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Feline fibroadenomatous hyperplasia and mammary carcinoma

Evaluation of the expression of growth hormone, growth hormone receptor, IGF-I and the progesterone receptor

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Table of contents

Abstract 3
Introduction 4
Materials and Methods 7
  Origin of mammary tissue 7
  RNA isolation 7
  cDNA synthesis 7
  QPCR 8
  Immunohistochemistry (IHC) 9
Results 10
  QPCR 10
    1. The difference in gene expression between FAH and carcinomas 12
    2. The effect of progestins 13
    3. The effect of ovariectomy 14
    4. The influence of the breed 15
  Immunohistochemical findings 17
    Standardization of the technique 17
    Feline fibroadenomatous change 19
  QPCR data compared with the immunohistochemical results 27
    The effect of progestins 27
    Grading system 27
Discussion 29
Conclusion 32
Acknowledgments 33
References 34
Appendixes 37

Abstract

Previous research has shown that feline mammary hyperplasia is related to progesterone-induced mammary growth hormone (GH) expression. The aim of the current study is to evaluate the expression of GH, growth hormone receptor (GHR), insulin like growth factor 1 (IGF-I) and the progesterone receptor (PR) in fibroadenomatous hyperplasia (FAH) and mammary carcinomas of cats using QPCR analysis.
A downregulation of GHR (p= 0,008), IGF-I (p= 0,001) and PR (p= 0,000) was found in the group of animals with carcinomas compared to animals with FAH. The effects of progestin treatment or removal of the endogenous progesterone source by ovary(hyster)ectomy on the gene expression were also analysed. Animals with FAH who were treated with progestins showed an upregulation of GH (p= 0,028) in comparison to animals with FAH wich were not treated. Intact queens showed an upregulation of GHR (p= 0,013) and PR (p= 0,038). Finally the influence of the breed was analysed showing that Maine Coons had a higher expression of the reference gene β_2 microglobulin (B2M) (p= 0,026), GH (p= 0,044) and GHR (p= 0,029) in compared to all breeds combined.
Additional eight FAH tissues were immunohistological analysed for the progesterone receptor and compared to the QPCR data. All eight tissue samples were positive for PR, but they differed in location and intensity. The number of positive nuclei and their intensity was found to be the best way of comparing the IHC results with the QPCR data.
Striking finding was that the positive nuclei were bigger and rounder in animals who had received progestins. This is interesting since progesterone and progestins normally have an autorepressing effect on the progesterone receptor expression. Further research is necessary to determine why this occurs.
In conclusion can be said that hormones play an important role in the etiology of FAH considering the results found in this study.

Introduction

Mammary tumours, both benign and malignant, are a topic of great interest for veterinary and human medicine. In the Netherlands in 2008 13.005 women were diagnosed with breast cancer (Kankerregistratie Nederland) and it is the third most common cancer in felines (Overley et al. 2005).
The queen and woman have a special relationship when it comes to mammary carcinomas since studies have shown that feline mammary carcinomas can provide valuable information and even serve as a model for hormone independent human breast cancer (Burrai et al. 2010, Munson, Moresco 2007, Martin et al. 1984). But despite those studies more research is necessary to develop the knowledge on this topic.
Besides mammary carcinomas there is another condition known in the queen that could possibly provide more information about mammary hyperplasia and the role of ovarian hormones herein. This condition is known as fibroadenomatous hyperplasia (FAH). Ovarian hormones as progesterone and estrogens play an important role in the development of both FAH and carcinomas. Below a short description of the conditions in the cat is given.

Fibroadenomatous hyperplasia (FAH)
FAH is a benign proliferation of the mammary gland. It is a condition that is only found in cats and usually affects young, sexually intact queens.
The disease was first described by H. Allen in 1973 (Allen 1973). A rapid proliferation of ductal epithelial and stroma leads to the proliferation of one or more mammary glands. FAH is a hormone dependent condition and is therefore often seen in animals that are under endogenous progesterone influence (sexually intact, pregnant or pseudo-pregnant queens) or receive exogenous progesterone (for instance megestrol acetate (MA) or medroxyprogesterone acetate (MPA)). The exact mechanism by which progesterone evokes FAH is not clear.
The condition is treated with aglepristone, a progesterone antagonist. Other treatment could be necessary when the affected glands are infected, which is a common side effect.

Feline mammary carcinomas
Mammary tumours are the third most diagnosed tumours in cats and 90% are malignant. One or more mammary glands can be affected and metastasis is frequently seen. The average age of affected animals is between 10 and 12 years of age. Here also a hormonal component is found. Intact queens have a higher chance of developing tumours (Misdorp 1991). Queens in which the endogenous progesterone source is removed by ovary(hyster)ectomy before six months of age have a 91% reduction of developing mammary tumours compared with intact queens (Overley et al. 2005).
Here also the complete mechanism by which hormones contribute to the tumour development is not fully understood.

In this study four different genes encoding hormones or hormone receptors are investigated: growth hormone (GH), growth hormone receptor (GHR), insulin-like growth factor I (IGF-I) and the progesterone receptor (PR). To define why these four are chosen they are briefly discussed, as is their role in mammary growth and how they interact with each other (Fig. 1).

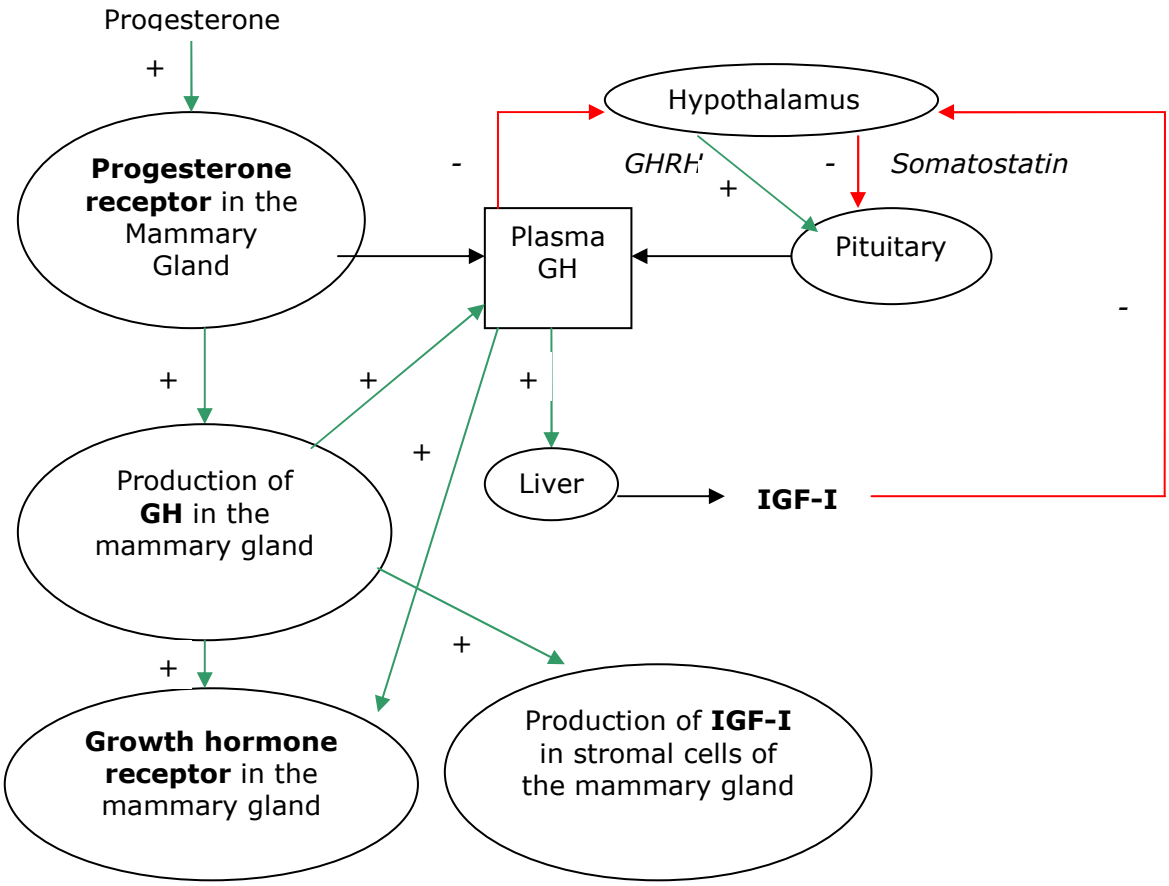

Figure 1 *Overview of the interactions between GH, GHR, IGF-I and PR. The green arrows are stimulating (+), the red arrows are inhibiting (-).*

Growth hormone (GH)
GH is synthesized, stored, and pulsatile secreted by the pars distalis of the feline pituitary gland. The release is stimulated by the GH releasing hormone (GHRH) and GH itself stimulates the release of IGF-I in the liver (Fig. 1). GH regulates cell growth and several aspects of metabolism.
GH mRNA is found in mammary tissue. The gene encoding GH in the mammary gland in the dog is identical to the pituitary-expressed gene and uses the same promoter (Mol et al. 2000). A difference between GH production in the pituitary and the mammary gland of the dog is that in the mammary gland Pit-1 is absent (Lantinga-van Leeuwen, Oudshoorn & Mol 1999). In the pituitary Pit-1 is an essential transcription factor that binds to the GH gene promoter. For instance a mutation of Pit-1 leads to deficiencies in mice and humans (Kooistra et al. 2000).
GH in the mammary gland is produced after activation of the progesterone receptor by progesterone. This local production of GH is necessary during pregnancy to prepare the mammary gland for lactation, but could also be part in the ethiology of FAH and mammary carcinomas in the cat.

Growth hormone receptor (GHR)
The GHR is a single-chain transmembrane receptor. The binding of GH leads to the activation of pathways that leads to growth of not only the targetcells, but also the surrounding cells.

Insulin like growth factor I (IGF-I)
IGF-I is mainly secreted in the liver after stimulation of plasma growth hormone. After binding to its receptor (type I IGF receptor) it plays a role in cell proliferation and apoptosis.
When stromal cell in the mammary gland are stimulated by (locally produced) GH they can produce IGF-I. By doing so local cell proliferation can occur.

Progesterone receptor (PR)
The PR is an intracellulair steroid receptor. It is activated by the binding of progesterone and is involved in the control of cell growth and proliferation. There are two isoforms of the progesterone receptor: PR-A and PR-B.
The amount of progesterone receptors is increased in FAH and some forms of carcinomas (Millanta et al. 2005b).
The aim of this study is to investigate the expression of GH, IGF-I, GHR and PR mRNA in FAH and feline mammary carcinomas. Additional the location of the PR in fibroadenomatous tissue is determined using immunohistochemistry and the QPCR data are compared with the immunohistochemical results.

Materials and Methods

Origin of mammary tissue

For this study mammary samples of 36 cats were used, 35 females and one male. The animals were presented at the Utrecht University Clinic for Companion Animals between 1987 and 2011. All cats had an abnormal growth of one or more mammary glands, with the exception of control cat 4. In total 35 tissues of mammary glands, one kidney sample and one ovarian and uterus sample were used. In appendix 1 the full background of the animals used in this study can be found.
The tissues were stored at -70°C for preservation. For the immunohistochemical analysis eight paraffin-embedded tissue samples were used.

RNA isolation

From each sample approximately 30 µg of tissue was used to obtain RNA. RNA isolation was done using the RNeasy Mini Kit according to the manufacturer's instructions. In short, the tissue was homogenized using 600 µL Buffer RLT. A DNase digestion step was included using a mix of 10 µL DNase I stock en 70 µL Buffer RDD. After this step buffer RPE was used and eventually approximately 30 µL RNA was obtained. The total amount of RNA obtained was then measured using the Nanodrop (appendix 2). Contamination of RNA with genomic DNA was verified using minus-RT controls in the QPCR.

cDNA synthesis

A concentration of 500 ng/ 15 µL RNA was required for the synthesis of cDNA. Samples 33-35 were excluded from the cDNA synthesis and further QPCR analysis due to the low concentration of mRNA. cDNA synthesis was performed in duplo using the reverse transcriptase and the iScript program.

Primer design and testing

The sequences of the cat were obtained from NCBI (http://www.ncbi.nlm.nih.gov/) and Ensembl (http://www.ensembl.org). At the time the sequence of IGF-I for the cat was not yet known, therefore a primerset that was originally developed for the dog was used.
The PR and GHR primers were created especially for the cat using Primer Select software. This was also done for GH, but despite positive QPCR results sequence analysis revealed that no usable product was formed. Therefore the decision was developed for canine GH was used. This means that both the IGF-I and GH primers were originally created for the dog, but also gave good results on feline tissue. Details of the primers can be found in table 1.

Table 1
Details of primers and their reaction conditions

Gene	Forward primer (*5'→3'*)	Reverse primer (*5'→3'*)	Amp (bp)	T_m (°C)
IGF-I	*TGTCCTCCTCGCATCTCTT*	*GTCTCCGCACACGAACTG*	122	60
PR	*CAATGGAAGGGCAGCATAAT*	*CAGCCTGGCAACACTTTCTAA*	111	57
GH	*CGAGGGACAGAGGTACTCCA*	*ACGACTGGATGAGCAGCAG*	143	63
GHR	*TCCCCAGGCCAAAAGAATAA*	*GCAGAAGTAGGCGTTGTCCAT*	105	59

Each primerset was optimised using tissue sample 12 previously shown to have high GH mRNA concentration (Mol et al. 1995).
First a gradient QPCR was performed to determine the optimum annealing temperature followed by a PCR to ensure that only one product was formed. An example of a gradient PCR result can be found in appendix 3. If only one line was visible in the PCR melting curve the product was sequenced to ensure that the right product was formed.
Figure 2 shows the very first PCR results. From here on the primerset for IGF-I (number 1 in figure 2) was further optimised and the GH primerset was replaced by another.

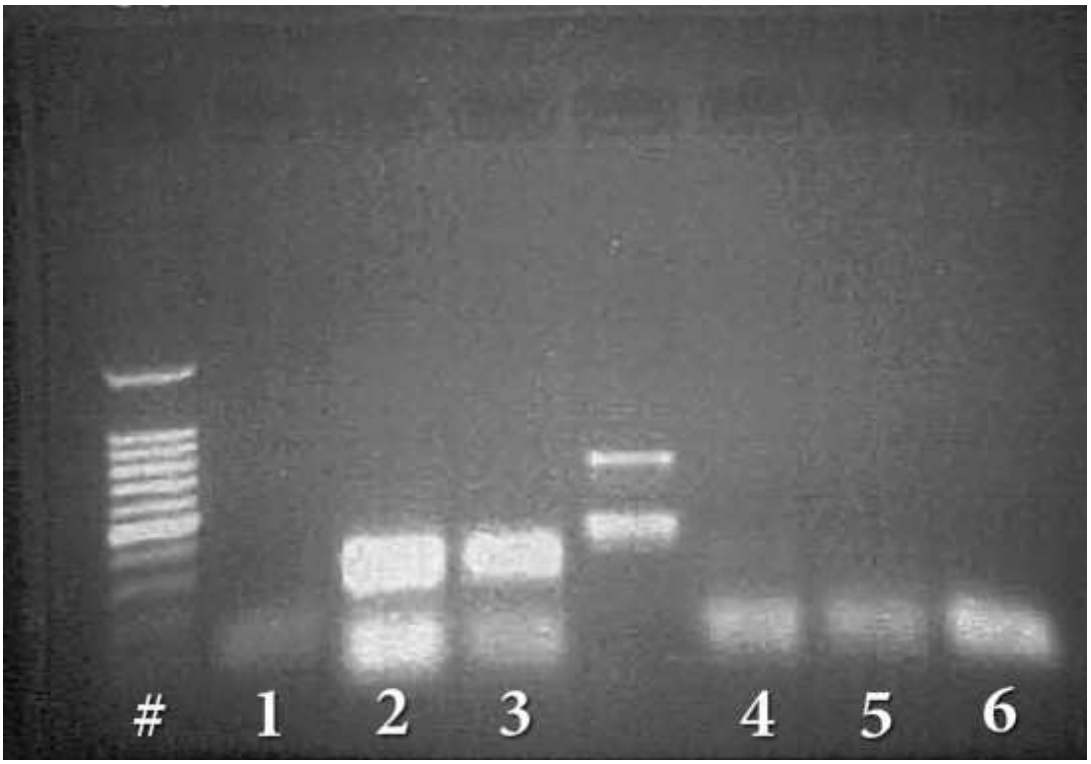

Figure 2 *PCR of all original primers. # ladder; 1 IGF-I; 2GH; 3 GH; 4 GHR; 5 GHR; 6 PR. Numbers 4 (GHR) and 6 (PR) were used in further analysis. Numbers 1-3 were further optimized.*

QPCR

To determine the gene expression in the QPCR the primers mentioned above were used. Six potential reference genes were selected from the database. Studies done by Penning et al. 2007 and Kessler et al. 2009 were taken into account. Both investigated the expression of several genes in feline tissue. Penning et al. investigated healthy mammary tissue (Penning et al. 2007), while Kessler et al. used different neoplastic tissues (Kessler et al. 2009). Since only one of the samples used in this study came from a healthy cat, genes from both studies were chosen.
As reference genes were used: beta-2-microglobulin (B2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyltransferase (HPRT), ribosomal protein L17 (RPL17), ribosomal protein L30 (RPL30) and ribosomal protein S19 (RPS19). The primers for these genes were created and tested in the study by Penning et al. (Penning et al. 2007). Details of these primers can be found in table 2.

Table 2
Details of primers of reference genes and their reaction conditions

Gene	Forward primer (5'→3')	Reverse primer (5'→3')	Amp (bp)	T_a (°C)	Fw exon	Rv exon	Genomic (bp)
B2M	*TTTGTGGTCTTGGTCC TGCTCG*	*TTCTCTGCTGGGT GACGGGA*	100	67	ex1	ex2	3187
GAPDH	*AGTATGATTCCACCA CGGCA*	*GATCTCGCTCCTGG AAGATGGT*	101	59	ex3	ex3/4	173
HPRT	*ACTGTAATGACCAGTCA ACAGGGG*	*TGTATCCAACACTTC GAGGAGTCC*	210	60	ex3/4	ex7	>12000
RPL17	*CTCTGGTCTTGAGCA CATCC*	*TCAATGTGGCAGGG AGAGC*	108	58	ex5	ex5	108
RPL30	*CCTCGGCAGATAAATTG GACTGTC*	*TGATGGCCCTCTGGA ATTTGAC*	111	64	ex4	ex4	111
RPS19	*TCATGCCCAGCCACTT TAGC*	*GAGGTGTCAGTTTGC GTCCC*	116	59	ex3	ex4	1538

Data gained from Penning et al. (Penning et al. 2007)

In total 32 samples were included in the QPCR analysis. From each sample 1.5 µL cDNA was used to create the sample pool for the standard line. Per target gene a mastermix was created and the plate was filled up and run in the QPCR. Details of the master mix, standard line and plate set up used in this study can be found in appendix 4. Analysis was performed with My-IQ software (Bio-Rad). Calculations to estimate expression stability and the pair wise variation were performed using geNorm. Statistical analysis was done using Rest-excel (http://www.rest.de.com).

Immunohistochemistry (IHC)

Eight formalin-fixed paraffin-embedded mammary tissue samples were used in the IHC. Part of the samples used in this study were collected before information was digital stored and therefore could not be found in the system (and were missing their pathology number in the written file). Others only had the pathological report without the pathological number that was needed to track down the paraffin-embedded tissue blocks in their digital files.
Due to the time limit the available tissue slides were only stained for the presence of the PR. The protocol used was optimized for the dog, but never used on feline tissue. Therefore canine mammary tissue, which was highly positive for the PR, was included as a positive control while the protocol was optimized for the cat. After this optimisation feline uterus and ovary were used as a positive control. The antibody against the PR (SC-539, Santa Cruz Biotechnology, Inc.) is a rabbit antibody.
The paraffin-embedded tissues were cut and transferred on to a slide. All slides were then first deparaffinized with xylene en rehydrated in decreasing concentrations of ethanol. Peroxidase-blocking was performed using 3% H_2O_2 in TBS for 15 min. Antigen retrieval was done using 50mM citrate buffer (pH 6.0). The slides were boiled in this solution for 20 min. and then left to cool down on the table for another 20 min. Nonspecific antibody binding was blocked using 10% normal Goat serum and 1% BSA for 30 min. at room temperature. Slides were then treated with the first antibody against PR, except for the negative control slide. The slides were incubated with a dilution of 1:1500 and kept overnight at 4°C. The second antibody (Envision (anti rabbit) K4003) was incubated for 30 min. and then washed off. The slides were then incubated with chromogen 3,3'-diaminobenzidine tetrahydrochloride (DAB) to visualize the immunoreactions. Finally they were covered with hematoxylin (Hematoxylin QS, Vector H3404) for five seconds, dehydrated and then mounted with Vecta mount (Vector H-5000). The complete protocol can be found in appendix 5.

Results

QPCR

Samples 9, 10, 11, 14, 15, 16, 18 and 25 showed unavailable data in one or more genes (reference as well as target genes). Due to the limited amount of time it was decided to exclude these samples from further analysis and carry on with the remaining samples (table 3).

Table 3
Details of all samples included in the QPCR analysis

Sample	Number	Tissue
1	GRK 10	Carcinoma
2	GRK 16	Carcinoma
3	GRK 21	Carcinoma
4	GRK 23	Normal
5	GRK 27	Carcinoma
6	GRK 28	Fibroadenoma
7	GRK 36	Carcinoma
8	GRK 40	FAH
12	K2R1	FAH
13	222492 P0208109	FAH
17	K01.1159.L P0113916	FAH
19	411283 P0411352	FAH
20	405166	FAH
21	401283 P0401311	FAH
22	306422	FAH
23	404392	FAH
24	301611 P0301833	Active mammary gland
26	503154	FAH
27	402312	FAH
28	300244 P0300337	FAH
29	302497	FAH
30	404020	FAH
31	406435	FAH
32	408631	FAH

geNorm
geNorm was used to determine expression stability and the pairwise variation of the reference genes. The pairwise comparison model of geNorm gives a combination of genes whose expressions are most correlated in the tested samples. The results indicated that the optimal number is three genes with a V2/3 value of 0,261 (Fig. 3). The cut-off value is 0,150, below this value the first number has to be taken, above it the second number. In this calculation the lowest result is given by V2/3 with 0,261. If this was lower than 0,150 two genes were enough, but now three reference genes have to be used.
The average expression stability of the genes showed that the least stable gene is B2M and the two most stable genes are RPL17 and RPS19 (Fig. 4).

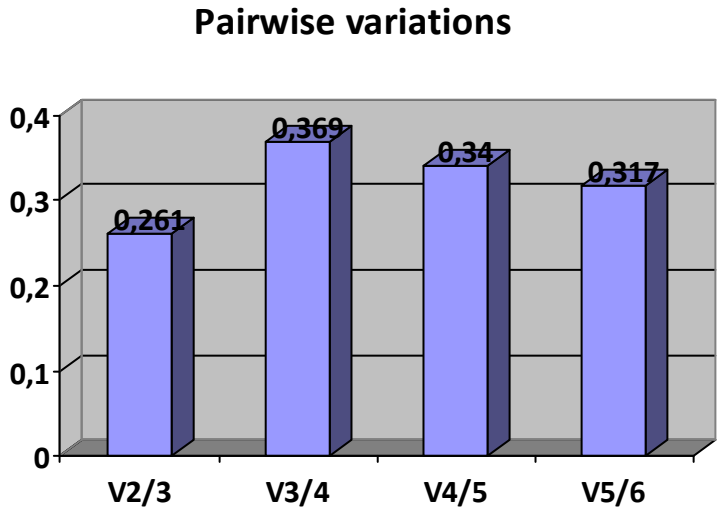

Figure 3 *Results of the pairwise variations test*

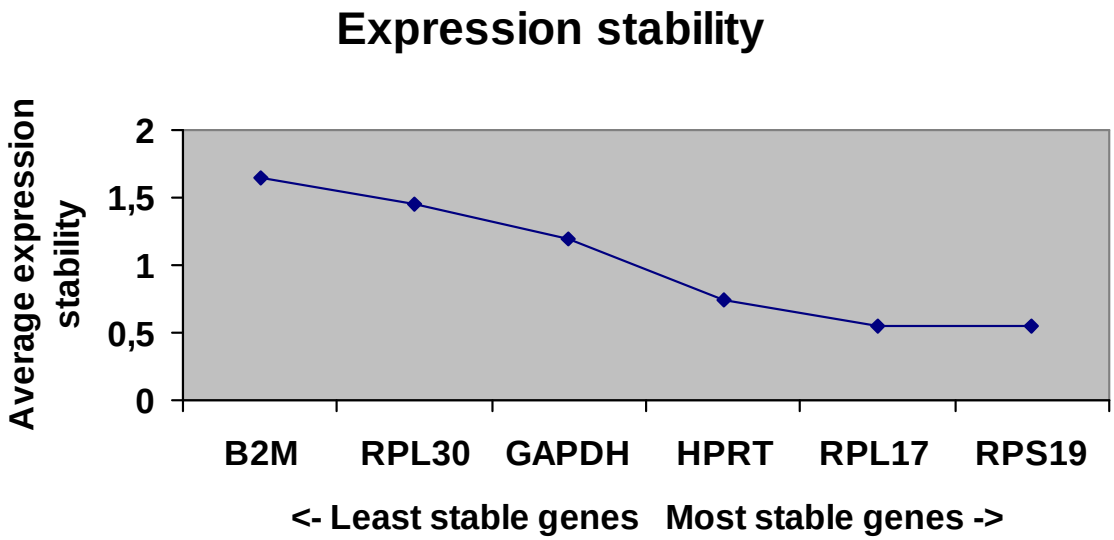

Figure 4 *Average expression stability values of control genes*

	B2M	GAPDH	HPRT	RPL17	RPL30	RPS19	Normalisation Factor
1	5,22E-03	6,71E-03	3,55E-02	1,16E-01	9,45E-02	3,91E-02	**0,3326**
12	4,50E-03	1,41E-02	1,00E+00	5,03E-01	2,11E-02	2,13E-01	**2,9100**
13	2,68E-02	5,10E-02	3,36E-01	2,22E-01	2,39E-02	1,73E-01	**1,4367**
17	6,57E-04	5,56E-02	2,72E-01	8,16E-02	1,53E-02	6,95E-02	**0,7078**
19	1,06E-02	2,15E-02	9,49E-02	7,31E-02	1,41E-02	4,16E-02	**0,4049**
2	1,78E-01	1,00E+00	5,39E-01	1,24E-01	2,82E-01	1,42E-01	**1,2985**
20	1,16E-01	7,44E-02	3,95E-01	1,95E-01	1,28E-02	1,47E-01	**1,3744**
21	2,14E-02	7,63E-02	1,73E-01	1,87E-01	1,87E-02	7,30E-02	**0,8155**
22	5,44E-03	3,81E-02	2,49E-02	2,07E-02	1,80E-03	1,49E-02	**0,1207**
23	2,52E-01	7,36E-02	6,90E-01	2,61E-01	2,24E-02	1,80E-01	**1,9533**
24	6,84E-03	6,70E-03	9,55E-02	1,30E-01	1,21E-02	3,57E-02	**0,4668**
26	1,47E-01	1,05E-01	1,73E-01	8,15E-02	1,14E-01	6,68E-02	**0,6001**
27	1,53E-01	5,74E-02	1,88E-01	8,07E-02	1,07E-01	5,69E-02	**0,5823**
28	4,20E-02	7,37E-02	2,54E-01	1,14E-01	1,34E-01	1,19E-01	**0,9239**
29	1,94E-03	1,60E-02	5,23E-02	3,33E-02	1,20E-01	2,32E-02	**0,2102**
3	4,54E-02	4,60E-01	4,63E-01	5,96E-01	7,03E-01	5,94E-01	**3,3503**
30	1,30E-01	2,32E-01	5,76E-01	2,07E-01	2,34E-01	1,81E-01	**1,7065**
31	2,06E-01	2,32E-01	7,55E-01	2,03E-01	2,96E-01	1,79E-01	**1,8469**
32	7,80E-02	1,78E-01	4,14E-01	1,79E-01	1,44E-01	1,24E-01	**1,2822**
4	5,76E-02	1,92E-02	4,83E-02	3,41E-02	3,74E-02	2,13E-02	**0,2006**
5	4,00E-01	5,30E-01	8,36E-01	1,00E+00	1,00E+00	1,00E+00	**5,7676**
6	2,15E-02	6,55E-02	2,49E-01	2,73E-01	2,22E-01	2,40E-01	**1,5522**
7	9,19E-02	5,07E-01	7,04E-01	6,20E-01	5,48E-01	5,98E-01	**3,9154**
8	4,52E-02	3,50E-01	4,98E-01	3,43E-01	3,72E-01	3,63E-01	**2,4251**
M < 1.5	**2,117**	**1,642**	**1,443**	**1,460**	**1,945**	**1,296**	

Figure 5 *Gene expression stability results using geNorm*

Three genes have a gene expression stability (M value) of < 1,5 (Fig. 5):

- RPL17: 1.460
- HPRT: 1.443
- RPS19: 1.296

This indicates that these three reference genes can be used in the analysis of the target genes. The three other genes were used as target genes in the further analysis with REST-excel.

REST- excel

The data obtained with the QPCR were analysed using the REST-excel program. There were four points of interest:

1. Carcinomas versus FAH
2. The effect of progestins
3. The effect of sterilization
4. The influence of breed

These four specific points were chosen because of the etiology of the diseases and their possible genetic background. Within each comparison the groups and the hypothesis are defined. Also the number of animals in each group is further specified.

1. <u>The difference in gene expression between FAH and carcinomas</u>

Group 1: animals with fibroadenomatous change
Group 2: animals with carcinomas

In group two all five animals with carcinomas are included. In group one 17 animals with FAH are included.
The animals with carcinomas had a significant lower expression of GHR, IGF-I and PR mRNA when compared to animals with FAH (Table 4). The almost 30-fold down regulation of GH mRNA just did not reach significance (p=0,057).

Table 4 *Gene expression of animals with carcinomas compared to animals with FAH*

GENE	TYPE	REACTION EFFICIENCY	EXPRESSION	STD. ERROR	95% C.I.	P VALUE
B2M	TRG	1.1417	0.475	0.093-6.204	0.00-69.828	0.633
GAPDH	TRG	1.0902	0.387	0.002-12.221	0.00-85.839	0.493
GH	TRG	1.1154	0.035	0.002-1.011	0.00-4.922	0.057
GHR	TRG	1.1599	0.038	0.027-0.0163	0.00-0.489	**0.008 DOWN**
HPRT	REF	1.0068	0.448			
IGF-I	TRG	1.1786	0.023	0.002-0.265	0.00-1.775	**0.001 DOWN**
PR	TRG	1.2599	0.006	0.000-0.092	0.00-1.201	**0.000 DOWN**
RPL17	REF	1.124	2.747			
RPL30	TRG	1.218	3.350	0.950-27.480	0.012-50.299	0.209
RPS19	REF	1.1416	0.813			

TRG: target gene; REF: reference gene

2. <u>The effect of progestins</u>

Group 1: animals which received no progestins
Group 2: animals which received progestins

Group two includes 14 cats. Table 5 gives an overview of the different progestins by which the animals were treated. Megestrol acetate (MA) was mostly prescribed to the animals as a contraceptive drug for queens that is commonly used in young animals.

Table 5 *Progestins used in group 2*

n (treated with progestins) = 14	
Name of medicine	**Number**
Medroxyprogesterone acetate (MPA)	3
Megestrol acetate (MA)	7
Tardak (anti androgen with progestin side effect given to a tomcat)	1
Melengestrol acetate (MGA)	2
Unknown	1

The progestin listed as unknown is Multado. The animal was treated with this medicine due to a skin condition. In the file it is listed as a progestin, but no further data of Multado can be found. Therefore it is listed as unknown.

Of all genes only GH showed a significant higher expression (Table 6).

Table 6 *Gene expression of animals which received progestins compared to animals who did not*

GENE	TYPE	REACTION EFFICIENCY	EXPRESSION	STD. ERROR	95% C.I.	P VALUE
B2M	TRG	1,076	5,270	0,563 - 26,191	0,039 - 4.868,264	0,062
GAPDH	TRG	0,9858	6,486	0,545 - 230,884	0,138 - 4.327,423	0,061
GH	TRG	1,1154	16,947	0,443 - 274,246	0,026 - 1.333.874,721	**0,028 UP**
GHR	TRG	1,1113	3,776	0,313 - 28,204	0,074 - 8.265,181	0,161
HPRT	REF	0,9114	1,460			
IGF-I	TRG	1,0384	3,208	0,160 - 66,986	0,019 - 737,362	0,220
PR	TRG	1,0123	2,136	0,048 - 89,217	0,002 - 20.407,371	0,577
RPL17	REF	0,99	0,465			
RPL30	TRG	0,9573	1,033	0,082 - 9,642	0,034 - 496,802	0,965
RPS19	REF	1,0491	1,473			

TRG: target gene; REF: reference gene

3. <u>The effect of ovariectomy</u>

Since the ethiology of carcinomas and FAH both include ovarian hormones the effect of removing the ovarian was analysed. Table 7 shows all the queens which were used in this study and their spaying background.

Table 7 *Spaying background of the queens used in this study*

n (queens) = 31		
	Number	**Percentage**
Sterilized	9	29.05%
Not sterilized	13	41.90%
Unknown	9	29.05%
Total	***31***	***100%***

All the animals with carcinomas in this study were spayed except for cat 2, where no data was available. Consequently no calculation could be performed within the group of animals with carcinomas. No information was available about the exact age of spaying as a result no judgments could be made about the relationship between the age of spaying and the development of mammary carcinomas.
When all the animals with FAH were compared no difference was found between the group that was sterilized and the group of animals that was not.
There was however a significant difference when all the animals were taken together (carcinomas and FAH). A significant higher expression of GHR (p= 0,013) and PR (p= 0,038) was found in the group of animals who were not sterilized (Table 8).

Group 1: animals which were spayed
Group 2: animals which were not spayed

Group one consist of nine queens, group two consist of eight queens. Animals with an unknown background were not included in the calculation.

Table 8 *Gene expression of animals which were intact compared to animals who were sterilized*

GENE	TYPE	REACTION EFFICIENCY	EXPRESSION	STD. ERROR	95% C.I.	P VALUE
B2M	TRG	1,076	3,136	0,172 - 20,513	0,032 - 4.813,928	0,339
GAPDH	TRG	0,9858	3,913	0,288 - 393,241	0,018 - 4.670,848	0,294
GH	TRG	1,1154	5,909	0,090 - 371,418	0,004 - 1.314.922,066	0,332
GHR	TRG	1,1113	9,089	0,725 - 30,637	0,296 - 58.422,191	**0,013 UP**
HPRT	REF	0,9114	1,380			
IGF-I	TRG	1,0384	6,906	0,422 - 110,080	0,027 - 961,843	0,065
PR	TRG	1,0123	19,383	0,364 - 551,419	0,043 - 293.499,537	**0,038 UP**
RPL17	REF	0,99	0,493			
RPL30	TRG	0,9573	1,322	0,103 - 13,018	0,043 - 540,917	0,767
RPS19	REF	1,0491	1,470			

TRG: target gene; REF: reference gene

4. <u>The influence of the breed</u>

It has been suggested the Siamese cats are predisposed to developing mammary tumours. In this study one Siamese cat with a carcinoma is included. From one animal with a carcinoma the breed was not known, therefore making the group too small to get a significant difference.

There were five Maine Coons represented in the FAH group. Two of them had the be excluded from QPCR analysis (cats 14 and 18), but an analysis could be performed with the remaining three samples (group two).

Group 1: all breeds except Maine Coons with FAH
Group 2: Maine Coons

A higher expression of B2M (0.026), GH (0.044) and GHR (0.029) was found (Table 9). This indicates that Maine Coons have a higher expression of GH and GHR. In this analysis only 3 Maine Coons were included. In order to make a statement about whether or not the breed is predisposed of developing FAH, healthy mammary tissue of Maine Coons has to be compared to mammary tissue of Maine Coons with FAH.

Table 9 *Gene expression of Maine Coons with FAH compared to other breeds with FAH*

GENE	TYPE	REACTION EFFICIENCY	EXPRESSION	STD. ERROR	95% C.I.	P VALUE
B2M	TRG	1,076	14,869	0,603 - 1.496,523	0,123 - 18.985,426	**0,026 UP**
GAPDH	TRG	0,9858	8,884	0,228 - 1.564,827	0,086 - 39.508,595	0,152
GH	TRG	1,1154	49,430	0,115 - 62.663,686	0,007 - 2.099.869,126	**0,044 UP**
GHR	TRG	1,1113	18,333	0,784 - 3.233,305	0,466 - 63.433,837	**0,029 UP**
HPRT	REF	0,9114	1,448			
IGF-I	TRG	1,0384	5,978	0,284 - 140,632	0,009 - 899,086	0,162
PR	TRG	1,0123	8,973	0,040 - 3.421,565	0,004 - 430.008,990	0,217
RPL17	REF	0,99	0,229			
RPL30	TRG	0,9573	5,392	0,437 - 108,155	0,114 - 1.588,073	0,089
RPS19	REF	1,0491	3,010			

TRG: target gene; REF: reference gene

Immunohistochemical findings

Standardization of the technique

- Positive control

During staining two highly PR positive organs were used as positive controls: ovary and uterus. Both tissues came from a six year old European Shorthair which was admitted to the clinic for reasons not related to either of these organs.
In the ovary most of the cells stained positive for the PR in their nuclei, cytoplasm or both. All the present follicles reacted, most positive staining was found in the cytoplasm of their cells (Fig.6). A present corpus luteum (not visible in fig. 6) was the slightest positive area of all present cells. In the stroma, theca interna and externa high numbers of positive nuclei and cytoplasm are notable.

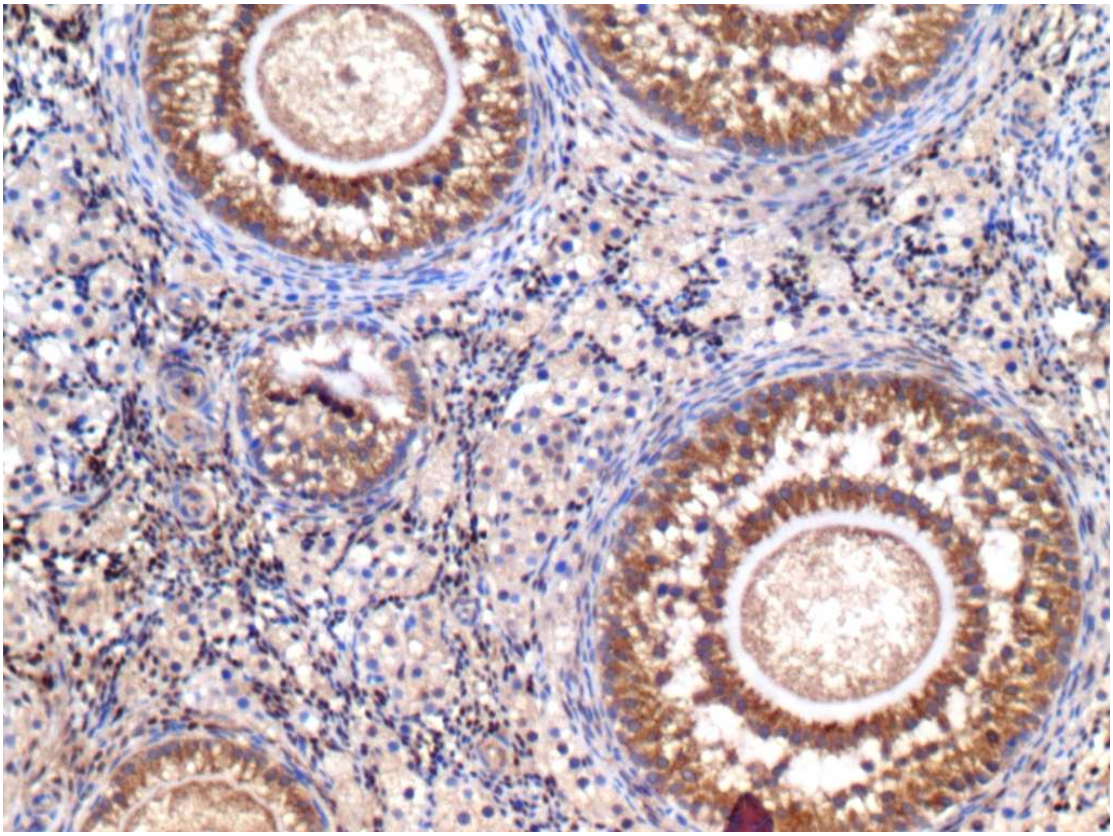

Figure 6 *Feline ovary (cat 37; 10x). Positive control PR expression.*

The other positive control that was used is part of an uterus. Another organ that is under progesterone influence. In the current study positive nuclei of epithelial and stoma cells in the endomytrium and myometrium, as well as positive cytoplasm was found. The cytoplasm of the epithelial cells of the endometrial glands and the contains of the endometrial glands were also positive (Fig. 7 and 8).

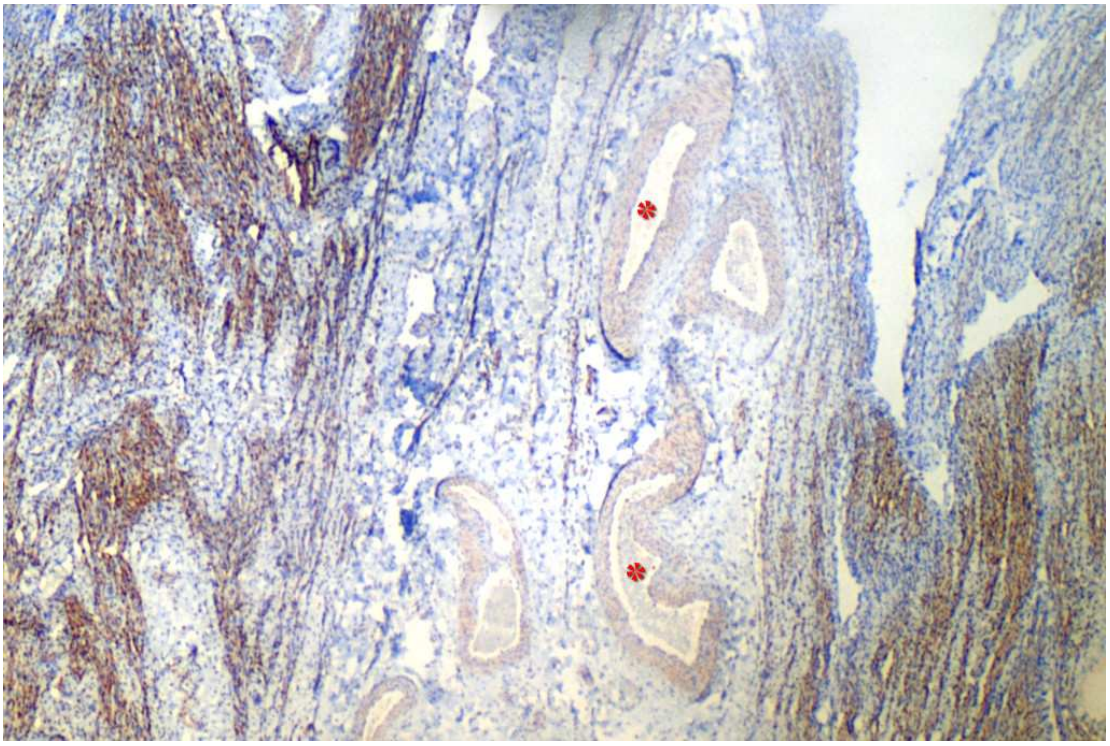

Figure 7 *Feline uterus (cat 37; 10x). Positive control for PR expression. The red* * *are placed in the lumen of some endometrial glands. Left and right lines of smooth muscle can be identified as the stand out against the bleu less positive/ non positive cells.*

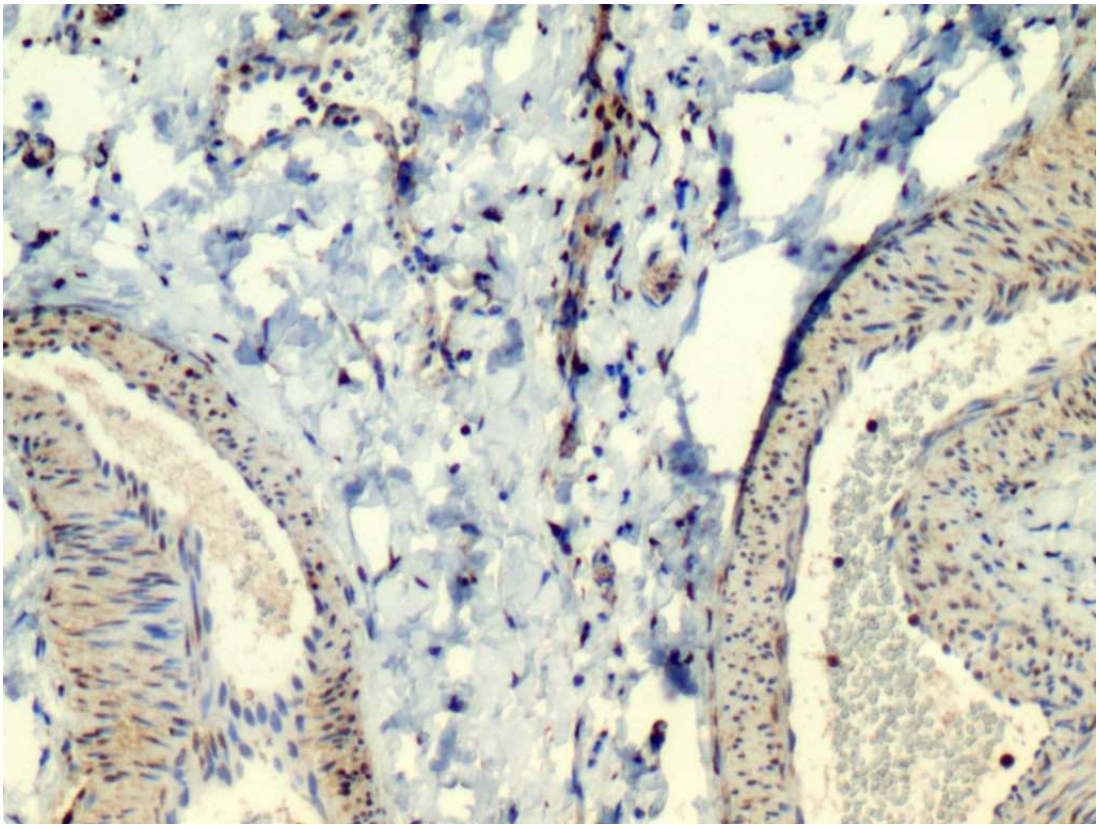

Figure 8 *Feline uterus (cat 37; 20x). Positive control for PR expression. Left and right lie endometrial glands from which the secrete also stained positive for the PR.*

- Negative control

Figure 9 shows an example of a negative control for the progesterone receptor staining. These tissue samples were not incubated with the SC-539 antibody. No brown staining is noticeable in the tissue (even with higher magnification), concluding that no reaction had taken place. This confirms that the antibody is specific for the PR and does not interact with other present substances.

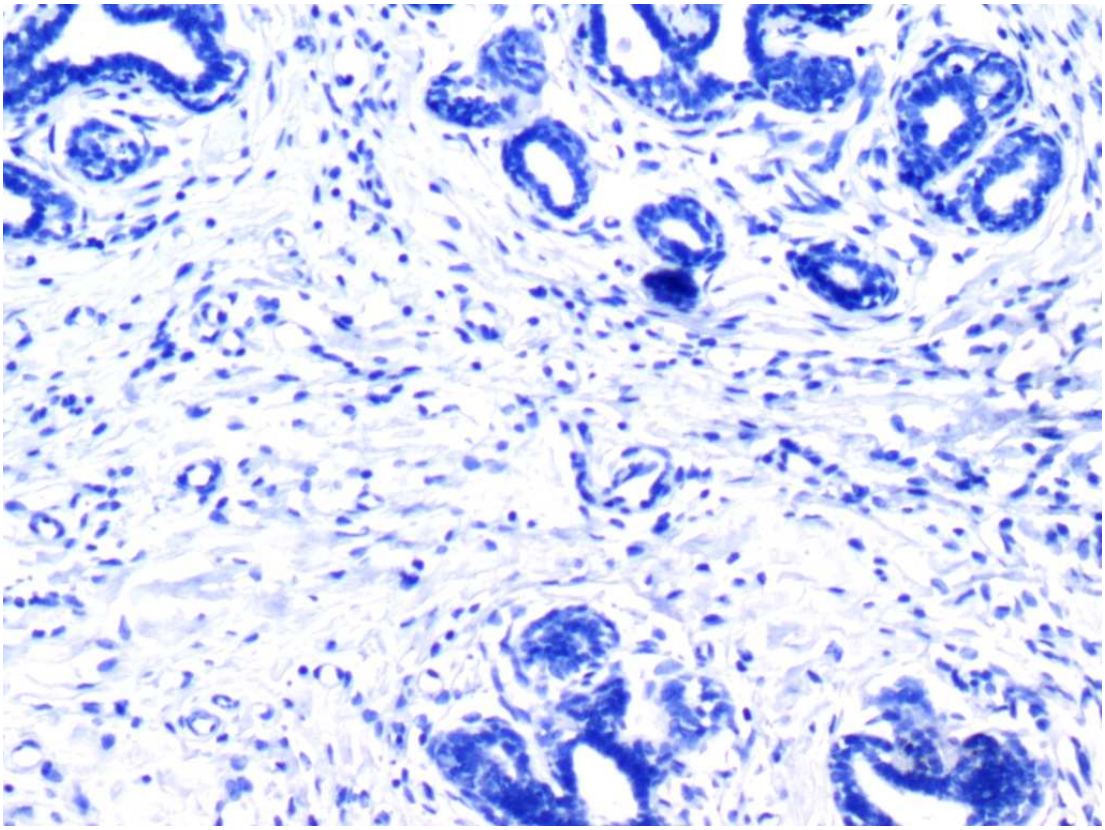

Figure 9 *Feline fibroadenomatous change in mammary tissue 10 x. Negative control*

Feline fibroadenomatous change

For the immunohistochemical analysis, eight tissues were used. The numbers of these tissues are: 15, 17, 18, 19, 21, 24, 28, and 34. Details of the animals can be found in appendix 1.

The slides are graded according to a four-tier grading system:

- 3+ : strong (>40%)
- 2+ : moderate (<40%)
- 1+ : faint (only a few cells)
- 0 : none

The nuclei, cytoplasm, stroma and secretion (if present) in the ducti are graded separately (Table 10).

Table 10 *IHC grading results*

Sample	Stroma	Cytoplasm	Nuclei	Secretum	Total	QPCR
Ovarian	3	3	3		9	
Uterus	3	3	3		9	
15	2	3	1	1	7	35.6
17	1	3	2	1	7	29.34
18	3	2	3		8	24.94
19	0	1	3		4	27.5
21	0	3	2		5	27
24	1	0	0	3	4	33.6
28	2	1	2		5	25.3
34	1	0	1	2	4	

The difference between the slides was the location and intensity of the positive reaction. Almost all tissues showed positive nuclei in the epithelial cells surrounding the ducts, although sample 15 was an exception. Here only a few positive nuclei can be found in the intermediate epithelial cells surrounding the ducts (Fig. 10), therefore a grading score of one was given. This tissue did show more convincing positive reactions in the cytoplasm of the epithelial cells (basal, intermediate and luminal) surrounding the duct and even in some stoma cells. Also some positive secretion was seen in some of the ducts.

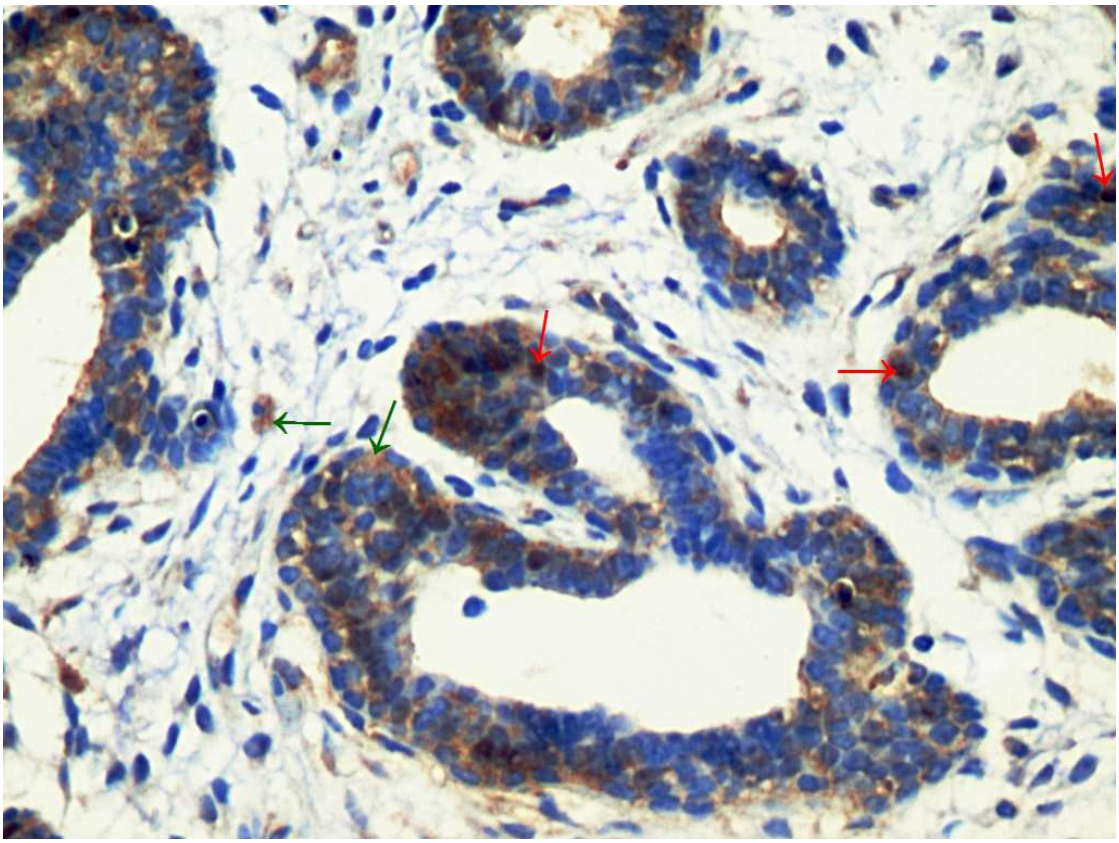

Figure 10 *(Cat 15; 20x). PR expression in feline fibroadenomatous change. The red arrows show positive nuclei, the green arrows show positive cytoplasm in epithelial (right) and stromal (left) cells.*

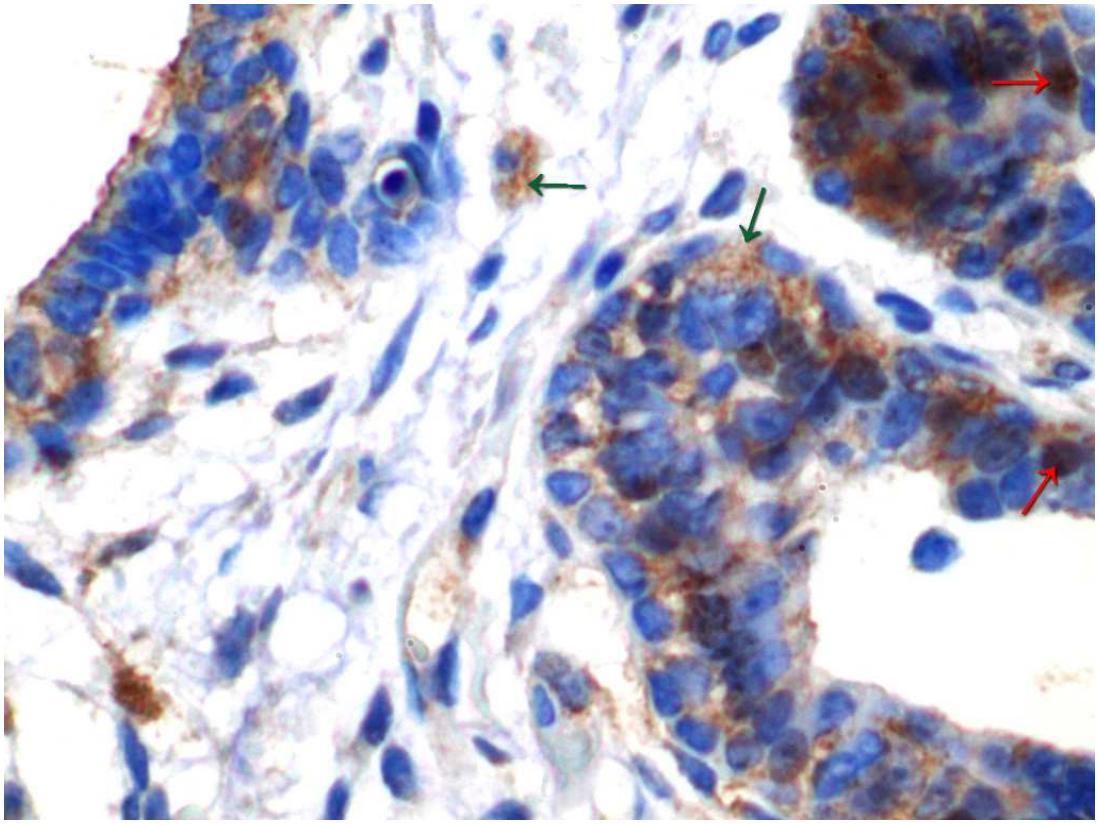

Figure 11 *(Cat 15; 40x). PR expression in feline fibroadenomatous change. The red arrows show positive nuclei, the green arrows show positive cytoplasm in epithelial (right) and stromal (left) cells.*

The nuclei of the epithelial cells of sample 18 scored the highest, not only in number, but also in colour (Fig 12). The deepest staining can be found in these nuclei. Almost all nuclei of the intermediary ductal epithelial cells were positive. These cells nearly all had positive cytoplasm as well. Besides the intermediary epithelial cells, the cytoplasm of the basal and some laminar epithelial cells was also positive. The stroma surrounding these highly positive areas had the tendency to stain positive for PR.

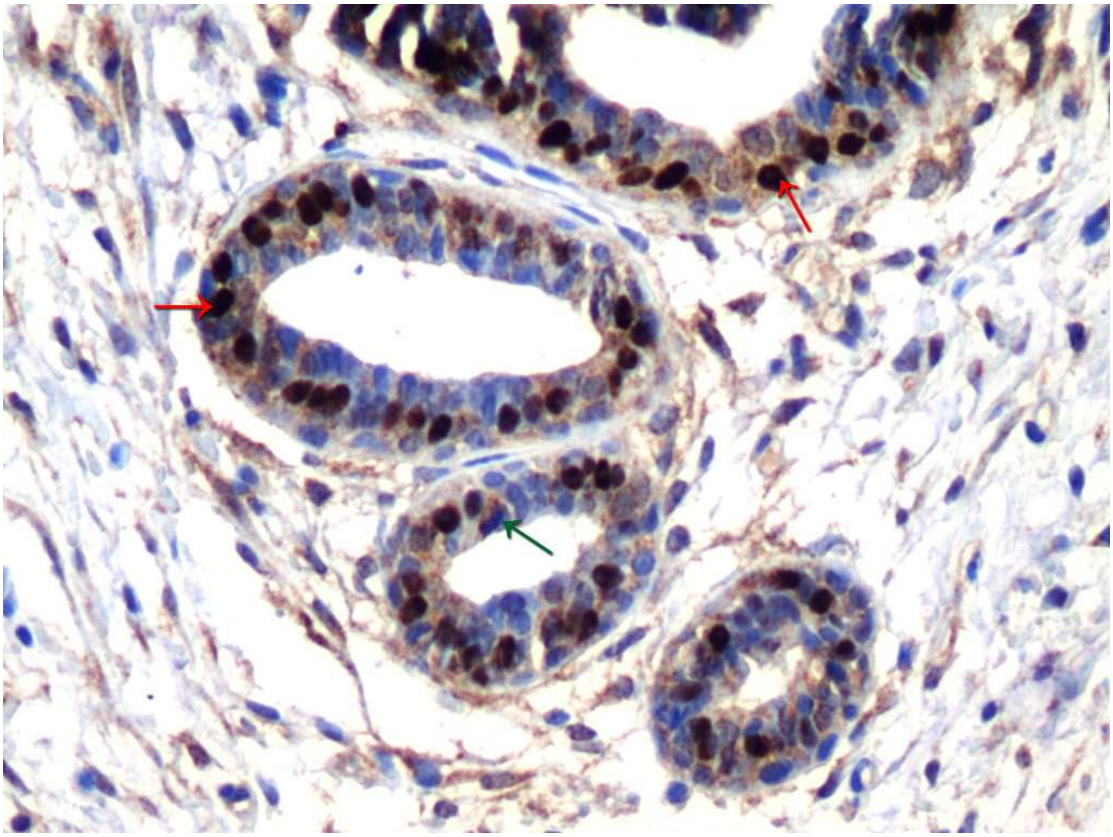

Figure 12 *(Cat 18; 20x). PR expression in feline fibroadenomatous change. The red arrows show positive nuclei, the green arrow show positive cytoplasm in a luminal epithelial cell.*

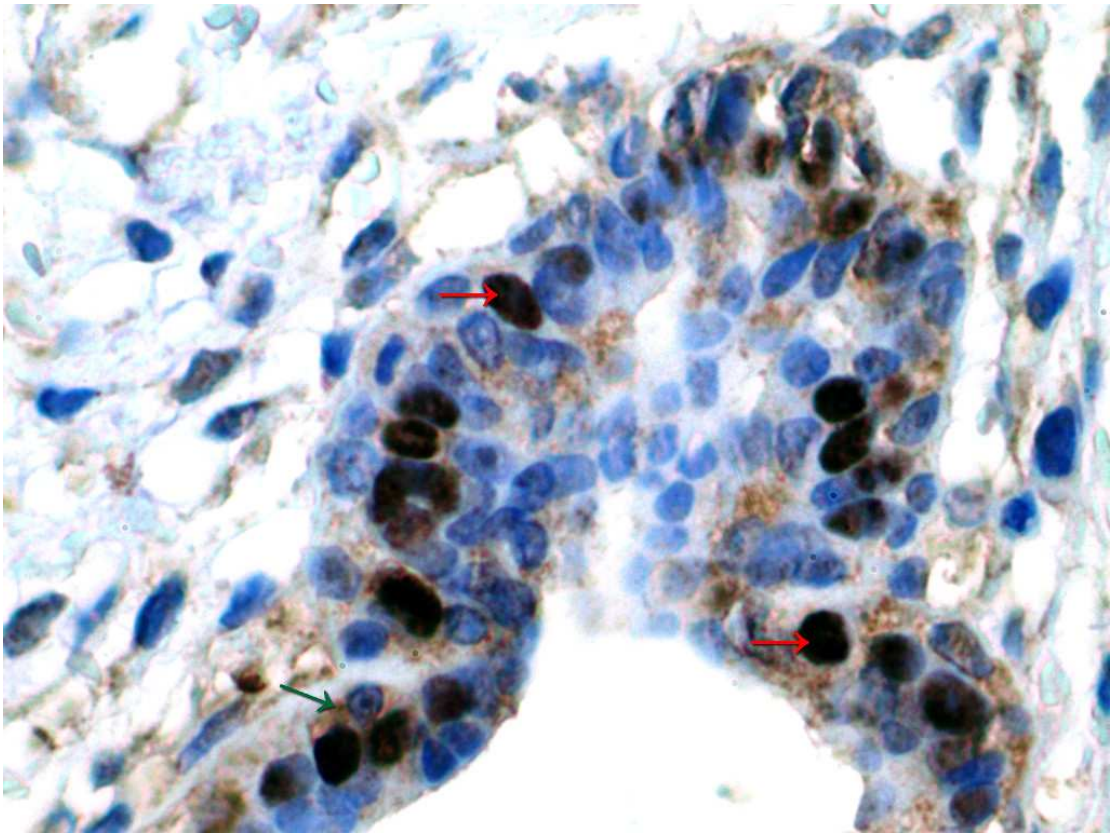

Figure 13 *(Cat 18; 40x).PR expression in feline fibroadenomatous change. The red arrows show positive nuclei, the green arrow show positive cytoplasm in an epithelial cell.*

Noteworthy is that sample 15 had the lowest QPCR value (35,6 CT) and showed hardly any positive nuclei whereas sample 18 had the highest QPCR value (24.94 CT) and the nuclei stained the deepest. Sample 24 was another sample with a low QPCR score (33.6 CT). This was a sample of an active mammary gland. The secretion inside the ducts stained positive for progesterone receptors as did the stroma at some places, but furthermore no nuclei or cytoplasm of any cell did (Fig. 14).

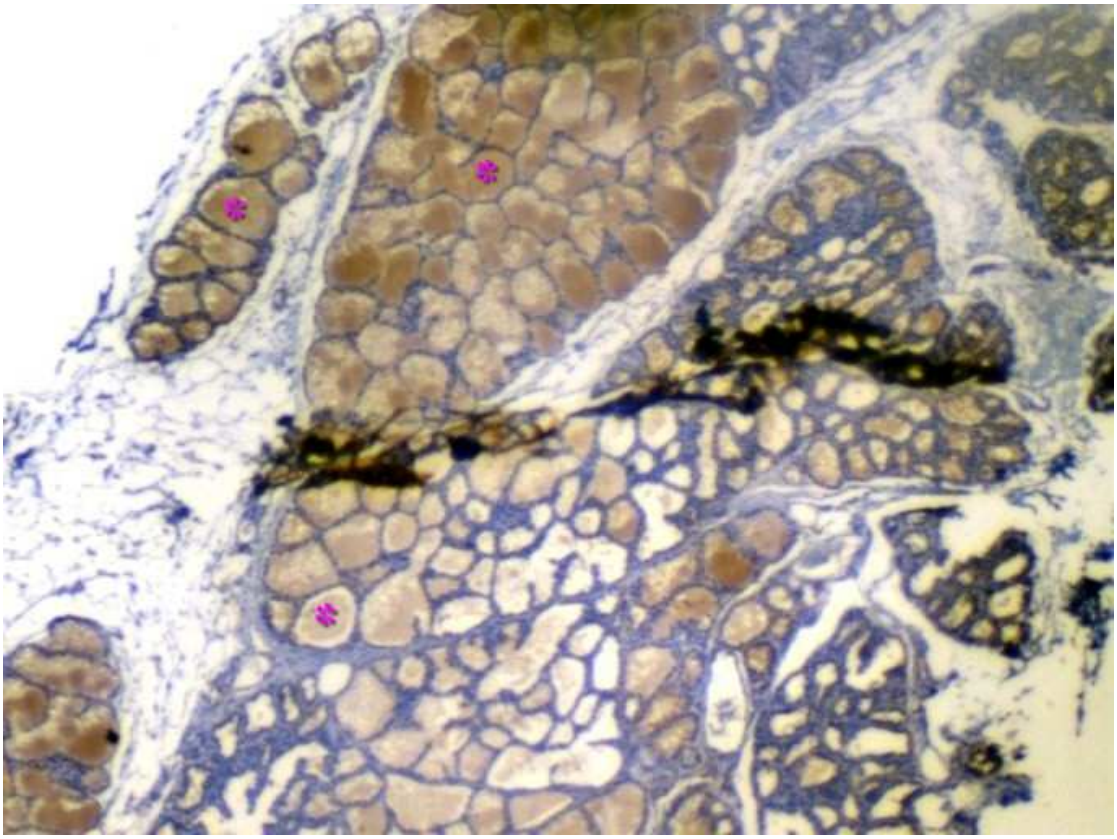

Figure 14 *(Cat 24; 2x).PR in a active mammary gland of a queen. The pink * indicate the positive cells in the ducti.*

Figures 15, 16 and 17 show tissue samples which scored a two for the nuclei reaction. Only samples 17 and 21 (Fig. 16 and 17) scored a three for the cytoplasm reaction whereas sample 28 (Fig. 15) scored one.
Sample 17 scored positive in the nuclei of the epithelial cells (2) and cytoplasm of epithelial (3) and stromal cells (1). In some ducti the secretion also stained slightly positive, this was scored 1.
Sample 19 was one of two samples that scored a three for the positive nuclei reaction. The only difference between sample 19 and 18 was the colour of the positive nuclei. In sample 18 these are dark brown, almost black, whereas in sample 19 also lighter positive nuclei can be seen (Fig. 18).

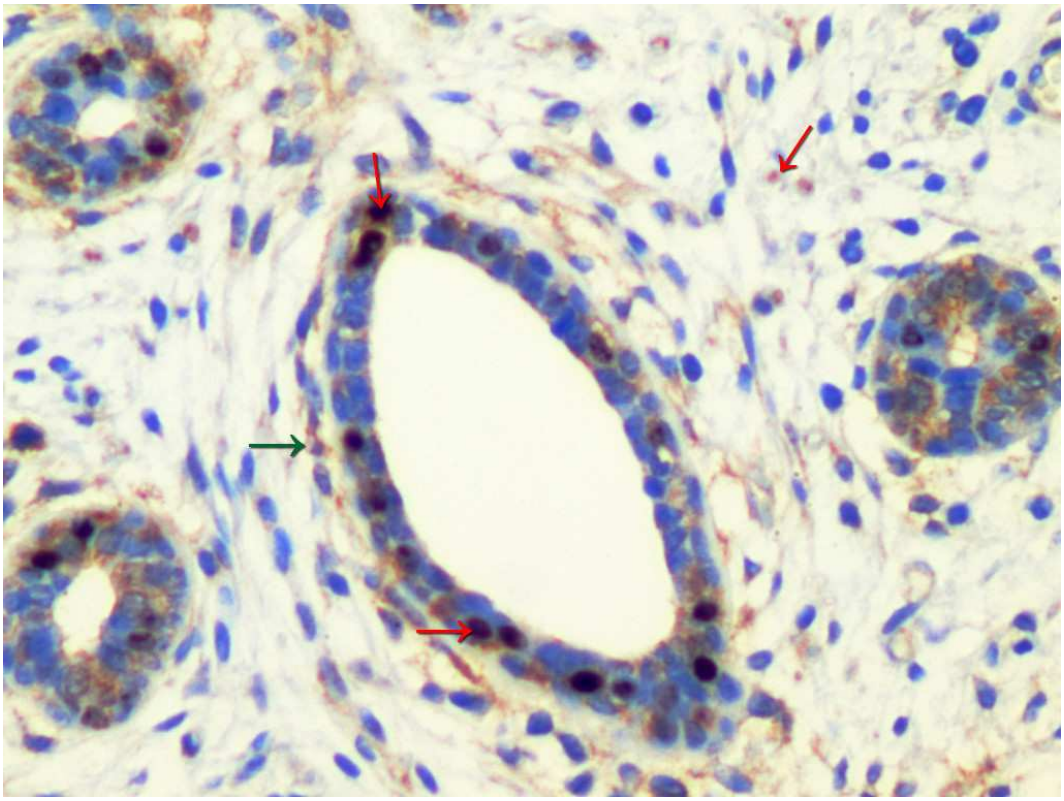

Figure 15 *(Cat 28; 20x). PR expression in feline fibroadenomatous change. The red arrows show positive nuclei in epithelial cells (left) and s stroma cell (right), the green arrow show positive cytoplasm in a stromal cell.*

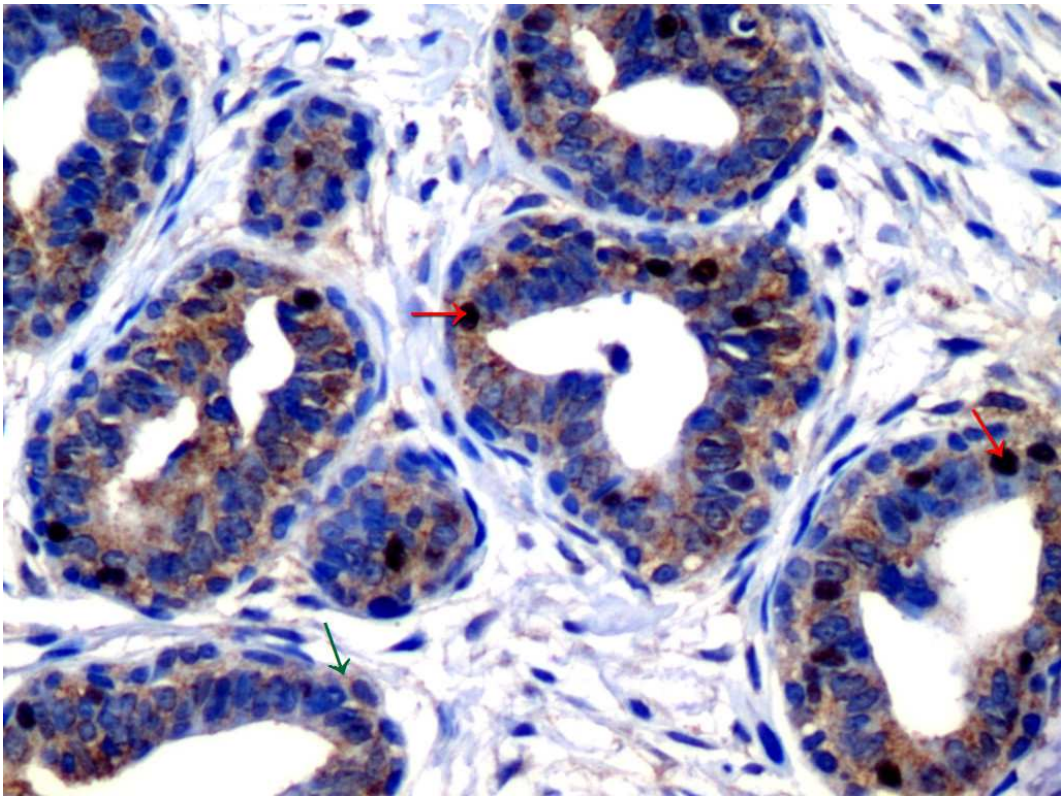

Figure 16 *(Cat 21; 40x). PR expression in feline fibroadenomatous change. The red arrows show positive nuclei, the green arrow show positive cytoplasm in an epithelial cell.*

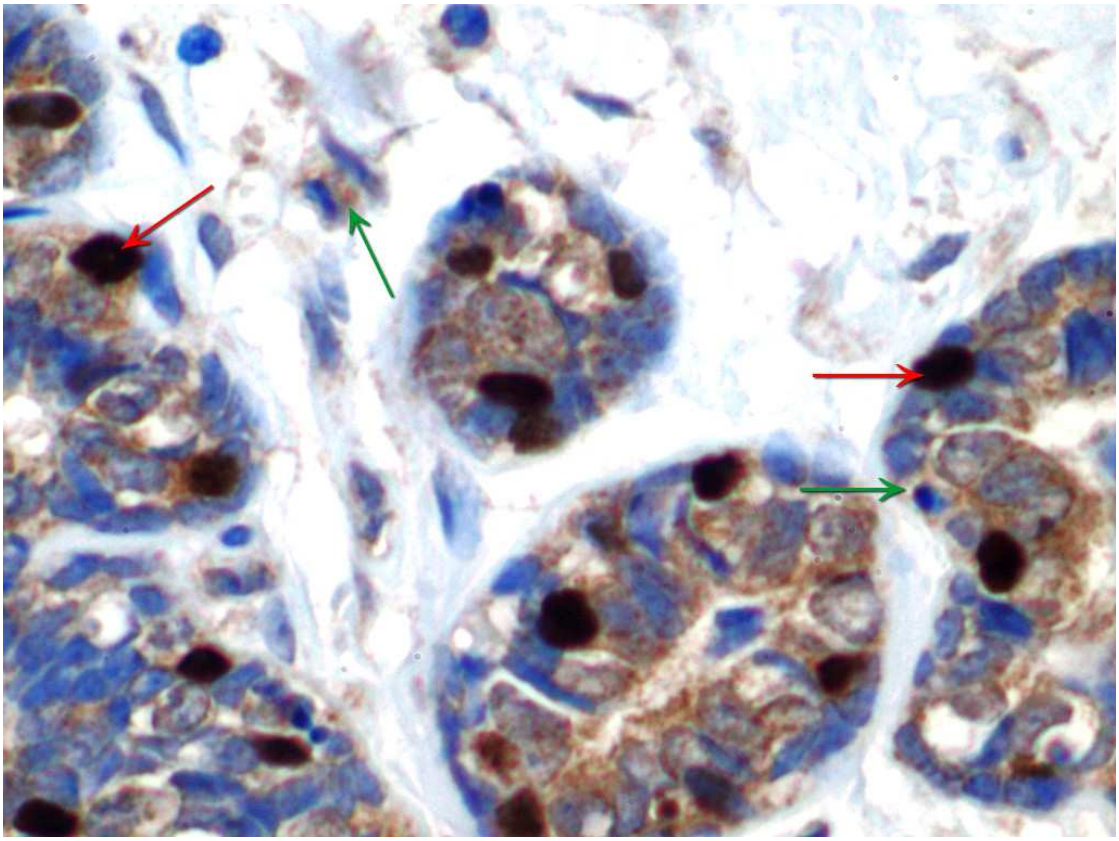

Figure 17 (*Cat 17; 40x). PR expression in feline fibroadenomatous change. The red arrows show positive nuclei in epithelial cells, the green arrows show positive cytoplasm in epithelial cells (right) and in a stroma cell.*

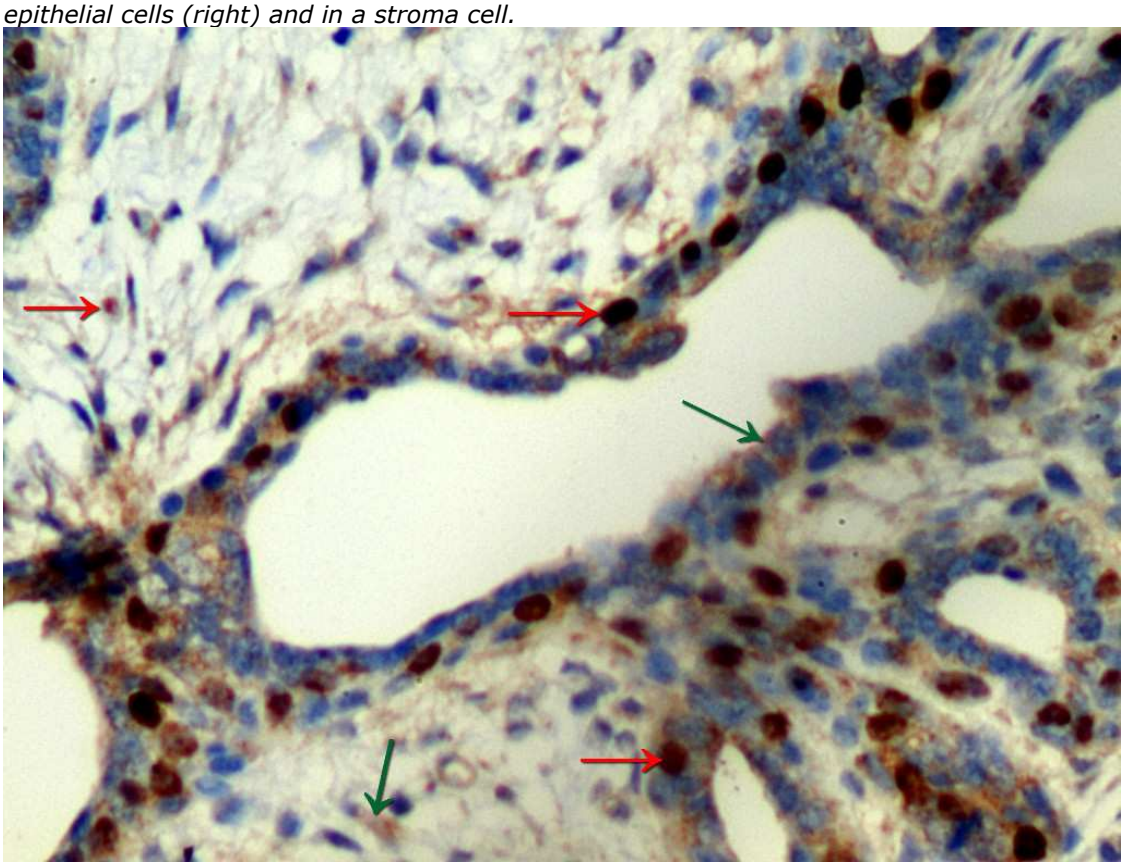

Figure 18 *(Cat 19; 20x).PR expression in feline fibroadenomatous change. The red arrows show positive nuclei in stromal (left) and epithelial cells (right), the green arrow show positive cytoplasm in an stromal cell.*

Sample 34 was excluded from analysis with QPCR due to the low quality mRNA. It was included in immunohistochemical evaluation and showed almost no positive cytoplasm, but remarkably the secretion in the ducti was slightly positive (score 1) (Fig. 19).

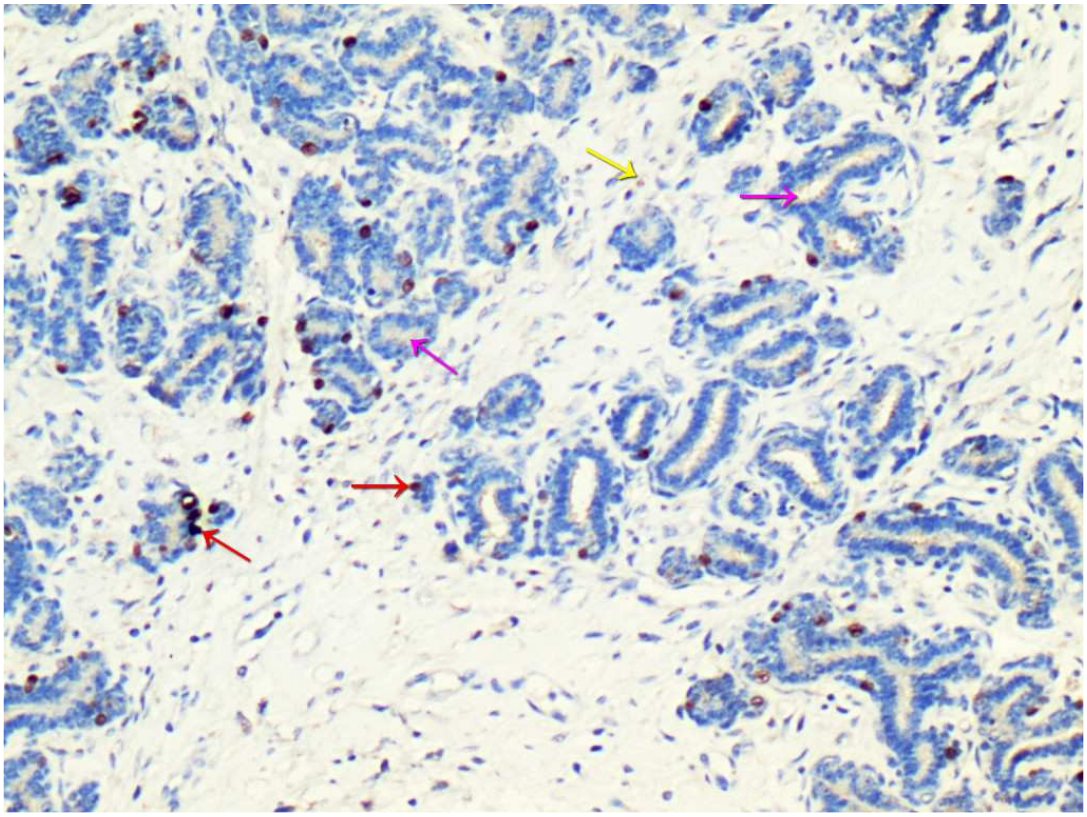

Figure 19 (*Cat 34; 10x). PR expression in feline fibroadenomatous change. The red arrows show positive nuclei in epithelial cells, the pink arrows show positive secretion in an the ducts and the yellow arrow shows a positive nuclei in the stroma.*

QPCR data compared with the immunohistochemical results

One of the aims of this study was to compare the QPCR data with the immunohistochemical results. When doing so it proved to be a difficult task. Not only just one of the four target genes stained for (PR), also of the four main topics of interest in the QPCR analysis (carcinomas compared with FAH, influence of progestins, influence of sterilization and influence of the breed) only the influence of progestins could be compared. There were no carcinomas present in the staining group, all of the animals with FAH were not sterilized and comparison of the breed proved to be not possible as the CT values were too different.
Therefore, besides the comparison of the effects of progestins, factors that could have influenced the results and comparison are looked at here.

<u>The effect of progestins</u>

When analysing the QPCR data an upregulation of GH was found in the group of animals with FAH that were treated with progestins. The PR had a p-value of 0.577, so this result was not significant. Though it is possible that the IHC could show a difference between an animal that was treated with progestins and one that was not.
Although this is also a personal interpretation it seems that indeed the animals which received some form of progestin treatment (samples 15, 17, 19, 21 and 34) have bigger and rounder nuclei.

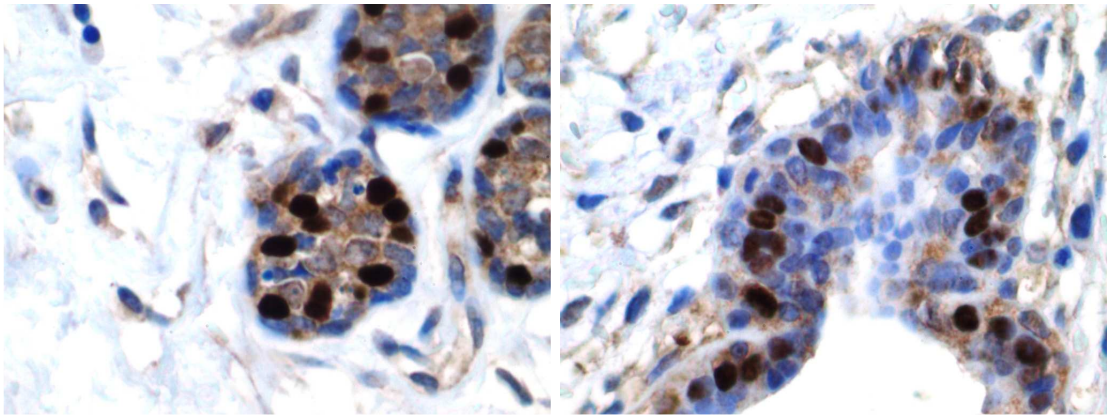

Figure 20 *Cats 17 (left) and 18 (right) 40x*

Figure 20 shows sample 17, which came from a cat with progestin treatment and sample 18, which came from a cat that did not had any progestin treatment. Both pictures were taken using the same magnification.
The positive nuclei of sample 17 does seem to be bigger and rounder compared with nuclei that remain bleu (not positive). There does not seem to be a difference between the positive and negative nuclei in the picture of sample 18.

<u>Grading system</u>

Apart from the positive control tissues (ovary and uterus) sample 18 had the highest total score PR. Sample 18 also had the highest QPCR value: 24,94 CT.
Tissue 15 had the lowest QPCR value: 35, 6 CT. However sample 15 scored a seven in the IHC grading, suggesting that this type of grading does not correspond with the QPCR results. The striking difference between samples 15 and 18 is the distribution of the scores over the diverse areas. For example sample 18 scored 3 for positive nuclei whereas sample 15 barely scored 1 (further details can be found in table 10). Sample 15 also had (faintly) positive secretion in some of the ducts as did sample 24, which also had a low QPCR result: 33,6

CT. This could indicate that the positive immunoreaction seen in the cytoplasm and secretion is more sensitive than what is seen in the nuclei. Therefore a positive reaction is faster seen with lower levels of progesterone receptor present at the location.
Samples which had a high QPCR value also had high scores for positive reactions in their nuclei. Samples 18 and 19 both scored 3 for their positive nuclei. Between those two there is almost no difference in the number of positive nuclei, but there seems to be a difference in intensity. In sample 18 almost all positive nuclei stain deep brown, almost black, whereas in sample 19 there are also somewhat more lighter brown coloured nuclei visible. Below are two photos of the samples displayed. The one on the left is sample 18, sample 19 is displayed on the right. Seeing the two samples besides each other makes the comparison of the positive nuclei easier. The amplification factor of both samples is the same.

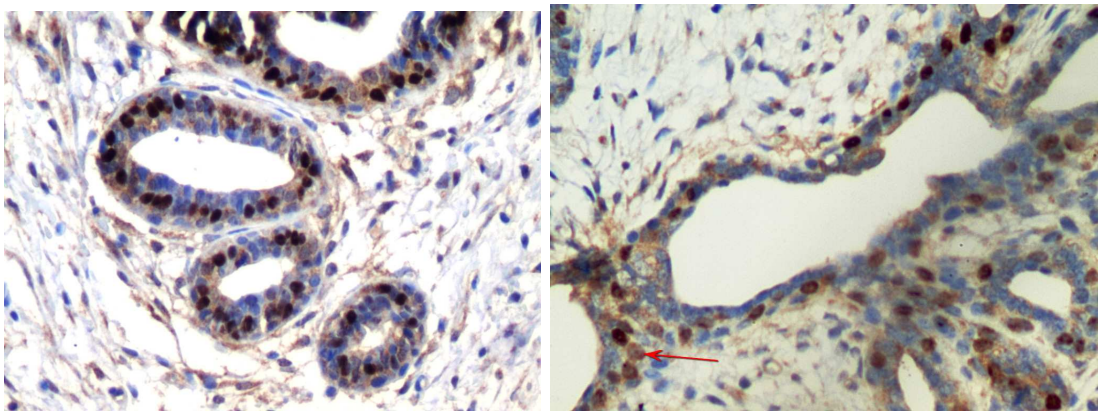

Figure 21 *Cats 18 (left) and 19 (right) 20x*

In sample 19 a red arrow indicates one of the lighter coloured positive nuclei, but the overall view shows more nuclei that are less intense.
The QPCR value of these samples are 24,94 CT (18) and 27,5 CT (19). This could indicate that the intensity of the positive reaction is a more reliable scoring factor than the number of positive cells alone. Downside of this system is that scoring of intensity is not a quantitative technique and could therefore differ between various pathologists.

Discussion

Due to the absence of normal mammary tissue two groups could be formed: the group of animals with carcinomas and the group of animals with FAH.

The group of animals with carcinomas
Unfortunately not much could be said about the group of animals with carcinomas, firstly because of the small size of the group (n=5) and secondly because some of the data of these animals was missing. It was however possible to evaluate the QPCR results manually. By doing so the expression of the progesterone receptor could be determined. The five carcinomas were all positive supporting a study done by Millanta et al. which suggested that feline mammary carcinomas are often PR positive (Millanta et al. 2005a). Nevertheless the CT-values of the carcinomas for PR were very low, ranging from 29,84 CT to 33,88 CT with an average of 32,00 CT. Consequently although they were all five positive, CT-values show that the expression is very low. This could support the data found by other studies as mentioned before who found that the expression of PR is indeed lower in carcinomas than in normal mammary tissue. Since not enough normal tissues could be included in this study no further statement could be made on this subject.

The group of animals with fibroadenomatous hyperplasia
Luckily more analysis could be performed within the group of animals with FAH. Even though there were some animals with lacking data, the group size was large enough to excluded them from that particular subject and calculate with the rest.
Since FAH is evoked through an endo- or exogenous source of progesterone two major progesterone source components were analysed: treatment with progestins and sterilization.
Several studies found a relation between treatment with MA and MPA and the development of mammary carcinomas or hyperplasia (MacDougall 2003, Nak et al. 2004, Keskin et al. 2009). In this study 14 of the 25 animals with FAH were treated with progestins. When this data was analysed using REST-excel a significant (p=0,028) upregulation of GH was found. The idea is that progesterone stimulates the local release of GH in the mammary gland, thereby leading to the hyperplasia (Mol et al. 2000, Mol et al. 1995, Mol et al. 1996). This data supports this theory. Another effect of progesterone is the autorepression of its receptor. Endogenous progesterone and progestins both have this effect (Bagamasbad, Denver 2011). This could explain why no significant effect was found for the expression of the PR.
The ovaries are an important exogenous source of progesterone. A case-control study done by Overley et al. showed that queens who had been spayed before six months of age only had a 9% chance of developing mammary carcinomas (Overley et al. 2005). The group of animals with carcinomas was too small to be analysed, but the group of animals with FAH was not. When these animals were compared no difference between the group that was sterilized and the group of intact animals was found. The group of animals with FAH in this study was very young, ranging from 7 months to 1,5 years old. Queens are usually spayed after their first heat, which occurs around one year of age. Therefore sterilization was not performed in most of these animals. With this data it is hard to demonstrate the effect of sterilization. Undoubtedly there must be an effect, since the main endogenous progesterone source is removed. The group of animals used in this study was simply too young to demonstrate the effect.

geNorm
The results of geNorm were used to determine which reference genes had to be used in the QPCR. In a study done by Kessler et al. in which they studied gene expression in feline tissue, they also looked at different methods for determining

reference genes. They analysed their data using geNorm, a program that looks at correlation between two genes and Normfinder, a program that uses an ANOVA-based calculation model. The geNorm results were further subdivided in the automatically calculated gene ranking and the M-value which they themselves ranked. They found that neither of the three options gave the same result (Kessler et al. 2009). In this study only geNorm is used to determine the reference genes. There were three genes which had a M-value below 1,5 (RPS19, HPRT and RPL17) which indicated that they were suitable as reference genes. Taken the results of Kessler into account it is possible that when the data were calculated with another programme other genes may had to be chosen, leading to other results.

Breed specifity
When all breeds included in this study with FAH were analysed, the Maine Coon showed an upregulation of B2M (p= 0,026), GH (p= 0,044) and GHR (p=0,029). B2M (beta-2-microglobulin) encodes for a protein that is a part of the MHC class I, therefore it plays a role in the immune response. The upregulation of B2M means that MHC I is more activated in Maine Coons with FAH than in other breeds with FAH. The reason for this is not known. In this study 5 Maine Coons were included and only 4 of them suffered from FAH. The upregulation of B2M could therefore just be an incident. It could also be that one of the cats had another underlying condition (not mentioned in the file) through which the MHC I could have been triggered, thereby chancing the group data. In order to make any statements more Maine Coons have to be investigated.

Immunohistochemical analysis
It was a disappointment when only eight paraffin-embedded tissue samples could be used for the immunohistochemical analysis. This limited the possibilities of comparing the QPCR data with the data obtained with the IHC. Furthermore due to the time limit only one of the initial target genes was stained for: the progesterone receptor.
In normal feline mammary tissue progesterone receptor is found in epithelial cells surrounding the ducts. Martin de Las Mulas et al. 2002 found that 5 -19% of all nuclei were positive in normal mammary tissue. In FAH this was 20-59% (Martin de las Mulas et al. 2002). Other studies found positive nuclei in the epithelium (mostly suprabasal) and some positive nuclei in fibroblast in the stroma of feline hyperplastic tissue (Martin de las Mulas et al. 2002, Martín De Las Mulas et al. 2000, Ordás et al. 2004).
In this study most of the positive reaction was found in the epithelial cells surrounding the ducti. The intensity of the positive nuclei and/ or cytoplasm differed between samples. Unfortunately no healthy mammary tissue was available for staining, therefor these results could not be compared to healthy tissue. The results of the current study support the results found by others considering the location of the positive reaction.
When the animals which had received a progestin were compared to animals which did not received any hormonal treatment an enlargement and rounding of the positive nuclei was noticeable. De Las Mulas et al. also found that the positive nuclei appear to be rounder and bigger in tissue of animals with FAH which were treated with a progestin (Martín De Las Mulas et al. 2000). The meaning of the enlargement of the positive cells is not clear. It could be that there are more receptors present in the cell thereby occupying more space and increasing the volume of the nuclei. This theory is however doubtable since progesterone is the auto-repressor of its own receptor. Meaning that a rise of progesterone leads to a decrease in PR. Endogenous progesterone and progestins both have this effect (Bagamasbad, Denver 2011).
Besides the grading system there are some other factors that could interfere with the comparison of the QPCR data with the staining results. Firstly the scoring of

the IHC can be influenced in many ways. It could depend on the slide itself or on the person grading the slides. When looked at the slide itself the expression could differ between different regions within the tissue. So the place of taking slides out of the paraffin embedded tissue could differ the result. Formalin fixation itself does not seem to be of any influence on preservation of the progesterone receptor according to a study done by Webster et al. (Webster et al. 2009). In this study the slides are graded by only one person which is not an experienced pathologist. This could also influence the results. Even so all slides are graded by the same person with help from people who are more experienced in assessing slides and other studies on this topic (Ordás et al. 2004, Loretti et al. 2005, Martin De Las Mulas et al. 2000). By doing so the aim was to create a more objective result.
Secondly not all tissues that were used in the QPCR data were stained. Therefore not all analyses made with the QPCR data could be made with the slides. The role of sterilization and breed could for instance not be compared due to the limited amount of samples that were available for the immunohistochemical analysis.

Conclusion

This study confirmed that the expression of GHR, IGF-I and PR is lower in carcinomas compared to FAH in cats. Unfortunately only one healthy tissue sample could be included in this study, therefore no comparisons could be made between healthy and diseased tissue.
The expression of growth hormone was significantly upregulated (p=0,028) in animals with FAH which received progestins. In the immunohistochemical analysis rounder and bigger positive nuclei were found in these animals. This could be an important feature. It could mean that the nuclei contains large numbers of progesterone receptor, thereby disproven the autorepression effect that progesterone has in normal tissue. The next step in this research would be to combine the QPCR data with the staining results of the PR and double stain for GH and PR to look for possible causes of these large positive nuclei.
No effect of sterilization could be found within the groups, but when all the animals were analysed together a significant upregulation of PR (p=0,038) and GHR (p=0,013) was found in the intact queens. This could lead to the conclusion that intact queens have a higher risk of developing mammary hyperplasia or carcinomas due to a higher expression of GHR and PR.
Finally the influence of the breed on developing FAH was analysed. The expression of B2M (p=0,026), GH (p=0,044) and GHR (p=0,029) was unregulated in Maine Coons. This could mean that this breed is more predisposed to developing FAH.
All results named are significant, but further research is necessary to make any conclusions. The group used in this study was relatively small: only 5 samples of carcinomas, 25 samples of FAH and only one normal mammary sample. Normal mammary tissue is needed to conclude whether or not the expression of the genes in FAH is higher than in normal mammary tissue. And normal mammary tissue of Maine Coons in needed to confirm whether or not this breed is indeed predisposed for developing FAH.

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Appendixes

Appendix 1

Sample data

Cat	Clinical/ Pathological number	Diagnosis	Sex	Breed	Age (years)	Spayed (age)	Hormonal Treatment
1.	87.10.162 GRK 10	Carcinoma	F	Siamese	19	Yes 12	No
2.	88.10.1163 GRK 16	Carcinoma			12		
3.	K88.0615.J 88.10.669 GRK 21	Carcinoma	F	ESH	12	Yes	Yes
4.	89.10.111 GRK 23	Normal	F		2.5	No	No
5.	K89.0230.C GRK 27	Carcinoma	F	ESH	10	Yes	No
6.	K89.0174.R GRK 28	Fibroadenoma	F	ESH	1.75	Yes 1.5	Yes
7.	K90.0037.A GRK 36	Carcinoma	F	ESH	10	Yes 5	Yes
8.	GRK 40	FAH	M	ESH	0.6	No	Yes
9.							
10.		Kidney					
11.*	KMT-56	FAH	F	Siamese	3	No	Yes
12.	83.3374 K2R1	FAH	F	ESH	1.2	No	Yes
13.	0222492 P0208109	FAH	F	ESH	0.9	No	Yes
14.	164254 K01.1039.S	FAH	F	Maine Coon	1.1	No	Yes
15.*	0300810 P0300906	FAH	F	ESH	0.6	No	Yes
16.	163911/ K01.0999.Y	FAH	F	BSH	0.3	No	
17.*	K01.1159.L	FAH	F	DSH	1.6	Yes 1.3	
18.*	226367 P0212250	FAH	F	Maine Coon	0.9	No	Yes
19.*	411283 P0411352	FAH	F	DSH	0.6	No	Yes
20.	405166	FAH	F	ESH	1.1	Yes	
21.*	401283 P0401311	FAH	F	ESH	1.3	No	Yes
22.	306422	FAH	F	ESH	0.9	No	
23.	404392	FAH	F	ESH	1.1	No	
24.*	301611 P0301833	Active mammary gland	F	Maine Coon	1.1	Yes 1	
25.	405248	FAH	F				
26.	503154	FAH	F	ESH	0.8	No	
27.	402312	FAH	F	Maine Coon	1.0	No	Yes
28.*	300244 P0300337	FAH	F	Somali	0.75	No	No
29.	302497	FAH	F	ESH	0.8	No	Yes

30.	404020	FAH	F	Maine Coon	0.8	No	
31.	406435	FAH	F	ESH	0.6	Yes	
32.	408631	FAH	F				
33.	GRK 12	FAH	F	ESH	7		
34.*	GRK 42	FAH	F	ESH			
35.	14 bt 59	FAH	F				
36.	304822 P0304790	FAH	F	ESH		No	Yes
37.*		Uterus & Ovarian	F	ESH	6	No	

** = sample was included in immunohistochemical staining*
BSH = British Short Hair
DSH = Domestic Short Hair
ESH = European Short Hair
When the box is blank, no background information could be found. The sample of cat 9 was known to be mammary tissue, but that was all available information.

Appendix 2

Results RNA isolation

Sample	RNA (ng/ µL)
1	103.3
2	315.5
3	978.9
4	1019.6
5	688.2
6	181.1
7	643.7
8	560.6
9	26.3
10	409.4
11	152.5
12	992.3
13	138.4
14	172.5
15	33.6
16	148.0
17	544.4
18	170.8

Sample	RNA (ng/ µL)
19	56.8
20	88.6
21	139.6
22	188.7
23	182.7
24	107.0
25	194.1
26	202.6
27	198.9
28	180.5
29	63.2
30	230.4
31	250.3
32	297.6
33	17.5
34	18.6
35	21.9
36	13.5

Appendix 3

PCR result of QPCR gradient of GH primerset

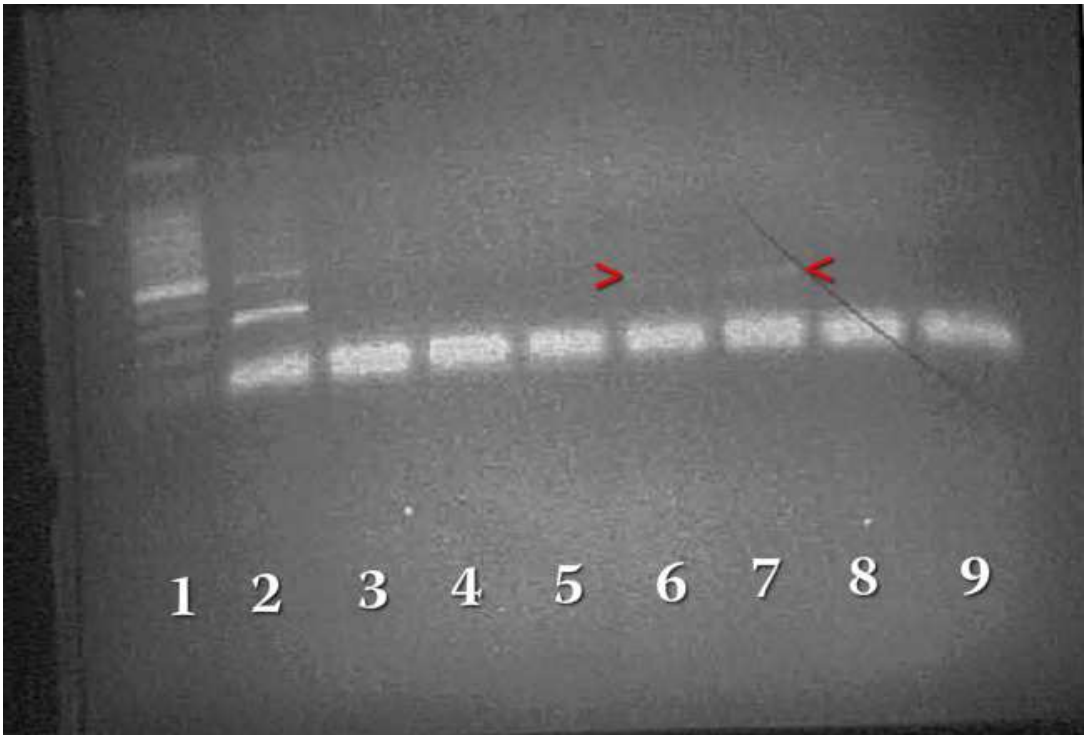

Figure 22 *PCR gradient of GH. Numbers 1 to 9 represent different tempetures in QPCR. Between the red arrows is a second line visible, indicating that more than one product is formed.*

Appendix 4

QPCR; Line of cups, plate set-up and protocol for performing duplo 25 µL reactions

Line of cups

Figure 23 *Schematic figure of a line of cups. This is for instant line 3 which contains samples 1-8.*

Plate set- up

1	2	3	4	5	6	7	8	9	10	11	12
S1	S1	1	1	9	9	17	17	25	25	33	33
S2	S2	2	2	10	10	18	18	26	26	34	34
S3	S3	3	3	11	11	19	19	27	27	35	35
S4	S4	4	4	12	12	20	20	28	28	36	36
S5	S5	5	5	13	13	21	21	29	29	37	37
S6	S6	6	6	14	14	22	22	30	30	38	38
S7	S7	7	7	15	15	23	23	31	31	39	39
MQ	MQ	8	8	16	16	24	24	32	32	40	40

Figure 24 *Schematic figure of a BioRad I-Cycler plate as used in this study. S1-9, standard line; MQ, milliQ; sample places 33-40 were not occupied.*

Master Mix

Master Mix	
MQ	1250 µL
iQ SYBR green SuperMix	1250 µL
Forward Primer	113 µL
Reverse Primer	113 µL

Figure 25 *Contains of the master mix used in this study*

Standard Line

Standard line		
S1	32 µL pool	
S2	8 µL S1	24 µL MQ
S3	8 µL S2	24 µL MQ
S4	8 µL S3	24 µL MQ
S5	8 µL S4	24 µL MQ
S6	8 µL S5	24 µL MQ
S7	8 µL S6	24 µL MQ

Figure 26 *Composition of the standard line used in this study*

Protocol for performing duplo 25 µL reactions

- 54 µL master mix used for each reaction to ensure 25 µL volume per reaction
- To each 2 µL cDNA 54 µL master mix is added
- This is mixed by carefully pipetting up and down several times
- 25 µL can then be pipetted into a BioRad I-Cycler plate in duplicate
- After finishing all samples put on a seal without gloves

- Spin the plate briefly to remove any air bubbles
- Place the plate in the QPCR machine without touching the top of the plate

Appendix 5

IHC Protocol for the progesterone receptor in feline tissue

- Dewaxing en rehydration

2x5 min xyleen
3 min ethanol 96%
3 min ethanol 80%
2 min ethanol 70%
2 min ethanol 60%

- 2x5 min TBS

- 15 min 3% H_2O_2 in TBS

- 2x5 min TBS

- Antigen retrieval: citrate boiled for 15-20 min, cooling down for 20 min on the lab table.

- 2x5 min TBS

- 30 min blocking 10% NGS in 1% BSA

- First antibody incubation

Dry slides and take off as much liquid as you can.
Draw circles round section with ImmEdgde pen.
200 ul, or as much as you need to cover the sections completely, 1^e antibody diluted 1:1500 on section in a special made container with a high humidity grade.

- Keep container at cold room overnight.

- 2x5 min TBS

- 30 min 2^e antibody at room temperature

Dry slides and take off as much liquid as you can.
Cover slides with a few drops of second antibody solution.

- 2x5 min TBS

- DAB

Dry slides and take off as much liquid as you can
Incubate each slide with 200 µl DAB-solution at room temperature for 5 minutes.

- Put the sections in a container with milliQ water.

- Hematoxylin

Dry slides and take off as much liquid as you can.
Cover sections for a few seconds with some drops of hematoxylin

- Drain hematoxylin from slide

- Flush sections during 10 min with tap water

- Dehydration

2 min ethanol 60%
2 min ethanol 70%
3 min ethanol 80%
3 min ethanol 96%
3 min ethanol 96%
2x5 min xyleen

- Mounting

Dry slides and take off as much liquid as you can.
Mount a cover slip with Vecta Mount.

Appendix 6

Effect of progestins

Animals treated with progestins compared to animals which did not receive progestins.

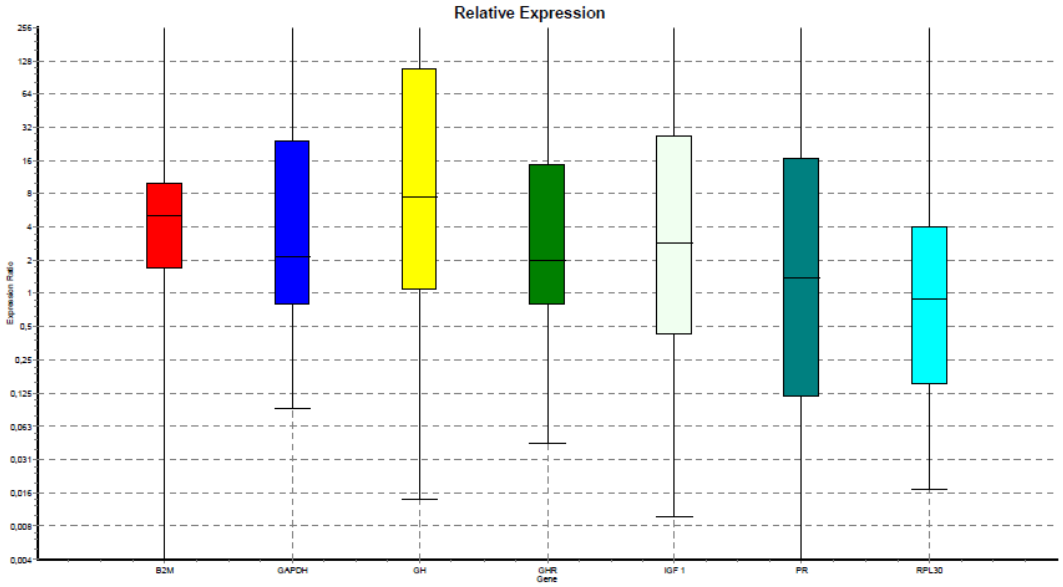

Figure 27 *Relative expression ratio animals treated with progestins vs. non treated animals. Y-axis: expression rate, x-axis: genes*

Appendix 7

Effect of sterilization

n (carcinomas) = 5		
	Number	**Percentage**
Sterilized	4	**80%**
Not sterilized	0	**0%**
Unknown	1	**20%**
Total	**5**	**100%**

Figure 28

n (FAH) = 26		
	Number	**Percentage**
Sterilized	3	**11.5%**
Not sterilized	18	**69.2%**
Unknown	5	**19.3%**
Total	**26**	**100%**

Figure 29

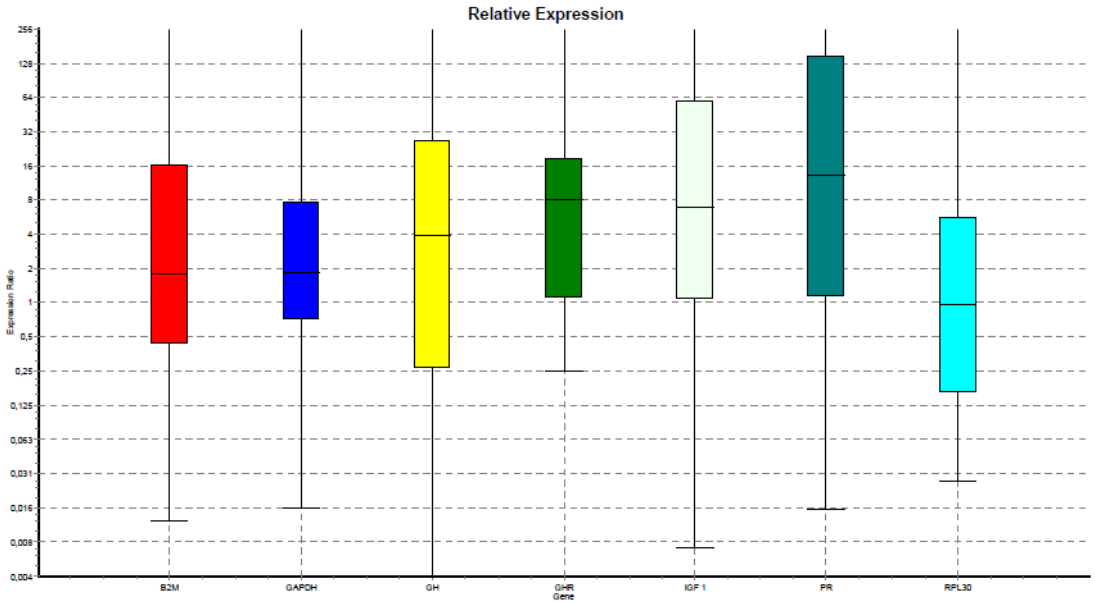

Figure 30 *Relative expression ratio sterilized queens vs. non sterilized queens*

Appendix 8

Influence of the breed

n (known breed) = 30		
Breed	**Number**	**Percentage**
Siamese	2	**6.67%**
European Shorthair	19	**63.33%**
British Shorthair	1	**3.33%**
Maine Coon	5	**16.67%**
Somali	1	**3.33%**
Domestic Shorthair	2	**6.67%**
Total	**30**	**100%**

Figure 31 *Overview of all breeds of cats included in this study*

n (breed cats with FAH) = 25		
Breed	**Number**	**Percentage**
Siamese	1	**2.5%**
European Shorthair	15	**72.5%**
British Shorthair	2	**5%**
Domestic Shorthair	2	**5%**
Maine Coon	4	**10%**
Somali	1	**5%**
Total	**25**	**100%**

Figure 32 *Overview of all breeds in the FAH group*

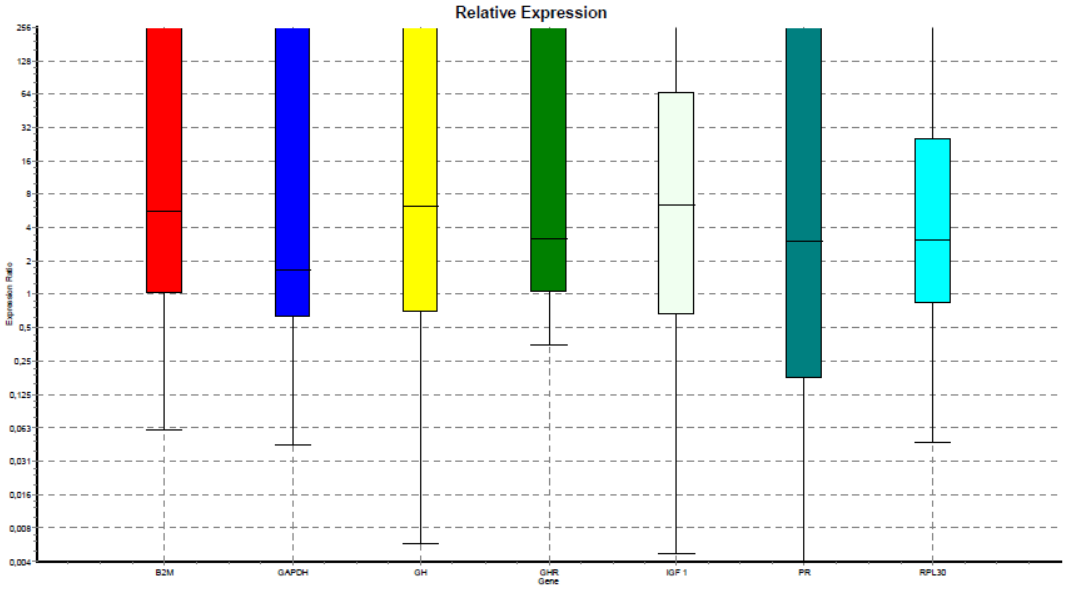

Figure 33 *Maine Coon vs. other breeds*