation Assay buffer (RIPA-buffer) was added. RIPA buffer is particularly used for nuclear membrane disruption to obtain nuclear extracts. It contains a detergent (sodium deoxycholate). RIPA-buffer is used to obtain cell lysis and to bring the proteins into solution. (Abcam WB1)
The cells were homogenized using an eppendorf vial potter stick. The lysate was left to incubate on ice. Due to lack of time, the protein measurement was performed on the next day. So, day 2 started with centrifuging the eppendorf vials in a cooled centrifuge for 5 minutes (10000 rotations per minute). The supernatant was taken off with a pipette and used for the protein measurement; the DC Protein Assay (Bio-Rad Laboratories). This is a colorimetric assay to determine protein concentration. (Bio-Rad 1) The DC protein Assay reagents contain both detergent and base. These are required for lysis of the cells prior to determination of the total cellular protein level. (Bio-Rad 2) A 96-well microplate was used. First off, BSA (Sigma-Aldrich) was diluted to the following concentrations; 1.50 mg/ml, 1.25 mg/ml, 1.00 mg/ml, 0.75 mg/ml, 0.50 mg/ml, 0.25 mg/ml and 0.00 mg/ml. Each of these concentrations was then pipetted in a 96-well microplate in triplicate (5µl). The same was done for the samples as follows:

	1	2	3	4	5	6	7	8	9	10	11	12
A	BSA 1.50	BSA 1.25	BSA 1.00	BSA 0.75	BSA 0.50	BSA 0.25	BSA 0.00	M3	M3 10X	M3 100X	30A	30A 10X
B	BSA 1.50	BSA 1.25	BSA 1.00	BSA 0.75	BSA 0.50	BSA 0.25	BSA 0.00	M3	M3 10X	M3 100X	30A	30A 10X
C	BSA 1.50	BSA 1.25	BSA 1.00	BSA 0.75	BSA 0.50	BSA 0.25	BSA 0.00	M3	M3 10X	M3 100X	30A	30A 10X
D								M3 5X	M3 50X	30A 5X	30A 50X	30A 100X
E								M3 5X	M3 50X	30A 5X	30A 50X	30A 100X
F								M3 5X	M3 50X	30A 5X	30A 50X	30A 100X

Table 1 overview of micoplate used to determine protein levels of M3 and 30A tissues through DC Protein Assay

As can be seen different dilutions of the tissue samples were tested (undiluted, 5x, 10x, 50x and 100x diluted). Then, 25µl of reagent A* (this is reagent S added to reagent A (20µl S to each ml of A) was added to each well, followed by the addition of 200µl of reagent B into each well. Reagent A, is an alkaline copper tartrate solution. Reagent B is a dilute Folin reagent. The DC protein assay is a colorimetric assay; two steps lead to color development; First, the reaction between the protein and copper in an alkaline medium. Second, the reduction of Folin reagent by the copper-treated protein which results in reduced species with a characteristic blue color. (Bio-Rad 3) Absorbance was read in the spectrophotometer at 655nm. Mean protein concentration was 90,1 µg/µl for M3 and 81 µg/µl for 30A. So in order to make sure that the same quantity of protein is being tested during Western Blotting, the amount of M3 times 1.112 should be used for 30A.

An 8% polyacrylamide gel was used and was produced in this laboratory. A polyacrylamide gel is formed from the polymerization of acrylamide and N,N-methylenebisacrylamide (Bis). Bis provides for the cross-linking within the gel. The addition of ammonium persulfate (APS) together with TEMED functions to initiate

the polymerization. Within the gel, pores are present, which is necessary for the separation of the molecules. The pore size of a gel is thus an important characteristic and is determined by 1) the total amount of acrylamide that is present (an increase of the total amount decreases the pore size) and 2) the amount of cross-linking (5% results in the smallest pore size, any in- or decrease increases the pore size). An 8% gel indicates that there is a total of 8 grams of acrylamide and bis per 100 ml of gel. The gel percentage determines the rate of migration and degree of separation between proteins. For small proteins, a higher percentage of mono/bis should be chosen. For larger proteins, a lower percentage of mono/bis should be chosen. (Abcam WB2) The inner chamber and part of the tank were filled with electrode buffer.

Protein size (kDa)	Gel percentage (%)
4-40	20
12-45	15
10-70	12.5
15-100	10
25-200	8

Figure 11 Protein sizes and advised corresponding gel percentage (Abcam WB2)

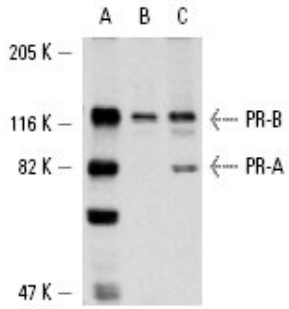


Figure 12 Protein sizes of PRAand PRB (SC 539 datasheet)

15µl of 30A and 13.5µl of M3, diluted in sample buffer, was loaded per slot. The sample buffer contains Sodium Dodecyl Sulfate (SDS, 2.0 ml 10%). The proteins attach to these SDS anions and become negatively charged. The negative charge of the protein is in proportion to its length. Each unit length of the protein has the same charge density, so that migration is determined by its molecular weight. SDS also denatures. Dithiothreitol (DTT) is added (30 mg/ml) to reduce disulphide bridging, glycerol (2.8 ml, Sigma G 7757) to prevent uneven gel loading and bromophenol blue to be able to monitor the progress of the separation. (Abcam WB2) To denature the protein, the SDS containing loading buffer with the sample dilution (loading buffer:sample = 1:1), is heated at 95˚C for 2 minutes. Following this the samples were loaded into the slots in this order:

standard	M3	30A	negative	standard	M3	30A	M3	30A	standard

Figure 13 Sequence in which samples were loaded into the slots

As can be seen, a standard (Precision plus Protein Standard, dual color, Bio-Rad) was loaded to 3 lanes to be able to follow the electrophoresis and to give an indication of protein size later on. The gel was then left to run at 100 Volts until the marker bands were sufficiently separated. When immunohistochemistry is performed before Western Blot, a tissue with a high expression of the protein (tissue M3 in this experiment) of interest and a tissue with a low expression of the protein of interest (tissue 30A in this experiment) can be chosen. If the same quantitative differences can be found using Western Blot, this helps support the hypothesis that the staining found in immunohistochemistry is indeed specific.

After the electrophoresis was finished we started with the blotting procedure. In blotting, a nitrocellulose membrane (Hybond C, Amersham) and the gel are

sandwiched in between filter paper and fiber pads and the goal is to transfer the proteins from the gel to the membrane. Before preparing the gel sandwich, the fiber pads, filter paper, the gel and the membrane were put in blot-buffer and left there to soak for approximately 10-15minutes. Blot-buffer (1L, pH 8.3) consisted of; 3.03 g Tris, 14.4 g glycine and 200 ml methanol diluted in milliQ. Then the gel sandwich was prepared. This was done in a cassette that was put in some buffer. The correct order of preparing a gel sandwich is: fiber pad (on black side of cassette), filter paper, gel, membrane, filter paper, fiber pad. We then blotted for 1 hour at 100 Volts.

After blotting, the membrane was blocked for 60 minutes in TBST0.1% and 4% blocking (ECL). The membrane was then incubated overnight at 4˚C in TBST0.1% buffer and primary antibody; KAQD dilution 1:1000, SC539 dilution 1:2000, and anti-actin (raised in mice, monoclonal Neomarkers) dilution 1:2000 (in 1% BSA). One piece of the blot was incubated without a primary antibody. An antibody to detect actin is being used because actin is present in all eukaryotic cells. When testing different samples of tissue belonging to the same sort (in this case mammary gland) actin is sure to be present in the same amount in each sample tested. This feature can be used in Western Blotting when quantitative differences are observed for the proteins of interest; since actin is present in the same amount in each sample tested, it should generate a signal that has the same intensity in every lane. If the signals caused by anti-actin differ in strength this means that the amount of protein loaded into those lanes was not equal. If the signal generated by anti-actin is as strong in every lane, and the intensity of the bands of interest differ it is fair to say that this is not caused by a difference in the total amount of protein that was loaded into the lanes, but a difference in the quantity of the proteins tested.

The next day started with washing the membrane; 3x5 minutes in TBST0.1%. After these wash steps the membrane was incubated with the HRP conjugated secondary antibody. For KAQD and SC539 goat anti-rabbit, HRP, R&D systems Minneapolis, was used, for anti-actin an anti-mouse secondary HRP conjugated antibody (dilution 1:20.000) was used. Secondary antibodies were dissolved in TBST0.1%, and then the membrane was incubated for 60 minutes. Secondary antibodies were ordered from R&D systems. The membrane was then washed again, 3x5 minutes in TBST0.1% and was then placed in the chemi-doc after incubating the membrane with the substrate.

Results

Immunohistochemistry

When using antibody **SC539** and looking at the 2x magnification it's already apparent that the glandular portion of the tissue that is rearranged in ductal and alveolar structures, has been positively stained. (Fig A-A, Fig C-A). The alveolar and ductal structure becomes even more clear when looking at the 10x magnification. When looking at Figure A-B, there is a stroke of stroma right in the center of the figure. On the left and right the distinct structure of the glandular tissue is visible. The stroma is remarkably blue compared to the epithelial cells of the glandular tissue surrounding it. And what's also clear now, is that the nuclei of the epithelial cells have stained strongly positive, resulting in a diffuse dark brown staining of some of the nuclei. There is a sharp contrast between these positive nuclei and clear blue nuclei that apparently did not stain at all. Staining appears to be located in luminal epithelial cells as well as myoepithelial cells, although the distinction between the two is hard and results should be reevaluated. (Fig A-B, Fig C-B) The 20x and 40x magnifications show us that not only the nuclei have positive staining, but there is also a mild to moderate staining visible in the cytoplasm as well. A 100x magnification shows the dark brown positively stained nuclei, where the staining fills the complete nucleus of the positive cells. The contrast between the nuclei and the cytoplasm can clearly be seen here, as well as the contrast between positive and negative nuclei. The stromal compartment only has very insignificant quantity of staining when compared to the epithelial cells, but some staining is present (Fig B). This staining is hardly ever found in the nuclei of the stromal compartment. It may represent cytoplasmic staining of the stromal cells, however, it looks like the brown discoloration is randomly scattered throughout the stromal compartment, not really being confined to individual cells. (Fig B)

With the use of antibody **KAQD** the same cells are stained compared to the use with SC539; epithelial cells (most likely luminal epithelial cells as well as myoepithelial cells) of the glandular epithelial tissue. As is seen with SC539, at 2x magnification islands of positively stained cells can already be seen (Fig E-A, H-A). Fig E-B shows a positively stained "island" which is surrounded by the stromal compartment which is considerably less positively stained. The ductal and alveolar structures can be seen better with the 10x magnification (E-B, G-A, HB) and indeed are shown to be the cause of the brown discoloration seen with magnification 2x. A closer look with a 20x magnification already shows the most important difference compared to SC539; KAQD stained cells are almost equally stained in the nuclear and cytoplasmic compartments. Both of these areas stain moderately. So staining is a bit stronger in the cytoplasm and weaker in intensity for the nuclei, compared to the use of SC539. There also seems to be more variation in intensity of nuclear staining, some cells being complete negative, others moderately stained (the maximal intensity seen with use of this antibody), but also cells with staining intensities in between. Staining is not as diffuse as was seen in the nuclei of SC539 stained cells, showing a bit of a speckled appearance in KAQD stained cells. (Fig E-C, Fig E-D, Fig F-C, Fig G-B, Fig H-C, Fig H-D). In the stromal compartment, infrequent staining can be seen. (Fig E-D) The addition of blocking peptide (BP) in a 5x excess very strongly decreases the general staining, which is most clearly seen when comparing the 2x magnifications which look completely negative after the use of BP. (Fig E-A, F-A, H-A, I-A) Only when looking closely at the 10x magnification and clearly visible in the 20x and 40x magnifications it becomes obvious that the use of BP does not result in negative slides. Staining of all compartments (nuclear, cytoplasmic, stromal) may remain, all of

which can be seen in Fig F-D. Most of the time this staining was only very mild. However, as is shown in figure G-E sometimes a part with stronger staining could be found. Figure G-E shows remaining cytoplasmic and nuclear staining after the use of BP. Figure I-D best depicts the general view after the use of BP; so even though staining in every compartment may remain and can be seen, these findings were infrequent. The situation shown in figure I-D shows what was most often seen after the use of BP. Here, nuclei appear to be all negative. The cytoplasm looks like it is completely negative but this may not be the case, as it does have a pinkish appearance at times. In this figure, some brown staining can be seen that looks like it neither nuclear, nor cytoplasmic.
Use of the third antibody, **AAEP**, again results in clear brown islands surrounded by blue tissue in the 2x magnification (Fig, J-A). The 10x magnification shows that the positively stained cells are epithelial cells of the glandular tissue, as was the case with SC539 and KAQD. The 20x and 40x magnifications however, show a major difference between this antibody and the previous two. AAEP stained slides result in very strong, dark brown, staining of the cytoplasm surrounding clear blue nuclei that appear to remain completely negative. (Fig J-C, Fig J-D) Stromal staining is seen with the use of this antibody as well, even though the stroma in general is considerably blue compared to the glandular epithelial cells. (Fig J-B, Fig J-C) After the use of BP (5x excess) no staining remains in any of the compartments. (Fig K-A, Fig K-B, Fig K-C, Fig K-D)

Results that have been described above have all been obtained using slides of a tissue called M3 (canine mammary gland tissue). We also used SC539 and KAQD on tissues called “Zagreb” tissues. In general staining observed when using these tissues was less intense, both for KAQD and SC539, but more obvious for SC539. Not only did SC539 stain the nuclei of cells with less intensity, the number of darkly stained nuclei was also considerably less compared to the number that was observed in tissue M3. Tissue 17B1 is a good example of one of these Zagreb tissues. It shows tissue that is strongly differentiated into lobuloalveolar tissue. For SC539, a small number of dark brown nuclei can be seen, the majority of the nuclei being only mildly stained, or negative. The cytoplasm is also mildly to moderately stained. The difference in observed intensity of staining thus seems to be more related to nuclear staining than cytoplasmic staining. (Fig L-A, Fig L-B, Fig L-C, Fig L-D, Fig L-E, Fig L-F)
The same tissue also shows a bit less intensity in nuclear staining when using KAQD, while cytoplasmic staining seems to be much less affected by loss of intensity between this tissue compared to tissue M3. The cytoplasm is mildly to moderately stained. The intensity in nuclear staining does show more variety, some nuclei being moderately stained, while others are negative. (Fig M-D, M-E)

Western Blot

As can be seen no band sizes fit the expected band sizes for PRA or PRB. The lanes that were incubated with primary antibody KAQD (lanes 8, 9 and 10), are expected to show only 1 band (PRB), however, as can be seen in lane 9, two bands are visible. Also remarkable findings are the bands observed in lanes 2 and 3. This part of the blot (lanes 1, 2, 3 and 4) was incubated without an primary antibody. Lanes 5, 6 and 7 were incubated with primary antibody SC539, which is known to function in Western Blotting procedures. However, no band sizes that fit the expected band size for PRB can be found for sure, while the lower band in lane 7 may come close to the expected band size for PRA. (Fig O)

Results

Immunohistochemistry

M3; SC539 (1:1000)

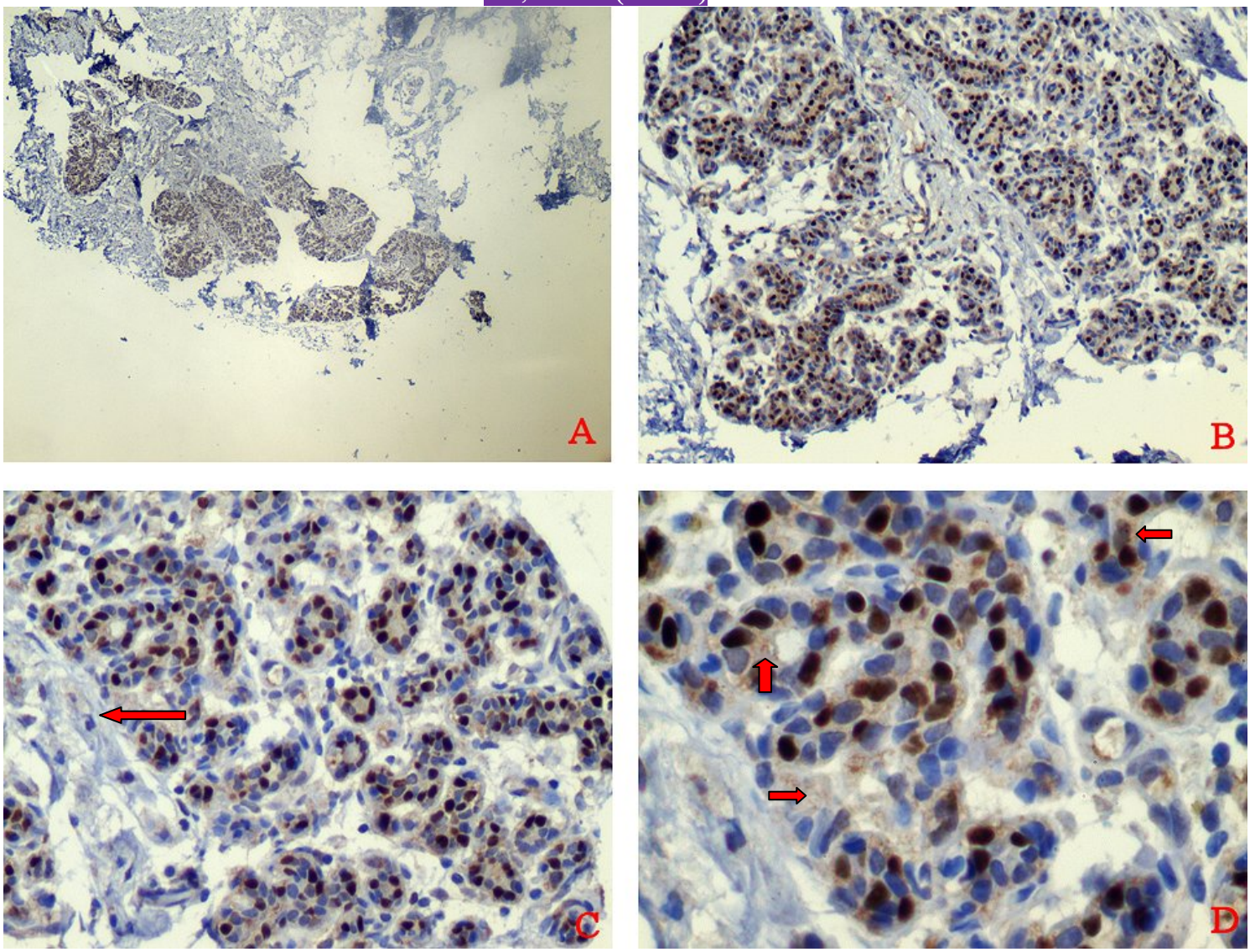


Figure A: Photographs taken of tissue called M3, primary antibody used was SC539 (dilution 1:1000), *, Figuuur11ozs
A: magnification 2x. Brown colored islands can already be seen in the blue tissue.
B: magnification 10x
C: magnification 20x At this magnification it's already apparent that the nuclei are responsible for the appearance of brown islands observed in A. Another thing that can be observed is that the brown nuclei are arranged in circular structures; these are the alveoli and ducts. The stroma appears blue, no positive nuclei can be found here (red arrow).
D: magnification 40x Strongly stained brown nuclei are clearly visible in between unstained blue nuclei. Some weak brown staining can be seen in the cytoplasm (red arrows). The nuclei however, give a significant stronger positive result compared to the weak brown staining found throughout the photograph.
The * means that the photographs in this figure were obtained from histologic sections that were stained on a certain day at a certain time. So every set of photographs indicated with one * means that these sections were stained at the same time as all the other sections indicated with one *. Photographs indicated with ** thus means that the sections that were photographed were stained on another day than the sections indicated with *.

M3; SC539 (1:1000)

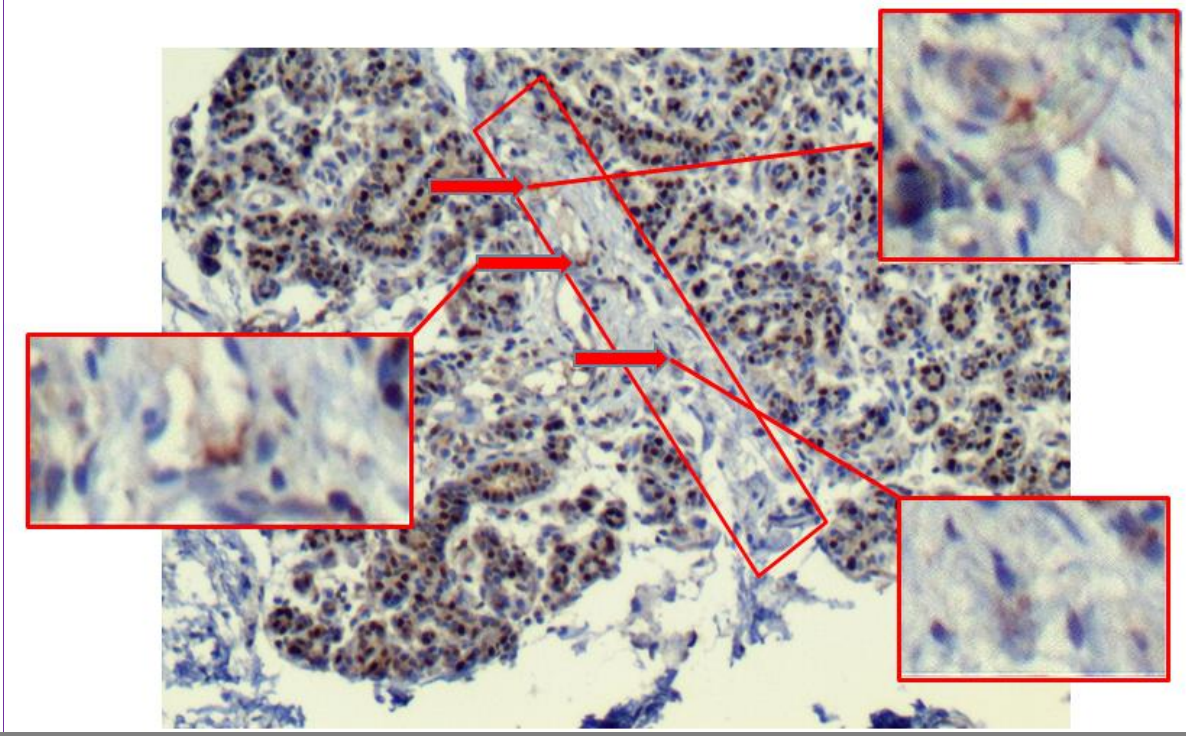


Figure B: Close-ups of the Staining of the stromal compartment

M3; SC539 (1:1000)

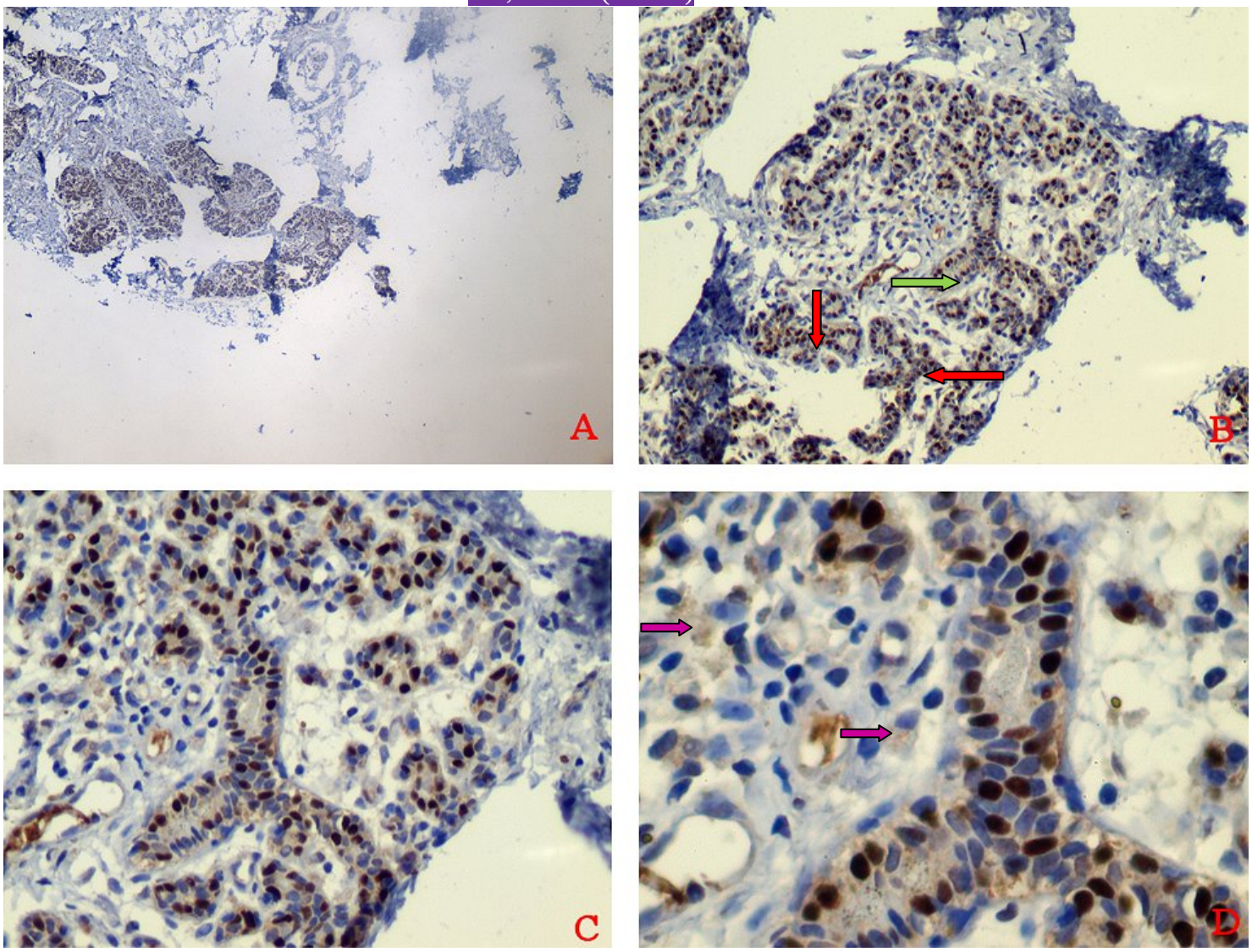


Figure C Same histologic section, same primary antibody tested (SC539, dilution 1:1000), different area photographed, *, figuur12ozs
A: magnification 2x
B: magnification 10x. In this photograph not only the small alveoli but also some larger structures that are positively stained can be seen (red and green arrows). This may be distended alveoli, or more likely ducts.
C: magnification 20x. A magnification of the area indicated by the green arrow in B.
D: magnification 40x. Again, like the figure A, clearly stained positive nuclei can be seen in between negative (blue) nuclei, mildly stained cytoplasm and the blue stroma. Some very slight brown staining can be seen in the stromal compartment (purple arrows).

M3; SC539 (1:1000)

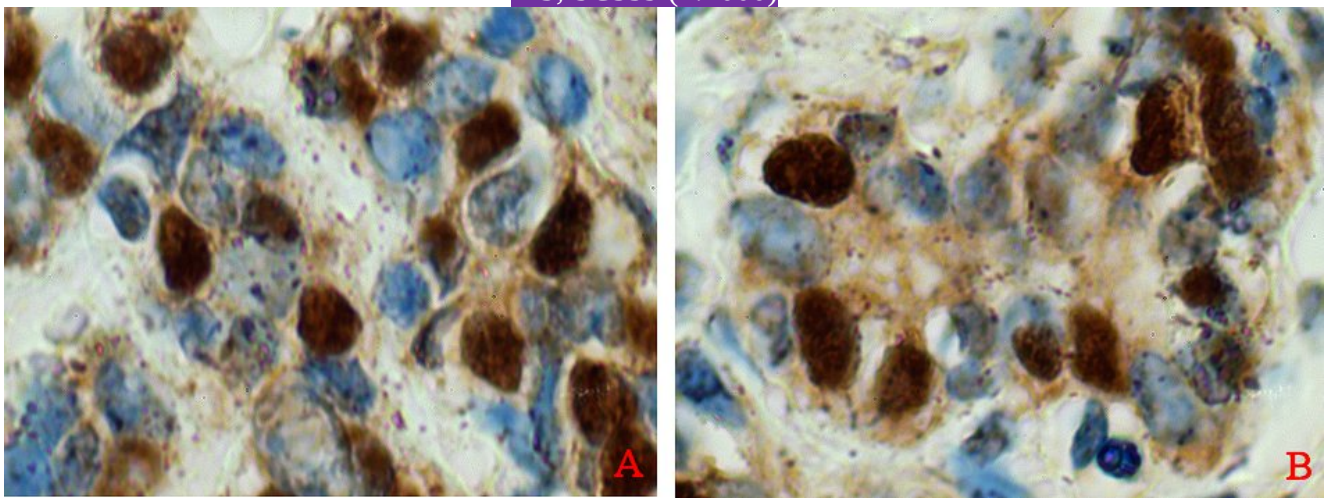


Figure D Photographs taken of tissue called M3, primary antibody used was SC539 (dilution 1:1000), **, figuur14ozs
A + B: magnification 100x. As can be seen the nuclei are strongly positive (dark brown). Around the nuclei, in the cytoplasm a weak staining is observed. However, the nuclei are significantly stronger stained and really stand out compared to the rest of the tissue.

M3; KAQD (1:100)

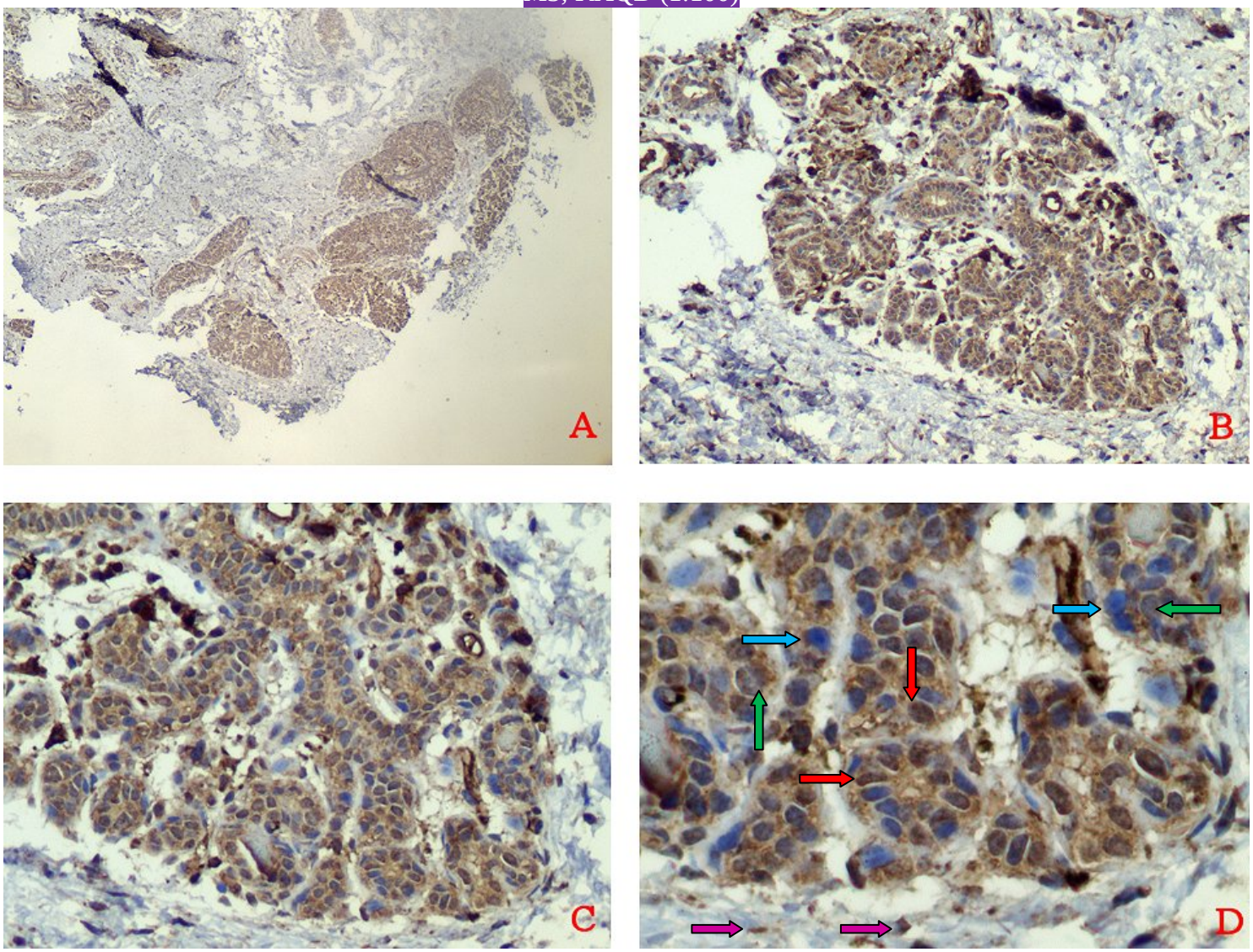


Figure E; Photographs taken of tissue called M3. Primary antibody used was KAQD (dilution 1:100), *, figuur13ozs
A: magnification 2x. As can already be seen, there are positively stained (brown) islands surrounded by blue tissue.
B: magnification 10x. A clear distinction between the positively stained (brown) tissue and the unstained tissue (blue).
C: magnification 20x. Magnification of a positively stained area. As can be seen, the positively stained structures appear to be alveolar/ductal structures, as was seen for primary antibody SC539 in figure A and figure C.
D: Magnification of positively stained cells. As can be seen, the areas that are positively stained are the ones to be expected (e.g. the areas containing alveolar/ductal structures, and are comparable to the areas that stain positive using primary antibody SC539). However, looking closer at the positively stained areas, even though the areas that are positive appear to be the same as observed using SC539, the staining of the cells is very different. Using KAQD, a moderate (compared to the strong, dark brown staining seen using SC539) brown staining of the cells can be seen. In contrast to what is observed using SC539, the staining is more apparent in the cytoplasm rather than the nuclei of cells. Looking closely however, the nuclei strongly differ in color. Some nuclei being extremely blue (blue arrows), while others vary between being less intense blue (green arrow) to being almost entirely brown (red arrows). But even the nuclei that are stained the most using KAQD are significantly less brown compared to the nuclei using SC539, where nuclei appear deep brown. Also the nuclei that are brown have a distinct speckled appearance, whether in the case of SC539 the nuclei appear to be diffusely stained. In the stromal compartment infrequent slight brown staining can be seen (purple arrows).

M3; KAQD (1:100) + BP (5x)

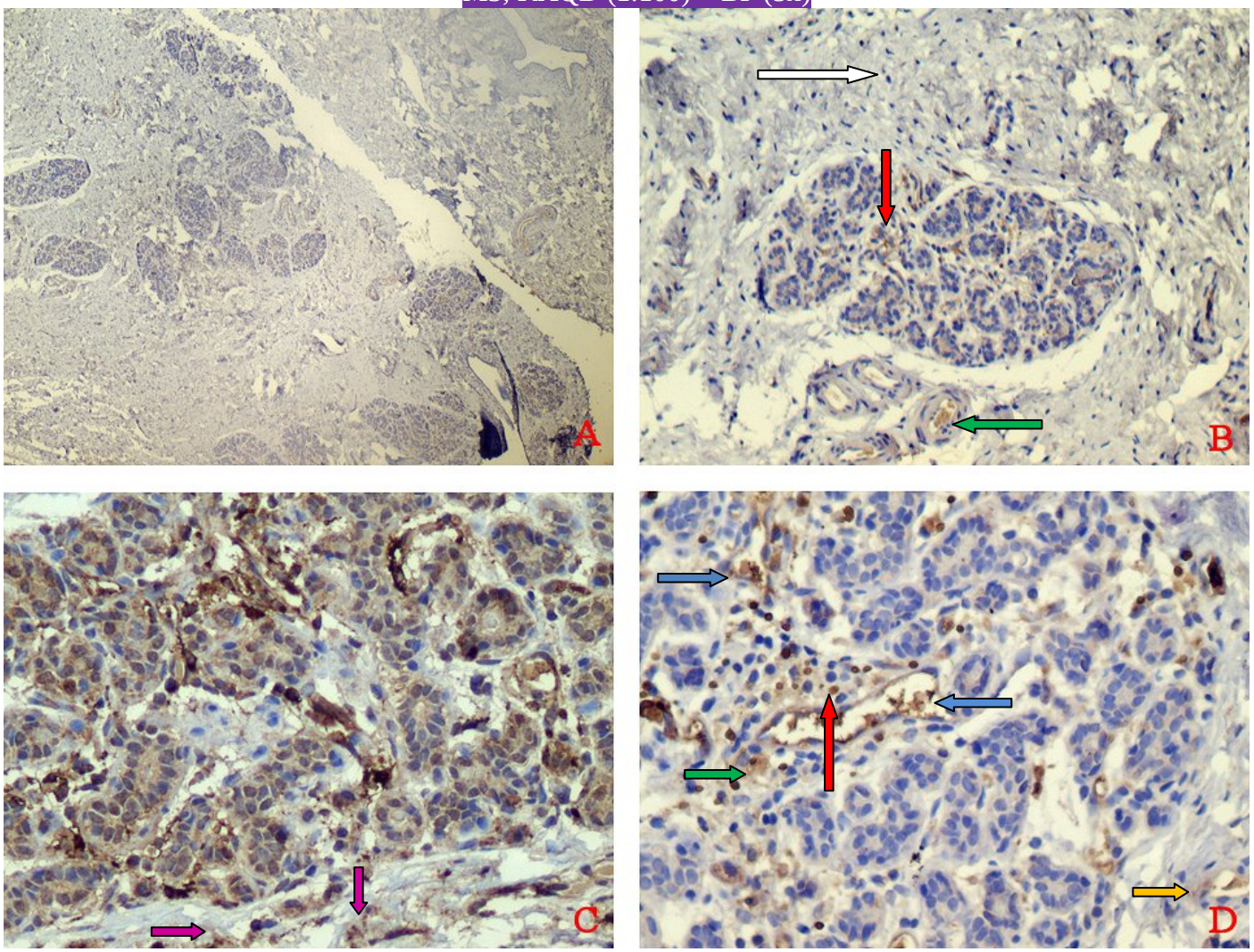


Figure F; Photographs taken of tissue M3. Primary antibody used was KAQD (dilution 1:100). Also blocking peptide in 5 times excess (BP 5x) was added, *, figuur20ozs
A: Magnification 2x. BP 5x added. At this magnification it is already clear that the addition of BP to the primary antibody solution results in a drastic decrease of the brown staining
B: magnification 10x. BP 5x added. Looking a little closer however, reveals that there is still some brown discoloration mainly concentrated around the ductal/alveolar structures (red and green arrows). The stroma (white arrow) seems to be completely blue.
C: Magnification 20x. No BP added. Distinct brown discoloration is present. In the stromal compartment as well, some brown staining can be seen (purple arrows).
D: Magnification 20x. BP 5x added. Approximately the same area as photograph C, so the difference between the addition of BP 5x (photograph D) and the addition of the non-neutralized antibody (photograph C) can be compared. As can be seen, the brown staining is significantly less in photograph D compared to photograph C, however, it is far from being absolutely negative. Even though most nuclei appear blue, some of them have stained brown (red arrow). Also, a light to moderate brown discoloration can be seen in the cytoplasm of some of the cells (green arrow). In what appears to be ductal structures a brown substance is visible (blue arrows). Finally, the yellow arrow point to staining in the stromal compartment.

M3; KAQD (1:100) + BP (5X)

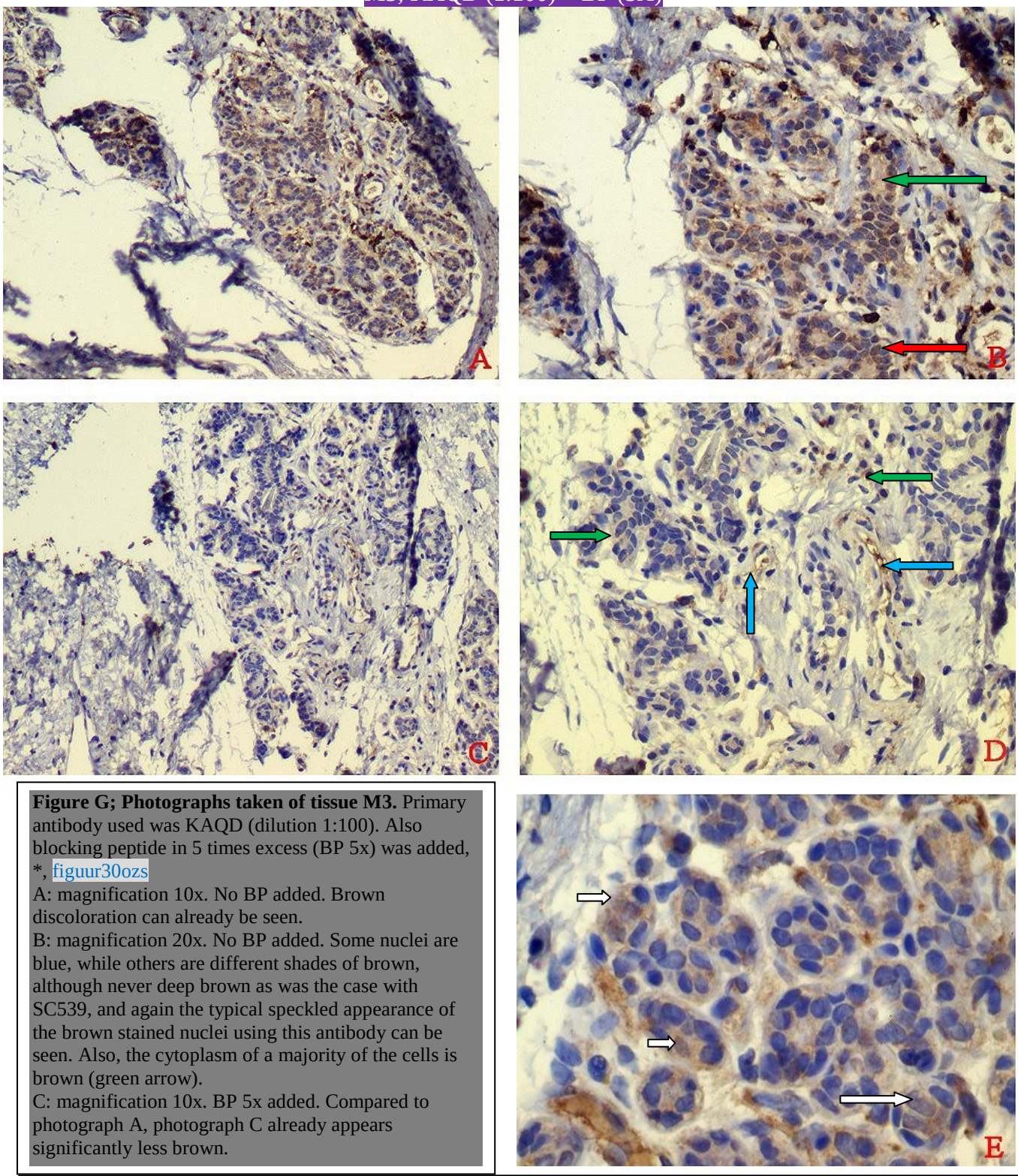


Figure G; Photographs taken of tissue M3. Primary antibody used was KAQD (dilution 1:100). Also blocking peptide in 5 times excess (BP 5x) was added, *, figuur30ozs
A: magnification 10x. No BP added. Brown discoloration can already be seen.
B: magnification 20x. No BP added. Some nuclei are blue, while others are different shades of brown, although never deep brown as was the case with SC539, and again the typical speckled appearance of the brown stained nuclei using this antibody can be seen. Also, the cytoplasm of a majority of the cells is brown (green arrow).
C: magnification 10x. BP 5x added. Compared to photograph A, photograph C already appears significantly less brown.
D: magnification 20x. BP 5x added. Approximately the area as in photograph C. Clear brown discoloration can no longer be found in the nuclei. The cytoplasm also, is significantly less brown, although not entirely negative (green arrows). A stronger brown discoloration can be seen scattered throughout the section in what could be the lumen of ducts or are just artefacts (blue arrows).
E: magnification 40x. BP 5x added. Figuur23ozs. A closer look at the cytoplasm of the alveoli indeed shows that there is still some light brown discoloration here. The nuclei in photograph D appear to be all blue, and the difference with photograph B is remarkable. When looking with a magnification of 40x however, it becomes clear that some nuclei still are not entirely blue (white arrows).

M3; KAQD (1:100)

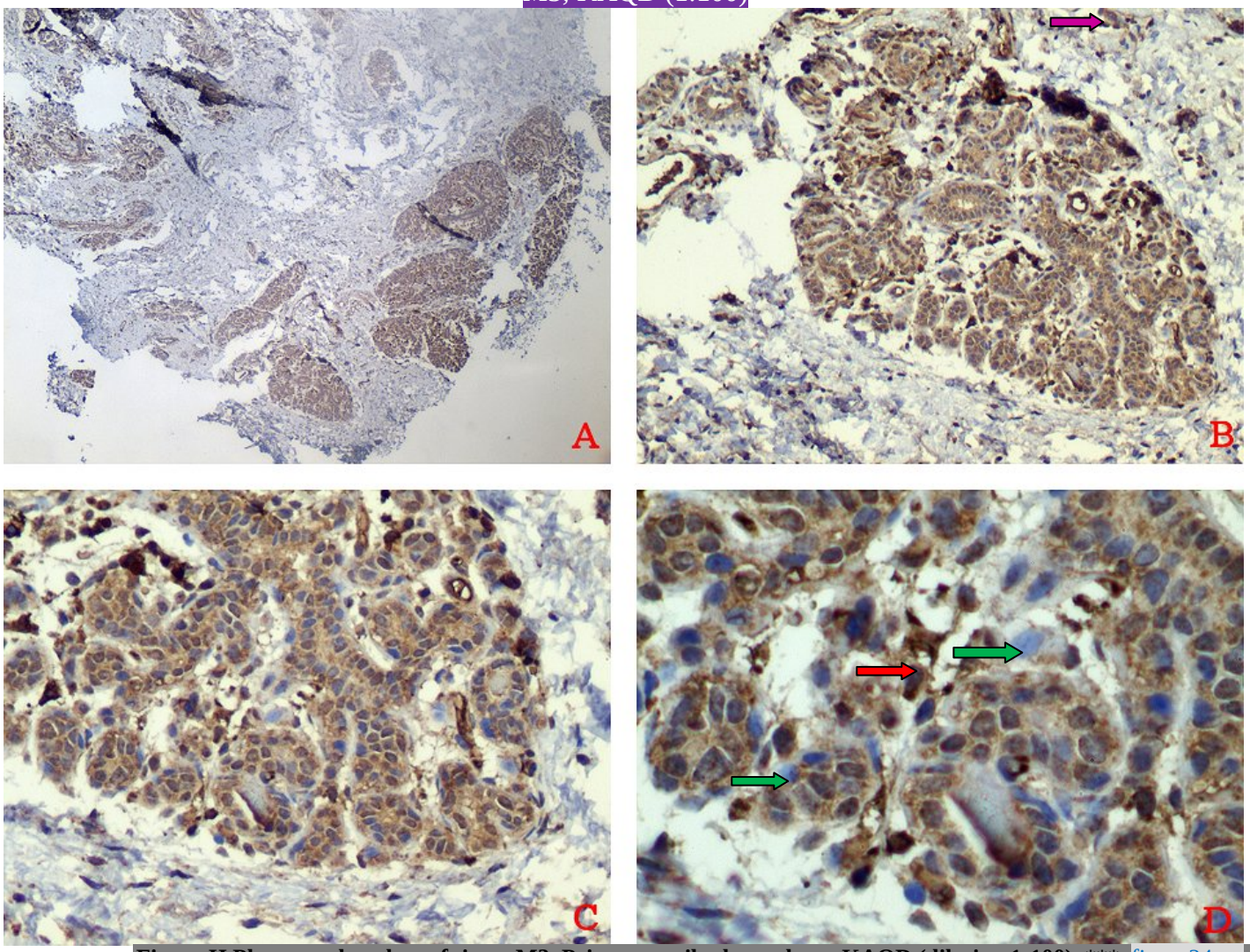


Figure H Photographs taken of tissue M3. Primary antibody used was KAQD (dilution 1:100), ***, figuur24ozs
A: Magnification 2x. Brown islands are already visible
B: Magnification 10x. The brown islands visible in photograph A, appear to be made up of alveolar and ductal structures. The stroma appears to be mostly blue, but slight infrequent staining can be seen (purple arrow).
C: Magnification 20x. In the brown discolored matter, now and then blue nuclei can be seen, whether other are stained brown.
D: Magnification 40x. The cytoplasm of the cells seems to be uniformly stained brown. The staining of the nuclei is not as uniform; clear blue nuclei can be found (green arrow), as well as dark brown nuclei (red arrow). The speckled appearance of the nuclei again is obvious. In general nuclei don't appear as dark brown as they do when using SC539.

M3; KAQD (1:100) + BP (5X)

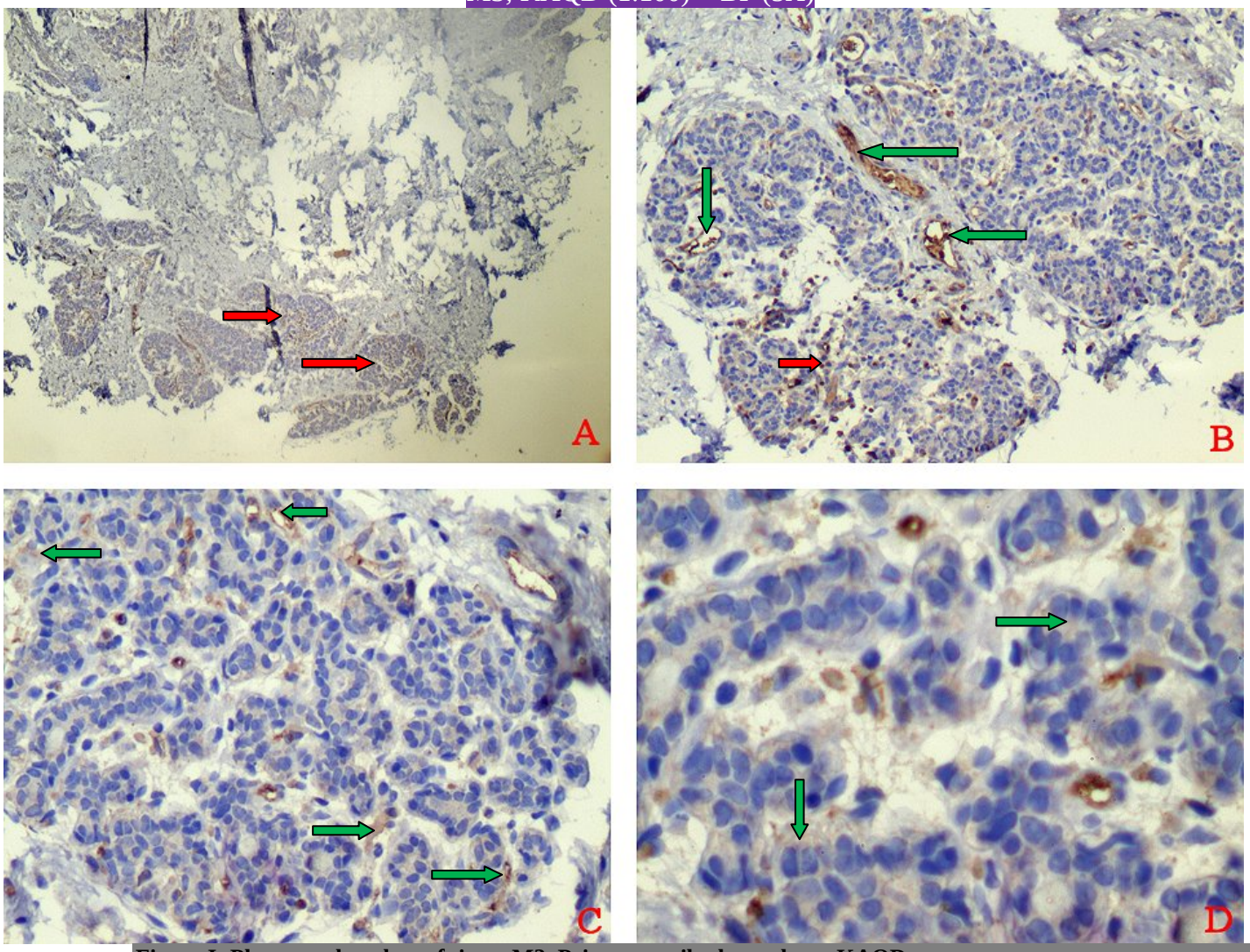


Figure I: Photographs taken of tissue M3. Primary antibody used was KAQD (dilution 1:100). Blocking peptide 5x has been added, ***, figuur25ozs
A: Magnification 2x. BP 5x added. Approximately the same area as the photographs in figure H. Comparing photograph A from this figure, with photograph A from figure H, it's already clear that the addition of blocking peptide significantly reduces the brown discoloration. However, it is also already visible that some brown staining did remain. This brown discoloration generally seems to be located in the alveolar/ductular islands (red arrows).
B: Magnification 10x. BP 5x added. When looking closer, brown discoloration indeed is present in the alveolar islands. Brown nuclei (red arrow) can be seen, a dark brown substance can also be seen in structures that could be ducts or artefacts (green arrows).
C: Magnification 20x. BP 5x added. Looking at a magnification of 20x there also proves to be brown coloration surrounding the alveolar structures (green arrows).
D: Magnification 40x. BP 5x added. The nuclei are remarkably blue. Whether the cytoplasm in general is stained very lightly or is negative is hard to say, it does appear a bit pinkish blue (green arrows).

M3; AAEP (1:400)

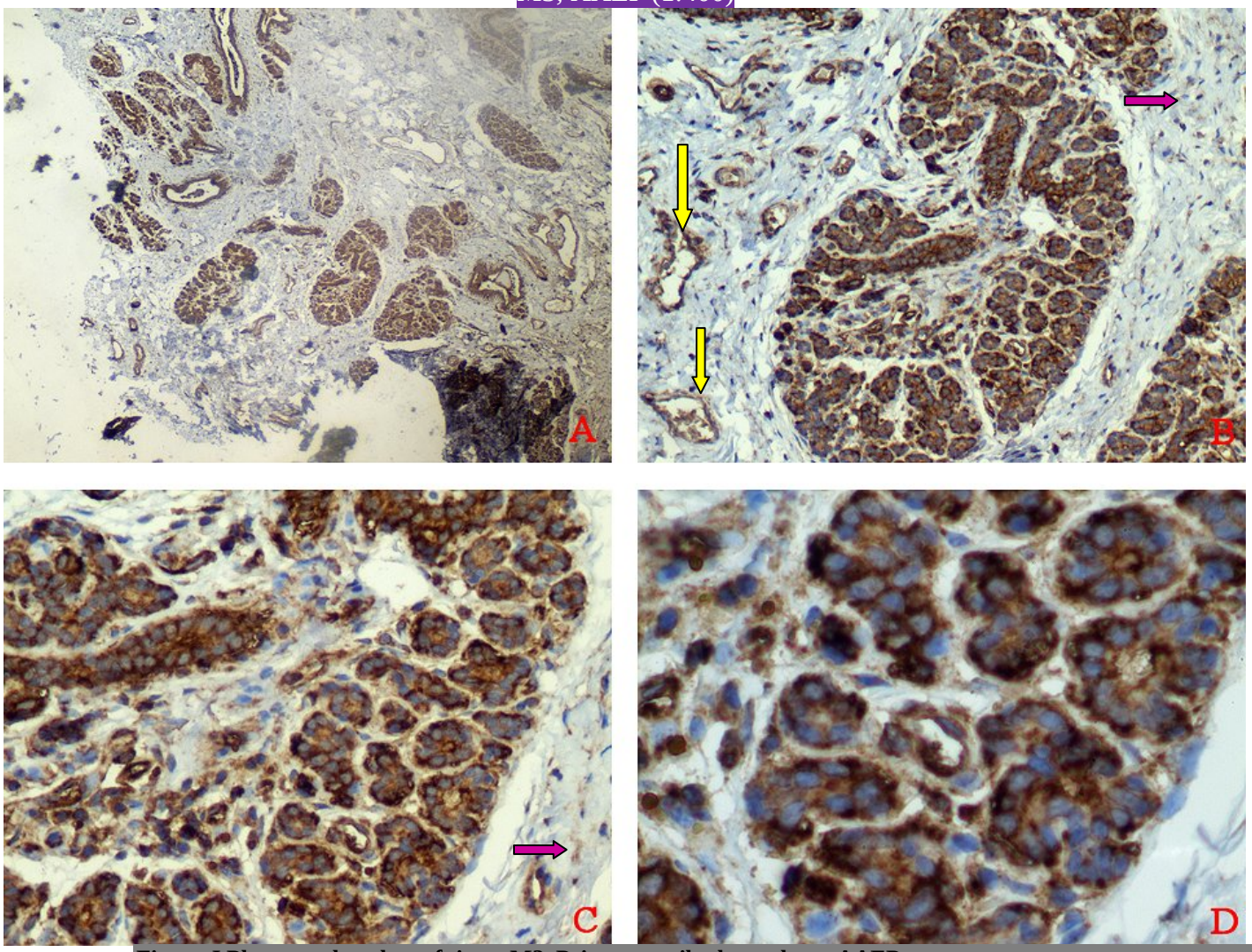


Figure J Photographs taken of tissue M3. Primary antibody used was AAEP (dilution 1:400),*, figuur18ozs
A: Magnification 2x. Very distinct brown islands can already be seen in the mammary gland tissue.
B: Magnification 10x. In between the islands composed of ducts and alveoli some brown staining, can be seen (yellow arrows). This may be collapsed ducts. Infrequent mild discoloration of the stromal compartment can also be seen (purple arrow)
C: Magnification 20x. The purple arrow points at brown staining in the stromal compartment.
D: Magnification 40x. Clear blue nuclei, surrounded by dark brown cytoplasm can be seen.

M3; AAEP (1:400) + BP (5x)

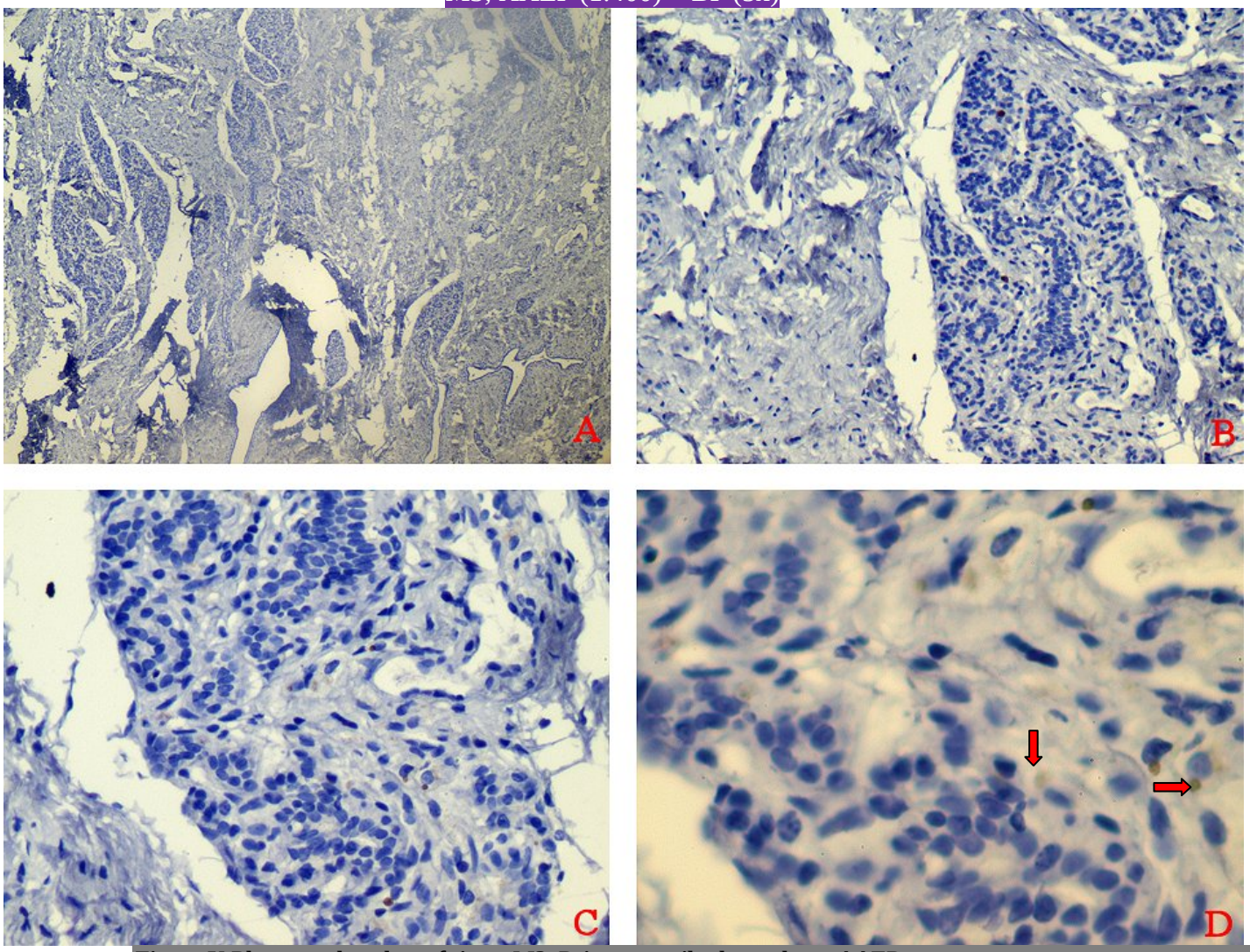


Figure K Photographs taken of tissue M3. Primary antibody used was AAEP (dilution 1:400). Blocking peptide in 5 times excess was added, *, figuur19ozs
A: Magnification 2x. No brown islands can be seen.
B: Magnification 10x.
C: Magnification 20x. At a magnification of 20x there is still no brown staining visible.
D: Magnification 40x. At this magnification the section still looks negative for immunohistochemical staining. Some yellow coloring can be seen (red arrows), but this does not resemble a typical staining pattern that can be seen using AAEP.

17B1; SC539 (1:1000)

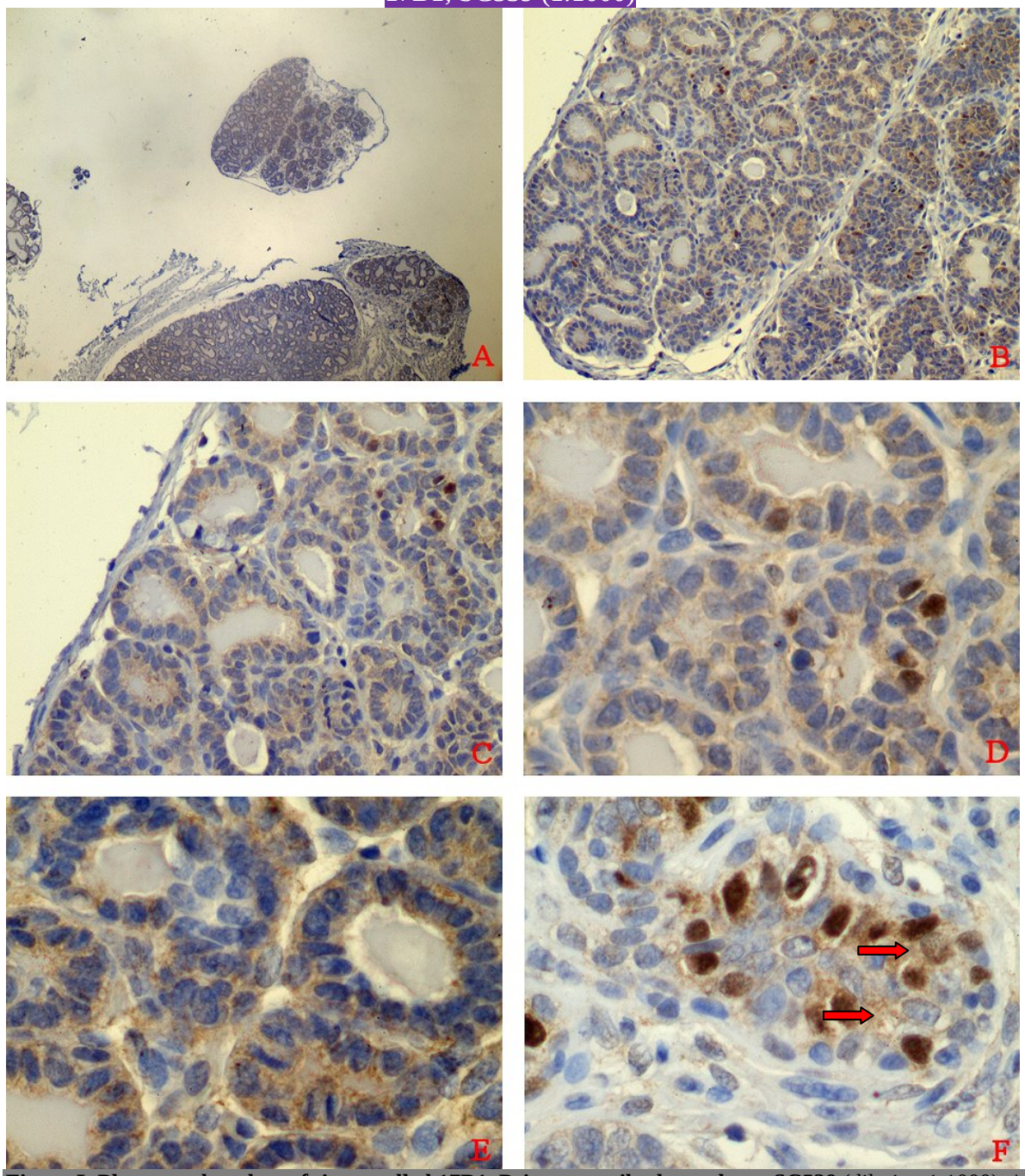


Figure L Photographs taken of tissue called 17B1. Primary antibody used was SC539 (dilution 1:1000), ****, figuur15ozs

A: Magnification 2x. A strongly differentiated piece of mammary gland tissue can be seen
B: magnification 10x.
C: magnification 20x. It is already clear here that the cytoplasm as well as a part of the nuclei have been stained brown.
D: magnification 40x. The cytoplasm has uniformly been mildly stained. Some dark brown nuclei can be found in the majority of blue or very mildly to moderately stained nuclei. The mildly and moderately stained nuclei do have a speckled appearance.
E: magnification 40x. The cytoplasm surrounding the nuclei has been moderately stained, while the nuclei appear clear blue and hardly any discoloration can be found here. Again the speckled appearance of the nuclei can be seen.
F: magnification 40x. Some nuclei appear moderate to dark brown, while others are negative. Also staining of the cytoplasm can be seen (red arrows). The blue nuclei and the ones that are only mildly stained, have a speckled appearance.

17B1; KAQD (1:100)

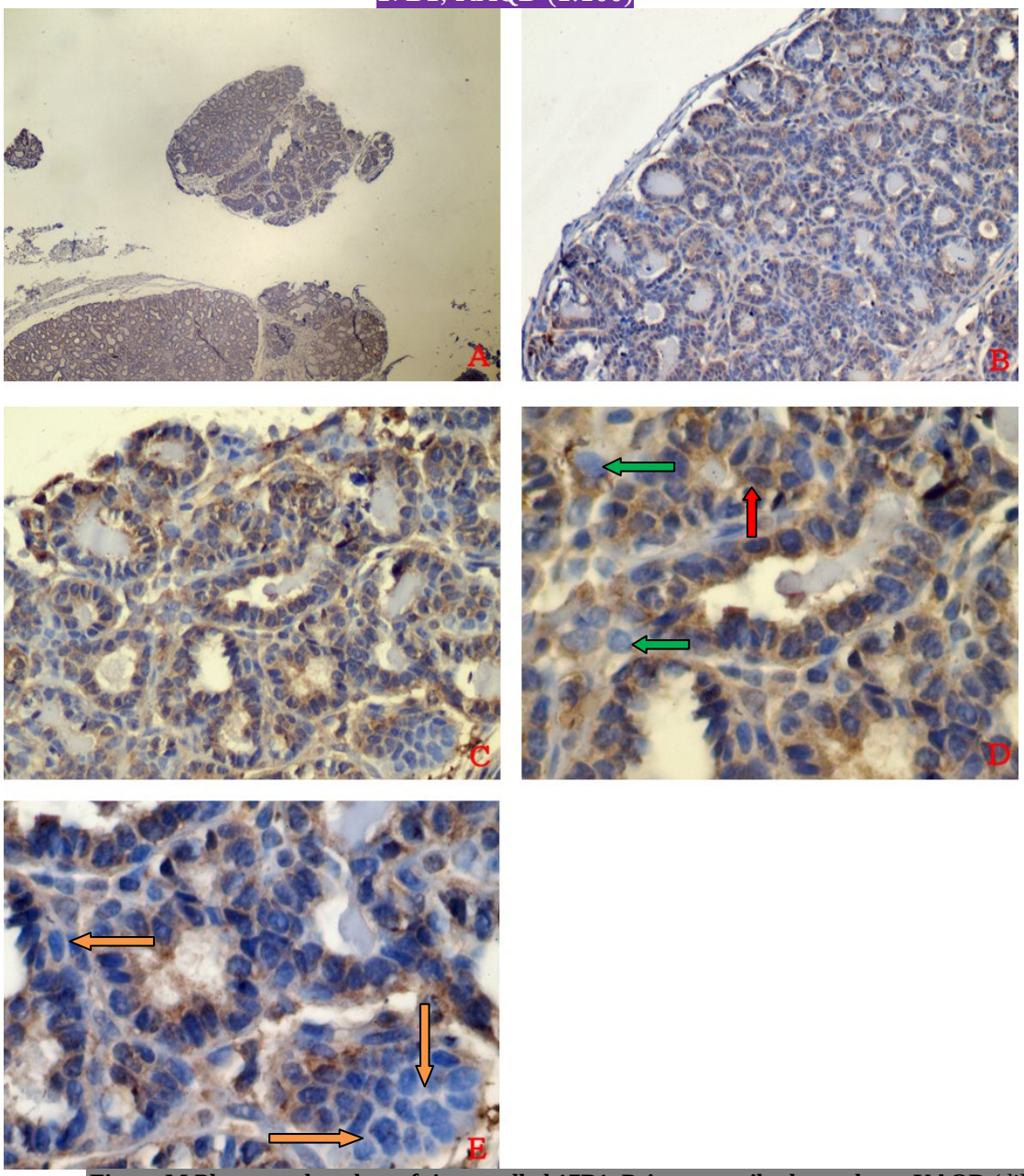


Figure M Photographs taken of tissue called 17B1. Primary antibody used was KAQD (dilution 1:100), ****, figuur16ozs

A: Magnification 2x. Well differentiated mammary gland tissue visible.
B: Magnification 10x.
C: Magnification 20x.
D: Magnification 40x. The brown staining seems to be mostly located in the cytoplasm. There is a mild to moderate staining uniformly spread through the alveolar structures. However, the nuclei are not uniform in color, some being moderately stained brown (red arrow), while others are not (green arrows).
E: Magnification 40x. Clear blue nuclei are pointed out by the orange arrows. The color of the nuclei is not uniform, varying from clear blue to moderately stained.

17B1; KAQD (1:100)

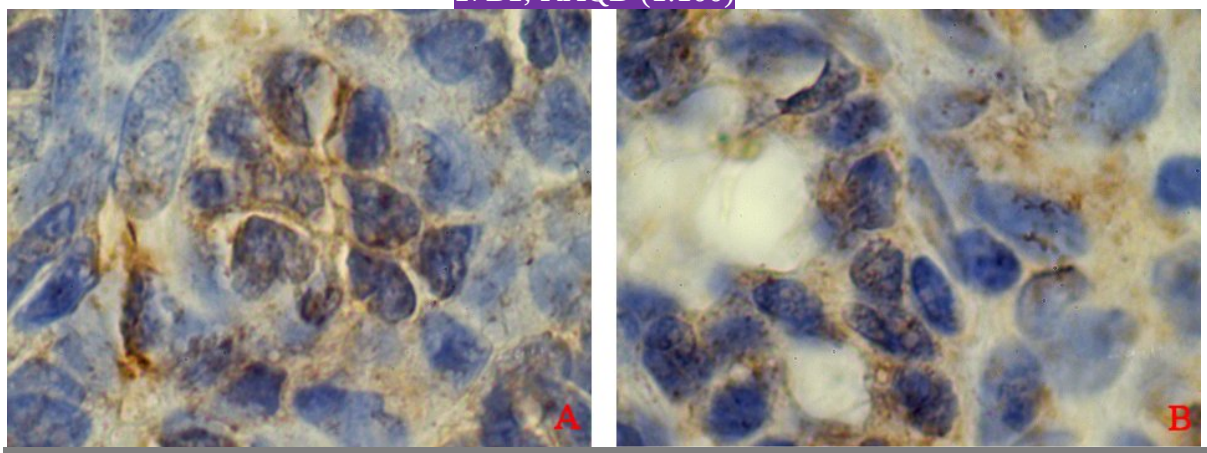


Figure N Photographs taken of tissue called 17B1. Primary antibody used was KAQD (dilution 1:100), ****, figuur17ozs
A: magnification 100x. Close up of the nuclei. The speckled appearance of some nuclei (mostly the ones in the centre) can be seen.
B: magnification 100x. Close up of the nuclei where nuclei with a speckled appearance (on the left) are situated next to nuclei that appear clear blue (on the right).

Western Blot

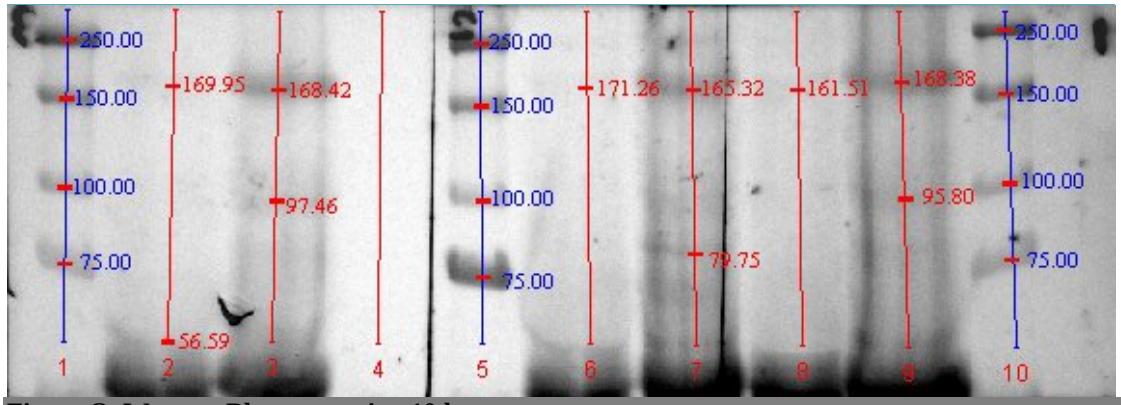


Figure O. Western Blot portraying 10 lanes.
Lane 1; standard, lane 2; M3, lane 3; 30A,
lane 4; negative control (no tissue loaded), lane 5; standard, lane 6; M3,
lane7; 30A, lane 8; M3, lane 9; 30A, lane 10; standard.
Lanes 8, 9 and 10 were incubated with primary antibody KAQD (dilution1:1000),
lanes 5, 6 and 7 were incubated with primary antibody SC539 (dilution 1:2000),
lanes 1, 2, 3 and 4 were incubated with no primary antibody.
The piece of blot that was incubated with anti-actin antibody is missing here, because the blot was cut in the wrong place (exactly where actin should be located on the blot).

Discussion

When consulting the literature concerning PRA and PRB, difficulties arise because of the differences that exist between species. Much of the research has been conducted in mice and human cell lines, while the present research aimed at clarifying the situation in dogs. For example, the previously described studies of Aupperlee 2005 and 2007, investigating the specific roles of PRA and PRB separately, used pregnancy levels of estrogen and progesterone in mice. However useful this research seems in clarifying PR's function, it should be kept in mind that species differences may exist. These restrictions apply to a lot of the literature used. It's also really important to consider certain factors that (may) influence PR expression, when analyzing the results. Examples of these factors are; ovariohysterectomy status, hormonal treatment and phase of the estrous cycle.

Immunohistochemistry

During our research, some problems did arise that may have affected the results. With the immunohistochemical staining, TBS was used to wash the slides. There were however different protocols for preparing the 10x stock of TBS, which we only found out by accident. The use of a wrongly prepared TBS may have had some influence on the obtained results. Too much, or not enough Tris does effect the capacity of the buffer. Worth mentioning also is that since immunohistochemical staining proceeded for several weeks, the slides that were being used varied in age. Sections of M3 varied more in age than the sections of Zagreb. In the beginning, slides that were in stock were being used, however, we ran out so freshly cut slides of M3 were used later on. The Zagreb tissues were all cut at once, however, a few weeks had passed between using the first and the last Zagreb slides. It is known that a long delay between cutting the sections and the immunohistochemical staining can decrease the intensity of the immunohistochemistry reaction and an increase in the frequency of falsely negative slides. (Mirlacher 2004) It would be advisable to standardize the age of the slides during future research, not only making sure that the slides being used are of comparable age, but also that not too much time has passed between cutting the sections and the staining procedure.

There is a marked difference between the results obtained with primary antibody KAQD (dilution 1:100) and AAEP (dilution 1:400) regarding localization of the staining within the cells, intensity of the staining and effect of blocking peptide on the staining. KAQD and AAEP both stain the same cells as SC539; epithelial cells located in ductal and alveolar structures. It looked as not only the luminal epithelial cells, but the myoepithelial cells as well, had been stained. However, these findings should be reevaluated by someone with more expertise. We also found staining in the stromal compartment for all three antibodies used. This stromal staining disappeared with the use of blocking peptide in the case of AAEP. In the case of KAQD, some stromal staining may remain. Obvious stromal nuclear staining appeared to be insignificant. If staining is observed in the stromal compartment, it more closely resembles cytoplasmic staining. However, staining in the stromal compartment in general, appeared to randomly localized, being confined neither to the nuclear or stromal compartment.

For the epithelial cells of the glandular tissue **KAQD** causes a mild to moderate staining of the cytoplasm as well as the nuclei. In general the cytoplasm does appear

to stain slightly stronger compared to the nuclei. Characteristic for staining with KAQD seems to be the speckled appearance of the nuclei, which is not found when using AAEP or SC539. After the use of blocking peptide (5x excess) a significant decrease in staining was seen, but slides never were completely negative. Using a 10x excess hardly made a difference. As can be seen in figure F-D, staining may remain in ductal structures as well as alveolar structures. In epithelial cells staining can be found in the nuclear and cytoplasmic compartment. Staining may also remain in the stromal compartment. So basically every structure that was positively stained, may remain positive after the use of blocking peptide. The overall staining, however, really is remarkably reduced. The use of **AAEP** results in prominent clear blue nuclei, surrounded by a strongly positive cytoplasm. After using blocking peptide (5x excess) the slides are completely negative for immunohistochemical staining. **SC539** strongly stains the nuclei, the intensity of staining being comparable with the staining seen in the cytoplasm when using AAEP. SC539 also mildly stains the cytoplasm. Blocking peptide experiments have not been performed for antibody SC539.

So the major disadvantage of antibody KAQD is the remaining of some staining after the use of blocking peptide. Most of the time this staining was only very mild, and as said, significantly less compared to the situation where no BP was added. However, as is shown in figure G-E sometimes a part with stronger staining could be found in the slides treated with blocking peptide. Even though these stronger positive areas were very rare, this finding does make it even harder to interpret the results when using this antibody. AAEP also has an important disadvantage; it seems to be unable to detect nuclear PRB. The cytoplasmic staining may still be specific though, since different anti-PR antibodies may detect epitopes that are exposed on the nuclear, cytoplasmic, or both forms of PR. (Aupperlee 2005) AAEP may only be detecting cytoplasmic PRB, and if it does prove to be specific staining, AAEP even performs this task with virtually no nonspecific staining, judging on the results with the use of blocking peptide. This is AAEPs major advantage. The major advantage of antibody KAQD is the distribution of staining within the cell, where positivity is observed in the cytoplasm as well as in the nucleus, and with a moderate intensity. In contrast, AAEP strongly stains the cytoplasm, at times the intensity of staining may even be stronger than the staining observed with SC539.

After carefully considering the advantages and disadvantages of both PRB specific primary antibodies, the decision was made to use antibody KAQD in the remaining experiments for the following reasons; from the literature we know that PRB is present in the cytoplasm as well as in the nucleus, at least in other species investigated. When using SC539 in canine mammary gland tissue also, we find staining in the cytoplasm as well as in the nucleus. So, KAQD does fit best with the results of other researches as well as with the results of SC539. When investigating the expression of a certain protein of interest, the ability of an antibody to detect this protein at all of its localizations clearly is an important advantage. A second reason to pick KAQD over AAEP was that AAEP staining was very strong in general. Since both AAEP and KAQD are supposed to be PRB-specific antibodies one would not expect stronger staining when using AAEP or KAQD compared to SC539.

In general, the Zagreb tissues stained less positively compared to M3. Of these tissues, 17B1 was a tissue with the strongest staining, but it is still clear that the use of SC539 in this tissue resulted in a different staining pattern compared to the one that is

seen in tissue M3. Most of the nuclei are only mildly to moderately stained, with a speckled appearance, and the color of the nuclei not as uniform as compared to use of the antibody in tissue M3. However, some strongly stained nuclei can be seen, in a smaller quantity compared to M3. KAQD staining in this tissue is actually comparable to that seen in tissue M3, but in general KAQD as well did result in weaker staining in the Zagreb tissues compared to the staining observed in M3. The different results between the used tissues may have been caused by a difference in differentiation; tissue 17B1 is stronger differentiated compared to tissue M3. Also, a difference in the fixation of the tissue, and/or a difference in the procedure preparing the slides that were being used, may help explain the observed differences. However, this should be investigated further.

Specificity control

When using antibodies, it's important that the antibody is known to or demonstrated to be specific, selective and reproducible. (Bordeaux)
Non-specific binding is more common with polyclonal antibodies compared to monoclonal antibodies. (Abcam BP1) Controls for antibody specificity are most important for the correct interpretation of its localization. (Burry)

Blocking peptide

Absorption controls, of which the use of blocking peptide is an example, is one option to test specificity. A very important limitation of this type of specificity control is that it only determines the specificity of the antibody for the (in this case) blocking peptide. It does not prove the antibody specificity for the protein of interest present in the tissue. An antibody is able to bind to a certain epitope, and this may include the blocking peptide but also any other protein with the similar epitope. So, binding of the antibody to the blocking peptide, does not prove that the protein to which the antibody binds in tissues, is the same protein. (Burry) The use of blocking peptide in specificity controls also fails to prove selectivity of the antibody; off-target binding of the antibody in the tissue is also inhibited as a result of the preincubation of antibody and blocking peptide. The use of blocking peptide thus merely demonstrates antibody specificity for the immunogen. (Bordeaux)

In theory complete inhibition of the antibody binding to the tissue should occur when appropriate concentrations of antibody and blocking peptide have been used. In practice however, dissociation of some of the antibody from the peptide occurs during the incubation period, resulting in the binding of antibody to the tissue of interest. This effect can be overcome; preincubation of the antibody with the peptide as well as preincubation of the tissue with the peptide reduces the chances of antibody binding to the tissue. Burry mentions another reason why the absorption control can be positive even when the antibody is indeed specific for the peptide or protein of interest in the tissue; as a result of protein-protein binding of the antibody-bound protein to protein in the tissue. (Burry)

In order to make this control useful, the blocking peptide must have been adequately purified, which means that it is free of contaminating proteins. (Burry) Abcam BP1 advices to incubate the antibody with the blocking peptide for 30 minutes at room temperature or overnight at 4°C. (AbcamBP1) We let the blocking peptide and antibody incubate for 2 hours at room temperature.

For antibody KAQD a significant decrease in staining was seen after the use of blocking peptide. Slides were however never completely negative. We tried a 10x excess and putting the antibody-blocking peptide solution in the freezer, 20 minutes before incubating the tissue with the solution, trying to lose the staining that remained in previous experiments with BP. Putting the solution in the freezer aims at stopping the dissociation of the antibody from the peptide. Both of these adjustments did not have a significant effect. In order to reduce the chances of dissociation of the antibody from the blocking peptide we could try to also preincubate the tissue with blocking peptide, and shortening the preincubation period of blocking peptide with KAQD. Two hours may be too long, possibly resulting in dissociation of the antibody from the peptide. Dissociation would then already have occurred at the time we put the slides in the freezer, which could explain why this adjustment did not have a significant effect.

When the adjustments in the experiments with blocking peptide don't result in slides without staining (thus indicating non-specific staining), the next step could be to try to reduce high background or non-specific staining (in general) in immunohistochemistry procedures as follows;
a) increasing the blocking incubation period. Abcam recommends 10% normal serum, which we did use, however, we incubated the tissue with normal goat serum for 30 minutes, while Abcam advises a period of 1 hour.
b)Primary or secondary antibody concentration may have been too high. Reevaluation of the optimal antibody concentration to be used may be necessary. One option is to reduce the antibody concentration and to increase the incubation period (however, we already incubated the slides overnight in case of the primary antibody), another option is to decrease the incubation period, with the same antibody concentration.
c) The washing may be insufficient, and should be optimized. Different detergents for example could be tried.
d) The incubation period for the chromogen (DAB) could have been too long and may be reduced, or a wash step (TBS) after incubation with DAB could be tried.

Negative control slides

We used "no primary antibody control" as negative control slides. Negative control slides in general are necessary to be able to evaluate non-specific staining. "No primary antibody control" is used to evaluate possible non-specific staining caused by sources other than the primary antibody. (Dako1)
Other options for negative control are; 1) replacing the primary antibody with normal serum from the same species which should be similarly diluted or 2) replacing the primary antibody with an unreactive antibody of the same isotype in the same concentration, using the same diluents. (Burry, Dako1) Bordeaux states that the use of no primary antibody control is valuable but insufficient and advices to 3) use a cell line or tissue that does not express the protein of interest, implicating that knockout cells are thus the best negative controls possible, but cell lines or tissues known not to express the protein can be used as an (less reliable) alternative. (Bordeaux) When using the "no primary antibody" negative control, non-specific binding of the primary antibody cannot be evaluated. Considering that KAQD and AAEP are brand new antibodies that have never been tested before, and the results obtained with the blocking peptide experiments, adding a second negative control to test for the

nonspecific binding properties of these secondary antibodies seems advisable for future research.

Positive control slides

The positive control confirms if the antibody is binding to the appropriate structure or not. (Burry) The positive control also verifies if the antigen retrieval procedure, used reagents, incubation time, temperature during differing steps etcetera were correct. If the positive controls do not turn out as was expected, the results of all the slides tested simultaneously should be considered invalid. (Dako1)
For the positive control a different tissue that is known to contain the protein should be used. (Burry)
Dako1 advices to select a tissue that contains a spectrum of weak to strong positive staining and if such tissue is not available to choose a tissue that is only weakly positive, since this makes it easier to evaluate whether the staining in another tissue is too weak or too strong. (Dako1) Another possibility is to use several different antibodies directed against the same protein and comparing the localization within cells and tissues, which should be the same. (Burry) According to Bordeaux, cells that do not express the protein of interest, that are transfected with this protein provide for the best positive control. Cells known to overexpress the protein of interest can be used as an (less reliable) alternative. (Bordeaux)
In our research, we started with uterine tissue to optimize the test conditions for primary antibody SC539. Later on, when we were testing primary antibodies KAQD and AAEP we used mammary gland tissue, called M3, because this tissue showed moderate to strong staining and consistent results when it was used in the prior phase (optimizing for SC539). As a second positive control we also used primary antibody SC539, to which primary antibodies KAQD and AAEP could be compared. The reason for choosing SC539 has been explained previously. Besides comparing the localization of the staining between the different antibodies used, we also looked at the intensity of the staining between the different antibodies.
The choice of positive control slides may not have been optimal, according to Dako1. M3 did not contain a spectrum of weak to strong positive staining and stained strongly in M3 and moderately in the Zagreb slides, not weakly as advised. However, we also used slides incubated with SC539 as positive control slides, the combination of both positive controls reinforcing one another.

Western Blot

The specificity of the antibody is best evaluated by immunoblotting according to Burry (Burry). Western Blot was thus mainly used as the ultimate means of specificity control of the results obtained using immunohistochemistry, aiming to confirm the hypothesis that the staining observed in the immunohistochemical sections was indeed caused by specific antibody binding to PRB.
Western Blot is often used to determine antibody specificity. Observing a single band at the correct molecular weight is a first indication that the antibody is specific for the protein of interest. (Bordeaux) Using Western Blotting as the ultimate type of specificity control does have some important practical limitations. Some antibodies may not perform as good as was hoped for when used with native proteins with intact 3D structure; these antibodies thus may not be useful for use in immunohistochemistry but may function in a SDS Western Blot procedure, where the protein of interest is being denatured and can then be bound. In contrast, there are also antibodies that work well with proteins in their native form, but not when they are

denatured. This ultimately means that Western Blot cannot be used as an absolute standardization for antibody binding in immunohistochemistry (or any other assay that contains antigen in its native form). (Bordeaux, Mote2001)

Our Blot evaluated

Looking at our blot, 4 observations are really notable:

1) Lane incubated with primary antibody KAQD does show 2 bands
2) Lanes incubated with primary antibody KAQD do not show band sizes that fit the expected band sizes for PRA and PRB
3) Lanes incubated with primary antibody SC539 do not show band sizes that fit expected band sizes for PRA and PRB
4) Lanes incubated without a primary antibody do show bands

There are multiple possible causes for the results obtained in the Western Blot. In the first place, of course, that indeed the observed staining in the immunohistochemical sections was not a result of specific antibody binding to PR and the Western Blot results were thus correct. However, Western Blot results could also be incorrect.

Bands of interest not detected

First of all the obtained band sizes do not fit the expected band sizes, for which several explanations can be found:
a) The treatment of tissues in the course of procedures such as Western Blot or immunohistochemistry can change the exposure of epitopes in the proteins, making it impossible to detect PRB. (Burry)
b) Storage/preparation of anything used in the Western Blotting procedure may have been incorrect, affecting results.
c) Blot transfer efficiency could have been poor, meaning that proteins did not transfer properly to the membrane. However, protein standards have clearly been transferred so this is unlikely to be the explanation for the obtained results.
d) In case protein transfer efficiency was good, insufficient binding to the membrane after transfer could have caused the problem. However, we used transfer buffer containing 20% methanol, which helps binding of protein to the membrane.
e) Excessive washing of the membrane can also result in loss of protein from the membrane. However, the same applies to the protein binding and excessive washing as it does to transfer efficiency; namely that protein standards are clearly visible in our blots, making insufficient binding of proteins to the membrane less likely to be the main cause.
f) Also, the concentration of primary and secondary antibodies could have been too low, or
g) that the antibodies had lost activity. Piercenet advices performing a dot blot to determine activity of the antibodies.
h) Another possibility is that not enough protein was loaded on to the gel. (Gelifesciences, Piercenet) This possibility is also mentioned in the literature; PR is only present in the epithelium according to Aupperlee 2007. The stromal, PR^- proteins, dilute the epithelial protein. This dilution limits the accuracy and sensitivity of a Western Blot when using whole mammary gland extracts. (Aupperlee 2007) So, the amount of protein loaded into each lane could be increased. Protease inhibitors were already used in our Western Blotting procedure, decreasing the chances of
i) losing protein through breakdown.

j) The antigen of interest could have been masked by the blocking buffer according to Piercenet. Abcam and GElifesciences also, advise trying different blocking buffers because a cross-reaction between the blocking agent and the primary antibody, secondary antibody or protein of interest can result in no or low signal. They also suggest using a mild detergent (such as Tween20) to reduce this cross-reaction. (AbcamWB3, Gelifesciences, Piercenet) A detergent is added to reduce non-specific binding. We already used a blocking solution containing a detergent (Tween), the concentration of which may be optimized to see if results improve; increasing the Tween concentration can be tried in order to reduce the possible cross-reaction between the blocking buffer and (one of) the antibodies or the protein of interest, but Tween concentration can also be reduced when excessive washing is a likely cause of why the protein of interest is not detected. Also another detergent (Triton X-100) instead of Tween20 can be tried. Usually, 0.05% to 0.1% Tween20 should be sufficient to reduce a high background staining without having adverse effects on the specific signal. (GElifesciences)

Non-specific bands

When looking at the blot, it's remarkable that the negative control lanes (the lanes that have not been incubated with a primary antibody) do show bands. Since there is no primary antibody present in these lanes, this must be the result of non-specific binding which results in the bands that can be seen. Piercenet, in the case of non-specific bands, advices to
a)reduce antibody concentrations because concentrations that are too high can result in non-specific binding and secondly to
b) wash more vigorously (first after proteins transfer, because SDS can cause non-specific binding and also after incubating with the primary antibody because the primary antibody can bind non-specifically to other proteins). (Piercenet) To these tips GElifesciences adds;
c) reducing the amount of protein loaded on the gel. For avoiding the development of low bands due to protein degradation only fresh samples kept on ice should be used, and protease inhibitors should be added. Electrophoresis should follow sample preparation as soon as possible.

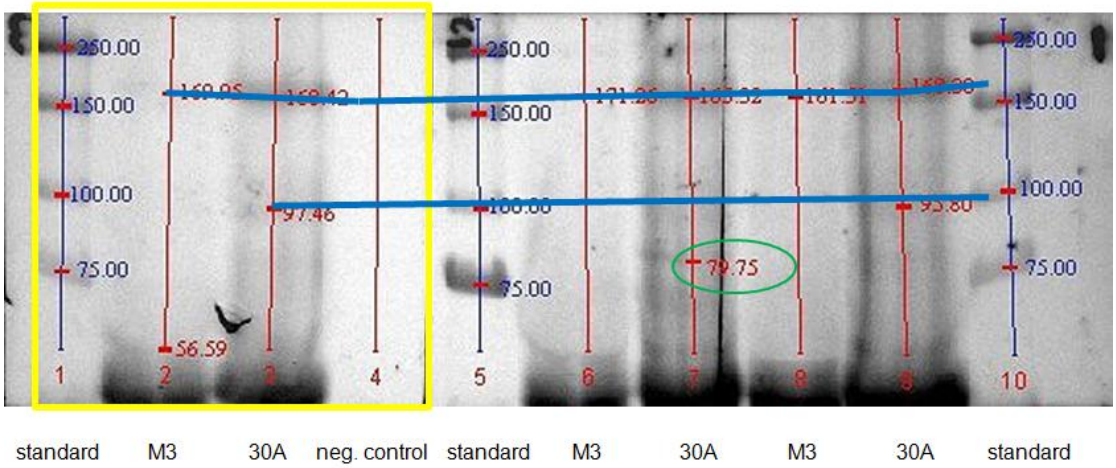


Figure P (compare to figure O) Western Blot interpretation. The lanes incubated without primary antibody are depicted within the yellow rectangle. The lanes closely resembling each other in size have been crossed out by the blue line, because they are most likely the result of nonspecific binding of the secondary antibody, since they can be found in the negative lanes as well. One band size in lane 7, encircled in green, is an exception. This may be a nonspecific band as well or a specific band that is a little smaller than the expected band size.

SC539

Lanes 5, 6 and 7 were incubated with primary antibody SC539. Here also, the expected band sizes were not found. The difference with the part that has been incubated with KAQD however is that SC539 has been tested before and is known to function using Western Blot. So, we should be able to obtain the expected results, at least for SC539. It is thus advisable to optimize the Western Blot procedure, aiming to obtain at least the expected bands for SC539, before concluding anything. As long as we do not find the expected results when using SC539, this may very well be caused by a suboptimal Western Blotting procedure.

So bands at the correct molecular weight for PRA and PRB did not show in our blots and so we were unable to confirm our hypothesis. In this case, the non-specific bands are most likely to be the result of an interaction caused by the secondary antibody. (Figure P) In order to lose the non-specific bands and to try to gain the bands we were looking for, the next step would be to try another secondary antibody, instead of using the same secondary antibody in a reduced concentration, which would increase the chance of losing the non-specific bands but not the chance of gaining the bands of interest. Adding a wash step after protein transfer or optimizing an existing wash step can be tried when non-specific bands appear again. As has been explained, it is advisable to keep optimizing Western Blotting procedure until the specific bands are found in the lanes incubated with SC539.

The most important thing to keep in mind however is that differences in the two methods used (Western Blotting versus immunohistochemistry) consequently may result in the antibody not functioning in one method, while performing well in the other. (Bordeaux, Mote 2001)

Conclusion

Progesterone Receptor in the literature

Progesterone and PR regulate the transcription of target genes. Progesterone acts through binding to PR, a nuclear ligand-activated transcription factor that exists in 2 isoforms; PRA and PRB that differ mainly in their amino-terminus, where PRB contains an additional amino acid sequence, also known as the BUS region. Both isoforms regulate a different (largely non-overlapping) subset of genes expressed by a cell. (Aupperlee 2005, Aupperlee 2007) The functional differences between PRA and PRB can be explained by structural differences; The BUS region contains (trans)activation function element 3 (AF3) which is thus only present in PRB, while PRA contains an inhibitory function that is not active in PRB. Also, PRB contains more phosphorylation sites compared to PRA and both isoforms differ in their affinity to the different co-regulators. (Camacho-Arroyo 2007, Scarpin 2009) Dimerization of the PR isoforms after ligand binding results in 3 possible forms: the PRA-PRA and PRB-PRB homodimers and the PRA-PRB heterodimer. The ratio of PRA to PRB is species-, cell type-, and tissue-specific, and in the reproductive tissues the ratios also vary as a consequence of developmental and hormonal status and during carcinogenesis. (Conneely 2001, Scarpin 2009)
Since each of the 3 dimers regulates a different set of genes, which one of these 3 dimers predominates within a cell has an important influence on the transcription. The dimers interact with co-regulators and general transcription factors. Disorders in expression or activity of PR co-regulators are linked to cancer. A shift in the predominance from one dimer to another, as is seen in human cells with pathological conditions of the breast, will most likely change the genes that are being transcribed following binding of the ligand. Besides a change of the PRA:PRB ratio within a cell, there also then appears to be a difference in expression of PRA and PRB between (neighbouring) cells, which is not normally the case.

Genomic (nuclear ligand-activated transcription factor; regulating the transcription of target genes) as well as non-genomic (cytoplasmic/membrane signal transduction pathways) actions of PR are known. The role of this non-genomic PR action is not entirely clear yet, but seems to also regulate gene transcription and may be relevant in tumorigenesis. (Lange 2008)

Progesterone proves to have an important role in proliferation and differentiation of the mammary gland, with a more distinct role for PRB being mentioned in the literature. A paracrine signal, through which PRA^+ and PRB^+ cells stimulate the proliferation of PR^- cells, seems to account for the majority of the proliferating cells. Conneely 2001, Conneely 2003 (2), Lange 2008, Robinson 2000)
The precise roles of progesterone and PR are not entirely clear yet, but they are likely to contribute to mammary gland tumorigenesis.
PR is likely to contribute to mammary gland tumorigenesis, which should explain why it was investigated in our project, but also that more research is needed to determine its definite role, as well as the specific roles for PRA and PRB.

The morphology that is considered to be normal, differs between the different species, which should be kept in mind when trying to extrapolate results from one species to another. It’s also important to keep in mind that fluctuations of progesterone and estrogen during the estrous cycle affect PR expression within an individual, and thus

knowledge of the estrous cycles of different species is important when interpreting the obtained results.

Expression of PR is influenced by many factors and differs between species (which can partly be explained by differences in degree of differentiation during the estrous cycle), between healthy and diseased tissue, between different diseased tissues, between different developmental stages of the mammary gland and between ovariectomized and non-ovariectomized animals, but it can also change in the course of disease or during a treatment. PR expression is regulated by estrogen and progesterone. Estrogen (acting through its receptor) up-regulates PRA, most likely through a direct mechanism and enhances the increase of the expression of PRB that is induced by progesterone through an indirect mechanism. Progesterone down-regulates PRA, weakens the up-regulation by estrogen, but up-regulates PRB; the net effect being a down-regulation of PR in general. Progesterone alone is enough to induce PRB expression (and proliferation). However, the addition of estrogen accelerates and enhances the up-regulation of PRB by progesterone. (Aupperlee 2007, Bagamasbad 2011, McGowan 2007)

Progesterone Receptor in this research

Conflicting results were found in the literature regarding the localization of PR; In our project staining of the stromal compartment was found infrequently, we also found staining in luminal epithelial as well as myoepithelial cells, and in alveolar as well as ductal structures. However someone with more experience in histology should evaluate these findings.
KAQD in a dilution of 1:100 was chosen as our primary PRB-specific antibody, because of the localization and intensity of the staining. Obtained results were compared with primary antibody SC539. KAQD resulted in mild to moderate staining of the cytoplasm and the nuclei. The staining of the nuclei was not as uniformly as was observed for SC539. SC539 strongly stains the nuclei and results in mild staining of the cytoplasm. The major disadvantage of KAQD was that slides were not negative after incubating them with blocking peptide, indicating a significant contribution of non-specific binding. The use of blocking peptide for specificity control has important limitations, however, it can be used to conclude that an antibody is not functioning properly. The experiments with blocking peptide could be performed again, with a few adjustments that have been described in the discussion, to see if results improve. When optimized conditions for the tests with blocking peptide still result in significant staining, a decision has to be made whether or not to continue on with KAQD. A significant portion of non-specific staining makes it hard to interpret the results concerning localization and intensity of staining, because part of that staining will be non-specific. If it is then decided to continue working with primary antibody KAQD, the emphasis should be on reducing the non-specific binding in immunohistochemistry. Possible adjustments have been described in the discussion.

Western Blot was used as the ultimate specificity control for the results of the immunohistochemical staining. Unfortunately, based on the Western Blot results, we were unable to conclude that the staining observed in immunohistochemistry, was indeed the result of specific staining caused by KAQD. However, Western Blot results may have been incorrect, therefore it may be too early to conclude that the observed staining was definitely not the result of specific staining of KAQD. The reasons for this and suggestions for possible adjustments for the Western Blot

procedure have been explained in the discussion. It seems advisable to optimize the Western Blot procedure, before rejecting our hypothesis. The most important reason not to reject our hypothesis just now is, as has been emphasized by Bordeaux, that Western Blotting and immunohistochemistry are two very different methods and the antibody may not function in Western Blotting while being fully functional in immunohistochemistry. Another reason is that SC539 also did not perform as expected in the Western Blot, while this antibody is known to function in Western Blotting as well as immunohistochemistry.
One important finding in our Western Blot results are the non-specific bands, also present in the negative control lanes, making a change of the secondary antibody the next logical step. Only changing the choice of secondary antibody will hopefully reduce the non-specific bands but will not necessarily result in detection of the protein of interest, so further optimizing the Western Blot procedure in addition to changing the secondary antibody would be the advice.

In summary; For now we did not soundly prove that the observed staining in immunohistochemistry is indeed the result of specific binding of our antibody to PRB. However, there are some important indications that do support specificity of our antibodies. Of our specificity controls the positive controls and the negative control did support the hypothesis that staining was caused by specific antibody binding. Using blocking peptide however showed a significant non-specific contribution to staining, and Western Blotting even showed no sign of specificity. The advice for now is to continue on with the experiments, optimizing them as has been described and see if results improve.

Future research

Specificity control could be improved. For negative control slides, a tissue or cell line known not to express the protein of interest should be used, to be able to investigate non-specific binding of the primary antibody. Blocking peptide and Western Blotting experiments may be optimized.

A good start in future research would be to continue on with Western Blotting, since only 2 Western Blots have been performed during this research project. Unfortunately there was not enough time left to change conditions in order to optimize Western Blot results. Therefore it seems fair to say that although PRB expression in canine mammary gland tissue has not been proven, there's not yet enough ground to completely dismiss the possibility that the staining observed in the immunohistochemical sections, have indeed been caused by antibody binding to PRB. Trying to optimize the Western Blotting procedure may lead to different results and optimizing any procedure should always be tried before drawing a conclusion.
In case Western Blotting results do show PR bands after optimization, results can be refined.

In immunohistochemistry it would be worth a try to optimize the experiments with blocking peptide, this time not only preincubating the antibody with the blocking peptide, but the tissue with the blocking peptide as well.
The results of AAEP (especially the complete loss of staining after the use of BP which would mean a major advantage), are interesting enough for us to advice to investigate this antibody further in the future, if possible.
Another interesting analysis would be to subdivide the histologic sections of this research project into the different phases (proestrus, estrus, diestrus, anestrus) and look at the expression of PRB in each specific phase. It may be particularly interesting to be able to investigate all four phases in the same dog.

When looking beyond restricted goals of this research project, the amount of future research that would be interesting seems endless. Investigating other species, comparing species and extrapolating results from one species to another. Especially the dog, a species in which mammary gland tumors are so common and prove to have a lot in common with human breast cancer, seems to be particularly interesting.

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Appendices

Appendix 1

Immunohistochemical staining of PR during different stages of estrous cycle

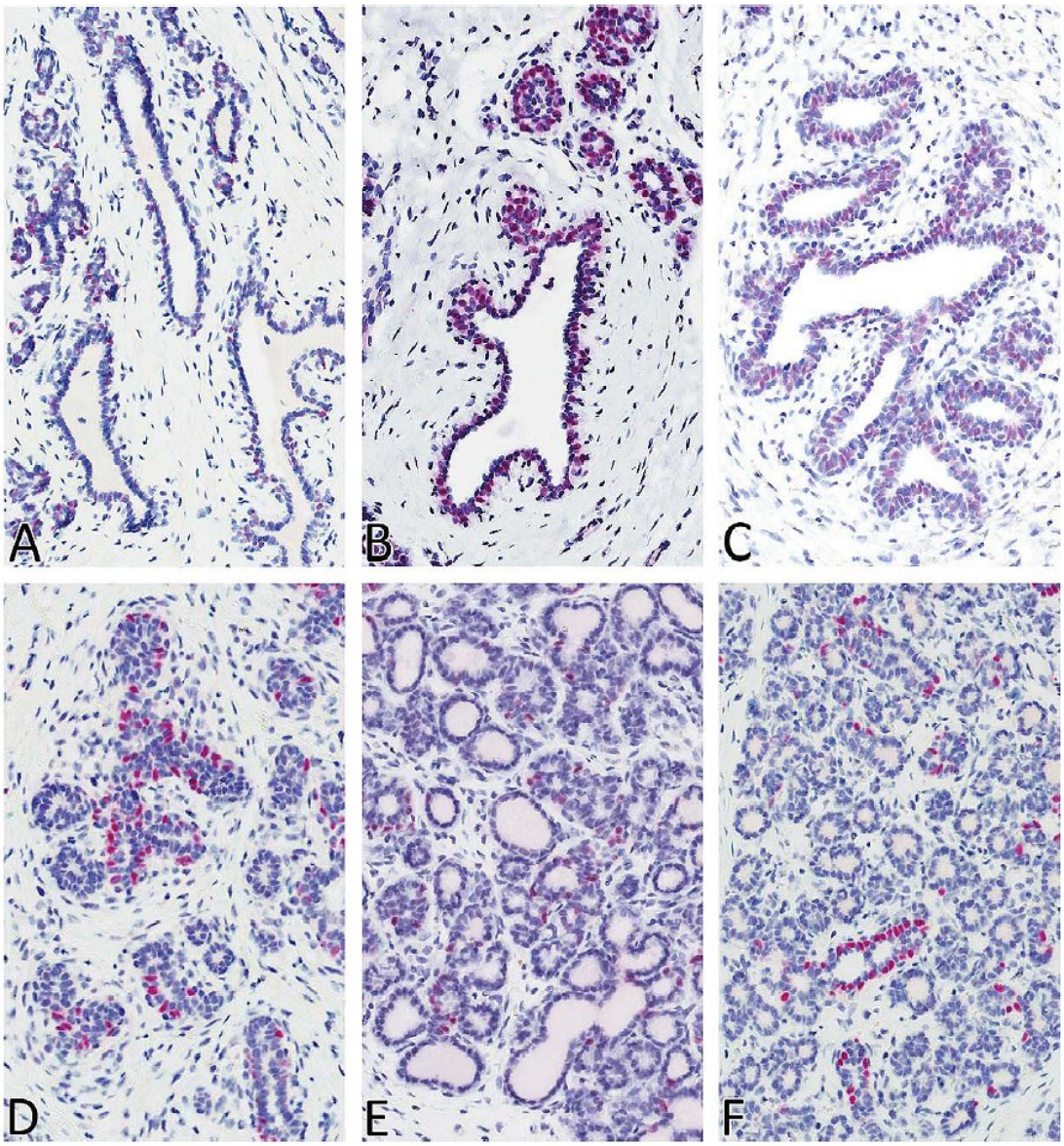
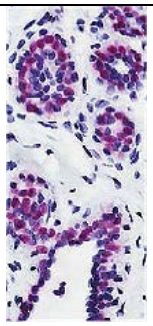
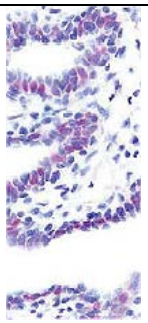
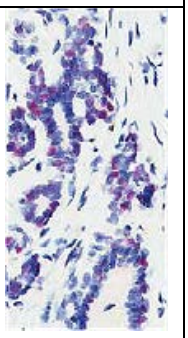
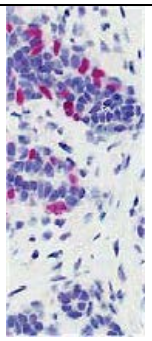
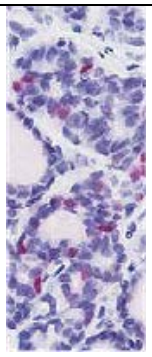


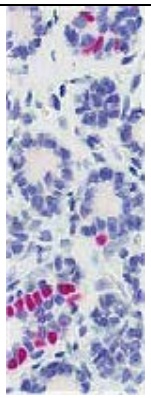
FIGURE 6.—Progesterone receptor (PR) expression in mammary gland of beagles in anestrus (A), estrus (B), diestrus I (C), diestrus II (D), diestrus III (E), and diestrus IV (F). Magnification 400×.

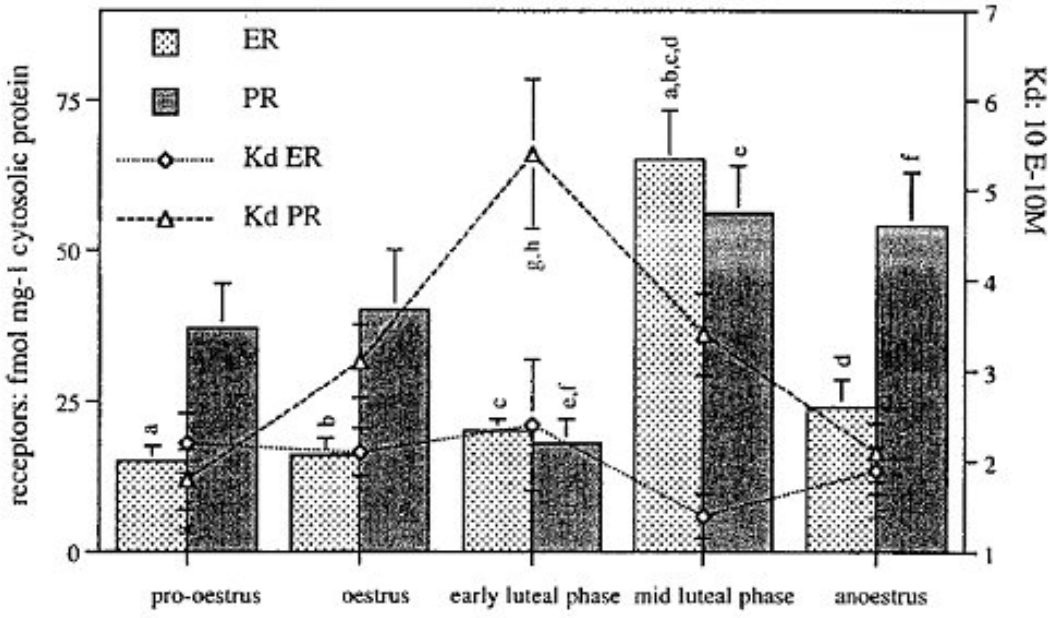
Original photographs of the immunohistochemical staining of PR. (Chandra 2010)

Appendix 2

Summary of PR expression during the canine estrous cycle

References	Localization	P	E	D	A
Chandra 2010 (dogs)	-Nuclear -Mainly acinar epithelium -Ductal epithelium to lesser extent	No data	+++	I: +++ II: ++ III: + IV: +	+
	Example*	No data	 1	 2	 6
				 3	
				 4	

References	Localization	P	E	D	A
				 5	
Rehm 2007 (Donnay 1995) (dogs)	No data	low	low	-Lower at beginning of luteal phase -Peak in mid-diestrus	Remaining elevated



Donnay 1995

Table 1 Overview of data obtained from two articles investigating the PR expression during the different phases of the estrous cycle of dogs.
*= this is an outtake of the original photograph. The original photographs can be found in Appendix 1. 1= estrus 2= diestrus I 3= diestrus II 4= diestrus III 5= diestrus IV 6= anestrus (all Chandra 2010). Semiquantative score of the immunoreactivity of the staining, scoring as follows: (+) = weak, (++) = moderate, (+++) = strong. (Chandra 2010) As can be seen Chandra 2010 found PR expression to be highest during estrus and diestrus I. In diestrus II, expression was already declining. Notable differences in the report of Rehm 2007 (reproducing the results of Donny 1995) are the maximal value of PR expression during the mid luteal phase, instead of during the estrus and diestrus I, and only a small decline of expression during anestrus. However, the results of Donnay 1995 have not been obtained by immunohistochemistry, the results of Chandra 2010 have. This may help explain the differences. Donnay 1995 also investigated the dissociation constant (Kd) of PR. The smaller the dissociation constant, the more tightly bound the ligand is. So Kd depicts the affinity between the ligand and the receptor.

Appendix 3

Estrous cycle

Progesterone as well as estrogen regulate the expression of PR. As a result of this, it's important to keep in mind that fluctuations of these hormones during the estrous cycle affect PR expression within an individual. Also, differing estrous cycles between different species, can be expected to have an effect on PR expression. The 3 most discussed species in this report (mouse/rat, human and dog) have significantly different estrous cycles, which will be described here in order to be able to compare them.

Canine

The dog is monoestric. In general the estrous cycle is subdivided into the anestrus, proestrus, estrus and diestrus. Rehm 2007, based on behavioral signs also uses metestrus, a phase following ovulation, when luteinization progresses towards diestrus. (Rehm 2007) (figure 1)

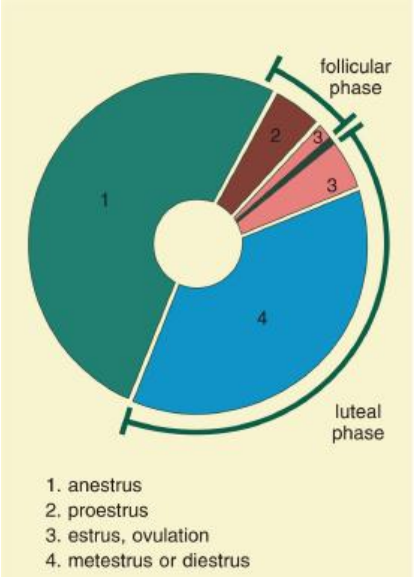


Figure 1 Estrous cycle dogs. The proestrus and a part of the estrus together constitute the follicular phase. Early in estrus, ovulation and early luteinization occurs. During diestrus (range 2-3 months) fully functional corpora lutea are present. Diestrus is followed by anestrus, which is highly variable in duration, lasting from 3-5 months. (Rehm 2007) Image from:
Schaefers-Okkens AC, Kooistra HS. Ovaries. In: Clinical Endocrinology of Dogs and Cats. Rijnberk A, Kooistra HS (Eds). Hannover, Schlütersche, 2010, pp. 203-234.

The dog has a unique estrous cycle among mammals. Characteristic of the canine estrous cycle is the long, progesterone dominated, luteal phase, which is similar in duration and hormonal profile (except for changes associated with parturition) in pregnant and non-pregnant bitches. A corpus luteum, secreting progesterone as it would in a pregnant bitch, is present in the ovary of the non-pregnant bitch during a period that is comparable to that in pregnant dogs (approximately 75 days). This is in contrast to most other mammalian species. (Chandra 2010, Kooistra 2002, Rehm 2007) Progesterone as described above has an important role in proliferation of the mammary gland. (Chandra 2010)

Progesterone levels stay low during early proestrus, but slowly start to rise during late proestrus and early estrus, rise faster during estrus and peak approximately 10 days after the LH peak. (Rehm 2007) During the second half of the luteal phase (mid to late diestrus) plasma progesterone levels decline. (Kooistra 2002, Rehm 2007) This decline promotes mammary gland differentiation through a change in the release of prolactin. (Rehm 2007)
Estrogen levels (in the form of estradiol) start to rise in early proestrus and peaks during the last 2 days of proestrus. Levels start to decline before the start of estrus and decline rapidly to reach basal levels approximately 5 days after the LH peak. Levels start to rise again in early diestrus and remain elevated during the luteal phase. In late anestrus, 1-2 weeks before the start of proestrus, estrogen levels start to rise again which happens in response to LH and marks the start of a new estrous cycle. (Rehm 2007) (Figure 2)

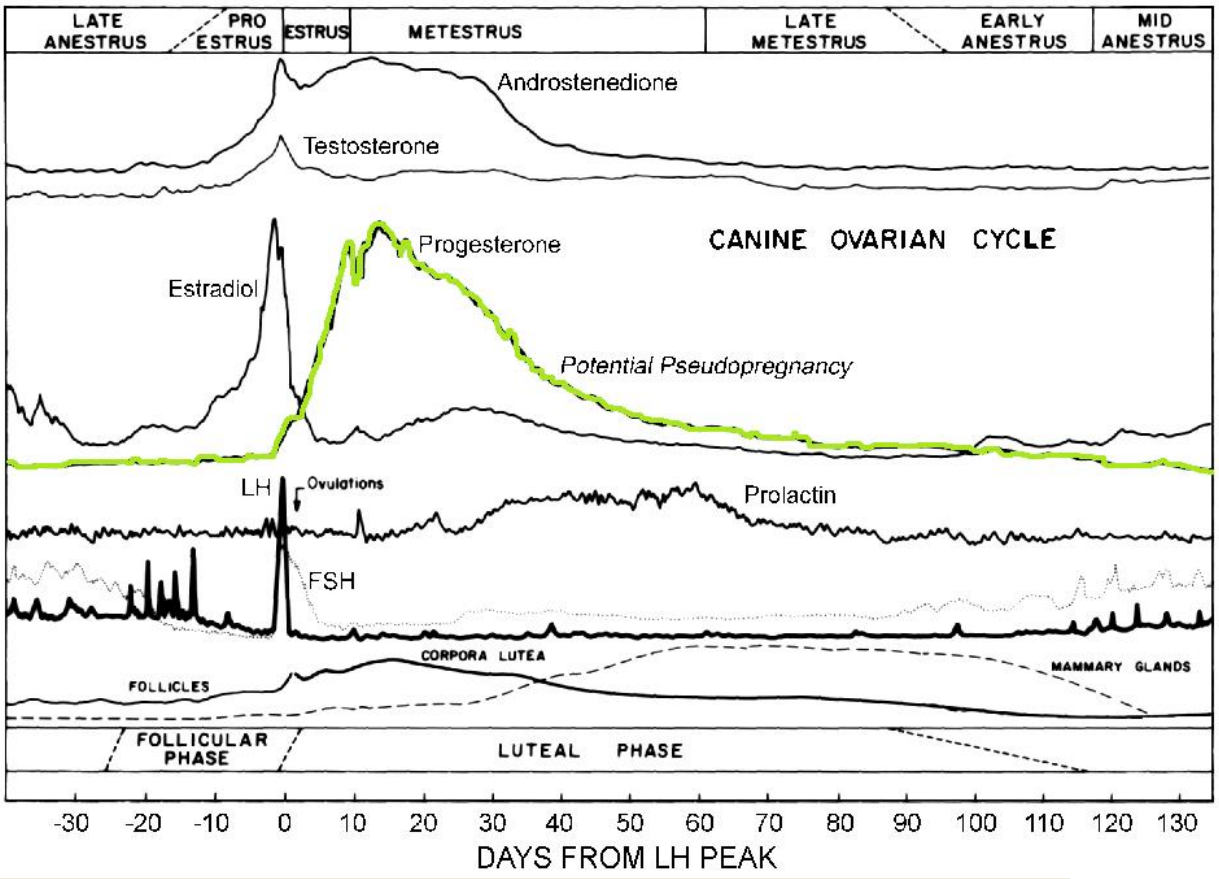


Figure 2 Overview of the hormonal changes during the canine estrous cycle. Changing hormone levels of progesterone and estrogen do affect mammary gland morphology and PR expression. In this figure a rise in progesterone can be seen before ovulation has occurred; this is the result of preovulatory luteinization. Progesterone continues to rise and reaches peak concentrations during early diestrus, approximately by 10 days after the LH peak. These high levels of progesterone remain for 25-30 days. This is followed by a slow decline over a 4 to 8 week period. (Concannon 2011, Rehm 2007) Note that in this figure a decline of estrogen from mid diestrus to late diestrus can be seen, which is contradictory with the article of Rehm 2007 which mentions a rise in early diestrus after which estrogen levels are supposed to remain elevated during the luteal phase.
Concannon, P.W. (2011) Reproductive cycles of the domestic bitch *Animal reproduction science* 124(3-4), 200-210.

The increasing progesterone levels in diestrus stimulate the synthesis of mammary gland growth hormone (GH) and it's receptor (GHR). GH and GHR promote

mammary gland proliferation in the dog and are considered to be key factors in hyperplasia and neoplasia of the mammary gland. (Rehm 2007)
The declining progesterone levels at mid to late diestrus increase the release of prolactin. Prolactin promotes differentiation of the mammary gland and is an inducing factor for mammary ER and PR. (Rehm 2007)

Rodent

The estrous cycle of the rodent is short, on average 4-5 days in rats and 5 days in mice. The estrous cycle can however be influenced by environmental conditions, such as illumination and presence/absence of males. Rats start cycling at an age of 32-36 days on average and will remain cyclic until approximately 10-12 months of age. (Chandra 2010, Goldman 2007)
In rodents, estrogen reaches its peak concentration during mid-proestrus before the LH surge. At late proestrus the progesterone level starts to rise and reaches its peak during the transition from proestrus to estrus. Ovulation occurs approximately 10-12 hours afther the LH surge, typically during the early morning hours of estrus. (Bagamasbad 2011, Goldman 2007) (Figure 3)

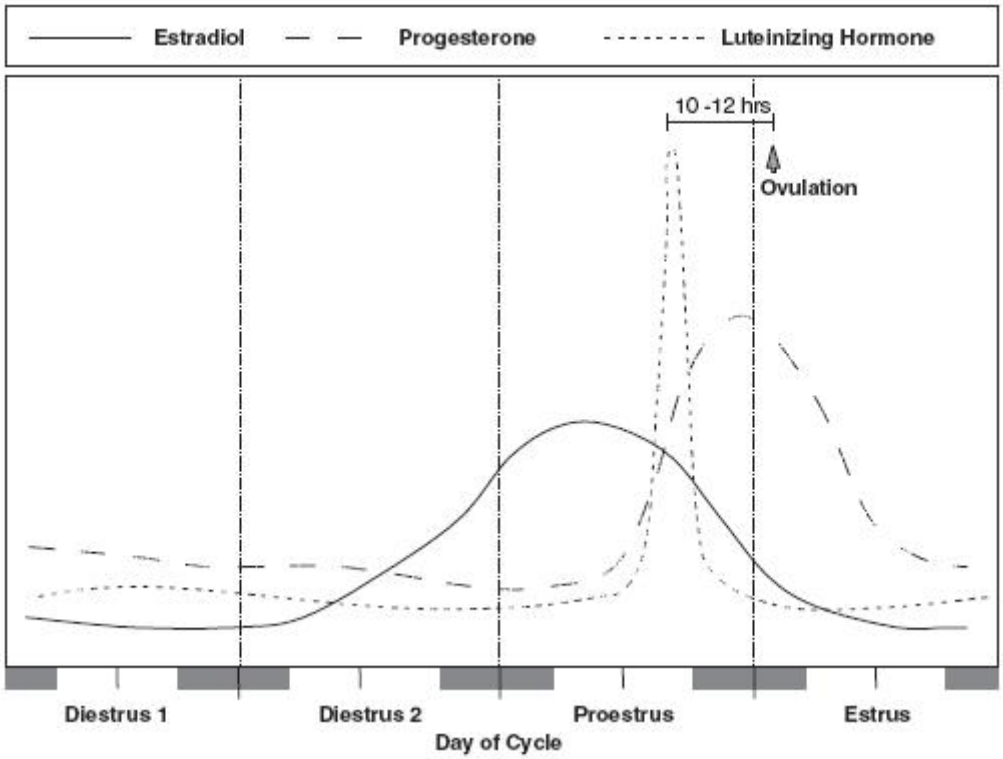


Fig 3: estrous cycle of the rat of 4 days duration. Estrogen reaches its peak value at mid-proestrus, progesterone at the transition of late proestrus to early estrus.The LH surge occurs between the estrogen and progesterone peaks. After the LH surge it takes 10 to 12 hours for ovulation to occur. (Goldman 2007)

Human

In humans the estrous cycle is more commonly called the menstrual cycle. On day 1 of the menstrual cycle menstrual bleeding occurs. This marks the first day of the follicular phase. The bleeding is a result of the decreased levels of estrogen and progesterone at the end of the previous cycle. At this time a slight increase in FSH occurs, which stimulates the development of several ovarian follicles. Once the level of FSH decreases only 1 of the follicles will continue to develop and produces estrogen. (Merck)

The ovulatory phase ranges from approximately day 13 to day 15 and starts with a surge in LH and FSH levels. 16-32 hours after the start of the LHsurge, ovulation can be expected. During the LH surge, estrogen levels peak and progesterone starts to increase. After the ovulatory phase comes the luteal phase, characterized by decreasing LH and FSH levels, increasing progesterone levels and a high estrogen level. (merck) In the luteal phase of the menstrual cycle, when progesterone levels are high, cell proliferation occurs. (nature) If there is no fertilization, the corpus luteum degenerates and stops producing progesterone, and estrogen levels also decline. (merck) (Fig 4)

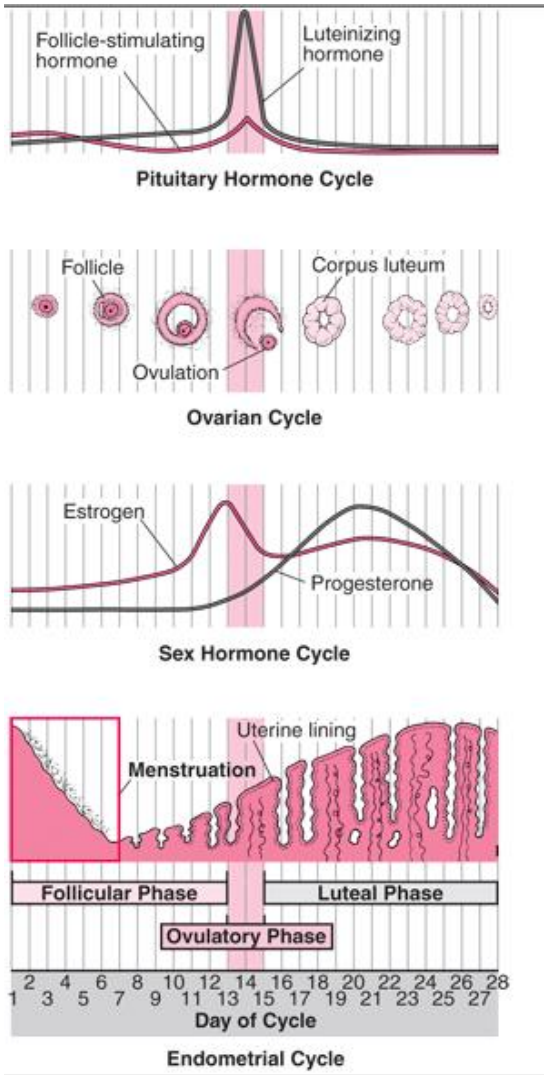


Fig 4: overview of the human menstrual cycle. The cycle is subdivided in a follicular, luteal and ovulatory phase. At the beginning of the follicular phase estrogen and progesterone have reached their minimum value. Estrogen rises to reach its maximum value at the transition from the follicular phase to ovulatory phase. Progesterone rises more slowly and reaches its peak value halfway the luteal phase. At the beginning of the follicular phase FSH slightly rises, then drops again and finally rises again to reach its peak during the ovulatory phase. The LH surge reaches its peak around the same time, only being much higher. Ovulation occurs 16 to 32 hours after the start of the LH surge. The ovulatory phase is followed by the luteal phase, where LH and FSH decrease while progesterone increases and estrogen remains high.

Appendix 4

Normal mammary gland/ breast morphology of the dog, rat/mouse and human

The mammary gland is composed of the epithelium and the stroma. The epithelium is bilayered; luminal cells (inner layer), are surrounded by basal cells (outer layer). Of these basal cells some are thought to be progenitor cells (the source of both luminal and myoepithelial cells), while the spindle shaped cells are called myoepithelial cells. (Beleut 2010, CSH perspectives) The basal lamina separates the epithelium from the stroma. The stroma is made up of several components including; fibrous connective tissue, fibroblasts, adipocytes, endothelial cells and innate immune cells (macrophages and mast cells). The stroma supports the epithelium through the delivery of nutrients, blood and immune defenses. (CSH perspectives)

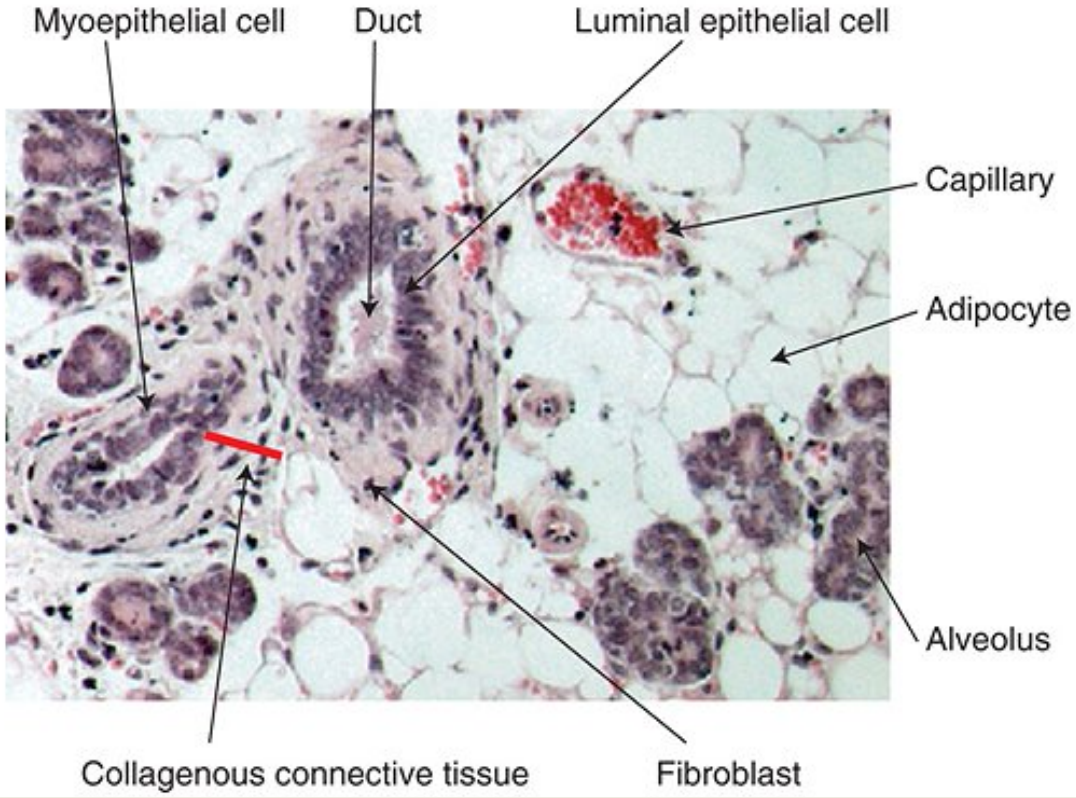


Figure 5 Histologic section of ducts and alveoli during early pregnancy. The ducts are surrounded by a thick layer of collagenous connective tissue, the alveoli are not. The bilayered epithelium, composed of luminal epithelial cells and myoepithelial cells is shown. The stroma is made up of connective tissue, fibroblasts and adipocytes. (CSH perspectives)

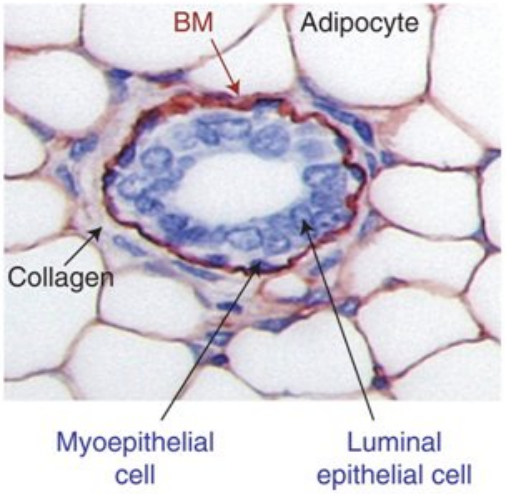


Figure 6 Basement membrane. Duct stained with an antibody to laminin to visualize the basement membrane surrounding the ductal epithelial cells. (CSH perspectives)

The morphology of the mammary gland changes in response to the ovarian hormones, so a different morphology can be seen in different stages of the estrous cycle. In the rat and human these changes in morphology are mild, while in canines there is a wide range of what is considered a normal histological appearance. This makes the morphology of the canine mammary gland unique among laboratory animals.

In mice a rudimentary mammary epithelial structure is present at birth, which then develops further to a mammary gland in 2 distinct growth phases. The first one occurs at the onset of puberty. At this time estrogen and growth factors regulate proliferation of the TEBs resulting in ductal elongation and branching. The second growth phase occurs at the onset of pregnancy at which time progesterone and prolactin stimulate epithelial proliferation, increased side branching, and differentiation into the milk producing tissue during lactation. In rats and mice, pregnancy is required for complete lobuloalveolar differentiation. (Aupperlee 2007, Beleut 2010, Conneely 2003 (2)) In between these two growth phases (adulthood), the virgin gland is relatively quiescent; however minimal side branching and alveolar development in response to cyclic changes in estrogen and progesterone secretion does occur. At weaning, apoptosis and remodeling occurs, resulting in involution of the lobuloalveolar system. The mammary gland then resembles the prepregnant mammary gland again. (Beleut 2010, Conneely 2003 (2))

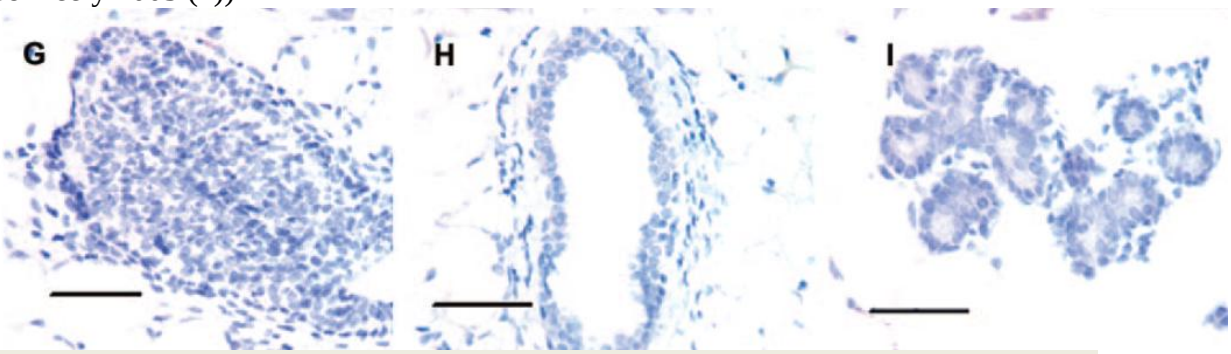


Fig7 mice Morphology of the mammary gland in the mouse. In photograph G the mammary gland of a 6 week old mouse is shown. It is composed immature end buds. Photograph H shows the mammary gland at 12 weeks of age (mature mammary gland) composed mostly of ducts, and finally, photograph I shows the pregnant mammary gland, the mammary gland being composed mostly of alveoli. (Aupperlee 2005)

The canine mammary gland completely differentiates into lobuloalveolar tissue during the luteal phase, whether the dog is pregnant or not. In dogs, in contrast to what is seen in nonhuman primates, humans and rats, the amount of epithelial tissue differs in the different phases of the cycle. (Chandra 2010)

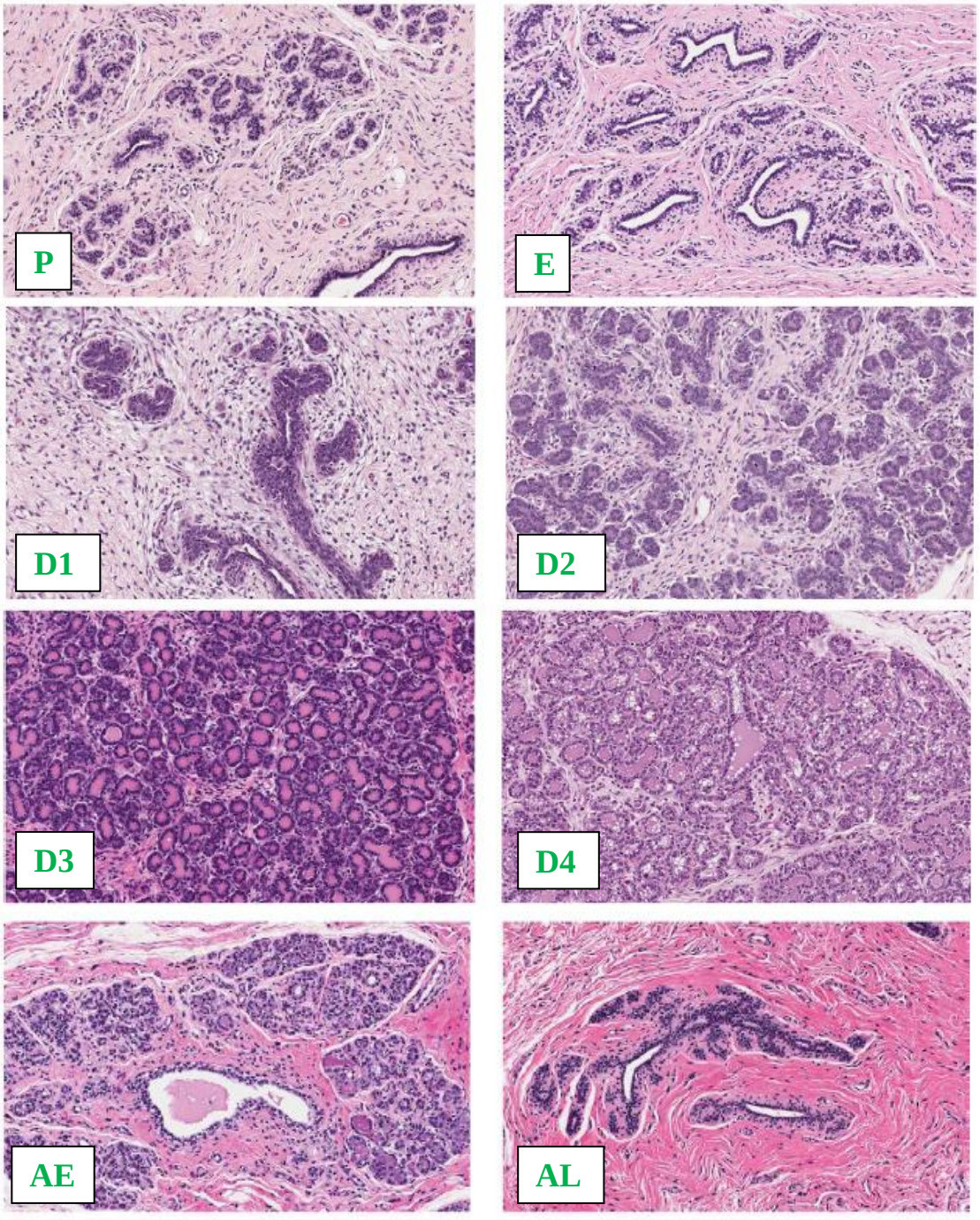


Fig 8 Physiological change of canine mammary gland morphology during the estrous cycle. P= proestrus, E=estrus, D1= diestrus 1, D2= diestrus 2, D3= diestrus 3, D4= diestrus 4, AE= early anestrus, AL= late anestrus. As can be seen the mammary gland tissue fully develops into lobuloalveolar tissue (D2, D3, D4) during the estrous cycle (Chandra 2010)

Nature however, in agreement with results of other morphologic studies, did find proliferative activity of the luminal epithelial cells in the luteal phase of human breast tissue cells. At the end of the cycle regression of the breast epithelium, through apoptosis, can be seen. The changes in lobuloalveolar development do appear to be significantly less obvious compared to the dog however. Nature divided the menstrual cycle in 4 stages; stage 1 (menstrual days 0-5), stage 2 (menstrual days 6-15), stage 3 (menstrual days 16-24), stage 4 (menstrual days 25-28).

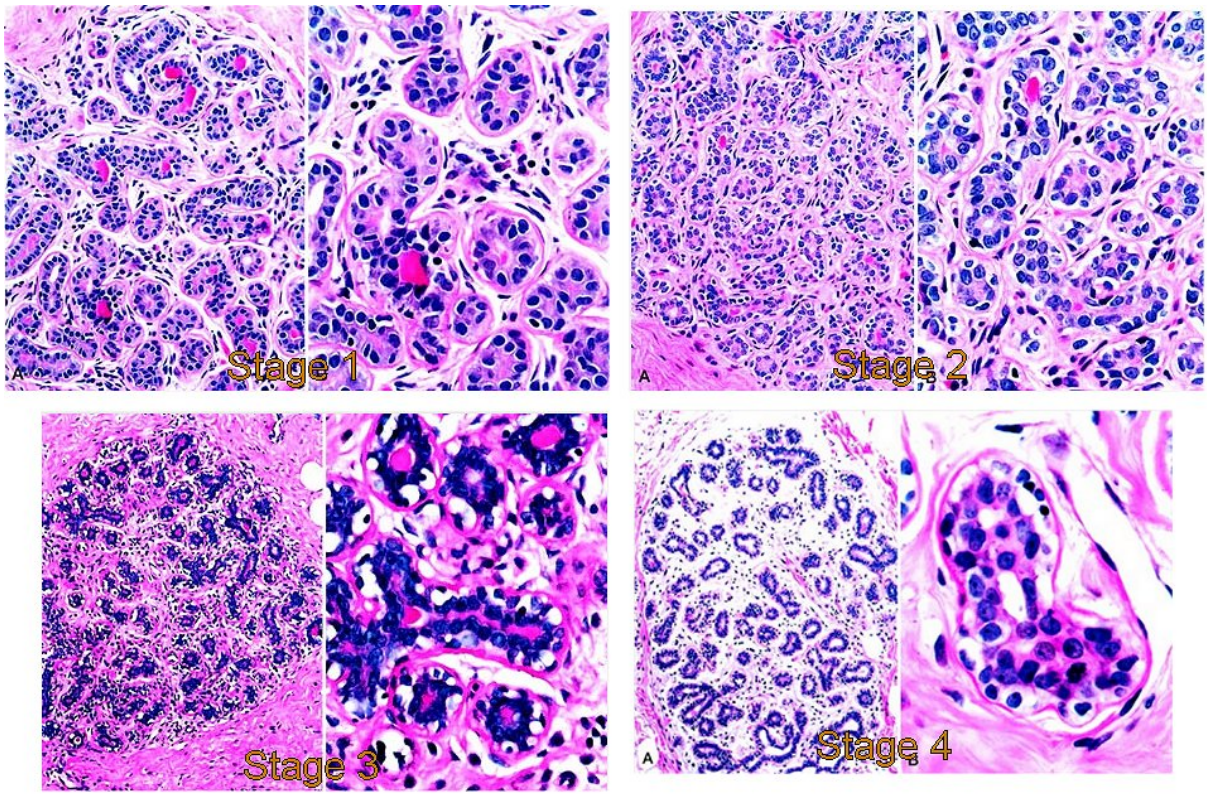


Fig. 9 Morphology of breast tissue during the menstrual cycle. During stage 1 the distinction between the epithelial and myoepithelial cells was not very clear. This changes in phase 2, where the distinction between the epithelial and myoepithelial layers of the acini becomes more obvious, so that clearly formed, double-layered alveoli can be seen within the lobules. Vacuolation in the myoepithelial layer is also present. In stage 3 larger lobules with increased number of terminal duct units were seen. These were lined with two distinct layers of cells. Again, vacuolation of the myoepithelial cells can be seen. In stage 4 the vacuolation within lobules is extensive. Frequent mitotic figures can be seen in the epithelial cells, but also an increase in apoptosis. (Nature)

Thus, in summary; in the non-pregnant mouse the mammary epithelium is mainly organized into ducts. In the adult (non-pregnant) human in contrast, the amount of lobules is higher. Mice and humans have in common that the morphological changes in response to the ovarian cycle are mild. In dogs the mammary gland completely differentiates into lobuloalveolar tissue during the luteal phase, pregnant or not. In dogs the amount of epithelial tissue differs strongly in the different phases of the cycle. Some changes can also be seen in the epithelium of the human breast during the menstrual cycle, but not as obvious as in the dog. In mice/rats, pregnancy is required for complete lobuloalveolar differentiation and only very slight differences in morphology of the gland as a result of the estrous cycle can be seen. (Aupperlee 2005, Chandra 2010, Nature)

Appendix 5

Changing mammary gland morphology during the canine estrous cycle

Chandra 2010 and Rehm 2007 investigated the change in morphology of the mammary gland during the estrous cycle in beagles. A summary of the results obtained by Chandra 2010 and Rehm 2007 is given here, because both articles have been used as a guideline in evaluating the histologic sections of the present study.

Proestrus (P):

*Gland inactive, mostly inactive ducts and stroma compact, leading to a relatively thin complex of skin and gland. No mitoses observed. Occasional apoptosis and hemosiderin pigment. (Chandra 2010)

*Atrophic glandular tissue is present. The ducts may be filled with eosinophilic secretions. The first proliferative changes may be observed in late proestrus. (Rehm 2007)

Estrus

Estrus(E)/Metestrus(M):

Metestrus=the later period of estrus after ovulation has occurred.

*Very similar to P. Edema of the stroma and/or periductal area's may be seen, although very slight and not consistent. While early proliferation during E has been described by other researchers this was not found in this research. (Chandra 2010)

*Proliferation of the ducts, alveoli and stroma can already be observed. The peri-epithelial connective tissue is edematous. Capillaries grow and extravasation of erythrocytes can be seen, as well as inflammatory cells. (Rehm 2007)

Diestrus

Diestrus I (DI):

*Start of proliferation; proliferation of the stroma; consisting mostly of fibroblasts around ductular structures. Extravasation of erythrocytes can be seen, as well as infiltration of lymphocytes though minimal. In the glandular tissue the formation of immature ducts was seen (formed by basophilic stratified epithelial cells) and the formation of structures that looked like terminal end buds (TEBs). In epithelial cells, mitoses were frequently present. In phase I there is still more stroma compared to glandular tissue. (Chandra 2010)

Diestrus II (DII):

*The stroma is less cellular. In the glandular tissue branching ducts and alveoli can be seen (early lobular development). In some of these alveoli an eosinophilic secretion can be seen. Compared to phase I, there were less mitoses and they were seen mostly in the acini. In phase II there is an equal amount of stroma compared to glandular tissue. (Chandra 2010)

Diestrus III (DIII):

*Characterized by large lobules composed of acini filled with eosinophilic secretions. The ducts also were distended because of the secretions. Mitoses in the acini were rare, as were apoptotic cells. In phase III the glandular tissue was abundant compared to the stroma. (Chandra 2010)

Diestrus IV (DIV):

Start of regression. variable morphology within the same dog as well as between different dogs. A difference in morphology within 1 histological section may even be observed. Early lobular atrophy can be seen in this phase and is characterized by a decrease in lobular size, an increase in connective tissue within as well as between lobules, different appearance of acinar epithelial cells and an increase in apoptosis. (Chandra 2010)

Early diestrus (DE):
*Mammary gland is still undifferentiated. Ducts and alveoli are rapidly growing. (Rehm 2007)

Later in diestrus:
*Maturation has occurred; well-developed secretory tissue can be seen. (Rehm 2007)

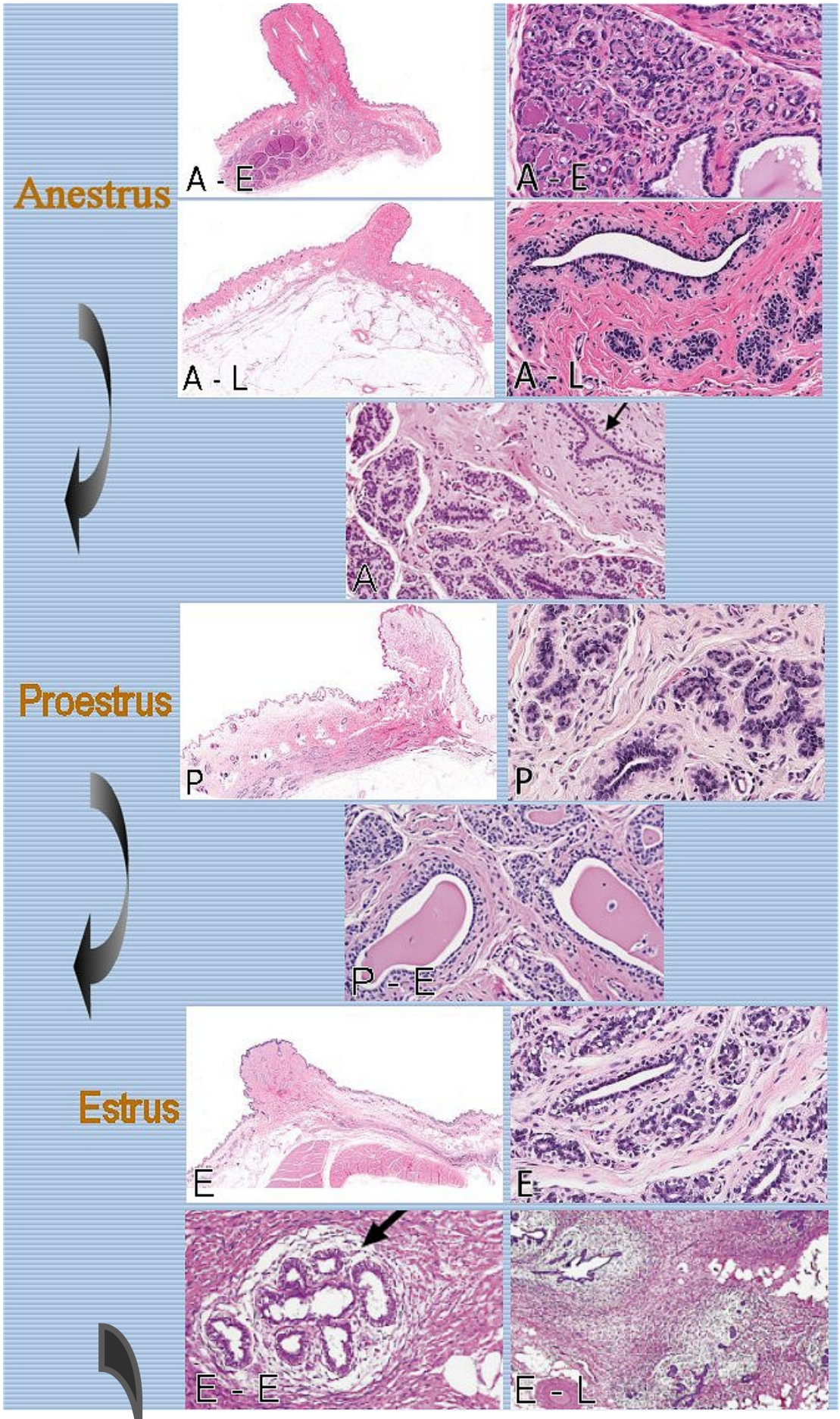
Late diestrus (DL):
Regression; apoptosis, declining size of alveoli, phagocytosis by macrophages of the secretum. (Rehm 2007)

Anestrus
Early anestrus (AE):
*Early anestrus still has distended ducts, and regression of the acini is not yet completed. Apoptosis of epithelium of the acini is frequently seen. So regression continues in anestrus. (Chandra 2010)

Late anestrus (AL):
*Collapsed ducts were present and the former lobular structure of the glandular tissue barely recognizable. Regression completed: inactive mammary tissue composed mainly of ducts, occasional alveolar remnants. Abundant connective tissue is present at this stage. Late anestrus is virtually similar to the morphology seen in proestrus and estrus. (Chandra 2010)

Anestrus
Results in mammary gland atrophy. Little alveolar structures remain and they surround ductular structures that are collapsed and shortened. Secretions inside these atrophic structures may still be visible. (Rehm 2007)

In summary, the major difference between both researches is:
According to Rehm 2007, the first proliferative changes may already be observed in late proestrus, while Chandra 2010 states that the mammary gland was inactive during late anestrus, proestrus and estrus. During diestrus (subdivided into diestrus I, II, III and IV) proliferation, differentiation and regression (in that order) were observed. (Chandra 2010).


Anestrus
A - E
A - E
A - L
A - L
A
Proestrus
P
P
P - E
Estrus
E
E
E - E
E - L

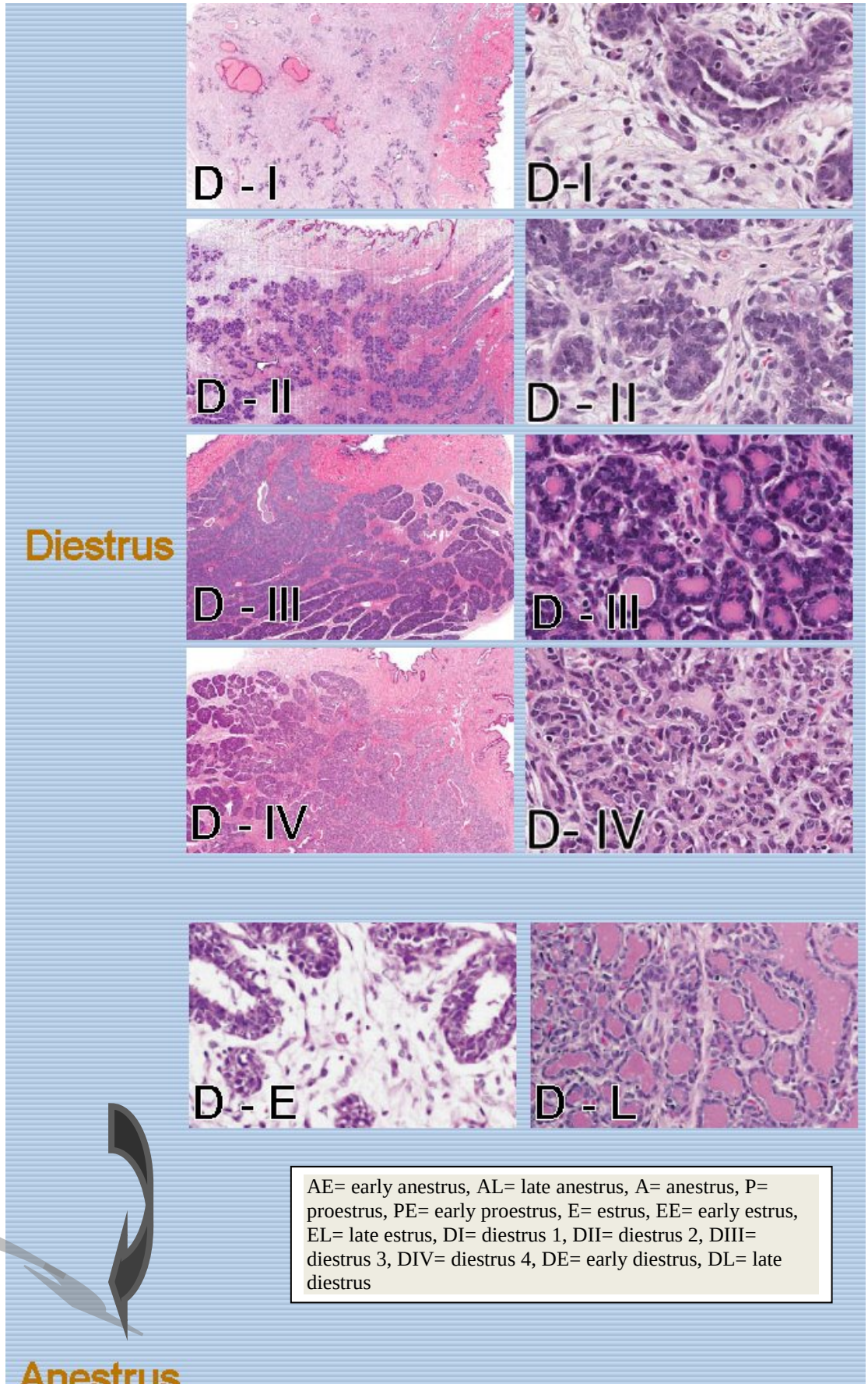


Figure 1 Overview of the morphological changes of the mammary gland in the course of the canine estrous cycle. Images obtained from Rehm 2007 and Chandra 2010